Journal of Biology and Earth Sciences

Volume 3 Number 1 January-June 2013

ISSN: 2084-3577

Affiliated scientific journal
of Free International Association of Researchers on Natural Substances 2009

TMKARPIŃSKI
PUBLISHER

http://www.journals.tmkarpinski.com/index.php/jbes
http://jbes.strefa.pl e-mail: jbes@interia.eu

Journal of Biology and Earth Sciences

Photo on the cover: *Doronicum austriacum* in the Tatry Mountains, Poland. Author: Tomasz M. Karpiński

Publisher and Editor's office:
Tomasz M. Karpiński, ul. Szkółkarska 88B, 62-002 Suchy Las, Poland, e-mail: jbes@interia.eu

Journal of Biology and Earth Sciences is abstracting and/or indexing in:

- Academic Journals Database
- AGRO
- ARIANTA
- Bielefeld Academic Search Engine
- Comsats
- Cornell University Library
- Directory of Research Journals Indexing
- DOAJ
- EBSCO
- e-journals.org
- Elektronische Zeitschriftenbibliothek
- Geo-Leo
- Geoscience e-Journals
- Google Scholar
- Index Copernicus
- Mendeley
- New Jour
- OARE
- Open Archives
- Open J-Gate
- Polish Scholarly Bibliography
- Public Knowledge Project
- Scirus
- WorldCat

Aims and Scope

The aim of the Journal of Biology and Earth Sciences (JBES) is to provide the platform for exchange of scientific progress in the field of Biology, Medicine and Earth Sciences, and to do so at the highest possible level. The JBES also aims to facilitate the application of new scientific knowledge to the daily practice of the concerned disciplines and addresses both researchers and academics.

The JBES publishes original contributions, case reports and review articles in the fields of biology, medicine and earth sciences.

Its scope encompasses:

1. in field of biology: botany, ecology, zoology, microbiology, molecular biology, cellular biology, genetics, taxonomy and allied,
2. in field of medicine: all medical aspects of human biology,
3. in field of earth sciences: geology, mineralogy, petrography, paleontology, geography, geophysics, soil sciences and allied.

Editorial Policy

The Journal of Biology and Earth Sciences (JBES) is a peer-reviewed, open-access, article-based, international, scientific Journal, that publishes full-length articles on biological, medical and earth sciences. Journal accepts original research articles, case reports and review articles.

All manuscripts received by the Editor are considered as confidential documents. A notification of receiving the manuscript is sent by e-mail to the corresponding author.

All manuscripts are subjected to pre-screening by the Editor in Chief and can be rejected at this stage or returned for corrections before evaluation (if they do not meet the criteria given in the Instruction to Authors, including language quality or are out of the scope of JBES). After passing the pre-screening stage the manuscripts are sent to at least two qualified outside referee, but the editors themselves may also act as reviewers. Reviewers are from behind of scientific unit in which is affiliated author of the publication. Papers that do not conform to the general aims and scope of the journal will, however, be returned immediately without review. Journal of Biology and Earth Sciences uses double blinded review process. The names of the reviewers will not be disclosed to the author submitting a paper, and the names of authors will not be disclosed to the reviewers.

The review process lasts approximately 4-8 weeks.

ITEMS EVALUATED

Referees, in confidence, evaluate the manuscript according to the following aspects: Present-day interest, Originality, Adequacy of methodology, Theoretical background, Essential level, Results and interpretation, Importance in its field, Conclusions, Language correctness.

All the above items can be evaluated as Excellent, Good, Fair or Poor. The manuscript can be evaluated as:

- acceptable in its present form
- acceptable after minor revision (no further review required)
- reconsidered after major revision
- not acceptable for publication.

When the review process is finished, a decision e-mail will be sent to the corresponding author, including the comments of the referee(s). Any corrections suggested by the reviewers, should be made within the period of time indicated in the e-mail. If the paper is accepted after major revisions, it will be submitted again for peer review to the same evaluator(s). After a second review of the corrected manuscript, a final decision e-mail concerning publication is sent to the authors. A proof is provided to the corresponding author before the article is published. The checked proof should be returned to the Editorial Office by e-mail within 7 working days.

The Journal is issued at least twice a year in electronic version. Each article is published online in PDF format at www.journals.tmkarpinski.com/index.php/jbes and jbes.strefa.pl as soon as it is ready for publication. Public access to articles in the Journal of Biology and Earth Sciences free of charge.

Instructions for authors

1. GENERAL

The Journal of Biology and Earth Sciences (JBES) is a peer-reviewed, Open-Access, article-based, international, scientific Journal, that publishes full-length articles on biological, medical and earth sciences. Journal accepts original research articles, case reports and review articles. The JBES is issued at least twice a year in electronic version. Public access to all articles is free of charge.

2. ETHICAL GUIDELINES

2.1. Authorship and Acknowledgements

Authors submitting a paper do so on the understanding that the manuscript have been read and approved by all authors and that all authors agree to the submission of the manuscript to the Journal.

2.2. Ethical Approvals

All experimental studies using human or animal subjects should include an explicit statement in the Material and Methods section identifying the review and ethics committee approval for each study.

2.3. “Ghostwriting” and “guest authorship” prevention

The editors of JBES quarterly lead the policy of “ghostwriting” and “guest authorship” prevention.

“Ghostwriting” is the case of contributing a publication without revealing one’s participation as one of the authors or without reference to their role in the acknowledgments in the publication. “Guest authorship” (honorary authorship) is the case of insignificant contribution of the author or its complete absence and nevertheless being the author or co-author of the publication. The editors reserve the right to reveal all aspects of scientific dishonesty, i.e. failure to comply with actions eliminating the occurrence of the aforementioned practices.

The editors require that Authors should reveal individual co-authors’ contribution to the publication. Potential contributions include:

Conception and design
Development of methodology
Acquisition of data
Analysis and interpretation of data
Writing, review and/or revision of the manuscript
Administrative, technical, or material support
Study supervision
Other

However, the author submitting the manuscript will chiefly be held responsible. (The ghostwriting and guest authorship prevention procedure in JBES was written according to the guidelines of the Ministry of Science and Higher Education).

2.4. Plagiarism

Plagiarism is when an author attempts to pass off someone else's work as his or her own. Duplicate publication, sometimes called self-plagiarism, occurs when an author reuses substantial parts of his or her own published work without providing the appropriate references. By submitting your manuscript to us you accept that your manuscript may be screened for plagiarism against previously published works.

Manuscripts that are found to have been plagiarized will incur plagiarism sanctions:

- immediate rejection of the submitted manuscript or published article
- no return of article-processing charge (if any)
- prohibition against all of the authors for any new submissions.

2.5. Conflict of Interest and Sources of Funding

Authors are requested to provide a statement concerning any commercial associations or patent licenses that might result in a conflict of interest with the work presented in the submitted paper.

2.6. Permissions

Materials copied from other sources must be accompanied by a written statement from both the author and publisher giving permission to Journal of Biology and Earth Sciences for reproduction. It is the author’s responsibility to ensure that such permissions are obtained.

2.7. Copyright Assignment

Authors submitting a paper do so on the understanding that the work and its essential substance have not been published before and is not being considered for publication elsewhere. The articles published in this journal are open-access articles distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

3. MANUSCRIPT SUBMISSION PROCEDURE

3.1. Cover Letter

Each manuscript should be accompanied by a Cover Letter. The cover letter should contain all important details such as: your full name (submitted by), full title of article and short title, full list of authors with affiliations, e-mail of the corresponding author, contact address, telephone/fax numbers of the corresponding author. In the cover letter Authors should propose 3-5 reviewers giving names, affiliations and e-mails.

All authors of the manuscript are responsible for its content; they must have agreed to its publication and have given the corresponding author the authority to act on their behalf in all matters pertaining to publication. The corresponding author is responsible for informing the coauthors of the manuscript status throughout the submission, review, and production process.

3.2. Manuscript Files Accepted

The final version of the manuscript, figures and tables should be submittedbysite
http://www.journals.tmkarpinski.com/index.php/jbes
Main text and tables must be in Microsoft Word (.doc) format (not write-protected).
Photographs must be in JPG or TIFF format, preferred resolution >300 dpi.
Drawings and graphs must be in JPG or EPS format, preferred resolution >300 dpi.

The manuscript must be accompanied by: a written Licence Form, and in case of experimental studies a statement that the protocol of study and informed consent were in compliance with the Helsinki Convention and were approved by local Ethics Committee. Upon acceptance of the manuscript, the author(s) will be asked to send Licence Form to the publisher.

3.3. Double-Blinded Review

All submitted manuscripts will be reviewed by two experts in the field, who are from behind of scientific unit in which is affiliated author of the publication. Papers that do not conform to the general aims and scope of the journal will, however, be returned immediately without review. Journal of Biology and Earth Sciences uses double blinded review process. The names of the reviewers will not be disclosed to the author submitting a paper, and the names of authors will not be disclosed to the reviewers.

3.4. E-mail Confirmation of Submission

In course of 5 working days after submission you will receive an e-mail to confirm receipt of your manuscript. If you do not receive the confirmation e-mail, please check your e-mail address carefully in the system. The error may be caused by some sort of spam filtering on your e-mail server.

3.5. Manuscript Status

The Journal will inform you by e-mail once a decision has been made.

3.6. Submission of Revised Manuscripts

To submit a revised manuscript, send it by e-mail:
jbes@interia.eu

4. MANUSCRIPT TYPES ACCEPTED

Journal of Biology and Earth Sciences publishes original research articles, case reports and reviews. Articles will be published under the heading of Biology, Medicine or Earth Sciences.

Original Research Articles must describe significant and original experimental observations and provide sufficient detail so that

Instructions for authors cont.

the observations can be critically evaluated and, if necessary, repeated.
Case Reports must describe an individual phenomenon, uncommon case or a new or improved method.
Reviews are selected for their broad general interest; should take a broad view of the field.

5. MANUSCRIPT FORMAT AND STRUCTURE

5.1. Language
The language of publications is English (in some cases we can publish in Polish). Authors whose native language is not English are strongly advised to have their manuscripts checked by a professional translator or a native speaker prior to submission.

5.2. Structure
All articles should include Title Page, Abstract, and References and in addition sections on Source of Funding and Conflict of Interests. Figures, Figure Legends and Tables should be included where appropriate.
Title Page: The title must contain no more than 100 characters including spaces. The title page should include a running title of no more than 40 characters; 5-10 key words, complete names of institutions for each author, and the name, address, telephone number, fax number and e-mail address for the corresponding author.
Conflict of Interest and Source of Funding: Authors are requested to provide a statement concerning any commercial associations or patent licenses that might result in a conflict of interest with the work presented in the submitted paper. Author's conflict of interest (or the absence of conflicts of interest) and the sources of funding for the research will be published under a heading "Conflict of Interest and Source of Funding Statement".
Abstract: is limited to 250 words in length and should not contain abbreviations or references.
Acknowledgements: Under acknowledgements please specify contributors to the article other than the authors accredited.

5.3. Original Research Articles
Main Text should be organized with Introduction, Materials and Methods, Results, Discussion (or Results and Discussion). The background and hypotheses underlying the study, as well as its main conclusions, should be clearly explained.

5.4. Case Report Articles
These should be divided into the following sections: Title page, Abstract, Introduction, Case presentation, Discussion, Conclusions.

5.5. Review Articles
Reviews should take a broad view of the field rather than merely summarizing the authors´ own previous work. The use of state-of-the-art evidence-based systematic approaches is expected. Main text should be organized with Introduction, Review, Conclusions.

5.6. References
Citations in the text should be marked by Arab numbers in square brackets.
a) The arrangement of the references should be in consecutive order (as they are cited in the text).
b) The first six authors should be presented; if are more than six, please use a phrase: et al.
c) The order of the items in each reference should be as in examples:
Nowak MA, Kowalski A. Natural antibiotics. J Biol Earth Sci. 1992; 4(1): E1-E6.
Nowak Z. 2003. Moss flora of Poznań. 4th edition: pp. 1014. Warszawa, Scientific Publishing.
Kowalski T. 1980. Skin infections. In: Microbiology. Ed. Nowak D. pp. 269-278. Warszawa, PublishMe.

5.7. Tables and Figures
Tables: should be with no vertical rulings, with a single bold ruling beneath the column titles. Units of measurements must be included in the column title. Max. format A4.
Figures: All figures should be planned to fit within either 1 column width (8.2 cm) or 2 column widths (17.0 cm). Lettering on figures should be in a clear typeface; the same typeface should be used for all figures.
Units should have a single space between the number and the unit, and follow SI nomenclature or the nomenclature common to a particular field (defined in the legend). Do not use pixel-oriented programmes.
Permissions: If all or parts of previously published illustrations are used, permission must be obtained from the copyright holder concerned. It is the author's responsibility to obtain these in writing and provide copies to the Publishers.

6. AFTER ACCEPTANCE
Upon acceptance of a paper for publication, the manuscript will be forwarded to the production of the journal.

6.1. Proof Corrections
The corresponding author will receive an email with the proof PDF file. Corrected proof must be scanned and returned to the Editor within 7 working days. Excessive changes made by the author in the proof, excluding typesetting errors, will be charged separately. Please note that the corresponding author is responsible for all statements made in his work, including changes made by the copy editor. Manuscripts submitted under multiple authorship are published on the assumption that the final version of the manuscript has been seen and approved by all authors.

6.2. Article-processing charge
Publication in JBES is free of charge.

6.3. Online publication
Online publication will normally be within 2 weeks of receipt of corrected proof by the TMKarpinski Publisher. Authors should note that online articles are complete and final and thus no changes can be made after online publication.

Contents

i-ii Editorial sites
iii-iv Instructions for authors
v-vi Contents

BIOLOGY

B1-B7 **Seasonal variation in abiotic factors and toxicity of thymol against the snail *Lymnaea acuminata***
Shefali Srivastava, Pradeep Kumar, V. K. Singh, Dinesh Kumar Singh

B8-B16 **Solid waste management with the help of vermicomposting and its applications in crop improvement**
Nandita Mehta, Arun Karnwal

B17-B23 **Alterations in some metabolic activities of *Scenedesmus quadricauda* and *Merismopedia glauca* in response to glyphosate herbicide**
Ahmed Abd El-Salam Issa, Mahmoud Salama Adam, Mustafa Ahmed Fawzy

B24-B29 **Diversity of tribe Anthemideae (Asteraceae) in flora of Jammu and Kashmir State**
B. L. Bhellum

B30-B41 **Nitrate-drought interactions on growth, osmoprotectants and antioxidant system of the oil accumulating desert plant jojoba**
Fatma Ali Farghaly, Hanaa Kamal Galal, Abeer Ahmed Farag Radi, Refat Abdel-Basset

B42-B53 **Allelopathic effects of some weeds on rhizosphere algae at El-Kharga Oasis (New Valley), Egypt**
Awatief F. Hifney, M. S. Adam, G. Ghareib, A. A. Issa

B54-B64 **Abiotic stress induced production of ß-carotene, allophycocyanin and total lipids in *Spirulina* sp.**
Awatief F. Hifney, Ahmed A. Issa, Mustafa A. Fawzy

B65-B71 **Reproduction of *Lymnaea acuminata* fed to bait containing binary combination of amino acids with molluscicide**
Pradeep Kumar, Vinay Kumar Singh, Dinesh Kumar Singh

B72-B88 **Physiological and biochemical responses of salt-tolerant and salt-sensitive wheat and bean cultivars to salinity**
Abeer Ahmad Radi, Fatma Ali Farghaly, Afaf Mohamed Hamada

B89-B92 **Earthworms: ecofriendly environmental engineers**
Seema Rani, Nitish Bansal, Vineeta Shukla

B93-B99 **Seasonal variation in the toxicity of umbelliferone against *Fasciola* larvae**
Kumari Sunita, Pradeep Kumar, Dinesh Kumar Singh

B100-B109 **Range extension of mugger crocodile *Crocodylus palustris* (Lesson, 1831) in upper Ganges and tributaries, lesser Himalayan zone, north India**
Ritesh Joshi

B110-B119 **Toxicological and biochemical alterations of apigenin extracted from seed of *Thevetia peruviana*, a medicinal plant**
Sunil Kumar Singh, Shailendra K. Singh, Ajay Singh

B120-B128 **Improvement of growth and some metabolites of the salt affected *Anabaena circinalis* by calcium**
Awatief F. Hifney

B129-B134 **Chemical composition and amino acid profile of *Sardinella longiceps* collected from Western coastal areas of Kerala, India**
Sheril Ann Shaji, Hindumathy Chelvaraya Kannan

B135-B143 **Ecophysiological studies on three desert plants growing in Wadi Natash, Eastern Desert, Egypt**
Suzan A. Sayed, Mohamed A. A. Gadallah, Fawzy M. Salama

B144-B153 **Role of antioxidant enzymes in amelioration of water deficit and waterlogging stresses on *Vigna sinensis* plants**
Abdelwahab Elsadek El-Enany, Abdullah D. AL-Anazi, Nahla Dief, Wafa'a A. Al-Taisan

B135-B143 **Effect of papain and piperonyl butoxide/MGK-264 on the reproduction/development of *Lymnaea acuminata***
Farhat Hanif, Dinesh Kumar Singh

MEDICINE

M1-M13 **Współczesna farmakoterapia astmy oskrzelowej. Contemporary pharmacotherapy of asthma**
Joanna Szkaradkiewicz

M14-M20 **Microbiology of chronic periodontitis**
Anna K. Szkaradkiewicz, Tomasz M. Karpiński

M21-M24 **Microbiology of dental caries**
Tomasz M. Karpiński, Anna K. Szkaradkiewicz

M25-M41 **The biological and medical significance of poisonous animals**
Hossam El-Din Mohamed Omar

M42-M47 **Probiotics and prebiotics**
Anna K. Szkaradkiewicz, Tomasz M. Karpiński

EARTH SCIENCES

E1-E11 **Wstępna charakterystyka skał żyłowych występujących w Dolinie Małaja Biełaja w Chibinach. Preliminary characterization of the mineral veins occurring in the Malaya Belaya Valley in the Khibiny**
Miłosz A. Huber

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Seasonal variation in abiotic factors and toxicity of thymol against the snail *Lymnaea acuminata*

Shefali Srivastava, Pradeep Kumar, V. K. Singh, D. K. Singh

Malacology Laboratory, Department of Zoology, DDU Gorakhpur University, Gorakhpur, India

ABSTRACT

Toxicity of thymol against *Lymnaea acuminata* was conducted in each month of the year 2010-2011. The 24, 48, 72 and 96 h LC_{50} values of a molluscicide thymol were determined, with the concomitant estimation of levels of temperature, pH, dissolved oxygen, carbon dioxide and electrical conductivity, both in control and test water. On the basis of a 24h toxicity assay, it was observed that 24h LC_{50} value of 6.41 mg/l in month of May, was most effective in killing the snails, while the thymol was least effective in month of April, when its 24h LC_{50} was 15.25 mg/l. There was a significant positive correlation between LC_{50} of thymol and levels of carbon dioxide/ pH of water in corresponding months. On the contrary, a negative correlation was noted between LC_{50} of thymol and dissolved oxygen/temperature of test water in the same months. In order to confirm that relationship between toxicity and abiotic factors is not coincidental, activity of acetylcholinesterase (AChE), acid phosphatase (ACP) and alkaline phosphatase (ALP) in the nervous tissue of control as well as sublethal thymol (60% of 24h LC_{50}) treated snail, were assayed during each of the 12 months of the same year. A significant positive rank correlation was noted between AChE/ACP/ALP activity and corresponding sublethal treatment of thymol. Maximum inhibition of AChE, ACP and ALP activity was observed in the month of May. This study shows conclusively that the best time to control the *L. acuminata* population with thymol is during the month of May to July.

Key words: Environmental factors; Thymol; Molluscicides; Temperature; pH; Enzymes.

J Biol Earth Sci 2013; 3(1): B1-B7

Corresponding author:
Prof. D. K. Singh
Malacology Laboratory, Department of Zoology,
DDU Gorakhpur University, Gorakhpur,
273009, U.P., India.
Tel: +91-551-2202187(O)/200509(R);
9454211574 (Mobile).
E-mail: dksingh_gpu@yahoo.co.in

Original Submission: 07 October 2012; Revised Submission: 14 December 2012; Accepted: 18 December 2012

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Fasciolosis is a worldwide zoonotic disease [1], caused by the liver flukes *Fasciola hepatica* and *F. gigantica* [2]. Incidence of fasciolosis is very high in eastern part of the state of Uttar Pradesh in India. The Fresh water snail *Lymnaea acuminata* is the intermediate host of *F. gigantica* [3-7]. One way to tackle the problem of fasciolosis is to control the population of vector snails and, their by delink the life cycle of these flukes [8-10]. Plant derived molluscicides are gaining the global acceptance in comparison to synthetic molluscicides. The molluscicides of plant origin are of special importance owing to lower cost, safely and eco-friendliness [5, 10-13]. Earlier studies have shown that thymol is an active molluscicidal component of *Trachyspermum ammi* [14]. It has also been conclusively shown that acetylcholinesterase (AChE), acid phosphatase (ACP) and alkaline phosphatase (ALP) in the nervous tissue of *L. acuminata* are very sensitive enzymes influenced by molluscicides [6, 15-17].

The objective of the present study was to explore the possibility that seasonal changes in abiotic factors, viz. temperature, pH, dissolved oxygen, carbon dioxide and conductivity of the test water can influence the activity of AChE, ACP and ALP assayed in each month of the year (2010-2011) in *L. acuminata*, following exposure to sublethal concentration (60% of 24h LC_{50}) of thymol.

MATERIALS AND METHODS

Test Materials

Thymol (2-isopropyl 5-methyl phenol, IPMB), a natural monoterpene phenol derivative of cymene was obtained from Sigma Chemical Co. U.S.A. Temperature, pH and electrical conductivity of water were measured by thermometer, digital pH and conductivity meters, respectively. Dissolved oxygen and carbon dioxide were estimated according to methods described by APHA [18].

Bioassays for LC_{50}

Adult *L. acuminata* (length 2.30 ± 0.25 cm in length) were collected from Ramgarh lake, located almost adjacent to Gorakhpur University campus. Snails were acclimatized in dechlorinated tap water for 72 h. The snails were exposed to different concentration of thymol in glass aquaria, each containing 3 litres of dechlorinated water. Ten experimental snails were kept in each glass aquarium. Control snails were kept in equal volumes of dechlorinated water under similar conditions. Mortality of snails was observed after 24, 48, 72 and 96h. No response to a needle probe was taken as evidence of death. Dissolved oxygen, carbon dioxide and conductivity, temperature and pH of water were measured simultaneously with toxicity tests, every 24h for 96h. Bioassay for the determination of LC_{50} was performed in each month of the year. Lethal concentration (LC_{50}) values, lower and upper confidence limits (LCL and UCL) and slope values were calculated by the method of POLO computer programme of Robertson et al. [19]. The product moment coefficient was determined between LC_{50} and temperature/pH/conductivity/dissolved oxygen/carbon dioxide of water in each month to observe any significant correlation of thymol against *L. acuminata*. Toxicity of thymol at corresponding LC_{50} of thymol against *L. acuminata* in each month of the year 2010-11 was also conducted against the fish *Colisa fasciatus*.

Enzyme assays

Twenty experimental snails kept in glass aquarium containing 3 litres of dechlorinated water, were exposed to sublethal concentration (60% of 24h LC_{50}) of thymol in each month. Six aquaria were set up for each concentration. After the 24h exposure, the snails were washed with water and the nervous tissue was dissected out from the buccal mass for the measurement of AChE, ACP and ALP activity.

Acetylcholinesterase

Acetylcholinesterase (AChE) activity was measured according to the method of Ellman et al. [20] as modified by Singh et al. [21]. Nervous tissue (50 mg) was homogenized in 1 ml of 0.1M phosphate buffer pH 8 for 5 min in an ice bath and centrifuged at 1000 g for 30 min at 4˚C. The supernatant was used as enzyme source. The change in optical density at 412 nm was recorded for 3 min after every 30 second interval. Enzyme activity was expressed as µ mol 'SH' hydrolysed/min/mg protein.

Phosphatases

Acid (ACP) and alkaline (ALP) phosphatase activity

were measured by the method of Bergmeyer [22] as modified by Singh and Agarwal [23]; p-nitrophenyl phosphate was used as substrate. Tissue homogenate (2% w/v) was prepared in ice-cold 0.9% NaCl and centrifuged (5000 g) for 20 minute at 4ºC. The supernatant was used as an enzyme source. The yellow colour developed due to the formation of p-nitro phenol was determined colorimetrically at 420 nm. The ACP and ALP activity were expressed as µ mol substrate hydrolysed/30min/mg protein.

Protein Estimation

Protein in the enzyme sources were estimated by the method of Lowry et al. [24].

Statistical analysis

Results were expressed as mean ± SE of six replicates. Rank correlations were applied between LC_{50} and corresponding changes in enzyme activity in different months of the year 2010-2011 [25].

RESULTS

There was a significant ($p<0.05$) time dependent variation in the toxicity of thymol against the *L. acuminata* in different months of the year (Table 1); usually highest toxicity was observed in months of May, June and July (24h LC_{50} 6.41-8.75 mg/l) and lowest in the month of April (24h LC_{50} 15.25 mg/l). A significant positive correlation between LC_{50} and water pH/carbon dioxide concentration was noted for each month and at each interval of 24h exposure (Table 1). Contrary, a negative correlation between LC_{50} and dissolved oxygen and water temperature was noted (Table 1). No marked correlation was observed between the LC_{50} and conductivity of water. The slope values were steep and separate estimations of LC_{50}, based on each of the six replicates, were found within the 95% confidence limits of LC_{50}. The t-ratio was greater than 1.96 and the heterogeneity factor was less than 1.0. The g value was less than 0.5 at all probability levels. There was a positive rank correlation between LC_{50} of thymol in different months and corresponding AChE/ACP/ALP activity in the nervous tissue of treated snail. Maximum inhibition in AChE (66.6% of control), ACP (69.9% of control) and ALP (78.72% of control) activity were noted in snails exposed to 60% of 24h LC_{50} of thymol in the month of May (Table 2). There was no mortality in the fish *Colisa fasciatus* exposed to LC_{50} of thymol against snail *L. acuminata* in each month.

DISCUSSION

It is evident from the result section that the toxicity of thymol varies with changes in abiotic factors in water. The temperature of the water is one of the significant abiotic factor which alters the toxicity of thymol during each month of the year. Higher temperature of water in the month of May caused maximal toxicity of thymol, which may be due to the overlap of negative effects. Singh and Singh [26] have also noted that toxicity of cypermethrin against *L. acuminata* was maximum in the month of May. Usually, temperatures between 25-30°C are optimal for the production of cercaria [2]. The production of cercariae in vector snail is a fundamental component of the parasite's overall transmission success [27]. Higher temperature in May to July is suitable for transmission of cercaria [2]. Toxicity of thymol against *L. acuminata* is also higher in these months. Consequently, use of thymol in month of May to July will be more effective in control of fasciolosis. Dissolved oxygen is also a factor that alters the toxicity of thymol. In winter, water holds more O_2 [28, 29] and as a result less mortality of snails occurs during this period. At higher temperature the dissolved oxygen concentration decreases, which is reflected by higher mortality of the snails. Dissolved oxygen is one of the major components required by snails during metabolic activity [30, 31]. At higher temperature, increasing rate of metabolism may release more CO_2, which affects the pH of water [32, 33]. The high concentration of carbon dioxide in water may synergize the lethality of molluscicide.

Thymol based commercial insecticides are used in control of *Varrora destructor* (Acari: Mesostigmata) in honey bee colony [34]. Earlier, it has been reported that thymol is toxic against the tick larvae *Rhipicephalus* (*Boophilus*) *microplus* [35] and cutworms, *Spodoptera litura* (Lepidopteran: Noctuidae) [36]. Thymol has also showed promising results when evaluated for larvicidal, oviposition deterrent, vapour toxicity and repellant activity against malarial vector, *Anopheles stephensi* [37].

Thymol adversely affect the both cholinergic and monoaminergic neuron [38]. Butcher [39] has demonstrated that in rat acetylcholine facilitates the release of dopamine which subsequently act as a

Table 1. Alterations in toxicity (LC_{50} mg/l) of thymol against *L. acuminata* and abiotic factors in pond water in different month of the year 2010-2011.

Parameters	MAY	JUNE	JULY	AUGUST	SEPTEMBER	OCTOBER	NOVEMBER	DECEMBER	JANUARY	FEBRUARY	MARCH	APRIL
24h												
LC_{50} (mg/l)	6.41	6.73	8.75	13.07	13.40	12.47	10.07	14.60	13.73	13.14	14.44	15.25
Temperature (C) *	37±0.51	32±0.30	32±0.30	31±0.21	31±0.16	31±0.22	25±0.16	18±0.47	16±0.16	21±0.16	33±0.33	34±0.16
pH^+	7.11±0.03	7.52±0.02	7.81±0.01	7.91±0.01	7.84±0.01	8.30±0.01	7.54±0.08	9.71±0.02	9.71±0.01	7.79±0.05	7.95±0.05	7.96±0.01
DO (ppm) *	1.2±0.04	2.0±0.07	1.2±0.03	1.9±0.04	2.2±0.05	1.9±0.04	2.1±0.06	1.2.8±0.06	2.6±0.09	4.4±0.01	1.3±0.06	2.0±0.06
DCO_2 (ppm) +	20.8±0.31	15.5±0.04	18.2±0.85	22.7±0.05	20.5±0.04	17.6±0.05	13.8±0.07	27.8±0.63	18.8±0.04	20.1±0.03	20.0±0. 08	13.8±0.47
CONDUCTIVITY (µmhos/cm)	39.4±0.05	31.5±0.17	40±0.00	45.2±0.5	43.7±0.5	28.2±0.09	27.9±0.03	31.3±0.07	32.5±0.18	34.3±0.05	38.0±0.01	36.1±50.07
48h												
LC_{50} (mg/l)	5.49	3.38	2.60	11.06	7.84	4.81	5.60	12.49	8.35	8.76	12.90	13.09
Temperature (C) *	36±0.05	33±0.30	37±0.55	32±0.21	33±0.17	31±0.22	25±0.11	17±0.65	16±0.04	21.1±0.16	33±0.23	34±0.9
pH^+	7.13±0.03	7.66±0.03	7.24±0.3	7.58±0.05	7.76±0.04	8.31±0.04	7.83±0.09	9.93±0.01	9.91±0.05	9.63±0.04	8.01±0.08	7.52±0.00
DO (ppm) *	1.2±0.04	1.3±0.07	0.8±0.04	1.3±0.05	2.1±0.05	1.2±0.03	1.0±0.07	1.0±0.05	1.9±0.50	4.2±0.01	1.3±0.06	1.9±0.04
DCO_2 (ppm) +	22.1±0.31	20.5±0.04	19±0.03	24.8±0.04	22.6±0.03	20.1±0.52	15.9±0.009	32.0±0.04	23.7±0.04	24.1±0.04	22±0.08	16.0±0.57
CONDUCTIVITY (µmhos/cm)	40.5±0.06	32.5±0.16	41±0.06	46.2±0.06	43.7±0.5	28.9±0.09	28.8±0.03	32.3±0.07	35.6±0.18	34.3±0.05	38.2±0.01	34.2±0.3
72h												
LC_{50} (mg/l)	3.17	1.04	1.41	8.99	3.03	1.90	2.82	11.33	5.64	3.37	11.76	10.76
Temperature (C) *	36±0.01	33±0.30	36.9±0.09	32±0.57	33±0.18	32±0.27	24±0.12	17±0.65	16±0.06	21.1±0.16	32±0.23	34±0.00
pH^+	7.33±0.03	7.73±0.03	7.23±0.5	7.63±0.08	7.54±0.03	8.27±0.04	7.83±0.03	9.01±0.02	9.03±0.04	9.61±0.04	8.23±0.01	7.56±0.02
DO (ppm) *	1.2±0.04	0.8±0.06	0.5±0.03	1.0±0.56	1.9±0.03	1.0±0.02	0.8±0.08	0.9±0.08	1.7±0.50	3.8±0.01	1.3±0.01	1.6±0.05
DCO_2 (ppm) +	24±0.31	22.3±0.04	20.3±0.09	26. 9±0.45	25.8±0.03	21.1±0.73	22±0.08	31.0±0.04	24.8±0.04	25.1±0.04	25±0.63	20±0.57
CONDUCTIVITY (µmhos/cm)	41.5±0.07	33.5±0.16	42.6±0.02	47.6±0.5	43.7±0.04	30.6±0.10	28.8±0.03	32.3±0.07	34.6±0.18	35.2±0.01	30.1±0.02	35.3±0.03
96h												
LC_{50} (mg/l)	2.00	0.63	0.91	6.92	2.11	1.11	1.22	10.63	4.39	1.74	10.93	8.65
Temperature (C) *	37±0.02	33.0±0.30	36±0.08	32±0.57	33±0.13	33±0.33	24±0.16	17±0.77	16±0.06	21.1±0.18	33±0.23	34±0.00
pH^+	7.46±0.04	7.89±0.04	7.58±0.87	7.93±0.04	7.17±0.03	8.03±0.02	7.93±0.01	8.89±0.07	9.04±0.04	9.14±0.03	8.16±0.01	7.73±0.60
DO (ppm) *	0.8±0.11	0.3±0.06	0.4±0.89	0.6±0.45	1.4±0.02	0.6±0.02	0.6±0.01	0.6±0.86	1.4±0.50	3.2±0.02	1.2±0.53	1.3s±0.06
DCO_2 (ppm) +	26±0.32	23.9±0.05	21.3±60.25	28±0.45	26.6±0.03	22.2±0.22	24±0.09	31.0±0.05	25.8±0.04	28.2±0.04	28.3±0.03	22±0.66
CONDUCTIVITY (µmhos/cm)	42.5±0.07	33.5±0.17	42±0.00	48.9±0.8	43.7±0.04	30.9±0.10	29.1±0.03	32.3±0.07	34.5±0.17	36.1±0.01	30.0±0.08	35.4±0.03

Each experiment was replicated six times and values are the Mean±SE of six replications. Temperature, pH, dissolved oxygen, free carbon dioxide and conductivity were measured at intervals of 24h upto 96h.a- Significant ($P<0.05$). When Two way ANOVA was applied in between the LC50 value of different month and time i.e. 24, 48, 72 and 96h. Product moment correlation coefficient in between the LC50 and different parameters indicate significance ($P<0.05$) positive (+) and negative (*) correlation.

Table 2. Effect of 24h exposure of 60% of 24h LC_{50} of thymol in different months of the year 2010-2011 on acetylcholinesterase, alkaline and acid phosphatase activity in the nervous tissue of *L. acuminata*.

MONTHS	24h LC_{50} mg/l	AChE -µ moles 'SH' hydrolyzed /min/mg protein		ACP-µ moles substrate /30min/mg protein		ALP-µ moles substrate /30min/mg protein	
		Control[a,+]	60%of 24h LC_{50}	Control[a,+]	60%of 24h LC_{50}	Control[a,+]	60%of 24h LC_{50}
May	6.41	0.048±0.03 (100)	0.032±0.006[+] (66.66)	2.73±0.007 (100)	1.91±0.44[+] (69.96)	0.47±0.01 (100)	0.37±0.07[+] (78.72)
June	6.73	0.074±0.00 (100)	0.050±0.01[+] (67.56)	1.71±0.06 (100)	1.47±0.08[+] (85.96)	3.00±0.02 (100)	2.50±0.05[+] (83.33)
July	8.75	0.089±0.02 (100)	0.068±0.07[+] (76.40)	3.88±0.01 (100)	2.76±0.02[+] (71.13)	4.14±0.01 (100)	3.70±0.07[+] (89.37)
August	13.07	0.054±0.02 (100)	0.040±0.00[+] (74.07)	4.28±0.03 (100)	3.11±0.05[+] (72.64)	3.49±0.03 (100)	3.01±0.09[+] (86.24)
September	13.40	0.139±0.03 (100)	0.102±0.02[+] (73.38)	3.84±0.002 (100)	2.82±0.009[+] (73.43)	2.67±0.01 (100)	2.27±0.02[+] (85.01)
October	12.47	0.208±0.03 (100)	0.150±0.03[+] (72.11)	2.33±0.08 (100)	2.22±0.09[+] (95.27)	4.49±0.02 (100)	3.78±0.07[+] (84.18)
November	10.07	0.146±0.03 (100)	0.121±0.00[+] (82.87)	3.45±0.08 (100)	3.09±0.08[+] (89.56)	4.17±0.00 (100)	3.76±0.02[+] (90.16)
December	14.60	0.128±0.01 (100)	0.114±0.03[+] (89.06)	3.03±0.006 (100)	2.99±0.09[+] (98.67)	3.94±0.02 (100)	3.36±0.02[+] (85.28)
January	13.73	0.194±0.01 (100)	0.174±0.00[+] (89.69)	2.71±0.08 (100)	2.58±0.02[+] (95.20)	3.59±0.01 (100)	2.97±0.06[+] (82.72)[+]
February	13.14	0.173±0.02 (100)	0.153±0.00[+] (88.43)	2.91±0.02 (100)	2.77±0.01[+] (95.39)	4.45±0.02 (100)	3.68±0.05 (82.76)
March	14.44	0.080±0.01 (100)	0.060±0.02[+] (75.00)	4.87±0.02 (100)	3.81±0.11[+] (78.23)	3.73±0.02 (100)	3.32±0.00[+] (89.00)
April	15.25	0.076±0.01 (100)	0.053±0.02[+] (69.73)	3.54±0.08 (100)	2.99±0.02[+] (84.46)	2.67±0.02 (100)	2.37±0.00[+] (88.76)

Values are mean ± S.E of six replicates. Value in parenthesis indicates % enzyme activity with untreated control taken as 100%.Rank correlation coefficient in between LC_{50} and AChE/ALP/ACP activity in treated group indicate significant ($p<0.05$) positive (+)correlation a, significant ($p<0.05$) when one way ANOVA was applied in between the enzymatic activity in different month of the year in control as well as treated group.

neurotransmitter. These findings raise the possibility that continued cholinergic stimulus caused by reduction in AChE activity might caused increased release of monoamine in the neurons. Alternatively, it is possible that it may affect the cholinergic, tryptaminergic and dopaminergic neurons independently [38]. However, data regarding in interaction between cholinergic and other kind of neurons in snails are not available. Earlier it has been reported that sublethal treatment 80% of 24h LC_{50} of thymol caused significant inhibition of AChE activity (81.01% of control) in nervous tissue of *L. acuminata* [38]. The high anti AChE, activity of thymol was observed in month of May.

ACP plays an important role in catabolism, pathological necrosis, autolysis and phagocytosis [40, 41]. Alkaline phosphatase (ALP) plays a critical role in protein synthesis [42] and shell formation [43]. The rank correlation coefficient applied between the LC_{50} values of different months and the corresponding inhibition of enzyme activity indicates a positive correlation between the LC_{50} and the inhibition of AChE, ALP and ACP. Besides its anticholinergic effect, thymol also inhibits ALP and ACP activity.

The precise prediction of consequences of molluscicidal toxicity in the water medium against the molluscs are interrupted due to shortage of appropriate information that how abiotic factors in aqueous medium influence the molluscicidal activity. The present study clearly demonstrates that variant abiotic factors can significantly alter the

toxicity of thymol. It is also obvious that most suitable period for the control of *L. acuminata* in India is during the months of May to July. It is suggested that treatment of thymol for control of *L. acuminata* and ultimately fasciolosis, is not only cost effective during these months than spending more money in rest the months of the year. Use of thymol as molluscicide needs a proper study regarding the abiotic factors of water body at the time of treatments. Adequate monitoring, its release in aquatic medium will be more useful in control of vector snail in any part of the world. No mortality in fish *C. fasciatus* exposed to LC_{50} of thymol against *L. acuminata* in each month clearly indicate that concentrations used to kill the snail is safe for the fish, which is showing the same habitat with snails.

CONCLUSION

The present study shows conclusively that variant abiotic in the aquatic habitat can significantly alter the toxicity of thymol. Most suitable period to control the snail in India is during the months of May to July. It is suggested that the treatment of the water body with thymol for snail control and ultimately fasciolosis is more potent and cost effective during May to July, rather than spending the more thymol in rest months of the year. Release of thymol in the aquatic medium is safe to other animals showing the same habitat.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. WHO. 2006. Report of the WHO informal meeting on use of triclabendazole in fasciolosis control. 17-18 October, Geneva, Switzerland.
2. Mas-Coma S, Valero MA, Bargues MD. Climate change effects on trematodiasis with emphasis on zoonotic fasciolosis and schistosomiasis. Vet Parasitol. 2009; 163(4): 264-280.
3. Singh O, Agarwal RA. Toxicity of certain pesticides to two economic species of snails in northern India. J Econ Entomol. 1981; 74(5): 568-571.
4. Agarwal RA, Singh DK. Harmful gastropods and their control. Acta Hydrochim Hydrobiol. 1988; 16(2); 113-138.
5. Upadhyay A, Singh DK. Molluscicidal activity of *Sapindus mukorossi* and *Terminalia chebula* against the fresh water snail *Lymnaea acuminata*. Chemosphere. 2011; 83: 468-474.
6. Kumar P, Singh VK, Singh DK. Enzyme activity in the nervous tissue of *Lymnaea acuminata* fed to different bait formulations. Am J Chem. 2012; 2(2): 89-93.
7. Sunita K, Kumar P, Singh DK. Abiotic environmental factors and infection of *Fasciola gigantica* in vector snail *Lymnaea acuminata*. Researcher. 2012; 4(8): 49-53.
8. Singh P, Singh VK, Singh DK. Effect of binary combination of some plant-derived molluscicides with MGK-264 or piperonyl butoxide on reproduction of snail *Lymnaea acuminata*. Pest Management Sci. 2005; 61: 204-208.
9. Kumar P, Singh DK. Molluscicidal activity *Ferula asafoedita*, *Syzygium aromaticum* and *Carum carvi* and their active components against the snail *Lymnaea acuminata*. Chemosphere. 2006; 63: 1568-1574.
10. Marston A, Hostettmann K. Plant molluscicides. Phytochemistry. 1985; 24: 639–652.
11. Singh A, Singh DK, Misra TN, Agarwal RA. Molluscicides of plant origin. Biol Agric Hortic. 1996; 13: 205-252.
12. Singh A, Singh VK. Molluscicidal activity of *Saraca asoca* and *Thuja orientalis* against the fresh water snail *Lymnaea acuminata*. Vet Parasitol. 2009b; 164: 206-210.
13. Singh KL, Singh DK, Singh VK. Toxicity of *Bauhinia variegata* and *Mimusops elengi* with plant molluscicides against *Lymnaea acuminata*. J Biol Earth Sci. 2012; 2(2); B76-B82.
14. Singh S, Singh VK, Singh DK. Molluscicidal activity of some common spice plants, Biol Agric Hortic. 1997; 14(3): 237-249.
15. Tripathi SM, Singh VK, Singh S, Singh DK. Enzyme inhibition by molluscicidal agent *Punica granatum* Linn. bark and *Canna indica* Linn. root. Phyther Res.

2004; 18: 501-506.
16. Jaiswal P, Singh VK, Singh DK. Enzyme inhibition by molluscicidal component of *Areca catechu* and *Carica papaya* in the nervous tissue of vector snail *Lymnaea acuminata*. Pestic Biochem Physiol. 2008; 92: 164-168.
17. Kumar P, Singh VK, Singh DK. Kinetics of enzyme inhibition by active molluscicides against ferulic acid, umbelliferone, eugenol and limonene in the nervous tissue of snail *Lymnaea acuminata*. Phyther Res. 2009; 23: 172-177.
18. APHA. 2005. Standard methods for the examination of water, sewage water and industrial waste. 21st Edition, Washington, D. C.
19. Robertson JL, Russell RM, Priesler HK, Savin NE. 2007. Bioassay with arthropods POLO computer program for analysis of bioassay data. 2nd Edition, Talor and Francis, CRC Press. pp: 1-224.
20. Ellman GL, Courteny KD, Andre V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961; 7: 88-95.
21. Singh DK, Singh O, Agarwal RA. Comparative study of cholinesterase in two snails. *Pila globosa* and *Lymnaea acuminata*. J Physiol. 1982; 78: 467-472.
22. Bergmeyer UH. 1967. Methods of enzymatic analysis. Academic Press, New York, pp: 1129.
23. Singh DK, Agarwal RA. Toxicity of piperonyl butoxide carbaryl synergism on the snail *Lymnaea acuminata*. Int Revue gesamten Hydrobiol Hydrographie. 1989; 74: 689-699.
24. Lowry OH, Rosebough NJ, Farr AL, Randall RJ. Protein measurement with folin phenol reagent. J Biol Chem. 1951; 193: 265-275.
25. Sokal RR, Rohlf FJ. Introduction to Biostatistics. W. H. Freeman & CO., Ltd., San Francisco. 1973; 271-273.
26. Singh V, Singh DK. The effect of abiotic factors on the toxicity of cypermethrin against the snail *Lymnaea acuminata* in the control of fasciolosis. J Helminthol. 2009a; 83: 39-45.
27. Poulin R. Global warming and temperature-mediated increases in cercarial emergence in trematode parasite. Parasitol. 2006; 132: 143-151.
28. Ingram BA, Hawking JH, Shiel RJ. 1997. Aquatic life in fresh water ponds, A Guide to identification and ecology of life in aquaculture ponds and farm dams in south eastern Australia. Co-operative research center for fresh water ecology, Albury, NSW, Australia.
29. Waterwatch Australia. 2002. National Technical Manual, Module 4: Physical and Chemical Parameters Waterwatch Australia Steering Committee Environment Australia.
30. Ishak MM, Mohammad AM. Effect of sublethal doses of copper sulphate and bayluscide on survival and oxygen consumption of the snail *Biomphalaria alexandrina*. Hydrobiol. 1975; 47: 449-512.
31. Watten BJ. 2004. Method and apparatus for control of aquatic vertebrates invasive species. US Patent No. 6821442, 23 November 2004.
32. Toews KL, Shroll RM, Wai CM, Smart NG. pH-Defining equilibrium between water and supercritical CO^{2-} influence on SFE of organics and metal chelate. Anal Chem. 1995; 67: 4040-4043.
33. Berge JA, Bjerkeng B, Petterson O, Schaanning MT, Oxnevad S. Effects of increased sea water concentrations of CO_2 on growth of the bivalve *Mytilus edulis* L. Chemo. 2006; 62: 681-687.
34. Adamczyk S, Lazaro R, Perez-arquillue C, Corchello P, Herrerce A. Evaluation of residues of essential oil components in honey after different anti-Varroa treatments. J Agric Food Chem. 2005; 53(26): 10085-10090.
35. Novelino MAS, Daemon E, Soares GLG. Evaluation of the acaricide effect of thymol, menthol, salicylic acid, and methyl salicylate on *Boophilus microplus* (Canestrini, 1887) (Acari : Ixodidae) larvae. Parasitol Res. 2007; 101(3): 809-811.
36. Hummelbrunner LA, Isman MB. Acute, sublethal, antifeedant, and synergistic effects of monoterpenoid essential oil compounds on tobacco cutworms, *Spodoptera litura* (Lep., Noctuidae). J Agric Food Chem. 2001; 49: 715-720.
37. Pandey SK, Upadhyay S, Tripathi AK. Insecticidal and repellent activities of thymol from the essential oil of *Trachyspermum ammi* (Linn) Sprague seeds against *Anopheles stephensi*. Parasitol Res. 2009; 105: 507-512.
38. Singh S, Singh VK, Singh S, Singh DK. Effect of active molluscicidal component of spices on different enzyme activities and biogenic amines levels in the nervous tissue of *Lymnaea acuminata*. Phyther Res. 1999; 13: 649-654.
39. Butcher LL. Nature and mechanism of cholinergic monoaminergic interaction in the brain. Life Sci. 1997; 21: 1207-1226.
40. Abou-Donia MB. Increased acid phosphatase activity in hens following oral dose of leptophos. Toxicol Lett. 1978; 2: 199-203.
41. Aruna P, Chetty CS, Naidu RC, Swami KS. Acid phosphatase activity in Indian apple snail *Pila globosa* (Swainson) during aestivation and starvation stress. Proc Indian Acad Sci (India). 1979; 88: 363-365.
42. Pilo B, Asnani MB, Shah RV. Studies on wound healing and repair in pigeon liver. Histochemical studies on acid and alkaline phosphatase activity during the process. J Ani Morphy. 1972; 19: 205-212.
43. Timmermans LPM. Studies on shell formation in mollusks. J Zool. 1969; 19: 17-36.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Solid waste management with the help of vermicomposting and its applications in crop improvement

Nandita Mehta, Arun Karnwal

Bhojia Institute of Life Sciences, Budh, Teh. Baddi, Solan, India

ABSTRACT

Management of solid waste has become one of the biggest problems that we are facing today. Vermicomposting is the better option to tackle with this problem. Vermicomposting is the process of conversion of organic wastes by earthworms to valuable humus like material which is used as a natural soil conditioner. Vermicomposting is environment friendly and cost effective technique for solid waste management. Vermicomposting serves two main purposes for the welfare of humans as it helps in the degradation of solid waste and the cast produced during this process is used as a natural fertilizer. Vermicompost is much better than chemical fertilizer because it is not associated with any kind of risk. Earthworms are potentially important creatures that are capable of transforming garbage into gold. *Eisenia fetida* is the most commonly used species of earthworms for vermicomposting. Vermicomposting is a mesophilic process and should be maintained up to 32°C with the moisture content of 60-80%. Earthworms break down organic matter and leave behind castings that are an exceptionally valuable fertilizer. Vermicomposting has many applications in crop improvement such as pathogen destruction, water holding capacity of soil, improved crop growth and yield, improved soil physical, chemical and biological properties and production of plant growth regulators.

Key words: Vermicomposting; *Eisenia fetida*; Solid waste; Vermicompost; *Vinca rosea*; Sadabahar.

J Biol Earth Sci 2013; 3(1): B8-B16

Corresponding author:
Arun Karnwal
Bhojia Institute of Life Sciences, Budh, Teh. Baddi,
Solan, H.P. India, 173205
Ph. +911795247621, Fax: +911795247621
Email: arunkarnwal@gmail.com

Original Submission: 16 November 2012; Revised Submission: 17 December 2012; Accepted: 27 December 2012

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Solid waste is defined as the unwanted organic and inorganic waste materials disposed of by humans from different sources. The living standard of people has increased across the world due to the rapid economic development which directly leads into more material consumption and more waste generation.

Vermicomposting is a simple biotechnological process of composting in which certain species of earthworms are used to convert the biodegradable solid wastes or organic wastes into a nutrient-rich end product, vermicompost, which is used as a soil conditioner. At the present time, vermicomposting is the better option of solid waste management than all the other options because it is a biological process, it is not associated with any kind of soil or groundwater pollution, no risk of gas leakage, cheaper method, less land requirements, resulting earthworm castings (worm manure) are rich in microbial activity and plant growth regulators, and fortified with pest repellence attributes as well. During vermicomposting, the important plant nutrients such as N, P, K and Ca are converted to more soluble forms which are easily utilized by plants. Wastes are the misplaced valuable resources, which can be utilized by proper composting. The composted waste, the vertices which has higher qualities of manure can be used to feed our “Nutrient-organic matter-hungry” soils [1].

There are an estimated 1800 species of earthworm worldwide [2]. But the most commonly used is *Eisenia fetida*, commonly known as the “compost worm”, “manure worm”, “red worm”, and “red wiggler”. Earthworms derive their nourishment from the microorganisms that grow upon organic materials, only few microorganisms support earthworms while others can cause toxic effects [3-5]. Microbial groups that are of nutritive value to earthworms are fungi, prototozoa, algae, bacteria and actinomycetes, in their decreasing order of importance [6]. Retention time of the waste in earthworms is short. Worms can digest several times than their own weight each day and large quantities of excreta are passed out through an average population of earthworms. Amount of substrate consumed depends upon substrate properties and environmental conditions [7]. Vermicomposting systems should be maintained at temperatures of 15 to 25°C and moisture content must be 60 to 80%. Earthworms are active and consume organic materials at a relatively narrow layer of 15 to 25 cm below the surface of compost bed.

MATERIALS AND METHODS

Preparation of Vermibeds

Four different types of vermibeds were prepared by adding different types of wastes with soil in four plastic tubs (Table 1). Pre-composting was done for about one week and after that earthworms were introduced into the bed. To 5 kg of substrate and soil mixture about 50 healthy adults were added.

Maintenance of Moisture Content

Moisture content was maintained in all the vermibeds by sprinkling enough amount of water at regular intervals.

Collection of Samples

Vermicomposting was done for seven weeks and samples were collected every week from all the vermibeds and analysed for their nutrient composition.

Soil and Sample Analysis

Samples were analysed for different nutrients. pH and moisture content were estimated for different samples. Moisture content was estimated by the difference between the initial and final weight (w1 – w2) of soil as mg of moisture/g soil.

Table 1. Composition of different Vermibeds

Vermibeds	Substrate	Total amount of soil and substrate mixture (in kg)	Number of adult earthworms added
First	Food Waste	5	50
Second	Agriculture waste	5	50
Third	Medical Waste	5	50
Fourth	Industrial Waste	5	50

Inorganic Phosphate content

Inorganic phosphate was determined by Ammonium molybdate reduction method and absorbance was noted in spectrophotometer at 690 nm.

Total organic content

Organic content was estimated by titration against ferrous ammonium sulphate. The end point was noted as dull green through turbid blue to brilliant green.

Chloride content

Chloride content was estimated by silver nitrate titration method. End point was noted as persistent red tinge.

Organic carbon

Organic carbon was estimated by modified Walkly-black procedure. Initially the colour was dark brown (colour depend on organic matter content of the sample). Then the solution colour changes to greenish and then changes to dark green or greenish blue. At the end point it flashes quickly from greenish blue to reddish brown.

Sulphate Content

Sulphate content was determined by turbidimetric method using barium chloride crystals. Absorbance was noted at 420 nm in spectrophotometer.

Calcium Carbonate Content

Calcium carbonate was estimated by titrimetric method. End point was noted as faint pink colour against NaOH solution.

Effect of vermicompost of different substrates on plant

Soil was added in five flower pots and pots were labeled as SS for control containing only soil, FW for soil mixed with vermicompost containing food waste as substrate, AW for soil mixed with vermicompost containing agriculture waste as substrate, MW for soil mixed with vermicompost containing medical waste as substrate, IW for soil mixed with vermicompost containing industrial waste as substrate. Small, young plants (about 3-4 cm) of *Vinca rosea* (common name – Prewinkle or Sadabahar) were planted in different pots and their initial features such as plant height, numbers of leaves were noted down (Table 2). Vermicompost of different substrates was sprinkled in respective pots.

Table 2. Initial Observation of Plants

Flower pots	Plant height (in cm)	Number of leaves
SS	4.7	12
FW	4	12
AW	3	10
MW	3.5	10
IW	3	8

RESULTS

Different samples were collected weekly for seven weeks from all the vermibeds and analysed for their nutrient composition. These samples were labeled as:

S0 – Sample collected after pre-incubation
S1 – Sample collected after one week of treatment
S2 – Sample collected after two weeks of treatment
S3 – Sample collected after three weeks of treatment
S4 – Sample collected after four weeks of treatment
S5 – Sample collected after five weeks of treatment
S6 – Sample collected after six weeks of treatment
S7 – Sample collected after seven weeks of treatment.

For soil nutrient analysis various parameters such as pH, moisture content, inorganic phosphate, total organic content, carbon, chloride, sulphate and calcium carbonate were analysed by different chemical tests to determine the nutrient composition of untreated soil sample and vermicompost samples of different wastes.

pH

Minimum pH of 6.9 was observed in simple soil. Among the various types of wastes maximum pH of 8.04 was observed in agriculture and industrial waste and of the various samples of different wastes maximum pH was observed in S7 (samples collected after 7th week of treatment).

Moisture Content

Moisture content was calculated as mg/g soil and minimum moisture content of 0.25 mg/g soil was observed in simple soil. In different vermicompost samples moisture content was increased to 60% which is the minimum moisture necessary for by earthworms. Earthworms help in retaining moisture.

Table 3. pH results of vermicompost samples of different wastes

Waste Materials	pH at different Sampling points							
	S0	S1	S2	S3	S4	S5	S6	S7
Food Waste	6.95	7.17	7.28	7.32	7.41	7.52	7.78	7.98
Agriculture Waste	7.08	7.21	7.23	7.32	7.43	7.51	7.84	8.04
Medical Waste	6.99	7.19	7.31	7.41	7.54	7.56	7.85	7.99
Industrial Waste	7.01	7.09	7.10	7.27	7.31	7.38	7.46	8.04

Table 4. Moisture content of different vermicompost samples of different wastes

Waste Materials	Moisture content (mg/g soil) at different Sampling points							
	S0	S1	S2	S3	S4	S5	S6	S7
Food waste	0.73	0.84	0.91	0.94	0.67	0.61	0.60	0.68
Agriculture waste	0.67	1.08	1.405	1.405	0.64	1.065	1.27	1.25
Medical waste	0.48	0.85	0.72	0.74	0.74	0.71	0.81	0.97
Industrial waste	0.73	0.59	0.74	0.68	0.63	0.71	0.75	0.72

Inorganic Phosphate Content

Inorganic content was calculated in mg/l and minimum inorganic content of 0.0004 mg/l was observed in simple soil. Inorganic phosphate content was increased gradually increased during earthworm's action. Among the different types of wastes maximum content of inorganic phosphate was observed in food waste. Of the different vermicompost samples of different wastes maximum content of inorganic phosphate was observed in S7 (sample collected after seventh week treatment).

Total Organic Content

Total organic content was calculated in mg/g soil. Simple soil contains only 66.55 mg/g soil of organic matter. Organic content was increased during processing by earthworms. Among the different types of wastes maximum organic content was determined in food waste followed by agriculture waste, medical waste and industrial waste. Of the different samples maximum organic content was determined in S7 (sample collected after seventh week).

Chloride Content

Chloride content was determined in mg/100 g soil. Simple soil resulted in minimum chloride content of 68.16 mg/100g soil. Chloride content increased during treatment by earthworms. Among the different types of wastes maximum chloride content was determined in food waste followed by

Table 5. Inorganic phosphate content (mg/l) of different vermicompost samples of different wastes

Waste Materials	Inorganic phosphate content (mg/l) at different Sampling points							
	S0	S1	S2	S3	S4	S5	S6	S7
Food waste	0.0009	0.001	0.00195	0.002	0.0043	0.0049	0.0075	0.0080
Agriculture waste	0.0008	0.00094	0.00169	0.00199	0.0042	0.0046	0.0064	0.0070
Medical waste	0.0008	0.00084	0.00153	0.0039	0.0047	0.0049	0.0052	0.0053
Industrial waste	0.00081	0.0009	0.00189	0.0044	0.0046	0.0048	0.0049	0.0052

Table 6. Total Organic content (mg/g soil) of different vermicompost samples of different wastes

Waste Materials	Total Organic content (mg/g soil) at different sampling points							
	S0	S1	S2	S3	S4	S5	S6	S7
Food waste	66.55	68.02	73.41	78.78	82.46	87.20	88.94	90.55
Agriculture waste	66.70	69.46	71.71	79.32	82.23	86.32	86.55	89.64
Medical waste	67.93	70.48	70.69	74.55	77.46	80.64	82.59	88.48
Industrial waste	67.10	70.32	72.85	74.43	76.87	79.10	81.22	82.37

Table 7. Chloride content (mg/100g soil) of different vermicompost samples of different wastes

Waste Materials	Chloride content (mg/100g soil) at different sampling points							
	S0	S1	S2	S3	S4	S5	S6	S7
Food waste	69.45	71	74	79.3	82.9	85.2	87.20	92.3
Agriculture waste	69.24	71	71.95	74.42	78.1	78.56	79.24	81
Medical waste	68.16	71.24	71.55	72.6	72.98	74.7	76.49	78.1
Industrial waste	69.10	70.96	71.54	72.44	72.68	74.95	76.10	78.1

agriculture waste, medical waste and industrial waste. Of the different samples maximum chloride content was determined in S7 (sample collected after seventh week).

Organic Carbon by Wet Digestion

Organic carbon was calculated as %C. Simple soil contains only 3.07% of organic carbon. Content of organic carbon was increased during processing by earthworms. Among the different types of wastes maximum percentage of organic carbon was determined in food waste followed by industrial waste, agriculture waste and medical waste. Of the different samples maximum organic content was determined in S7 (sample collected after seventh week).

Calcium Carbonate Content

Calcium carbonate was calculated as percentage of $CaCO_3$. Calcium carbonate content was less in simple soil only 3%. Calcium carbonate content varies with pH of the soil. Alkaline soils contains large amount of $CaCO_3$ compared to acidic soils. Among the different types of wastes CaCO3 content was higher in industrial waste followed by agriculture waste, food waste and medical waste. Of the various samples $CaCO_3$ content was higher in S7 (sample collected after seventh week) of all the wastes.

Sulphate Content

Sulphate content was measured as mg/100g soil. Sulphate content was very less in simple soil only

Table 8. Organic carbon content (%) of different vermicompost samples of different wastes

Waste Materials	Organic Carbon content (%C) at different sampling points							
	S0	S1	S2	S3	S4	S5	S6	S7
Food waste	3.33	3.33	3.56	4.74	4.87	5.90	9.23	16.41
Agriculture waste	3.33	3.56	4.10	4.36	4.65	5.13	5.64	7.43
Medical waste	3.08	3.31	4.10	4.28	4.79	5.08	5.64	6.92
Industrial waste	3.28	3.51	3.86	4.69	4.79	5.36	5.64	7.59

Table 9. Calcium carbonate content (mg/100g soil) of different vermicompost samples of different wastes

Waste Materials	Calcium Carbonate content (%$CaCO_3$) at different sampling points							
	S0	S1	S2	S3	S4	S5	S6	S7
Food waste	3.5	5	6	6.5	7	7.9	8.7	9.5
Agriculture waste	3	4.9	5.5	6.5	7	8.1	9.2	11
Medical waste	4	5	6	6.25	7.5	7.7	8	10
Industrial waste	4	4.5	5	6	7.5	8.9	9.7	11.5

Table 10. Sulphate content (mg/100g soil) of different vermicompost samples of different wastes

Waste Materials	Sulphate content (mg/100g soil) at different sampling points							
	S0	S1	S2	S3	S4	S5	S6	S7
Food waste	3.39	4.575	6.355	7.29	7.542	8.31	9.152	18.73
Agriculture waste	3.305	3.815	4.575	5.594	5.762	6.27	8.66	16.66
Medical waste	3.34	4.675	5.254	5.34	6.44	7.035	7.46	12.71
Industrial waste	3.712	3.85	3.932	3.712	5	5.085	5.677	5.93

3.25mg/100g soil and it increases gradually during processing by earthworms. Among the different types of wastes sulphate content was higher in food waste followed by agriculture waste, medical waste and industrial waste. Of the different samples sulphate content was higher in S7 (sample collected after seventh week of treatment).

Among all the different substrates food waste was best utilized by earthworms in seven weeks period. Food waste vermicompost gives best results with plants and contains higher amounts of nutrients as compared to other types of wastes (Figure 1).

Fig. 1. Vermicompost tubs after seven weeks

Use of Vermicomposting in Crop Improvement

Vermicompost provides sufficient nutrients to the plants and help them to grow faster. Among the various types of wastes best results was observed with the food waste vermicompost. After seven weeks of treatment food waste was fully utilized by earthworms. Food waste vermicompost contains higher amounts of nutrients. A simple comparison was made between simple soil and vermicompost of different waste materials by growing small plants of Sadabahar (*Catharanthus roseus*) in different pots with different vermicompost. After 32 days of observation plant grown in food waste vermicompost was most nourished with much increased plant height (Figure 2), increased number

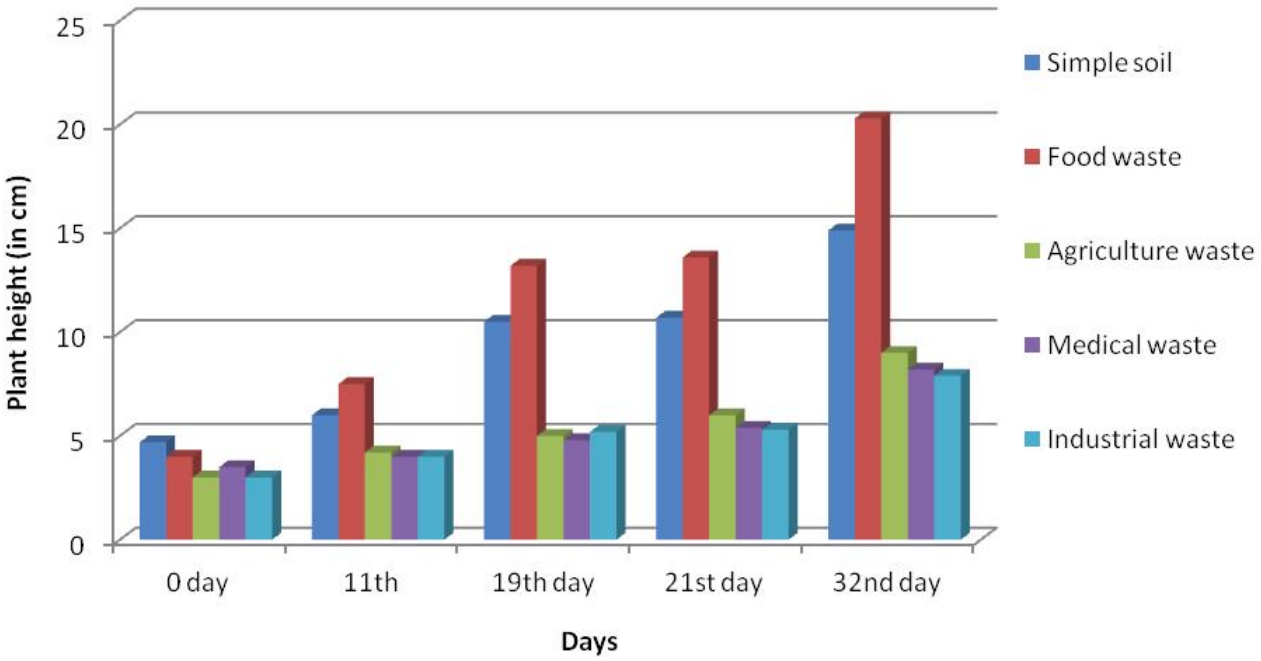


Fig. 2. Comparison of varying plant height during different days of observation of different vermicomposts and simple soil

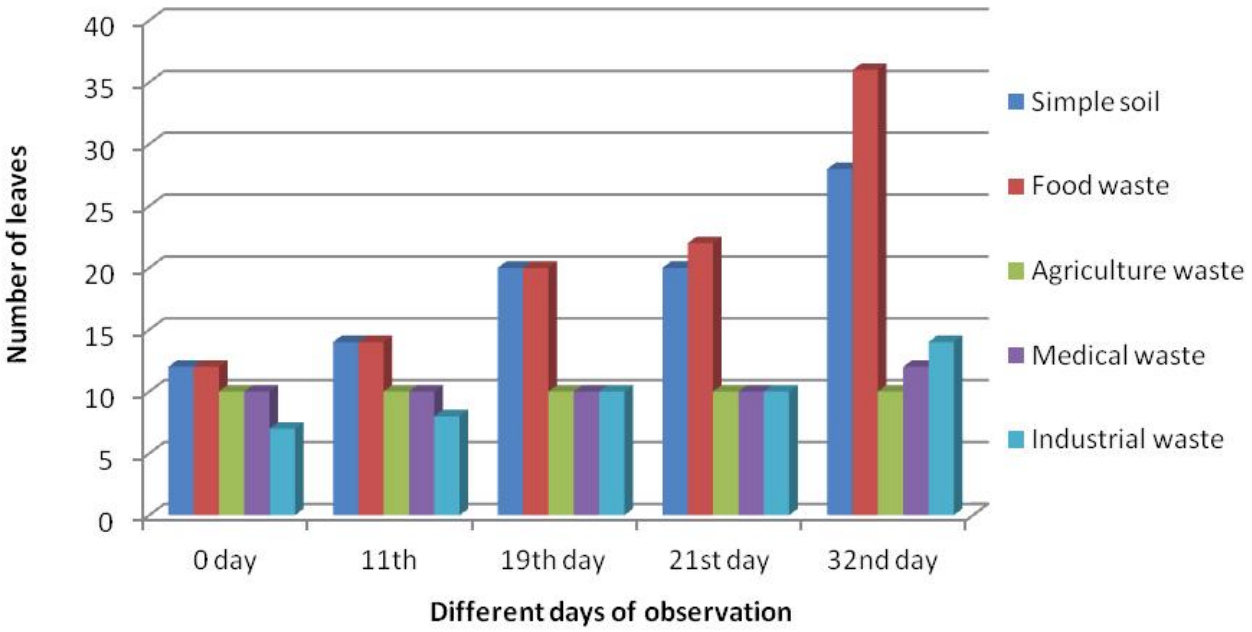


Fig. 3. Comparison of varying number of leaves during different days of observation of different vermicomposts and simple soil

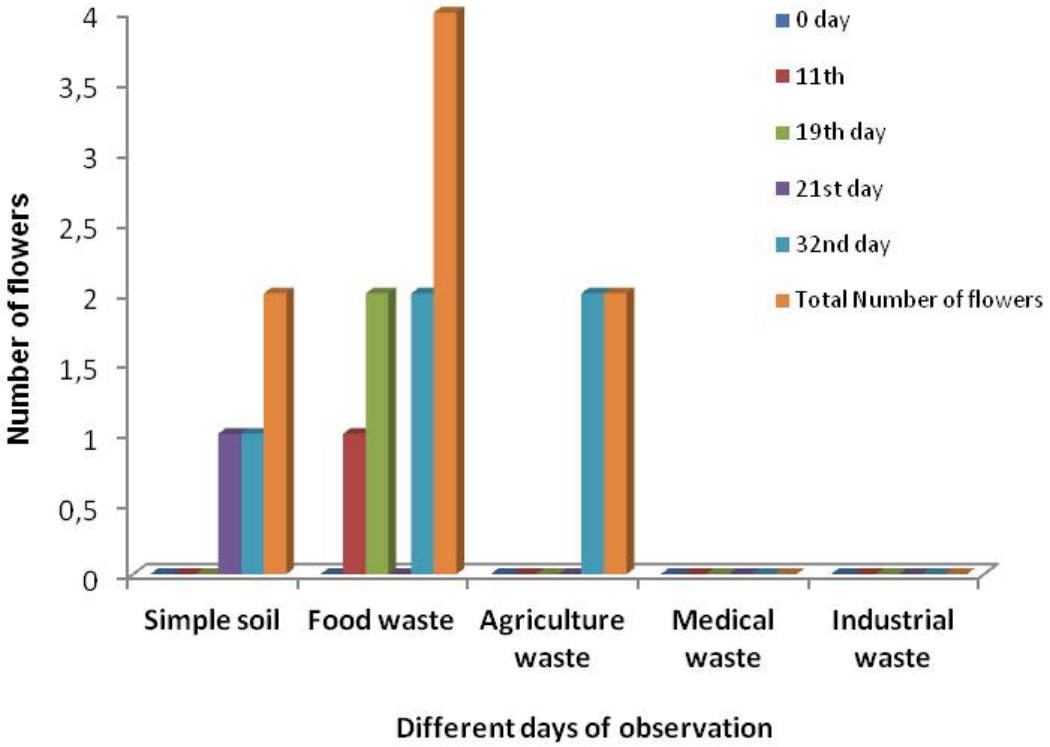


Fig. 4. Comparison of varying number of flowers during different days of observation of different vermicomposts and simple soil

of flowers (Figure 3) and leaves (Figure 4) than other plants grown in different vermicomposts. Plant height, number of leaves and number of flowers at different observation days for all the different types of vermicompost and simple soil were compared in table shown below In the table SS, FW, AW, MW and IW stands for Simple soil, Food waste, Agriculture waste, Medical waste, Industrial waste respectively.

DISCUSSION

Vermicomposting of different wastes such as food waste, agriculture waste, medical waste and industrial waste was done for the degradation of solid wastes and the nutrient rich end product vermicompost was used as a very effective natural fertilizer with no environmental hazards. Different vermibeds were prepared for different wastes and are treated for seven weeks. Every week vermicomposts were analysed for the presence of nutrients such as inorganic phosphate, total organic matter, chloride, carbon, calcium carbonate and sulphate. Moisture content and pH were also determined every week for different vermicomposts. Rate of mineralization and decomposition becomes faster with optimal moisture content [8]. Moisture content of 60-70% was proved having maximal microbial activity and 50% was the minimal requirement for rapid rise in microbial activity [9]. pH was increased gradually during vermicomposting and it was attributed to the progressive utilization of organic acids and increase in mineral constituents of wastes [10]. Near neutral pH of vermicompost may be attributed by the secretion of NH^{4+} ions that reduce the pool of H^{+} ions [11]. In earthworms the activity of calciferous glands containing carbonic anhydrase that catalyzes the fixation of CO_2 as $CaCO_3$, thereby prevent the fall in pH [12]. Inorganic phosphate content was increased in vermicomposts of different wastes than simple soil. Even when phosphorus is added to the soil in organic materials such as dung it may become mineralized, often as a chemical precipitate of orthophosphate or adsorbed onto the surface of minerals. Vermicomposts prepared from their respective organic wastes possessed considerably higher levels of major nutrients - N, P, K, Ca, and Mg compared to that of the substrates [13, 14]. Vermicomposting helps in crop improvement. It was

analysed by growing plants in different pots containing different vermicomposts and simple soil. Vermicompost contains plant growth promoters with other nutrients and improves physical, chemical and biological properties of soil on repeated application [15]. Many important factors, such as the presence of beneficial microorganisms or biologically active plant growth influencing substances such as phytohormone are released by beneficial microorganisms present in the vermicompost rich soil [6, 16]. Root initiation, increased root biomass, enhanced plant growth and development and sometimes, alterations in plant morphology are among the most frequently claimed effects of vermicompost treatment [17]. Stem elongation, dwarfing and early flowering have been found to be because of the hormone effect in a wide variety of plants and in a number of physiological situations, stem elongation is promoted (or inhibited) by endogenous phytohormones, a class of growth regulating substances which inhibited stem elongation without affecting leaf or flower development [18].

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Ismail SH, Joshi P, Grace A. The waste in your dustbin is scarring the environment. The technology of composting. Adv Biotechnol. 2003; 5(II): 30-34.
2. Edwards CA, Lofty JR. 1970. Biology of Earthworms. p. 283. Chapman and Hall Publications.
3. Newhauser EF, Kalpan DL, Malecki MR, Hartenstein R. Materials supporting weight gain by earthworm *Eisenia fetida* in waste conversion systems. Agric Wastes. 1980; 2: 43-60.
4. Flack FM, Hartenstein R. Growth of the earthworm Eiseniafetida on microorganisms and cellulose. Soil Biol Biochem. 1984; 16(5): 491-495.
5. Morgan MH. 1988. The role of microorganisms in the nutrition of Eiseniafetida. Earthworms in organic waste and environmental management. p. 71-72. The Hague, SPB Publishing Cooperation.
6. Edwards CA. 1988. Breakdown of animal, vegetable and industrial organic wastes by earthworms. Earthworms in waste and environmental management. p. 21-31. The Hague, SPB Academic Publishing.
7. Zazonc I. 1992. Breeding and production of earthworms in vermicompost. p. 59. Europe, Anima press.
8. Singh NB, Khare AK, Bhargava DS, Bhattacharya S. Optimum moisture requirement during vermi-composting using *Perionyx excavates*. Appl Ecol Environ Res. 2004; 2: 53–62.
9. Liang C, Das KC, McClendon RW. The influence of temperature and moisture contents regimes on the aerobic microbial activity of a biosolids composting blend. Bioresource Technol. 2003; 86(2): 131–137.
10. Fares F, Albalkhi A, Dec J, Bruns MA, Bollag JM. Physicochemical characteristics of animal and municipal wastes decomposed in arid soils. J Environ Qual. 2005; 34(4): 1392–1403.
11. Haimi J, Huhta V. Comparison of composts produced from identical wastes by vermistabilization and conventional composting. Pedobiologia. 1987; 30(2): 137–144.
12. Kale RD, Bano K, Krishnamoorthy RV. Potential of *Perionyx excavatus* for utilizing organic wastes. Pedobiologia. 1982; 23(6): 419–425.
13. Edwards CA. 2004. Earthworm Ecology. Florida, CRC Press.
14. Kitturmath MS, Giraddi RS, Basavaraj B. Nutrient changes during earthworm (*Eudrilus eugeniae*) mediated vermicomposting of agro-industrial wastes. Karnataka J Agric Sci. 2007; 20: 653–654.
15. Jayakumar B, Senthivelu M, Pandian SK, Paramasivam M. Vermicomposting of organic wastes. Kisan World. 2005; 32(12): 54.
16. Tomati U, Galli E. Earthworms, Soil fertility and plant productivity. Acta Zool Fenn. 1995; 196: 11-94.
17. Tomati U, Grapelli A, Galli E. The hormone-like effect of earthworm casts on plant growth. Biol Fertil Soils. 1987; 5(4): 288-294.
18. Wirwille JW, Mitchell JW. Six new plant growth inhibiting substances. Bot Gazette. 1950; 111: 491-494.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Alterations in some metabolic activities of *Scenedesmus quadricauda* and *Merismopedia glauca* in response to glyphosate herbicide

Ahmed Abd El-Salam Issa, Mahmoud Salama Adam, Mustafa Ahmed Fawzy

Botany Department, Faculty of Science, Assiut University, 71516 Assiut, Egypt

ABSTRACT

The effect of glyphosate herbicide concentrations on the growth and some metabolic activities of the green alga *Scenedesmus quadricauda* and the blue-green alga (cyanobacteria) *Merismopedia glauca* isolated from soil (Assiut, Egypt) were assessed. The growth of *Scenedesmus quadricauda* and *Merismopedia glauca* was negatively affected by glyphosate herbicide. In addition, decreasing of the cell number as well as chlorophyll a, b and the dry weight of *Scenedesmus* were occurred with increasing the herbicide concentrations, while the dry weight and chlorophyll a of *Merismopedia* was increased by low concentration of glyphosate herbicide. The photosynthesis and respiration were increased in the two algae with increasing glyphosate herbicide. Increasing of soluble and insoluble carbohydrates with increasing of the herbicide in *Scenedesmus*. But in case of *Merismopedia glauca* the soluble sugars only were enhanced by all concentrations used of the herbicide. Decreasing soluble and insoluble proteins was occurred in *Scenedesmus* with increasing glyphosate. Also, glutelins, globulins and prolamines were decreased with increasing of glyphosate herbicide. The insoluble proteins, glutelins, globulins were increased and soluble proteins as well as prolamines were decreased with increasing of glyphosate herbicide to *Merismopedia*. Free amino acids were increased by increasing glyphosate herbicide in *Scenedesmus* and *Merismopedia*. Glyphosate herbicide inhibited the growth and some metabolic activities in the tested algae, this effect depend on the algal species, the type and concentration of the herbicide.

Key words: *Scenedesmus quadricauda*; *Merismopedia glauca*; Glyphosate; Glutelins; Globulins; Prolamines.

J Biol Earth Sci 2013; 3(1): B17-B23

Corresponding author:
Mustafa Ahmed Fawzy
Botany Department, Faculty of Science, Assiut University, 71516 Assiut, Egypt
Tel: +201002931424, Fax: +2088342708
E-mail: Drmustafafawzy81@yahoo.com

Original Submission: 11 November 2012; Revised Submission: 17 December 2012; Accepted: 31 December 2012

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

The widespread use of herbicides in modern agriculture might adversely affect algal flora. Algae and cyanobacteria are quite sensitive to herbicides because they share many common characters with higher plants, which are target organisms. Herbicides have detrimental effects on growth, photosynthetic pigments, protein content and oxidative stress in cyanobacterial cells [1, 2]. The use of herbicides may decrease pigment content, destroy chloroplasts, thylakoids and PSII, and even cause DNA damage in organisms [3-5]. Some herbicides cause metabolic alterations in algae due to oxidative stress [6], and others function via binding to the exchangeable quinone site in the photosystem II (PSII) reaction center, thus blocking electron transfer [7], or by changing fluorescence emission and electron transport activities of the bio-samples [5].

Glyphosate is a broad-spectrum, nonselective and systemic herbicide used for the control of annual and perennial plants [8]. Glyphosate mechanism of action appears to be the inhibition of aromatic amino acid synthesis, which results in the inhibition of nucleic acid metabolism and protein synthesis. The use of glyphosate may result in significant population losses of a number of terrestrial species through habitat and food supply destruction and thus cause a threat to endangered species and biodiversity. Glyphosate can become a source of inorganic phosphorus in systems that are phosphorus limited. Bacterial degradation removes the phosphate group resulting in sarcosine. The sarcosine is further degraded to form glycine and ultimately carbon dioxide [9].

The soil algae have variable responses to specific herbicides ranging from stimulatory via tolerance to inhibitory. Some authors have explained the inhibitory effect of certain herbicides as due to the inhibition of photosynthesis through blocking of photolysis of water or possibly to interference with some primary energy transfer [10]. Although photosynthesis is a primary site of action for many herbicides, there is no evidence to suggest that this is an important site of action for glyphosate. While there are many studies about the effect of herbicides on algal growth, biochemical composition and the metabolic activities [11], little is known about the effect of herbicides on *Merismopedia glauca*. Thus, it is important to determine the effect of glyphosate herbicide on the growth and some metabolic activities of *Merismopedia glauca* (Cyanophyta) and *Scenedesmus quadricauda* (Chlorophyta).

MATERIALS AND METHODS

The green alga *Scenedesmus quadricauda* and the blue green alga *Merismopedia glauca* were subjected to various concentrations of glyphosate herbicide. Three concentrations of herbicide were used in this study according to the performed preliminary experiments. These concentrations calculated as ppm active ingredient (High 600, Medium 300 and Low 150 ppm for *Scenedesmus* and High 150, Medium 75 and Low 37.5 ppm for *Merismopedia*). Rippka and Herdman [12] modified medium was used for culturing of blue-green algae, and Bold's basal medium [13] was used for culturing green algae.

The growth rate and generation time of a test alga grown with various herbicides concentrations were followed by daily measurements of absorbance at 750 nm. Optical density was used as a parameter for algal growth. The growth rate μ (d^{-1}) was determined from the following formula:

$$\mu \quad (d^{-1}) \quad \frac{\ln A_1 - \ln A_0}{t_1 - t_0}$$

Where

A_1 = Optical density at time t_1.

A_0 = Optical density at time t_0.

t_1 - t_0 = The time elapsed in days between two determinations of optical density.

The generation time (G) can be calculated as follows:

$$G = \frac{\ln 2}{\mu} d$$

After 8 days of growth in case of *Scenedesmus quadricauda*, and 7 days in case of *Merismopedia glauca* the algal cells were harvested for some metabolic estimations, in the late of exponential phase or beginning of the stationary phase according to algal growth curves as shown in (Fig. 1 and 2).

The optical density of the algal suspension was measured at 750 nm as one of the growth indicators as described by Lefort-Tran et al. [14]. The cell numbers (in case of green alga) were determined by counting of the cell number microscopically

using 1 mm deep haemocytometer slide. For determination of dry matter 10 ml of algal suspension, after filtered through Whatman No. 1 filter paper, was dried in oven at 105°C until constant weight. The data were given as mg/ml algal suspension.

Photosynthetic pigments (chl.*a* and chl.*b*) were extracted in hot methanol (70°C) for 10 minutes, this made daily during the period of experiment [15]. It is worthy to mention that, in the case of Cyanophyceae species, ch1.a only is measured.

The photosynthetic activity was measured as oxygen evolution using (O_2 Meter CG 867 Germany). The data obtained were calculated as (µmoles O_2 mg^{-1} chl. h^{-1}) After photosynthesis was estimated in the system described, the lamps were switched off and O_2 uptake in the dark was estimated. For the determination of carbohydrate, the anthrone sulfuric acid method was used [16]. All carbohydrates including polysaccharides were spectrophotometrically determined. Soluble, insoluble and total proteins were determined according to the method adapted by Lowry et al. [17]. The protein fractions were estimated by the methods of Moureaux and Landry [18]. Free amino acids were estimated according to the method adopted by Lee and Takahashi [19].

RESULTS

The effect of glyphosate herbicide on the growth of Scenedesmus quadricauda and Merismopedia glauca was shown in Fig. 1 and 2, and Table 1, they were inhibited by the concentrations of herbicide applied. The maximum growth rate calculated as chlorophyll a for *Scenedesmus* was (1.56 $\mu_{maxBBB}.d^{-1}$) and minimum generation time (0.44 $G.d^{-1}$) was recorded in control culture. The maximum growth rate for *Merismopedia* was (1.33 $\mu_{max}.d^{-1}$) and minimum generation time (0.52 $G.d^{-1}$) was recorded in control culture.

DISCUSSION

The cell number and dry weight as well as chlorophyll a and b for *Scenedesmus* were markedly decreased with increasing the concentrations of herbicide. In *Merismopedia* the dry weight and chlorophyll a were decreased with increasing the concentrations of the herbicide, and

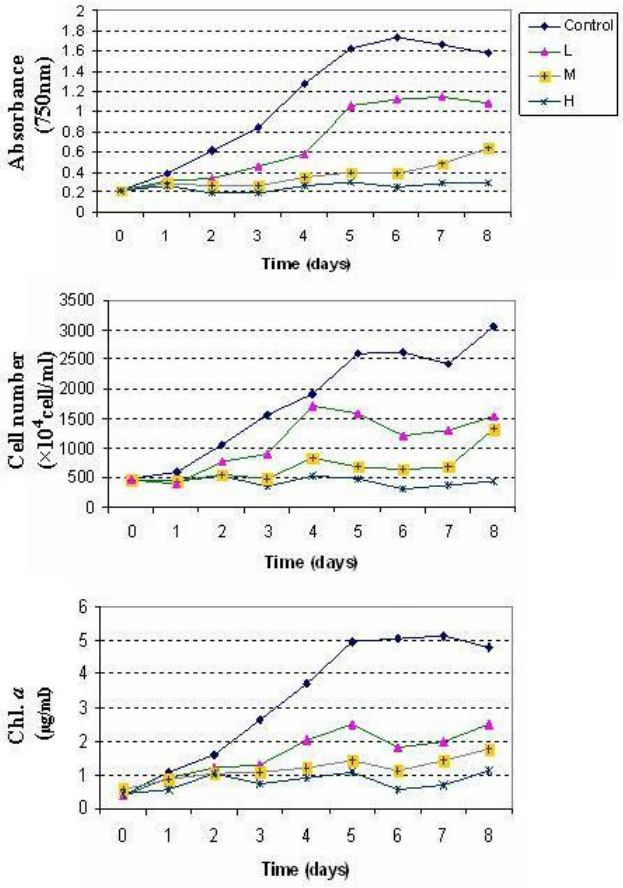


Fig. 1. Effect of glyphosate on the growth curve of *Scenedesmus quadricauda* calculated as absorbance, cell number and chlorophyll *a*

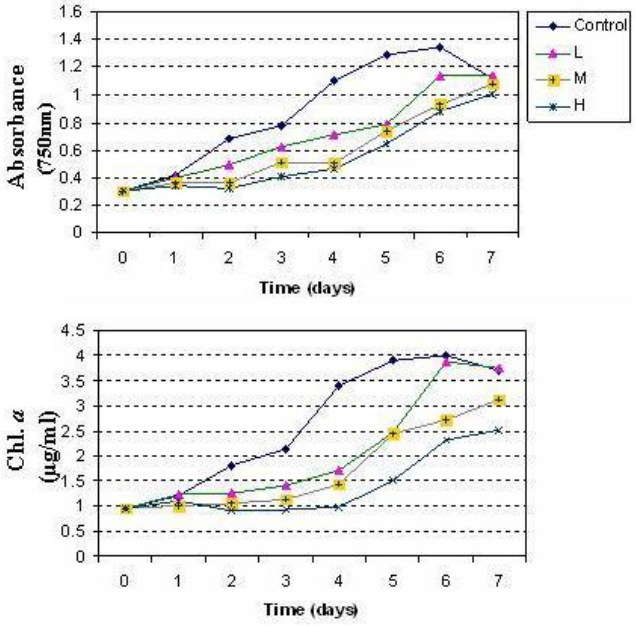


Fig. 2. Effect of glyphosate on the growth curve of *Merismopedia glauca* calculated as absorbance and chlorophyll *a*

Table 1. Effect of Glyphosate herbicide concentrations on the growth and some metabolic activities of *Scenedesmus quadricauda* and *Merismopedia glauca*

Treat.		Cell no. $\times 10^4$ cell/ml	μ_{max} (Chl.*a*) (d^{-1})	G (d^{-1})	Dry wt. mg/ml	Chl. *a* µg/ml	Chl. *b* µg/ml	$O_2\uparrow$*	$O_2\downarrow$**	Carbohydrates (mg/g dry wt.)			Proteins (mg/g dry wt.)						A.A.
										S.C.	Ins.C.	T.C.	S.P.	Ins.P.	Glut.	Glob.	Prol.	T.P.	
Control		3066	1.56	0.44	0.362 ±0.00	4.77 ±0.55	4.42 ±0.14	16.4 ±0.05	5.91 ±0.05	3.20 ±1.1	11.4 ±0.86	14.6 ±0.7	9.97 ±0.57	49.8 ±1.20	23.86 ±0.40	35.6 ±2.00	52.56 ±0.95	171.8 ±1.0	9.40 ±0.50
Scenedesmus quadricauda	L	1541	0.89	0.78	0.360 ±0.00	2.52 ±0.02	1.98 ±0.06	5.89 ±0.02	4.05 ±0.03	1.10 ±0.1	7.60 ±1.40	8.70 ±1.5	14.5 ±1.30	49.1 ±5.60	37.39 ±0.60	40.0 ±0.63	86.00 ±0.74	226.9 ±1.6	4.30 ±0.46
	M	1317	0.55	1.26	0.350 ±0.00	1.75 ±0.33	1.29 ±0.12	9.66 ±0.09	5.39 ±0.01	2.05 ±0.1	9.15 ±1.00	11.2 ±0.8	11.8 ±1.30	27.9 ±0.60	23.16 ±0.50	23.8 ±1.50	62.80 ±0.70	149.4 ±1.5	12.3 ±0.06
	H	442	0.12	5.8	0.232 ±0.00	1.11 ±0.15	0.69 ±0.06	15.5 ±0.06	18.2 ±0.02	3.78 ±0.5	10.5 ±1.70	14.3 ±2.3	8.60 ±0.06	21.5 ±0.16	10.00 ±1.20	18.6 ±0.23	41.75 ±0.16	100.4 ±1.9	20.9 ±0.07
		μ_{max} (Chl.*a*) (d^{-1})				Chl. *a*													
Control		1.33		0.52	0.280 ±0.00	3.68 ±0.16		26.8 ±0.01	14.4 ±0.01	8.60 ±0.4	21.4 ±0.77	30.0 ±1.1	36.7 ±0.79	36.2 ±0.79	10.0 ±0.24	12.0 ±0.90	20.0 ±0.19	114.9 ±0.1	11.6 ± 1.2
Merismopedia glauca	L	1.31		0.53	0.29 ±0.0	3.75 ±0.15		49.4 ±0.05	41.5 ±0.02	10.1 ±0.8	4.30 ±0.03	14.4 ±0.9	55.7 ±0.42	18.3 ±0.1	6.00 ±0.5	8.70 ±0.80	26.2 ±0.31	114.9 ±0.2	8.00 ± 0.58
	M	1.09		0.63	0.27 ±0.0	3.12 ±0.05		91.5 ±0.05	49.7 ±0.02	10.3 ±1.6	6.70 ±0.8	17.0 ±1.0	54.0 ±0.99	39.6 ±0.53	9.90 ±0.17	13.2 ±0.70	26.0 ±0.11	142.7 ±0.6	10.3 ± 3.4
	H	0.88		0.79	0.17 ±0.0	2.50 ±0.12		108 ±0.01	92.0 ±0.01	13.9 ±0.7	0.93 ±0.01	14.8 ±0.9	52.0 ±0.57	43.7 ±0.48	12.0 ±0.56	15.0 ±0.64	24.4 ±0.34	147.1 ±0.1	16.4 ± 1.7

Treat. = treatment with three replicates, G = generation time, $O_2\uparrow$* (µmoles O_2 ↑ mg chl.a h^{-1}); $O_2\downarrow$** (µmoles O_2 ↓ mg chl.a h^{-1}), S.C. = soluble carbohydrates, Ins.C. = insoluble carbohydrates, T.C. = total carbohydrates, S.P. = soluble proteins, Ins.P. = insoluble proteins, Glut. = glutelins, Glob. = Globulins, Prol. = Prolamines, T.P. = Total proteins, A.A. = amino acids, L = Low, M = Medium, H = High (High 600, Medium 300 and Low150 ppm for *Scenedesmus* and High 150, Medium 75 and Low 37.5 ppm for *Merismopedia*).

were only enhanced by low concentration of glyphosate herbicide. Many reports indicate herbicide affects on soil algal growth, photosynthesis, nitrogen fixation, and metabolic activities which in accordance with the obtained results [20]. Wakabayashi et al. [21] studied the influence of cyclic imide herbicides and oxyfluorfen on growth, content of photosynthetic pigments and degradation of chloroplast components using autotrophic (light grown) and heterotrophic (glucose-grown, dark grown) *Scenedesmus acutus* cells. The compounds inhibited growth and decreased the content of chlorophylls and carotenoids in both types of cells. The reduction of chlorophyll may be due to sensitivity of the enzymes of chlorophyll biosynthesis towards the herbicides. Hernando et al. [22] stated that glyphosate decreased cell density and photosynthetic pigment contents for *Chlorella pyrenoidosa*. The inhibition of chlorophyll appearance might be an indirect effect whereby glyphosate could be envisaged to interference with the development of the chloroplast in some way, thus preventing insertion of the chlorophyll molecule into the thylakoid membrane [8]. Couderchet and Vernet [23] investigated the effects of one of the sulfonylurea herbicides, flazasulfuron, on *Scenedesmus obliquus*. They found that the low concentration of flazasulfuron induced a reduction in chlorophyll content of the culture. In this respect, the influence of simazine on the growth and pigment content of *Chlorella vulgaris*, *Scenedesmus acutus* and *Arthrospira fusiformis* elucidated by Fournadzhieva et al. [24]. They found that the herbicide at concentrations of 0.1 to 1.0 mg/L nutrients medium, inhibited the photosynthetic activities of all studied strains. The activity of *Scenedesmus acutus* was the least affected, while that of *Arthrospira fusiformis* was the most affected. The dry weight and chlorophyll a of *Merismopedia* was increased by low concentration of glyphosate herbicide. Megharaj et al. [25] reported stimulatory effect of certain herbicides when applied in low concentrations. Such stimulatory effect was also reported by Ibrahim [26]; Ahmed and Venkataraman [27]. Younis et al. [28] observed progressive great reduction in growth of *Chlorococcum humicula* and dry weight accumulation by increasing fluometuron concentration. They demonstrated that low concentration of this herbicides increased photosynthetic pigment content, while the reverse was true at high concentration. In this respect, Wong and Wong [29] determined the effect of 2,4-D, glyphosate and paraquat herbicides on growth, photosynthesis and chlorophyll a synthesis by freshwater alga *Scenedesmus quadricauda* Berb 614. Algal growth, photosynthesis and chlorophyll a synthesis were stimulated by the presence of low concentrations (0.02 or 0.2 mg/L, respectively) of 2,4-D and glyphosate. Glyphosate stimulation may have resulted from its use after degradation as carbon or nitrogen source for algal growth [30]. Algal susceptibility to herbicides differs among species [31] and even also among strains [32]. These confirm the results obtained in this investigation.

The photosynthesis and respiration were increased in *Scenedesmus* and *Merismopedia* with increasing glyphosate herbicide. In this respect, although glyphosate reduced chlorophyll content, it stimulated slightly respiration [8]. Abu-Iramaileh et al. [33] reported that an increase in respiration could be detected within 24 hours in bean plants sprayed with a 20 mM of glyphosate.

With respect to the effect of glyphosate herbicide on the soluble and insoluble carbohydrates, they were increased with increasing the herbicide in *Scenedesmus* while, soluble carbohydrates only were increased in *Merismopedia*. The increase in carbohydrates level in this study was associated with stimulation of photosynthesis. In this respect, Fournadzhieva et al. [24] studied the influence of simazine on *Chlorella vulgaris*, *Scenedesmus acutus* and *Arthrospira fusiformis* and reported that the herbicide concentrations of 0.1 to 1.0 mg/L nutrients medium increased carbohydrate level.

As regards, the soluble and insoluble proteins as well as glutelins, globulins and prolamines it found that they decreased in *Scenedesmus* with increasing glyphosate herbicide. It was suggested that the increase in amino acids may reflected a decrease in protein synthesis [34]. The insoluble proteins, glutelins, globulins were increased and soluble proteins as well as prolamines were decreased with increasing of glyphosate herbicide in *Merismopedia*. In this respect, Marsalek and Rojickova [30] reported that a higher increase of proteins than carbohydrates was observed after addition of glyphosate to algal cultures. The changes in protein synthesis in algae grown under stress could be due to changes in gene expression [35]. The high concentrations of herbicides may

affect the enzymatic reactions responsible for protein biosynthesis [36]. The mode of action of glyphosate seems to inhibit the aromatic amino acid synthesis, which results in the inhibition of nucleic acid metabolism and protein synthesis.

Glyphosate kills plants by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which catalyzes the reaction of shikimate-3-phosphate (S3P) and phosphoenolpyruvate to form 5-enolpyruvyl-shikimate-3-phosphate (ESP). ESP is subsequently dephosphorylated to chorismate an essential precursor in plants for the aromatic amino acids: phenylalanine, tyrosine and tryptophan [37].

The free amino acids were increased with increasing glyphosate herbicide in *Scenedesmus* and *Merismopedia*. In this respect, a gradual increase in total amino acids content of *Scenedesmus bijuga* was generally a function of trichlorfon concentration increase from 0.1 to 0.4 mM [38].

CONCLUSION

Scenedesmus quadricauda was considered more tolerant to the herbicide than *Merismopedia glauca*, this effect depend on the algal species, as well as the herbicide concentration. The variability in sensitivity of the two photosynthetic organisms to the same chemical substance may be explained by different characteristics of the organisms such as morphology, cytology, physiology and genetics.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Cedergreen N, Spliid NH, Streibig JC. Species-specific sensitivity of aquatic macrophytes towards two herbicides. Ecotoxicol Environ Saf. 2004; 58: 314–323.
2. Chen Z, Juneau P, Qiu B. Effects of three pesticides on the growth, photosynthesis and photoinhibition of the edible cyanobacterium Ge-Xian-Mi (*Nostoc*). Aquat Toxicol. 2007; 81: 256–265.
3. Nielsen LW, Dahllof I. Direct and indirect effects of the herbicides Glyphosate, Bentazone and MCPA on eelgrass (*Zostera marina*). Aquat Toxicol. 2007; 82: 47–54.
4. Manas F, Peralta L, Raviolo J, Garcıa OH, Weyers A, Ugnia L, Gonzalez CM, Larripa I, Gorla N. Genotoxicity of glyphosate assessed by the comet assay and cytogenetic tests. Environ Toxicol Pharmacol 2009; 28: 37–41.
5. Ventrella A, Catucci L, Agostiano A. Herbicides affect fluorescence and electron transfer activity of spinach chloroplasts, thylakoid membranes and isolated Photosystem II. Bioelectrochem. 2010; 79: 43–49.
6. Romero DM, de Molina MCR, Juarez AB. Oxidative stress induced by a commercial glyphosate formulation in a tolerant strain of *Chlorella kessleri*. Ecotoxicol Environ Saf. 2011; 74: 741–747.
7. Rutherford AW, Krieger-Liszkay A. Herbicide-induced oxidative stress in photosystem II. Trends Biochem Sci 2011; 26: 648–653.
8. Grossbard E, Atkinson D, (Eds.). 1985. The herbicide glyphosate. Butterworth, London, p. 490.
9. Giesy JP, Dobson S, Solomon KR. Ecotoxicological risk assessment for roundup herbicide. Rev Environ Contam Toxicol. 2000; 167: 35-120.
10. Moreland DE. Mechanisms of action of herbicides. Ann Rev Plant Physiol. 1980; 31: 597.
11. Liu H, Xiong M. Comparative toxicity of racemic metolachlor and *Smetolachlor* to *Chlorella pyrenoidosa*. Aquat Toxicol. 2009; 93, 100–106.
12. Rippka R, Herdmann M. 1993. Pasteur culture collection of cyanobacterial strains in axenic culture. Vol. 1 Catalogue of Strains 103 P., Institut Pasteur, Paris, France.
13. Bischoff HW, Bold HC. Phycological studies. 4 - Some soil algae from enchanted rock and related algal species. Uni. Texas Publ. 1963; 6318: 32-36.
14. Lefort-Tran M, Pouphile M, Spath S, Packer L. Cytoplasmic membrane changes during adaptation of the freshwater cyanobacterium *Synechococcus* 6311 to salinity. Plant Physiol. 1988; 87: 767-775.
15. Marker AFH. The use of acetone and methanol in the estimation of chlorophyll in the presence of Phaeophytin. Fresh Wat Biol. 1972; 2: 361-385.
16. Badour SSA, 1959. Analytische-Chemische unter-

suchung des kalimangels bei Chlorella in vergleich mit anderen manget zamstanden. Ph.D. Dissertation Goettingen.
17. Lowry DH, Rosebrough NJ, Farr AL, Randall, R.J. Protein measurement with the folin phenol reagent. J Biol Chem. 1951; 193: 265-275.
18. Moureaux T, Landry J. Extraction selective des proteins du grain de mais et en particular de la fraction “glutelines”. C. R. Acad Sci. 1968; 266: 2302-2305.
19. Lee YD, Takahashi T. An improved colorimetric determination of amino acids with use of ninhydrin. Anal Biochem. 1966; 14: 71-77.
20. Bhunia AK, Basu NK, Roy D, Chakrabarti A, Banerjee, SK. Growth and chlorophyll a content, nitrogen-fixing ability and certain metabolic activities of *Nostoc muscorum*: effect of methylparathion and benthiocarb. Bull Environ Contam Toxicol. 1991; 47: 43-50.
21. Wakabayashi K, Sandmann G, Ohta H, Böger, P. Peroxidising herbicides: comparison of dark and light effects. J Pesticide Sci. 1988; 13(3): 641.
22. Hernando F, Poyuela M, Munoz-Rueda A, Gonzalez-Murua, C. Effects of glyphosate on the greening process and photosynthetic metabolism in *Chlorella pyrenoidosa*. Plant Physiol. 1989; 134: 26-31.
23. Couderchet M, Vernet G. Pigments as biomarker of exposure to the viney and herbicide flazasulfuron in freshwater algae. Ecotoxicol Environ Saf. 2003; 55(3): 271-277.
24. Fournadzhieva S, Kassabov P, Andreeva R, Petkov G, Dittrit F. Influence of the herbicide simazine on *Chlorella*, *Scenedesmus* and *Arthrospira*. Archiv-fur-Hydrobiologie, Supplementband, Algological studies 1995; 106: 97-109.
25. Megharaj M, Venkateswarlu K, Rao AS. Growth response of four species of soil algae to monocrotophos and quinalphos. Environ Pollut Ser. A. 1986; 42: 15.
26. Ibrahim AN. Effect of certain herbicides on growth of nitrogen fixing algae and rice plants. Symp Biol Hung. 1972; 11: 445.
27. Ahmed MH, Venkataraman GS. Tolerance of *Aulosira fertilissima* to pesticides. Current Sci. 1973; 42: 180.
28. Younis ME, Osman MEH, Soliman AI. Determination and characterization of the effects of fluometuron on growth and metabolism of *Chlorococcum humicula*. Microbiol. 1991; 67(271): 75-86.
29. Wong PK, Wong MH. Effect of 2,4-D, glyphosate and paraquat on growth, photosynthesis and chlorophyll (a) synthesis of *Scenedesmus quadricauda* Berb 614. Environ Contam Toxicol Health Chemosphere. 2000; 41(1-2): 177-182.
30. Marsalek B, Rojickova R. Stress factors enhancing production of algal exudates: a potential self-productive mechanism. Zeitschrift für Naturforschung, Section C, Biosci. 1996; 51(9-10): 646-650.
31. Torres AM, Oflaherty L. Influence of pesticides on *Chlorella*, *Chlorococcum*, *Stigoclonium* (Chlorophyceae), *Tribonema*, *Vaucheria*, (Xanthophyceae) and *Oscillatoria* (Cyanophyceae). Phycol. 1976; 15: 25.
32. Kasai F, Hatakeyama S. Herbicide susceptibility in two green algae in acid forest and nursery soils. Can J Microbiol. 1993; 14: 1179.
33. Abu-Iramaileh BE, Jordan LS, Kumamoto J. Enhancement of CO_2 and ethylene production and cellulose activity by glyphosate in *Phaseolus vulgaris*. Weed Sci. 1979; 27: 103-105.
34. Ashton FM, Crafts AS. 1981. Mode of Action of Herbicides. A Wiley-Interscience Publication. New York.
35. Tiwari DN, Pandey AK, Mishra AK, Srivastava, V. Role of 2,4-D on growth and macromolecular synthesis on a cyanobacterium *Anabaena cylindrical*. Indian J Bot. 1982; 5(2): 111-118.
36. Eladel HM, Henley WJ, Kobbia EA. Effect of thiobencarb on growth and photosynthesis of the soil algae *Protosiphon botryoides*. J Appl Phycol. 1999; 10(6): 547-554.
37. Andrea MM, Peres TB, Luchini LC Bazarin S, Papini S, Matallo, MB, Savoy VLT. Influence of repeated applications of glyphosate on its persistence and soil bioactivity. Pesq. agropec. bras. Brasília. 2003; 38 (11): 1329-1335.
38. Fathi AA. Some metabolic activities in the green alga *Scenedesmus bijuga* as affected by the insecticide trichlorfon. Protistology. 2003; 3 (2): 92-98.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Diversity of tribe Anthemideae (Asteraceae) in flora of Jammu and Kashmir State

B. L. Bhellum

Department of Botany, Govt. College for Women, Parade, Jammu, India

ABSTRACT

Introduction: The present communication deals with tribe Anthemideae comprising of 47 species belonging to 11 genera based on the collections from Jammu and Kashmir State. Many of these species are mostly confined to the alpine zones of Kashmir Himalayas. The relevant data, number of species in Jammu and Kashmir, key to the species, diversity of the tribe anthemideae is indicated under enumeration in each genus.

Material and Methods: The collections were made from the area under study to identify the different genera and species.

Results: The present communication of tribe Anthemideae is first report of its kind from Jammu and Kashmir State comprising of 11 genera and 47 species of the tribe.

Key words: Anthemideae; Asteraceae; Jammu and Kashmir; Himalayas.

J Biol Earth Sci 2013; 3(1): B24-B29

Corresponding author:
B. L. Bhellum
Department of Botany, Govt. College for Women,
Parade, Jammu – 180001, India
E-Mail: blbhellum@gmail.com

Original Submission: 24 October 2012; Revised Submission: 20 December 2012; Accepted: 02 January 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Asteraceae is one of the largest families of the flowering plants having the estimated size comprising of 1314 genera representing 2100 species [1]. Anthemideae is the large tribe of family Asteraceae comprising of 109 genera and ca 1740 species [2]. Asteraceae of Jammu and Kashmir is quite rich. Many of the species are confined to alpine zones. The endemic flora of Asteraceae is dominant. The author has recorded 90 endemic species of Asteraceae from Jammu and Kashmir State [3]. The concentration of species of this family is more in hilly areas than plains. In India this tribe represents as many as 12 genera and 66 species [4]. In Jammu and Kashmir state, the author has studied 11 genera and 47 species of this tribe as enumerated below. Of these 11 genera, 6 species of the genera, namely *Anthemis* L., *Centipeda* Lour., *Chrysanthemum* L., *Leucanthemum* Mill., *Soliva* Ruiz & Pav. and *Waldheimia* are represented by a single species (Fig. 1). The highest number of species in tribe Anthemideae is found in *Artemisia* L. is 400 in the world, 31 in India and 21 in Jammu and Kashmir (Fig. 2). Many species belonging to tribe Anthemideae of family Asteraceae find their application in therapy of their being high content of pharmacologically action, compounds produced by secretary tissues such as *Matricaria chamomilla* is one of the oldest species of this tribe. Some other species are used mainly as remedies as wild sedative, spasmolytic and pharmaceutical preparations, anti-inflamatory, carminative and also as perfumes and cosmetics [5].

MATERIALS AND METHODS

Field trips were made in Jammu and Kashmir to study flora general and Asteraceae in particular of Jammu and Kashmir State. The relevant data was collected and key characters were studied. Key characters were found very useful in preparation of key to genera of this tribe.

TRIBE: ANTHEMIDEAE

Key to the Genera

1 + Receptacles paleaceous; heads usually rayed ---------- 2.
- Receptacles naked or with fimbriate pits; heads rayed or disciforma ---------- 3.

2 + Heads corymbose; achenes margined ---------- 1. *Achillea*
- Heads solitary peduncled; achenes 4-5 angled or many ribbed ---------- 2. *Anthemis*

3 + Heads rayed ---------- 4.
- Heads disciform ---------- 7.

4 + Herbs diffuse; peduncles short; achenes 5-angled ---------- 11. *Waldheimia*
- Herbs erect; peduncles long; achense angled or ribbed ---------- 5.

5 + Annual herbs; achenes ventrally 3- 5 ribbed, dorsally many ribbed or smooth -------- 8. *Matricaria*
- Perennial herbs; achenes ribbed or angled ---------- 6.

6 + Achenes 3-ribbed; mucilage cells absent ---------- 4. *Chrysanthemum*
- Achenes usually prominently 10- ribbed; mucilage cells present ---------- 7. *Leucanthemum*

7 + Heads many, racemose, penciled or corymbose ---------- 8.
- Heads solitary ---------- 9.

8 + Heads racemose or panicled; involucral bracts few seriate ---------- 3. *Artemisia*
- Heads in corymbs; involucral bracts many seriate ---------- 10. *Tanacetum*

9 + Herbs stoloniferous; heads sessile; corolla of outer florets wanting---------- 9. *Soliva*
- Herbs non-stoloniferous; heads usually peduncled; corolla of outer florets usually present ---------- 11.

10 + Heads sessile or subsessile; involucral bracts 2-serriate; leaves toothed or lobed--- 5. *Centipeda*
- Heads peduncled; involucral bracts sub-2-serriate; leaves pinnatifid or pinnatisect -- 6. *Cotula*

RESULTS

In Jammu and Kashmir, the author has studied 11 genera and 47 species of this tribe as enumerated below. As many as 9.42% species of tribe Anthemideae are endemic to Jammu and Kashmir Himalayas.

ANTHEMIDEAE CHARACTERISTICS

Herbs, subshrubs or shrubs; latex absent; leaves usually alternate, usually variously dissected with characteristic smell; heads various; invlucres often hemispheric; bracts 2-7 seriate, imbricate, margin almost scarious; rays of variously coulured, limb 3-lobed; pollen echinate rarely rugose or smooth; style slender, apex truncate, stigmatic surfaces parallel; achenes shapes various; pappus never capillary.
Distribution: South Africa, Central Asia and Mediterranean region.

ENUMERATION

1. *Achillea* L.
200 species approximately; 2 in India and 1 in Jammu and Kashmir: *Achillea millefolium* L.

2. *Anthemis* L.
200 species approximately; 1 in India and 1 in Jammu and Kashmir: *Anthemis cotula* L.

3. *Artemisia* L.
400 species approximately; 32 in India and 21 in Jammu and Kashmir: *Artemisia absinthium* L., *A. amygdalina* Decne., *A. banihalensis* Kaul et Bakshi, *A. biennis* Willd., *A. brevifolia* Wall. ex DC., *A capillaris* Thunb., *A. cashmirica* Kaul & Bakshi, *A. desertorum* Spreng., *A. dranculus* L., *A. glauca* Pall. ex Willd., *A. japonica* Thunb., *A. laciniata* Willd., *A. maritima* L., *A. nilagirica* (Clarke) Pump., *A. parviflora* Roxb., *A. roxburghiana* Wall. ex Bess., *A. sacrorum* Ledeb., *A. sieversiana* Ehrh. ex Willd., *A. stracheyi* Hk. ex Thoms., *A. vestita* Wall. ex DC., *A. wallichiana* Bess.

4. *Centipeda* Lour.
6 species approximately; 1 in India and 1 in Jammu and Kashmir: *Centipeda minima* (L.) A. Br. & Aschers.

5. *Chrysanthemum* L.
200 species approximately; 4 in India and 4 in Jammu and Kashmir: *Chrysanthemum parthenium* (L.) Berth., *C. pyrethroides* (Kar. & Kir.) B. Fedtsch-Rostit., *C. stoliczkai* Clarke, *C. tibeticum* Hk.f. & Thoms.,

6. *Cotula* L.
75 species approximately; 4 in India and 2 in Jammu and Kashmir: *Cotula anthemoides* L., *C. aurea* Loeffl.

7. *Leucanthemum* Mill.
20 species approximately; 1 in India and 1 in Jammu and Kashmir: *Leucanthemum vulgare* Lam.

8. *Matricaria* L.
60 species approximately; 2 in India and 1 in Jammu and Kashmir: *Matricaria recutita* L.

9. *Soliva* Ruiz & Pav.
12 species approximately; 1 in India and 1 in Jammu and Kashmir: *Soliva anthemifolia* (A. Juss.) R. Br.

10. *Tanacetum* L.
70 species approximately; 12 in India and 9 in Jammu and Kashmir: *Tanacetum artemisioides* Sch.-Bip. ex Hook., *T. dolichophyllum* (Kitam.) Kitam., *T. falconeri* Hk. f., *T. fruticulosum* Ledeb., *T. gracile* Hk. f. & Thoms., *T. griffithii* (Clarke) Muradyan, *T. nanum* Clarke, *T. tibeticum* Hk. f. & Clarke, *T. tomentosum* DC.

11. *Waldheimia* Kar & Kir
8 species approximately; 5 in India and 5 in Jammu and Kashmir: *Waldheimia glabra* (Decne.) Regel, *W. nivea* (Hk.f. & Thoms. ex Clarke) Regel, *W. stoliczkai* (Clarke) Ostanf., *W. tomentosa* (Decne.) Regel, *W. vestita* (Hk.f. & Thoms.) Pamp.

DISCUSSION

The family Asteraceae shows a remarkable morphological diversity among angiosperms which ruled over 250 years as a basis on morphology for classification of various tribes. Therefore, in order to know about the tribes of this family, Anthemideae is selected to find out the diversity of species in the flora of Jammu and Kashmir State. The members of Asteraceae exceed over 1314 genera and 2100

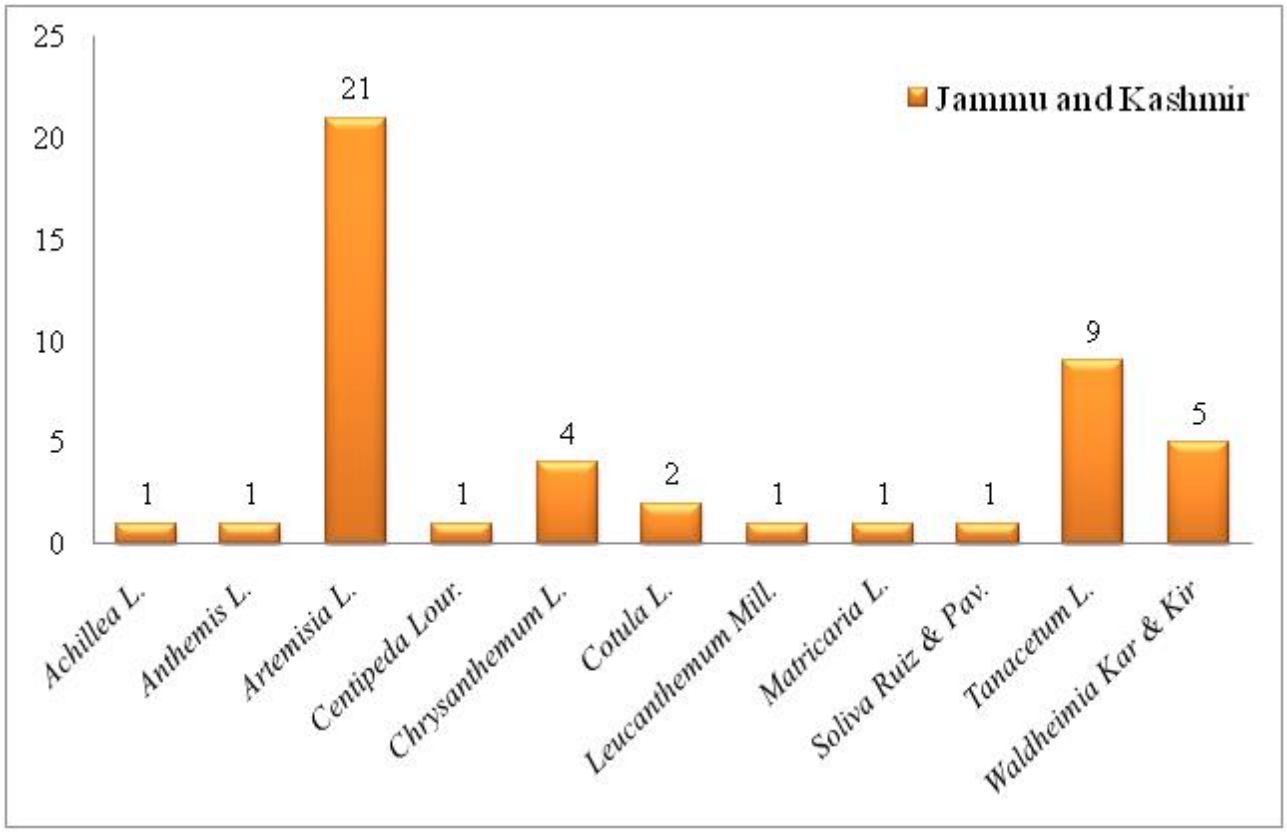


Fig. 1. Bar Graph represents number of species of tribe Anthemideae in Jammu and Kashmir

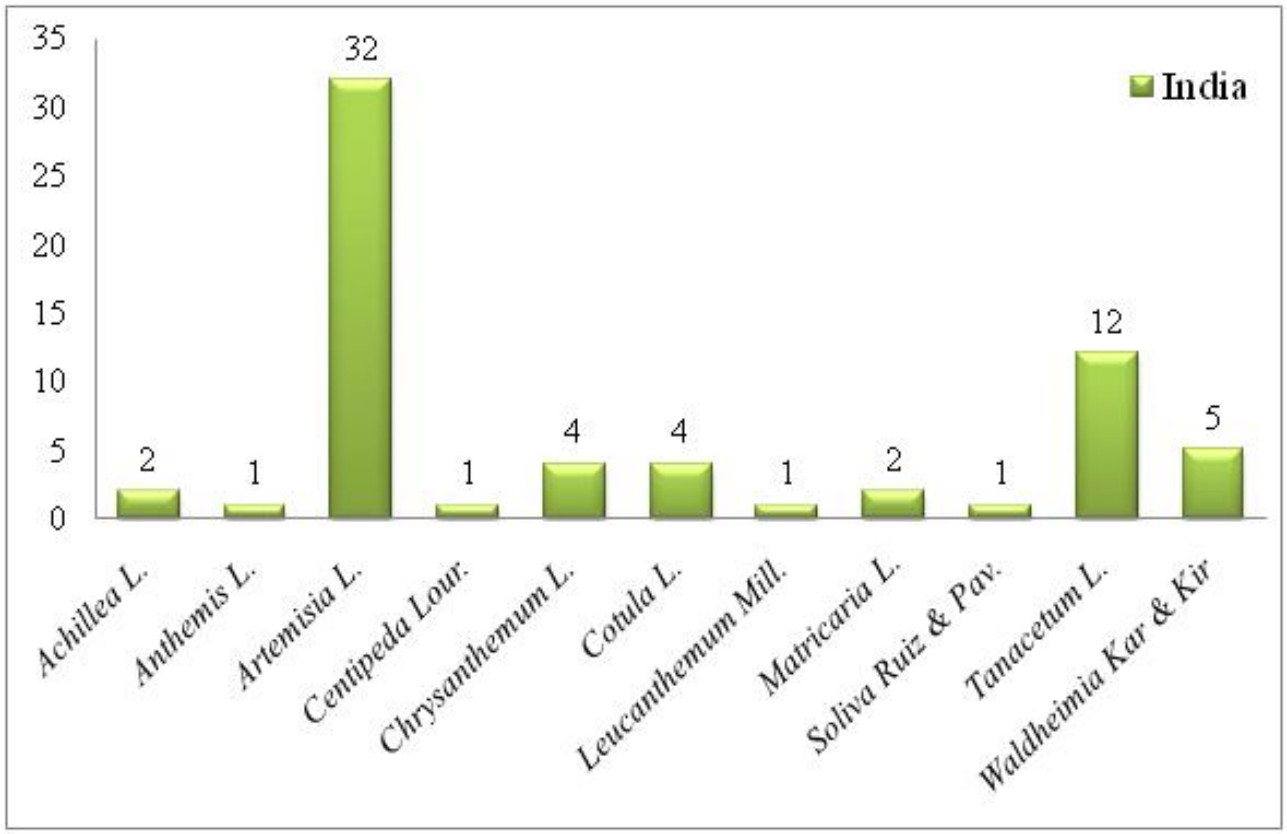


Fig. 2. Bar Graph represents number of species of tribe Anthemideae in India

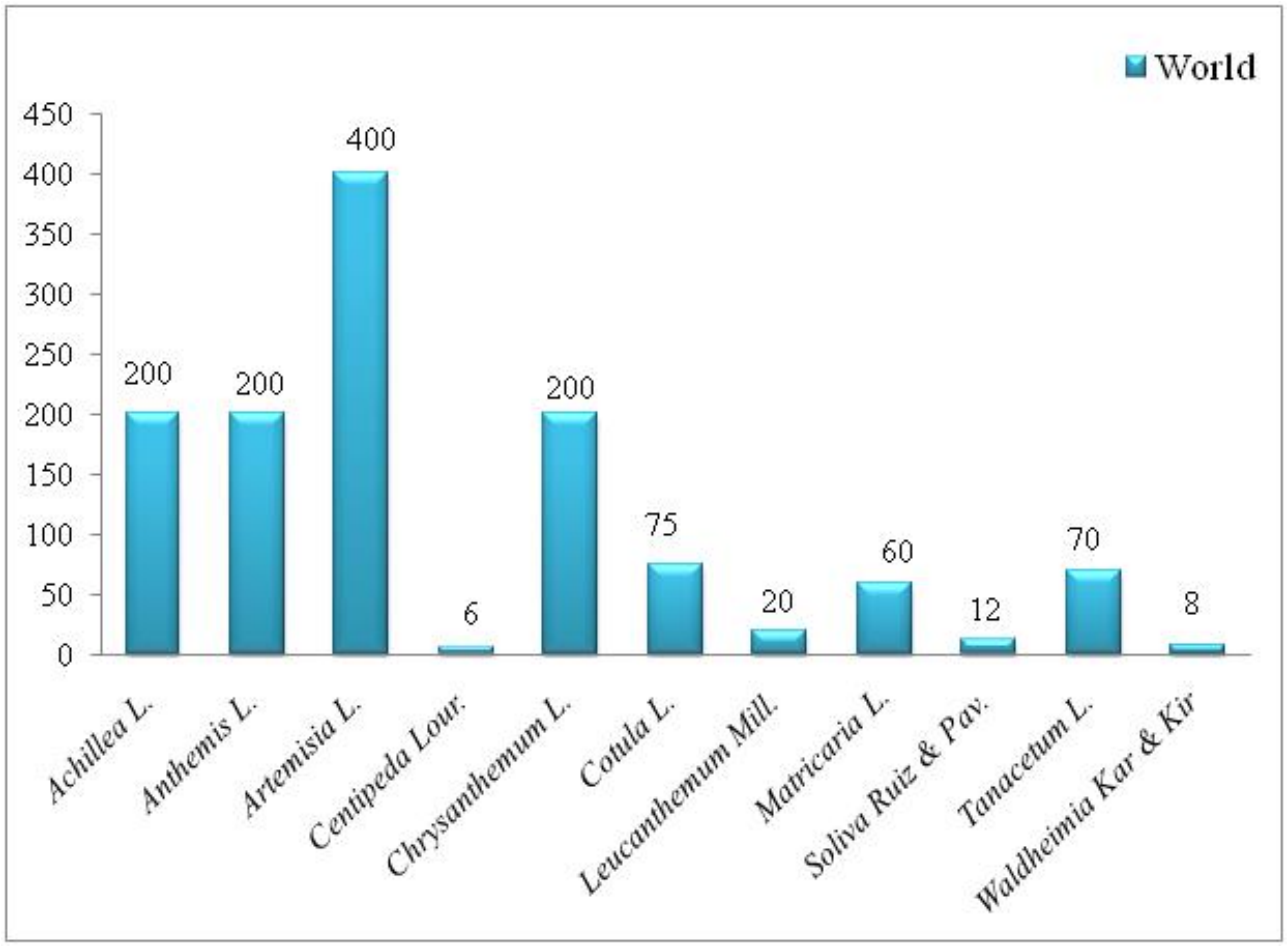


Fig. 3. Bar Graph showing global representation of species under each genus in tribe Anthemideae

species [1]. A single tribe of this family, anthemideae is comprised of 109 genera and 1740 species [2]. While publishing the Flora of India, family Asteraceae [4] described 66 species of the tribe anthemideae belonging to 12 genera from India. The family is of a great economic value and plays a significant role in various ailments [5].

Bhellum [3] enumerated 90 species of Asteraceae endemic to Kashmir Himalayas of Jammu and Kashmir State. The most dominant genus of this tribe in world, India and Jammu and Kashmir State is Artemisia L. Of the total 11 genera of tribe Anthemideae in Jammu and Kashmir, 5 genera in India and Jammu and Kashmir viz. *Anthemis* L., *Centipeda* Lour., *Chrysanthemum* L., *Leucanthemum* Mill. and *Soliva* Ruiz & Pav. are represented by a single species. All the 5 species *Waldheimia* (Fig. 2) of Indian flora are found in the flora of Jammu and Kashmir (Fig. 1) which indicates Anthemideae of J & K State is very rich.

As many as 9.42% species of tribe Anthemideae are endemic to Jammu and Kashmir Kashmir Himalayas. The global representation of 10 genera of this tribe is indicated in bar graph (Fig. 3).

CONCLUSION

Anthemideae comprising of 47 species belonging to 11 genera based on the collections from Jammu and Kashmir State. The author has enumerated 90 species of Asteraceae endemic to Kashmir Himalayas of Jammu and Kashmir.

ACKNOWLEDGEMENTS

The author is grateful to Prof. A. K. Koul Dean, Centre for Biodiversity, Baba Gulam Shah Badshah (BGSB) University Rajouri and Prof. Rani Magotra Department of Botany, University of Jammu, Jammu for encouragement.

TRANSPARENCY DECLARATION

The author declares no conflicts of interest.

REFERENCES

1. Mabberley DJ. 1987. The Plant Book. A Portable Dictionary of the Higher Plants. Cambridge Univ. Press Cambridge.
2. Bremer K. 1994. Asteraceae: Cladistics and classification. Timber Press, Portland, Oregon.
3. Bhellum BL. Endemic Species of Asteraceae in the Flora of Kashmir Himalayas, J & K State, India J Res Plant Sci. 2012; 2: 67-70.
4. Hajra PK, Rao RR, Singh DK and Uniyal BP. 1995. Flora of India. Vol. 12. BSI. Calcutta.
5. Hegnauer R. 1964. Chemotaxonomie der Pflanzen Vol. 3. Birkhauser Verlag, Basel & Stuttgart.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Nitrate-drought interactions on growth, osmoprotectants and antioxidant system of the oil accumulating desert plant jojoba

Fatma Ali Farghaly[1], Hanaa Kamal Galal[1,2], Abeer Ahmed Farag Radi[1], Refat Abdel-Basset[1]

[1]Botany Department, Faculty of Science, Assiut University, 71516 Assiut, Egypt

[2]Biological Science Department, Faculty of Science, King Abdull Aziz University, KSA

ABSTRACT

To cope better with desert harsh conditions or to improve their growth magnitude and productivity, jojoba plants might need some aid. One week drought, over a period of two months, was stressful as it induced severe growth inhibition compared with maintained 50% field capacity. In addition, jojoba sustained growth throughout the whole experimental period (4 months) relying on a minimum of nitrate (tap water or 25% Hoagland concentration). Extra nitrate (125%) was stimulatory to growth in full hydration and may overcome growth inhibition in partial hydration but not in drought. The osmoregulants (soluble sugars, proline and amino acids), membrane properties (leakage of K, electrolytes and UV absorbing metabolites as well as lipid peroxidation) and antioxidants (phenolics, catalase, guiacol peroxidase and superoxide dismutase activity) were assessed to evaluate drought stress impacts. They did not respond identically to drought leading to confusion which is most-expressing manifestation of drought stress. From which, enhanced proline accumulation and lipid peroxidation with inhibited ascorbate peroxidase displayed a clear cut and reliable stress biomarkers. The antioxidant phenolics increased with increasing nitrate in water-stressed jojoba leaves (50% field capacity or one week drought). Nitrate may participate in scavenging electrons since lipid peroxidation was lowered at high nitrate levels.

Key words: Drought; Jojoba; NRase; Proline; Phenolics; SOD.

J Biol Earth Sci 2013; 3(1): B30-B41

Corresponding author:
Refat Abdel-Basset
Botany Department, Faculty of Science, Assiut University, 71516 Assiut, Egypt
Tel: +20 88 2314386, Fax: +20 88 2342708
E-mail: rbasset@aun.edu.eg

Original Submission: 22 November 2012; Revised Submission: 18 December 2012; Accepted: 27 December 2012

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

Abbreviations:
FC - field capacity, OWD - one week drought, FW - fresh weight, DW - dry weight, EDTA - ethylenediamine tetraacetic acid, PVP - polyvinylpyrrolidone, APX - ascorbate peroxidase, SOD - superoxide dismutase, NRase - nitrate reductase

INTRODUCTION

The anticipated fuel crisis advocated finding out alternatives, preferably renewable and clean ones. Plant and microbial hydrocarbons may participate in narrowing the gap between production and needs. Jojoba shrubs got an abrupt importance in recent years because of their ability to accumulate waxes in their seeds (up to 50%); from which bio-diesel would be synthesized by alkylation. Substituting fossil fuels with methyl esters of jojoba oils, or of other plants implies no further accumulation in the atmosphere of greenhouse gases or sulfur contaminants. World production of jojoba oil is currently around 3,500 metric tons per year. The cosmetic industry appears to be the principal market for jojoba oil, equating to almost 80% of the total market, followed by pharmaceutical sector and lubricants [1].

Jojoba is a perennial shrub which does not require much care and produce well for decades after establishment. This plant is strong and tolerates harsh conditions of salinity, drought and heat. It grows in both arid and saline conditions [2, 3]. Indeed, shrubs of jojoba grow satisfactorily with brackish water having 2000 ppm concentration of salts in the Pacific Ocean and in California. Despite that, growth magnitude and its components were reported to vary with the intensity of drought stress [4]. In addition, severe drought has induced an extensive pulse of adult plant mortality over broad ranges of elevation and latitude across the southwest US [5-7].

Jojoba has been first introduced into the Egyptian agricultural map in 1990's around the city of Assiut (375 Km south of Cairo). Since a considerable proportion of the Egyptian soil (more than 90%) is desert with high temperature at days, inhabiting jojoba for oil production in the Egyptian part of the African Sahara is extremely preferable, not only for oil production but may also be protective against desertification. Mass cultivation of jojoba in the desert excludes competition with other crops on the very limited and crowded Nile valley and Delta. To cope better with harsh conditions in the desert or to improve their growth magnitude and productivity, jojoba plants might need some aid. In this work, we supplemented several nitrate concentrations at three levels of water availability. Growth, primary metabolites (osmoregulants), membrane properties and antioxidant enzymes of jojoba have been analyzed to assess nitrate–drought interactions. In addition, nitrate reductase has been followed to elucidate drought impact on nitrate reduction and vice versa i.e. nitrate availability to overcome drought stress.

MATERIALS AND METHODS

Plant growth and harvest

Jojoba (*Simmondsia chinensis* (Link) Schneider, Buxaceae) was grown in an outdoor green house at the Faculty of Science, Assiut University (Egypt) under natural conditions of temperature, humidity, light and day/night rhythm in the period of February – May, 2009. Ten seeds of jojoba were cultivated in pots containing 4 Kg acid washed sandy soil (suspended for 24 hrs in 0.1M HCl and rinsed several times with tap followed by distilled water until the pH becomes neutral). At this stage, nutrients content of the soil becomes trivial or negligible. After developing 4-5 true leaves (50 days later on planting), plants were subdivided into three subsets. The first (control) was maintained at 100% FC (Field Capacity) using full strength modified Hoagland solution [8]. Two other subsets were, since then subjected to partial or complete drought; the first by daily compensation to 50% of the field capacity (50% FC) and the other to one week drought (OWD) by withholding irrigation for one week and then re-watering up to full field capacity (OWD) at due times.

Stocks of Hoagland medium contain $Ca(NO_3)_2$ and KNO_3; the culture medium supplemented to plants contains 0.84 mM $Ca(NO_3)_2$ and 2mM KNO_3 giving a final concentration of 3.68mM NO_3^-. Lower percentages of 75%, 50% and 25% and the higher percentage of 125% were constructed by decreasing or increasing the concentration of both salts containing nitrate [$Ca(NO_3)_2$ and KNO_3]. Ca^{2+} and K^+ were compensated by equimolar concentrations of $CaCl_2$ and KCl, respectively.

Analytical Methods

At the end of the experimental period (fifty days after treatments application), plants become about 4

months old. Fresh plants (three replicates, 6-7 plants per each replicate) were separated into shoots and roots; their fresh weights (FW) were recorded, and the samples were then oven dried at 70ºC for 48 hrs to determine their dry weight (DW). Some other leaves were freshly frozen in liquid nitrogen and stored at -80ºC for assaying the activity of some selected enzymes.

Determination of photosynthetic pigments

The pigment fractions (chlorophyll a, chlorophyll b and the carotenoids) were estimated in 95% ethanol extracts at 60ºC following the spectrophotometric method (Unico UV-2100 spectrophotometer) recommended by Lichtenthaler [9].

Assessment of membrane stability properties

A number of criteria have been used to evaluate membrane stability and integrity. Electrolyte leakage was carried out in leaf discs as given by Lutts et al. [10]. Potassium leakage was determined in the same solution of conductivity measurements using the flame photometric method of Williams and Twine [11]. The leakage of UV absorbing metabolites was also assessed according to Navari-Izzo et al. [12]. The data of K and UV leakage are given as percentage of components leaked out of leaf discs in 24 hrs relative to total cellular contents after autoclaving the same discs. Lipid peroxidation was determined according to Madhava Rao and Sresty [13] and the concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Determination of some metabolites acting as osmoregulants (soluble sugars, proline and free amino acids)

The anthrone sulphuric acid method [14] was used for the determination of soluble sugars. Proline contents were determined according to Bates et al. [15] in fresh samples of leaves and roots. Free amino acids and soluble proteins were determined in dry mass extracts according to the methods of Moore and Stein [16] and Lowry et al. [17]; respectively. Calibration curves using glucose (soluble sugars), proline (proline), glycine (amino acids), and bovine serum albumin (soluble proteins) were constructed. Free and cell wall-bound phenolics were determined according to Kofalvi and Nassuth [18] in 50% methanol extracts of fresh leaves using the Folin-Ciocalteu's phenol reagent. A calibration curve using galic acid was done.

Preparation of enzyme extracts and assay of enzyme activities

Leaf tissues (0.5 g) were ground to a fine powder in liquid nitrogen and then homogenized in 5 ml of 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA and 0.1 g PVP. The homogenate was centrifuged at 12000 xg for 10 min at 4ºC, the supernatants were collected and used for enzyme assays. All enzyme activities were assayed at 25ºC using a UV visible Unico UV-2100 spectrophotometer. The specific activity of the enzymes was expressed as mmole mg^{-1} protein min^{-1}. Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed as described by Misra and Fridovich [19]. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to the method of Nakano and Asada [20]. Guaiacol peroxidase (POD, EC 1.11.1.7) activity was assayed by following the method of Tatiana et al. [21]. Catalase (EC 1.11.1.6) activity was assayed by following the consumption of H_2O_2 for 1 min [22]. Nitrate reductase activity (NR, EC 1.6.6.1) was assayed similar to that of Heuer and Plaut [23] and modified by Sym [24]. A standard curve using $NaNO_2$ has been conducted.

Statistical analysis

All data obtained have been subjected to one-way analysis of variance (ANOVA) using the SPSS statistical package. For comparison of means, the Duncan's multiple range tests ($p < 0.05$) were used. Also, factorial ANOVA analysis was performed to partition the variance into the main effects of nitrate, drought as well as their interaction using the MSTATC test.

RESULTS

Growth

Fully irrigated jojoba plants exhibited significantly higher fresh weight of shoots than 50% FC or OWD plants (Fig. 1); both treatments similarly inhibited about 30% of shoot fresh weight. Extra nitrate (125%) significantly enhanced the fresh weight of shoots at all water regimes; enhancement was proportional with water level supplied. Dry mass of jojoba shoots (Fig. 2) responded to nitrate and water supply quite similar to their fresh weight. However, 50% FC exhibited higher values of dry

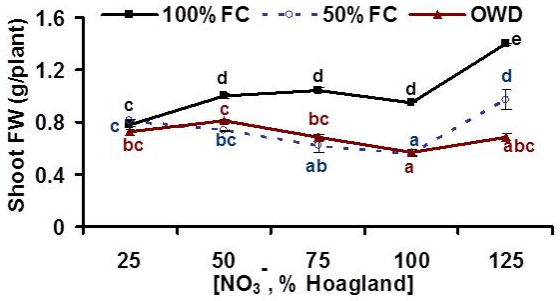


Fig. 1. Fresh weight of jojoba shoots as influenced by drought-nitrate interactions for 2 months at Assiut University natural conditions. Presented data are means of three replicates ± SE (n = 3). Different letters indicate statistical significance at P < 0.05 between, controls (100% FC "field capacity"), 50% FC and one week drought (OWD); respectively. The following caption is applied for all figures 100%FC 50%FC OWD

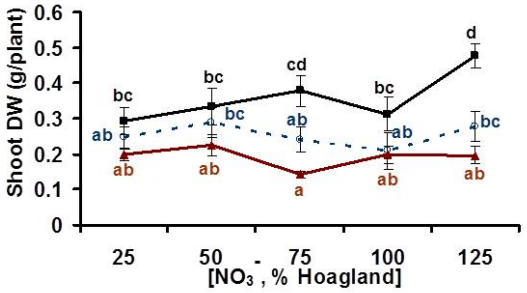


Fig. 2. Dry weight of jojoba shoots as influenced by drought-nitrate interactions for 2 months at Assiut University natural conditions. Presented data are means of three replicates ± SE (n = 3). Different letters indicate statistical significance at P < 0.05 between, controls (100% FC "field capacity"), 50% FC and one week drought (OWD); respectively.

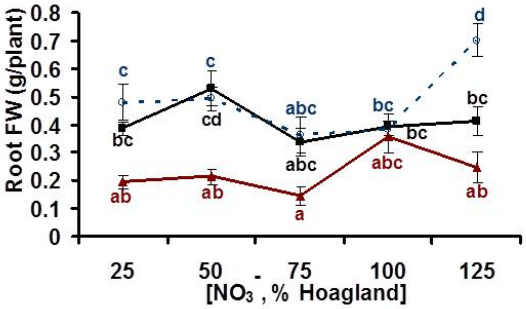


Fig. 3. Fresh weight of jojoba roots as influenced by drought-nitrate interactions for 2 months at Assiut University natural conditions. Presented data are means of three replicates ± SE (n = 3). Different letters indicate statistical significance at P < 0.05 between, controls (100% FC "field capacity"), 50% FC and one week drought (OWD); respectively.

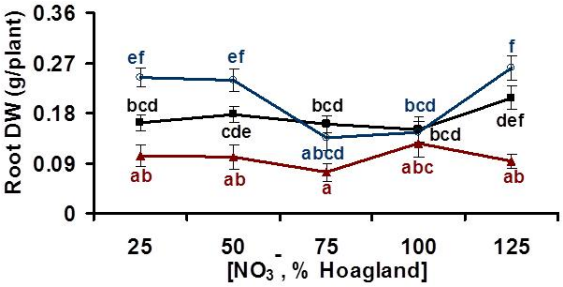


Fig. 4. Dry weight of jojoba roots as influenced by drought-nitrate interactions for 2 months at Assiut University natural conditions. Presented data are means of three replicates ± SE (n = 3). Different letters indicate statistical significance at P < 0.05 between, controls (100% FC "field capacity"), 50% FC and one week drought (OWD); respectively.

mass than OWD. Roots, unlike shoots, exhibited similar fresh weights in control and 50% FC plants, both of them were significantly higher than those of OWD plants (Fig. 3). Dry mass of control roots become less than those of 50% FC indicating higher water content (Fig. 4). Extra nitrate (125%) significantly stimulated root growth (fresh and dry mass) at 100% FC and 50% FC but not in OWD plants.

Total pigments (chlorophylls a & b as well as the carotenoids) have been poorly varied in response to water limitation (Fig. 5). Nitrate tended to decrease pigment contents, depending on water status, however. Fully irrigated (control), OWD and 50% FC plants exhibited their highest pigment contents at 100%, 50% and 25% nitrate; respectively. It seems that decreasing water availability decreased nitrate levels at which pigment contents become highest.

Membrane Properties

Several means (leakage of electrolytes, potassium and UV absorbing metabolites) have been assessed to evaluate membrane properties in variously treated jojoba leaves. Electrolyte leakage of control and 50% FC plants was not significantly affected by water availability; only OWD significantly enhanced electrolyte leakage at lowest nitrate levels (to conserve space data not shown). K leakage out of leaf discs was minimally altered by water status at which jojoba was grown (Fig. 6).

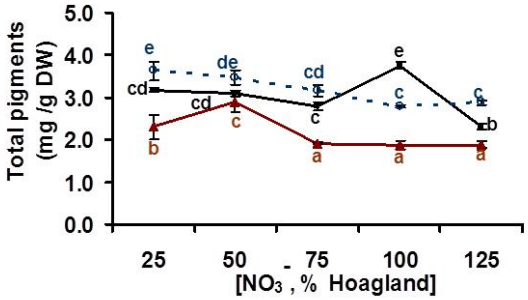


Fig. 5. Total pigments (chlorophyll a, b and carotenoids) of jojoba plants as influenced by drought-nitrate interactions for 2 months at Assiut University natural conditions. Presented data are means of three replicates ± SE (n = 3). Different letters indicate statistical significance at P < 0.05 between, controls (100% FC "field capacity"), 50% FC and one week drought (OWD); respectively.

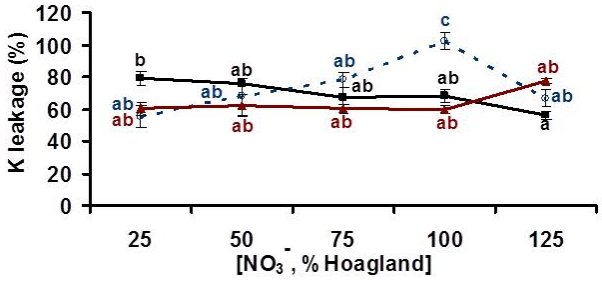


Fig. 6. Potassium leakage of jojoba as influenced by drought-nitrate interactions for 2 months at Assiut University natural conditions. Presented data are means of three replicates ± SE (n = 3). Different letters indicate statistical significance at P < 0.05 between, controls (100% FC "field capacity"), 50% FC and one week drought (OWD); respectively.

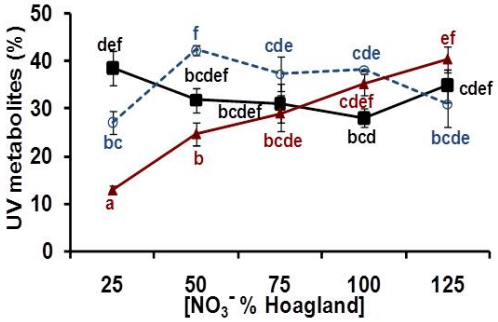


Fig. 7. UV absorbing metabolites of jojoba as influenced by drought-nitrate interactions for 2 months at Assiut University natural conditions. Presented data are means of three replicates ± SE (n = 3). Different letters indicate statistical significance at P < 0.05 between, controls (100% FC "field capacity"), 50% FC and one week drought (OWD); respectively.

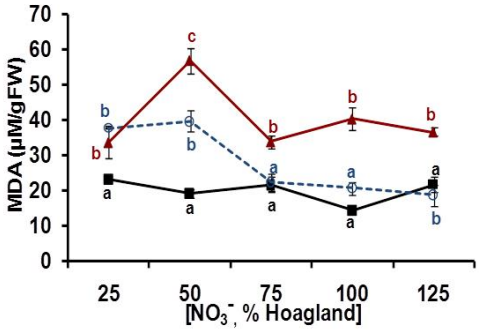


Fig. 8. Malondialdehyde contents of jojoba as influenced by drought-nitrate interactions for 2 months at Assiut University natural conditions. Presented data are means of three replicates ± SE (n = 3). Different letters indicate statistical significance at P < 0.05 between, controls (100% FC "field capacity"), 50% FC and one week drought (OWD); respectively.

However, nitrate (up to 100%) was stimulatory to K leakage at 50% FC while inhibitory in control plants. The efflux of the UV absorbing metabolites from jojoba leaves exhibited continuous and significant rise with increasing nitrate in one week drought-stressed plants (OWD). In control, as well as in 50% FC plants, leakage of UV absorbing metabolites was minimally altered; so were those of 50% FC in most cases (Fig. 7).

Lipid peroxidation

Malondialehyde (MDA), the indicator of lipid peroxidation and membrane damage, was also followed in jojoba leaves (Fig. 8). Lipid peroxidation was significantly affected by drought; OWD induced the highest levels of MDA compared with 50% FC plants or least at controls. Increasing supplemental nitrate was of insignificant effect on MDA accumulation in control plants.

Osmoregulants

Soluble sugars: Leaf soluble sugars after one week drought were highest whereas those of 50% FC were lowest (data not shown). Varying nitrate levels (50 - 100%) induced significantly little variation in soluble sugars. Extra nitrate (125%) lowered soluble sugars at all water regimes whereas the lowest nitrate concentration significantly altered them. In roots, soluble sugars were significantly accumulated under inadequate water supply (highest at 50% FC followed by OWD) at low nitrate levels of 25% and 50% (Fig. 9). Fully irrigated

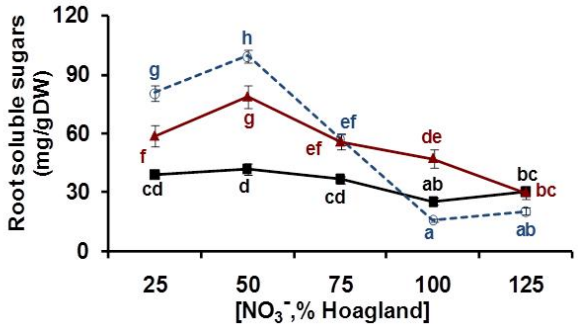


Fig. 9. Soluble sugar contents of jojoba roots as influenced by drought-nitrate interactions for 2 months at Assiut University natural conditions. Presented data are means of three replicates ± SE (n = 3). Different letters indicate statistical significance at P < 0.05 between, controls (100% FC "field capacity"), 50% FC and one week drought (OWD); respectively.

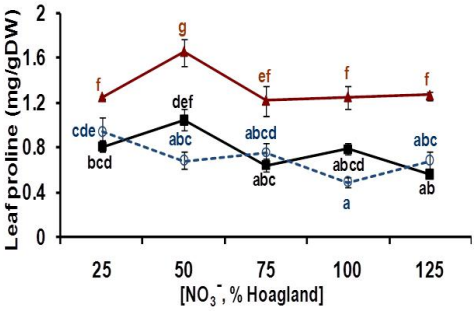


Fig. 10. Proline contents of jojoba leaves as influenced by drought-nitrate interactions for 2 months at Assiut University natural conditions. Presented data are means of three replicates ± SE (n = 3). Different letters indicate statistical significance at P < 0.05 between, controls (100% FC "field capacity"), 50% FC and one week drought (OWD); respectively.

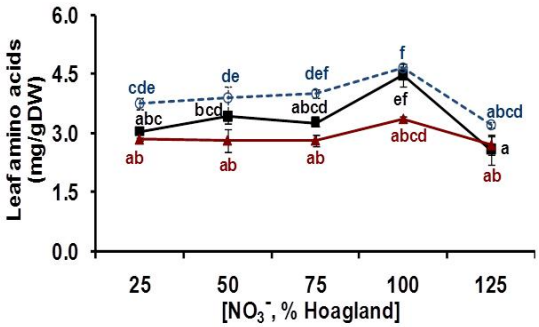


Fig. 11. Free amino acid contents of jojoba leaves as influenced by drought-nitrate interactions for 2 months at Assiut University natural conditions. Presented data are means of three replicates ± SE (n = 3). Different letters indicate statistical significance at P < 0.05 between, controls (100% FC "field capacity"), 50% FC and one week drought (OWD); respectively.

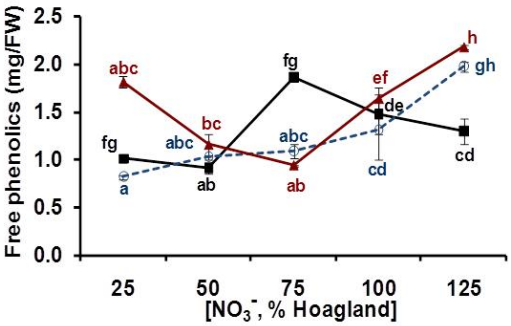


Fig. 12. Free phenolics in jojoba as influenced by drought-nitrate interactions for 2 months at Assiut University natural conditions. Presented data are means of three replicates ± SE (n = 3). Different letters indicate statistical significance at P < 0.05 between, controls (100% FC "field capacity"), 50% FC and one week drought (OWD); respectively.

(control) plants did not display significant alteration of root soluble sugars in response to nitrate concentration. Generally, soluble sugars were lowered by increasing nitrate.

Proline: One week drought (OWD) induced significantly highest leaf proline contents. Control (100% FC) and 50% FC leaves displayed similarly and significantly lower levels than OWD plants (Fig. 10). Thus, 50% FC was not stressful enough as it did not enhance proline accumulation over the control content. Increasing supplemental nitrate was of slightly decreasing impact on proline accumulation in variously irrigated plants. Root proline, however, exhibited markedly less obvious responses to drought and nitrate concentration compared with the leaves' proline contents (data not shown).

Free amino acids: Drought imposition for one week significantly depressed free amino acid levels in jojoba leaves to be least whereas 50% FC elevated them even relative to those of the control (Fig. 11). Amino acid contents have been significantly increasing as supplemented nitrate was increased up to 100% in control and 50% FC plants. Extra nitrate (125%) did not induce further accumulation of free amino acids; otherwise it was relatively suppressive. However, in roots of fully irrigated plants, amino acids continued to increase by increasing the concentration of external nitrate (data not shown). One week drought imposed the opposite whereas 50% FC did not alter amino acid contents significantly.

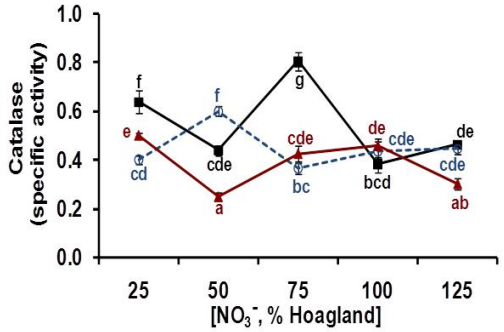


Fig. 13. Specific activity of catalase in jojoba as influenced by drought-nitrate interactions for 2 months at Assiut University natural conditions. Presented data are means of three replicates ± SE (n = 3). Different letters indicate statistical significance at P < 0.05 between, controls (100% FC "field capacity"), 50% FC and one week drought (OWD); respectively.

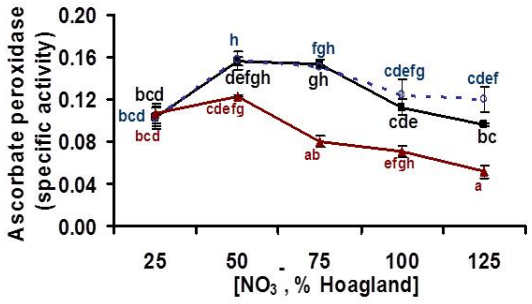


Fig. 14. Specific activity of ascorbate peroxidase in jojoba as influenced by drought-nitrate interactions for 2 months at Assiut University natural conditions. Presented data are means of three replicates ± SE (n = 3). Different letters indicate statistical significance at P < 0.05 between, controls (100% FC "field capacity"), 50% FC and one week drought (OWD); respectively.

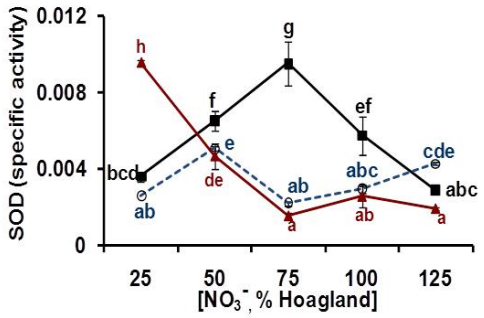


Fig. 15. Specific activity of superoxide dismutase in jojoba as influenced by drought-nitrate interactions for 3 months at Assiut University natural conditions. Presented data are means of three replicates ±SE (n= 3). Different letters indicate statistical significance at P < 0.05 between, controls (100% FC "field capacity"), 50% FC and one week drought (OWD); respectively.

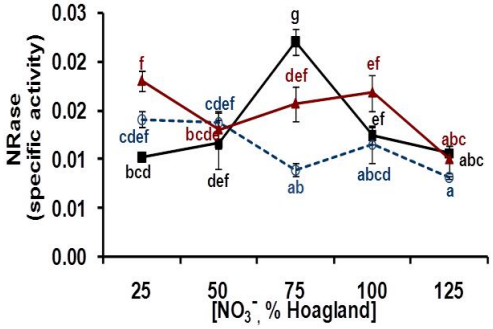


Fig. 16. Specific activity of nitrate reductase in jojoba as influenced by drought-nitrate interactions for 2 months at Assiut University natural conditions. Presented data are means of three replicates ±SE (n= 3). Different letters indicate statistical significance at P < 0.05 between, controls (100% FC "field capacity"), 50% FC and one week drought (OWD); respectively.

Antioxidant system

Phenolic compounds

In fully irrigated plants (control) free phenolic contents have been increasing as supplemental nitrate increased until a peak at 75% nitrate while decreasing thereafter (Fig. 12). An opposite attitude has been recorded in OWD plants. At 50% FC, contents of free phenolic compounds continually increased with the rise of supplemental nitrate. Bound phenolics were generally of higher levels than those of the free fraction. However, the applied treatments did not cause considerable alterations compared with those in the free phenol fraction (data not shown).

Enzymes

Catalase: Catalase activity has not been noticeably affected by varying levels of provided water; so was mostly the effect of nitrate (Fig. 13). However, the activity of catalase was enhanced only in moderate nitrate concentrations (50% & 75% in 50% FC and 100% FC; respectively) whereas highest concentrations (100% & 125%) looked slightly inhibitory.

Guiacol Peroxidase: Guiacol peroxidase activity displayed attitudes more or less similar to those of catalase activity in response to water and nitrate availability (data not shown).

Ascorbate peroxidase (APX): Ascorbate peroxidase activity has been significantly inhibited in OWD

plants relative to either the control or 50% FC plants which exhibited comparable rates (Fig. 14). Such inhibition of ascorbate peroxidase by drought is a conspicuous difference between ascorbate peroxidase and other antioxidant enzyme systems (namely, catalase and Guiacol peroxidase). Increasing nitrate concentrations more than 75% seems inhibitory to ascorbate peroxidase, highest in OWD plants and least in 50% FC.

Superoxide dismutase (SOD): Superoxide dismutase activity has been variably altered by nitrate concentrations provided depending on the water status at which plants were grown. It exhibited highest rates of activity in fully irrigated plants while lowest rates in 50% FC and OWD plants, both at 75% nitrate (Fig. 15).

Nitrate reductase (NRase)

Nitrate reductase activity of variously treated jojoba plants were not exhibiting markedly altered rates in response to varied water levels (Fig. 16). Nitrate at concentrations of 50-100% was stimulatory to NRase activity in fully irrigated as well as in OWD plants. Highest nitrate concentration of 125% was mostly inhibitory at all water regimes. Increasing nitrate supplementation was almost depressing to NRase activity in 50% FC plants.

ANOVA factorial analysis

Factorial analysis (Table 1) revealed that nitrate, drought as well as their interaction exerted significant effects on almost all parameters examined in this study. Few exceptions of non-significant effects of nitrate on shoot dry mass and leakage, and the interaction on shoot dry mass, root fresh mass and leaf amino acids were resulted.

Table 1. Two-way ANOVA to determine the influence of nitrate, drought and their interaction on jojoba according to MSTATC test.

Parameter	Treatments		
	Nitrate	Drought	Interaction
Shoot Fresh weight	19.20**	71.232**	9.070**
Shoot Dry weight	1.950^{NS}	22.349**	1.345^{NS}
Root Fresh weight	2.768*	18.368*	1.858^{NS}
Root Dry weight	4.324**	30.994*	2.408*
Total pigments	13.858*	79.019*	16.791*
K Leakage	1.317^{NS}	3.162*	4.334**
UV metabolites	2.788*	4.374*	4.708**
MDA	3.601*	40.338**	7.702**
Root soluble Sugars	100.887**	55.715**	22.641**
Leaf Proline	5.499**	66.363**	2.722*
Leaf amino acids	12.209**	20.503**	0.981^{NS}
Free phenolic	29.541**	21.837**	15.904**
Catalase	13.101**	40.866**	25.77**
Ascorbate peroxidase	27.659**	52.926**	5.658**
SOD	10.350**	22.90**	28.181**
NRase	6.899**	7.029**	7.136**

* = Significant ** = Highly significant NS = None-significant

DISCUSSION

The oil accumulating plants are acquiring great attention because of the foreseen fuel and energy crisis. Combustion of plant oils, furthermore, do not add to the atmosphere extra oxide emissions of carbon, nitrogen or sulfur. It is well known that shortage of irrigation or precipitation is one of the main factors limiting crop production in arid and semiarid soils. Jojoba (*Simmondsia chinensis* (Link) Schneider, Buxaceae) seems fascinating because it tolerates harsh environmental conditions (edaphic and atmospheric). Furthermore, jojoba may better cope with high light under drought conditions [25]. Jojoba plants have exceptionally deep tap root system that helps to survive in drought conditions. Despite such resistance, severe drought induced an extensive pulse of adult mortality over broad ranges of elevation and latitude across the southwest US [5-7]. Also, salinity was reported to cause reduction of the elongation and the thickening of stems [26, 27], the reduction of the shoot system and leaf size as well as an increase in the thickening of leaves [28]. Salinity, in addition, also influenced cutinisation, reduced the development of vascular tissues and increased the density of the trichomes [29] and the chemical composition of fatty acids and fatty alcohols in the grains of jojoba [30]. For normal growth and development of aerial plant parts, proper and suitable environment must be provided to the root system [31].

In this work, growth of jojoba was not significantly inhibited by maintaining water supply at 50% field capacity (50% FC), confirming the resistance of the shrub to water deficiency. One week drought (OWD), however, significantly dropped the fresh and dry mass. Similar observations have been recorded by other authors [32]. However, drought did not impose significant differences in pigment contents under varied water statuses; as it has been also reported in olive [33, 34]. As well, jojoba plants could survive relying on a minimum of supplemental nitrate for an extended period of time (4 months). Extra nitrate (125%) relatively improved plants response; it was significantly stimulatory to shoot and root growth (FW & DW) in control and 50% FC but not in OWD plants. The number of leaves per plant was significantly increased with increasing supplemental nitrate in 50% FC plants only (data not shown). Rates of NRase activity were not dropped in OWD plants, indicating that reduced nitrogen was not limiting to growth of jojoba plants.

Diversion of metabolites to accumulate osmoregulants might enable plants to withstand desiccation. This process, however, takes place at the expense of primary metabolism and production. Restoration of normal metabolism would save energy for growth and production. Proline accumulation that is frequently used as a stress bio-marker has been also found so in jojoba [35]. Only OWD induced proline accumulation whereas 50% FC did not, coinciding with attitudes of fresh and dry mass. It can be inferred, then, that only OWD was stressful to jojoba plants but the maintained 50% FC was not. Soluble sugars in relation to nitrate displayed the conventional C/N negative relationship i.e. increasing nitrogen decreases sugars and carbohydrates, most obvious under water stress [36]. Nitrogen metabolism is usually impaired by drought [37]. Free amino acids dropped by water deficiency but increased by nitrate mostly opposite to proline.

Biotic and abiotic stresses are known to induce the formation of reactive oxygen species (ROS). These exert harmful effects such as lipid peroxidation and protein and DNA oxidation, leading in many cases to cell death [38]. Despite that, the biosynthesis of ROS cannot be completely eliminated due to their function as signaling molecules, being important parts of plant defense against stresses [39]. Negative impacts of ROS as well as plant defense mechanisms are concerned with in this work. Plasmalemma and organelle membranes are a prime site of damage by ROS produced by drought and other stresses. Response to limited water supply confirmed the settled notion that jojoba is genetically resistant. Drought did not significantly change membrane properties in terms of leakage (K, electrolytes and UV absorbing metabolites) whereas tended to increase by nitrate. The metabolites absorbing the UV spectra comprise proteins, organic acids and nucleic acids that their leak would starve the cells. Lipid peroxidation, unlike leakage properties, was severely enhanced in OWD plants i.e. lipid peroxidation and leakage were not coinciding. A similar observation has been reported in cucumber roots exposed to 1 µM $Al_2(SO_4)_3$ [40]. MDA, a biomarker for lipid peroxidation and membrane damage, increases with environmental stresses [41]. MDA pools, however, decreased at high nitrate levels. Nitrate, in

this concert, can act as a major sink scavenging electrons, indirectly protecting membranes.

Plants developed enzymatic as well as non-enzymatic antioxidant defenses that work efficiently to reduce excess ROS production and hence its subsequent damaging effect. Phenolic compounds (bound and free), have been enhanced up to similar levels by increasing nitrate under drought (partial or full). The importance of phenolics as antioxidant compounds have been reported in variously stressed plants [42].

A negative relationship between SOD, CAT and GPX activity and lipid peroxidation or MDA content has been described [41, 43]. The various antioxidant enzymes (CAT, GPX and SOD) assayed in jojoba plants were mostly decreased by nitrate while minimally enhanced by drought. Specifically, ascorbate peroxidase gave conclusive indication as a stress biomarker. It is severely inhibited by drought and such inhibition was increased by increasing nitrate.

CONCLUSIONS

- Maintaining soil water content at 50% field capacity was much less drastic to jojoba than supplying 100% FC following one week drought.
- Enhanced proline accumulation, lipid peroxidation as well as inhibited ascorbate peroxidase exhibited conclusive response and can be used as biomarkers for drought stress in jojoba. Other osmoregulants, membrane damage properties and antioxidant enzymes were not decisive in their response to drought.
- Jojoba survived harsh conditions in agreement with previous reports. Growth and production magnitudes, however, might deter by diversion of energy and metabolites into survival metabolism (accumulation of osmoregulants, antioxidant system and membrane repair mechanisms).
- Nitrate as an electron sink may be considered part of the non-antioxidant system.

ACKNOWLEDGEMENTS

Sincere thanks are due to Dr. T. Ramadan (Professor of Plant Ecology, Botany Department, Faculty of Science, Assiut University, 71516 Assiut Egypt) for assistance in factorial analysis.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Bashir MA, Ahmad M, Anjum M.A. Effect of various potting media on growth of rooted jojoba (*Simmondsia chinensis*) cuttings. Int J Agric Biol. 2007; 9: 147–151.
2. Jensen WA, Salisbury FB. 1988. Botanica. 2nd ed. Libras McGraw-Hill de Mexico, Mexico. pp: 722.
3. Brown CA, Palzkill D, Whittaker C. 1996. The jojoba industry 1994, a status and up date. In: Princen LH, Rossi C (Eds.), Proceedings of the Ninth International Conference on New Industrial Crops and Products, 25-30 September 1994, Catamarca Argentina. 150-154.
4. Osman HE, Abo-Hassan AA. Effect of drought stress on flowering and on seed yield and its components in jojoba *Simmondsia chinesis* (Link Schneider) established in Western Saudi Arabia. IKAU Met Env Arid Land Agric Sci. 2002; 8: 121-131.
5. Bowers JE. Effects of drought on shrub survival and longevity in the northern Sonoran Desert. J Torrey Bot Soc. 2005; 132: 421-431.
6. Breshears DD, Cobb NS, Rich PM, Price KP, Allen CD, Balice RG, Romme WH, Kastens JH, Floyd ML, Belnap J, Anderson JJ, Myers OB, Meyer CW. Regional vegetation die-off in response to global-changetype drought. Proceedings of the National Academy of Sciences of the United States of America 2005; 102: 15144–15148.
7. Miriti MN, Rodriguez-Buritica S, Wright SJ, Howe HF. Episodic death across species of desert shrubs. Ecology. 2007; 88: 32-36.
8. Hoagland DR, Arnon DI. The water-culture method for growing plants without soil. Cal Agric Exp Sta Cir. 1950; 347: 1-32.
9. Lichtenthaler HK. 1987. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. In: Methods in Enzymology, vol. 148: 183-350. Academic Press, Orlando, USA.
10. Lutts S, Kinet JM, Bouharmont J. NaCl–induced senescence in leaves of rice (*Oryza sativa* L.) cultivars differing in salinity resistance. Ann Bot. 1996; 78: 389-398.
11. Williams V, Twine S. 1960. Flame photometric method for sodium, potassium and calcium. In: modern methods of plant analysis. Eds. Peach K. and Tracey MV. Springer-Verlag Berlin. 56, 6-12.
12. Navari-Izzo F, Izzo R, Quartacci MF, Lorenzini G. Growth and solute leakage in *Hordeum vulgare* exploded to long-term fumigation with low concentration of CO_2. Physiol Plant. 1989; 76: 445-

450.

13. Madhava Rao KV, Sresty TVS. Antioxidative parameters in the seedlings of pigeonpea (*Cajanus cajan* L. Millspaugh) in response to Zn and Ni stresses. Plant Sci. 2000; 157: 113-128.
14. Fales FW. The assimilation and degradation of carbohydrates by yeast cells. J Biol Chem. 1951; 193: 113-118.
15. Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water stress studies. Plant Soil. 1973; 39: 205-207.
16. Moore S, Stein WW. Amino acid free photometric ninhydrin method for use in chromatography of amino acids. J Biol Chem. 1948; 176: 367-388.
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with folin phenol reagent. J Biol Chem. 1951; 193: 265-275.
18. Kofalvi SA, Nassuth A. Influence of wheat streak mosaic virus infection phenyl propanoid metabolism and the accumulation of phenolics and lignin in wheat. Physiol Mol Plant Pathol. 1995; 47: 365-377.
19. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem. 1972; 247: 3170-3175.
20. Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 1981; 22: 867-880.
21. Tatiana Z, Yamashita K, Matsumoto H. Iron deficiency induced changes in ascorbate content and enzyme activities related to ascorbate metabolism in cucumber roots. Plant Cell Physiol. 1999; 40: 273-280.
22. Aebi H. 1984. Catalase. In: Methods in Enzymology (ed.1. Paker), vol. 105: 121-126. Academic Press, Orlando, USA.
23. Heuer B, Plaut Z. Reassessment of the in vivo assay for nitrate reductase in leaves. Physiol Plant. 1978; 43: 306-212.
24. Sym GJ. Optimization of the in vivo assay conditions for nitrate reductase in barely. J Sci Food Agric. 1983; 35: 725-730.
25. Hamerlynck EP, Huxman TE. Ecophysiology of two sonoran desert evergreen shrubs during extreme drought. J Arid Environ. 2009; 73: 582-585.
26. Bartolini G, Mazuelos C, Troncoso A. Influence of Na_2S0_4 and NaCl salts on survival, growth and mineral composition of young olive plants in inert sand culture. Adv Hortic Sci. 1991; 5: 73-76.
27. Franco JA, Esteban C, Rodriguez C. Effects of salinity on various growth stages of muskmelon cv Revigal. J Hortic Sci. 1993; 68: 899-904.
28. Botti CI. 1996. Evaluacion de clones seleccionados de jojoba en ensayos realizodos en chile desan-ollo y rendimiento de semilla. Resistencia en campo a condiciones salinas. In: Proc. Reunion Internacional, 29-30 October 1996, Coquimbo, Chile, pp. 1-10.
29. Botti C, Palzkill D, Prat L. Morphological and anatomical characterization of six jojoba clones at saline and non-saline sites. Industrial Crops Products. 1988; 9: 53-62.
30. Kayani SA, Nakvi HR, Ting IP, Kumamoto J. Effect of Salinity on Fatty Acid and Fatty Alcohol Composition during the Germination of Jojoba (*Simmondsia chinensis*) Seeds. J Agric Food Chem. 1990; 38: 1269-1271.
31. Wazir MG, Amin NU, Khan I, Khan MI. Effects of different potting mixtures and nitrogen source on the performance of Brassaia seedlings-II. Sarhad J Agric. 2004; 20: 25-31.
32. Benzioni A, Nerd A. Effect of irrigation and fertilization on vegetative growth and yield of jojoba in relation to water status of the plant population. Appl Res Inst. 1985; 44: 1-18.
33. Galmes J, Abadia A, Cifre J, Medran OH, Flexas J. Photoprotection processes under water stress and recovery in Mediterranean plants with different growth forms and leaf habits. Physiol Plant. 2007; 130: 495-510.
34. Roussos AP, Kleio Denaxa N, Damvakaris T, Stournaras V, Argyrokastritis IA. Effect of alleviating products with different mode of action on physiology and yield of olive under drought. Sci Horticult. 2010; 125: 700-711.
35. Roussos PA, Pontikis CA. Long term effects of sodium chloride salinity on growing in vitro, proline and phenolic compound content of jojoba explants. Eur J Hort Sci. 2003; 68: 38–44.
36. Remans T, Nacry P, Pervent M, Girin T, Tillard P, Lepetit M, Gojon A. A central role for the nitrate transporter NRT2.1 in the integrated morphological and physiological responses of the root system to nitrogen limitation in *Arabidopsis*. Plant Physiol. 2006; 140: 909–921.
37. Fresneau C, Ghashghaie J, Cornic G. Drought effect on nitrate reductase and sucrose-phosphate synthases activities in wheat (*Triticum durum* L.): role of leaf internal CO_2. J Exp Bot. 2007; 58: 2983–2992.
38. Strozycki PM, Skąpska A, Szcześniak K, Sobieszczuk E, Briat JF, Legocki AB. Differential expression and evolutionary analysis of the three ferritin genes in the legume plant *Lupinus luteus*. Physiol Plant. 2003; 118: 380-389.
39. Gratão PL, Polle A, Lea PJ, Azevedo RA. Making the life of heavy metal-stressed plants a little easier. Func. Plant Biol. 2005; 32: 481-494.
40. Pereira LB, Mazzanti CMA, Gonçalves JF, Cargnelutti D, Tabaldi LA, Becker AG, Calgaroto, NS, Farias JG, Battisti V, Bohrer D, Nicoloso FT, Morsch VM, Schetinger MRC. Aluminum-induced oxidative stress in cucumber. Plant Physiol Biochem. 2010; 48: 683-689.

41. Soleimanzadeh H, Habibi D, Ardakani MR, Paknejad F, Rejali F. Effect of Potassium Levels on Antioxidant Enzymes and Malondialdehyde Content under Drought Stress in Sunflower (*Helianthus annuus* L.). Am J Agricult Biol Sci. 2010; 5(1): 56-61.
42. Weidner S, Karmac M, Amarowicz R, Szypulska E, Golgowska A. Changes in composition of phenolic compounds and antioxidant properties of *Vitis amurensis* seeds germinated under osmotic stress. Acta Physiol Plant. 2007; 29: 283-290.
43. Esfandiari E, Shakiba MR, Mahboob S, Alyari H, Toorchi M. Water stress, antioxidant enzyme activity and lipid peroxidation in wheat seedling. J Food Agric Environ. 2007; 5: 149-153.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Allelopathic effects of some weeds on rhizosphere algae at El-Kharga Oasis (New Valley), Egypt

Awatief F. Hifney, M.S. Adam, G. Ghareib, A.A. Issa

Botany Department, Faculty of Science, Assiut University, 71516 Assiut, Egypt

ABSTRACT

El-Kharga oasis soils tend to alkaline, very poor in N, P and rich in Ca, Mg, Na, and K. The total algal counts fluctuated, in the study sites (16), and ranged between 3.333 colonies/g soil for the plant *Hyoscyamus muticus* and 4978.3 colonies/g soils for *Plantago major*. The high percentage of cyanophyta (97.7%) was recorded around *Oxalix erniculata*, chlorophyta (84.15%) around *Solanum nigrum* and diatoms (53.3%) around *Langonychium farctum*. On the other hand, the number of total algal species in the study sites ranged between 2 and 16 species for *Hyoscyamus muticus* and *Langonychium farctum*, respectively. No species belong to chlorophyta and bacillariophyta around the root of *Hyoscyamus muticus*, *Calendula micrantha* and *Ambrosia martema* plants. The only one species encountered high occurrence remark was *Pseudoanabaena papilloterminat* (Cyanophyta). Treatments of *Nostoc carneum* with various soaked root extracts of *Ambrosia maritima* and *Hoscyamus muticus* caused enhancement of the growth, especially with high concentration of the extract (5%).

Key words: Allelopathy; Rhizosphere algae; Soil analysis; Weeds.

J Biol Earth Sci 2013; 3(1): B42-B53

Corresponding author:
Awatief F. Hifney
Botany Department, Faculty of Science, Assiut University, 71516 Assiut, Egypt
Tel: +201002931424, Fax: +2088342708
E-mail: hifney@yahoo.com

Original Submission: 22 November 2012; Revised Submission: 27 December 2012; Accepted: 05 January 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Allelopathy is formulated as any process involving secondary metabolites produced by plants, algae, bacteria, and fungi that influence the growth and development of agricultural and biological systems [1]. The allelopathic organism releases chemicals that inhibit the growth of a competing organism and thus indirectly prevents it from using common resources. For a long time, it has been recognized that several compounds that are present in the soil in replant situations, actively or passively alter the prevailing soil conditions, inducing a plant population, microorganisms and insects reduction that may inhabit that soil environment. Therefore, due to their allelopathic characteristics, these substances could somehow act as a pesticide [2]. Exudates from roots have long been recognized as a major potential source of energy for many saprophytic bacteria in soil, being the prime cause of the rhizosphere effect. There is considerable amount of evidence suggesting that loss of soluble organic substances from roots is significantly stimulated by the presence of microbes around them [3]. Because all soil borne nutrients obtained by the plant root must pass through the rhizosphere, the potential for microbes to alter these compounds in a way that will affect plant growth is great [4]. Plant species had strong influence on soil microbial organisms and their activity. According to Merckx et al. [5], obviously the input of nutrient by the roots into surrounding soil as well as the mineral nutrients levels in the soil is of considerable importance. Rovira [6] was convinced that root exudates play a key role in the selective stimulation of microorganisms and the view has shared by others [7]. Plants have an important effect on soil microbiology, due releasing different nutrients and organic compounds into the soil. Indeed, rhizosphere microorganisms can both mobilize and immobilize plant nutrients (C, N and S) and can produce growth promoting substances, such as phytohormones, as well as phytotoxins. The rhizosphere is a centre of intense biological activity due to the food supply provided by the root exudates. Soil chemistry and pH can influence the species mix and functions of microbes in the rhizosphere. However, there are some microorganisms that do interact with specific plants. Most soil microorganisms do not interact with plant roots, possibly due to the constant and diverse secretion of antimicrobial root exudates. It contains root-specific metabolites that has critical ecological impacts on soil macro and microbiota as well as on the whole plant itself. Through the exudation of a wide variety of compounds, roots impact the soil microbial community in their immediate vicinity, influence resistance to pests, support beneficial symbioses, alter the chemical and physical properties of the soil, and inhibit the growth of competing plant species [8]. Many phytoplankton cells are known to release elevated amounts of organic compounds under nutrient limitation. Algal species that can compete successfully for available growth-limiting nutrient(s) have the potential to become dominant and form blooms. The stress conditions imposed by the shifted nutrient supply ratios can, in some algae, stimulate production of allelochemicals that inhibit potential competitors [9]. No attempts had been made in New Valley, Egypt to study the soil and rhizosphere algae with relation to the ecology and biology of the Egyptian weed plants. Therefore, the present work aims at the elucidation of quantitative and qualitative study of the rhizosphere algae, of the sixteen common weed plants in El-Kharga oasis area (New Valley), Egypt. Moreover, the growth as well as some metabolic activity of *Nostoc carneum* under various concentrations of soaked plants (*Ambrosia maritima* or *Hoscyanus muticus*) were also followed.

MATERIALS AND METHODS

This investigation was devoted to study the algal diversity in the rhizosphere of 16 weeds plant naturally found in El-Kharga oasis at eight sites (two plants for each), the sites and weeds names are listed in Table 1.

Two replicates of each sample around the roots of 16 studied weeds at depth 5 cm were collected in December, 2010. Physical and chemical analyses of the collected soil samples were carried out as the following, pH value measured by using digital pH meter (pH Pen Jenco Electronics, U.S.A). Electrical conductivity was measured in soil water suspension using conductemeter (YSI Model 35 yellow springs, OH, U.S.A), The mechanical analysis of soil texture was carried out using hydrometer method as described by Russell [10]. The versene (disodium dihydrogen ethylene diaminetetra acetic acid) titration method [11] was employed for both Ca^{+2} and Mg^{+2} determinations, Na^{+} and K^{+} were

Table 1. Study sites and name of weed plants (W)

Plant symbol	Sites	Weeds plant		Plant symbol	Sites	Weeds plant	
		Latin name	English name			Latin name	English name
W1	Elbeleada	*Brassica sp.*		W9	Ein Pameis	*Calendula micrantha*	Field marigold
W2		*Sonchus oleraceus*	Smooth Sow Thistle	W10		*Ambrosia martem*	
W3	Kharga (1)	*Solanum nigrum*	Black Nightshade	W11	Elkalakan	*Plantago major*	Greater Plantain
W4		*Hyoscyamus muticus*	stinking nightshade	W12		*Mentha microphylla*	
W5	Hepet	*Euphorbia geneculata*		W13	Ein Ahmed	*Convolvulus arvensis*	Field Bindweed
W6		*Angonychium farctum*		W14		*Conyza linifolia*	narrowleaf whitetop aster
W7	Elnadora	*Portulaca oleracea*	Purslane	W15	Elparamody	*Cichorium pumilum*	Small Chicory
W8		*Oxalix erniculata*		W16		*Rumex dentatus*	

determined by the flame photometric technique [12] using Dr Lange Flame Photometer M 71 D type Nr/LPG 075. The method described by Dewis and Freitas [13] was used for the determination of orthophosphate. The method described by Sheen et al. [14] was used for determination of sulphate. For isolation of the rhizosphere algae, the soil sample were cultivated in three synthetic media, BG11 medium was used for isolation of blue-green algae [15], Chu,10 medium for isolation of diatoms [16], while Bold's basal medium [17] was used for isolation of eukaryotic and green algae. Petri-dishes were incubated at 30ºC for prokaryotic algae and 20±2ºC for green algae and diatoms. Counts of colonies were made after 20 days of inoculation and the number of colony were proportioned to dry weight soil (colonies/gram soil). Algal colonies were examined and species identified according to the following references [18-21].

The growth rate and generation time of Nostoc carneum grown with various concentrations of soaked plants (100 gram dry mass plant/200 ml dist water for three days of *Ambrosia maritima* or *Hyoscyamus muticus*) were followed by daily measurements of absorbance at 620 nm as described by Lefort-Tran et al. [22]. Chlorophyll-a was extracted in acetone (90%) over night and determined according to Marker [23]. Photosynthetic O_2 evolution and respiratory O_2 uptake were determined by O_2 electrode (O_2 Meter CG 867 Germany) and calculated as μ moles O_2. mg^{-1} chlorophyll-a.h^{-1}. For the determination of carbohydrate, the anthrone sulfuric acid method was used [24]. Proteins were determined according to Lowry et al. [25]. Free amino acids were estimated according to the method adopted by Lee and Takahashi [26].

RESULTS

Changes in the physico-chemical character of the 16 soils sample collected from El-Kharga oasis around root zone of 16 weed plants, (Table 1) as described in material and method, were illustrated in (Table 2). The results pointed out that the soils of El-Kharga oasis tend to be alkaline in all tested sites; pH value ranged between 7.88 and 8.14. The texture of all tested soil sample of El-Kharga oasis was characterized by sand to sandy loam. There were large difference in the electrical conductivity value of the all tested sample, minimum (20.2) and maximum values (336 μmoh/cm) recorded around *Hoscyamus muticus* and *Convolvulus arvensis*, respectively. Rhizosphere sample of Convolvulus arvensis had the lowest total soluble salts, while the highest value was 21.5 around *Hoscyamus muticus*. The soil sample around *Conyza linofolia* has lowest moisture content, where the highest moisture

Table 2. Physico-chemical characteristics (%) of various soil samples collected from rhizosphere soil sample of 16 weeds plant, valid presented in El-Kharga oasis during December 2010

Sites / Parameter	W1	W2	W3	W4	W5	W56	W7	W8	W9	W10	W11	W12	W13	W14	W15	W16
Air temp.	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
Soil temp.	18	18	16	18	17	17.5	17.5	17.5	18	18	17	18	17.5	19	18	17
pH	8.01	7.88	8.03	7.89	8.02	8.29	8.04	8.1	8.08	8.02	8	8.04	7.99	8.14	8.09	8.01
Total alkalinity	085	0.7	0.75	0.8	0.35	1.25	1.05	1.3	0.85	0.9	0.65	0.85	0.5	1.05	0.65	0.75
E.C. ($\mu moh.cm^{-1}$)	52.9	74.5	97	336	25.2	44.3	47.1	29.9	30.5	37.6	37.2	29.9	20.2	34.5	300	89.8
T.S.S.	3.38	4.76	6.2	21.5	1.61	2.83	3.01	1.91	1.95	2.4	2.38	1.91	1.29	2.2	19.2	5.74
M.C.	17.2	16.2	8.8	7.4	16.2	14	18.8	19	10.4	12.4	33.8	23.2	11.2	3.4	20.8	22.2
O.C.	1.64	1.686	1.62	1.59	1.69	1.63	1.62	1.617	1.559	1.513	1.49	1.582	1.732	1.536	1.617	1.605
Cl^-	2.84	1.78	3.916	2.67	1.335	1.246	2.314	1.068	0.89	0.623	0.80	0.356	0.534	0.445	0.623	0.712
T. N.	0.01	0.008	0.00755	0.0083	0.013	0.0165	0.016	0.01	0.011	0.011	0.02	0.019	0.011	0.014	0.114	0.014
NO_3-N	0.01	0.007	0.045	0.1505	0.012	0.0064	0.1128	0.005	0.009	0.012	0.01	0.021	0.004	0.0055	0.001	0.005
PO_4-P	0.11	0.0270	0.0642	0.0773	0.139	0.1489	0.1510	0.114	0.157	0.067	0.17	0.115	0.0678	0.0700	0.226	0.075
SO_4-S	0.19	0.1203	0.31216	0.2905	0.357	0.4702	0.1892	0.346	0.189	0.176	0.53	0.562	0.3189	0.1216	0.203	0.284
Ca^{++}	0.6	0.45	0.4	0.6	0.25	0.25	0.35	0.2	0.2	0.5	0.2	0.45	0.2	0.3	0.4	0.45
Mg^{++}	0.31	0.61	0.31	0.915	2.44	1.22	0.915	1.83	1.83	0.915	1.83	1.83	1.525	0.915	3.355	1.22
K^+	0.42	0.788	0.77	0.129	0.57	0.0769	0.426	0.46	0.262	0.265	0.89	0.679	0.652	0.473	0.615	0.831
Na^+	0.11	0.294	0.44	0.16	0.04	0.02	0.08	0.04	0.03	0.070	0.13	0.04	0.03	0.1	0.11	0.181

E.C. = Electrical conductivity, M.C. = Moisture content, O.C. = Organic carbon, T.N. = Total nitrogen, W = Weed.

Table 3. The total algal biomass (Kg ha^{-1}) and counts (Colonies/g) of the rhizosphere soil sample collected around the roots of 16 weeds plants presented in El-Kharga oasis during December 2010

Sites / Taxa	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12	W13	W14	W15	W16
Count of Chlorophyta	760	760.33	230	0.00	90.00	10.00	1326.6	21.66	0.00	0.00	235	386.6	5.00	0.00	640	50
%Chlorophyta	60.64	51.71	83.84	0.00	36.49	1.89	49.32	0.64	0.00	0.00	4.72	9.407	1.786	0.00	27.55	25.01
Count of Cyanophyta	483.33	710.00	43.33	3.33	153.3	236.6	1305	3320	101.6	155	4688.3	3523.3	238.33	1515	1630	91.6
%Cyanophyta	38.56	48.29	15.79	100	62.16	44.79	48.51	97.74	100	100	94.17	85.72	85.139	97.02	70.16	45.81
Count of Bacillariophyta	10	0.0	1.00	0.00	3.33	281.66	58.3	55.00	0.00	0.00	55	200	36.6	46.6	53.3	58.3
%Bacillariophyta	0.797	0.0	0.004	0.00	1.35	53.31	2.167	1.62	0.00	0.00	1.104	4.86	13.07	2.98	2.29	29.17
Total algal count	1253.33	1470.33	274.33	3.33	246.63	528.26	2689.9	3396.66	101.6	155	4978.3	4109.9	279.93	1561.6	2323.3	199.93
Total algal biomass	0.37	0.075	0.062	0.064	0.01	0.44	0.42	0.3	0.28	0.008	0.26	0.19	0.23	0.24	0.24	0.14

W = No and names of weeds plants as described in Material and Methods

content 33.8 was record around *Plantago major*. The soil samples under testing were very poor in sodium and potassium contents and rich in calcium and magnesium as in Table 2. No remarkable difference was recorded between the values of nitrogen in all tested soil samples. Highest NO_3-N was detected around the rhizosphere samples of *Hyoscyamus muticus*. Phosphate–phosphorus was fluctuated within 0.027 around the rhizosphere soil sample of *Sonchus oleraceus* to 0.22% around *Cichorium pumilum* weeds plant. Generally, there was no noticeable difference in the percentage of the organic carbon of all investigated soil samples as in Table 2.

The present study was also conducted to elucidate the qualitative and quantitative variations in the various rhizosphere algae of 16 weeds plants valid presented in El-Kharga oasis, the total algal biomass in all sites ranged from 0.008 to 0.446 around the rhizosphere of *Ambrosia maritima* and *Angonychium farctum*, respectively (Table 3). While the total algal counts ranged between 3.33 colonies g^{-1} around *Hyoscyamus muticus* and 4978.3 around the rhizosphere soil sample of *Plantago major*. It has been found that, three algal groups namely cyanophyta, chlorophyta and bacillariophyta were recorded in this investigation.

On the basis of total algal counts, chlorophyta (colonies g^{-1}) ranged from a minimum of 0.00 around *Hyoscyamus muticus*, *Calendula micrantha*, *Ambrosia maritima* and *Conyza linifolia* to a maximum 1326.6 of soil sample collected around the roots of *Portulaca orraceae* (Table 3), this group accounted for a maximum percent of chlorophyta (83.84) around *Solanum nigrum*. Also, cyanophyta counts fluctuated within 3.33 colonies g^{-1} soil around the roots of *Hoscyamus muticus* to (4688.3) around *Plantago major*, the highest % of cyanophyta 100% were recorded around *Hyoscyamus muticus*, *Calendula micrantha*, *Ambrosia martema*, the only algal group which recorded. With respect to bacillariophyta, the highest counts recorded 281 colonies/gram soil from the rhizosphere soil of *Angonychium farctum*, while soil sample collected around *Euphorbia geneculata* recorded the lowest counts (3.33) and noticeable that no species of bacillariophyta was appeared around the rhizosphere soil sample of *Sonchus oleraceus*, *Hyoscyamus muticus*, *Calendula micrantha* and *Ambrosia maritima*, as in Table 3.

Twenty three genera (35 species) of algae identified, isolated and purified from El-Kharga city around the rhizosphere soil sample of 16 weeds plants. Twenty five species (14 genus) belonged to cyanophyta, 6 species (5 genus) belonged to chlorophyta and 4 species (4 genus) belonged to bacillariophyta (Table 4, Fig. 1).

The highest algal taxa were recorded around the roots of *Angonychium farctum*, while only two taxa belonged to cyanophyta were recorded around the roots of *Hyoscyamus muticus*. No species belonging to chlorophyta and bacillariophyta was recorded around the roots of *Hyoscyamus muticus*, *Calendula micrantha* and *Ambrosia martema*. The blue green alga *Pseudoanabena papilloterminat* was identified as the dominant cyanophyta in all studying sites (occurrence remark were 75%), while the other algal taxa were presented in moderate, frequency or rarely occurrence as shown in Table 4.

It has been found that, *Pseudoanabena papilloterminat* and Wollea saccata were the only algal species presented around *Hyoscyamus muticus* (Table 4). The response of growth and metabolic activities of *Nostoc carneum* isolated from rhizosphere soil sample of El-Kharga oasis to various soaked root extracts (1%, 3%, 5% to original medium) of *Hyoscyamus muticus* and *Ambrosia maritima* were assessed. It has been found from the results shown in Fig. 2, Table 5 that the growth of *Nostoc carneum* expressed as absorbance (750 nm) and chlorophyll-a content were stimulated with the addition of the various concentration of *Ambrosia martima* and *Hyoscyamus muticus* especially with high concentration of *Ambrosia maritima* (Fig. 2).

Photosynthetic oxygen evolution were increased with addition of *Ambrosia maritima*, especially at high concentration used (Table 5). With respect to the carbohydrates content of *Nostoc carneum*, the addition of both crude extract of the plant under testing caused increase in the soluble carbohydrates contents (soluble, insoluble, total) especially with the high concentration used. Also, alleviation of protein content in *Nostoc* cells cause by addition of *Ambrosia maritima* extract, especially with high concentration as shown in Table 5. In conclusion, the results found that the addition of soaked water extract of both of *Ambrosia maritima* and *Hyoscyamus muticus* roots improved the growth rate and its metabolites of *Nostoc carneum*, especially with high concentration 5 %.

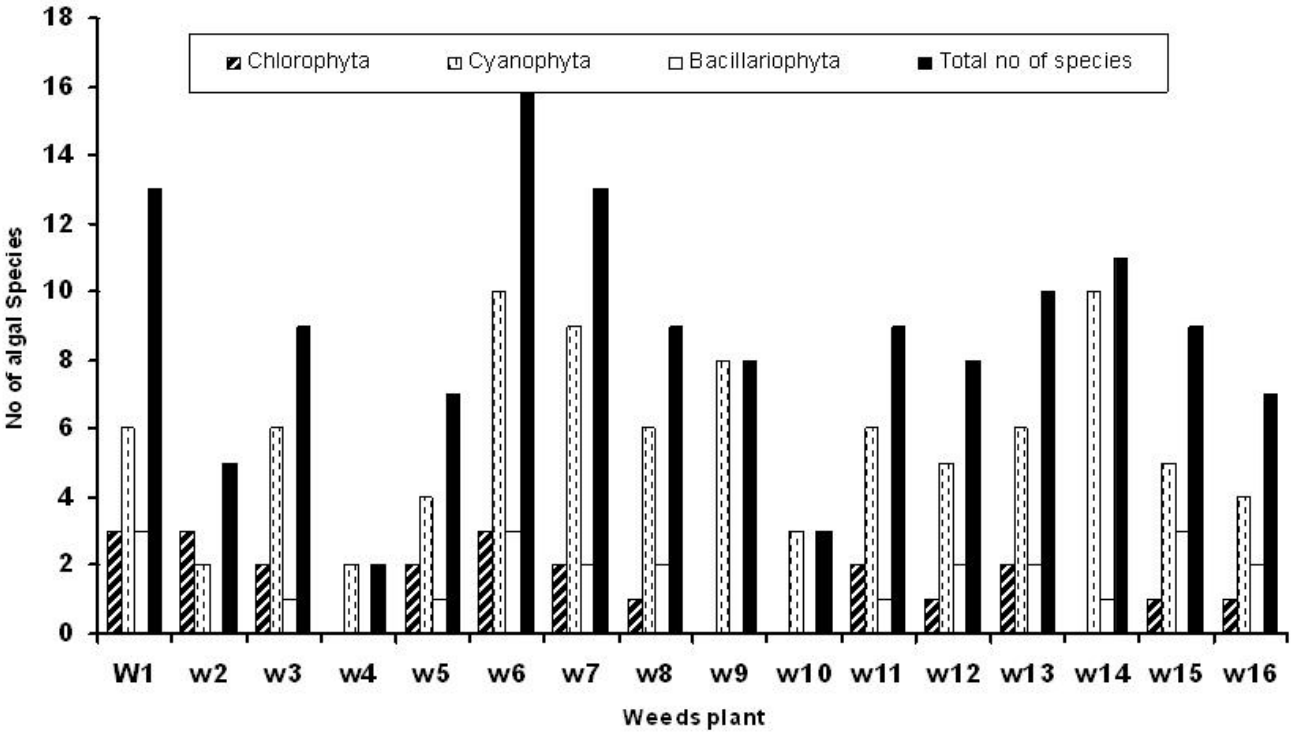


Fig. 1. Number of algal species collected from the rhizosphere soil sample of 16 weeds plants valid represented in El-Kharga oasis during December, 2010

Table 5. Response of growth and their metabolic activities of *Nostoc carneum* to various soaked root extract concentrations of *Hyoscyanus muticus* and *Ambrosia martima*

	Treatments	μ.max (h^{-1})	G (h^{-1})	Chl.a (μg/ml)	O_2↑*	O_2↓**	F.A.A.	Proteins (mg/L)			Carbohydrates (mg/L)		
								S.P.	Ins. P.	T.P.	S.C.	Ins. C.	T.C.
	Control	0.044	15.85	0.92	40.76	40.76	262.25	1.21	0.18	1.4	0.342	0.57	0.914
Hyoscyanus muticus	1%	0.041	16.85	0.69	45.29	54.34	86.317	1.101	0.223	1.32	0.93	0.08	1.007
	3%	0.072	9.67	1.43	48.07	43.7	116.03	1.93	0.241	2.17	1.035	0.58	1.62
	5%	0.066	10.46	1.73	50.57	28.9	122.64	2.045	0.49	2.53	1.83	1.37	3.21
Ambrosia martima	1%	0.046	15.10	1.36	32.16	45.95	168.86	1.06	0.38	1.44	1.164	1.24	2.41
	3%	0.085	11.98	1.49	37.75	41.94	184.42	1.67	0.65	2.32	1.32	1.53	2.85
	5%	0.055	12.66	2.10	41.66	32.73	256.59	1.98	0.72	2.70	2.005	1.76	3.77

μ = maximum growth rate, G = generation time, * O_2 ↑ (μmoles O_2 ↑ mg chl.a h^{-1}); ** O_2 ↓ (μmoles O_2 ↓ mg chl.a h^{-1}), S.C. = soluble carbohydrates, Ins.C. = insoluble carbohydrates, T.C. = total carbohydrates, S.P. = soluble proteins, Ins.P. = insoluble proteins, T.P. = Total proteins, F.A.A. = free amino acids.

DISCUSSION

Soils of El-Kharga oasis tend to be alkaline in all tested sites; pH value ranged between 7.88 and 8.14. The texture of all tested soil sample of El-Kharga oasis was characterized by sand to sandy loam. Dakhla and Kharga are sand stone desert oases [27]. There were large difference in the electrical conductivity value of the all tested sample, minimum (20.2) and maximum values (336 μmoh/cm) recorded around *Hoscyamus muticus* and *Convolvulus arvensis*, respectively.

Rhizosphere sample of *Convolvulus arvensis* had the lowest total soluble salts, while the highest value was 21.5 around *Hoscyamus muticus*. The soil sample around *Conyza linofolia* has lowest moisture content, where the highest moisture content 33.8 was record around *Plantago major*. Microbial activity in soils (desert soils) is highly dependent on characteristics such as temperature, moisture and availability of organic carbon [28, 29] of this moisture availability is the major constraint effecting microbial activity. The soil samples under testing were very poor in sodium and potassium

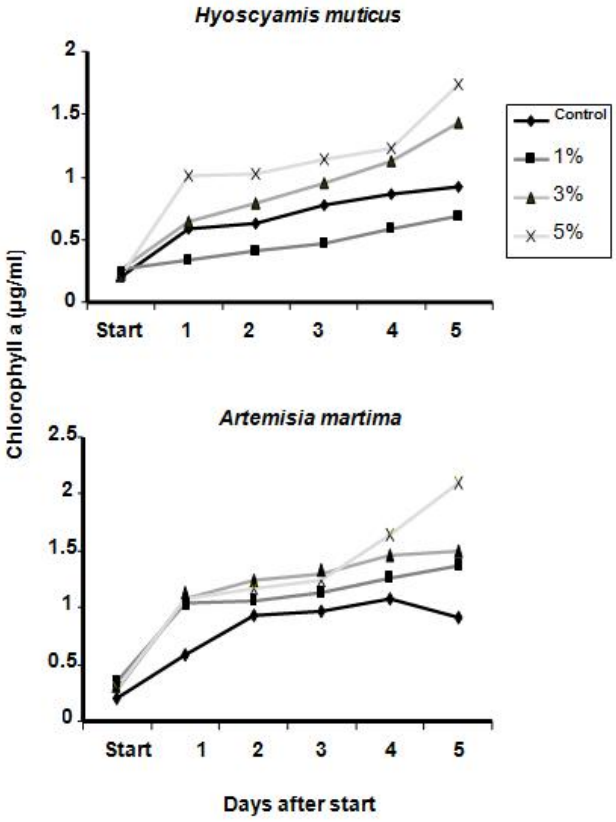


Fig. 2. Enhancement of chlorophyll-a content for *Nostoc carneum* to various soaked root extract concentrations of *Hyoscyanus muticus* and *Ambrosia martima*

Table 4. List of rhizosphere algae and occurrence remark of algal species collected from the rhizosphere soil sample of 16 weeds plants valid represented in El-Kharga oasis during December, 2010

site sample / Taxa	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12	W13	W14	W15	W16	Occurrence Remark
Cyanophyta																	
Anabaena viguierii **Denis and Fremy**					+	+	+	+	+	+	+	+	+	+		+	**M**
Calothrix artcha **Fermy**												+					**R**
C. braunii **Bornet&Flahault**								+			+		+	+			**L**
Chroococcus subnarius **(Hansgirg) Kovacik**					+										+		**R**
C.turjidus **(Kutz) Naeg**		+	+		+		+	+						+	+	+	**M**
Gloeocapsapsis magma **(Breebisson) Komarer**						+											**R**
Lyngbya biebliana **Claus**						+									+		**R**
Nostoc carneum **Agardh**			+			+	+	+	+		+	+	+			+	**M**
N. microscopicum **Carmichael**	+								+		+	+					**L**
N. piscinale **Kutz**							+		+		+			+			**L**
N. pruniforme **C.Agardh**													+	+			**R**
Oscillatoria subbrevis **Schmidle**						+											**R**
Phormidium californicum **Drouet**	+		+													+	**R**
P. formosum **(Bory ex Gomont) Anagnostidis et Komarek**									+						+		**R**
P. tergestinum **(Kutzing)**			+			+	+										**R**
P. pachydermaticum **Fremy**						+	+		+	+			+	+			**L**
Pseudoanabaena balatonica **Scherffel et Kol**						+											**R**
P. papilloterminat **(Kiselev) Kukk**	+	+		+	+	+	+	+			+	+	+	+	+		**H**

Table 4. Continue

P. starmachii **Anagnosstidis.**							+										**R**
Schizothrix radius **Komare**									+								**R**
S. semiglobosa **Geitler**	+		+					+	+	+							**L**
Scytonema coactil **Montagne**														+			**R**
Spirulina nodosa **Schiller**	+						+										**R**
Stigeoclonium tenue **Kutz**														+			**R**
Wollea saccata **(Wolle) Bornet&Flahult**	+		+	+		+								+			**L**
Total no. of Cyanophyta sp.	**6**	**2**	**6**	**2**	**4**	**10**	**9**	**6**	**8**	**3**	**6**	**5**	**6**	**10**	**5**	**4**	
Chlorophyta																	
Chlorella emersonii **Shih&Krauss**		+	+		+	+	+	+				+			+	+	**M**
Chlamydomonas gallica **Ettl**	+				+	+					+						**L**
C. ***platyrhyncha*** **Korsck**						+					+						**R**
Chlorococcum humicola **Nag**		+											+				**R**
Gloeocystis vesiculosa. **Scnsu Skuja**	+	+	+				+						+				**M**
Oocystis borgei **Snow**	+																**R**
Total no. of Chlorophyta sp.	**3**	**3**	**2**	**0**	**2**	**3**	**2**	**1**	**0**	**0**	**2**	**1**	**2**	**0**	**1**	**1**	
Bacillaryophyta																	
Achnanthus lanceolata **Breb**	+		+					+					+				**R**
Dentecola teinuis **Kutz**	+				+	+	+	+			+	+			+	+	**M**
Hantzschia amphioxys **(E)Grun**	+					+	+					+	+	+	+	+	**M**
Nitzschia linears **(Ag)W.Smith**						+									+		**R**
Total no. of Bacillariophyta sp.	**3**	**0**	**1**	**0**	**1**	**3**	**2**	**2**	**0**	**0**	**1**	**2**	**2**	**1**	**3**	**2**	

contents and rich in calcium and magnesium.No remarkable difference was recorded between the values of nitrogen in all tested soil samples. Highest NO_3-N was detected around the rhizosphere samples of *Hyoscyamus muticus*.

Phosphate–phosphorus was fluctuated within 0.027 around the rhizosphere soil sample of *Sonchus oleraceus* to 0.22% around *Cichorium pumilum* weeds plant.

The present study was also conducted to elucidate the qualitative and quantitative variations in the various rhizosphere algae of 16 weeds plants valid presented in El-Kharga oasis, the total algal biomass in all sites ranged from 0.008 to 0.446 around the rhizosphere of *Ambrosia maritima* and *Angonychium farctum*, respectively. While the total algal counts ranged between 3.33 colonies g^{-1} around *Hyoscyamus muticus* and 4978.3 around the rhizosphere soil sample of *Plantago major*. Schweitzer et al. [30] found that intra specific plant genetic variation can affect soil microbial biomass and microbial community composition, feedbacks from plant may be an important ecological factor affecting microbial dynamics. It has been found that, three algal groups namely cyanophyta, chlorophyta and bacillariophyta were recorded in this investigation. Kumar and Kumar [31] recorded that, nearly 12 species of algae living in close proximity of the rhizoidal system of mosses. It included members of cyanophyceae, bacillariophyceae and xanthophyceae. On the basis of total algal counts, chlorophyta (colonies g^{-1}) ranged from a minimum of 0.00 around *Hyoscyamus muticus*, *Calendula micrantha*, *Ambrosia maritima* and *Conyza linifolia* to a maximum 1326.6 of soil sample collected around the roots of *Portulaca oleracea*, this group accounted for a maximum percent of chlorophyta (83.84) around *Solanum nigrum*. Also, cyanophyta counts fluctuated within 3.33 colonies g^{-1} soil around the roots of *Hoscyamus muticus* to (4688.3) around *Plantago major*, the highest % of cyanophyta 100% were recorded around *Hyoscyamus muticus*, *Calendula micrantha*, *Ambrosia martema*, the only algal group which recorded. With respect to bacillariophyta, the highest counts recorded 281 colonies/gram soil from the rhizosphere soil of *Angonychium farctum*, while soil sample collected around *Euphorbia geneculata* recorded the lowest counts (3.33) and noticeable that no species of bacillariophyta was appeared around the rhizosphere soil sample of *Sonchus oleraceus*, *Hyoscyamus muticus*, *Calendula micrantha* and *Ambrosia maritima*. Twenty three genera (35 species) of algae identified, isolated and purified from El-Kharga city around the rhizosphere soil sample of 16 weeds plants. Twenty five species (14 genus) belonged to cyanophyta, 6 species (5 genus) belonged to chlorophyta and 4 species (4 genus) belonged to bacillariophyta.

Increase accumulation of microorganism in the root soil of various plants was not accident, it was caused by the biological activity of the roots, and that the differences in qualitative and relationship depend on the species of plant and soil climate [32].

The highest algal taxa were recorded around the roots of *Angonychium farctum*, while only two taxa belonged to cyanophyta were recorded around the roots of *Hyoscyamus muticus*. No species belonging to chlorophyta and bacillariophyta was recorded around the roots of *Hyoscyamus muticus*, *Calendula micrantha* and *Ambrosia martema*. A negative rhizosphere effect on algae was reported by Hodifield [33] for the tea brush, and for *Solanum* sp. [34, 35]. The blue green alga *Pseudoanabena papilloterminat* was identified as the dominant cyanophyta in all studying sites (occurrence remark were 75%), while the other algal taxa were presented in moderate, frequency or rarely occurrence.

It has been found that, *Pseudoanabena papilloterminat* and *Wollea saccata* were the only algal species presented around *Hyoscyamus muticus*. Hifney et al. [35] suggested that there is a direct relationship between root growth of some plants (7 tested plants) and diversity of algae. In this respect, [36] recorded a release of chemical compound by plant inhibit algal growth these bioactive substance are called allelochemicals, and their mechanism involved are called allelopathy.

The response of growth and metabolic activities of *Nostoc carneum* isolated from rhizosphere soil sample of El-Kharga oasis to various soaked root extracts (1%, 3%, 5% to original medium) of *Hyoscyamus muticus* and *Ambrosia maritima* were assessed. The growth of *Nostoc carneum* expressed as absorbance (750 nm) and chlorophyll-a content were stimulated with the addition of the various concentration of *Ambrosia maritima* and *Hyoscyamus muticus* especially with high concentration of *Ambrosia maritima*. Woodbine and Cullimore [37] showed that the seedling of the tea

plant could significantly increase the algal population on the surface of the soil. Photosynthetic oxygen evolution were increased with addition of *Ambrosia maritima*, especially at high concentration used. With respect to the carbohydrates content of *Nostoc carneum*, the addition of both crude extract of the plant under testing caused increase in the soluble carbohydrates contents (soluble, insoluble, total) especially with the high concentration used. Also, alleviation of protein content in *Nostoc* cells cause by addition of *Ambrosia maritima* extract, especially with high concentration.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Anonymous (International Allelopathy Society). 1996. First World Congress on Allelopathy. A science for the future. Cadiz, Spain.
2. Rice EL. 1974. Plant parts that contains inhibitors and ways in which inhibitors enter the environments. In: TT Kozlowsky (ed.) Allelopathy. University of Wisconsin. pp. 237-244.
3. Lynch JM. Phytotoxicity of acetic acid produced in the anaerobic decomposition of wheat straw. J Appl Bacteriol. 1997; 42: 81–87.
4. Chanway PC, Bent E. Potential for misidentification of a spore-forming *Paenibacillus polymyxa* isolate as an endophyte by using culture-based methods. Appl Environ Microbiol. 2002; 68: 4650-4652.
5. Merckx R, Dukstra A, Den Hartog A, van Vees JA. Production of root-derived material and associated microbial growth in soil at different nutrient levels. Biol Fertil Soils. 1987; 5: 126-132.
6. Rovira AD. Interactions between plant roots and soil microorganisms. Annu Rev Microbiol. 1965; 19: 241-266.
7. Atkinson TG, Neal JL, Larson RI. 1975. Genetic control of the rhizosphere microflora of wheat. In: Biology and control of soil borne plant pathogens. Ed. GW Bruehl. American Phytopathological Society, St.Paul. pp. 116-122.
8. Bertin C, Yang XH, Weston LA. The role of root exudates and allelochemicals in the rhizosphere. Plant Soil. 2003; 256: 67-83.
9. Granéli E, Weberg M, Salomon SP. Harmful algal blooms of allelopathic microalgal species: The role of eutrophication. Harmful Algae. 2008; 8: 94–102.
10. Russell EW. 1973. Soil conditions and plant growth. ELBS and Longman. p. 848.
11. Schwarzenbeck G, Biederman W, Komplexone X. Erdalkalikomplexe von o,6-Dioxyazofarbstoffen. Helv Chim Acta. 1948; 31: 678-687.
12. Williams CH, Twine JR. 1960. Flame photometric method for sodium, potassium and calcium. In: K. Peach & Tracey (Eds.). Modern Methods of Plant Analysis, 5: 3-5. Springer Verlag, Berlin, Goettingen, Heidelberg.
13. Dewis J, Freitas F. 1970. In physical and chemical methods of soil and water analysis. Soil Bulletin, No. 10. Food and Agriculture Organization of the United Nations, Rome. Weinheim.
14. Sheen RT, Kahler HL and Ross EM. Turbidmetric determination of sulfate in water. Ind Eng Chem Anal Ed. 1953; 7: 262.
15. Rippka R, Herdmann M. 1993. Pasteur culture collection of cyanobacterial strains in axenic culture. Vol. 1 Catalogue of Strains. P103, Institut Pasteur, Paris, France.
16. Chu SP. The influence of the mineral composition of the medium on the growth of planktonic algae. 1- Methods of culture media. I Ecol. 1942; 30: 284-325.
17. Bischoff HW, Bold HC. Phycological studies. 4- Some soil algae from enchanted rock and related algal species. Univ Texas Publ. 1963; 6318: 32-36.
18. Philipose MT. Chlorococcals. Indian Council of Agric. Res., New Delhi. 1967, pp. 363.
19. Sreenivase MR, Duthie HC. Diatom flora of the grand river, Ontario, Canada. Hydrobiol. 1973; 42: 161-224.
20. Tell G. Soil algae from around Buenos Aires (Argentina). Darwiniana. 1976; 20: 491-548.
21. Lund HC, Lund JWG. 1995. Freshwater algae their microscopic world explored. Biopress Limited (Hong Kong). pp. 1-36.
22. Lefort-Tran M, Pouphile M, Spathj S, Packer L. Cytoplasmic membrane changes during adaptation of the fresh water *Cyanobacterium synchococcus* 6311 to salinity. Plant Physiol. 1988; 87: 767-775.
23. Marker AFH. The use of acetone and methanol in the estimation of chlorophyll in the presence of Phaeophytin. Fresh Wat Biol. 1972; 2: 361-385.
24. Fales FW. The estimation and degradation of carbohydrates by yeast cell. J Biol Chem. 1951; 193: 113-118.
25. Lowry DH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem. 1951; 193: 265-275.
26. Lee YD, Takahashi T. An improved colorimetric determination of amino acids with use of ninhydrin. Anal Biochem. 1966; 14: 71-77.
27. Abd El-Ghani MM, Fawzy MA. Plant diversity around springs and well in five Oases of the western Desert, Egypt. Int J Agricult Biol. 2006; 8: 294-255.
28. Parker LW, Miller J, Steinberger Y, Whitford WG. Soil respiration in a chihuahuan desert rangeland. Soil

Biol Biochem. 1983; 15: 303-309.
29. Parker LW, Freckman DW, Steinberger Y, Driggers L, Whitford WG. Effect of stimulated rainfall and litter quantitative on desert soil biota: Soil respiration, microflora and protozoa. Pedobiologia. 1984; 27: 185-195.
30. Schweitzer JA, Bailey JK, Fischer GD, LeRroy JC. Plant–Soil–Microorganism Interaction: Heritable relationship between plant genotype and associated soil microorganism. Ecology. 2008; 89: 773-781.
31. Kumar GE, Kumar GP. Phycoflora associated with the rhizosphere of *Funaria hygrometrica* – A prelude. Ekologia. 2010; 10: 109-112.
32. Armstrong J, Afreen-Zobayed F, Blyth S, Armstrong W. *Phragmites australis*: Effects of shoot submergence on seedling growth and survival and radial oxygen loss from roots. Aquatic Bot. 1999; 64: 275-289.
33. Hodfield W. Rhizosphere effect on soil algae. Nature. 1960; 185: 178-179.
34. Kobbia IA. Ecophysiological studies on the rhizosphere algae of some Egyptian plants. Egypt Soc. Appl. Microbial. Proc. V., Conf., Microbial, Cairo. Soils Water Microbiol Paper. 1983; 49: 143-166.
35. Hifney AF, Issa AA, Mohammed AA. Distribution pattern of algae in the root zone of some Egyptian plants at Assiut region. Egypt J Union Arab Biol Sci. Cairo. 2004; 15: 81-99.
36. Morrius K, Paul IB, Lessa H. Alternative stable states in the aquatic vegetation of shallow urban lakes. Marine Fresh Water Res. 2003; 54: 185-215.
37. Woodbine M, Cullimore DR. A rhizosphere effect of the pea root on soil algae. Nature. 1963; 198: 304-305.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Abiotic stress induced production of β-carotene, allophycocyanin and total lipids in *Spirulina* sp.

Awatief F. Hifney, Ahmed A. Issa, Mustafa A. Fawzy

Botany and Microbiology Department, Faculty of Science, Assiut University, 71516 Assiut, Egypt

ABSTRACT

The present study examines the possibility of increasing the levels of some bioactive compounds (β-carotene, phycopiliprotein and total lipids) in *Spirulina* sp. isolated from Wadi El Natron lake (Egypt) and cultivated in modified Zarrouk's medium under standard growth condition and abiotic stress including change in culturing condition as (pH, temperature, light intensity), nutrient limitation (-N, -P, -S) and salinity. The yield production of β-carotene was enhanced with 0.9M NaCl, phosphorus deficiency and at pH 6. Total lipids were also enhanced in all conditions under tested especially with 0.9M NaCl, light intensity 14.52 µmol photon.m^{-2}.s^{-1}. Phycobiliproteins fractions (phycocyanin, allophycocyanine and phycoerethrine) were elevated with 0.3, 0.6M NaCl as well as phosphorus, nitrogen and sulphur deficiency. Antioxidant enzymatic activities of *Spirulina* sp. were elevated with all studied abiotic stress factor.

Key words: Allophycocyanin; β-carotene; Lipids; *Spirulina* sp.; Antioxidant enzymes.

J Biol Earth Sci 2013; 3(1): B54-B64

Corresponding author:
Awatief F. Hifney
Botany Department, Faculty of Science, Assiut University, 71516 Assiut, Egypt
Tel: +201002931424, Fax: +2088342708
E-mail: hifney@yahoo.com

Original Submission: 29 November 2012; Revised Submission: 09 January 2013; Accepted: 20 January 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Cyanobacteria have recently gained increasing interest as microorganisms because they are believed to be an attractive source of various bioactive substances, such as polyunsaturated fatty acids, β-carotene and other phytopigments (carotenoids, chlorophyll and phycocyanin) that function as antioxidants [1], polysulfated polysaccharides as antivirals [2], sterols as antimicrobials [3] and mycosporine-like amino acids (MAAs) and scytonemin as photoprotectants [4]. Phycobiliproteins (PBPs) are the unique photosynthesis pigments of this group and are water soluble pigments that can be divided into three types according to their maximum absorbance: phycocyanin (PC), phycoerythrin (PE) and allophycocyanin (APC) [5, 6].

Cyanobacteria, especially *Spirulina*, a blue green microalga, have been used since ancient times as a source of food because of its high nutritional value [7]. The cyanobacterium *Spirulina* platensis is rich in nutrients, such as proteins, vitamins, minerals, carbohydrates, and γ-linolenic acid. It is gaining more and more attention, not only for the foods aspects but also for the development of potential pharmaceuticals [8] and medicinal properties; thus, several studies have shown that *Spirulina* or its extracts could prevent or inhibit cancer in humans and animals, and has immuno-promoting effects [9, 10].

Spirulina is found in soil, marshes, freshwater, brackish water, seawater and thermal springs. Alkaline, saline water (>30 g/l) with high pH (8.5–11.0) favour good production of spirulina, especially where there is a high level of solar radiation at altitude in the tropics. *Spirulina platensis* and *Spirulina maxima* thrive in highly alkaline lakes where the cyanobacteria population is practically monospecific [11].

Spirulina appears to have considerable potential for development, especially as a small-scale crop for nutritional enhancement, livelihood development and environmental mitigation. In particular, the production and use of *Spirulina* has the following advantages: it provides an easily digestible high (c. 60 percent) protein product with high levels of β-carotene, vitamin B12, iron and trace minerals [12] and the rare essential fatty acid γ-linolenic acid (GLA). In addition, it has no obvious negative cultural or religious issues associated with its consumption; its production occupies only a small environmental footprint, with considerable efficiencies in terms of water use, land occupation and energy consumption when compared to traditional terrestrial crops; its production can be conducted at a number of different scales, from household “pot culture” to intensive commercial development over large areas; it has the potential for integration with rural organic waste treatment processes to improve both environmental conditions and improve energy transfer efficiencies [13].

Phycocyanin is a biliprotein pigment of the blue green algae like *Spirulina* with a variety of pharmacological properties such as antioxidant, anti-inflamatory, neuro and hepatoprotective [14] and antitumor activities [15-17]. Phycocyanin stimulates the antioxidant enzymatic defence systems to modulate the early radiation response, therefore phycocyanin may be of interest in the radioprotection of subjects exposed to low doses of radiations [18].

Cyanobacteria may contain significant quantities of lipids with the composition similar to those of vegetable oils [19]. The lipids of some cyanobacterial species are rich in essential fatty acids such as linoleic 18:2n6 and α-linolenic 18:3n3 acids and their C20 derivatives, eicosapentaenoic acids 20:5n3 and arachidonic acids 20:4n6 [20]. Some of the filamentous cyanobacteria tend to contain large quantities (20–60% of the total of fatty acids) of polyunsaturated fatty acids (PUFA) [21]. Where microalgae can be cultured, PUFA in algae have profound benefits and functions in dietetics and therapeutic uses [20, 22-24]. They are believed to have positive effects for the treatment of hypertension, premenstrual tension, various atopic disorders, diabetes and a number of other cases [25, 26].

The objective of this work is to investigate in more details the effect of abiotic factors as nutrient deficiency (Nitrogen, Phosphorus and Sulphur), change in culture condition as (Light intensity, Temperature and pH) and salinity stress take into our consideration on growth and other metabolic activities of *Spirulina*. To establish a particular cultivation strategy for enrichment of the biomass with some specific added value compounds (phycocyanin, β-carotene and/or fatty acid production) and some antioxidant enzymes from *Spirulina* sp. under testing.

MATERIALS AND METHODS

Microorganism and culture media

In this investigation *Spirulina* sp. was isolated from Wadi El-Natron salt lake (Egypt). Spirulina sp. was grown in modified Zarrouk's medium [27] which was used for culture maintenance and to prepare the inoculums as well as to study the growth of *Spirulina* sp. in batch culture.

Cultivation

Cultivation was done in 500 ml Erlenmeyer flask and the cultures were gassed with sterile air provided by a small air pump operating at a rate of 0.046 vvm (volumetric flow rate of air per volume of liquid per minute), at 30˚C. The pH of the medium was adjusted to pH 9.00 prior to autoclaving (controlled pH culture). The cultivated flasks were illuminated 24 h with continuous cool white fluorescent lamp at 48.4 µmole photon.$m^{-2}.s^{-1}$.The concentration of KH_2PO_4 (the phosphorus source), $NaNO_3$ (the nitrogen source), and K_2SO_4, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$ (the sulphur sources) in the medium being modified according to the experimental design explained below in section 2.3.

Experimental design

Nutrient deficiency: for this study a multilevel factorial design was used in which the concentrations of KH_2PO_4 in Zarrouk's medium were 0.5, 0.25, 0.125, 0.0 g/l for control, -50%, -75%, -100% P respectively, $NaNO_3$ concentrations in were 2.5, 1.25, 0.625 and 0.0 g/l for control, -50%, -75%, -100% N, respectively, the concentrations of K_2SO_4 were 1.0, 0.5, 0.25 and 0.0 g/l, the concentrations of $MgSO_4.7H_2O$ were 0.2, 0.1, 0.05, 0.0 g/l and the concentration of $FeSO_4.7H_2O$ were 0.01, 0.005, 0.0025, 0.0 g/l for control, -50%, -75%, -100% S respectively. In case of -100%S $MgSO_4.7H_2O$ was replaced by $MgCl_2$. Cultures were grown in different concentrations of NaCl (control, 0.3M, 0.6M and 0.9M). The prepared media were adjusted at different pH values at ((control 9), 6, 7, and 11). The temperature was adjusted at ((control 30˚C), 15˚C and 40˚C). The flasks were incubated at different light intensity levels ((control 48.4), 27.83 and 14.52 µmol.photon.$m^{-2}.s^{-1}$).

Growth measurements

The growth of the *Spirulina* sp. was daily followed by determination of optical density and/or chl. a content. In the late of exponential or beginning of the stationary phase according to the method described by Metzner et al. [28] and Marker [29]. The algal cells were harvested for some metabolic estimation. For determination of dry weight, 10 ml of algal suspension, after filtered through Whatman (GF/F) glass fiber paper, was dried in oven for 24 h at 80˚C, while filtering 20 ml of phosphate buffer (KH_2PO_4 0.01M, pH 7) was added to remove insoluble salts, the filter paper was then put in the oven, after cooling the filter paper was reweighed to evaluate the dry matter (mg/l algal suspension).

Determination of growth rate by optical density

Optical density of algal suspension were measured at 560nm [30] and the growth rate µ (h-1) was determined from the following formula:

$$\mu(h^{-1}) = \frac{\ln A_1 - \ln A_0}{t_1 - t_0}$$

Where: A1 = Optical density at time T1. A0= Optical density at time t0. t1-t0 = The time elapsed in days between two determinations of optical density. The generation time (G) can be calculated as follows:

$$G = \frac{\ln 2}{\mu} h^{-1}$$

Derermination of phycobiliproteins (phycocyanin, allophycocyanin and phycoerythrin)

Phycobiliproteins contents were determined according to the method described by Bennet and Bogorad [31]. Fifty ml of *Spirulina* culture were centrifuged at 4000 rpm and the pellet was resuspended in 20 ml of sterile distilled water. The quantitative extraction of biliproteins was achieved by combination of prolonged freezing and sonication, followed by centrifugation at 4000 rpm for 20 min. The crude extract was completed to 50 ml distilled water. The concentration of c-phycocyanin, phycoerythrin and allophycocyanin (mg/g D.wt.) in crude extracts was calculated by measuring the absorbance at 615, 652 and 526 nm, respectively using the following equations:

$PC = (E_{615} - 0.476\ E_{652}) / 5.34$

PC - c-phycocyanin

$APC = (E_{652} - 0.208\ E_{615}) / 5.09$

APC - allophycocyanin

$PE = E_{526} - 2.4\ (PC) - 0.849\ (APC) / 9.62$

PE - phycoerythrin
Total phycobiliproteins = PC+APC+PE

Estimation of antioxidant enzymes

Preparation of enzyme extract and assay of enzyme activity

Hundred ml of algal culture were centrifuged at 5000 rpm and the pellet was homogenized in 5 ml of 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM ethylenediamine tetraacetic acid (EDTA) and 0.1 g polyvinylpyrrolidone (PVP) by combination of prolonged freezing and sonication. The homogenate was centrifuged at 18000 rpm for 10 min. at 4ºC and the supernatants were collected and used for assays of catalase, ascorbate peroxidase and guaiacol peroxidase. All colorimetric measurements (including enzyme activities) were made at 20ºC using Unico UV-2100 spectrophotometer. The specific activity was expressed as units/mg protein. Protein concentrations in the enzyme extract were determined by the method of Lowry et al. [32].

Catalase

Catalase (CAT) activity was assayed by following the consumption of H_2O_2 for 1 min according to Aebi [33] and Matsumura et al. [34].

Guaiacol peroxidase

Guaiacol peroxidase (POD) activity was measured spectrophotometrically following the method of Tatiana et al. [35] with some modifications. The reaction mixture (3 ml) consisted of 30 mM potassium phosphate buffer (pH 7), 6.5 mM H_2O_2 and 1.5 mM guaiacol. The reaction was started by the addition of 100 µl enzyme extract. The formation of tetraguaiacol was measured at 470 nm.

Ascorbate peroxidase

Ascorbate peroxidase (APX) activity was determined according to the method of Nakano and Asada [36] with some modifications. The activity was determined by recording the decrease in A_{290} for 1 min. in 3 ml of reaction mixture containing 50 mM potassium phosphate buffer (pH 7), 0.5 mM ascorbic acid, 0.1 mM EDTA, 100 µl 0.1 mM H2O2 and 100 µl enzyme extract. The reaction was started with the addition of H_2O_2. Absorbance was measured at 290 nm in Unico UV-2100 spectrophotometer.

Determination of total lipids

Total lipid contents were determined by the sulfophosphovanilin method (SPV) Drevon and Schmit [37].

RESULTS

The growth of spirulina sp. expressed as daily change in chl. a content (µg/ml) were generally inhibited by all abiotic factors applied (Fig. 1). The maximum growth rate and minimum generation time were recorded at culture grown at pH 6.0 and 50% P (-), while the best biomass were recorded when the culture were grown at 30˚C, at high light intensity 48.4 µmol.photon.m^{-2}.s^{-1} and at 75% sulfur deficiency. High salinity (0.9M NaCl), completely removed nitrogen source from medium and growth at 15˚C caused severely drop in biomass (dry wt.) of *Spirulina* sp. (Table 1).

The phycobiliprotein fractions were depicted in Fig. 3 and it was found that the phycobiliprotein contents were decreased at 15˚C and 40˚C as well as at low light intensity. On the other hand, the pH 9 and the increase in NaCl concentration gave the maximum content of c-phycocyanin, allophycocyanin, phycoerythrin and total phycobiliproteins. In general, the phycobiliprotein fractions were decreased at 100% phosphorus, nitrogen and sulphur deficiency.

The production of β-carotene of *Spirulina* sp. under testing was stimulated when the culture grown in the presence of 0.9 M NaCl. Absence of phosphorus source from Zarrouk's medium caused significantly increase in β-carotene content while the deficiency of nitrogen, decreasing light intensities and temperature or pH caused decrease in β-carotene content (Fig. 2).

The total lipids content of *Spirulina* sp. under testing were shown in Fig. 2 and found that low light intensity, low temperature and high concentration of NaCl as well as nitrogen deficiency caused accumulation of total lipids content.

The activity of all antioxidant enzymes (catalase, peroxidase and guiacol peroxidase) was increased under all biotic factors and stress condition in comparison to control (Fig. 2).

DISCUSSION

The main environmental factors influencing microalgal growth and chemical composition are

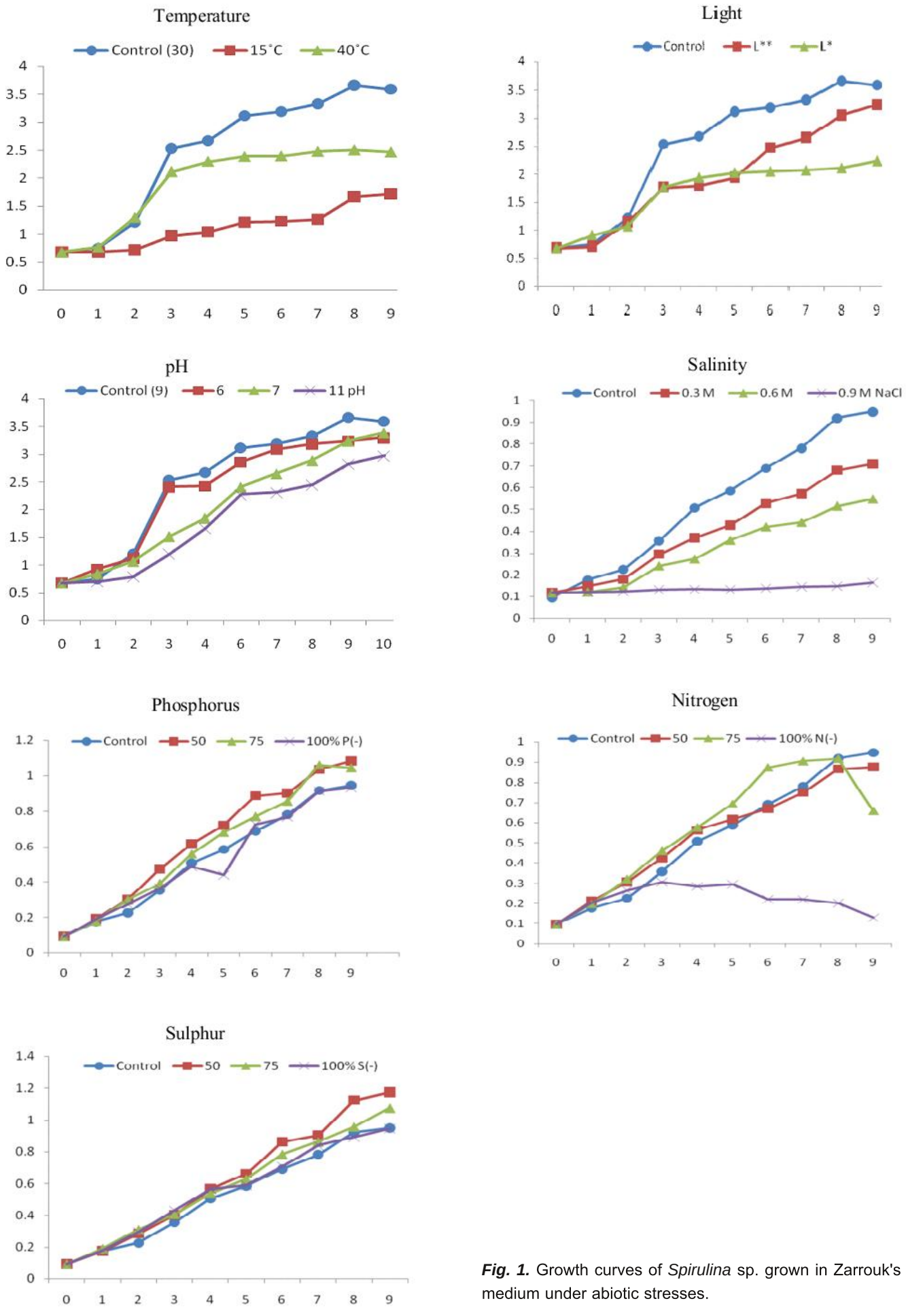


Fig. 1. Growth curves of *Spirulina* sp. grown in Zarrouk's medium under abiotic stresses.

Table 1. Effect of abiotic stresses on growth criteria of *Spirulina* sp.

	Temperature				Light					pH					Salinity				
	Chl. a (µg .ml^{-1})	**μ_{max} (h^{-1})**	**G (h^{-1})**	**Dry wt. gm/l**		**Chl. a (µg .ml^{-1})**	**μ_{max} (h^{-1})**	**G (h^{-1})**	**Dry wt. gm/l**		**Chl. a (µg .ml^{-1})**	**μ_{max} (h^{-1})**	**G (h^{-1})**	**Dry wt. gm/l**		**Chl. a (µg .ml^{-1})**	**μ_{max} (h^{-1})**	**G (h^{-1})**	**Dry wt. gm/l**
C. (30^{o}C)	3.59 ±.07^{c}	0.031	22.4	0.27	**C. (48.8)**	3.59 ±0.07^{b}	0.031	22.4	0.27	**C. (9)**	3.59 ±0.07^{b}	0.031	22.4	0.27	**C. (0.017M)**	3.11 ±0.15^{c}	0.031	22.2	0.23
15^{o}C	1.72 ±0.01^{a}	0.013	55.4	0.105	**L****	3.24 ±0.16^{b}	0.02	33.9	0.184	**6**	3.3 ±0.13^{ab}	0.032	21.6	0.203	**0.3M**	1.9 ±0.01^{b}	0.021	33.2	0.11
40^{o}C	2.47 ±0.08^{b}	0.02	33.9	0.264	**L***	2.24 ±0.08^{a}	0.021	33.2	0.148	**7**	3.39 ±0.14^{b}	0.015	46.7	0.224	**0.6M**	1.89 ±0.14^{b}	0.028	24.8	0.09
										11	2.97 ±0.04^{a}	0.018	39.3	0.282	**0.9M**	0.6 ±0.02^{a}	0.003	243	0.05

	Phosphorus				Nitrogen					Sulphur				
	Chl. a (µg.ml^{-1})	**μ_{max} (h^{-1})**	**G (h^{-1})**	**Dry wt. gm/l**		**Chl. a (µg.ml^{-1})**	**μ_{max} (h^{-1})**	**G (h^{-1})**	**Dry wt. gm/l**		**Chl. a (µg.ml^{-1})**	**μ_{max} (h^{-1})**	**G (h^{-1})**	**Dry wt. gm/l**
C.	3.11 ±0.15^{a}	0.031	22.2	0.23	**C.**	3.11 ±0.15^{c}	0.031	22.2	0.23	**C.**	3.11 ±0.15^{b}	0.031	22.2	0.23
50% P(-)	3.13 ±0.14^{a}	0.033	20.9	0.263	**50% N(-)**	1.76 ±0.01^{b}	0.014	50.1	0.212	**50% S(-)**	3.17 ±0.06^{b}	0.021	33.4	0.255
75% P(-)	2.68 ±0.14^{a}	0.018	39.3	0.254	**75% N(-)**	1.59 ±0.19^{b}	0.025	27.4	0.159	**75% S(-)**	3.02 ±0.2^{b}	0.014	50	0.261
100% P(-)	2.77 ±0.18^{a}	0.025	27.7	0.227	**100% N(-)**	0.29 ±0.03^{a}	0.017	41.6	0.11	**100% S(-)**	2.03 ±0.07^{a}	0.014	50	0.229

C.= Control, L** = light intensity (27.83 µmol.photon.m^{-2}.s^{-1}), L* = light intensity (14.52 µmol.photon.m^{-2}.s^{-1}), Chl. a (µg/ml), µmax = maximum growth rate and G = generation time. The data are given as averages of three replicates ± standard error. Values followed by the different letters are significantly different at $p < 0.05$.

light, nutrients, temperature and pH. Most of published literature on growth rates focuses on nutrient limitation, especially nitrogen, followed by phosphate and also silica deficiency for diatoms. The effect of temperature and light intensity on growth is also well studied in the literature when compared to the amount of information available on the effect of salinity [38]. The growth of *Spirulina* sp. was generally decreased by all applied abiotic factors. Ayachi et al. [39] found that an increase of NaCl concentration caused reduction of growth of *Spirulina* sp. and total inhibition of chlorophyll biosynthesis. Vonshak et al. [40] attributed this growth decrease to an energy shortage caused by pumping out the entering sodium ions and by the synthesis of sugar as osmoticum. A severely drop was occurred in biomass of *Spirulina* sp. expressed by dry weight when it was grown at 15˚C. In this respect, Rafiqul et al. [41] found that maximum specific growth rate of *Spirulina platensis* and *Spirulina fusiformis* was found at 32˚C and 37˚C respectively, both species showed negligible growth at 20˚C and 40˚C. The phycobiliprotein contents of *Spirulina* sp. were increased at 30˚C, pH 9 as well as 0.3M and 0.6M NaCl concentrations. Hemlata and Fatma [42] found that the optimum temperature for phycobiliproteins was obtained at 30˚C, there was 23.6 % decrease at 20˚C and 38% at 40˚C. Poza-Carrion, et al. [43] revealed that increasing pH (7-9) significantly increased the total phycobiliproteins content in *Nostoc* sp. UAM206. Abd El-Baky [44] reported that increasing in NaCl levels in nutrient medium led to significant increase in phycocyanin contents and soluble proteins in *Spirulina platensis* and *Spirulina maxima* cells. The phycobiliprotein fractions were decreased at 100% phosphorus, nitrogen and sulphur deficiency. Kassem et al. [45] reported that c-phycocyanin, allophycocyanin and total phycobiliproteins decreased by 14.89%, 26.63% and 18.64%, respectively in phosphorus starved cells in *Spirulina* sp. The content of β-carotene in *Spirulina* sp. was increased by the treatment with high salinity, but the deficiency of nitrogen, low light intensity and low temperature caused decrease in β-carotene content. Incharoensakdi and Phunpruch [46] found that nitrate deficiency in the growth medium did not cause an increase in β-carotene content, while increasing NaCl in the medium caused an increase in β-carotene content of *Spirulina platensis*. The total lipids content and the activity of all antioxidant enzymes of *Spirulina* sp. was increased under all biotic factors. Uslu et al. [47] found that nitrogen deficiency (50% and 100% N(-)) in *Spirulina* culture medium increased total lipid ratio (13.60 and 17.05%). Other researchers worked on other algae

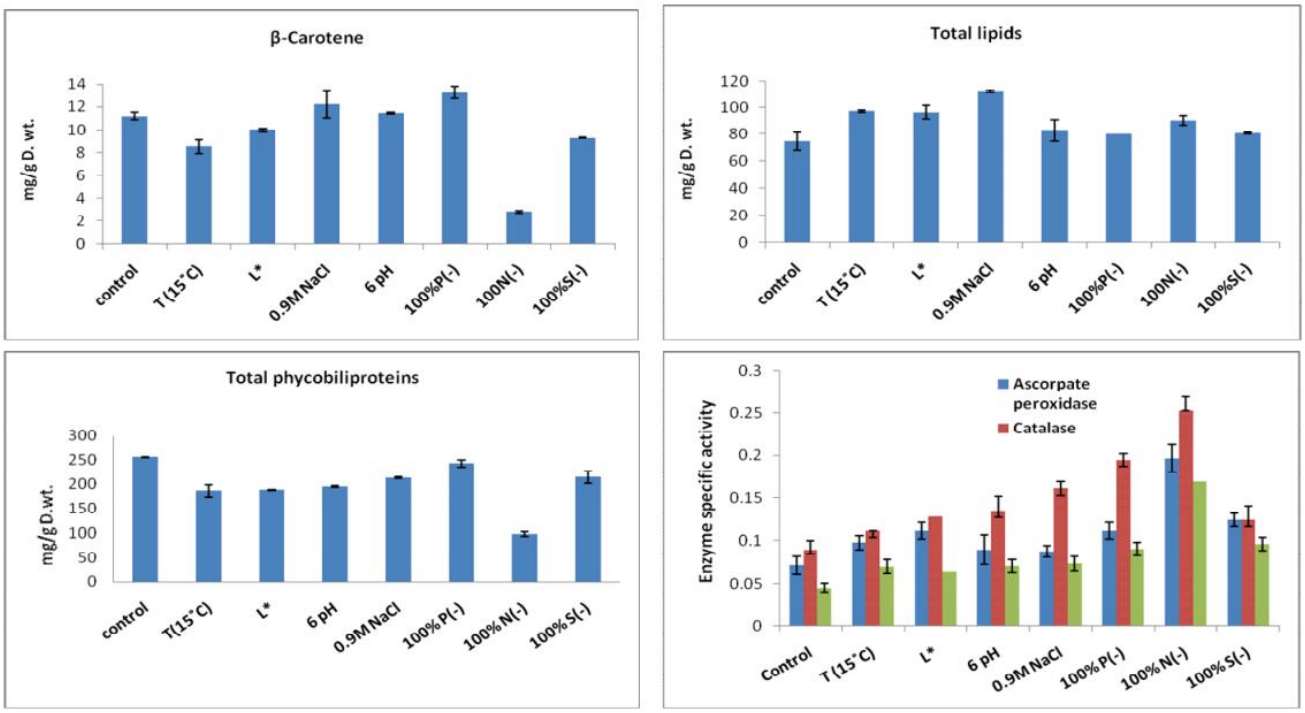


Fig. 2. Production of total lipids, β-carotene, total phycobiliprotein and antioxidant enzymes from *Spirulina* sp. under some abiotic stress.

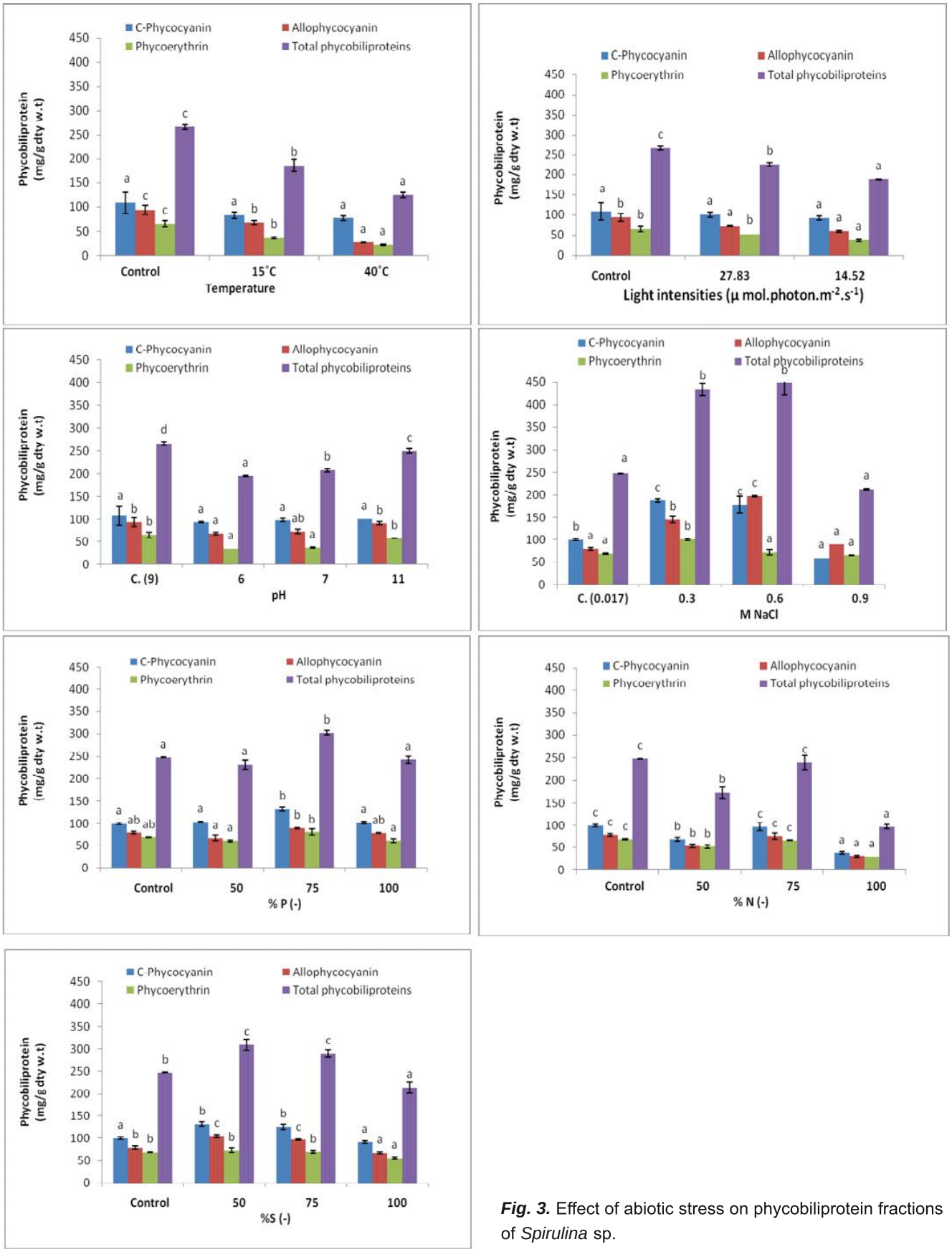


Fig. 3. Effect of abiotic stress on phycobiliprotein fractions of *Spirulina* sp.

and found that the reduction of nitrogen content alleviated the lipid content of algae as; Zhila et al. [48] on *Botryococcus braunii*, Pruvost et al. [49] on *Neoochloris oleobudas* and Bulut [50] on *Chlorella vulgaris*. Nitrogen deficiencies increased total lipids content; however biomass productivity did not fall significantly. For the feasible biodiesel studies with microalgae, the issue should determine the algal species which are resistant to environmental conditions, high biomass and high lipid productivity. There are some microalgae species having more lipid content but their low biomass productivity will restrict their large scale production for biodiesel. Ramadan et al. [51] reported that *S. platensis* gives considerable yield of lipids which are rich sources of essential fatty acids and lipid soluble antioxidants; moreover the recovered lipid may be suitable for commercial exploitation as a source of lipids for food use and cosmetics production. It has been recorded that polyunsaturated fatty acids, phycobiliproteins, β-carotene and other pigments function as antioxidants [52, 53]. Phycobiliproteins have been described as the strongest antioxidant such as in cases of PC in the studies of Bhat and Madyasth [54], [55] and [56]; APC in the studies of Ge et al. [57] and PE in the studies of Yuan et al. [58], therefore PBPs have gained a somewhat high level of expectation as a potential source of bioactive substances.

In conclusion *Spirulina* sp. can elevate the activity of all the antioxidant enzymes, the effect may be due to the high phytopigments (carotenoids, chlorophyll, phycocyanin). Therefore make *Spirulina* sp. under investigation good candidates for successful cultivation in artificial open ponds and/or in Wadi El-Natron lake under different environmental condition as a high value health foods, functional food and as a source of wide spectrum of nutrient especially these anticancer substances as β-carotene or can be used as a model for biotechnological production of antioxidant enzymes.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Wang L, Pan B, Sheng J, Juan Xu J, Hu Q. Antioxidant activity of *Spirulina plantasis* extracts by supercritical carbon dioxide extraction. Food Chem. 2007; 105: 36–41.
2. Ghosh P, Adhikari U, Ghosal PK. In vitro anti-herpetic activity of sulfated polysaccharide fractions from *Caulerpa racemosa*. Phytochem. 2004; 65: 3151–3157.
3. Prakash S, Sasikala SL, Aldous V, Huxley J. Isolation and identification of MDR-*Mycobacterium tuberculosis* and screening of partially characterized antimycobacterial compounds from chosen marine micro algae. Asian Pac J Trop Med. 2010; 3(8): 655–661.
4. Rastogi RP, Sinha RP. Biotechnological and industrial significance of cyanobacterial secondary metabolites. Biotechnol Adv. 2009; 27: 521–39.
5. Glazer AN, Apell GS, Hixson CS, Bryant DA, Rimon S, Brown DM. Biliproteins of cyanobacteria and Rhodophyta: homologous family of photosynthetic accessory pigments. Proc Natl Acad Sci USA. 1976; 73: 428–431.
6. Yamanaka G, Glazer AN, Williams RC. Cyanobacterial phycobilisomes characterization of the phycobilisomes of *Synechococcus* sp. 6301. J Biol Chem. 1978; 253: 8303–8301.
7. Dillon JC, Phuc AP, Dubacq JP. Nutritional value of the alga *Spirulina*. World Rev Nutr Diet. 1995; 77: 32–46.
8. Quoc KP, Pascaud M. Effects of dietary gammalinolenic acid on the tissue phospholipid fatty acid composition and the synthesis of eicosanoids in rats. Ann Nutr Metabol. 1996; 40: 99–108.
9. Hirahashi T, Matsumoto M, Hazeki K, Saeki Y, Ui M, Seya T. Activation of the human innate immune system by *Spirulina* augmentation of interferon production and NK cytotoxicity by oral administration of hot water extract of *Spirulina platensis*. Int Immunopharmacol. 2002; 2: 423–434.
10. Subhashini J, Mahipal SVK, Reddy MC, Reddy MM, Rachamallu A, Reddanna P. Molecular mechanisms in C-Phycocyanin induced apoptosis in human chronic myeloid leukemia cell line - K562. Biochem Pharmacol. 2004; 68: 453–462.
11. Sasson A. 1997. Micro Biotechnologies: Recent Developments and Prospects for Developing Countries. BIOTEC Publication 1/2542. Place de Fontenoy, Paris. France. United Nations Educational, Scientific and Cultural Organization (UNESCO): 11–31.
12. Costa JAV, Colla LM, Filho PFD. Improving *Spirulina platensis* biomass yield using a fedbatch process. Biores Technol. 2004; 92: 237–241.

13. Habib MAB, Huntington TC, Hasan MR. 2008. A review on culture, production and use of *Spirulina* as food for humans and feeds for domestic animals and fish. FAO Fisheries and Aquaculture Circular No. 1034.
14. Romay C, Gonzalez R, Ledon N, Remirez D, Rimbau V. C-phycocyanin: a biliprotein with antioxidant, anti-inflammatory and neuroprotective effects. Curr Protein Pept Sci. 2003; 4(3): 207-216.
15. Liu Y, Xu L, Cheng N, Lin L, Zhang C. Inhibitory effect of phycocyanin from Spirulina platensis on the growth of human leukemia K562 cells. J Appl Phycol. 2000; 12(2): 125-130.
16. Liu B, Zhang X, Gao M, Chu, X. Effects of CD59 on antitumoral activities of phycocyanin from *Spirulina platensis*. Biomed Pharmacother. 2005; 59(10): 551-560.
17. Pardhasaradhi BV, Ali AM, Kumari AL, Reddanna P Khar A. Phycocyanin-mediated apoptosis in AK-5 tumor cells involves down-regulation of Bcl-2 and generation of ROS. Mol Cancer Ther. 2003; 2(11): 1165-1170.
18. Ivanova KG, Stankova KG, Nikolov VN, Georgieva RT, Minkova KM, Gigova LG, Rupova IT, Boteva RN. The biliprotein C-phycocyanin modulates the early radiation response: A pilot study. Mutat Res. 2010; 695: 40-45.
19. Singh CS, Sinha PR, Hader PD. Role of lipids and fatty acids in stress tolerance in cyanobacteria. Acta Protozool. 2002; 41: 297–308.
20. Othes S, Pire R. Fatty acid composition of *Chlorella* and *Spirulina microalgae* species. J AOAC Int. 2001; 84: 1708–1714.
21. Kyle DJ, Boswell KD, Gladue RM, Reab SE. 1992. Designer oils from micro algae nutritional supplements. In: Bills DB, Kung S. (eds): Biotechnology and Nutrition. Proceeding 3rd International Symposium Butterworth, Heinemann, Boston: 468.
22. Carlson SE, Salem N. 1991. Essential of ω3 fatty acids in growth and development of infants. In: Simopoulus AP, Kifer RR, Barlow SM. (eds): Health Effects of ω3 Polyunsaturated Fatty Acids in Seafood. World Revue of Nutrition and Dietetics. Vol. 66. Karger, Basel: 74–86.
23. Innis SM. Essential fatty acids in growth and development. Progress Lipid Res. 1991; 30: 39–103.
24. Ogata J, Hagiwara Y, Hagiwara H, Shibamoto T. Inhibition of molonoldehyde formation by antioxidant from ω3 polyunsaturated fatty acids. J Am Oil Chem Soc. 1996; 73: 654–656.
25. Roughan GP. *Spirulina*: A source of dietary gamma-linolenic acid. J Agricult Food Chem. 1989; 47: 85–93.
26. Saito T, Saito T, Oka N. 1992. Scientific Reports on *Chlorella* in Japan. Japan *Chlorella* Treatment Association. Silpaque Publishing, Kyoto.
27. Zarrouk C. 1966. Contribution à l'étude d'une cyanophycée. Influence de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de *Spirulina maxima*. Ph.D. Thesis, Université De Paris.
28. Metzner H, Rau H, Senger H. Untersuchungen zur synchronisier-barkeit ein zelner pigment managel. Mutanten von *Chlorella*. Planta 1965; 65: 186-194.
29. Marker AFH. The use of acetone and methanol in the estimation of chlorophyll in the presence of Phaeophytin. Fresh Wat Biol. 1972; 2: 361-385.
30. Fatma T, Sarada R, Venkataraman LV. Evaluation of selected strains of *Spirulina* for their constituents. Pykos 1994; 33(1-2): 89-97.
31. Bennet A, Bogorad L. Complementary chromatic adaptation in filamentous blue-green algae. J Cell Biol. 1973; 58: 419-435.
32. Lowry DH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem. 1951; 193: 265-275.
33. Aebi H. Catalase in vitro. Methods in Enzymol. 1984; 105: 121-126.
34. Matsumura T, Tabayashi N, Kamagata Y, Souma C, Saruyama H. Wheat catalase expressed in transgenic rice can improve tolerance against low temperature stress. Physiol Plant. 2002; 116: 317-327.
35. Tatiana Z, Yamashita K, Matsumoto H. Iron deficiency induced changes in ascorbate content and enzyme activities related to ascorbate metabolism in cucumber root. Plant Cell Physiol. 1999; 40: 273-280.
36. Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 1981; 22: 867-880.
37. Drevon B, Schmit JM. 1964. La reaction sulfophosphovanillique dans l'ét.ude des lipides seriques. Bull. Trau. Soc. Pharm. Lyon 8: 173-178.
38. Rousch JM, Bingham SE, Sommaerfeld MR. Change in fatty acid profiles of thermo-intolerent and thermo tolerant marine diatoms during temperature stress. Exp Mar Biol Ecol. 2003; 295: 145-156.
39. Ayachi A, El-Abed A, Dhifi W, Marzouk B. Chlorophylls, proteins and fatty acids amounts of *Arthrospira platensis* growing under saline conditions. Pakistan J Biol Sci. 2007; 10: 2286-2291.
40. Vonshak A, Guy R, Guy M. The response of the filamentous cyanobacterium *Spirulina platensis* to salt stress. Arch Microbiol. 1988; 150: 417–420.
41. Rafiqul Islam Md, Hassan A, Sulebele G, Orosco C, Roustaian P. Influence of temperature on growth and biochemical composition of *Spirulina platensis* and *S. fusiformis*. Iranian Int J Sci. 2003; 4(2): 97-106.
42. Hemlata, Fatma T. Screening of Cyanobacteria for phycobiliproteins and effect of different environmental

stress on its yield. Bull Environ Contam Toxicol. 2009; 83: 509–515.

43. Poza-Carrion C, Fernadez-Valiente E, Fernadez-Pinas F, Leganes F. Acclimation of photosynthetic pigments and photosynthesis of the cyanobacterium *Nostoc* sp. Strain UAM 206 to combined fluctuations of irradiance, pH and inorganic carbon availability. Plant Physiol. 2001; 158: 1455–1461.
44. Abd El-Baky HH. Over production of phycocyanin pigment in blue green alga *Spirulina* sp. and its inhibitory effect on growth of Ehrlich ascites carcinoma cells. Med Sci. 2003; 3: 314–24.
45. Kassem DA. 2010. Possible therapeutic effect of *Spirulina* sp. on hepatotoxicity in rates. M.Sc. Thesis. Bot. Dept. Fac. of Sci. Tanta Univ., Egypt.
46. Incharoensakdi A, Phunpruch S. 1996. Environmental factors affecting the beta-carotene content in *Spirulina platensis*. In: "Proceedings of the 3rd Asia-Pacific Conference on Agricultural Biotechnology: Issues and Choices". Melia Hotel, Prachuabkhirikhan, 10-15 November: 587-592.
47. Uslu L, Isik O, Koc K, Göksan T. The effects of nitrogen deficiencies on the lipid and protein contents of *Spirulina platensis*. Afr J Biotechnol. 2011; 10(3): 386-389.
48. Zhila NO, Kalacheva GS, Volova TG. Influence of nitrogen deficiency on biochemical composition of the green alga *Botryococcus*. J Appl Phycol. 2005; 17: 309-315.
49. Pruvost J, Van Vooren G, Cogne G, Legrand J. Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor. Biores Technol. 2009; 10(23): 5988-5995.
50. Bulut Y. 2009. The investigations on the possibility of increase lipid content of *Chlorella* (Master Thesis). Cukurova Univ., Institute of Science and Technology, Biotechnology Department, 62 p. Turkey.
51. Ramadan MF, Asker MMS, Ibrahim ZK. Functional bioactive compounds and biological activities of *Spirulina platensis* lipids. Czech J Food Sci. 2008; 26: 211–222.
52. Reddy CM, Vadiraja BB, Kiranmai G, Reddy MN, Reddanna P, Madyastha KM. Selective inhibition of cyclooxygenase-2 by C-phycocyanin, a biliprotein from *Spirulina platensis*. Biochem Biophys Res Commun. 2000; 277: 599-603.
53. Estrada JE, Bescós P, Villar Del Fresno AM. Antioxidant activity of different fractions of Spirulina platensis protean extract. Il Farmaco 2001; 56: 497-500.
54. Bhat V B, Madyastha KM. C-phycocyanin: a potent peroxyl radical scavenger in vivo and in vitro. Biochem Biophys Res Communic. 2000; 275: 20–25.
55. Benedetti S, Benveuti F, Pagliarani S, Francogli S, Scoglio S, Canestrari F. Antioxidant properties of a novel phycocyanin extract from the blue green alga *Aphanizomenon flos-aquae*. Life Sci. 2004; 75: 2353-2362.
56. Huang Z, Guo BJ, Wong RNS, Jiang Y. Characterization and antioxidant activity of selenium-containing phycocyanin isolated from *Spirulina platensis*. Food Chem. 2007; 100: 1137-1143.
57. Ge B, Qin S, Han L, Lin F, Ren Y. Antioxidant properties of recombinant allophycocyanin expressed in *Escherichia coli*. J Photochem Photobiol B, Biol. 2006; 84: 175-80.
58. Yuan YV, Carrington MF, Walsh M. Extracts from dulse (*Palmaria palmata*) are effective antioxidants and inhibitors of cell proliferation in vitro. Food Chem Toxicol. 2005; 43: 1073-1081.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Reproduction of *Lymnaea acuminata* fed to bait containing binary combination of amino acids with molluscicide

Pradeep Kumar, Vinay Kumar Singh, D. K. Singh

Malacology Laboratory, Department of Zoology, DDU Gorakhpur University, Gorakhpur, India

ABSTRACT

Effect of sublethal (20% and 60% of 24h LC_{50}) feeding of molluscicides (eugenol, ferulic acid, umbelliferone and limonene) in bait containing binary combination (1:1 ratio) of amino acids (valine, aspartic acid, lysine and alanine) on the, hatchability and survival of young snails of *Lymnaea* (*Radix*) *acuminata* (Lamark) was studied. It was observed that the different amino acid combinations of bait formulation with molluscicides significantly reduced the reproductive capacity (fecundity, hatchability and survival of young snails) of the snail *Lymnaea acuminata*. Maximum in reduction of fecundity was observed in valine + aspartic acid + ferulic acid (28.89% of control). In withdrawal group there is a significant recovery observed in all feeded group (85.95% of control).

Key words: Amino acid; Molluscicides; Bait formulation; Fecundity; Hatchability; *Lymnaea acuminata*.

J Biol Earth Sci 2013; 3(1): B65-B71

Corresponding author:
Prof. D. K. Singh
Malacology Laboratory, Department of Zoology,
DDU Gorakhpur University, Gorakhpur,
273009, U.P., India.
Tel: +91-551-2202187(O)/200509(R);
9454211574 (Mobile).
E-mail: dksingh_gpu@yahoo.co.in

Original Submission: 09 December 2012; Revised Submission: 28 January 2013; Accepted: 01 February 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Fasciolosis is a dreadful to human as well as in animals, in developing countries [1, 2]. It is caused by the digenetic trematode *Fasciola hepatica* and *F. gigantica* having two hosts, a final mammalian hosts and a molluscan intermediate snail hosts [3-5]. *Lymnaea acuminata* breeds all the year round and lay eggs on the lower surface of the aquatic plants. Snail control is regarded by many experts as the best means to control the trematode infections [6-8]. Direct releases of molluscicides in aquatic environment have played a very significant role in controlling the population of molluscan pest problem [7]. With growing awareness of environmental pollution caused by synthetic molluscicides, now the recent research focus is turned on to plant products as alternatives, as they seemed to cause fewer side effects, more effective and readily available [7, 9-11]. Use of bait formulation containing an attractant and molluscicides is very effective in controlling the population of target molluscan pest [4, 12-15]. Recently, we have observed that different preparations of bait formulation of molluscicides (eugenol, ferulic acid, umbelliferone and limonene) in binary combination with different amino acids as an attractant are very effective against *L. acuminata* [4, 15]. It has been advocated that a molluscicide is very effective if it also kill snail eggs and reduce the reproductive capacity of snail [8, 16].

The aim of present study is to evaluate the effect of sublethal feeding of molluscicides (eugenol, ferulic acid, umbelliferone and limonene) in binary combination (1:1 ratio) of amino acid (valine, aspartic acid, lysine and alanine) in bait formulation on the fecundity, hatchability and survival of young snails of *L. acuminata*.

MATERIALS AND METHODS

Test Material

Adult snails (2.60 ± 0.30 cm in length) were collected locally from lakes; ponds and low lying submerged fields and were used as test animals. The snails were acclimatized for 72h in laboratory condition. The pH of the water was 7.1-7.3 and dissolved O_2, free CO_2 and bicarbonate alkalinity were 6.5-7.2 mg/l, 5.2-6.3 mg/l and 102.0-105.0 mg/l, respectively. Twenty experimental snails were kept in glass aquaria containing 3 L of dechlorinated tap water at 22ºC to 24ºC. *Lymnaea acuminata* laid their eggs on the lower surface of leaves of the aquatic plants in the form of elongated gelatinous capsules containing 5-180 eggs.

Pure compounds

Agar-agar, amino acids (valine, aspartic acid, lysine and alanine) and different active components (molluscicidel: eugenol, ferulic acid, umbelliferone and limonene) were used in bait formulations. The pure active components eugenol (2-Methoxy-4-(2-propenyl) phenol), ferulic acid (4-Hydroxy-3-methoxycinnamic), umbelliferone (7-Hydroxy coumarin; 7-hydroxy-2H-1-benzopyran-2-one) and limonene (R)-4-Isopropenyl-1-methyl-1-cyclohexene): were purchased from Sigma Chemical Co. (USA).

Preparation of bait formulation with molluscicides

Bait formulations containing binary combination (1:1 ratio) of amino acids (valine, aspartic acid, lysine and alanine 10 mM) and sub-lethal (20% and 60% of 24h and 96h LC_{50}) molluscicides (eugenol, ferulic acid, umbelliferone and limonene) were prepared in 100 ml of 2% agar solution by the method of Madsen [17]. Concentrations of amino acids were based on the earlier reports of Tiwari and Singh [18, 19]. Sublethal concentrations of molluscicides in bait were based on the study of Kumar et al. [5]. These solutions were spread at a uniform thickness of 5 mm. After cooling the bait containing sublethal molluscicides were cut out a corer measuring 5 mm in diameter. Six replicates were prepared for each concentration. Control snails were fed with bait without molluscicide.

Assay apparatus and procedure

The bioassay was performed by the method by Tiwari and Singh [18, 19]. The bioassay chambers consist of a clean glass aquarium having a diameter of 30 cm. Each aquarium was divided into four concentric zones; Zone 3 (Central zone), 2, 1 (Middle zone) and zone 0 (Outer zone) had diameters of 13, 18, 24 and 30 cm, respectively. A small annular elevation of 9 mm height and 2.4 cm diameter was made in the centre of aquarium (Zone 3). Zone 0 had an area of 254 cm^2 on the periphery of aquarium. The aquaria were then filled with 3L of dechlorinated tap water to a height of 8 mm and maintained at 25±1ºC. At the start of the assay twenty snails of uniform size were placed on the circumference of zone 0. Simultaneously, one of the prepared bait of different active component

(molluscicides) was added on the small annular elevation in the center (Zone 3). Six sets of experiments have been designed with twenty snails in each replicate.

Treatments

Snail fed to bait formulation containing sublethal molluscicide (eugenol, ferulic acid, umbelliferone and limonene) and binary combination (1:1 ratio) of amino acid (valine, aspartic acid, lysine and alanine) as attractant on snail reproduction was studied by the method of Kumar et al. [5]. Groups of 20 snails in 3L water were fed to sublethal concentrations (20% and 60% of 24h LC_{50}) of different binary combinations of amino acids with molluscicides.

The total number of eggs laid by bait fed and control snails were counted after every 24h for 96h. Since it is difficult to detect the mother snails for particular spawn, capsules containing eggs from each feeded group were incubated at 30ºC in covered petridishes. The development of embryos at regular intervals was observed under binocular microscope until hatching. Per cent hatching was studied only with eggs laid after the 24h feeding period. Dead embryo was removed to avoid any contamination. Survival of young snails was observed up to 72h. Snails were transferred to fresh water after a 96h feeding period to observe the effect of the above bait formulations after withdrawal.

Statistical analysis

Each experiment was replicated at least 6 times. Values were expressed as Mean±SE. Students 't' test was applied to determine the significant ($P<0.05$) difference between bait feeded and control animals. Product moment correlation coefficient was applied in between exposure time and different values of fecundity/survival of hatched snails [20].

RESULTS

Feeding of bait to snail *L. acuminata* caused significant ($p<0.05$) reduction in the fecundity of snail *L. acuminata* (Table 1). In control groups of 20 snails laid 180-184 eggs/day. Binary combination (1:1 ratio) of amino acids as a snail attractants with molluscicides in bait formulations caused significant reduction in fecundity of snail *L. acuminata* (Table 1). No egg lying after 48h feeding was observed in the snails feed to 60% of 24h LC_{50} of bait formulation in binary combination (1:1 ratio) of alanine + valine with active molluscicidal component eugenol. The hatching period was prolonged in treated group (11-23 days) with respect to control group (7-9 days) (Table 2). Withdrawal of snail after 96h feeding of bait for 72h in fresh water caused a significant ($P<0.05$) recovery in the fecundity of snail with respect to their corresponding treatment (Table 1).

Snails fed with 20% or 60% of 24h LC_{50} of valine + aspartic acid with eugenol and lysine + valine with eugenol 60% of 24h LC_{50} caused no survival of snails after 24h of hatching (Table 2). There was a significant ($P<0.05$) negative correlation between the feeding time and survival of young snails hatched from eggs laid by snail feeded to 20%, 60% of 24h LC_{50} of different preparation of bait formulation (Table 2).

DISCUSSION

It is clear from the result section that sublethal feeding (20% and 60% of 24h LC_{50}) of the binary combination (1:1 ratio) of amino acids (valine, aspartic acid, lysine and alanine) with molluscicides (eugenol, ferulic acid, umbelliferone and limonene) in bait formulations, significantly reduced the reproductive capacity of snail *L. acuminata*. Bait formulation with 60% of 24h LC_{50} of alanine + valine with active molluscicidal component eugenol in binary combination (1:1 ratio) reduced the fecundity of *L. acuminata* within 24h.

The effect of sublethal eugenol, ferulic acid, umbelliferone and limonene feeding in bait formulation containing attractant amino acids caused a significant reduction in fecundity, hatchability and survival of the young snails of *L. acuminata*. The plant derived active component eugenol (*Syzygium aromaticum*), ferulic acid/ umbelliferone (*Ferula asafoetida*) and limonene (*Carum carvi*) have dose dependent influence on the fecundity of snails [8]. A number of plant products have been effectively used for control of snail reproduction [16, 21-24]. Instead of increasing the molluscicidal activity of eugenol, ferulic acid, umbelliferone and limonene direct release in aquatic environment [8], it reduces the reproductive capacity of snail *L. acuminata*. Tripathi et al. [25] reported that the combinations of cow urine kept in sunlight with *Camellia sinensis* and *Ferula*

Table 1. Effect of sublethal (20% and 60% of 24h LC_{50}) molluscicide (eugenol, ferulic acid, umbelliferone and limonene) in bait containing binary combinations (1:1 ratio) of amino acids (valine, aspartic acid, lysine and alanine) on the fecundity of the snail *L. acuminata*.

Treatment (Sublethal concentration of 24h LC_{50} of 20 and 60%)	Fecundity after 24h (eggs/20 snail)	Fecundity after 48h (eggs/20 snail)	Fecundity after 72h (eggs/20 snail)	Fecundity after 96h (eggs/20 snail)	Withdrawal after 96h feeded Fecundity after 72h (eggs/20 snails)
Control (Agar)	175.22±0.32	176.13±0.45	180.21±0.26	184.63±0.65	186.40±0.32
Control (a) Vali+Aspa	165.28±0.26	163.42±0.68	166.20±0.44	168.39±0.24	167.20±0.33
Control (b) Lysi+Vali	164.46±0.48	165.23±0.76	163.31±0.27	164.84±0.72	165.48±0.32
Control (c) Lysi+Ala	165.32±0.68	166.45±0.37	164.29±0.28	165.72±0.82	164.36±0.83
Control (d) Ala+Vali	163.28±0.24	165.25±0.48	166.33±0.26	164.38±0.25	163.61±0.21
Vali+Aspa+Eug	65.23±0.29*+	60.85±0.88*	55.68±0.21*	52.73±0.23*	146.62±0.73
	30.85±0.42*+	26.76±0.32*	24.32±0.76*	23.75±0.85*	125.85±0.85
Vali+Aspa+Fer	50.63±0.82*+	48.80±0.63*	45.66±0.23*	40.85±0.91*	160.22±0.66
	38.48±0.96*+	25.73±0.92*	20.76±0.93*	19.32±0.81*	124.36±0.26
Vali+Aspa+Umb	50.64±0.72*+	48.81±0.73*	46.79±0.81*	42.53±0.39*	124.79±0.55
	30.62±0.82*+	27.62±0.11*	26.92±0.44*	23.13±0.71*	125.78±0.50
Vali+Aspa+lim	62.32±0.72*+	59.69±0.78*	55.77±0.26*	51.97±0.23*	156.66±0.21
	36.22±0.76*+	35.26±0.93*	31.82±0.76*	28.66±0.28*	134.81±0.18
Lysi+Vali+Eug	64.45±0.85*+	60.81±0.49*	51.65±0.24*	36.95±0.72*	140.85±0.62
	32.72±0.92*+	28.67±0.32*	22.76±0.52*	-	130.62±0.71
Lysi+Vali+Fer	51.68±0.76*+	48.69±0.48*	40.92±0.23*	38.67±0.23*	142.72±0.23
	35.72±0.28*+	31.76±0.94*	28.42±0.69*	-	135.26±0.29
Lysi+Vali+Umb	51.72±0.92*+	49.72±0.48*	46.55±0.31*	42.63±0.29*	148.62±0.28
	28.63±0.29*+	27.69±0.77*	25.66±0.30*	24.96±0.49*	128.68±0.81
Lysi+Vali+Lim	60.82±0.36*+	57.48±0.92*	55.42±0.97*	51.26±0.72*	157.64±0.24
	30.76±0.85*+	28.38±0.64*	26.92±0.46*	24.85±0.46*	129.84±0.85
Lysi+Ala+Eug	64.41±0.65*+	58.63±0.92*	55.76±0.26*	48.64±0.65*	150.65±0.11
	40.82±0.38*+	38.46±0.82*	35.79±0.23*	-	140.52±0.08
Lysi+Ala+Fer	52.68±0.72*+	48.63±0.58*	45.23±0.96*	36.11±0.28*	142.65±0.31
	37.61±0.98*+	32.82±0.36*	25.38±0.72*	22.81±0.76*	126.72±0.92
Lysi+Ala+Umb	51.74±0.78*+	48.66±0.48*	43.26±0.24*	38.71±0.42*	146.12±0.90
	28.62±0.30*+	26.73±0.92*	22.76±0.81*	21.48±0.72*	124.86±0.24
Lysi+Ala+Lim	60.42±0.82*+	58.92±0.48*	55.64±0.42*	48.96±0.21*	157.24±0.72
	38.52±0.63*+	36.80±0.36*	32.75±0.72*	29.65±0.56*	132.62±0.73
Ala+Vali+Eug	64.38±0.75*+	60.45±0.96*	58.42±0.44*	-	160.56±0.32
	28.58±0.76*+	-	-	-	132.64±0.72
Ala+Vali+Fer	50.98±0.62*+	48.64±0.93*	48.72±0.28*	43.81±0.26*	148.77±0.44
	29.11±0.64*+	28.06±0.48*	26.92±0.72*	23.96±0.33*	126.56±0.72
Ala+Vali+Umb	54.38±0.82*+	53.96±0.55*	50.48±0.26*	46.72±0.66*	152.85±0.36
	28.81±0.57*+	26.33±0.76*	24.76±0.92*	22.76±0.32*	127.68±0.90
Ala+Vali+Lim	60.76±0.26*+	55.48±0.48*	52.73±0.38*	48.62±0.71*	154.64±0.33
	27.82±0.86*+	24.90±0.82*	23.66±0.48*	23.06±0.82*	125.86±0.72

Each value is mean±SE of six replicates. Each replicates represents the egg laid by the group of 20 snails. (*) significant (P<0.05) when student "t" test was applied to treated and control groups. (+) product moment correlation coefficient showed that there was significant (P<0.05) negative correlation in between the exposure period and fecundity of snail *L. acuminata*. (-) No fecundity was observed, Abbreviations: Vali = valine, Aspa = aspartic acid, Lysi = lysine, Ala = alanine, Eug = eugenol, Fer = ferulic acid, Umb = umbelliferone, Lim = limonene.

Table 2. Effect of sublethal (20% and 60% of 24h LC_{50}) molluscicide (eugenol, ferulic acid, umbelliferone and limonene) in bait containing binary combinations (1:1 ratio) of amino acids (valine, aspartic acid, lysine and alanine) on the hatchability and survival of the snail *L. acuminata* eggs obtained after 24h in table1.

Treatment (Sublethal feeded of 24 h LC_{50} of 20 and 60%)	Hatchability percentage (hatching period)	Percent survival after 24h	Percent survival after 48h	Percent survival after 72h
Control (Agar)	100 (7-9)	100	100	100
Control (a) Vali+Aspa	100 (7-8)	100	100	100
Control (b) Lysi+Vali	100 (7-10)	100	100	100
Control (c) Lysi+Ala	100 (7-9)	100	100	100
Control (d) Ala+Vali	100 (7-9)	100	100	100
Vali+Aspa+Eug	50.62±0.92 (11-15)	-	-	-
	26.92±0.81(11-14)	-	-	-
Vali+Aspa+Fer	40.62±0.52 (13-13)	36.52±0.12*+	26.63±0.23	-
	32.42±0.92 (14-15)	24.81±0.81*+	-	-
Vali+Aspa+Umb	46.72±0.62(13-15)	38.62±0.12*+	31.42±0.72	24.82±0.81
	37.25±0.51 (15-18)	36.41±0.31*+	28.62±0.92	22.72±0.44
Vali+Aspa+lim	55.81±0.32 (11-14)	48.81±0.92*+	45.67±0.81	38.72±0.32
	36.32±0.42 (12-17)	33.92±0.30*+	27.92±0.38	24.62±0.81
Lysi+Vali+Eug	55.62±0.62 (17-19)	44.86±0.72*+	36.24±0.63	-
	31.42±0.90 (16-18)	-	-	-
Lysi+Vali+Fer	38.13±0.42 (14-18)	36.81±0.38*+	31.75±0.81	26.62±0.72
	36.42±0.72 (12-16)	32.72±0.81*+	28.42±0.71	-
Lysi+Vali+Umb	48.42±0.66 (13-15)	38.92±0.75*+	36.22±0.61	30.65±0.32
	38.61±0.92 (14-18)	37.55±0.09*+	32.70±0.81	25.62±0.72
Lysi+Vali+Lim	55.92±0.81 (11-14)	50.63±0.13*+	47.32±0.92	38.29±0.16
	41.38±0.92 (13-16)	39.72±0.63*+	35.22±0.31	30.08±0.18
Lysi+Ala+Eug	60.42±0.31 (18-20)	40.29±0.42*+	36.23±0.40	-
	31.85±0.24 (19-23)	28.39±0.72*+	-	-
Lysi+Ala+Fer	40.76±0.48 (14-17)	36.75±0.81*+	31.20±0.72	-
	31.74±0.42 (15-18)	28.92±0.46*+	-	-
Lysi+Ala+Umb	46.09±0.36 (13-16)	41.42±0.72 *+	38.40±0.72	31.42±0.12
	41.42±0.26 (14-17)	38.42±0.62*+	33.92±0.42	28.42±0.92
Lysi+Ala+Lim	55.36±0.72 (11-14)	41.48±0.83*+	38.66±0.19	30.19±0.66
	35.13±0.42 (12-16)	30.98±0.16*+	25.13±0.17	23.06±0.94
Ala+Vali+Eug	50.42±0.72 (19-20)	38.42±0.72*+	-	-
	39.42±0.11 (20-21)	32.26±0.41*+	28.42±0.13	22.42±0.36
Ala+Vali+Fer	36.40±0.72 (14-16)	31.92±0.36*+	25.95±0.18	-
	25.48±0.81 (15-18)	24.42±0.81*+	22.66±0.70	21.92±0.13
Ala+Vali+Umb	48.90±0.42 (13-16)	47.32±0.22*+	39.04±0.61	30.24±0.42
	44.93±0.60 (14-18)	39.42±0.15*+	31.32±0.48	26.40±0.23
Ala+Vali+Lim	50.80±0.23 (15-19)	47.38±0.23*+	40.69±0.81	37.92±0.24
	40.82±0.13 (17-20)	38.28±0.24*+	35.42±0.70	34.72±0.24

Each value is mean±SE of six replicates. Each replicates represent the egg laid by the group of 20 snails. (*) significant ($P<0.05$) when student "t" test was applied to treated and control groups. (+) product moment correlation coefficient showed that there was significant ($P<0.05$) negative correlation in between survival period and survival of the snail *L. acuminata*. (-) No fecundity was observed.

asafoetida are more effective in reducing the fecundity of *L. acuminata*. Although, the binary combinations (1:1 ratio) of amino acids with bait formulation of molluscicides (eugenol, ferulic acid, umbelliferone and limonene) are more effective, it reduce the free amino acids, proteins, DNA and RNA level in the ovotestis of *L. acuminata* [26]. It may be possible that in the present study bait formulations of amino acids with molluscicides direct enter in the snail body through alimentary canal and affect the caudo darsal cells (CDSs), reducing the release of the ovulation hormone that resulted a decrease in the fecundity of bait fed snails. Caudo darsal cell is responsible for the fecundity of snail *L. acuminata* [16, 27, 28].

The reduced hatchability of *L. acuminata* fed to the different baits may be due to interference of molluscicides with the embryonic growth and development of the snails. In bait fed snails, young larvae were weak, unable to break the egg capsule, and died owing to starvation. Young snails hatched from the treated egg masses showed delay in attaining maturity in comparison with the control groups. Most of these eggs were attached to the wall of the aquarium and were apathetic toward feeding. In general, the egg shells were thinner, and the hatchlings had shorter tentacles and slower movement and were smaller in size as compared with control group. Mortality and low reproduction in the bait fed snails suggest the active molluscicidal components in bait formulation was able to control the population of snail *L. acuminata* by inhibiting development at any stage of growth.

Transfer of mother snails to fresh water for the next 72h after 96h feeding all the feeded snails leads to a significant recovery in the fecundity of snails. Withdrawal experiments also showed that the feeding of bait formulation were reversible as the levels of free amino acids, proteins, DNA and RNA [26] activity were restored within 7 days. Thus, reversibility of the effects would be an added advantage in their use against aquatic target snails as they would cause only short-lived effects. The above finding suggested that the use of bait formulation with sublethal molluscicides will be helpful in control program of target snails.

ACKNOWLEDGEMENTS

One of the authors (Dr. Pradeep Kumar - Post Doctoral Fellow) is thankful to University Grants Commission, New Delhi, India for financial assistance.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. W H O. Report of the WHO Informal Meeting on use of triclabendazole in fascioliasis control. WHO/CDS/NTD/PCT/2007.1.
2. Mas-Coma S. 2004. Human fascioliasis. In: World Health Organization. Waterborne Zoonoses: Identification causes and control. Eds, JA Cotruvo, A Dufour, G Rees, J Bartram, R Carr, DO Cliver, GF Craun, R Fayer, VPJ Gannon. IWA Publishing, London, UK. pp. 305-322.
3. Mas-Coma S. Epidemiology of fascioliasis in human endemic areas. J Helminthol. 2005; 79: 207-216.
4. Kumar P, Singh VK, Singh DK. Attractant food pellets containing molluscicides against the fresh water snail *Indoplanorbis exustus*. Global Vet. 2012; 8(2): 105-110.
5. Kumar P, Singh VK, Singh DK. Enzyme activity in the nervous tissue of *Lymnaea acuminata* fed to different bait formulations. Am J Chem. 2012; 2(2): 89-93.
6. Agarwal RA, Singh DK. Harmful gastropods and their control. Acta Hydrochim Hydrobiol. 1988; 16: 113-138.
7. Kumar P, Singh DK. Molluscicidal activity of *Ferula asafoetida*, *Syzygium aromaticum* and *Carum carvi* and their active components against the snail *Lymnaea acuminata*. Chemosphere. 2006; 63: 1568-1574.
8. Kumar P, Singh VK, Singh DK. Kinetics of enzyme inhibition by active molluscicidal against ferulic acid, umbelliferone, eugenol and limonene in the nervous tissue of snail *Lymnaea acuminata*. Phyto Res. 2009; 23: 172-177.
9. Jaiswal P, Singh DK. Molluscicidal activity of Nutmeg and Mace (*Myristica fragran*s Houtt.) against the vector snail *Lymnaea acuminata*. J Herbs Spice Med Plants. 2009; 15: 117-186.
10. Upadhyay A, Singh DK. Molluscicidal activity of Sapindus mukorossi and *Terminalia chebula* against the freshwater snail *Lymnaea acuminata*. Chemosphere. 2011; 83: 468-474.
11. Singh KL, Singh DK, Singh VK. Characterization of the molluscicidal activity of *Bauhinia variegata* and *Mimusops elengi* plant extracts against the *Fasciola* vector *Lymnaea acuminata*. Rev Inst Med Trop Sao Paulo. 2012; 54(3): 135-140.
12. Tiwari F, Singh DK. Toxicity of plant derived molluscicides in attractant food pellets against snail

Lymnaea acuminata. Iranian J Pharm Therapeutics. 2007; 6: 103-107.

13. Singh P, Singh DK. Binary combination of carbohydrates and amino acids as snail attractant in pellets containing molluscicides against the snail *Lymnaea acuminata*. Pesti Biochem Physiol. 2008; 92: 120-124.
14. Kumar P, Singh VK, Singh DK. Binary combination of amino acids and plant molluscicide in bait formulation against *Lymnaea acuminata*. World J Zool. 2010; 5(2): 143-147.
15. Kumar P, Singh VK, Singh DK. Combination of molluscicides with attractant carbohydrates and amino acids in bait formulation against the snail *Lymnaea acuminata*. Eur Rev Med Pharmacol Sci. 2011; 15: 550-555.
16. Singh A, Singh DK. Effect of herbal molluscicides and their combinations on the reproduction of the snail *Lymnaea acuminata*. Environ Contam Toxicol. 2004; 46: 470-477.
17. Madsen H. A comparative study on the food-locating ability of *Helisoma duryi*, *Biomphalaria camerunensis* and *Bulinus truncatus* (Pulmonata: planorbidae). J Appl Ecol. 1992; 29: 70-78.
18. Tiwari F, Singh DK. Attraction of amino acids by Lymnaea acuminata, snail host of *Fasciola* species. Braz J Med Biol Res. 2004; 37: 587-90.
19. Tiwari F, Singh DK. Behavioural responses of the snail *Lymnaea acuminata* to carbohydrates in snail attractant pellets. Naturwissenschaften. 2004; 91: 378-380.
20. Sokal RR, Rohlf FJ. 1973. Introduction of biostatistics. pp. 185-207. WH. Freeman, San Francisco.
21. Marston A, Hostettmann K. Plant molluscicides. Phytochem. 1985; 24: 639-652.
22. Singh S, Singh VK, Singh DK. Effect of spices ginger (*Zingiber officinale*) and ajowan (*Trachyspermum ammi*) on the reproduction of the vector snail *Lymnaea acuminata*. Biol Mem. 2004; 30(1): 14-19.
23. Bacchetta R, Mantecca P, Vailati G. Oocyte degeneration and altered ovipository activity induced by paraquat in the fresh water snail *Physa fontinalis* (Gastropoda: Pulmonata). J Moll Stud. 2002; 68: 181-186.
24. Mello-Silva CC, Vilar MM, Bezerra JCB, de Vasconcellos MC, Pinheiro J, Lerdes Rodrigues M. Reproductive activity alterations on the *Biomphalaria glabrata* exposed to *Euphorbia splendens* var. hislopi latex. Mem Inst Oswaldo Cruz. 2007; 102(6): 671-674.
25. Tripathi R, Kumar P, Singh VK, Singh DK. Cow urine and their formulations with plant molluscicide against the reproduction of vector snail *Lymnaea acuminata*. Drug Inven Today. 2010; 2(8): 376-380.
26. Kumar P, Singh VK, Singh DK. Bait formulations of molluscicides and their effects on biochemical changes in the ovotestis of snail *Lymnaea acuminata* (Mollusca; Gastropoda: Lymnaeidae). Rev Inst Med Trop Sao Paulo. 2011; 53(5): 271-275.
27. Roubos EW, Boer HH, Schot LPC. Peptidergic neurons and the control of neuroendocrine activity in the freshwater snail *Lymnaea stagnalis* L. Proc Int Sym Neurosecret. 1981; 8: 119-127.
28. Singh K, Singh DK. Effects of Neem (*Azadirachta indica*) on fecundity, hatchability and survivality of snail (*Lymnaea acuminata*). Malaysian Appl Biol. 1995; 24(1): 19-22.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Physiological and biochemical responses of salt-tolerant and salt-sensitive wheat and bean cultivars to salinity

Abeer Ahmad Radi[1], Fatma Ali Farghaly[1], Afaf Mohamed Hamada[1,2]

[1]Botany Department, Faculty of Science, Assiut University, Assiut, 71516 Egypt
[2]Department of Botany, Stockholm University, SE-106 91 Stockholm, Sweden

ABSTRACT

Salinity stress caused a marked reduction in dry matter gain in roots and shoots, and transpiration rate of all the test wheat and bean cultivars. Although the accumulation of malondialdehyde was stimulated in roots of sensitive cultivars at second phase of NaCl stress (160 mM), there were no appreciable differences in the production of it in roots of tolerant cultivars of wheat and bean plants. The electrical conductivity of the selected wheat and bean cultivars was stimulated by salt stress. All fractions of photosynthetic pigments in the test plants decreased gradually with the rise of salt level (80-160 mM NaCl). A notable sensitivity in the biosynthesis of carbohydrates and proteins in different organs of the selected wheat and bean cultivars was displayed in the presence of salinity. The presence of NaCl did not affect the phenylalanine ammonia-lyase activity in leaves of the selected wheat and bean cultivars, except in salt-tolerant bean cultivar (cv. Sakha1) salinity stress exhibited an inhibitory effect. NaCl stress failed to induce appreciable variations in the production of free phenolic compounds in leaves of the wheat and bean cultivars, except in case of cv. Sakha1. Salinity stress caused an inhibitory effect on the production of bound phenolic compounds in wheat and bean cultivars.

Key words: Bean; Sensitive cultivar; Tolerant cultivar; Wheat.

J Biol Earth Sci 2013; 3(1): B72-B88

Corresponding author:
Prof. Afaf M. Hamada
Department of Botany, Stockholm University
SE-106 91 Stockholm, Sweden
Fax: +46 (0)8- 165525, Tel.: +46 (0)8- 163759
e-mail: afafhamada@yahoo.com

Original Submission: 21 December 2012; Revised Submission: 26 January 2013; Accepted: 06 February 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Soil salinity is a prevalent abiotic stress that limits the productivity and geographical distribution of plants. The impact of salinity is most serious in countries where all or most of agricultural production is based on irrigation (Egypt, Pakistan) and when agriculture is a substantial part of the national economy [1-3]. High concentrations of soluble salt in the soils of arid and semi-arid regions adversely affect plant growth and yield [4, 5]. Excessive salts in soil affect all major living processes such as growth, photosynthesis, protein, and lipid metabolism [4].

Indeed, salinity is a major environmental stress and is a substantial constraint to crop production. Increased salinization of arable land is expected to have devastating global effects, resulting in 30% land loss within next 25 years and up to 50% by the middle of 21st century [6]. High salinity causes both hyperionic and hyperosmotic stress and can lead to plant demise. Salinity can affect growth in a number of ways; the first phase of the growth response is due to the osmotic effect of the salt in the soil solution and produces a suite of effects identical to those of water stress caused by drought. Later, there may be an additional effect on growth, if excessive amounts of salt enter the plant they will eventually rise to toxic levels in the older transpiring leaves, causing premature senescence. This will reduce the amount of assimilate that the plant can produce, and a reduction in this assimilate transported to the growing tissue may further limit growth. This is the second phase of the growth response, and is the phase that clearly separate species and genotypes that differ in the ability to tolerate saline soil. Thus, growth is limited predominantly by osmotic stress, but in species that have a high rate of salt uptake, or cannot compartmentalize salt effectively in vacuoles, salt-specific effects develop with time, impose an additional stress on the plant through failing capacity to produce photoassimilate, and give rise to the categories of salt-sensitive and salt-tolerant [7].

Soil salinization is one of the biggest threats to inland agriculture [8]. Therefore, a concerted effort to understand the effects of salinity on plants and to develop salt-tolerant species is essential to combat soil salinization problems [9]. The objectives of this research were to investigate the salt stress tolerance of monocot and dicot plants and, further to dissect how far these responses are correlated with the salt-tolerance mechanisms. To achieve these goals, the growth criteria, transpiration rate, membrane integration, photosynthetic pigments, sugars, proteins, phenyl ammonia-lyase enzyme activity and phenolic compounds were analyzed in Sakha93 (wheat cultivar) and Sakha1 (broad bean cultivar) which were ranked as the most salt-tolerant cultivars and Gemmeza10 (wheat cultivar) and Giza716 (broad bean cultivar) the most salt-sensitive ones out of 14 cultivars under at two phases of NaCl (80 and 160 mM).

MATERIALS AND METHODS

Plant culture and treatments

All the studies were carried out on salt-tolerant cultivar (cv. Sakha93) and salt-sensitive cultivar (cv. Gemmeza10) wheat (*Triticum aesativum* L.) and salt-tolerant cultivar (cv. Sakha1) and salt-sensitive cultivar (cv. Giza716) broad bean (*Vicia faba* L.) exposed to two phases of salt stress treatments. The whole experiment was replicated twice. Plants were grown hydroponically in aerated Hoagland's solution [10] in a greenhouse under natural conditions. Salt treatment (16-d-old) was performed by supplementing the nutrient solution with NaCl first phase (80 mM) and second phase (160 mM). Control plants were kept in Hoagland's solution without NaCl.

Harvesting and plant growth

After treatment (7 days) the fresh weight (FW) was determined directly and the dry weight (DW) was obtained after drying the shoots and roots tissues for 48 hours at 72ºC.

Determination of transpiration rate

Transpiration rate was measured as described by of Bozcuk [11]. The transpired water was determined by weighing the plants together with pots and experimental nutrient solution at daily intervals for 7 days. After each weighing, the experimental solution was completed to the initial level by addition of nutrient solution. Transpiration rate was calculated as g/dm^2/day after subtractions the amount of water lost from pots.

Determination of photosynthetic pigments

The fractions of pigments (chlorophyll a,

chlorophyll b and carotenoids) were estimated using the spectrophotometric method recommended by Lichtenthaler [12]. Chlorophylls and carotenoids concentrations were calculated as mg/g FW at 663, 644 and 452 nm.

Membrane integrity

Cell membrane integrity was carried out as given by Premachandra et al. [13].

Determination of lipid peroxidation

Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation using the thiobarbituric acid reaction as described by Madhava Rao and Sresty [14]. The concentration of MDA was calculated by using an extinction coefficient (155 mM^{-1} cm^{-1}) and the results expressed as µM MDA/g FW.

Determination of reducing sugars

Reducing sugars were extracted from dry seedling tissues (powder form) and Nelson's method [15] was used. A calibration curve was constructed using pure glucose and the reducing sugars were determined as mg/g DW.

Determination of soluble carbohydrates

The anthrone sulphuric acid method [16, 17] was used for the determination of carbohydrates. A calibration curve using pure glucose was made.

Determination of soluble and total proteins

Soluble proteins were determined in the plant extract using Folin reagent, according to Lowry et al. [18]. A calibration curve was constructed using egg albumin and the data were expressed as mg protein/g DW.

Phenylalanine ammonia-lyase (EC 4.3.1.5)

The activity of phenylalanine ammonia-lyase (PAL) was assayed according to the method described by Havir and Hanson [19]. Calibration curve was constructed with known concentration of cinnamic acid. The activity was expressed as mg/g FW.

Determination of free and cell wall-bound phenolics

Free and cell wall-bound phenolics were determined according to Kofalvi and Nassuth [20]. Phenolic concentration in the extract was determined from standard curve prepared with gallic acid.

Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) using the SPSS statistical package. Duncan's multiple range test ($P<0.05$) was used to compare the means.

RESULTS

Shoot and root growth

In the light of the previous paper [21], the wheat Sakha93 and bean Sakha1 were ranked as the most tolerant, while wheat Gemmeza10 and bean Giza716 were ranked as the lowest tolerant out of 7 wheat and 7 bean cultivars. The role of salt stress on growth criteria of the lowest and highest sensitive wheat and bean cultivars is presented in Fig. 1. Increasing salinity level from first phase (80 mM) to second phase (160 mM) reduced dry weight gain in roots and shoots of all the test cultivars.

The adverse concentration effects of salinity stress were clearly demonstrated by wheat and bean cultivars treated with 160 mM NaCl. The results indicate that roots were more sensitive to salt stress compared to shoots. In addition, it could be noticed that the successive increases in salinization level did not attenuate the dry matter gain in wheat cv. Sakha93 and bean cv. Sakha1 as severely as wheat cv. Gemmeza10 and bean cv. Giza716.

Transpiration rate

The role of salt stress on the transpiration rate of the lowest and highest sensitive wheat and bean cultivars were considered in the current studies. The data presented in Fig. 2 reveal that the transpiration rate decreased in the selected sensitive (Gemmeza10 and Giza716) and tolerant cultivars (Sakha93 and Sakha1) as salinity increased. In addition, the presence of NaCl in the culture media at the second phase (160 mM) greatly attenuated the transpiration rate of the tolerant cv. of both wheat (Sakha93) and bean (Sakha1) plants.

Lipid peroxidation

The lipid peroxidation was measured in terms of malondialdehyde (MDA) contents, a product of lipid peroxidation. The level of MDA is often used as an

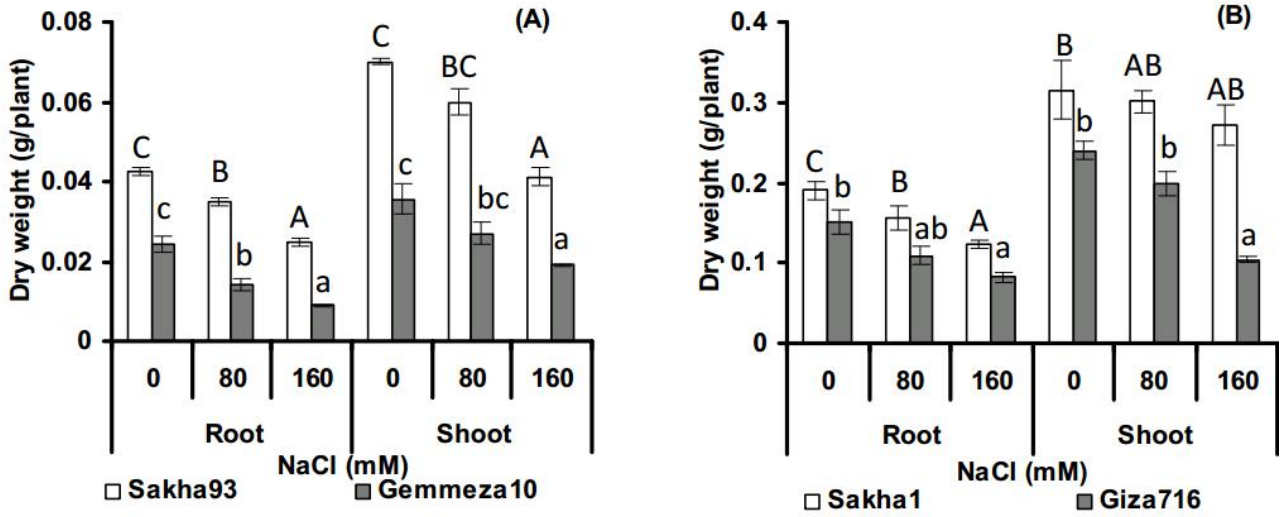


Fig. 1. Effect of two phases of NaCl stress on dry weight (A) of wheat and bean cultivars (B). [The data are given as average of six replicates ± SD]. Bars carrying different letters are significantly different at P< 0.05.

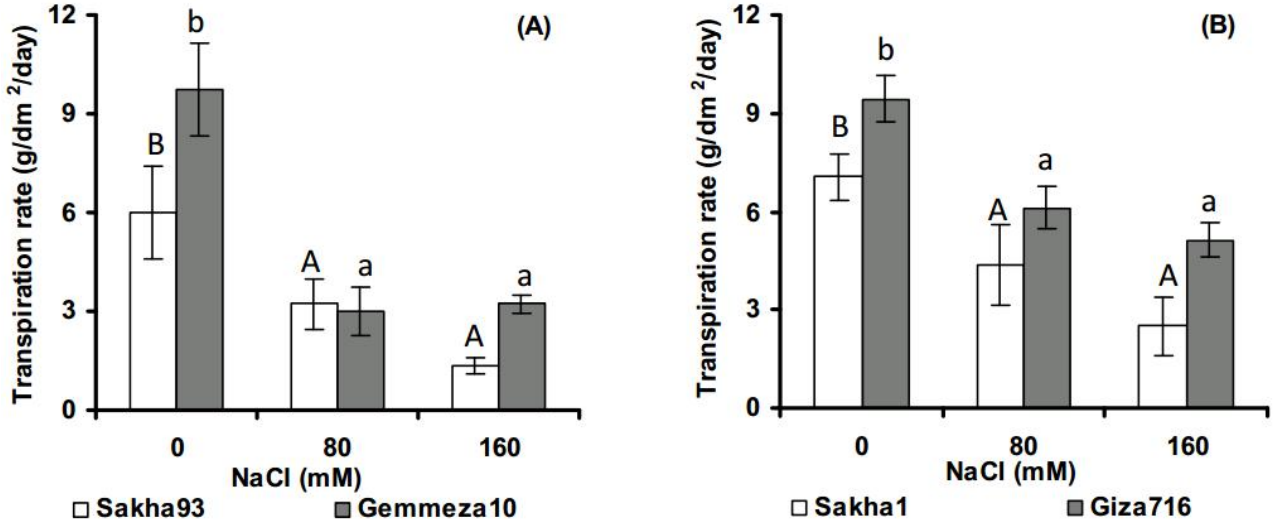


Fig. 2. Effect of two phases of NaCl stress on transpiration rate of wheat (A) and bean (B) cultivars. [The data are given as average of six replicates ± SD]. Bars carrying different letters are significantly different at P< 0.05.

indicator of oxidative damage. The results presented in Fig. 3 demonstrate that NaCl at 80 mM could not exert a significant change in MDA concentration in root system of both sensitive and tolerant wheat and bean cultivars. At the second phase of NaCl stress, although the accumulation of MDA was significantly stimulated in roots of wheat Gemmeza10 and bean Giza716 (NaCl-sensitive cultivars), there were no appreciable differences in the production of MDA in roots of wheat Sakha93 and bean Sakha1 (NaCl-tolerant cultivars).

Membrane permeability

Membrane permeability (electrical conductivity, EC %) was assessed by measuring the electrolytes, leaching from leaf tissue into distilled water. The data herein obtained clearly demonstrate that, in most cases, the EC% of wheat and bean cultivars was significantly stimulated by the two phases of salt stress and EC% increased with the rise of salt level (Fig. 4). It is worth to mention that the percentage of injury in NaCl-sensitive wheat Gemmeza10 was more than in NaCl-tolerant wheat Sakha93. However, the opposite trend was exhibited in bean plants; EC% of NaCl-sensitive Giza716 was lower as compared to that reached in NaCl-tolerant Sakha1.

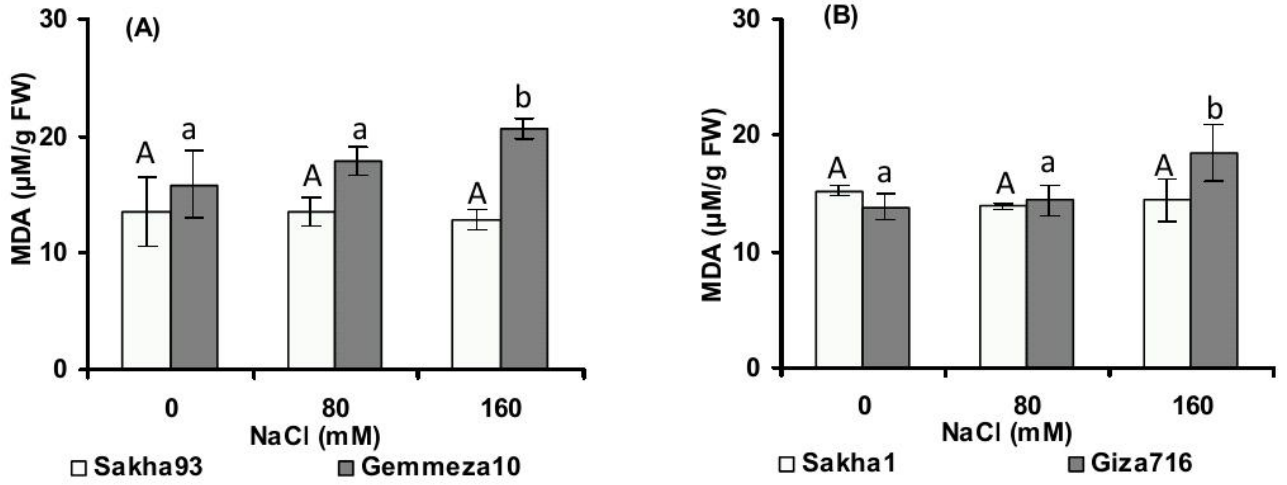


Fig. 3. Effect of two phases of NaCl stress on malondialdehyde (MDA) concentration in roots of wheat (A) and bean (B) cultivars. [The data are given as average of six replicates ± SD]. Bars carrying different letters are significantly different at P< 0.05.

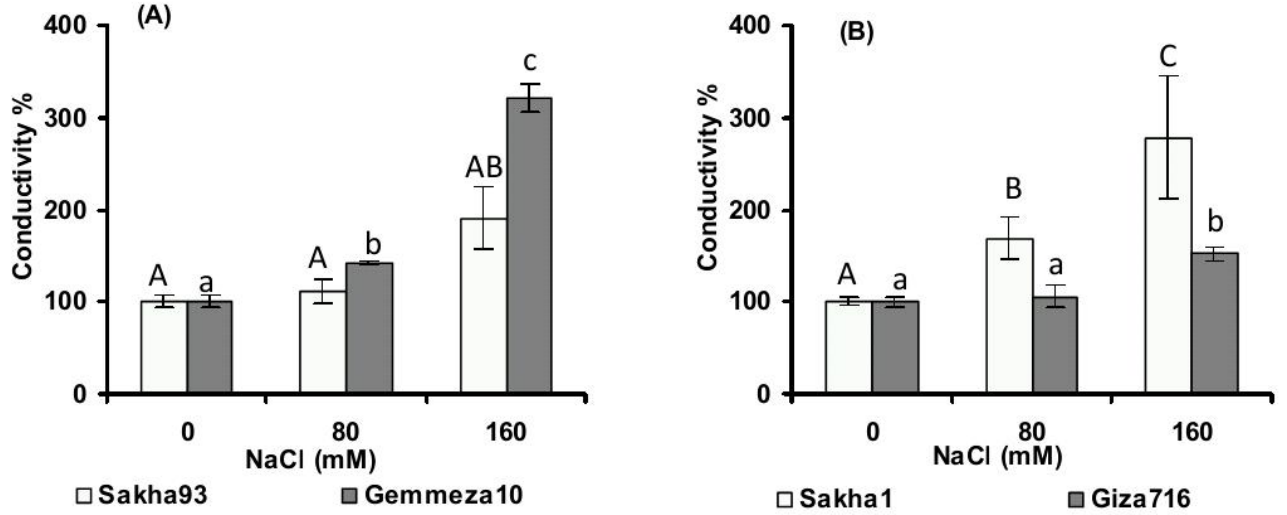


Fig. 4. Effect of two phases of NaCl stress on membrane damage (% of electrical conductivity) of leaves of wheat (A) and bean (B) cultivars. [The data are given as average of six replicates ± SD]. Bars carrying different letters are significantly different at P< 0.05.

Photosynthetic pigments

The concentrations of the pigments fractions (chlorophyll a, chlorophyll b and carotenoids) in the leaves of salt stressed plants are presented in Fig. 5. The data herein obtained clearly demonstrate that the biosynthesis of photosynthetic pigments was affected by NaCl stress. The biosynthesis of all fractions decreased with the rise of salt level from 80 to 160 mM in the test wheat and bean cultivars. Moreover, photosynthetic pigments concentration was highly inhibited at the second phase of salt-stress in wheat Gemmeza10 and bean Giza716 (sensitive cultivars) as compared to wheat cv. Sakha93 and bean cv. Sakha1 (tolerant cultivars).

Reducing sugars

A notable sensitivity in the accumulation of reducing sugars in different organs of the selected wheat and bean cultivars was displayed by salt stress. Data in Fig. 6 reveal that NaCl at 80 mM did not induce a stimulatory effect on the accumulation of reducing sugars in roots or shoots of the two wheat cultivars, except roots of Gemmeza10 where a significant stimulation was detected. On the other hand, when NaCl was provided at 160 mM more reducing sugars were accumulated in roots and shoots of two wheat cultivars (Sakha93 and Gemmeza10) as compared to control. With respect to bean plants, NaCl at 80 mM could not exert a

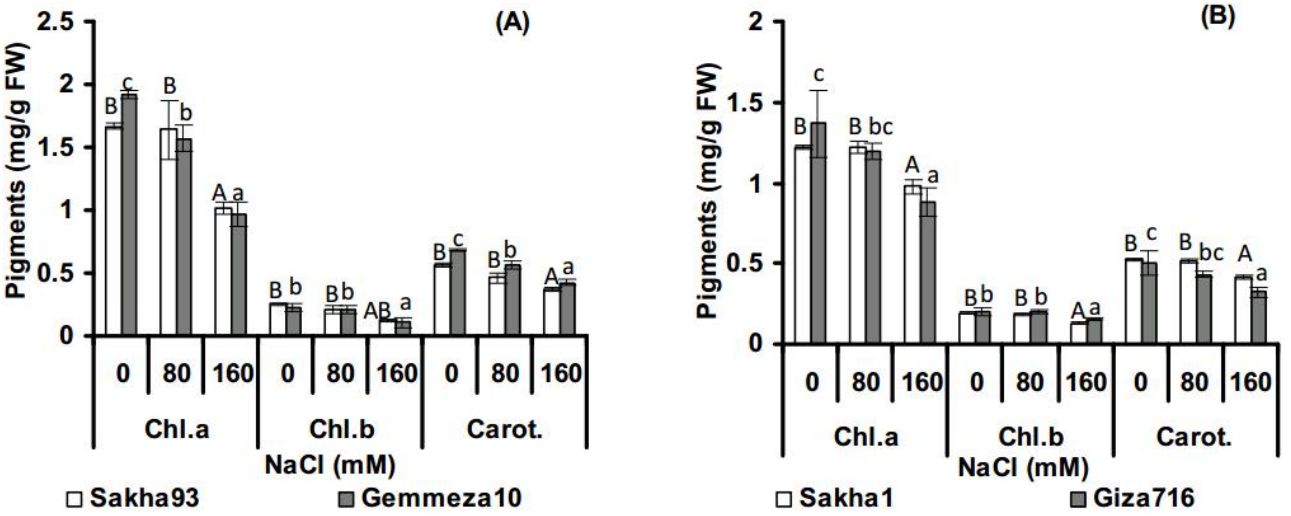


Fig. 5. Effect of two phases of NaCl stress on photosynthetic pigments fractions in leaves of wheat (A) and bean (B) cultivars. [The data are given as average of six replicates ± SD]. Bars carrying different letters are significantly different at P< 0.05.

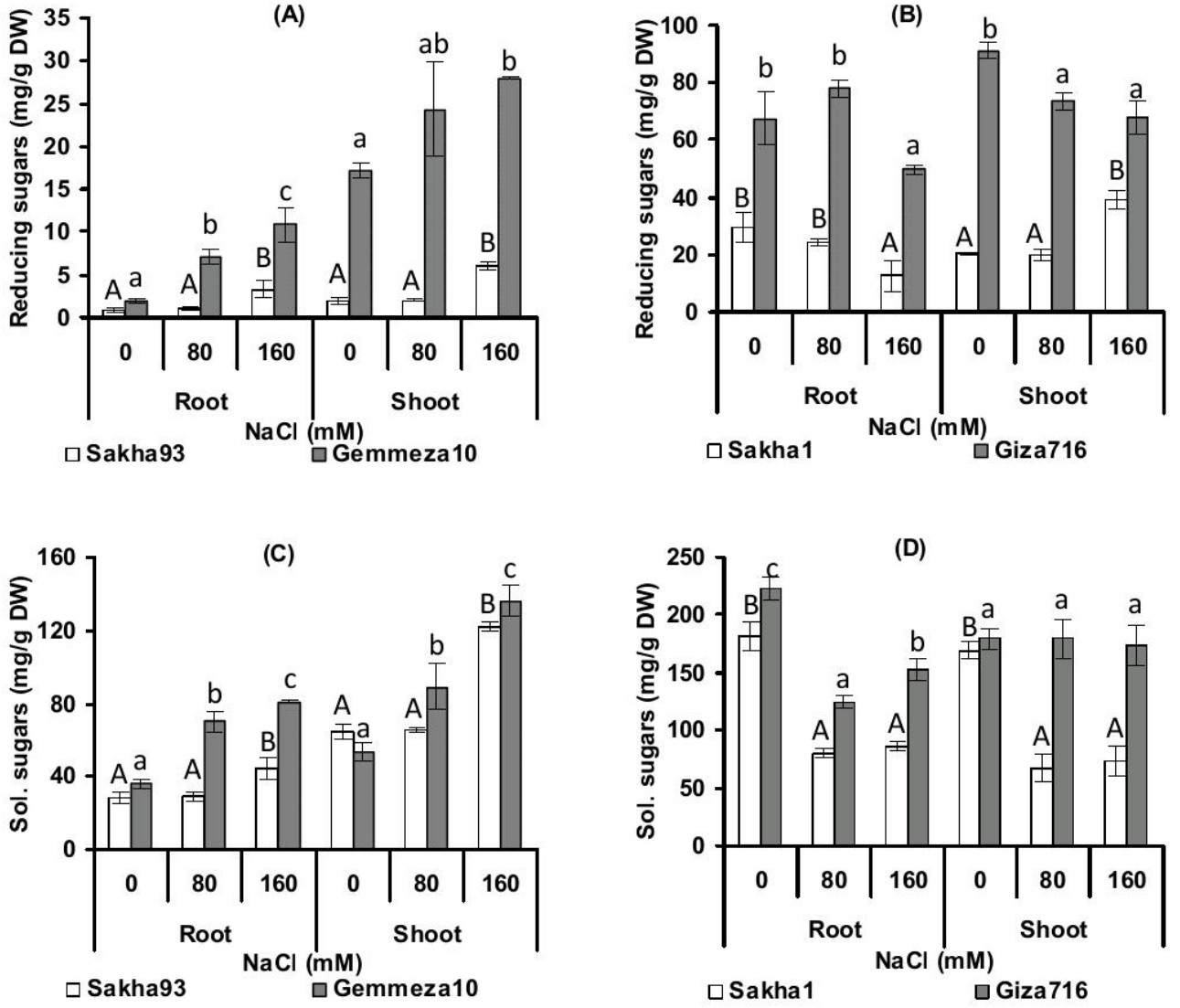


Fig. 6. Effect of two phases of NaCl stress on reducing sugars (A; B) and total soluble sugars (C; D) in roots and shoots of wheat and bean cultivars. [The data are given as average of six replicates ± SD]. Bars carrying different letters are significantly different at P< 0.05.

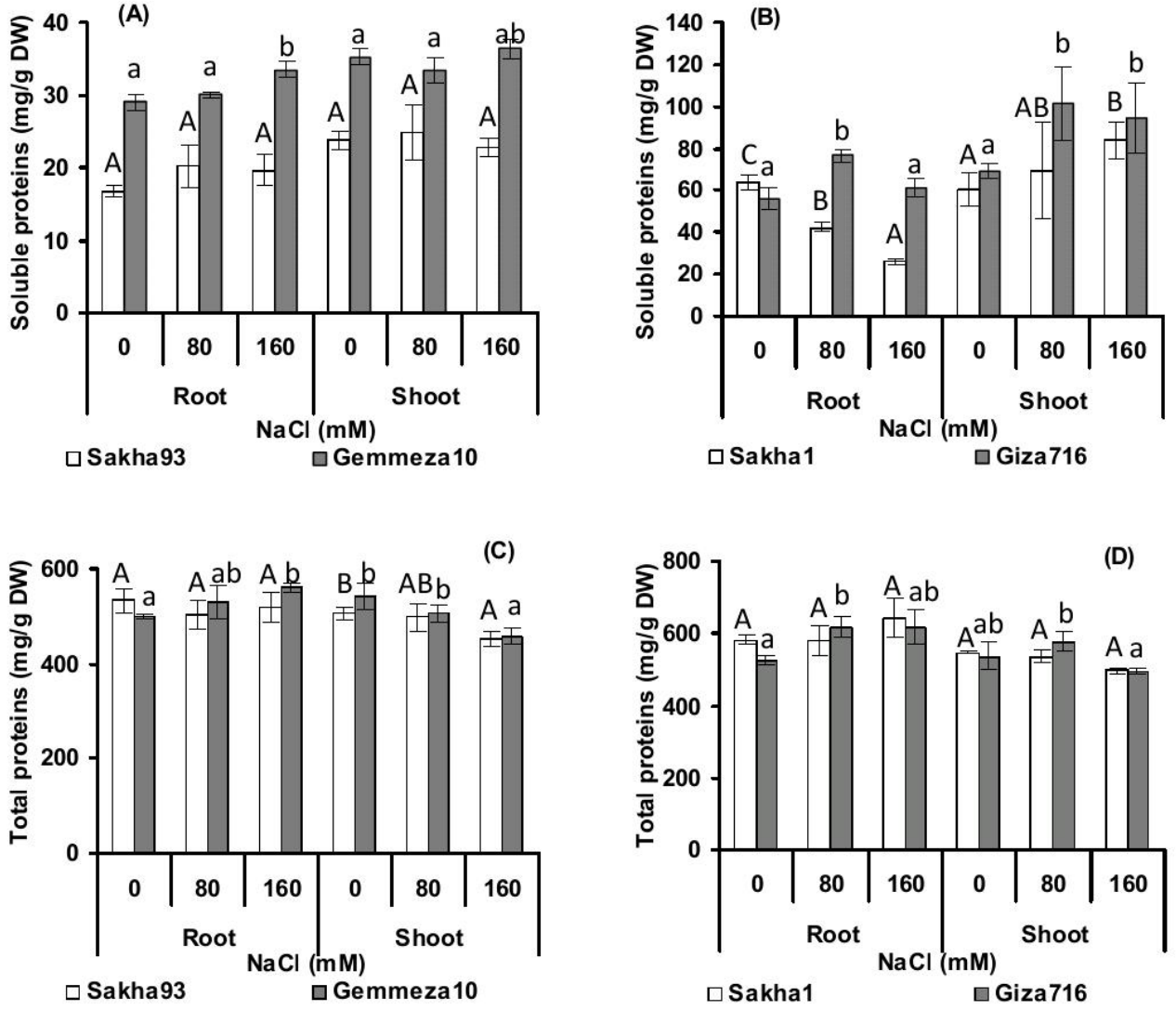


Fig. 7. Effect of two phases of NaCl stress on soluble proteins (A; B) and total proteins (C; D) in roots and shoots of wheat and bean cultivars. [The data are given as average of six replicates ± SD]. Bars carrying different letters are significantly different at $P< 0.05$.

significant change in reducing sugars in roots or shoots of test cultivars, except shoots of Giza716, where a significant decrease was detected. However, the second phase of NaCl stress was of depressive effect on the accumulation of reducing sugars in roots of bean cultivars and shoots of Giza716 and the opposite trend was detected in shoots of Sakha1.

Soluble sugars

The results presented in Fig. 6 reveal that the role played by salt stress on the accumulation of soluble sugars in the different organs of wheat and bean cultivars was somewhat complicated. In case of Sakha93 cv., the first phase of NaCl stress (80 mM) failed to stimulate the accumulation of soluble sugars in shoots and roots. The data herein obtained also demonstrate the capability of the first phase in stimulating the production of soluble sugars in the shoots and roots of Gemmeza10. On the other hand, at second phase of NaCl, the shoots and roots of both salt tolerant and salt sensitive wheat cultivars showed a noticeable increase in the soluble sugars as compared with the control.

In case of bean plant, the investigated levels of NaCl induced an inhibitory effect on the accumulation of soluble sugars in Sakha1 roots and shoots. In Giza716, although the accumulation of soluble sugars was significantly inhibited in roots at the two phases of salt stress, there were no appreciable differences in the production of the soluble fraction in shoots at low (80 mM) and high (160 mM) NaCl levels.

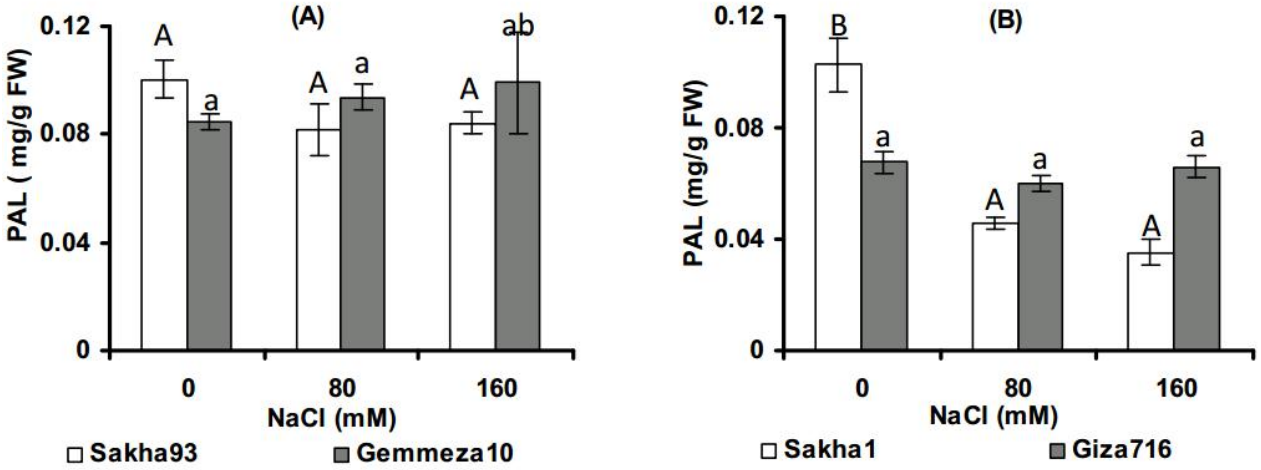


Fig. 8. Effect of two phases of NaCl stress on phenyl ammonia-lyase activity in leaves of wheat (A) and bean (B) cultivars. [The data are given as average of six replicates ± SD]. Bars carrying different letters are significantly different at P< 0.05.

Soluble proteins

The concentrations of the soluble proteins in the roots or shoots of salt stressed wheat and bean cultivars are presented in Fig. 7. In case of wheat (sensitive and tolerant) cultivars soluble proteins concentration values approached that of the control at the two salt levels. However, constitutive soluble proteins content of roots or shoots was higher in Gemmeza10 (NaCl-sensitive cv.) compared to Sakha93 (NaCl-tolerant cv.). On the other hand, a biphasic action of salinization treatments involving inhibition of soluble proteins and non-significant stimulation was clearly manifested by Sakha1 (NaCl-tolerant bean cv.) roots or shoots. In case of Giza716 (NaCl-sensitive bean cv.), it is clearly shown that at the two phases of salt stress, roots and shoots showed a noticeable increase in the soluble proteins accumulation as compared with the control, except roots, where a significant decrease was detected.

Total proteins

Values of total proteins concentrations in roots and shoots of the relatively NaCl-tolerant (Sakha93 and Sakha1) versus NaCl-sensitive (Gemmeza10 and Giza716) wheat and bean cultivars respectively are presented in Fig. 7. The first phase of NaCl stress in the culture medium resulted in non-significant stimulation in the accumulation of total proteins in the roots and shoots of the selected wheat and bean cultivars, except in case of Giza716 roots where a slight increase but significant was detected. Similarly, the presence of NaCl in the culture media at a second phase (160 mM) slightly increased or decreased the total proteins accumulation in wheat and bean cultivars. This phase of NaCl stress did not stimulate the accumulation of total proteins in roots of Sakha93 (NaCl-tolerant wheat cv.), while in roots of Gemmeza10 (NaCl-sentsitive wheat cv.) stimulatory effects were manifested. However, the presence of NaCl in the culture media at a concentration 160 mM significantly attenuated the accumulation of total proteins in shoots of both test wheat cultivars. Bean cultivars, displayed no appreciable differences in the total proteins accumulation at 160 mM.

Phenylalanine ammonia-lyase (PAL) activity

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is widely distributed in higher plants and has been shown to play an important role in the biosynthesis of many phenolic compounds. In this investigation, the presence of NaCl at 80 and 160 mM in the culture media generally did not affect the PAL activity in leaves of test wheat and bean cultivars, except in Sakha1 (NaCl-tolerant bean cv.) where the presence of NaCl at the two phases of salinity exhibited an inhibitory effect (Fig. 8).

Free phenolic compounds

The production of free phenolic compounds in the leaves of NaCl stressed wheat and bean cultivars are presented in Fig. 9. The presence of NaCl in the culture media at the first phase (80 mM) failed to induce appreciable variations in the production of free phenolic compounds in leaves of

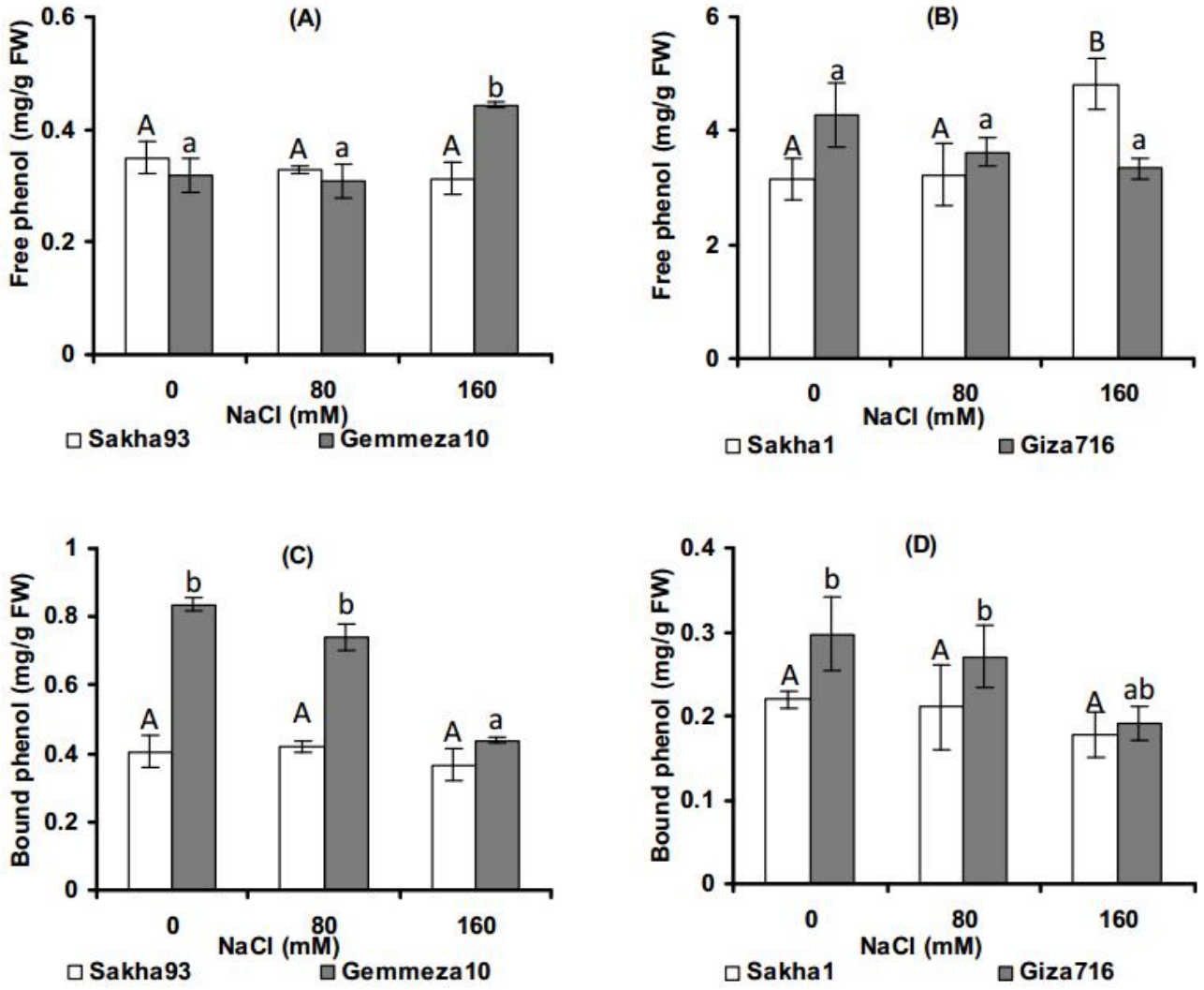


Fig. 9. Effect of two phases of NaCl stress on free and bound phenolic compounds in leaves of wheat (A; C) and bean (B; D) cultivars. [The data are given as average of six replicates ± SD]. Bars carrying different letters are significantly different at P< 0.05.

the test wheat and bean cultivars. Moreover, NaCl at 160 mM could not exert a significant change in the content of free phenolic compounds of the tolerant wheat cv. (Sakha93), while the sensitive one exhibited a significant accumulation of these compounds. In bean cultivars, a biphasic action of the second phase of NaCl treatment was manifested involving significant stimulation and non-significant inhibition of the production of free phenolic compounds in leaves of Sakha1 (NaCl-tolerant cv.) and Giza716 (NaCl-sensitive cv.) respectively.

Bound phenolic compounds

The results presented in Fig. 9 reveal that in leaves of the relatively NaCl-tolerant wheat and bean cultivars, the two phases of NaCl stress induced non-significant inhibitory effects on bound phenolic compound content. While in NaCl-sensitive ones, the second phase of NaCl stress (160 mM) reduced the concentration of bound phenolic compounds, and at the first phase (80 mM) the retarding effect was partially or completely alleviated.

DISCUSSION

Shoot and root growth

Shoot and root growth inhibition is a common response to salinity and plant growth is one of the most important agricultural indices of salt stress tolerance as indicated by certain studies [7, 22-24]. The dry matter yield of root and shoot systems of the test cultivars showed marked decrease as the

salinity level was increased. The data herein clearly demonstrate that treatments involving NaCl at second phase (160 mM) strongly inhibited dry matter gain in roots and shoots. The effectiveness of the two phases of NaCl in the culture media varied in the different test wheat and bean cultivars, wheat cv. Sakha93 and bean cv. Sakha1 were relatively more tolerant to NaCl stress, compared with wheat cv. Gemmeza10 and bean cv. Giza716, which were relatively more sensitive ones. In this respect, Murillo-Amador et al. [25] found that biomass decreased more severely at 170 mM NaCl than in case of 85 mM NaCl in all groups of cowpea genotypes and decreased as salinity increased.

Salinity induces injuries to plant growth by causing physiological water stress [26], so it mainly damages plant roots [27]. Salt stress might inhibit cell division and expansion directly [28]. Munns [29] hypothesized that plant growth is initially inhibited (phase 1) by cellular responses to the decreased availability of soil water. In a later, second response (phase 2), growth is further inhibited by the toxic effects of excessive salt accumulation within the plant. Varietals diversity in plant growth responses is only expected to appear after long-term exposure to salinity. Yasseen et al. [30] also suggested that NaCl inhibits growth by reducing both cell division and cell enlargement. The cell expansion process also depends on the hydraulic conductivity of the water uptake pathway, uptake of solutes to maintain osmotic potentials, and the yielding of the surrounding cell walls [31, 32]. Reduced rates of new cell production may show additional contribution to the inhibition of growth as reported by Shabala et al. [33]. The reduction in growth could be attributed to increasing stiffness of the cell wall, due to altered cell wall structure induced by salinity as reported by Sweet et al. [34].

Transpiration rate

The transpiration rate decreased in the four selected wheat and bean cultivars as salinity increased. In addition, the presence of NaCl in the culture media at 160 mM greatly attenuated the transpiration rate of the tolerant cv. of both wheat (Sakha93) and bean (Sakha1) plants. Other investigators [35-38] also reported the inhibition of transpiration activity as affected by salt stress. The reduced stomatal conductance under high salinity conditions typically promotes considerable reduction in the transpiration rate as previously reported with barley genotypes differing in salt tolerance [39], tomatoes [40] and many other halophytes and glycophytes species. The reduction in transpiration rate under salt stress conditions was probably due to a partial closure of stomata.

Membrane damage

The cell membrane, being at the interface, is the first organelle to be affected by salt stress [41]. The integrity of the membrane is disrupted due to peroxidation of lipids in the membrane system resulting in increased membrane permeability [42]. Polyunsaturated fatty acids are the main membrane lipid components susceptible to peroxidation and degradation by free radicals generated due to salt stress [43]. Accordingly, several studies have shown that lipid peroxidation in salt sensitive plants is more pronounced than in salt-tolerant ones [23, 44]. In this investigation, MDA formation was used as an index of lipid peroxidation. The data herein demonstrated that MDA content of wheat Sakha93 and bean Sakha1 (tolerant cultivars) was not changed at the two phases of salt stress, but wheat Gemmeza10 and bean Giza716 (salt sensitive cvs.) accumulated more MDA at the second phase of salinity. These results agree with those given by Azevedo-Neto et al. [45] using two maize genotypes one is salt-tolerant and other is salt-sensitive. In this regard, salinity induced increase in lipid peroxidation as reported by Ying et al. [46], Koca et al. [23], Keutgen and Pawelzik [47] and Falleh et al. [48].

Data of the present study reveal that membrane permeability (electrical conductivity; EC%) was increased in wheat and bean cultivars with increasing NaCl stress. The percentage of injury in NaCl-sensitive wheat cv. (Gemmeza10) was more than in NaCl-tolerant one Sakha93. However, the opposite trend was reflected by bean plant; EC% of NaCl-sensitive cv. (Giza716) was lower than in case of NaCl-tolerant cv. (Sakha1). In this connection Tuna et al. [24, 49] reported that electrolyte leakage increased in the leaves of tomato plants grown under salt stress. In addition, Lutts et al. [50, 51]; Ghoulam et al. [52] and Kaya et al. [53] obtained similar results.

Photosynthetic pigments

The present findings clearly state that pigment fractions were significantly reduced by NaCl stress in both tolerant and sensitive wheat and bean

cultivars. This is similar with the results obtained by Tuna et al. [24] who claimed that both chlorophyll a and b contents of maize plant decreased in response to salinity stress. The depressive effect of salt stress on chlorophyll biosynthesis may be due to the formation of proteolytic enzymes such as chlorophyllase, which is responsible for the chlorophyll degradation [54] and /or damaging the photosynthetic apparatus [55, 56]. On the other hand, Jaleel et al. [57] suggested that the reduction in leaf chlorophyll under salinity could be ascribed to the destruction of the chlorophyll pigments and the instability of the pigment protein complex. Lakhdar et al. [58] reported that a decrease in the content of photosynthetic pigments could be attributed either to a decrease of synthesis and/or an increase of degradation of chlorophyll and to chlorophyllase activity stimulation. Also, Santos [59] who pointed out that the induced decrease in chlorophyll content in severely NaCl stressed leaves is mainly due to a decrease of ALA (5-aminolinolic acid) synthesis, and therefore to limitations of chlorophyll synthesis. This acid is a precursor of protochlorophyllide, which converts to chlorophyll when exposed to light [60]. On the other hand, Sabra et al. [61] concluded that the highest salt concentration (100 mM NaCl) reduced Chl a, Chl b and carotenoid contents in *Echinacea purpurea* and *E. angustifolia*, and overall in both species, this reduction was correlated with shoot Na^+ content rather than Cl^-, suggesting that Na^+ was the major ion causing pigment reduction. Nevertheless, in other plant species like *Vicia faba*, the decline in the leaf Chl was strictly attributed to Cl^- accumulation in the leaves [62].

Sugars

The accumulation of soluble carbohydrates in plants has been widely reported as a response to salinity or drought, despite a significant decrease in net CO_2 assimilation rate [63, 64]. In this investigation, the second phase of NaCl stress induced stimulatory effect on the accumulation of soluble sugars in shoots and roots of tolerant and sensitive wheat cultivars, whereas the first phase of NaCl stimulated the accumulation of soluble sugars in the sensitive wheat (Gemmeza10) and inhibited their synthesis in the tolerant one Sakha93. In bean cultivars, the two phases of NaCl induced an inhibitory effect on the accumulation of soluble sugars in Sakha1 roots and shoots. In case of Giza716, although the accumulation of soluble sugars was significantly inhibited in roots at the two test salt levels, there were no appreciable differences in their production in shoots. Biochemical responses are often correlated with physiological adaptations. Accumulation of sugars in plant was considered as one of the most notable consequences for osmotic adjustment under salt stress [65-67]. In this context, significant increases in soluble sugar content of shoots and roots of both salt tolerant and sensitive wheat cultivars were reported by Zheng et al. [68] in response to NaCl stress.

According to Bohnert and Jensen [69], carbohydrates may act also as ROS scavengers and contribute to increase in membrane stabilization. The accumulation of sucrose under salt stress supported the well-established role of this sugar as an osmoprotectant that stabilizes cellular membranes and maintains turgor [70-73]. Jouve et al. [73] concluded that the observed variations in the endogenous content of mono saccharides might be due to slowdown in sugar transfer from the mesophyll cells to phloem. On the other side, other authors reported impairment of sugars biosynthesis [74] or no appreciable differences [75] during stress conditions. Accumulation of soluble sugars and proline under salt stress protect the cell by balancing the osmotic strength of cytosol with that of vacuole and external environment [76-78]. In addition to their role as cytosolic osmolytica, these solutes may interact with cellular macromolecules such as enzymes and stabilize the structure and function of such macromolecules [79-80].

Proteins

Several salt-induced proteins have been identified in plant species and classified into two categories [81-83]: proteins, which accumulate only due to salt stress, and stress associated proteins, which also accumulate in response to heat, cold, drought, water logging, and high and low mineral nutrients. In the current study, salinity stress induced insignificant change in the soluble proteins of the tolerant and sensitive wheat cultivars. However, constitutive soluble protein content of roots or shoots was higher in Gemmeza10 (NaCl-sensitive cv.) relative to Sakha93 (NaCl-tolerant cv.). In case of the bean cultivars, the soluble proteins increased insignificantly with salinity in shoots. With respect to

the production of total protein, salinity stress at the first phase resulted in non-significant stimulation in the accumulation of total proteins in the roots and shoots of the selected wheat and bean cultivars, except in roots of Giza716 (sensitive bean cv.) where a slight increase but significant was detected. The presence of NaCl in the culture media at the second phase slightly activated or reduced the total protein accumulation in wheat and bean cultivars. Proteins that accumulate in plants grown under saline conditions may provide a storage form of nitrogen that is re-utilized when stress is over [84]. The increase in protein carbonylation has been utilized as an indicator of oxidative damage in plants exposed to several abiotic stresses [38, 85-88].

A higher content of soluble proteins has been observed in salt tolerant than in salt sensitive cultivars of barley [81], sunflower [89], finger millet [90] and rice [91]. In contrast, in lentil Ashraf and Waheed [92] reported that leaf soluble proteins decreased due to salt stress in all lines, irrespective of their salt tolerance. On the other hand, Ashraf and Fatima [93] found that salt tolerant and sensitive accessions of safflower did not differ significantly in leaf soluble proteins. Ashraf and Harris [94] reported that the proteins produced under salt stress are not always associated with salt tolerance, thus using proteins as salt tolerant indicators depending on the nature of the plant species or cultivar. The protein degradation under saline environment has been attributed to the decrease in protein synthesis, accelerated proteolysis, decrease in availability of amino acids and denaturation of enzymes involved in protein synthesis [57, 58, 95].

Phenylalanine ammonia-lyase (PAL) activity

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) is the first enzyme in the phenylpropanoid biosynthetic pathway [96, 97]. This enzyme catalyzes the non-oxidative deamination of phenylalanine to cinnamic acid [98]. PAL has been shown to play an important role in the biosynthesis of many phenolic compounds. In addition, PAL has been shown to play an important role in plant resistance [99]. In the current study generally, salt stress at the two phases did not affect the PAL activity in leaves of test wheat and bean cultivars, except in Sakha1 (NaCl-tolerant bean cv.) an inhibitory effect was detected. El-Shora [98] found that higher concentration of NaCl caused progressive inhibition of the enzyme activity of marrow. Supra optimal concentrations of NaCl may cause inactivation of the enzyme and a decrease in its activity [100].

Phenolic compounds

Phenolic compounds such as phenolic acids, flavonoids and proanthocyanidins play an important role in scavenging free radicals [101, 102]. Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors, from the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron (chain-breaking function), and from their ability to chelate transition metal ions [103]. It has been shown in some recent studies that polyphenols synthesis depends on abiotic factors [104-106]. Particularly, when plants were submitted to saline treatment, variation in antioxidant pools, notably in polyphenols, were found. The present data demonstrated that no appreciable variations in their production in leaves of salt-tolerant wheat and bean cultivars at the two phases of salt stress, except in Sakha1 (NaCl-tolerant bean cv.) where a stimulatory effect was induced in the presence of second phase of NaCl. On the other hand, NaCl at first phase induced insignificant inhibitory effect on the production of free phenolic compounds in both NaCl-sensitive test wheat and bean cultivars, while, a biphasic action of the second phase of NaCl treatment was exhibited involving stimulation of their production in leaves of Gemmeza10 (NaCl-sensitive wheat cv.) and marked inhibition in Giza716 (NaCl-sensitive bean cv.). In leaves of NaCl-tolerant wheat and bean cultivars, the two phases of salt stress induced non-significant inhibitory effects on the synthesis of bound phenolic compounds. With respect to NaCl-sensitive wheat and bean cultivars, NaCl at second phase significantly reduced the content of bound phenolic compounds, while at first phase the retarding effect was, partially or completely alleviated. Salinity induced disturbances of the metabolic process leading to an increase in phenolic compounds have been reported by Dhingra and Varghese [107] and Ayaz et al. [108]. In addition, the ability to response to salt stress by the synthesis of phenolic compounds has been observed in the tolerant and sensitive strawberry genotypes Keutgen and Pawelzik [109]. Such a decrease in total polyphenol

level under salinity has been reported in a number of species including *Raphanus sativus* [110]. Actually, the accretion of ROS under salt stress is generally coupled with changes in net carbon gain which may strongly affect the biosynthesis of carbon-based secondary compounds, particularly leaf polyphenols [111].

CONCLUSIONS

The assessment of the effect of salinity on the growth parameters in wheat and bean cultivars allow to conclude that all of the considered parameters were affected by salinity with a varietals difference. The tolerant wheat (Sakha93) and bean (Sakha1) cultivars recorded the highest dry weight as compared with the sensitive ones (Gemmeza10) and (Giza716). Growth responses are often correlated and paralleled with biochemical and physiological adaptations. By reduction in transpiration rate, the tolerant cultivars might keep its water content. In the tolerant wheat and bean cultivars, MDA did not show any change, so the lipid peroxidation was lower in tolerant plants and consequently limiting the damaging within the cell membranes especially in tolerant wheat.

The accumulation of the carbohydrate and protein fractions plays an important role in osmotic adjustment in plants under salt stress, which is clear in tolerant wheat (Sakha93).

From the precedent discussions it could be concluded that although, it is somewhat difficult to follow the gross disturbance induced by salt stress in plant growth and the relevant metabolic activities.

This work secured additional data on the biochemical and physiological responses to salinity in wheat and bean cultivars, as an attempt to find the areas of difference between them, which might be of importance in elucidating the intricacies of mechanisms underlying and controlling salt resistance. There may be more changes difficult to perceive in cellular metabolism that are not so readily detected as those listed in this work but in the light of the present investigation, it could be assumed that wheat cultivar Sakha93 and bean Sakha1 have a certain affinity to tolerate saline condition.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. McWilliam JR. The national and international importance of drought and salinity effects on agricultural production. Austral J Plant Physiol. 1986; 13: 1-13.
2. Ghassemi F, Jakeman AJ, Nix HA. 1995. Salinization of Land and Water Resources. University of New South Wales Press Ltd, Canberra, Australia.
3. Mashali AM. 1999. FAO global network in soil management for sustainable use of salt affected soils. 32 p. International Workshop, September 6-9, Menemen, Izmir, Turkey.
4. Evelin H, Kapoor R, Giri B. Arbuscular mycorrhizal fungi in alleviation of salt stress: a review. Ann Bot. 2009; 104: 1263–1281.
5. Hammer EC, Nasr H, Pallon J, Olsson PA,Wallander H. Elemental composition of arbuscular mycorrhizal fungi at high salinity. Mycorrhiza. 2011; 21: 117–129.
6. Wang W, Vinocur B, Altman A. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta. 2003; 218: 1-14.
7. Munns R. Comparative physiology of salt and water stress. Plant Cell Environ. 2002; 25: 239-250.
8. Wahid A, Ghazanfar A. Possible involvement of some secondary metabolites in salt tolerance of sugarcane. J Plant Physiol. 2006; 163: 723-730.
9. Rengasamy P. World salinization with emphasis on Australia. J Exper Bot. 2006; 57: 1017-1023.
10. Hoagland DR, Arnon DI. The water-culture method for growing plants without soil. Calif Agric Exper Stat Circul. 1950; 347: 1-32.
11. Bozcuk S. 1975. Effect of sodium chloride upon growth and transpiration in *Statice* sp. and *Pisum sativum* L. Proc. of the third MPP Meeting IZMIR: 37-42.
12. Lichtenthaler HK. 1987. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. In: Methods in Enzymology vol. 148, pp. 183-350. Academic Press, Orlando, Fl, USA.
13. Premachandra GS, Saneoka AH, Fujta K, Ogata S. Leaf water relations, osmotic adjustment, cell membrane stability, epicuticular wax load and growth as affected by increasing water deficits in sorghum. J Exper Bot. 1992; 43: 1569-1576.
14. Madhava Rao KV, Sresty TVS. Antioxidative parameters in the seedlings of pigeonpea (*Cajanus cajan* L. Millspaugh) in response to Zn and Ni stresses. Plant Sci. 2000; 157: 113-128.
15. Nelson N. A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem. 1944; 153: 375-380.
16. Fales FW. The assimilation and degradation of carbohydrates by yeast cells. J Biol Chem. 1951;

193: 113-118.
17. Schlegel HG. Die Verwertung organischer sauren duch Chlorella in licht. Planta. 1956; 47: 510-526.
18. Lowry OH, Rosebough NJ, Farr AL, Randall RJ. Protein measurement with folin phenol reagent. J Biol Chem. 1951; 193: 265-275.
19. Havir EA, Hanson KA. L-Phenylalanine ammonia lyase I. purification and molecular size of the enzyme from potato tubers. Biochem. 1968; 7: 1896-1903.
20. Kofalvi SA, Nassuth A. Influence of wheat streak mosaic virus infction phrenyl propanoid metabolism and the accumulation of phenyl propanoid metabolism and the accumulation of phenolics and lignin in wheat. Physiol Mol Plant Pathol. 1995; 47: 365-377.
21. Radi AA, Abdel-Wahab DA, Hamada AM. Evaluation of some bean lines tolerance to alkaline soil. J Biol Earth Sci. 2012; 2(1): B18-B27.
22. Ruiz JM, Blasco B, Rivero RM, Romero L. Nicotine-free and salt tolerant tobacco plants obtained by grafting to salinity-resistant rootstocks of tomato. Physiol Plant. 2005; 124: 465-475.
23. Koca H, Bor M, Özdemir F, Türkan I. The effect of salt stress on lipid peroxidation, antioxidative enzymes and proline content of sesame cultivars. Environ Exper Bot. 2007; 60: 344-351.
24. Tuna AL, Kaya C, Dikilitas M, Higgs D. The combined effects of gibberellic acid and salinity on some antioxidant enzyme activities, plant growth parameters and nutritional status in maize plants. Environ Exper Bot. 2008; 62: 1-9.
25. Murillo-Amador B, Troyo-Dieguez E, Garcia-Hernandez JL, Lopez-Aguilar R, Avila-Serrano NY, Zamra-Salgado S, et al. Effect of NaCl salinity in the genotypic variation of cowpea (*Vigna unguiculata*) during early vegetative growth. Scien Horticul. 2006; 108: 432-431.
26. Calatayud A, Iglesias DJ, Talon M. Effects of 2-month ozone exposure in spinach leaves on photosynthesis, antioxidant systems and lipid peroxidation. Plant Physiol Biochem. 2003; 41: 839-845.
27. Zheng YH, Li X, Li YG, Miao BH, Xu H, Simmons M, Yang XH. Contrasting responses of salinity-stressed salt-tolerant and intolerant winter wheat (*Triticum aestivum* L.) cultivars to ozone pollution. Plant Physiol Biochem. 2012; 52: 169-178.
28. Zhu JK. Plant salt tolerance. Trends Plant Sci. 2001; 6: 66-71.
29. Munns R. Physiological processes limiting plant growth in saline soils: some dogmas and hypotheses. Plant Cell Environ. 1993; 16: 15-24.
30. Yasseen BT, Jurjee JA, Sofajy SA. Changes in some growth processes induced by NaCl in individual leaves of two barley cultivars. Ind J Plant Physiol. 1987; 30: 1-6.
31. Cosgrove DJ. Wall extensibility: its nature, measurement and relationship to plant cell growth. Tansley review 46. New Phytol. 1993; 124: 1-23.
32. Neumann PM. The role of cell wall adjustment in plant resistance to water deficits. Crop Sci. 1995; 35: 1258-1266.
33. Shabala S, Babourina O, Newman I. In specific mechanisms of osmoregulation in bean mesophyll cells. J Exper Bot. 2000; 51: 1243-1253.
34. Sweet WJ, Morrison JC, Labaritch JM, Matthews MA. Altered synthesis and composition of cell wall of grapevines *Vitis vinifera* L. during expression and growth inhibiting water deficits. Plant Cell Physiol. 1990; 31: 407-414.
35. Jeschke WD, Wolf O. Effect of NaCl salinity on growth, development, ion distribution and ion translocation in castor bean (*Ricinus communis* L.). J Plant Physiol. 1988; 132: 45-53.
36. Subbarao GV, Johansen C, Jana MK, Kumar Rao JVDK. Effects of the sodium/calcium ratio in modifying salinity response of pigeonpea (*Cajanus cajan*). J Plant Physiol. 1990; 136: 439-443.
37. Abbas MA, Younis ME, Shukry M. Plant growth, metabolism and adaptation in relation to stress conditions. XIV. Effect of salinity on the internal solute concentrations in *Phaseolus vulgaris*. J Plant Physiol. 1991; 138: 722-727.
38. Ferreira-Silva SL, Voigt EL, Silva EN, Maia JM, Aragão TCR, Silveira JAG. Partial oxidative protection by enzymatic and non-enzymatic components in cashew leaves under high salinity. Biol Plant. 2012; 56: 172-176.
39. Jiang Q, Roche D, Monaco TA, Durham S. Gas exchange, chlorophyll fluorescence parameters and carbon isotope discrimination of 14 barley genetic lines in response to salinity. Field Crop Res. 2006; 96: 269-278.
40. Maggio A, Raimondi G, Martino A, De Pascale S. Salt stress response in tomato beyond the salinity tolerance threshold. Environ Exper Bot. 2007; 59: 276-282.
41. Xu F, Li L, Huang X, Cheng H, Wang Y, Cheng S. Antioxidant and antibacterial properties of the leaves and stems of *Premna microphylla*. J Med Plant Res. 2010; 4: 2544–2550.
42. Kaya C, Ashraf M, Sonmez O, Aydenir S, Tuna AL, Cullu MA. The influence of arbuscular mycorrhizal colonization on key growth parameters and fruit yield of pepper plants grown at high salinity. Scient Horticul. 2009; 121: 1–6.
43. Elkahoui S, Hernandez AJ, Abdelly C, Ghrir R, Fb L. Effects of salt on lipid peroxidation and antioxidant enzyme activities of *Catharanthus roseus* suspension cells. Plant Sci. 2005; 168: 607–613.
44. Ksouri R, Megdiche W, Debez A, Falleh H, Grignon

C, Abdelly C. Salinity effects on polyphenol content and antioxidant activities in leaves of the halophyte *Cakile maritime*. Plant Physiol Biochem. 2007; 45: 244-249.

45. Azevedo-Neto AD, Prisco JT, Eneas-Filho J, Braga de-Abreu CE, Gomes-Filho E. Effect of salt stress on antioxidive enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. Environ Exper Bot. 2006; 56: 87-94.
46. Ying HC, Chen YM, Huang CY. Role of glutathione reductase and related enzymes in salt tolerance mechanism of soybean plants grown under salt stress condition. Taiwania. 1995; 44: 21-34.
47. Keutgen AJ, Pawelzik E. Quality and nutritional value of strawberry fruit under long term salt stress. Food Chem. 2008; 107: 1413-1420.
48. Falleh H, Jalleli I, Ksouri R, Boulaaba M, Guyot S, Magné C, Abdelly C. Effect of salt treatment on phenolic compounds and antioxidant activity of two *Mesembryanthemum edule* provenances. Plant Physiol Biochem. 2012; 52: 1-8.
49. Tuna AL, Kaya C, Ashraf M, Altunlu H, Yokas I, Yagmur B. The effects of calcium sulphate on growth, membrane stability and nutrient uptake of tomato plants grown under salt stress. Environ Exper Bot. 2007; 59: 173-178.
50. Lutts S, Kinet JM, Bouharmont J. NaCl-induced senescence in leaves of rice (*Oryza sativa* L.) cultivars differing in salinity resistance. Ann Bot. 1996; 78: 389-398.
51. Lutts S, Bouharmont J, Kinet JM. Physiological characterization of salt-resistant rice *(Oryza sativa* L.) somaclones. Austral J Bot. 1999; 47: 835-849.
52. Ghoulam C, Foursy A, Fares K. Effects of salt stress on growth, inorganic ions and proline accumulation in relation to osmotic adjustment in five sugar beet cultivars. Environ Exper Bot. 2002; 47: 39-50.
53. Kaya C, Tuna AL, Ashraf M, Altunlu H. Improved salt tolerance of melon (*Cucumis melo* L.) by the addition of proline and potassium nitrate. Environ Exper Bot. 2007; 60: 397-403.
54. Sabater B, Rodriguez MI. Control of chlorophyll degradation in detached leaves of barley and oat through effect of kinetine on chlorophyllase levels. Physiol Plant. 1978; 43: 274-276.
55. Singh G, Jain S. Effect of some growth regulators on certain biochemical parameters during seed development in chickpea under salinity. Ind J Plant Physiol. 1981; 20: 167-179.
56. Yasseen BT. 1983. An analysis of the effects of salinity on leaf growth in Mexican wheat. Ph.D. Thesis, University of Leeds.
57. Jaleel CA, Manivannan P, Lakshmanan GMA, Sridharan R, Panneerselvam R. NaCl as a physiological modulator of proline metabolism and antioxidant potential in *Phyllanthus amarus*. Comp Rend Biol. 2007; 330: 806-813.
58. Lakhdar A, Hafsi C, Rabhi M, Debez A, Montemurro F, Abdelly C, Jedidi N, Ouerghi Z. Application of municipal solid waste compost reduces the negative effects of saline water in (*Hordeum maritimum* L.). Bioresour Technol. 2008; 99: 7160-7167.
59. Santos CV. Regulation of chlorophyll biosynthesis and degradation by salt stress in sunflower leaves. Scient Horticul. 2004; 103: 93-99.
60. Stobart A, Griffiths W, Ameen-Bukhari I, Sherwood R. The effect of Cd^{2+} on the biosynthesis of chlorophyll in leaves of barley. Physiol Plant. 1985; 63: 293-298.
61. Sabra A, Daayf F, Renaulta S. Differential physiological and biochemical responses of three *Echinacea* species to salinity stress. Scient Horticul. 2012; 135: 23–31.
62. Tavakkoli E, Rengasamy P, McDonald GK. High concentrations of Na^+ and Cl^- ions in soil solution have simultaneous detrimental effects on growth of faba bean under salinity stress. J Exper Bot. 2010; 61: 4449-4459.
63. Popp M, Smirnoff N. 1995. Polyol accumulation and metabolism during water deficit. In: Smirnoff N. (Ed.), Environment and Plant Metabolism: Flexibility and Acclimation. Bios Scientific, Oxford. pp. 199-215.
64. Murakeozy EP, Nagy Z, Duhaze C, Bouchereau A, Tuba Z. Seasonal changes in the levels of compatible osmolytes in three halophytic species of inland saline vegetation in Hungary. J Plant Physiol. 2003; 160: 395-401.
65. Cheeseman JM. Mechanisms of salinity tolerance in plants. Plant Physiol. 1988; 87: 547-550.
66. Kameli A, Losel DM. Contriution of carbohydrates and other solutes to osmotic adjustment in wheat leaves under water stress. J Plant Physiol. 1995; 145: 363-366.
67. Kameli A, Losel DM. Growth and sugars accumulation in durum wheat plants under water stress. New Phytol. 1996; 132: 57-62.
68. Zheng Y, Jia A, Ning T, Xu J, Li Z, Jiang G. Potassium nitrate application alleviates sodium chloride stress in winter wheat cultivars differing in salt tolerance. J Plant Physiol. 2008; 165: 1455-1465.
69. Bohnert HJ, Jensen RG. Strategies for engineering water stress tolerance in plants. Trends in Biotechnol. 1996; 14: 89-97.
70. Lineberger DR, Steponkus P. Cryoprotection by glucose, sucrose and raffinose to chloroplast thylakoids. Plant Physiol. 1980; 65: 298-304.
71. Norwood M, Truesdale MR, Richter A, Scott P. Metabolic changes in leaves and roots during dehydration of the resurrection plant *Craterostigma plantagineum* (Hochst). S Afri J Bot. 1999; 65: 421-427.
72. Whittaker A, Bochicchio A, Vazzana C, Lindsey G, Farrant J. Changes in leaf hexokinase activity and

metabolite levels in response to drying in the desiccation-tolerant species *Sporobolus staphianus* and *Xerophyta viscose*. J Exper Bot. 2001; 1: 961-969.
73. Jouve L, Hoffmann L, Hausman JF. Polyamine, carbohydrate, and proline content changes during salt stress exposure of Aspen (*Populus tremula* L.): involvement of oxidation and osmoregulation metabolism. Plant Biol. 2004; 6: 74-80.
74. Hanson AD, Hitz WD. Metabolic responses of plant water deficit. Annu Rev Plant Physiol Plant Mol Biol. 1982; 33: 163-203.
75. Morgan JM. Osmotic components and properties associated with genotypic differences in osmoregulation in wheat. Austral J Plant Physiol. 1992; 19: 67-76.
76. Hellebust JA. Osmoregulation. Annu Rev Plant Physiol Plant Mol Biol. 1976; 27: 485-505.
77. Greenway H, Munns R. Mechanisms of salt tolerance in nonhalophytes. Annu Rev Plant Physiol Plant Mol Biol. 1980; 31: 149-190.
78. Gadallah MAA. Effects of proline and glycinebetaine on *Vicia faba* response to salt stress. Biol Plant. 1999; 42: 249-257.
79. Rhodes D. 1987. Metabolic responses to stress. In: Stumpf PK, Priess J (Eds). The Biochemistry of Plants. Academic Press San Deigo, CA. 12: 201-241.
80. Smirnoff N, Cumbes QT. Hydroxy radicals scavenging activity of compatible isolates. Phytochem. 1989; 28: 1057-1060.
81. Hurkman WJ, Fornari CS, Tanaka CK. A comparison of the effect of salt on polypeptides and translatable mRNA in roots of a salt tolerant and salt sensitive cultivar of barley. Plant Physiol. 1989; 90: 1444-1456.
82. Ali G, Srivasta PS, Iqbal M. Proline accumulation, protein patteren and photosynthesis in regenerants grown under NaCl stress. Biol Plant. 1999; 42: 89-95.
83. Mansour MMF. Nitrogen containing compounds and adaptation of plants to salinity stress. Biol Plant. 2000; 43: 491-500.
84. Singh NK, Bracken CA, Hasegawa PM, Handa AK, Buckel S, Hermodson MA, Pfankoch F, Regnier FE, Bressan RA. Characterization of osmotin. A thaumatin-like protein associated with osmotic adjustment in plant cells. Plant Physiol. 1987; 85: 529-536.
85. Moran JF, Becana M, Iturbe-Ormaetxe I, Frechilla S, Klucas RV, Aparicio-Tejo P. Drought induces oxidative stress in pea plants. Planta. 1994; 194: 346-352.
86. Gomez JM, Hernandez JA, Jimenez A, Del Rio LA, Sevilla F. Differential response of antioxidative enzymes of chloroplasts and mitochondria to long-term NaCl stress of pea plants. Free Rad Res. 1999; 31: 11-18.
87. Boscolo PRS, Menossib M, Jorgea RA. Aluminum induced oxidative stress in maize. Phytochem. 2003; 62: 181-189.
88. Miller G, Suzuki N, Rizhsky L, Hegie A, Koussevitzky S, Mittler R. Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses. Plant Physiol. 2007; 144: 1777-1785.
89. Ashraf M, Tufail M. Variation in salinity tolerance in sunflower (*Helianthus annuus* L.). J Agron Crop Sci. 1995; 174: 351-362.
90. Uma S, Prasad TG, Kumar MU. Genetic variability in recovery growth and synthesis of stress proteins in response to polyethylene glycol and salt stress in finger millet. Ann Bot. 1995; 76: 43-49.
91. Pareek A, Singla SL, Grover A. 1997. Salt responsive proteins/genes in crop plants. In: Jaiwal PK, Singh RP, Gulati A (Eds). Strategies for improving salt tolerance in higher plants. Oxford and IBH Publication Co, New Delhi. pp. 365-391.
92. Ashraf M, Waheed A. Responses of some local/exotic accessions of lentil (*Lens culinaris* Medic.) to salt stress. J Agron Crop Sci. 1993; 170: 103-112.
93. Ashraf M, Fatima H. Responses of some salt tolerant and salt sensitive lines of safflower (*Carthamus tinctorius* L.). Acta Physiol Plant. 1995; 17: 61-71.
94. Ashraf M, Harris PJC. Potential biochemical indicators of salinity tolerance in plants. Plant Sci. 2004; 166: 3-16.
95. Dagar JC, Bhagwan H, Kumar Y. Effect of growth performance and iochemical contents of *Salvadora persica* when irrigated with water of different salinity. Ind J Plant Physiol. 2004; 9: 234-238.
96. Hahlbrock K, Scheel D. Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol. 1989; 40: 347-369.
97. Dorey S, Baillieul F, Pierel MA, Saindrenan P, Fritig B, Kauffmann S. Spatial and temporal induction of cell death, defense genes, and accumulation of salicylic acid in tobacco leaves reacting hypersensitivity to a fungal glycoprotein elicitor. Mol Plant Micr Inter. 1997; 10: 646-655.
98. El-Shora HM. Properties of phenylalanine ammonia-lyase from marrow cotyledons. Plant Sci. 2002; 162: 1-7.
99. Mauch-Mani B, Slusarenko AJ. Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. The Plant Cell. 1996; 8: 203-212.
100. Yanagisawa H, Hamasima N, Kato T. Polyamine oxidase from leaves of *Lilium longiflorum*: Purification and properties. J Plant Physiol. 1996; 149: 657-662.
101. Ksouri R, Megdiche W, Koyro HW, Abdelly C. Responses of halophytes to environmental stresses with special emphasis to salinity. Advan Botan Res. 2010; 53: 117-145.

102. Falleh H, Oueslati S, Guyot S, Ben Dali A, Magné C, Abdelly C, Ksouri R. LC/ESI-MS/MS characterisation of procyanidins and propelargonidins responsible for the strong antioxidant activity of the edible halophyte *Mesembryanthemum edule* L. Food Chem. 2011; 127: 1732-1738.
103. Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J Agricul Food Chem. 2005; 53: 1841-1856.
104. Ksouri R, Megdiche W, Falleh H, Trabelsi N, Boulaaba M, Smaoui A, Abdelly C. Influence of biological, environmental and technical factors on phenolic content and antioxidant activities of Tunisian halophytes. Comp Rend Biol. 2008; 331: 865-873.
105. Megdiche W, Ben Amor N, Debez A, Hessini K, Ksouri R, Abdelly C. Physiological and biochemical traits involved in the genotypic variability to salt tolerance of Tunisian *Cakile maritime*. Afr J Ecol. 2009; 47: 774-783.
106. Naffeti M, Sriti J, Hamdaoui G, Kchouk EM, Marzouk B. Salinity impact on fruit yield, essential oil composition and antioxidant activities of *Coriandrum sativum* fruit extracts. Food Chem. 2011; 124: 221-225.
107. Dhingra H R, Varghese TM. Effect of salt stress on viability germination and endogenous levels of some metabolites and ions in maize (*Zea mays* L.) pollen. Ann Bot. 1985; 55: 415-420.
108. Ayaz FA, Kadioglu A, Turgut A. Water stress effects on the content of low molecular weight carbohydrates and phenolic acids in *Ctenanthe setosa* (Rosc.) Eichler. Can J Plant Sci. 2000; 80: 373-378.
109. Yuan G, Wang X, Guo R, Wang Q. Effect of salt stress on phenolic compounds, glucosinolates, myrosinase and antioxidant activity in radish sprouts. Food Chem. 2010; 121: 1014-1019.
110. Tattini M, Remorini D, Pinelli P, Agati G, Saracini E, Traversi ML, Massai R. Morpho-anatomical, physiological and biochemical adjustments in response to root zone salinity stress and high solar radiation in two Mediterranean evergreen shrubs *Myrtus communis* and *Pistacia lentiscus*. New Phytol. 2006; 170: 779-794.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY REVIEW

Earthworms: ecofriendly environmental engineers

Seema Rani[1], Nitish Bansal[2], Vineeta Shukla[1]

[1]Department of Zoology, Maharshi Dayanand University, Rohtak, Haryana, India
[2]Department of Zoology, Hisar Agricultural University, Hisar, Haryana, India

ABSTRACT

Earthworms are important biological sources that have a tremendous potential agro system. India is a diverse country harbouring a very high diversity of earthworms. Earthworms have gained renewed scientific attention in India and abroad because of their wide application in various fields. These have been used for centuries as a means of decomposing of wastes and improving soil structure. Earthworms serve versatile natural bio reactors to harness the beneficial soil micro flora and destroy pathogen, thus converting organic waste into valuable products such as bio fertilizers, bio pesticides, vitamins, enzymes, antibiotics, growth hormones and proteinaceous worm biomass. These have wide usages for various toxicological studies as test worms. Earthworms promise to provide cheaper solutions to several social, economic and environmental problems plaguing the human society. Earthworms can safely manage all municipal and industrial organic wastes including sewage sludge and divert them from ending up in the landfills. Their body works as a 'biofilter' and they can 'purify' and also 'disinfect' and 'detoxify' municipal and several industrial wastewater. Earthworms can bio-accumulate and bio-transform many chemical contaminants including heavy metals and organic pollutants in soil and clean-up the contaminated lands for re-development. The present paper reviews the knowledge on earthworms in various technologies and heavy metal toxicity.

Key words: Bioengineering; Vermicomposting; Environmental engineers; Biomarkers.

J Biol Earth Sci 2013; 3(1): B89-B92

Corresponding author:
Seema Rani
Department of Zoology, Maharshi Dayanand University, Rohtak, Haryana, India
Tel: 9466170085
E-mail: sj181191@gmail.com

Original Submission: 10 December 2012; Revised Submission: 31 January 2013; Accepted: 11 February 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Vermiculture is sustainable technology to manage most organic wastes; treat waste water; clean up chemically contaminated soils; improve soil fertility and produce food crops. Uses of earthworms in life saving medicines for industries are some 'new discoveries'. The global scientific community today is searching for a technology which should be 'economically viable; environmentally sustainable' and 'socially acceptable'. Vermiculture Technologies based on the use of earthworms combine all the virtues together. Earthworms have over 600 million years of experiance as 'environmental engineers'. Vermiculture scientists all over the world know about their role as 'waste and soil engineers' and 'plant growth promoters' for long time.

REVIEW

DIFFERENT TECHNOLOGIES

Various technologies for environmental protection can be operated by use of useful earthworm species which are cheaper solutions to various social economic, environmental and health problem of society. These are:

1) THE VERMICOMPOSTING TECHNOLOGY (Worms as WASTE ENGINEERS) for efficient management of municipal and industrial solid wastes.

2) a. THE VERMIFILTRATION TECHNOLOGY (Worms as WASTE WATER ENGINEERS) for treatment of municipal and some industrial wastewater.

b. THE VERMIREMEDIATION TECHNOLOGY (Worms as BIOCHEMICAL ENGINEERS) for cleaning up chemically contaminated lands.

3) THE VERMI-AGROPRODUCTION TECHNOLOGY (Worms as SOIL ENGINEERS) for restoring and improving soil fertility to produce safe and chemical free food for society.

Bioengineering technologies based on earthworms are self promoted, self regulated, self improved and self enhanced, low or no energy requiring zero- waste technologies. They excel all 'bioconversion', 'biodegradation' and 'bioproduction' technologies. They involve about 100-1000 times higher 'value addition' than other biological technologies [1, 2].

Vermiremediation (using chemical tolerant earthworm species) is emerging as a low-cost and convenient technology for cleaning up the chemically polluted/contaminated sites/lands in world. Earthworms in general (specially *E. fetida*) are highly resistant to many chemical contaminants including heavy metals and organic pollutants in soil. They have been reported to bio-accumulate them in their tissues and either biodegrade or bio-transform them to harmless products with the aid of enzymes.They have also been reported to host microbes in their gut which can biodegrade chemicals. Ramteke and Hans [3] isolated hexachlorocyclohexane (HCH) degrading microorganisms from the gut of earthworms.

Earthworms have been used for land recovery, reclamation and rehabilitation of sub-optimal soils such as poor mineral soils, polder soils, open cast mining sites, closed landfill sites and cutover peat [4, 5]. Within the soil environment, an earthworm's sphere of influence is known as the 'drilosphere system'. This incorporates the burrow systems, surface and belowground earthworm casts, internal earthworm gut and process, the earthworm surface in contact with the soil, and associated biological, chemical and physical interactions, in addition to the soil microorganisms [6].

Earthworms in general are highly resistant to many chemical contaminants including heavy metals and organic pollutants in soil and have been reported to bio-accumulate them in their tissues. After the Seveso chemical plant explosion in 1976 in Italy, when vast inhabited area was contaminated with certain chemicals including the extremely toxic TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) several fauna perished but for the earthworms that were alone able to survive. Earthworms which ingested TCDD contaminated soils were shown to bio-accumulate dioxin in their tissues and concentrate it on average 14.5 fold. [7].

Vermi-agroproduction technology promises to usher in the 'Second Green Revolution' by completely replacing the destructive agro-chemicals which did more harm than good to both the farmers and their farmland during the 'First Green Revolution' of the 1950-60's. Earthworms restore and improve soil fertility, and boost crop productivity by the use of their metabolic product - 'vermicast'. They excrete beneficial soil microbes, and secrete polysaccharides, proteins and other nitrogenous compounds into the soil. They promote soil fragmentation and aeration, and bring about 'soil

turning' and dispersion in farmlands.

EARTHWORM SPECIES

Visvanathan et al. [8] found that most earthworms consume half their body weight of organics in the waste in a day. These farmer friends no eyes, legs and anus. Worms tunnel deeply in soil and bring subsoil closer to the surface mixing it with the topsoil. Slime, a secretion of earthworms, contains nitrogen which is an important nutrient for plants. The sticky slime helps to hold clusters of soil particles together forming aggregates.

These hermaphrodite tillers of soil can produce 3 to 80 cocoons/year depending on the species. Surface dewellers produce more cocoons than deep dwellers. Generally species have the potential to live 4-8 years. Some species under ideal conditions live for 10-14 years. *Eisenia fetida* lives for 4 to 5 years. It can consume organic matter at the rate equal to their body weight everyday.

Earthworm participation enhances natural biodegradation and decomposition of organic waste from 60-80%. As the worms double their population every 60-70 days, the process becomes faster with time. With temperature (20º-30ºC) and moisture (60-70%), about 5 kg of worms can vermiprocess 1 ton of waste in to vermicompost in 30 days. The Indian Blue Worm (*Perionyx excavates*) is also best suited for vermicomposting of variety of organic wastes [9,10,11]. Earthworm species may be increased or decreased by various agricultural practices like cultivation, cropping, fertilizers and pesticides etc. They are a measure of soil fertility helping in soil management practices.

EARTHWORMS AS BIO–INDICATORS

Earthworm species can be used as bio-indicators for monitoring of ecosystem state and change. Various workers identified the earthworms for evaluating the effect of soil contamination with heavy metals and pesticides [12]. There are numerous studies about the heavy metal influence on growth, reproduction and mortality of earthworms. Earthworm skin is a significant route of contaminant uptake and thus investigation of earthworm biomarkers in ecological risk assessment (ERA) can be helpful. Cadmium (sp. gravity 8.65) and lead (sp. gravity 11.34) are very toxic metals having fatal effects on living beings. If these metals are deposited on surface sediments, they become incorporated in plants, food crops and animals [13-15]. Earthworms are efficient accumulators of heavy metals. These not only convert the waste into nutrients but also remediate the persistant heavy metals from the wastes by bioaccumulation in their body during vermicomposting [16, 17].

CONCLUSION

Earthworms are tireless tillers of our soils and their castings are richest and best of all fertilizers. They have full potential to have a crown of "ecofriendly environmental engineers". They can replace the chemical fertilizers for production of safe organic foods which can help in prevention of various health hazards of modern world. As ecofriendly engineers, earthworms are both 'protective' and 'productive' for society.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Appelhof M. 1997. Worms eat my garbage. 2nd (ed); Flower Press, Kalamazoo, Michigan, U.S. (http://www.wormwoman.com).
2. Wang ZW. Research advances in earthworms bioengineering technology. Medica. 2000; 31(5): 386-389.
3. Ramteke PW, Hans RK. Isolation of hexachlorocyclohexane (HCH) degrading microorganisms from earthworm gut. Environ Sci Health. 1992; 27(8): 2113–2122.
4. Lowe CN, Butt KR. 2003. Inoculation of earthworms into reclaimed soils: experiences from Britain. Proc. Of Ninth International Waste Management and Landfill Symposium.
5. Butt KR, Lowe CN, Frederickson J, Moffat AJ. The development of sustainable earthworm populations at Calvert landfill site, UK. Land Degrad Develop. 2004; 15: 27–36.
6. Brown GB, Doube BM. 2004. On earthworms assisted bioremediation. In: CA. Edward (Ed.), Earthworm Ecology (2nd Ed.). pp. 213–239. CRC Press, Boca Raton, Fl.
7. Satchell JE. 1983. Earthworm ecology - from Darwin to Vermiculture. pp. 1-5. Chapman and Hall Ltd., London.
8. Visvanathan C, Trankler J, Jospeh K, Nagendran R. 2005. Vermicomposting as an Eco-tool in Sustainable Solid Waste Management. Asian Institute of Technology, Anna University, India.

9. Graff O. 1981. Preliminary experiment of vermicomposting of different waste materials using *Eudrilus eugeniae* Kingberg. In: M. Appelhof (ed.) Proc. of the workshop on 'Role of Earthworms in the Stabilization of Organic Residues'; pp. 179-191. Malanazoo Pub. Michigan, USA.
10. Beetz A. 1999. Worms for composting (vermicomposting). ATTRA-National Sustainable Agriculture Information Service, Livestock Technical Note, June 1999.
11. Sinha RK, Herat S, Valani D. 2010. Earthworms - The Environmental Engineers: Review of Vermiculture Technologies for Environmental Management & Resource Development. Int J Environ Engin. In: RK Sinha, S. Herat, S. Agarwal (Eds.).
12. Paoletti MG, Faveretto MR, Stinner BR, Purrington FF, Bater JE, Invertebrates as bioindicators of soil use. Agricult Ecosystem Environ. 1991; 34: 341-62.
13. Neuhauser EF, Callahan CA. Growth and reproduction of the earthworm *Eisenia foetida* exposed to sub lethal concentration of organic chemicals. Soil Biol Biochem. 1990; 31: 363-366.
14. Fitzpatrick LC Muratti-Ortiz JF, Venables BJ, Goven AX. Comparative toxicity in earthworms *Eisenia foetida* and *Lumbricus terrestris* exposed to cadmium nitrate using artificial soil and filter paper protocol. Bull Environ Contam Toxicol. 1996; 57: 63-68.
15. Neuhauser EF, Callahan CA. Growth and reproduction of the earthworm *Eisenia foetida* exposed to sub lethal concentration of organic chemicals. Soil Biol Biochem. 1990; 31: 363-366.
16. Gupta SK, Singh SB, Sundararaman V. Cadmium toxicity in earthworm, Mataphire posthums: Ultra structural changes in secretary cells of clitellar epithelium. Ind J Exp Biol. 1997; 35(7): 780-786.
17. Edwards CA, Bohlen PJ. (Eds.). 1996. Biology and Ecology of earthworm. pp. 426. Chapman and Hall, London.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Seasonal variation in the toxicity of umbelliferone against *Fasciola* larvae

Kumari Sunita, Pradeep Kumar, D. K. Singh

Malacology Laboratory, Department of Zoology, DDU Gorakhpur University, Gorakhpur, India

ABSTRACT

Snail is one of the important components of aquatic ecosystem it act as intermediate host of *Fasciola* species. The life cycle of parasite can be interrupted by killing the vector snail or *Fasciola* larva (sporocyst, redia and cercaria) inside the snail body. Effect of abiotic factors on the toxicity of umbelliferone against *Fasciola* larvae in different months of the year 2011-2012. In highest *in vivo* toxicity is noted of umbelliferone against, redia and cercaria larva was noted in month of May and July (redia - 8h LC_{50}=0.93, and 0.89 mg/L; cercaria - 8h LC_{50}=0.70 and 0.92 mg/L). In *in vitro* treatment highest toxicity against redia and cercaria larva was absorbed June, July and August (redia - 8h LC_{50}= 0.26, 0.30 and 0.28 mg/L; cercaria - 8h LC_{50}=0.09, 0.11 and 0.18 mg/L). The lowest toxicity was observed the month of November to January. The highest temperature, free carbon dioxide, and lowest pH, dissolved oxygen were observed in the months of June to August. The present study conclusively shows that variant abiotic factor can significantly alter the *in vitro* and *in vivo* toxicity of umbelliferone against sporocyst redia and cercaria larva.

Key words: Fascioliasis; Sporocyst; Redia; Cercaria; Umbelliferone; *Lymnaea acuminata*.

J Biol Earth Sci 2013; 3(1): B93-B99

Corresponding author:
Prof. D. K. Singh
Malacology Laboratory, Department of Zoology,
DDU Gorakhpur University, Gorakhpur,
273009, U.P., India.
Tel: +91-551-2202187(O);
9454211574 (Mobile).
E-mail: dksingh_gpu@yahoo.co.in

Original Submission: 02 January 2013; Revised Submission: 20 February 2013; Accepted: 21 February 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Fascioliasis is a zoonotic disease caused by trematode parasite, *Fasciola* sp. During the last few decades the public health importance of human fascioliasis has significantly increased, including several areas of true human endemic ranging from low to very high prevalence and intensities [1-3].

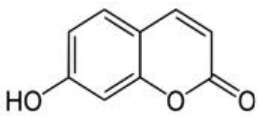
Two *Fasciola* species, *Fasciola hepatica* and *F. gigantica* are involved in both animal and human fascioliasis [4]. Fascioliases due to *F. gigantica* in major endemic areas are large tropical regions of Africa, and many areas of Asia [5-7]. Snail *Lymnaea acuminata* serves as intermediate host of *Fasciola* species [8]. Incidence of endemic fascioliasis is very common in the eastern region of the state of Uttar Pradesh [9, 10]. In India human fascioliasis has been reported in state of Assam, Bihar, Maharashtra, Uttar Pradesh, Arunachal Pradesh and West Bengal [11-15]. One of the possible approaches to control fascioliasis is to interrupt the life cycle of the parasitic trematodes by eliminating the larva (sporocyst, redia and cercaria) inside the snail body. The massive use of chemical drugs to control helminth parasites has led to the alarming development of parasite resistance in all livestock species [16]. Natural plant products are eco-friendly and easily biodegradable [17].

Recently, Sunita and Singh [10] have reported that the phytotherapy of snails by products different plant have significant larvicidal activity. *Ferula asafoetida* (Umbelliferone) has molluscicidal/ larvicidal effect against *L. acuminata*/*Fasciola* larvae [10, 18, 19]. The objective of the present study is explore the possibility that seasonal change in abiotic factors such as temperature, pH, dissolved oxygen and free carbon dioxide in test water can influence the *in vivo* and *in vitro* larvicidal activity of umbelliferone against different *Fasciola gigantica* larvae in infected snails in each month of the year 2011-2012.

MATERIALS AND METHODS

Test component

Umbelliferone (7-Hydroxycoumarin, 7-hydroxy-2H-1-benzopyran-2-one) is punched from Sigma chemical Co. USA.



Animals

Adult *L. acuminata* (2.6±0.20 cm in length) were collected locally, cercaria shedding infected and uninfected snails were separated in two groups. The snails were allowed to acclimatize for 24 in laboratory condition.

Each infected snail was dissected in a glass petridish containing 10 ml of dechlorinated water at 22-24ºC. The pH of the water was 7.1-7.3 and dissolved oxygen, free carbon dioxide and bicarbonate alkalinity were 6.5-7.2 mg/L, 5.2-6.3 mg/L and 102.0-105.0 mg/L, respectively. After dissection sporocyst, redia and cercaria larva were separated in different petridish containing 10 ml of dechlorinated water by the method of Sunita and Singh [10]. These larvae were kept in dechlorinated tap water where they survive up to 48h in laboratory condition.

Toxicity determination

In vivo

In vivo toxicity of umbelliferone against larvae of *Fasciola* in infected *L. acuminata* was done by the method of Sunita and Singh [10]. Physical parameters of water such as temperature, pH, dissolve oxygen and free carbon dioxide were measured in each months of the year (2011- 2012). Dissolved oxygen and CO_2 were estimated according to methods described by APHA [20]. After 2h, 4h, 6h and 8h of treatment infected snails were dissected. Live and dead sporocyst, redia and cercaria were counted. Per cent mortality of larvae at each concentration for 2h, 4h, 6h and 8h were used for determination of LC_{50}.

In vitro

In vitro toxicity of umbelliferone was performed in the petridis by the method of Sunita and Singh [10]. Ten sporocyst, redia and cercaria larva of *Fasciola* were separated in different petridish containing 10 ml dechlorinated tap water. Treatment of umbelliferone was made directly in the petridis containing 10 sporocyst/ redia/ cercaria. Mortality of sporocyst, redia and cercaria were observed after 2h, 4h, 6 h and 8h of treatment. Counting of larvae were done with help of microscope.

Lethal value (LC_{50}), lower and upper confidence limits (LCL and UCL), slop-values, t-ratio, g value and heterogeneity factors were calculated with the help of POLO computer programme of Robertson et al. [21]. One way ANOVA and product moment correlation coefficient were done by the method of

Sokal and Rohlf [22].

RESULTS

In *in vivo* and *in vitro* larvicidal activity of umbelliferone against the sporocyst, redia and cercaria larva of *F. gigantica* was time and concentration dependent in each month of year 2011-2012 (Table 1, 2). In *in vivo* treatment, highest toxicity of umbelliferone was noted against redia and cercaria larva in month of May and July (redia -8h LC_{50}=0.93, and 0.89 mg/L; cercaria - 8h LC_{50}=0.70 and 0.92 mg/L May and August, respectively) and lowest in between December to February (redia - 8h LC_{50}=2.34, 2.24 and 2.23 mg/L; cercaria - 8h LC_{50}=1.56, 2.51 and 1.53 mg/L, respectively (Table 1). In the same month abiotic factors temperature/carbon dioxide were high (23.33-25.78/27.32-27.18) and pH/dissolved oxygen were low.

In vitro treatment, highest toxicity of umbelliferone against *F. gigantica* larva was highest in June, July and August (redia 8h LC_{50}=0.26, 0.30 and 0.28 mg/L; cercaria 8h LC_{50}=0.09, 0.11 and 0.18 mg/L, respectively). The lowest toxicity was observed in month of November to January (Table 2). The slope values were steep and separate estimation of LC_{50} based on each six replicate were found to be within the 95% confidence limit of LC_{50}. The t-ratio was greater than 1.96 and the heterogeneity factor is less than 1.0. The g value was less than 0.5 at all probability levels (90, 95 and 99 respectively) (Table1, 2).

DISCUSSION

Ferula asafoetida (umbelliferone) commonly known as “hing”, is routinely added as a flavoring agent in India way of food preparation. *In vivo* and *in vitro* toxicity of umbelliferone against sporocyst, redia and cercaria larva was significantly ($p<0.001$) altered with respect to change in abiotic factors in each month of the year 2011-2012. Temperature, pH, dissolved oxygen and free carbon dioxide are important factors, which alter the toxicity of umbelliferone. When the water temperature is higher in the summer season (June to August), toxicity of umbelliferone is high *Fasciola* larva. Contrarily, in the winter season (November-February) the temperature of water is low and the toxicity of umbelliferone is less, as evident by a higher LC_{50} value. Temperature is one of crucial factor for snail and *Fasciola* larval growth [23]. High temperate is the suitable for umbelliferone to become more soluble, low pH and free carbon dioxides causes more larval (sporocyst, redia and cercaria) mortality. However, dissolved oxygen is also one of the factors that alter the toxicity of umbelliferone. In winter, water holds more oxygen and as a result, less mortality of larvae [24].

According to their variant abiotic factors (temperature, pH, CO_2, O_2 and conductivity) in different months of the year (2009-2010) can significantly alter the infection rate and development process of *Fasciola gigantica* larvae (sporocyst, redia and cercaria) in the snail *Indoplanorbis exustus*. There has been many reports on phytotoxic, fungitoxic, insecticide, antibacterial and nematociadal activity of different coumarins [25]. When sweet potato is attacked by *Fusarium oxysprum*, umbelliferone is produced in plant tissue, a phytoalexn which is considered as a defense tool for plants against pathogenic fungi [26]. It also have a strong insecticidal activity and caused high percentage of mortality on eggs and larvae of insects and regarded as an ovicidal agent [25]. Umbelliferone is used as molluscicidal [17]/larvicidal to kill snail/*Fasciola* larvae in snail body [10]. Kumar et al. [27] has reported that snail fed with a sub lethal dose (20% and 60% of 24h and 96h LC_{50}) umbelliferone inside snail attractant pellets caused a significant inhibition in ALP and AChE activity in the nervous tissue of snail *L. acuminata*.

Temperature is considered as an important abiotic factor. Pathogen replication rate and dynamics may be affected by the invertebrate body temperature and as a result, the infection process in the different organs of the invertebrate and their duration cycle is altered [28]. In nature abiotic factors of freshwater bodies vary considerably. However, organisms living in these environments have adopted themselves and live happily despite change in their ambient environment. Abiotic factors of the environment vary from one season to other [29, 30], the aquatic environment has numerous physical and chemical parameters that may influence the physiology of fresh water organism [31]. Like all other animals in the ecosystem, the distribution and abundance of fresh water snails and life cycle of trematodes are also influenced by the environmental components, such as water qualities, aquatic vegetation, soil substratum, depth

Table 1. *In vitro* alteration in toxicity (LC_{50} mg/L) of umbelliferone against *Fasciola* larva (sporocyst, redia and cercaria) in different months of year (2011-2012).

Exposure time	Larvae	March	April	May	Jun	July	August	September	October	November	December	January	February
2h	Sporocyst	-	-	-	-	1.48	1.58	2.60	5.81	6.85	-	-	-
	Redia	4.53	3.16	1.12	0.79	1.43	1.16	2.02	2.54	2.62	3.89	3.42	7.76
	Cercaria	1.49	0.89	1.32	0.26	2.33	0.64	1.97	1.52	2.28	2.37	2.46	5.21
4h	Sporocyst	-	-	-	-	1.11	1.31	1.43	3.92	4.41	-	-	-
	Redia	1.85	1.42	0.73	0.45	0.67	0.64	0.93	1.51	1.60	2.44	2.10	2.21
	Cercaria	1.03	0.53	1.02	0.17	0.11	0.24	0.97	0.98	1.33	1.41	1.56	3.45
6h	Sporocyst	-	-	-	-	1.00	0.94	0.84	2.44	2.57	-	-	-
	Redia	0.77	0.62	0.51	0.29	0.41	0.31	0.52	0.85	0.98	1.38	1.19	1.28
	Cercaria	0.46	0.31	0.38	0.14	0.14	0.23	0.46	0.60	0.72	0.82	0.99	2.28
8h	Sporocyst	-	-	-	-	0.74	0.72	0.55	1.51	1.47	-	-	-
	Redia	0.63	0.47	0.39	0.26	0.30	0.28	0.27	0.57	0.60	0.78	0.68	0.71
	Cercaria	0.27	0.25	0.37	0.09	0.11	0.18	0.23	0.37	0.39	0.41	0.65	1.21

Each experiment was replicated six times. Six batches of 15 snails were exposed different concentration of the above molluscicidal treatments. Mortality of redia was recorded every 2h. Concentration given are the final concentration (W/V) in the glass aquarium water. t-ratio was more than 1.96. The heterogeneity factor was less than 1.0. The g-values were less than 0.5. Significant negative regression ($p < 0.05$) was observed between exposure time and LC50 of treatments. TS - testing significant of the regression coefficient. March - sporocyst/redia/cercaria (0, -7.55++, -6.39++), April (0, -9.92++, -9.34++), May (0, -8.28++, -8.25++), June (0, -2.27++, -0.25+), July (-0.07+, -0.57++, -2.42++), August (-9.10+, -1.39+, 0.74+), September (-47.38+, -3.82++, -1.46++), October (-17.90+, -4.04++, -9.30++), November (-17.81+, -5.14+, -6.63+), December (0, -9.64+, -7.34+), January (0, -20.36+, -4.70+), February (0, -31.89+, -8.62++): linear regression between x and y., ++: non-linear regression.

Table 2. *In vivo* alteration in toxicity (LC_{50} mg/L) of umbelliferone against *Fasciola* larva and different abiotic parameter in different month of year 2011-2012.

Exposures LC_{50} mg/ml	Larvae	Mar	April	May	Jun	July	August	September	October	November	December	January	February
2h	Sporocyst	-	-	-	-	2.42	7.09	9.11	6.27	8.83	-	-	-
	Redia	3.70	6.24	3.51	5.92	5.09	4.48	9.62	6.23	12.38	11.38	9.32	8.43
	Cercaria	3.76	5.19	2.79	4.37	3.37	3.04	6.44	6.12	6.81	7.09	8.48	6.55
4h	Sporocyst	-	-	-	-	1.72	6.17	6.12	3.88	5.93	-	-	-
	Redia	2.48	3.72	2.47	2.41	1.52	1.87	3.30	3.90	4.15	7.51	7.11	6.34
	Cercaria	1.64	3.03	1.33	2.49	2.21	1.81	4.04	3.80	4.34	4.48	6.81	4.15
6h	Sporocyst	-	-	-	-	1.02	3.05	3.95	2.39	3.73	-	-	-
	Redia	1.52	1.95	1.28	1.76	1.29	1.14	2.48	2.39	2.55	4.40	4.33	3.91
	Cercaria	1.09	1.46	1.01	1.44	1.23	1.10	2.16	2.18	2.51	2.68	3.14	2.55
8h	Sporocyst	-	-	-	-	0.99	2.13	2.54	1.45	2.35	-	-	-
	Redia	1.11	1.14	0.93	1.10	0.89	0.93	1.50	1.45	1.61	2.34	2.24	2.23
	Cercaria	0.92	1.06	0.70	1.12	1.00	0.92	0.98	1.25	0.12	1.56	2.51	1.53
Physical parameters	Temp	20.26± 0.30*	22.10± 0.25*	23.33± 0.33*	35.3± 0.33*	28.6± 0.21*	25.78± 0.30*	24.96± 0.42*	23.16± 0.30*	12.33± 0.33*	11.30± 0.33*	11.50± 0.34*	16.50± 0.22*
	pH	8.78± 0.16*	7.81± 0.23*	7.50± 0.11*	7.11± 0.83*	7.09± 3.06*	8.00± 4.76*	8.07± 0.02*	8.18± 0.01*	8.09± 4.20*	8.85± 0.76*	8.99± 0.03*	9.00± 0.01*
	DO	4.00± 0.02*	3.09± 5.15*	2.05± 5.76*	1.85± 5.76*	1.11± 5.15*	1.30± 0.01*	2.49± 4.27*	2.99± 6.13*	4.99± 0.01*	5.12± 0.01*	6.00± 0.02*	5.14± 0.01*
	CO_2	14.40± 0.45*	25.11± 0.33*	25.96± 0.18*	27.58± 0.34*	30.10± 0.33*	25.31± 0.23*	22.15± 0.17*	22.51± 0.36*	20.86± 0.17*	16.98± 0.21*	15.98± 0.04*	15.78± 0.26*

Each experiment was replicated six times. Temperature, pH, dissolve oxygen and free carbon dioxide were measured at intervals of 2h, 4h, 6h and 8h. Product movement correlation coefficient in between the LC_{50} and different parameters indicate significant ($p<0.05$) (*) positive correlation. Each experiment was replicated six times. Six batches of 15 snails were exposed different concentration of the above molluscicidal treatments. Mortality of redia was recorded every 2h. Concentration given are the final concentration (W/V) in the glass aquarium water. t-ratio was more than 1.96. The heterogeneity factor was less than 1.0. The g-values were less than 0.5. Significant negative regression ($p < 0.05$) was observed between exposure time and LC50 of treatments. TS - testing significant of the regression coefficient. March - sporocyst/redia/cercaria (0, -1.21++, -11.06), April (0, -6.20+, -9.00++), May (0, -4.79+, -5.15++), June (0, -14.69++, -7.16++), July (-5.93+, -11.58+, -7.17++), August (-13.83+, 0.21+, -9.73++), September (-10.72+, -1.23++, -7.36++), October (-8.54+, -2.68+, -10.08+), November (-8.79+, -6.92+, -9.71+), December (0, -7.98+, -9.39+), January (0, -13.55+, -2.72+), February (0, -16.35+, -6.15++): linear regression between x and y., ++: non-linear regression.

of water, temperature, pH, turbidity, dissolved oxygen, hardness, carbon dioxide [32, 33]. At higher temperature, increase rate of snail metabolism many release more CO_2 which affect the pH of water [34, 35]. This was evident from the elevated concentration of CO_2 which decreases the pH of water during summer season. Trematode parasite is highly sensitive to abiotic factors. Earlier it has been reported that *in vitro* treatment of umbelliferone redia (8h LC_{50}=0.63mg/ml) and cercaria (8h LC_{50}=0.27mg/ml) was more effective to control *F. gigantica* larvae [10]. The concentration used to kill sporocyst, redia and cercaria in not toxic to snail, even in 24h exposure period. So that use of umbelliferone in killing the sporocyst, redia and cercaria of *F. gigantica* within or outside snail body directly, without killing the host snail is important. In *in vivo* and *in vitro* killing of sporocyst, redia and cercaria of *F. gigantica* is beneficial as it kills directly target larva of *F. gigantica*. Earlier, it has been reported the acetylcholinesterase and cytochrome oxides system in cercaria is well responsible for efficient release of energy [36, 37].

The steep slope value indicates that a small increase in the concentration of larvicidal caused higher larval mortality in different month of year 2011-2012 in respect of physical parameter. A t-ratio value greater than 1.96 indicates that the regression is significant. Heterogeneity factor value less than 1.0 denote that in the replicate test of random sample the significance of the potency estimation g indicates that the value of mean is within the limit at all probability level (90, 95, and 99, respectively) since it is less than 0.5.

CONCLUSION

It can be concluded from the present study that sublethal treatment of umbelliferone significantly killed the sporocyst, redia and cercaria larva of *F. gigantica* inside the body of vector snail *Lymnaea acuminata*. Phytotherapy of infected snails by umbelliferone is one of the new approaches to control the fascioliasis. The high temperature and free carbon dioxides and low pH and dissolved oxygen were observed in the month of the May-August. The variant abiotic factor significantly altered the *in vivo* and *in vitro* toxicity of umbelliferone against *Fasciola* larvae. Effective control of fascioliasis can be done by killing *Fasciola* larva in between the month of year 2011-2012.

ACKNOWLEDGEMENTS

One of the authors (Kumari Sunita) is thankful to Rajiv Gandhi National Fellowship (RGNF), University Grants Commission (UGC), New Delhi for financial assistance.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Mas-Coma S, Bargues MD, Valero MA. Fascioliasis and other plant-borane trematode zoonoses. Int J Parasitol. 2005; 35: 1255-1278.
2. Mas-Coma S, Valero MA, Barguses MD. Chapter 2. *Fasciola*, Lymnaeacids and human fascioliasis, with a global overview on disease transmission, epidemiology and control. Adv Parasitol. 2009; 69: 41-146.
3. Lewin MR. The Johns Hopkins Microbiology Newsletter, 26 No.03. http://www. Hopkinsmedicine, Og/microbiology/newsletter/index. 2007.
4. Mas-Coma S, Bargues MD, Esteban JG. 1999. Human Fascioliasis. In: Fascioliasis. Dalton JP. Ed., Wallingford: CAB International. pp. 411-434.
5. Shelmon E, AL-Barwari. A survey on liver infection with *Fasciola gigantica* among slaughtered animals in Iraq. Bull Endemic Dis. Baghdad. 1978; 20: 75-82.
6. Mas-Coma S. 2003. The 9th Chamlong-Tranakchit Harinasuta Lecture - Human fascioliasis: epidemiological patterns in human endemic areas of South America, Africa and Asia. In: 4th Seminar on Food-and Water-borne Parasitic zoonises and Joint International Tropical Medicine Meeting. Bangkok. pp. 44-60.
7. Sunita K, Kumar P, Singh DK. Abiotic environmental factors and infection of *Fasciola gigantica* in vector snail *Lymnaea acuminata*. Researcher. 2012; 4(8): 49-53.
8. Osman EA, Mohamed EM, Abu Elreesh BI, Elegami AA. Molluscicidal activity of *Cobretum glutinosum*. Int J Mole Medand Adv Sci. 2007; 3(4): 151-154.
9. Singh O, Agarwal RA. Toxicity of certain pesticides to two economic species of snails in northern India. J Econ Entomol. 1981; 74: 568-571.
10. Sunita K, Singh DK. Fascioliasis control: In vivo and in vitro phytotherapy of vector snail to kill *Fasciola* larva. J Parasitol Res. 2011; 2011: 240807.
11. Narain K, Biswas D, Rajguru SK, Mahanta J. Human distomatosis due to *Fasciola hepatica* infection in

Assam, India. J Commun Dis. 1997; 29: 161-165.
12. Elhence V, Mehta B, Gupta RK. Fascioliasis: A case from central Uttar Pradesh. Indian J Gastroenterol. 2001; 20: 164.
13. Vatsal DK, Kapoor S, Venkatesh V, Vatsal P, Husain N. Ectopic fascioliasis in the dorsal spine: Case report. Neurosurgery. 2006; 59: E706-E707.
14. Gandhi V, Jain P, Rathod V. Nagral S. Endoscopic ultrasound in biliary fasciolosis. Indian J Gastroenterol. 2010; 29: 128.
15. Ramachandran J, Ajjampur SSR, Chandramohan A, Varhese GM. Case of human fascioliasis in Indian: Tip of the iceberg. J Postgrad Med. 2012; 58(2): 150-152.
16. Waller PJ. Anthelmintic resistance. Vet Parasitol. 1997; 72(3-4): 145-168.
17. Kumar P, Singh DK. Molluscicidal activity of *Ferula asafoetida*, *Syzygium aromaticum* and *Carum carvi* and their active components against the snails *Lymnaea acuminata*. Chemosphere. 2006; 63: 1568-1574.
18. Kumar P, Singh VK, Singh DK. Kinetics of enzyme inhibition by active molluscicides against ferulic acid, umbelliferone, eugenol and limonene in the nervous tissue of snail *Lymnaea acuminata*. Phyther Res. 2009; 23: 172-177.
19. Kumar P, Singh VK, Singh DK. Attractant food pellets containing molluscicidal against the fresh water snail *Indoplanorbis exustus*. Golbal Veterinaria. 2012; 8(2): 105-110.
20. APHP. 2005. American Public Health Association standard methods for the examination of water and waste water. 21st ed. Washington, DC, APHA.
21. Robertson JL, Russell RM, Preciter HK, Savin NE. 2007. Bioassay with arthropods data. 2nd Eds. Taylar and Francis. CRC Press. pp. 1-224.
22. Sokal RR, Rohlf FJ. 1996. Introduction of biostatistics, W.H. Freeman, San Francisco, Co, USA.
23. Njoku-Tony RF. Effect of some physico-chemical parameters on abundance of intermediate snails of animal trematodes in Imo state, Nigeria. Researcher. 2011; 3(4): 1-20.
24. Singh N, Kumar P, Singh DK. Variant abiotic factor and the infection of *Fasciola gigantica* larval stage in vector snail *Indoplanorbis exustus*. J Biol Earth Sci. 2012; 2(2): B110-B117.
25. Razavi SM. Plant coumarins as allelopathic agents. Int J Biol Chem. 2011; 5: 86-90.
26. Brooker NL, Kuzimichev Y, Laas J, Pavlis L. Evaluation of coumarin derivatives as anti-fungal agents soil-borne fungal pathogens. Commun Agric Appl Biol Sci. 2007; 72: 785-793.
27. Kumar P, Singh VK, Singh DK. Enzyme activity in the nervous tissue of *Lymnaea acuminata* fed to different bait formulations. Am J Chem. 2012; 2(2): 89-93.
28. Reiter P. Climate change and mosquito-borne disease Environ Health Perspect. 2001; 109(1): 141-161.
29. Mas-Coma S, Valero MA, Bargues MD. Effect of climate change on animal and zoonotic helminthiases. In: De La Rocque, S., Hendrickx, G., Morand, S., (Eds.), Climate change: Impact on the epidemiology and control of animal diseases. World Organization for Animal Health (OIE), Paris. Sci Tech Rev. 2008; 27: 443-452.
30. Mas-Coma S, Valero MA, Bargues MD. Climatic change effects on trematodiases, with emphasis on zoonotic fascioliasis and schistosomiosis. Vet Parasitol. 2009; 163: 264-280.
31. Khangarot BS, Ray PK. Zinc Sensitivity of a freshwater snail, *Lymnaea luteola* L; in relation to seasonal variation in temperature. Bull Environ Contam Toxicol. 1987; 39: 45-49.
32. Jahan MS, Rahman MR. Breeding biology of freshwater snail *Lymnaea* (*Radix*) *acuminata* Lamarck (Gastropoda; Lymnaeidae). J Zool Rajshahi Univ. 1996; 14&15: 47-54.
33. Sarkar SK, Saha M, Takada H, Bhattacharya A, Mishra P, Bhattacharya B. Water quality management in the lower stretch of the river Ganges, east coast of India: an approach through environmental education. J Clean Prod. 2007; 15: 1559-1567.
34. Toews KL, Shroll RM, Wai CM, Smart NG. pH-Defining equilibrium between water and supercritical CO_2 influence on SFE of organics and metal chelate. Anal Chem. 1995; 67: 4040-4043.
35. Berge JA, Bjerkerg B, Oettersen O, Schannnig MT, Oxnemad S. Effect of increased sea water concentration of CO_2 on growth of the bivalve *Mytilus edulis* L. Chemosphere. 2006; 62: 681-687.
36. Humiczewska M. Oxidative enzymes in the development of *Fasciola hepatica* L. Folia Histochem Cytochem. 1975; 13: 37-130.
37. Schmidt GD, Roberts LS. 2000. Trematoda: From function, and classification of Digeneans foundation of Parasitology. MC Graw-Hill, New York, NY, USA, 6th ed.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Range extension of mugger crocodile *Crocodylus palustris* (Lesson, 1831) in upper Ganges and tributaries, lesser Himalayan zone, north India

Ritesh Joshi

Conservation and Survey Division, Ministry of Environment and Forests, New Delhi, India

ABSTRACT

In Uttarakhand State, mugger crocodiles *Crocodylus palustris* are distributed in Corbett Tiger Reserve and in Baan-Ganga wetland, which is situated across the upper Gangetic plains. However, their presence has been recently recorded from Rajaji National Park and adjoining protected areas, which has put on record their new habitat and range. This study is illustrating about their movement in some annual rivers, which are tributaries to river Ganges especially at higher elevation of upper Gangetic plains in north India. Two specimens of mugger crocodile were recorded in 2011, from different location of the Lansdowne and Haridwar forest divisions, which revealed that their range is extending in upper Gangetic plains. Study also given an account on the natural history of the animal's presence in lesser Himalayan zone and placed some technical notes on its range extension. As some habitats, which are available across the Ganges have huge potential to introduce this threatened species, hatchlings could be introduced/released to some potential sites; however, detailed scientific based protocols are needed to be taken into account to take any conservation initiative. This study is the first effort of its kind, which highlights about the new habitat and range of mugger crocodile, especially at higher elevations of Ganges. Field observations indicated that the distributional range of the mugger crocodile is increasing in upper catchment of river Ganges near to Rajaji National Park and in parts of Haridwar and Lansdowne forest divisions. However, studies are required to be carried out to propose some long-term conservation actions.

Key words: Mugger Crocodile; range extension; upper Ganges; conservation; north India.

J Biol Earth Sci 2013; 3(1): B100-B109

Corresponding author:
Ritesh Joshi
Conservation and Survey Division, Ministry of Environment and Forests, Govt. of India, CGO Complex, New Delhi – 110 003, India
E-mail: ritesh_joshi2325@yahoo.com

Original Submission: 24 January 2013; Revised Submission: 20 February 2013; Accepted: 22 February 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Of the three species of crocodiles found in India, the most common and widely distributed is the Mugger Crocodile *Crocodylus palustris*, which inhabits all kinds of fresh water habitats such as rivers, lakes, reservoirs, hill streams and village ponds. This broad-snouted mugger crocodile is restricted to the Indian sub-continent (India, Nepal, Pakistan, Iran and Sri Lanka) and is categorized under vulnerable category in IUCN Red List of Threatened Species [1] and protected under Schedule I of the Indian Wildlife Protection Act, 1972 [2]. By 1974, this species, formerly widespread and very abundant, was greatly depleted in numbers and considered rare in most, if not all, of its former ranges [3].

Available historical records revealed that in Bangladesh wild population of crocodile was extinct and only two wild crocodiles were known to live in community ponds (except in zoos). Similarly in Bhutan, mugger crocodiles are considered to have become extinct in the 1960s and in Myanmar last record of the species was observed in 1867-68 [4]. Increase in population and intensification of agricultural practices, construction of dams and diversion of water channels for irrigation purpose are considered acute reason for shrinkage of the population of marsh crocodiles in Pakistan however, presence of this species is limited to certain area of Sindh and coastal areas of Balochistan and further degradation in its habitat may lead to extinction in Pakistan [5].

In India, mugger crocodiles are reported from over ten states and its wild population is tentatively estimated as 2500 to 3500 non-hatchlings [6]. In Uttarakhand state, this threatened species is known to found in Corbett Tiger Reserve (Ram-Ganga river and Sonanadi reservoir) and in Baan-Ganga wetland (Laxar area, district-Haridwar) adjoining to river Ganges. However, a study has recorded the presence of mugger crocodile in river Ganges flowing across the Rajaji National Park and in Haridwar forest division, which was the first record from this area [7]. But to till date, any record doesn't exist, which confirm the presence of crocodiles in Laldhang forest of the Lansdowne forest division and in the Sarai village, which is adjacent to Haridwar city. This study put on record the presence of mugger crocodile in these areas, which further articulate the range extension of crocodiles in upper Ganges and some of its tributaries spread within upper Gangetic plains at higher elevation.

MATERIALS AND METHODS

Study area

Lansdowne forest division lies in lesser Himalayan zone and is well connected with Rajaji National Park in the north-west axis and Corbett Tiger Reserve in the south-east axis (Fig. 1). Haridwar forest division is connected with the Rajaji National Park on the north and with the Lansdowne forest on the east. Some parts of this protected habitat are also adjoined to Uttar Pradesh forests (Najibabad forest division). Both of these protected areas falls under Shivalik landscape and comprises of foothills dominant areas. Several annual water streams are also flowing across this landscape, which further ensures the faunal distribution in myriad types of the habitats. Lokmanipur village is situated at 29º76' N, 78º48' E in between the Lansdowne and Haridwar forest divisions. Sigaddi canal is flowing adjoining to this village, which holds the water from Kotawali rau (water stream) – an annual river present in Chiriapur forest. Sarai village is adjoined to Haridwar city and situated at 29º58' N, 78º13' E (Fig. 2). A small water channel is flowing across this area, the water of which is bifurcated from Ganga canal flowing across the city heart. This water channel is finally mingling in Pathri rau, which is a tributary to river Ganges. Haridwar–Bijnor railway track is also running across this area, besides, Haridwar-Delhi national highway is also running parallel to this area (±7 kilometers).

Methods

In both cases, field surveys were carried out to document ground based facts behind animal's presence in new habitat and ranges. A total of 17 discussions were made with the local people of Sigaddi and adjoining villages, besides, 23 discussions were held with locals of Sarai and adjoining villages. Literature was also searched to see and cross-check, whether crocodile was reported earlier from these areas or not. Consultations were held with the forest officers of Haridwar and Laldhang forest range. The distance was measured from main Ganges to the spot from where the specimens were reported by walking on foot along the annual river beds and the location map was prepared for documenting notes of its

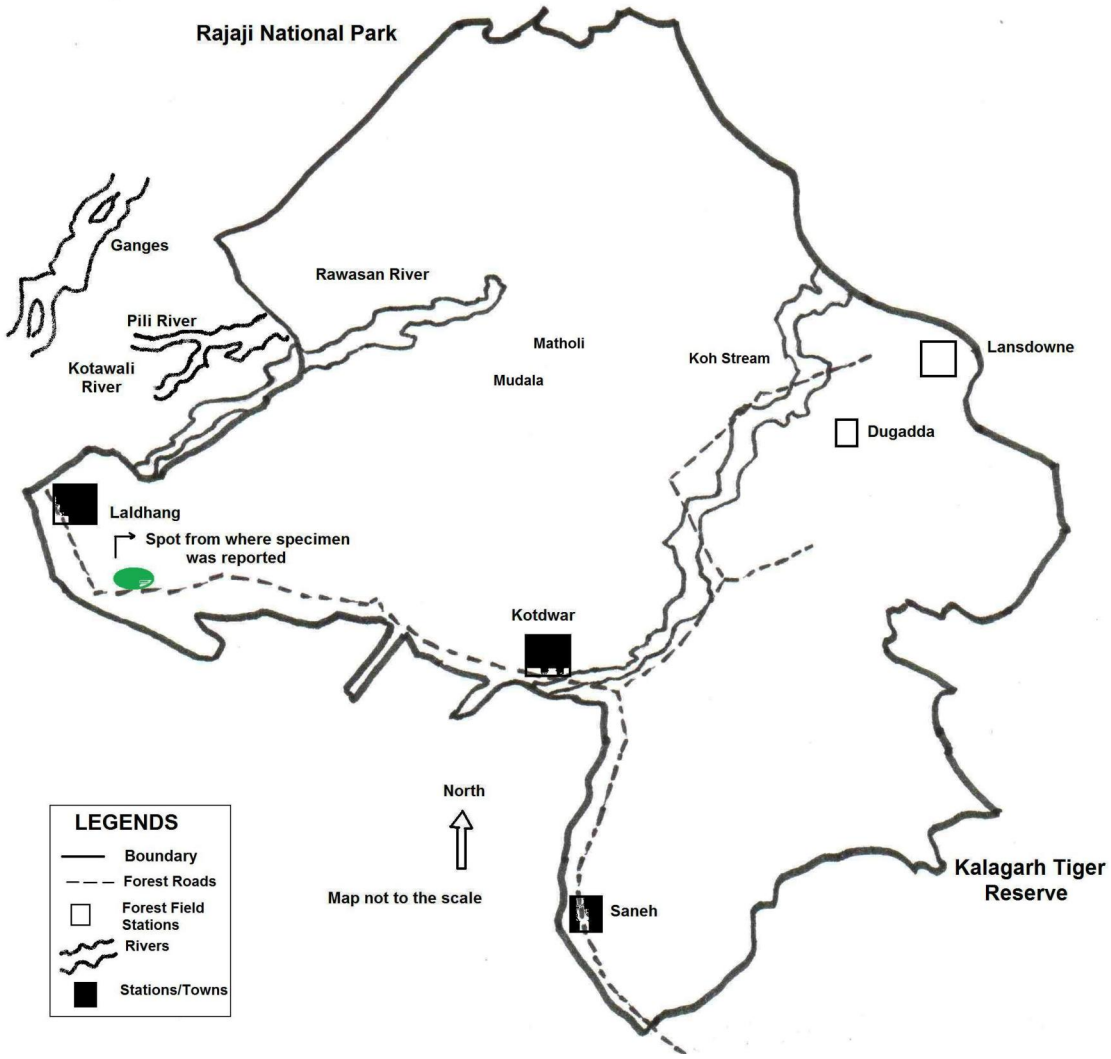


Fig. 1. Map of the Lansdowne forest division showing location from where the specimen was recorded.

natural history. Possible reasons for the movement of animal from the Ganges to tributaries were also proposed. Field binocular (Nikon Action series, 10x50 CF) and Nikon Coolpix 8700 Camera was used for field observations and to document photographic evidences. Garmin made GPS was also used to denote geographical coordinates.

RESULTS AND DISCUSSION

On 13th September, 2011 (07:30 h), a juvenile specimen of mugger crocodile was sighted near to Sigaddi canal, which falls in Laldhang forest of the Lansdowne forest division (29º47'21" N, 78º24'23" E, elevation 373.6 m, Fig. 3). The animal was 1.25 meter long. The specimen was captured by forest staff and was translocated to river Ganges. Notably, before to this record, crocodile was never seen or reported from Laldhang forest. This site is close to Lokmanipur village, in between which Sigaddi canal is flowing; this entire area falls under west Papidanda forest beat of the Laldhang forest range. I investigated all the potential routes and sites to trace out the route, which was followed by animal to reach that spot. It was found after carrying out extensive field work that animal had moved there from the Kotawali river flowing in between this forest, which is a tributary to river Ganges. It was also observed that animal moved up more than 18 kilometers in Kotawali river from river Ganges to Sigaddi forest, in which huge water flow could be seen during monsoon.

Later to it, on 20th September, 2011 (07:40 h), a juvenile crocodile died on Haridwar–Laxar railway track near to Sarai village, Jwalapur (29º53'14" N, 78º05'54" E, elevation 271.2 m, Fig. 4). Animal's head portion was badly crushed by the train. The animal was 1.5 meter long. While documenting the death cause, it was revealed that animal had moved towards Pathri rau from a nallah (running water

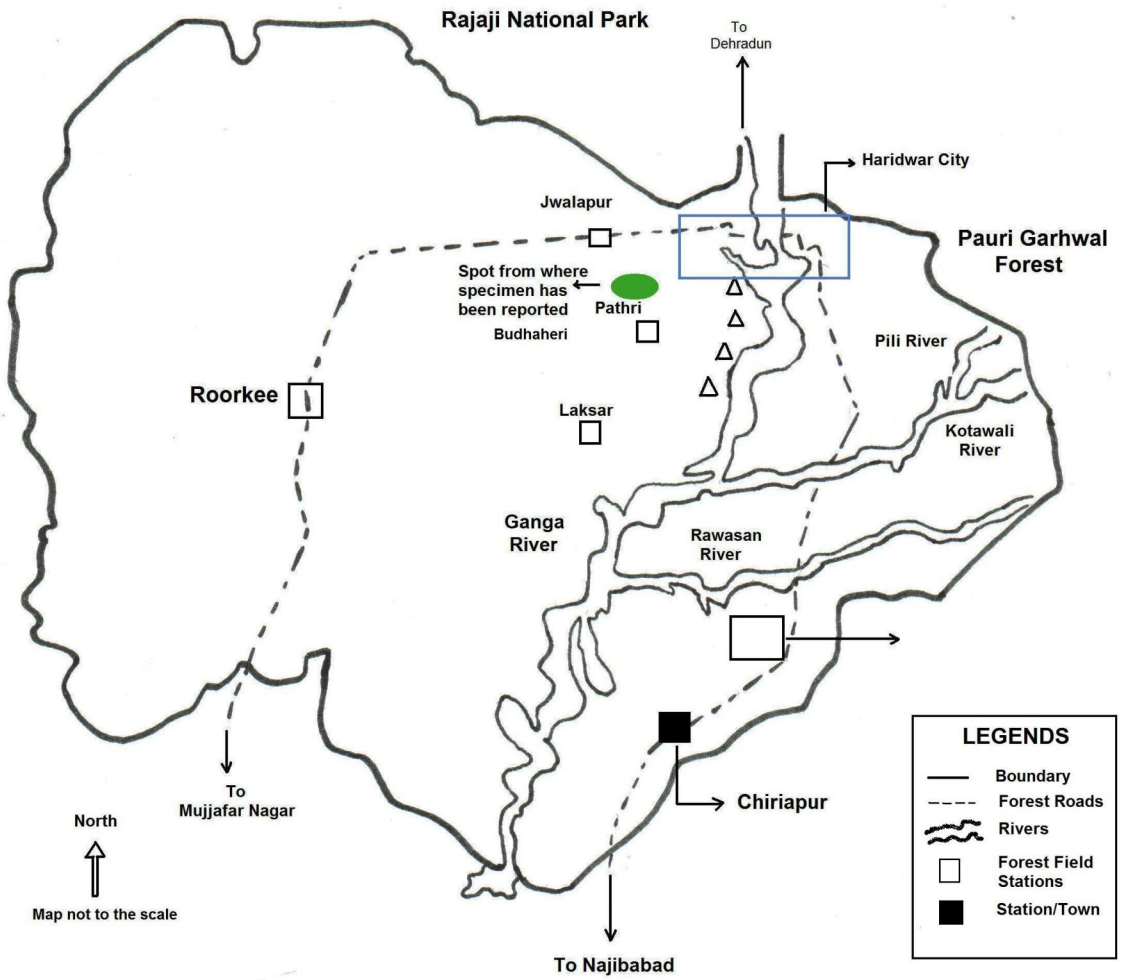


Fig. 2. Map of the Haridwar forest division showing location from where the specimen was recorded.

Fig. 3. Extending home range: a juvenile of mugger crocodile captured by Lansdowne forest division's staff from Sigaddi canal, which was further released to Ganges.

channel), which finally drains into Ganges. After examining the carcass and post mortem, forest officials burned the animal on the spot. Notably, this was happened for the first time, when any crocodile was sighted in Sarai village, Jwalapur area just adjacent to Haridwar city (±10 kilometer distance). Examination of the spot further revealed that animal had moved upward nearly 20 kilometers in the Pathri rau and entered to a water channel flowing parallel to the railway track. This water channel is arriving from Haridwar city and finally mingles with Pathri rau.

The dominant vegetation of the forest block in Sigaddi area, Lansdowne forest division comprises of *Shorea robusta* Sal, *Mallotus phillipinensis* Rohini, *Acacia catechu* Khair, *Adina cordifolia* Haldu, *Terminalia bellirica* Bahera, *Ficus bengalensis* Bar, *Schleichera oleosa* Kusum and *Dalbergia sissoo* Shisham. Whereas, among mammalian fauna, elephant *Elephas maximus*, tiger *Panthera tigris*, leopard *Panthera pardus*, sloth bear *Melursus ursinus*, barking deer *Muntiacus muntjak*, spotted deer *Axis axis*, sambar *Cervous unicolor*, wild boar *Sus scrofa*, etc. shows the forest's diverseness. Sarai village is entirely covered with agriculture land with stands of mango *Mangifera indica*, sagaun *Tectona grandis*, eucalyptus *Eucalyptus* spp., etc. The major cash crop cultivated here are wheat, paddy, sugarcane and some seasonal vegetables. Among fauna, only wild avian species, which includes several migratory species, were recorded from the area.

Noticeably, one site falls under protected habitat, whereas another site falls under civil area (covered with villages), which is adjoining to protected habitats of Haridwar division's forests. Since, both of these forests are important biological area and holds a rich faunal wealth, therefore, there is a scope to study these areas and the riparian corridors of Ganges flowing across the Haridwar forest division and Rajaji National Park. Based on these documentations, it was revealed that the range of this genus is extending in upper Gangetic plains and in some of the tributaries of Ganges. This documentation could be considered as first ever study, which highlighted the range extension of mugger crocodile in higher elevations of Ganges and represented notes on its natural history.

Crocodile's range extension in upper Ganges: technical points

In Uttarakhand, crocodiles are commonly distributed in river Ganges near to Laxar area and its adjoining hamlets, which are having large water reservoirs. Besides, they are commonly found in Baan-Ganga wetland adjacent to Laxar area. On

Fig. 4. Corpse of juvenile crocodile on Haridwar–Laxar railway track, near to Sarai village.

the other hand, they are fairly distributed in some parts of Corbett National Park in Ram-Ganga river and in some parts of Kalagarh Tiger Reserve.

Rivers possess a delicate ecology that depends on a regular cycle of disturbance within certain tolerances. The plant and animal communities that inhabit the river and river margins have evolved to adapt to their rivers own peculiar pattern of flood and drought, slow and fast current [8]. Organisms too, move through river and stream ecosystems. These range from regular movements necessary to access food, shelter, mates, nesting areas, or other resources, to significant shifts in response to extreme conditions brought about by natural disturbances [9].

During monsoon, when water flow in Ganges is considered at its higher level, most of the aquatic faunal species could be seen easily migrating in various tributaries. For example, during monsoon several cold water fishes, which are commonly found in high altitude areas like mahaseer Tor tor could be seen in Song river, which is also a tributary to river Ganges. Similarly, various species of turtles can be observed within small torrential rivers spread within the protected habitats. Strong water current, huge flowing water, accumulation and flowing of silt and garbage in main stream and riparian corridors etc. are few reasons behind this occasional migration.

Crocodile's specimens were observed in parts of upper Ganges and in some tributaries maximum during monsoon and post monsoon season; therefore, this can be attributed with huge flow of water during monsoon, which sometimes carries uncontrolled floods. Notably, tremendous patches of the forest were logged-off in floods of 2010. In some pockets of Rajaji National Park, the free flowing river has created small reservoirs and streams within its premise, which has influenced the physical and chemical characteristics of the natural water (changes in the level of dissolved oxygen, temperature, turbidity, salinity, acidity, etc.). Besides, about eight hectare forests present in Dudhia island and in the Jhabargarh forest had been washed-off completely.

On the other hand, wastes/organic matter have created eutrophic conditions in some of the potential sites (large reservoirs). Massive fish kills were also observed during the same period. Besides, tremendous litter, household wastes (clothes, etc.) and the immersion of idols and disposal of material used for worship further affected the water quality of the river and reservoirs. The decomposition of these organic matters, especially in some potential reservoirs, might originated the low oxygen level in river water. As oxygen is needed for decomposition process, this had caused the aquatic environment hypoxic. Extensive floods during 2010 and 2012 had made a significant impact on crocodile's basking. As most of the sandy banks of river were washed off and covered with huge flow, there were no such sites available for the crocodiles upon which they can bask comfortably except some forest pockets, which also consists rough terrain with irregular ground features.

Most of the irregular movements of the animal were observed at the places where disturbances have been found in natural water system. However, this has strengthened the occurrence of crocodiles in some potential tributaries of Ganges and in higher elevations. Study revealed that crocodiles are resilient to the occurrence of natural disturbances, as like other animals and were found preferring to replace their habitat to more suitable areas, where water flow and large reservoirs would be comfortable for them to survive and for the survival of their nests. For an organism to survive it must have access to appropriate habitats. Habitat is a combination of physical and biological characteristics of an area (or areas) essential for meeting the food and other metabolic needs, shelter, breeding, and over-wintering requirements of a particular species [9].

Previous significant and confirm records of crocodile's presence in the area

In November, 2007, I noticed a specimen in Chiriapur forest of the Haridwar forest division, which was basking on a sand bank of Ganges near Tatwala village. Later on, in January 2008, a local person had seen an adult specimen early in the morning on Haridwar–Chilla–Rishikesh motor road near to old Chilla check-post, close to Bhimgora barrage. I carried out extensive surveys there and adjoining areas across river Ganges in Chilla forest of the Rajaji National Park and observed the movement signs (crawling signs) of an individual. In continuation to this, during January 2009, a juvenile individual was observed basking in sub-merged position (sand-grass patch) on a small island, which falls under Shyampur forest of the Haridwar forest

division. Later on, in September 2008, a juvenile specimen was also observed in Song river in between Raipur and Lacchiwala near to Doiwala; noticeably, this spot is approximately 17 kilometers far from main Ganges. Song river is having huge flow year round, however, sometimes heavy rain fall creates flood conditions especially during monsoon. As several small torrential rivers also mingle in this river, tremendous flow could be seen during monsoon.

Evidently, during September 2009, two individuals were seen randomly in the large water channels flowing in between the Haridwar city (Birla ghat and Chandi ghat area). This incident has caused tremendous rumor and panic among devotees and locals as happened near to Har ki Pauri–the main bathing site for pilgrims. As both the area falls under bathing sites, caused difficulty for district administration especially at the time when Maha-Kumbh 2010 fair had to start from January 2010. Recently, this genus has been documented from Rajaji National Park and from Haridwar forest division (new range and habitat) for the first time, which could be added in the list of reptilian fauna of the Rajaji National Park [7] (Fig. 5).

Some reports of crocodile's presence were also came forward earlier during 1970s–1980s from Rishikesh, Ganga Bhogpur village (situated along the Ganges, in between Haridwar and Rishikesh and adjoined to Rajaji National Park) and from Jagjeetpur village (4 kilometers far from Haridwar, towards Laxar); however, only old people, who are residing in this area traditionally, knowing these facts and because of lacking of proper documentation and unawareness towards ecological conservation and threatened texa, it could not prove scientifically (V.P. Upadhyay, personal communication). Since last one decade, villager's complaints about the crocodile's presence in ponds spread across the Ganges came forward time to time and slowly these reports are increasing.

Potential habitats in the State

Limited work has been carried out on mugger crocodile in Uttarakhand state, which still needs some field-based studies to explore its geographical distribution in the state. Presently, protected habitats of Corbett Tiger Reserve (some parts of

Fig. 5. An adult mugger is basking on sand bank of Ganges in Rajaji National Park.

Jim Corbett National Park and Sonanadi Wildlife Sanctuary) and Ganges stretch from Baan-Ganga wetland to Laxar area are considered as their potential habitats in the state. However, Sharda river, which is flowing across Kumaun Himalaya could be also a potential habitat for mugger crocodile, therefore, a need is felt to conduct preliminary assessment across potential sites in Sharda river to trace out the presence of this genera.

Both Corbett Tiger Reserve and Baan-Ganga wetland falls under reserve forests and therefore protected, whereas some pockets of Laxar and its adjoining areas exists partially under reserve forests, therefore, needs proper conservation-based monitoring especially during February–June, which is supposed as egg-laying and hatching time. Available records on mugger crocodile indicated that significant work has been carried out in southern India and some other parts of the country but still studies are required to be carried out in Uttarakhand State, which would be helpful in proposing conservation actions.

Based on these recent observations and evidences, I put on record the presence of the Mugger Crocodile *Crocodylus palustris* in some tributaries of river Ganges and confirm its range extension in upper Gangetic plains. Mining activities ongoing in some annual rivers, shrinking of small watersheds/reservoir and accumulation of silt in some upper parts of Ganges could be the reasons behind their upward movements. A study carried out in Koshi Tappu Wildlife Reserve in eastern Nepal highlighted that siltation of river beds during the monsoons, high water velocity of swift current during floods, and change of mesotrophic marshes to eutrophic marshes are the natural factors for the low survival and sighting of crocodiles in the Koshi River [10]. Further, downward movement of crocodiles during the monsoon period has been reported from Koshi River to Bihar (India) because one Gharial was seen in the marshes in Bhimnagar (Bihar) where there was no previous record.

Possible approaches to introduce mugger crocodile in upper Ganges

In dynamic environments, like rivers and streams, the location and quality of habitats are ever-changing Flooding and woody debris work together to shape river and stream channels, water depth, and flow characteristics, creating a shifting mosaic of habitats within riverine systems. For a time, fisheries biologists thought that fish like trout generally stayed put, except for specific periods of movement for breeding or to avoid unfavorable conditions. However, it now appears that a significant proportion of these fish make regular movements that allow individuals to locate and exploit favorable habitat within this ever-shifting mosaic [9]. Based on the conservation activities carried out under Gharial Rehabilitation Project, which was the subunit of the umbrella Crocodile Conservation Project; some possible rehabilitation approaches could be adopted to re-introduce mugger crocodile. In between 1982 and 1994, more than 250 gharials were released to Ram-Ganga river in Corbett National Park and still their population is surviving there and conservation efforts are being carried out by the Government and International non-governmental agencies.

Some parts of the Rajaji National Park consists potential habitats that would be suitable for establishing new populations of mugger crocodile. Reporting of this species from different locations in upper Gangetic plains and tributaries during last few years, supports for its introduction in this area. Water reservoir available in Dogadda rau in Chilla forest, which is adjoining to Ganges, Andheri Chaur in Jhabargarh forest adjoining to Dudhia forest (island) and Ganges, bank of Ganges near to Ghasiram rau, parts of Song river are some potential locations in Rajaji, where mugger crocodile's hatchlings could be introduced. Besides, hatchlings could be introduced in some parts of Ganges flowing across Haridwar forest division (from Shyampur to Chiriapur forest), which is also having large reservoirs at some places. As Haridwar forest division also consists most of the potential sites (riparian corridors of Ganges, which are situated near to Tatwala village, Rawasan, Pili and Kotawali annual rivers), the hatchlings can also be released to these sites. All these sites are having vast sand plains and forests, however at some places villages are situated along the Ganges.

Restoration and finding of suitable habitat are the largest hurdles that the Crocodile Conservation Project faces; besides many factors are essential for survival and success of the mugger's release and rearing; sites must be protected and without severe environmental degradation [11]. Establishing new populations needs base-line surveys, consultations with locals, planning and excessive

monitoring. Besides, a scientific protocol is requires to be follow to carry out such conservation initiatives. However, the technical guidelines given by the IUCN (SSC)–CSG and the experience, which was received in between 1978-1992 (when 1,193 mugger crocodiles were released into 28 National Parks, Wildlife Sanctuaries and Crocodile Sanctuaries), could be used to take initiatives in this direction. A group of experts, which include experts from Wildlife Institute of India, WWF, IUCN-CSG and forest officers, could also be constituted for this initiative. As of now, we have learned too much about the successful and unsuccessful trials, from the results of Crocodile Conservation Project, we can move forward considering the results perceived from previous actions in planning for this conservation initiative.

As human–crocodilian conflict has been also reported from some parts especially in the areas, which are situated along the Ganges near to Laxar area, ensuring the community participation is one of the important aspect, which should be secured before initiating for this action. Besides, this is also highly requires for obtaining success in these types of conservation actions. One major problem in crocodile conservation in India is the ignorance of the people, who considered that crocodiles are either man-eaters or competitors, which should be eliminated [3].

CONCLUSION

Field observations indicated that the distributional range of the mugger crocodile is increasing in upper catchment of river Ganges near to Haridwar city. The increasing movements of this species in some tributaries of Ganges, which includes river Song, Pathri and Kotawali, all of which falls under protected habitats (parts of Rajaji National Park and Haridwar and Dehradun forest divisions) revealed that this species is getting favourable habitat in some upper regimes of these reserve forests. However, their upward movement in Ganges was more frequently observed after the tremendous flood in monsoon of 2010, when Ganges water was found to flow above than 294.60 m asl (225000 cusec), which has also created flood conditions in plains. These new records on range extension of mugger crocodile in some tributaries of Ganges, flowing across Lansdowne and Haridwar forests and in the higher elevations requires further studies to reach some firm conclusion:

1. Ground-based surveys are required to be carried out in upper Gangetic plains especially during monsoon and winter, in some potential tributaries of Ganges and in the reservoirs spread across forests adjoining to river Ganges.
2. Monitoring of potential habitats to trace out presence of animal, recording of basking sites, documentation of indirect evidences (crawling sings, etc.) are some possible ways through which we can reevaluate its range extension.
3. As females are known to lay eggs in sandbanks between February and April, and hatching occurs between April and June, proper monitoring of habitat should be needed on regular basis.
4. An interview based assessment is needed to be carried out in entire belt from Laxar to Aikkar/Pathri area, which includes more than twenty villages. Reports of crocodile's presence were noted year round from this belt. As numerous small ponds, which are mostly rainfed are present in this area besides, man-crocodilian conflict was also observed from some pockets, documentation of field based notes on regular basis including animal's natural history could be considered as a valuable conservation tool.
5. Hatchlings of mugger crocodile could be released in some potential perennial rivers flowing across this area, which are the tributaries to river Ganges for example in Song and Rawasan. As perennial streams consist of water pools and muddy bases, these sites can act as favourable basking sites, which are also connected with main river flow. Besides, some potential large reservoirs, which are present in Rajaji National Park, could be considered for this purpose.

ACKNOWLEDGEMENTS

I am thankful to Mr. M.S. Negi, Forest Range Officer, Rajaji National Park for providing inputs on topography of the area. Thanks are due to Mr. Alok Dixit, Doon Institute of Engineering and Technology, who has assisted in collection of field data and made dialogues with local people. I also would like to thanks Mr. Shivang Agarwal for providing assistance in collecting photographic evidences.

TRANSPARENCY DECLARATION

The author declares no conflicts of interest.

REFERENCES

1. IUCN. 2011. IUCN Red List of Threatened Species. Version 2011.2. www.iucnredlist.org. Downloaded on 26th March, 2012.
2. Anon. 1992. The Wildlife (Protection) Act, 1972. Natraj Publishes, Dehradun, India.
3. De Vos, A. Crocodile conservation in India. Biol Conserv. 1984; 29: 183-189.
4. Da Silva A, Lenin J. 2010. Mugger Crocodile *Crocodylus palustris*. In: Crocodile: Status Survey and Conservation Action Plan (Third ed.), Eds. Manolis SC, Stevenson C.: pp. 94-98. Crocodile Specialist Group, Darwin.
5. Javed HI, Rehman H. Status of marsh Crocodile (*Crocodylus palustris*) in Sindh. Rec Zool Surv Pakistan. 2004; 15: 22-30.
6. Whitaker R, Andrews H. Crocodile conservation, Western Asia Region: an update. J Bombay Nat Hist Soc. 2003; 100(2&3): 432-445.
7. Joshi R, Singh R, Negi MS. First record of mugger crocodile *Crocodylus palustris* (Lesson, 1831) from the Rajaji National Park, North India. Int J Biodivers Conserv. 2011; 3(9): 444-450.
8. Stream Biology and Ecology (SBE). 2012. The ecology of dams. http://chamisa.freeshell.org/dam.htm. Downloaded on 14th December, 2012.
9. Jackson SD. 2003. Ecological Considerations in the Design of River and Stream Crossings. In: Proceedings of the International conference on ecology and transportation. eds. Leroy IC, Garrett P, McDermott KP.: pp. 1-10. Center for Transportation and the Environment, North Carolina State University, Raleigh, NC.
10. Goit RK, Basnet K. Status and conservation of crocodiles in the Koshi Tappu Wildlife Reserve, eastern Nepal. J Threat Taxa 2011; 3(8): 2001-2010.
11. Jacobson C. Reintroduction of the mugger crocodile, *Crocodylus palustris*, in India. Restor Reclam Rev. 1999; 4(3): 1-7.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Toxicological and biochemical alterations of apigenin extracted from seed of *Thevetia peruviana*, a medicinal plant

Sunil K. Singh[1], Shailendra K. Singh[2], Ajay Singh[1]

[1]*Department of Zoology, D.D.U. Gorakhpur University, Gorakhpur, India*
[2]*Department of Zoology, T.D. College, Jaunpur, India*

ABSTRACT

The active compound apigenin extracted from the seed of *Thevetia peruviana* (Pers.) L. Schum. plant (family: Apocynaceae) was administered for 24h or 96h to the freshwater target snail *Lymnaea* (*Radix*) *acuminata* (Lamarck) to evaluate its molluscicidal activity. The molluscicidal activity of apigenin was time as well as dose dependent at all exposure periods. There was a significant ($P < 0.05$) negative correlation between LC values of apigenin and exposure periods, thus LC_{50} values decrease from 3.65 mg/L (24h); > 2.98 mg/L (48h); > 1.63 mg/L (72h); > 0.73 mg/L (96h) against the snail *L. acuminata*. The LC_{90} dose of apigenin extract had no toxic effect on freshwater non-target fish *Channa punctatus* (Bloch) which shares the same aquatic habitat with snails. Exposure of sub-lethal doses (40% and 80% of LC_{50}) of apigenin over 24h caused significant ($P < 0.05$) alterations in carbohydrates and nitrogenous metabolism in nervous, hepatopancreas and ovotestis tissues of the snail *L. acuminata*. The fish *C. punctatus* also exposed at the same sub-lethal doses of apigenin for 96h in order to measure potential effects on non-target organisms also showed significant ($P < 0.05$) alterations in carbohydrates and nitrogenous metabolism in muscle, liver and gonadal tissues. Seven days of withdrawal of treatment showed partial recovery in these parameters in both snail and fish, which supports the view that apigenin can be used as molluscicide for controlling harmful snail population in aquatic bodies without harming their fish fauna.

Key words: Apigenin; Snail; Fish; Metabolism.

J Biol Earth Sci 2013; 3(1): B110-B119

Corresponding author:
Sunil K. Singh
Department of Zoology, D.D.U. Gorakhpur University, Gorakhpur - 273 009 (U.P.), India,
E-mail: sunil_shrinet@rediffmail.com
singhdr.sunilkumar@yahoo.com

Original Submission: 20 January 2013; Revised Submission: 28 February 2013; Accepted: 12 March 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Many aquatic snails act as vectors for the larvae of trematodes and thereby cause a number of diseases [1]. Two diseases carried by aquatic snails, schistosomiasis and fascioliasis, cause immense harm to man and his domestic animals.

Schistosomiasis is caused by *Schistosoma* it is a devastating disease of mankind, second only to malaria in its deleterious effect. In India the freshwater harmful snail *Lymnaea* (*Radix*) *acuminata* (Lamarck) is the intermediate host of *Fasciola hepatica* and *F. gigantica* [2]. They cause endemic fascioliasis in sheep, cattle, goat and other herbivorous animals in low-lying areas. A sure way to eliminate the problem of fascioliasis was to destroy the carrier snails and thus remove an essential link in the life cycle of causative flukes. This can be accomplished by several ways including the use of many synthetic or plant origin molluscicides [3-6].

Thevetia peruviana (Pers.) L. Schum. (family: Apocynaceae) is a common medicinal plant of India, used as cathartic and febrifuge and also in different kinds of intermittent fever while the latex of this plant is used in teeth cavities for relief from toothache [7]. The toxicological actions of *T. peruviana* may be due to the presence of apigenin-5-methyl ether (flavonoid) and several other triterpenoid, glycosides etc. [8-10]. The leaves of *Polygonum senegalense* have a known flavonoid, quercetin. This compound possesses significant molluscicidal activity at 10 ppm, causing 100% mortality of three species of snails, *Lymnaea natalensis*, *Biomphalaria peifferi* and *B. glabrata* within 24h. Likewise, an eupatorin compound isolated from the plant *Baccharis timera* killed 100% of *B. glabrata* at 100 ppm, but other flavone glycosides from *Asparagus plumosus* were found to be completely harmless to snails [11]. Earlier studies indicated that *T. peruviana* plant extracts have potent molluscicidal activity against the freshwater harmful snail *L. acuminata* [12-14] but their doses were high, so their further purification was needed.

The aim of the present communication is to observe the toxicological and biochemical alterations induced by apigenin extracted from seeds of *T. peruviana* plant against the freshwater target snail *L. acuminata* and also observe biochemical parameters of the non-target freshwater fish *Channa punctatus*, which shares the same habitat with these target snails, for measure their environmental toxicity, if any.

MATERIALS AND METHODS

Animals

The freshwater target snail *Lymnaea acuminata* [2.6 ± 0.3 cm in total shell height] and freshwater fish *Channa punctatus* [10.5 ± 0.9 cm in total length] were collected from the local freshwater bodies of Gorakhpur district, India. The collected animals were stored in glass aquaria containing 100L dechlorinated tap water for acclimatization to laboratory conditions. Experimental conditions of water were determined in the beginning of the experiments by the method of APHA [15]. Average sized adult animals were used for the biochemical experiments. Atmospheric and water temperature was ranging from 30.4–31.6°C and 26.0–27.0°C, respectively, pH of water was 7.3–7.5, while dissolved oxygen, free carbon dioxide and bicarbonate alkalinity were ranging from 6.9–7.8 mg/L, 4.4–6.7 mg/L and 104.0–108.0 mg/L, respectively, during the experiments.

Plant

The plant *Thevetia peruviana* (family: Apocynaceae) was collected from Botanical Garden of D.D.U. Gorakhpur University, Gorakhpur, Uttar Pradesh, India and identified by a Plant taxonomist, Department of Botany, D.D.U. Gorakhpur University, where a voucher specimen is deposited.

Extraction of active compound apigenin

Fresh seeds of *T. peruviana* were collected, dried in an incubator at 37°C and powdered with electrical device. Dried powdered seeds (500 g), extracted with hot EtOH yielded a crude yellow powder in the concentrated extract. The powder was purified by dissolving it in dilute NaOH solution, and it was precipitated by the addition of dilute HCl solution. The precipitate was crystallized from methanol, m.p. 323–324°C (reported 325–327°C); yield 0.15% [8]. The following derivatives were prepared diacetate, m.p. 203–205°C (Lit. 199–200°C); dibenzoate, m.p. 203–205°C; dimethyl ether, m.p. 155–156°C (Lit. 157–158°C); diethyl ether, m.p. 171–173°C [8]. Demethylation of crystals obtained from methanol yielded apigenin (500 mg, yield 0.10%). Identification of the extracted

compound was further done by composition with an authentic sample of apigenin ($C_{15}H_{10}O_5$) (Fig. 1), supplied by Sigma Chemical Co., St. Louis, USA.`

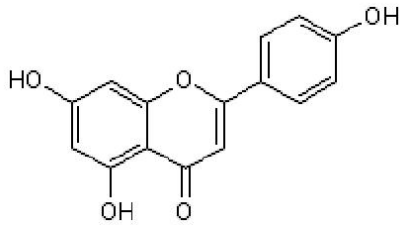


Fig. 1. Chemical structure of apigenin.

Toxicological experiments

Toxicity experiments were performed by the method of Singh and Agarwal [16]. Ten experimental animals of *L. acuminata* were kept in glass aquaria containing 3L de-chlorinated tap water for 24h, 48h, 72h or 96h. Control groups were kept in de-chlorinated tap water without any treatment. Each set of experiment was replicated six times. Mortality was recorded after every 24h up to 96h exposure period. The LC values were calculated by the probit log analysis by the method of Robertson et al. [17].

To test the environmental toxicity, if any, the toxic effect of apigenin was also studied in mixed population of target organisms (snails) and non-target organisms (fish). In this experiment, groups of 10 *L. acuminata* and 10 *C. punctatus* were put together in 2 L de-chlorinated tap water and exposed to 12.27 mg/L (24h LC_{90} of *L. acuminata*) apigenin for 24h.

Biochemical experiments

The acclimatized animals were treated with 40% and 80% of 24h LC_{50} of apigenin for 24h or 96h exposure periods. Six aquaria were set up for each dose, and each aquarium contained 20 snails in 3 L and 10 fishes in 6L de-chlorinated tap water. After termination of treatment, the test animals were removed from aquaria and washed with water and killed. Dissection of the treated animals, nervous, hepatopancreas and ovotestis tissues of snail and liver, muscle and gonadal tissues of fish were collect in ice trays and used for biochemical analysis. Control animals were held under similar condition without any treatment.

Each experiment was replicated at least six times and the values have been expressed as means ± SE of six replicates. Student's' test was applied to locate significant changes with controls [18].

Total protein levels were estimated according to the method of Lowry et al., [19] using bovine serum albumin as standard. Homogenates (5 mg/mL, w/v) were prepared in 10% Tri Chloro Acetic acid.

Estimation of total free amino acid was made according to the method of Spices [20]. Homogenates (10 mg/mL, w/v) were prepared in 95% ethanol, centrifuged at 6000 x g and used for amino acid estimation.

Nucleic acids (DNA and RNA) were estimated by the methods of Schneider [21]. Homogenates (1 mg/mL, w/v) were prepared in 5% TCA at 90°C, centrifuged at 5000 x g for 20 min, and the supernatant was used for the estimation. Glycogen was estimated by the anthrone method of Van der Vies [22]. In the present experiment 50 mg of tissue were homogenized with 5 mL of cold 5% TCA. The homogenate was filtered and 1.0 mL of filtrate was used for the assay.

The pyruvate level was measured according to Friedemann and Haugen [23]. Homogenate (50 mg/mL, w/v) was prepared in 10% TCA. Sodium pyruvate was taken as standard.

Lactate was estimated according to Huckabee [24]. Homogenate (50 mg/mL, w/v) was prepared in 10% cold TCA. Sodium lactate was taken as standard.

The protease activity was estimated by the method of Moore and Stein [25]. Homogenate (50 mg/mL, w/v) was prepared in cold distilled water.

The acid and alkaline phosphatase activity was determined by the method of Anderson and Szcypinski [26]. Homogenates (2%, w/v) were prepared in ice-cold 0.9% NaCl solution and centrifuged at 5000 x g at 0°C for 15 min. Lactic dehydrogenase activity (LDH) activity was measured according to the method of Anon [27]. Homogenates (50 mg/L, w/v) were performed in 0.1 M phosphate buffer (pH 7.5) for 5 min at 4°C and the supernatant was taken as enzyme source. Succinic dehydrogenase activity (SDH) activity was measured by the methods of Arrigoni and Singer [28]. Homogenate (50 mg/L, w/v) was prepared in 0.5 M potassium phosphate buffer (pH 7.6) for 5 min in an ice bath and centrifuged at 10,000 x g for 30 min at 4°C.

Cytochrome oxidase activity was measured according to Cooperstein and Lazarow [29].

Homogenates (50 mg/mL, w/v) were prepared in 1.0 mL of 0.33 M phosphate buffer (pH 7.4) for 5 min in an ice bath.

Acetylcholinesterase (AChE) activity was measured by the method of Ellman et al. [30]. Homogenates (50 mg/ml) were prepared in 0.1 M phosphate buffer, pH 8.0, for 5 min in an ice bath and centrifuged at 1000 x g for 30 min at -4ºC.

Withdrawal experiment

In order to see effect of withdrawal of apigenin treatment, both snail and fish were exposed for 96h to 80% of the LC_{50} (24h) and one half of the animal was sacrificed and the level, of total protein, total free amino acids, DNA, RNA, glycogen, pyruvate, lactate and activity of protease, phosphatase, LDH, SDH, cytochrome oxidase and AChE enzymes were measured in liver and muscle tissue of fishes. The other half was transferred to fresh, treatment free water, which was changed every 24h for the next 6 day. Following to this, all above biochemical parameters were also measured in liver and muscle tissues of fishes. Control animals were kept in dechlorinated tap water under similar conditions without any treatment.

RESULTS

Effects on behavioural changes and poisoning symptoms

Exposure to apigenin caused significant behavioural changes in the freshwater target snail *L. acuminata*. Behavioural changes appeared after 5 to 10 min of exposure. The initial 30-40 min was a period of hyperactivity during which if sluggish snails moved rapidly in the aquarium water. After some time they started crawling on each other. As the poison entered in the snails body a muscular twitching and the snails became spirally twisted, which resulted in ataxia, convulsion, paralysis and finally death of the snails. Prior to death, there was complete withdrawal of the body inside the shell indicating nerve poisoning.

Dose-mortality response

In the present study the toxicity of apigenin (Fig. 1) was tested against the freshwater target snail *L. acuminata*. Snail mortality was used as a bioassay for monitoring the molluscicidal activity of the apigenin extracts.

The LC_{50} values of apigenin for periods ranging from 24h to 96h throughout are shown in (Table 1). The toxicity was both times and dose dependent, as there was a significant negative correlation between LC_{50} values and exposure times. Thus, the LC_{50} values of apigenin extracts for *L. acuminata* decreased from 3.65 mg/L (24h) to 0.73 mg/L (96h), respectively (Table 1).

The slope values given in Table 1 were steep and the heterogeneity factor was less than 1.0

Table 1. Toxicity (LC_{10}, LC_{50} and LC_{90}) of apigenin extracted from seed of *Thevetia peruviana* plant against freshwater harmful snail *Lymnaea acuminata* at different time intervals.

Exposure period [h]	Effective dose [mg/L]	Limit [mg/L]		Slope value	'g' factor	't' ratio	Hetero.
		LCL	UCL				
24	LC_{10}= 1.08 **LC_{50}=3.65** LC_{90}=12.27	3.05	4.87	2.43 ± 0.44	0.12	5.53	0.16
48	LC_{10}=0.69 **LC_{50}=2.98** LC_{90}=11.86	2.47	3.92	2.02 ± 0.39	0.14	5.08	0.07
72	LC_{10}=0.32 **LC_{50}=1.63** LC_{90}=8.40	1.18	2.02	1.80 ± 0.37	0.16	2.55	0.13
96	LC_{10}=0.16 **LC_{50}=0.73** LC_{90}=3.31	0.33	1.03	1.95 ± 0.42	0.18	1.72	0.35

There was no mortality in control groups.

Batches of ten snails were exposed to four different concentrations of apigenin. Concentrations given were the final concentrations (w/v) in aquarium water. Regression coefficient showed that there was significant ($P < 0.05$) negative correlation between exposure time and different LC values.

LCL: lower confidence limit; UCL: upper confidence limit; Hetero.: Heterogeneity.

Table 2. Changes in total protein, total free amino acids, nucleic acids (DNA and RNA) (µg/mg) level and activity of protease (mmol of tyrosine equivalents/mg protein/h) and acid and alkaline phosphatase (mmol substrate hydrolysed/ 30 min/mg protein) in nervous (NT), hepatopancreas (HP) and ovotestis (OT) tissues of freshwater harmful snail *Lymnaea acuminata* after exposure to sub-lethal doses of 40% and 80% of LC_{50} (1.46 mg/L and 2.92 mg/L) of apigenin extract after 24h.

Parameter	Tissue	Control	40% of LC_{50} (24h) 1.46 mg/L	80% of LC_{50} (24h) 2.92 mg/L	7 day of withdrawal
Protein	NT	63.0±0.20 (100)	35.91±0.11^{acde} (57)	18.90±0.06^{acdf} (30)	61.11±0.48^{bcef} (97)
	HP	65.0±1.98 (100)	36.40±1.10^{acde} (56)	23.40±0.71^{acdf} (36)	62.30±1.90^{bcef} (96)
	OT	70.0±0.50 (100)	37.10±0.26^{acde} (53)	19.60±0.14^{acdf} (28)	66.50±0.48^{bcef} (95)
Amino acid	NT	30.2±1.20 (100)	49.53±0.92^{acde} (164)	51.34±0.58^{acdf} (170)	31.10±0.62^{bcef} (103)
	HP	28.8±1.40 (100)	41.76±0.98^{acde} (145)	43.48±0.72^{acdf} (151)	29.95±0.68^{bcef} (104)
	OT	32.2±0.70 (100)	53.77±0.54^{acde} (167)	55.70±0.36^{acdf} (173)	32.84±0.42^{bcef} (102)
DNA	NT	74.2±0.30 (100)	44.52±0.14^{acde} (60)	29.68±0.12^{acdf} (40)	69.74±0.16^{bcef} (94)
	HP	71.6±0.20 (100)	47.97±0.18^{acde} (67)	23.62±0.16^{acdf} (33)	68.02±0.18^{bcef} (95)
	OT	80.2±0.98 (100)	42.50±0.76^{acde} (53)	23.25±0.52^{acdf} (29)	75.38±0.58^{bcef} (94)
RNA	NT	50.2±0.40 (100)	25.60±0.34^{acde} (51)	18.07±0.28^{acdf} (36)	48.18±0.32^{bcef} (96)
	HP	48.2±0.40 (100)	27.95±0.32^{acde} (58)	19.28±0.29^{acdf} (40)	45.79±0.32^{bcef} (95)
	OT	54.2±0.60 (100)	23.86±0.54^{acde} (44)	16.27±0.51^{acdf} (30)	49.90±0.68^{bcef} (92)
Protease	NT	0.468±0.048 (100)	0.665±0.002^{acde} (142)	0.706±0.001^{acdf}(151)	0.505±0.002^{bcef} (108)
	HP	0.462±0.002 (100)	0.647±0.001^{acde} (140)	0.665±0.002^{acdf}(144)	0.443±0.003^{bcef} (102)
	OT	0.470±0.006 (100)	0.662±0.004^{acde} (141)	0.714±0.003^{acdf}(152)	0.494±0.004^{bcef} (105)
Acid phosphatase	NT	0.274±0.007 (100)	0.254±0.006^{acde} (93)	0.205±0.005^{acdf} (75)	0.249±0.007^{bcef} (91)
	HP	0.272±0.006 (100)	0.263±0.005^{acde} (97)	0.220±0.004^{acdf} (81)	0.252±0.005^{bcef} (93)
	OT	0.278±0.007 (100)	0.261±0.006^{acde} (94)	0.211±0.003^{acdf} (76)	0.256±0.004^{bcef} (92)
Alkaline phosphatase	NT	0.468±0.002 (100)	0.379±0.001^{acde} (81)	0.299±0.007^{acdf} (64)	0.421±0.006^{bcef} (90)
	HP	0.470±0.003 (100)	0.376±0.002^{acde} (80)	0.314±0.002^{acdf} (67)	0.427±0.002^{bcef} (91)
	OT	0.472±0.007 (100)	0.382±0.005^{acde} (81)	0.302±0.004^{acdf} (64)	0.424±0.006^{bcef} (90)

Values are mean ± SE of six replicates. Values in parenthesis are % level with control taken as 100%.

[a]Significant ($P < 0.05$) student's 't' test was applied between control and treated groups.

[b]Significant ($P < 0.05$) student's 't' test was applied between treated and withdrawal groups.

ANOVA and LSD test show significant ($P < 0.05$), when [c]treated groups were compared with controls, [d]treated groups were compared with each other, [e]40% treated groups were compared with withdrawal and [f]80% treated groups were compared with withdrawal.

indicating that the result found was within the 95% confidence limits of the LC_{50} values. The regression test ('t' ratio) was greater than 1.96 and the potency estimation test ('g' value) was less than 0.5 at all probability levels.

Biochemical experiments

Exposure of sub-lethal doses (40% and 80% of LC_{50}) of apigenin for 24h against the freshwater target snail *L. acuminata* and for 96h against the freshwater non-target fish *C. punctatus* caused significant ($P < 0.05$) alterations in carbohydrates and nitrogen metabolism in various tissues (Tables 2 to 5).

Total protein levels were reduced to 57%, 56% and 53% of controls in nervous, hepatopancreas and ovotestis tissues, respectively, after exposure to 40% of LC_{50} (24h) of apigenin. The maximum decrease in protein level (28% of control) was observed in snails treated with 80% of LC_{50} (24h) of apigenin. Same trend was also observed in case of DNA, RNA, acid and alkaline phosphatase activity in various tissues of *L. acuminata*, (Table 2). Glycogen, pyruvate, LDH and AChE activity was also reduced of controls in nervous, hepatopancreas and ovotestis tissues of the snail *L. acuminata* (Table 3).

Total free amino acid levels were induced to 164%, 145% and 167% of controls after treatment with 40% of LC_{50} (24h) of apigenin in various tissues of the snail *L. acuminata*, respectively. The maximum increase in total free amino acid levels (173% of control) was observed in snails treated with 80% of LC_{50} (24h) of apigenin. Same trend was also observed in case of protease, activity (Table 2). Lactate levels and SDH enzyme activity was also increased of controls in nervous, hepatopancreas and ovotestis tissues of the snail *L. acuminata*, (Table 3).

Table 3. Changes in glycogen (mg/g), pyruvate (µmol/g), lactate (mg/g) level and activity of LDH (µmol/mg protein/h), SDH (µmol of dye reduced/min/mg protein), Cytochrome oxidase (arbitrary unit/min/mg protein) and AChE (µmol 'SH' hydrolysed/min/mg protein) after 24h of exposure to sub-lethal doses of 40% and 80% of LC_{50} (1.46 mg/L and 2.92 mg/L) of apigenin extract in nervous (NT), hepatopancreas (HT) and ovotestis (OT) tissues of freshwater harmful snail *Lymnaea acuminata* after 24h.

Parameter	Tissue	Control	40% of LC_{50} (24h) 1.46 mg/L	80% of LC_{50} (24h) 2.92 mg/L	7 day of withdrawal
Glycogen	NT	5.6±0.06 (100)	2.5±0.03^{acde} (45)	1.79±0.02^{acdf} (32)	5.09±0.06^{bcef} (91)
	HP	6.4±0.12 (100)	3.2±0.06^{acde} (50)	2.36±0.04^{acdf} (37)	5.89±0.11^{bcef} (92)
	OT	8.6±0.06 (100)	3.9±0.03^{acde} (45)	2.75±0.02^{acdf} (32)	7.83±0.05^{bcef} (91)
Pyruvate	NT	0.562±0.02 (100)	0.191±0.01^{ace} (34)	0.157±0.01^{acf} (28)	0.505±0.02^{bef} (90)
	HP	0.602±0.04 (100)	0.246±0.02^{acde} (41)	0.229±0.02^{acdf} (38)	0.535±0.04^{bef} (89)
	OT	0.524±0.05 (100)	0.157±0.02^{acde} (30)	0.136±0.01^{acdf} (26)	0.472±0.05^{bef} (90)
Lactate	NT	2.98±0.06 (100)	4.79±0.09^{acde} (161)	5.24±0.11^{acdf} (176)	3.33±0.07^{bcef} (112)
	HP	3.12±0.04 (100)	5.36±0.07^{acde} (172)	5.87±0.07^{acdf} (188)	3.61±0.04^{bcef} (116)
	OT	3.62±0.06 (100)	5.82±0.09^{acde} (161)	6.37±0.11^{acdf} (176)	4.02±0.06^{bcef} (111)
LDH	NT	0.078±0.003(100)	0.062±0.002^{acde} (80)	0.040±0.001^{acdf} (51)	0.069±0.003^{bcef} (88)
	HP	0.092±0.002(100)	0.075±0.001^{acde} (82)	0.047±0.002^{acdf} (56)	0.083±0.002^{bcef} (90)
	OT	0.082±0.003(100)	0.066±0.002^{acde} (81)	0.039±0.001^{acdf} (48)	0.073±0.003^{bcef} (89)
SDH	NT	30.02±0.12 (100)	37.82±0.15^{acde} (126)	49.23±0.20^{acdf} (164)	33.02±0.13^{bcef} (110)
	HP	41.03±0.10 (100)	50.02±0.12^{acde} (122)	61.09±0.15^{acdf} (149)	43.46±0.11^{bcef} (106)
	OT	26.28±0.08 (100)	32.58±0.09^{acde} (124)	42.04±0.13^{acdf} (160)	28.90±0.09^{bcef} (110)
Cytochrome oxidase	NT	17.28±0.10 (100)	10.71±0.06^{acde} (62)	9.33±0.05^{acdf} (54)	15.55±0.09^{bcef} (90)
	HP	15.20±0.12 (100)	10.33±0.08^{acde} (68)	9.12±0.07^{acdf} (60)	13.53±0.11^{bcef} (89)
	OT	16.32±0.14 (100)	10.77±0.09^{acde} (66)	9.13±0.08^{acdf} (56)	15.01±0.13^{bcef} (92)
AChE	NT	0.062±0.007 (100)	0.038±0.004^{acd} (62)	0.055±0.006^{acd} (90)	0.055±0.006^{b} (89)
	HP	0.078±0.004 (100)	0.052±0.002^{acde} (67)	0.031±0.002^{acdf} (89)	0.071±0.004^{bef} (91)
	OT	0.070±0.003 (100)	0.045±0.001^{acde} (64)	0.064±0.003^{acdf} (91)	0.063±0.003^{bef} (90)

Details are same as given in Table 2.

Table 4. Changes in total protein, total free amino acids, DNA and RNA (µg/mg) level and activity of protease (µmol of tyrosine equivalents/mg protein/h) and acid and alkaline phosphatase (mmol substrate hydrolysed/30min/mg protein) in muscle (MU), liver (LI) and gonadal (GO) tissues of the freshwater fish *Channa punctatus* after 96h exposure to sub-lethal doses of 40% and 80% of LC_{50} (1.46 mg/L and 2.92 mg/L) of apigenin extract and 7 day after withdrawal.

Parameter	Tissue	Control	40% of LC_{50} (24h) 1.46 mg/L	80% of LC_{50} (24h) 2.92 mg/L	7 day of withdrawal
Protein	MU	170.4±0.80 (100)	102.24±0.48^{acde} (60)	68.16±0.32^{acdf} (40)	160.18±0.75^{bcef} (94)
	LI	150.2±0.68 (100)	126.16±0.57^{acde} (84)	117.15±0.53^{acdf} (78)	144.19±0.65^{bcef} (96)
	GO	146.2±0.98 (100)	110.96±0.74^{acde} (76)	74.56±0.50^{acdf} (51)	133.04±0.89^{bcef} (91)
Amino acid	MU	36.28±0.24 (100)	39.90±0.26^{cde} (110)	45.71±0.30^{acdf} (126)	39.04±0.30^{bcef} (108)
	LI	20.08±0.38 (100)	24.09±0.45^{acde} (120)	28.11±0.53^{acdf} (140)	21.28±0.40^{bcef} (106)
	GO	31.60±0.68 (100)	42.97±0.92^{acde} (136)	46.45±0.99^{acdf} (147)	35.70±0.77^{bcef} (113)
DNA	MU	150.42±0.76 (100)	120.33±0.62^{acde} (80)	90.24±0.46^{acdf} (60)	144.40±0.72^{bcef} (96)
	LI	148.06±0.72 (100)	127.33±0.62^{acde} (86)	103.69±0.50^{acdf} (70)	134.73±0.66 ^{bcef} (91)
	GO	141.00±0.77 (100)	107.16±0.58^{acde} (76)	78.96±0.43^{acdf} (56)	126.90±0.69^{bcef} (90)
RNA	MU	102.00±0.24 (100)	81.6±0.19^{acde} (80)	67.32±0.16^{acdf} (66)	97.92±0.23^{bcef} (96)
	LI	100.00±0.26 (100)	91.0±0.23^{acde} (91)	70.00±0.18^{acdf} (70)	94.00±0.24^{bcef} (94)
	GO	105.62±0.62 (100)	80.27±0.47^{acde} (76)	63.37±0.37^{acdf} (60)	96.11±0.56^{bcef} (91)
Protease	MU	0.562±0.012 (100)	0.764±0.016^{acde}(136)	0.854±0.018^{acdf}(152)	0.590±0.010^{bcef}(105)
	LI	0.678±0.014 (100)	0.813±0.017^{acde}(120)	0.949±0.020^{acdf}(140)	0.719±0.013^{bcef} (108)
	GO	0.622±0.014 (100)	0.796±0.018^{acde}(128)	0.970±0.022^{acdf}(156)	0.640±0.012^{bcef} (103)
Acid phosphatase	MU	0.322±0.010 (100)	0.099±0.003^{acde}(31)	0.090±0.002^{acdf}(28)	0.293±0.009^{bcef} (91)
	LI	0.298±0.012 (100)	0.116±0.005^{acde}(39)	0.077±0.007^{acdf} (26)	0.280±0.011^{bcef} (94)
	GO	0.268±0.014 (100)	0.085±0.004^{acde} (32)	0.056±0.005^{acdf} (21)	0.257±0.013^{bcef} (96)
Alkaline phosphatase	MU	0.468±0.008 (100)	0.196±0.003^{acde} (42)	0.122±0.012^{acdf} (26)	0.421±0.007^{bcef} (90)
	LI	0.412±0.018 (100)	0.156±0.007^{acde} (38)	0.091±0.010^{acdf} (22)	0.374±0.016^{bcef} (91)
	GO	0.438±0.010 (100)	0.205±0.005^{acde} (47)	0.131±0.013^{acdf} (30)	0.416±0.009^{bcef} (95)

Details are same as given in Table 2.

Table 5. Changes in glycogen (mg/g), pyruvate (µmol/g), lactate (mg/g), level and activity of LDH (µmol/mg protein/h), SDH (µmol of dye reduced/min/mg protein), cytochrome oxidase (arbitrary unit/min/mg protein) and AChE (µmol 'SH' hydrolysed/min/mg protein) in muscle (MU), liver (LI) and gonadal (GO) tissues of the fish Channa punctatus after 96h exposure to sub-lethal doses of 40% and 80% of LC_{50} (1.46 mg/L and 2.92 mg/L) of apigenin extract and 7 day after withdrawal.

Parameter	Tissue	Control	40% of LC_{50} (24h) 1.46 mg/L	80% of LC_{50} (24h) 2.92 mg/L	7 day of withdrawal
Glycogen	MU	2.26±0.04 (100)	1.92±0.03^{acd} (85)	1.37±0.02^{acdf} (61)	1.94±0.03^{bcf} (86)
	LI	2.58±0.03 (100)	1.83±0.02^{acde} (71)	1.70±0.01^{acdf} (66)	2.12±0.02^{bcef} (82)
	GO	3.12±0.04 (100)	2.68±0.03^{acde} (86)	2.18±0.03^{acdf} (70)	2.80±0.02^{bcef} (90)
Pyruvate	MU	3.42±0.03 (100)	1.64±0.02^{acde} (48)	1.03±0.09^{acdf} (30)	3.08±0.03^{bcef} (90)
	LI	4.12±0.04 (100)	2.43±0.03^{acde} (59)	1.56±0.01^{acdf} (38)	3.74±0.04^{bcef} (91)
	GO	2.98±0.04 (100)	1.66±0.03^{acde} (56)	0.89±0.01^{acdf} (30)	2.68±0.03^{bcef} (90)
Lactate	MU	3.28±0.02 (100)	3.93±0.01^{acd} (120)	5.77±0.04^{acdf} (176)	3.60±0.02^{bf} (110)
	LI	2.68±0.04 (100)	3.64±0.03^{acde} (136)	4.56±0.07^{acdf} (170)	2.97±0.04^{bcef} (111)
	GO	3.66±0.06 (100)	4.24±0.06^{acde} (116)	5.89±0.09^{acdf} (161)	3.95±0.06^{bef} (108)
LDH	MU	426.3±0.06 (100)	366.61±0.73^{acde} (86)	255.78±0.52^{acdf} (60)	379.40±0.76^{bef} (89)
	LI	559.0±0.98 (100)	514.28±0.90^{acde} (92)	391.30±0.69^{acdf} (70)	508.69±0.89^{bcef} (91)
	GO	498.2±0.82 (100)	438.41±0.72^{acde} (88)	358.70±0.59^{acdf} (72)	448.38±0.74^{bcef} (90)
SDH	MU	56.2±0.22 (100)	67.44±0.26^{acde} (120)	76.43±0.30^{acdf} (136)	60.69±0.24^{cef} (108)
	LI	60.2±0.20 (100)	68.62±0.23^{acde} (114)	82.47±0.27^{acdf} (137)	66.22±0.22^{bcef} (110)
	GO	63.3±0.24 (100)	68.36±0.26^{acde} (108)	93.68±0.36^{acdf} (148)	66.46±0.25^{bcef} (105)
Cytochrome oxidase	MU	26.82±0.22 (100)	22.79±0.19^{acde} (85)	16.09±0.14^{acdf} (60)	24.67±0.20^{bcef} (92)
	LI	23.68±0.09 (100)	17.04±0.06^{acde} (72)	16.10±0.06^{acdf} (68)	21.31±0.08^{bcef} (90)
	GO	31.28±0.06 (100)	26.90±0.05^{acde} (86)	22.20±0.10^{acdf} (71)	28.46±0.05^{bcef} (91)
AChE	MU	0.096±0.010 (100)	0.046±0.004^{ace} (48)	0.035±0.003^{acf} (36)	0.089±0.009^{bef} (93)
	LI	0.098±0.009 (100)	0.052±0.005^{acde} (53)	0.034±0.003^{acdf} (35)	0.088±0.008^{bef} (90)
	GO	0.089±0.018 (100)	0.043±0.008^{acde} (48)	0.025±0.005^{acdf} (28)	0.081±0.016^{bef} (91)

Details are same as given in Table 2.

Apigenin also caused a similar alteration in carbohydrates and nitrogen metabolism of the non-target freshwater fish *C. punctatus* (Table 4 and 5).

Seven days of withdrawal of the treatment of 80% of 24h LC_{50} of apigenin (Tables 2 to 5) showed significant ($P < 0.05$) recovery in the protein, amino acids, nucleic acid, glycogen, pyruvate and lactate levels and in protease, acid phosphatase, alkaline phosphatase, LDH, SDH, cytochrome oxidase and AChE enzyme activity in nervous, hepatopancreas and ovotestis tissues of snail and liver, muscle and gonadal tissues of fish, respectively.

DISCUSSION

It is evident from the results section presented here that the extracted compound apigenin is toxic to freshwater target snail *L. acuminata*. The apigenin caused significant behavioural changes in the freshwater target snail *L. acuminata*. The most obvious sign of distress in the treated snails were muscular twitching and spiral twisting of the body, followed by crawling on each other. The nature and rapid onset of these behavioural responses indicates that, the extract perhaps contains some neurotoxins, which amongst other things, might be active at the neuromuscular system of the exposed animals. Similar behavioural responses were also observed [31] in their study on acute toxicity of lattices of *Euphorbia royleana*, *E. antisyphliatica* and *Jatropha gossypifolia* on snail *L. acuminata*. No such behavioural symptoms and death occurred in control groups indicating that no factor other than apigenin was responsible for altered behaviour and mortality.

The toxicity data indicate that apigenin was lethal to snail *L. acuminata*. The present study also demonstrates that the apigenin has higher molluscicidal activity i.e. more effective at low doses than any other prevalent synthetic molluscicides like carbamate, organophosphate and synthetic pyrethroids. Thus, the 24h LC_{50} of aldicarbe (30.00 mg/L) against the freshwater harmful snail *L. acuminata* [32] was much higher than that of the LC_{50} (24h) of apigenin (3.65 mg/L).

The depletion of protein fraction in various tissues may have been due to their degradation and possible utilization of degraded products for

metabolic purposes. Mommensen and Walsh [33] reported that proteins are mainly involved in the architecture of the cell, which is the chief source of nitrogenous metabolism and during chronic period of stress they are also a source of energy. The quantity of protein depends on the rate of protein synthesis its degradation. It also affected due to impaired incorporation of amino acids into polypeptide chains [34]. The synthesis of RNA plays an important role in protein synthesis. The inhibition of RNA synthesis at transcription level, thus may affect the protein level. In this study, a significant decline in RNA level in exposed snail was observed. The decrease in the RNA concentration may also have been a cause of protein depletion. Alternatively, the increase in protease activity may be the cause of increased protein degradation.

Apigenin has also significantly decreased the level of nucleic acids in the various tissues. Nordenskjold et al., [35] also reported reduction in DNA and RNA level after exposure to different pesticides. Data attained in this study make it clear that apigenin are potential inhibition of DNA synthesis, resulting in reduction of the RNA level. The increase in free amino acid level suggests tissues damage probably due to the increased proteolytic activity under apigenin toxic stress. However, the elevated levels of FAA can be utilised for energy production by feeding them in to the TCA cycle through aminotransferase reaction. The increase in the levels of free amino acid can also be attributed to the synthesis of amino acids in addition to their elevation by protein hydrolysis. A third possibility for increased free amino acid level might be their increase due to transamination and amination of keto acids [36].

Carbohydrates are the primary and immediate source of energy. In stress condition, carbohydrates reserve depleted to meet energy demand. Depletion of glycogen may be due to direct utilization for energy generation, a demand caused by active moiety-induced hypoxia. Pesticides are also inhibiting energy production by suppressing aerobic oxidation of carbohydrate leading to energy crisis in animals [37]. Carbohydrate metabolism is broadly divided into anaerobic segment or glycolysis in which break down of glucose or glycogen through Embden Meyerhaf pathway occur and aerobic segment which consist oxidation of pyruvate to acetyl co-A to be utilized through citric acid cycle. The end product of glycolysis under anaerobic condition in tissue is lactic acid where if the pyruvate level in tissue can be taken as a measure of aerobic condition of tissue depending on the availability of molecular oxygen. The level of tissue lactate content act as an index of anaerobiosis, which might be beneficial for animal to tolerate hypoxic condition [38] under pesticide exposure condition.

Under stress condition, with the increases of lactate content there was a decrease in pyruvate content in all the tissue. The decrease in liver and muscle pyruvate level and increase in lactate content suggest a shift towards anaerobiosis as a consequence of hypoxia, created under pesticides toxic impact leading to respiratory distress [39]. The increase in tissue lactate content may be due to its involvement in osmoregulation. During stress condition there was a decrease in osmolarity of internal body media of animal by loss of mono as well as divalent cations which is compensated by the animal with the increase of organic ions like lactate, amino acid etc. [40]. The decrease of pyruvate level may be due to its conversion to lactate or due to its mobilization to form amino acids, lipids, triglycerides and glycogen synthesis in addition to its role as a detoxification factor [41].

Vorbrodt [42] has reported that phosphatase is an important enzyme of animal metabolism, which plays an important role in the transport of metabolites across membranes. Since, apigenin used in the present study may also have antiphosphatase activity. So the reduction in protein level may be due to the inhibition of acid and alkaline phosphatase activity, as it plays an important role in protein synthesis [43].

Higher activity of lactic dehydrogenase (LDH) enzyme in control animals than treated suggested the inherent capacity of tissue to oxidize lactate to pyruvate and thus testifying themselves as aerobic tissue. LDH forms the centre for a delicately balanced equilibrium between catabolism and anabolism of carbohydrates [44]. LDH is present in most animal tissues and catalyzes the last step in glycolysis as it reduces pyruvic acid to lactic acid. It serves as a pivotal enzyme between the glycolytic pathway and tricarboxylic acid cycle. Lipid peroxidation is known to disturb the integrity of cellular membranes, leading to the leakage of cytoplasmic enzymes such as LDH, into the media. The decrease in LDH activity with a consequent increase in lactic acid levels suggests the

predeominanace of anaerobic segment of glycolysis. In present study apigenine might have forced to fish to rely on anaerobiosis by imposing anoxic or hypoxic condition.

The higher activity of succinic dehydrogenase (SDH) is due to higher distribution of mitochondria, since SDH is mitochondrially localized enzyme. SDH is active regulatory enzyme of the tricarboxlic acid cycles. The toxicity of apigenin decreased the ability of animals to respire thereby decreasing oxygen uptake. At the same time, the requirements of energy are geared up by starting the mobalisation of the carbohydrate reserves for production of energy. So SDH activity was increased in all the tissues of test animals in the present study. Stress produced by toxicant has adverse effect on energy metabolism and cause the SDH activity to rise; it seems that it is a compensatory effect [45].

Cytochrome oxidase transfers electrons to their final acceptor oxygen in electron transfer system (ETS). It produces ATP molecules there by influencing other cellular metabolic process. Sub-lethal experiment with apigenin causes decrease in cytochrome oxidase activity in liver and muscle tissues, which shows a profound impact on the oxidative metabolism. Results section indicates that apigenin, significantly inhibit the activity of enzyme AChE in different tissues. Apigenin may be promote, activity of the enzyme protein kinase C, which specifically phosphorylates serine and threonine residues in proteins [46]. Since the active site of enzyme acetylcholinesterase contains a serine residue, it is possible that the inhibition of this enzyme is due to the phosphorylation of the active site.

CONCLUSION

It is believed that the plant origin apigenin compound may eventually be of great value for the control of aquatic target organisms i.e. freshwater harmful snails and other molluscan pests. Plant origin apigenin compounds are less expensive, easily available, easily solubility in water and safer for the non-target animals than synthetic molluscicides.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Bali HS, Singh S, Sharma S. The distribution and ecology of vectors snails of Punjab. Indian J Ecol. 1986; 13: 31-37.
2. Hyman LH. 1970. The invertebrate. Vol. VI. Mollusca I. Mc Graw Hill, New York.
3. Ndamba J. Response of the molluscicidal berry plant (*Phytolacca dodecandra*) to different climatic and edaphic conditions. Trop Agric. 1995; 72: 135-140.
4. Singh SK, Yadav RP, Tiwari S, Singh A. Toxic effect of stem bark and leaf of *Euphorbia hirta* plant against freshwater vector snail *Lymnaea acuminata*. Chemosphere. 2005; 59: 263-270.
5. Singh SK, Yadav RP, Singh A. Molluscicides from some common medicinal plants of eastern Uttar Pradesh, India. J Appl Toxicol. 2010; 30: 1-7.
6. Hanif F, Singh DK. Molluscicidal activity of *Morus nigra* against the freshwater snail *Lymnaea acuminata*. J Biol Earth Sci. 2012; 2(2): B54-B62.
7. Rama R. 1967. Poisonous plants of India. Vol. I: A. ICAR, New Delhi, pp. 201.
8. Voigtlander HW, Balsam G. Apigenin 5-methyl ether, ein neues Flavon aus *Thevetia peruviana*. Arch Pharmacol. 1970; 303: 792.
9. Graf J. Herbal anti-inflammatory agents for skin disease. Skin Ther Lett. 2000; 5: 3-5.
10. Patel D, Shukla S, Gupta S. Apigenin and cancer chemoprevention: progress, potential and promise (Review). Int J Oncol. 2007; 30: 233-245.
11. Marston A, Hostettmann K. Plant molluscicides. Phytochem. 1985; 24: 639-652.
12. Singh SK. 2000. Studies on molluscicidal properties of some local plants of eastern Uttar Pradesh against harmful snails. Ph.D. thesis, D.D.U. Gorakhpur University, Gorakhpur (U.P.) India.
13. Singh SK, Yadav RP, Singh D, Singh A. Toxicological effects of stem bark of *Thevetia peruviana* and *Alstonia scholaris* on the freshwater snail *Lymnaea acuminata*. Malay Appl Biol. 2004; 33: 61-68.
14. Singh A, Singh SK. Molluscicidal evaluation of three common plant species from India. Fitoterapia. 2005; 76: 747-751.
15. APHA. 2005. Standard method for the examination of water and waste water. American Public Health Association, Washington, DC.
16. Singh A, Agarwal RA. Possibility of using latex of euphorbiales for snail control. Sci Tot Environ. 1988; 77: 231-236.
17. Robertson JL, Russell RM, Preisler HK, Savin NE. 2007. Bio-assays with arthropods: A POLO computer program. Taylor and Francis CRC Press, pp. 224.
18. Sokal RR, Rohlf FJ. 1973. Introduction of Biostatistics. W.H. Freeman, San Francisco, pp. 368.
19. Lowry HW, Rosebrough NJ, Farr AL, Randall RJ.

Protein measurement with folin phenol reagent. J Biol Chem. 1951; 193: 265-275.
20. Spices JR. 1957. Colorimetric procedures for amino acids. In: Methods of Enzymology. Calowick SP, Kaplon NO. (eds.). Academic Press, New York, pp. 468.
21. Schneider WC. 1957. Determination of nucleic acids in tissue by pantose analysis. In: Enzymology. Calowick SP, Kaplon NO. (eds.) Academic Press, New York, pp. 680.
22. Van der Vies J. Two methods for determination of glycogen in liver. J Biochem. 1954; 57: 410-416.
23. Friedmann TE, Haugen GF. Pyruvic acid. I. Collection of blood for the determination of pyruvic acid and lactic acid. J Biol Chem. 1943; 144: 267-277.
24. Huckabee WE. 1961. Blood analysis, determination of lactic acid. In: Hawk's Physiological Chemistry. Oser BL. (ed.): Tata McGraw-Hill, New Delhi, India, pp. 1103.
25. Moore S, Stein WH. A modified ninhydrine reagent for the photometric determination of amino acids and related compounds. J Biol Chem. 1954; 211: 907-913.
26. Anderson MA, Szcypinski AJ. Method of Enzymatic Analysis. Bergmeyer UH. (Ed.). Academic Press. J Clinic. 1947; 17: 571.
27. Anon, Sigma diagnostics TM. 1984. Lactic dehydrogenase (quantitative, colorimetric determination in serum, urine and cerebrospinal fluid) at 400-450 nm. Procedure No. 500, Sigma, St. Louis, USA.
28. Arrigoni O, Singer TP. Limitations of the phenazine methosulphate assay for succinic and related dehydrogenase. Nature. 1962; 193: 1256-1258.
29. Cooperstein SJ, Lazarow A. Microspectrophotometric method for the determination of cytochrome oxidase. J Biol Chem. 1957; 189: 665-670.
30. Ellman GL, Courtney KD, Andres Jr. V, Featherstone RM. A new and rapid Colorimetric determination of acetylcholinesterase activity. Biol Pharmacol. 1961; 7: 88.
31. Singh A, Agarwal RA. Molluscicidal and anti-cholinesterase activity of euphorbiales. Biol Agric Hort. 1990; 7: 81-91.
32. Sahay N, Singh DK, Agarwal RA. Synergistic effect of piperonyl butoxide the toxicity of synthetic pyrethroids in the snail *Lymnaea* (*Radix*) *acuminata*. J Med Appl Malacol. 1991; 3: 107-111.
33. Mommensen TP, Walsh PJ. Biochemical and metal perspectives on nitrogen in fishes. Experimentia. 1992; 48: 583-593.
34. Singh A, Singh DK, Mishra TN, Agarwal RA. Molluscicides of plant origin. Biol Agric Hortic. 1996; 13: 205-252.
35. Nordenskjold M, Soderhall J, Moldeus P. Studies on DNA trand break induced in human fibroblast by chemical mutagens and carcinogens. Mutat Res. 1979; 63: 393-400.
36. Jayantha Rao K, Madhu CH, and Murthy VSR. Histopathology of malathion on gills of freshwater teleost *Tilapia mossambica* (Peters). J Environ Biol. 1983; 4: 9-13.
37. Kohli KK, Sharma SC, Bhatia SC, Venkita Subramonian TA. Biochemical effect of chlorinated insecticides: DDT and dieldrin. J Sci Ind Res. 1975; 34: 462.
38. Thoye RA. Effect of halothan, anoxia and hemorhage upon canine whole body skeletal muscle and splanchine excess lactate production. Anaes. 1971; 35: 394-400.
39. Sambasiva Rao KRS. 1999. Pesticide impact on fish metabolism. Discovery Publishing House, New Delhi, India.
40. Kabeer Ahammad Sahib I, Siva Prasad Rao K, Sambasiva Rao KRS, Ramana Rao KV Sub-lethal toxicity of malathion on the protease and free amino acid composition in the liver of the teleost, *Tilapia mossambica* (Peters). Toxicol Lett. 1984; 20: 59-62.
41. Sathya Prasad K. 1983. Studies on the toxic impact of lindane on tissue metabolic profiles in the fresh water fish, *Tilapia mossambica* (Peters) with emphasis on carbohydrate metabolism. Ph.D. Thesis, S.V. University, Tirupati, India.
42. Vorbrodt A. The role of phosphatase in intracellular metabolism. Post Hig Med Dosw. 1959; 13: 200-206.
43. Pilo B, Asnani MV, Shah RV. Studies on wound healing and repair in pigeon liver: II. Histochemical studies on acid and alkaline phosphatase during the process. J Ani Morp Physiol. 1972; 19: 205-212.
44. Everse T, Kaplan NO. Lactate dehydrogenase: Structure and function. In: Advances in Enzymology. Meister A. (ed.). John Wiley, New York pp., 1973; 61-133.
45. Singh A, Srivastava VK. Toxic effect of synthetic pyrethroid permethrin on the enzyme system of the freshwater fish *Channa striatus*. Chemosphere. 1999; 39: 1951-1956.
46. Aitken A. The activation of protein kinase C by dephnane, ingenane and tigliane diterpenoid esters. Bot J Linn Soc. 1987; 94: 247-263.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Improvement of growth and some metabolites of the salt affected *Anabaena circinalis* by calcium

Awatief F. Hifney

Botany and Microbiology Department, Faculty of Science, Assiut University, 71516 Assiut, Egypt

ABSTRACT

The growth and some metablic activites of *Anabaena circinalis*, which were grown under different salinity stress levels were followed and it has been found that these species can tolerate NaCl salinity to the level of 80 mM and survived to the level of 0.5 M of NaCl. Higher doses of salinity caused reduction in growth criteria (Absorbance, chlorophyll content) with the time elapsed, reduction of protein content, and caused increasing in carbohydrate content, significance increasing in proline content was also recorded. Addition of Ca^{+2} to the salinized culture caused improvement in growth and improved K^{+}/Na^{+} ratio which increase the osmoprotectant, as well as protect this organism from the toxic effect of NaCl.

Key words: *Anabaena circinalis*; salinity; calcium.

J Biol Earth Sci 2013; 3(1): B120-B128

Corresponding author:
Awatief F. Hifney
Botany and Microbiology Department, Faculty of Science, Assiut University, 71516 Assiut, Egypt
Tel: +201002931424, Fax: +2088342708
E-mail: hifney@yahoo.com

Original Submission: 31 January 2013; Revised Submission: 10 March 2013; Accepted: 14 March 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Osmotic stress exerts alteration on cellular activities, photosynthesis was commonly affected by NaCl salinity. Due to such alterations exerted by stress conditions, the soluble and insoluble organic constituents were variably accumulated or consumed. In this respect salinization was recorded to affect nitrogen metabolism and consequently the nitrogen compounds [1]. In particular the content of free proline increased markedly under stress condition [2].

Exogenous application of suitable concentration of Ca^{+2} to the salinized media improved the growth and consequently the relative metabolites and retained the broken salt tolerance of many algae [3]. Also they found that the growth of *Anabaena subcylindrica* and *Nostoc linckia* is markedly inhibited with the rise of NaCl level. However, marked growth stimulation was observed under certain combination of NaCl and $CaCl_2$. The physiology of osmotic adjustment is not adequately understood in Cyanobacteria. Additionally, the beneficial effect of supplemented calcium still complicated which different with the different dose of calcium, the different salinity levels, algal species, as well as the environmental conditions. However there are still many points which need to be studied to clarify the role played by Ca^{+2} to counter the adverse effects of NaCl salinization.

Thus the aim of the present study to follow the salt stress and/or calcium on growth as well as some metabolites of *Anabaena circinalis*.

MATERIALS AND METHODS

Anabaena circinalis was isolated from Great-subgroup Egyptian soil sample (Entisols Torriorthents, EOTT), for details Hifney et al. [4], grown in gas wash bottle containing 250 ml BG11 modified medium [5] and continuously bubbled with CO_2 enriched air (2% CO_2) at 30ºC, with continuous light intensity of 120 µE m^{-2} S^{-1} under regular and salinity stress condition as following: 40, 80, 160, 200, 300, 400, 500 mM salinization with or without addition of 50, 100, 200, 300 ppm $CaCl_2$.

Absorbance at 750 nm was measured and Chlorophyll a content was determined according to Lefort-Tran et al. [6] for six days to monitor the changes in the growth conditions. The photosynthetic activities in various treatments determined as an oxygen evolution in an oxygen gas electrode after six days according to Michel et al. [7]. The carbohydrates (total soluble, insoluble) were determined using anthrone sulphoric acid method [8-10]. Free amino acids were extracted from algal suspension and colorimetrically determined using the method of Lee and Takahashi [11]. Protein contents (total, soluble, insoluble) were determined as described by Lowry et al. [12] using Folin reagent. Proline content was determined according to Bates et al. [13]. The concentration of Na^+, K^+ was determined using flame photometer [14] M71D type Nr/LPG 075. The data was obtained by three independent experiments and measured as means ± s. error using Excel 2001 program (statistical analysis).

RESULTS AND DISCUSSION

The result obtained in this study showed that A. circinalis tolerate NaCl salinity to the level of 80 mM NaCl and survived to the level of 0.5 M of NaCl, (Fig. 1a and Table 1). The addition of NaCl caused remarkable decrease in the growth criteria (Absorbance at 750 nm, chlorophyll a content) with increasing salinity and the time elapsed especially at 500 mM NaCl as shown in Fig. 1a, 2a, this result in accordance with Lee et al. [15] they found that optimum growth of *Porphyridium cruentum* was affected by the concentration of NaCl. Optimum growth was found with salinities ranging between 0.45 M and 0.8 M NaCl, a further increased in medium salinity to 1.5 M NaCl result in drastic drop in algal growth. Ahmed et al. [16] found that *Chlorella vulgaris* grown for 7 days in various levels of NaCl exhibited a gradual growth reduction with the rise of salinization levels.

This reduction in growth criteria at higher doses of salt was also recorded by other investigators using of other algae [3, 17, 18]. Loebich [19] found that the cessation of pigment synthesis under salt stress probably due to inhibition of the use of radiant energy by the chlorophyll molecule and the chlorophyll may be become oxidized and bleached leading to lethal photodynamic reaction.

Addition of $CaCl_2$ to the normal media improved growth criteria (Absorbance 750 nm and chlorophyll a content) as shown in Fig. 1b, 2b, when compared with those of the corresponding reference culture (of the same osmotic level but containing only NaCl) as shown in Fig. 1c, Fig. 2c. These results in

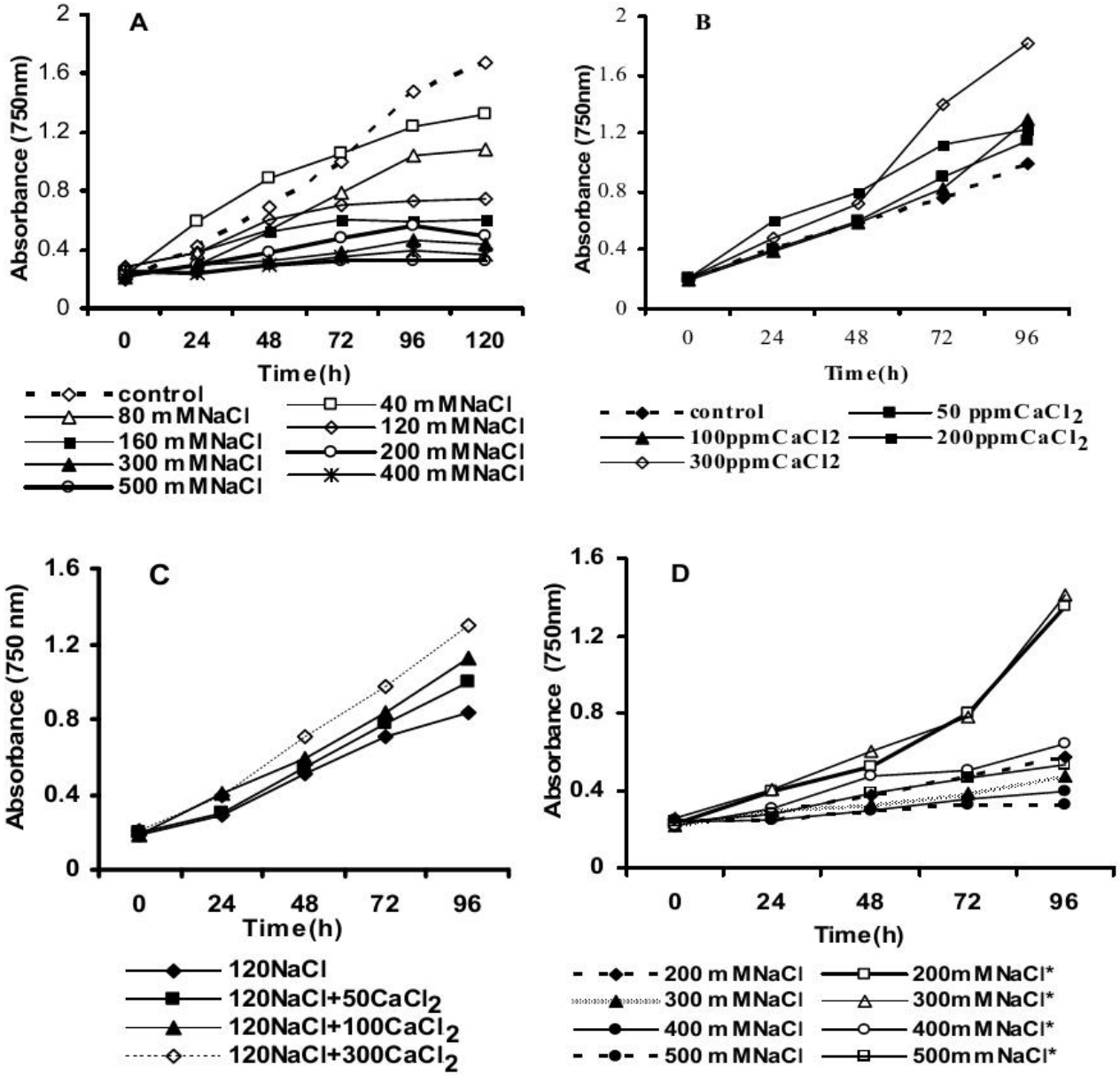


Fig. 1. The interaction effect of different concentration of NaCl and $CaCl_2$ on growth (absorbance 750nm) of *Anabaena circinalis*.

A: Culture grown under salinity stress

B: Culture grown with CaCl2 and without salinity stress

C: Culture grown under 120 mM NaCl with (50, 100, 300) ppm $CaCl_2$

D: Culture grown under salinity stress without or supplemented with $CaCl_2$(*)

accordance with those obtained by Mokhaled et al. [3], Ahmed et al. [16] and Issa [17].

The photosynthetic activities (O_2) evolution increased progressively by increasing NaCl up to the level of 80 mM NaCl, and then gradually decreased by increasing the salinity stress (Table 1). Allakhverdiev et al. [20] demonstrated that salt stress due to 0.5 M NaCl inactivated both PSII and PSI mediated electron transport, in the *Cyanobacterium synechococcus* sp. PCC 7942.

Addition of $CaCl_2$ (300 ppm) enhanced the photosynthetic activity and completely alleviated the inhibitory effect of 300 mM NaCl (Table 1). In accordance with this result Ahmed et al. [16] observed decrease in cell number, dry weight, and photosynthetic oxygen evolution in culture of *Chlorella vulgaris* with the rise of NaCl level. They also found that inclusion of Ca^{+2} to the media enhanced the growth of salinized cultures, the maxima of cell number, dry matter, and pigment

Table 1. The interaction effect of different concentration of Nacl and $CaCl_2$ on growth (chlorophyll a content), photosynthesis and accumulation of Na^+, K^+ and K^+/Na^+ ratio of *Anabaena circinalis*.

Treatments	Chlorophyll (µg/ml algal suspension)	Photosynthesis (µ mol $O_2\uparrow$/ µg chlorophyll.h^{-1})	Na^+	K^+	K^+/Na^+ ratio
			(mg/ml algal suspension)		
control	6.35±0.45	643.13±0.036	0.095±0.002	2.14±0.053	22.50
40 mM NaCl	6.64±0.13	888.75±0.87	0.105±0.009	2.21±0.040	21.05
80 mM NaCl	5.63±0.40	900.05±0.79	1.135±0.036	2.11±0.003	15.70
120 mM NaCl	4.03±0.23	586.9±2.32	1.136±0.033	1.68±0.012	1.48
160 mM NaCl	3.72±0.07	404.03±1.35	1.50±0.014	1.41±0.004	0.94
200 mM NaCl	3.57±0.24	342.10±2.09	2.03±0.022	1.21±0.023	0.60
300 mM NaCl	2.94±0.03	312.52±0.135	2.70±0.044	1.12±0.196	0.41
400 mM NaCl	2.62±0.02	269.20±1.03	4.90±0.081	0.82±0.004	0.17
500 mM NaCl	2.39±0.31	243.98±1.64	5.60±0.08	0.71±0.003	0.13
control*	7.23±0.05	792.50±11.3	0.042±0.001	2.88±0.256	68.6
120 mM NaCl*	5.96±0.05	780.40±0.28	0.11±0.002	2.24±0.045	20.4
160 mM NaCl*	5.92±0.13	670.33±0.75	0.22±0.006	2.42±0.004	11.00
200 mM NaCl*	5.93±0.25	668.75±0.17	0.52±0.04	2.41±0.097	4.60
300 mM NaCl*	4.90±0.40	591.70±0.95	0.81±0.043	2.3±0.089	2.84
400 mM NaCl*	3.30±0.32	524.31±0.33	1.92±0.15	2.12±0.045	1.12
500 mM NaCl*	3.14±0.16	441.12±0.55	2.32±0.21	2.72±0.131	1.173

*Culture supplemented with 300 ppm $CaCl_2$.

were recorded under a Na^+/Ca^{+2} ratio of 13.5. The partial substitution of Ca^{+2} for Na^+ improved photosynthetic oxygen evolution, Issa [17] found that Ca^{+2} has a positive role in photosynthetic oxygen evolution and dark oxygen uptake in salinity stressed - *Dunaliella* cells supplemented with $CaCl_2$, in comparison to the culture containing NaCl alone.

The data for Na^+ and K^+ ion content reveal that while NaCl salinity stress enhanced the accumulation of sodium ion progressively (Table 1), it retarded the accumulation of K^+ especially at moderate and higher salt levels (Table 1). K^+/Na^+ ratio decreased progressively, which associated with the marked reduction in growth criteria of A. circinalis (Table1). This increase in Na^+ over K^+ might be responsible for the break down of the salt tolerance of *A. circinalis* as also recorded in nitrogen fixing cyanobacteria [22]. Interestingly, $CaCl_2$ reversed these trends; while Na^+ content was reduced markedly, K^+ content was enhanced progressively in $CaCl_2$ treated salinized-culture as compared with that of only NaCl treated culture as given in (Table 1). Our result also in accordance with [3] who found that Na^+ ions accumulated in *Nostoc linckia* and *Anabaena subcylindrica* by increasing salinity and the cultures showed progressive increase with increasing the incubation time, he also found that the addition of $CaCl_2$ to the salinized culture caused remarkable decrease in the Na^+ ions accumulation in both organisms as compared with salinized culture. Thus $CaCl_2$ treatment partially alleviated the inhibitory effect of salinity of *A. circinalis* which found to be associated with the marked and progressive increase in K^+/Na^+ ratio and consequently the growth yield.

Kaushik and Nagar [23] found that when halotolerant Cyanobacterium *Westiellopsis prolifica* grown in graded concentration of NaCl up to 350 mg/L, there was a progressive increase in Na^+ uptake up to 350 mg/L of NaCl. In accordance with the present result [24] and [3] reported that Na^+ exclusions achieved primarily by curtailment of net Na^+ uptake, appears to be important mechanisms of combating salt stress in Cyanobacteria. Calcium is an important factor in the resistance of plants to salinity. Abdel-Basset [25] found that Ca^{+2} in certain ratios to Na^+ reversed most of NaCl stress symptoms in *Chlorella vulgaris*.

Carbohydrate content (soluble, total) tended to increased up to the level of 80 mM NaCl, and then

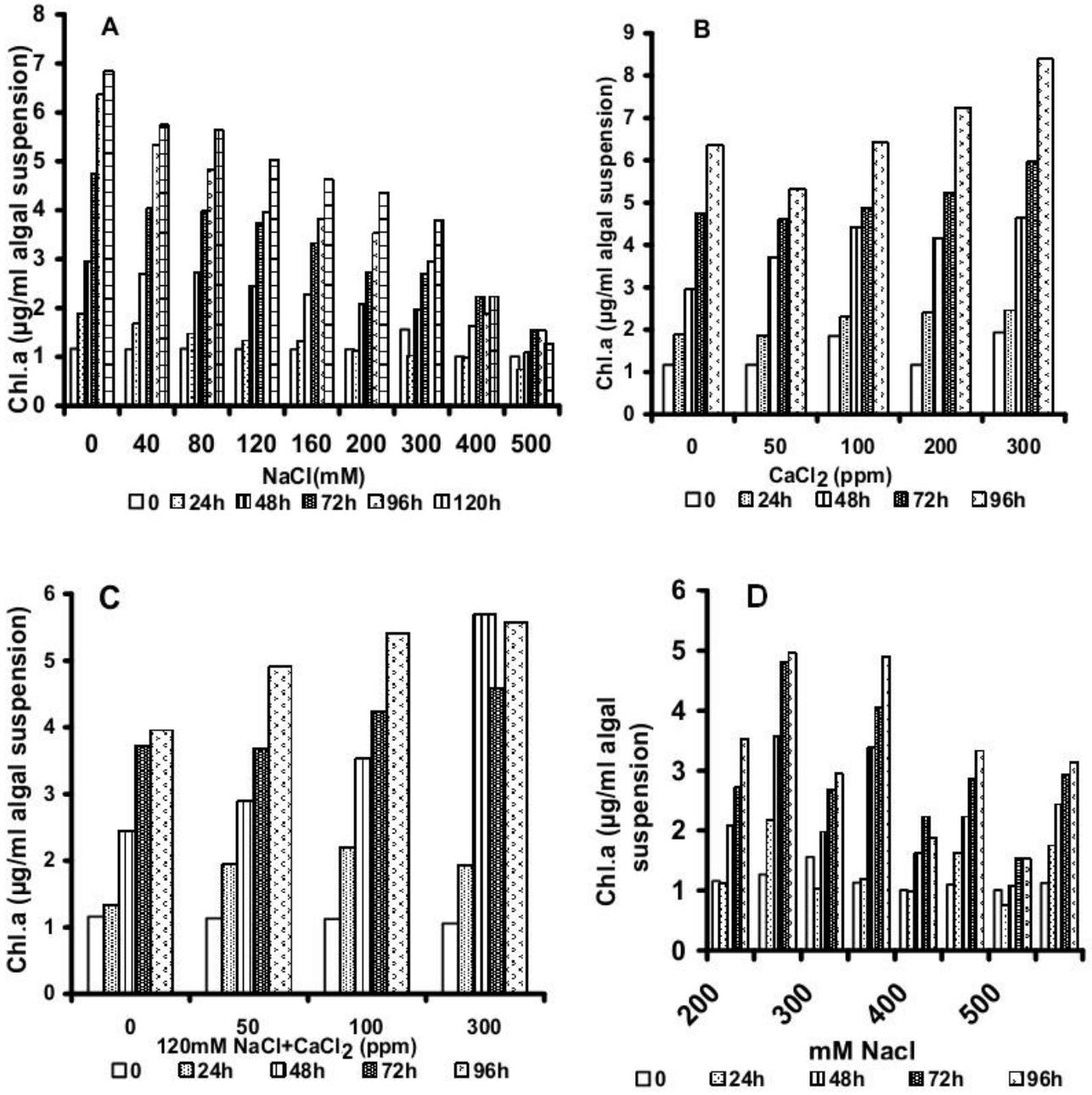


Fig. 2. The interaction effect of different concentration of NaCl and $CaCl_2$ on concentration of chlorophyll a of *Anabaena circinalis*

A: Culture grown under salinity stress

B: Culture grown with $CaCl_2$ and without salinity stress

C: Culture grown under 120 mM Nacl with (50, 100, 300) ppm $CaCl_2$

D: Culture grown under salinity stress without or supplemented with $CaCl_2$(*)

Table 2. The interaction effect of various concentration of NaCl and $CaCl_2$ on some metabolic activities of *Anabaena circinalis* grown in batch culture.

Treatments	Carbohydrate			Proteins			Amino acids	Proline
	S.C.	Ins.C.	T.C.	S.P.	In.P.	T.P		
	mg/g dry wt.							
Control	35.43±0.3	102±0.59	137.43±0.28	36.88 ± 0.75	85.6±0.91	122.48±0.16	22.2 ± 0.57	5.61 ± 0.06
40 mM Nacl	38.65±0.55	111.49±0.21	150.14±0.76	36.96 ± 0.42	86.2±0.50	123.16±0.91	23.2 ± 0.53	5.66 ± 0.17
80 mM Nacl	39.73±0.60	111.3±0.5	151.03±0.11	37.1 ± 0.50	85.9±0.11	123±0.61	24.4 ± 0.53	5.57 ± 0.50
120 mM Nacl	20.51±0.26	69.8±1.1	90.31±1.4	28.24 ± 0.09	74.5±0.37	102.74±0.46	38.6 ± 0.61	12.12 ± 0.45
160 mM Nacl	12.42±0.11	62.37±0.09	74.79±0.22	25.6 ± 0.39	74.1±0.64	99.7±0.10	41.2 ± 0.45	13.82 ± 0.30
200 mM Nacl	11.42±0.04	64.82±0.67	76.23±0.71	19.1 ± 0.64	66.2±0.76	85.3±0.14	44.5 ± 1.06	14.17 ± 0.30
300 mM Nacl	11.51±0.19	52.99±0.51	64.5±0.33	15.2 ± 0.86	50.1±0.097	65.3±1.8	45.6 ± 0.41	15.9 ± 0.20
400 mM Nacl	7.9±0.41	45.6±0.36	53.5±0.37	16.3 ± 0.31	33.2±0.19	49.5±1.6	45.6 ± 0.37	16.03 ± 0.01
500 mM Nacl	4.9±0.09.	37±0.23	41.9±0.13	13.36 ± 0.57	26.2±0.45	39.56±0.12	48.2 ± 0.78	27±0.69
Control*	54.74±4.6	111.4±0.65	176.14±0.42	39.2 ± 0.33	94.2±0.61	133.4±0.93	21.6 ± 0.33	5.17 ± 0.10
120 mM Nacl*	61.44±0.54	110.9±0.67	172.34±0.40	40.1 ± 0.82	91.1±0.59	131.2±1.4	21.7 ± 0.61	5.11 ± 0.03
160 mM Nacl*	39.9±0.44	112.2±0.41	152.1±0.94	40.4 ± 0.39	88.6±0.77	129±1.16	19.5 ± 0.12	5.1 ± 0.13
200 mM Nacl*	42.75±0.16	112.6±0.6	155.65±0.38	37.2 ± 0.40	88.8±0.66	126±1.1	19.3 ± 0.02	5.7 ± 0.15
300 mM Nacl*	41.6±0.15	102.62±0.73	144.2±0.53	36.3 ± 0.79	86.6±0.84	122.9±0.01	18.2 ± 0.08	5.8 ± 0.25
400 mM Nacl*	24.9±0.09	101.51±0.86	126.41±0.67	37.5 ± 0.54	84.6±0.54	122.1±2.29	19.1 ± 0.04	5.34 ± 0.11
500 mM Nacl*	21.2±0.08	81.22±1.1	102.42±1.51	35.9 ± 0.64	84.4±0.06	120.3±0.65	19.2 ± 0.33	6.39 ± 0.07

S.P.= soluble proteins, Ins.P.= insoluble proteins, T.P.= Total proteins.S.C = soluble carbohydrates, Ins.C.= insoluble carbohydrates, T.C.= total carbohydrates.
*:Culture supplemented with 300 ppm $CaCl_2$.

reduced severely by the further increasing of the salt (Table 2). This could be due to the alteration on cellular activities, photosynthesis which commonly affected by NaCl, Osmotic adaptation in most organisms involves intracellular accumulation of low-molecular-weight organic solutes or inorganic ions. Among Cyanobacteria, carbohydrates such as glucosylglycerol, sucrose [26] and trehalose [27] along with the quaternary ammonium compound glycine and betaine [27] constitute the major internal osmotica during salt stress. Biochemical, physiological and genetic studies have revealed how cyanobacteria are able to adapt to high salt environments, initially by active ion export followed by accumulation of compatible solutes [28, 29].

Protein content (soluble, total) slightly increased by the effect of NaCl up to the level of 80 mM and then reduced with increasing salinity levels (Table 2). This result in accordance with Hagemann et al. [30] who demonstrated that salt stress by NaCl reduced the synthesis of most protein to approximately 30% to 35% of the control level, moreover Mikkat et al. [31] observed that addition of NaCl at concentration of 513 mM, caused gradually decrease in the protein content of the *Cyanobacterium synechocystis* sp. The immediate, or shock, responses include the accumulation of compatible solutes and active export of inorganic ions, with these being regarded as largely protein synthesis independent [25].

Treatment with $CaCl_2$ resulted in a remarkable increase in content of both carbohydrate and protein (Table 2). At the level of 0.5 M NaCl and treated with 300 ppm $CaCl_2$, protein content was around 3 fold compared to the culture treated with 0.5 M NaCl only (Table 2). On the other hand, inhibition of protein by accumulation of NaCl alone may be attributed its inhibitory effect on the enzymatic reaction responsible for protein biosynthesis [18, 32].

On the other hand the concentration of free amino acids increased by salinity stress (Table 2). Stewart et al. [33] found that salinity stimulated the conversion of saccharide to amino acids and/or inhibit amino acids incorporation into protein [34].

Proline content was negatively correlated with the growth of *A. circinalis*. That while the growth was considerably reduced the proline content increased progressively by the salt stress.The highest accumulation of proline was recorded at the highest salinity level used (500 mM NaCl), at this level proline content was 5 fold that of the absolute control (normal culture) (Table 2).

Calcium treatment retarded the accumulation of proline and other free amino acids especially at the high doses of the salt compared with the corresponding salinized cultures (Table 2). In $CaCl_2$ treated culture the improved growth was accompanied with the pronounced retardation in proline, it was reduced by 1.5 fold at the level of 500 mM NaCl supplemented by 300 ppm $CaCl_2$ as compared with the corresponding level of the only NaCl stressed culture. Hifney et al. [4] and Ahmed et al. [16] found also the addition of 0.05 M $CaCl_2$ to NaCl-treated culture caused significance recovery of the biosynthesis of all amino acid of *Nostoc linckia* and *Anabaena subcylindrica*, thus the addition of Ca^{+2} seems to countered the toxic effects of Na^+ [25, 35]. The protective action of calcium in stressed plant could be achieved through membrane stability [36]. Low calcium increased membrane permeability at high external NaCl [37]. Also Leopald and Willing [38] found that calcium served partially to protect tissue from NaCl damage and lessens the leakiness of organic metabolites. Therefore it can be generalized that calcium relief occurs in the following sequence stabilization and repaired NaCl damage membranes (including thylakoids), less uptake of Na^+ (less toxicity) and preservation of cell metabolites from leakiness.

CONCLUSION

It can be reported that in *Anabaena circinalis* soluble saccharide, amino acids, and protein seemed to be the organic solutes used for osmotic adjustment under moderate salinization while the contribution of proline to osmotic adjustment in salt stressed A. circinalis up to the level of 80 mM NaCl seemed to be insignificant). Besides osmoregulation proline probably serve to improve salt tolerance in plant cell [39].

The addition of Ca^{+2} to the salinized cultures of *A. circinalis* improved K^+/Na^+ ratio which consequently increased the osmoprotectant (soluble saccharides, proteins, K^+) which consequently improved the growth of this algae and protect the organism from the toxic effect of NaCl.

ACKNOWLEDGEMENT

The author thank both of Prof Dr MAK. Shadad and Prof Dr AA. Issa (Botany Department, Faculty of Science, Assiut University) for their critical reading and discussion of the manscript and for their encouragement.

TRANSPARENCY DECLARATION

The author declares no conflicts of interest.

REFERENCES

1. Imamul-Hug SM, Larher F. Osmoregulation in higher plants. Effect of NaCl salinity on non-nodulated *Phaseoleus aureus* L. II. Changes in organic solutes. New Phytol. 1983; 93: 206-216.
2. Imamul-Hug SM, Larher F. Dynamics of Na^+, K^+ and proline accumulation in salt treated *Vigna siensis* (L.) and *Phaseolus aureus* L. J Plant Physiol. 1985; 119: 133-147.
3. Mokhaled MF, Osman MH, El-Sheekh MM, El-Naggar AH. 2003. Influence of salinity stress on growth and some metabolic activities of *Anabaena subcylindrica* and *Nostoc linckia*. Ph. D. Thesis, Faculty of Science, Menoufia University, Egypt.
4. Hifney AF, Issa AA, Adam MS, AL-Abssy AA. The influence of the heavy metals (Cd^{+2},Ni^{+2},Pb^{+2}) concentrations on the algal distributions as well as species composition in relation to the soil types in Assiut, Egypt. Assiut Univ J Bot. 2009; 38(2): 121-134.
5. Rippka R, Herdmann M. 1993. Pasteur culture collection of cyanobacterial strains in axenic culture. Vol. 1 Catalogue of Strains 103 P. Institut Pasteur, Paris, France.
6. Lefort-Tran M, Pouphile M, Spath S, Packer L. Cytoplasmic membrane changes during adaptation of the freshwater cyanobacterium *Synechococcus* 6311 to salinity. Plant Physiol. 1988; 7: 767-775.
7. Michel K-P, Thole HH, Pistorius EK. IdiA, a 34 KDa protein in the cyanobacterium *Synechococcus* sp. strain PCC 6301 and PC C 7942 is required for growth under iron and manganese limitation. Microbiol. 1996; 142: 2635-2645.
8. Fales FW. The estimation and degradation of carbonhydrates by yeast cell. J Biol Chem. 1951; 193: 113.
9. Schlegel HG. Die verwertung organischer sauren duch *Chlorella* in licht. Planta. 1956; 117: 510-526.
10. Badour SSA. 1959. Analytisch Chemische unter suchung des kalimangels bei Chlorella in vergleich mit anderen manget zamstanden. Ph. D. Dissertation, Goettingen, Germany.
11. Lee YD, Takahashi T. An improved colorimetric determination of amino acids with use of ninhydrin. Anal Biochem. 1996; 14: 71-77.
12. Lowry DH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem. 1951; 193: 265-275.
13. Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water-stress studies. Short communication. Plant Soil. 1973; 39: 205-207.
14. Williams CH, Twine JR. 1960. Flame photometric method for sodium, potasium and calcium. In: K. Peach & Tracey (eds.). Modern Methods of Plant Analysis, 5: 3-5. Springer Verlag, Berlin, Goettingen, Heidelberg.
15. Lee YK, Tan HM, Low CS. Effect of salinity of medium on cellular fatty acid composition of marine alga *Porphyridium cruentum* (Rhodophyceae). J Appl Phycol. 1989; 1: 19-23.
16. Ahmed AM, Radi AF, Heikal MD, Abdel-Basset R. Effect of Na-Ca combination on photosynthesis and some related processes of *Chlorella vulgaris*. J Plant Physiol. 1989; 135: 175-178.
17. Issa AA. The role of calcium in the stress response of halotolerant green algae *Dunaliella bardawil* (Ben-Amotz et Avron). Phyton. 1996; 36: 295-302.
18. Khatoon H, Banerjee S, Yusoff FM, Shariff M. Effects of salinity on the growth and proximate composition of selected tropical marine periphytic diatoms and cyanobacteria. Aquacult Res. 2010; 41: 1348–1355.
19. Loebich I. Photosynthesis and bigment influence by light intensity and salinity in the halophile *Dunaliella salina* (Chlorophyta). J Mar Biol ASS U.K. 1982; 62: 493-508.
20. Allakhverdiev SI, Sakamoto A, Nishiyama Y, Inabe M, Murata N. Ionic and osmotic effects of NaCl-induced inactivation of photosystem I and II in *Synechococcus* sp. Plant Physiol. 2000; 123: 1047-1056.
21. Kawasaki S, Borchert C, Deyholos M, Wang H, Brazille S, Kawai K, Galbraith D, Boherneh HJ. Gene expression during the initial phase of salt stress in rice. Plant Cell. 2001; 13: 889-905.
22. Apte SK, Reddy BR, Thomas J. Relationship of sodium influx and salt tolerance of nitrogen fixing cyanobacteria. Appl Environ Microbiol. 1987; 53(8): 1934-1939.
23. Kaushik BO, Nagar AP. Sodium uptake of halotolerant *Westiellopsis prolifica* in presence of K^+, Ca^{+2}, Mg^{+2}, Fe^+ and Li^+. Indian J Microbiol. 1993; 33(2): 93-96.
24. Sanders D, Brownlee C, Harper JF. Communication with calcium. Plant Cell. 1999; 11: 691-706.
25. Abdel-Basset R. Role calcium and calmodulin antagonist in photosynthesis and salinity tolerance in *Chlorella vulgaris*. Biol Planet. 1993; 35: 237-244.
26. Blumwald E, Mehlahorn RJ, Packer I. Studies on

osmoregulation in salt adaptation of cyanobacteria with ESR spin probe techniques. Proc Nalt Sci USA. 1983; 80: 2599-2602.
27. Reed RH, Chudek JA, Foster R, Stewart WDP. Osmotic adjustement in cyanobacteria from hypersaline environment. Arch Microbiol. 1984; 138: 333-337.
28. Hagemann M, Zuther E. Selection and characterization of mutants of the cyanobacterium *Synechocystis* sp. PCC 6803 unable to tolerate high salt concentrations. Arch Microbiol. 1992; 158(6): 429-434.
29. Pandhal J, Wright CP, Biggs CA. Proteomics with a pinch of salt: A cyanobacterial perspective (review). Saline Systems. 2008; 4: 1.
30. Hagemann M, Techel D, Rensing L. Comparison of salt-and heat-induced alteration of protein synthesis in the cyanobacterium *Synechocystis* sp. PCC 6803. Arch Microbiol. 1991; 155: 587-592.
31. Mikkat S, Effmert U, Hagemann M. Uptake and use of the osmoprotective compounds trehalose, glucosylglycerol, and sucrose by the cyanobacterium *Synechocystis* sp. PCC6803. Arch Microbiol. 1997; 167(2-3): 112-118.
32. Sinha RP, Hader DP. Response of a rice field Cyanobacterium *Anabaena* sp. to physiological stressors. Environ Exp Bot. 1996; 36(2): 147-155.
33. Stewart CR, Morris CJ, Thompson JF. Change in amino acid contents of excised leaves during incubation II-Role of sugar in the accumulation of proline in wilted leaves. Planta. 1966; 120: 279.
34. Handa S, Bressan RA, Handa AK, Carpita NC, Hasegawa PM. Solutes contribution to osmotic adjustement in culture plant cells adapted to water stress. Plant Physiol. 1983; 73(3): 834-839.
35. Jacob A, Kirst GO, Wiencke C, Lehmann H. Physiological responses of the antarcetic green algae *Prasiola crispa* sp. antractica to salinity stress. J Plant Physiol. 1991; 139: 57-62.
36. Munns R, Greenway H, Kirst GO. 1983. Halotolerant eukaryotes. In: Physiological Plant Ecology. III. Responses to the Chemical and Biological Environment (eds. Lange OL, Nobel PS, Osmond CB, Zeigler HH). Encyclopedia of Plant Physiology. New Series, Vol. 12C, pp. 59–135. Springer-Verlag, Berlin.
37. Greenway H, Munns R. Mechanisms of salt tolerance in nonhalophytes. Ann Rev Plant Physiol. 1980; 31: 149–190.
38. Leopold AC, Willing RP. 1984. Evidence for toxicity effects of salt on membranes. In: Staples RC, Toenniessen GH (eds.). Salinity Tolerance in Plants. Strategies for Crop Improvement. pp. 67-76. A Wiley-Interscience Publication, Toronto, Singapore.
39. Uedo A, Yamato-Yamane Y, Takabe T. Salt stress enhanced proline utilization in the apical region of the barly roots. Biochem Biophys Res Commun. 2007; 355: 61-66.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Chemical composition and amino acid profile of *Sardinella longiceps* collected from Western coastal areas of Kerala, India

Sheril Ann Shaji, C.K. Hindumathy

V.M.K.V Engineering College, Salem, Tamil Nadu, India

ABSTRACT

The proximate, mineral composition, moisture content, fatty acid and amino acid profile of *Sardinella longiceps* has been investigated. Four different forms of same sample were taken for various analyses. They were sardine fresh meat (SFM), sardine cooked meat (SCM), sardine whole extract (SWE) and sardine cooked dried meat (SCDM). Amino acid profiles of the samples showed that cystine and arginine was lost due to oxidation and absorption in the SCM and SCDM samples, during the cooking process. The amino acid tryptophan was analyzed separately due to its tendancy to degrade in acidic medium. It was found that tryptophan was present in high level in the SCDM samples. The moisture content of SFM and SCM was 64.8% and 55.7% respectively. SWE and SCDM had negligible moisture content. The total crude protein content in SFM was little less than SCM. And protein content was 23.63% in SFM and incase of SDM, it was 29.31%. The crude protein content in SWE was 2.22% and in SCDM, it was negligible. The crude fat content in SFM was 41.25% and in SCM, it was 46.25%. No trace of crude fat content was seen in SWE, but SCDM showed an appreciable amount of 37.15% crude fat content. Ash content estimation of five different samples including of bone were done. The ash content was maximum (27.48%) in bone. The ash content in SFM, SCM and SCDM was 3.94%, 2.49% and 1.77% respectively. SWE showed negligible ash content. Potassium content was found to be maximum in all the samples except bone. Mineral study showed potassium content was maximum in all the samples except in bone, where percentage of calcium was analyzed as maximum.

Key words: *Sardinella longiceps*; Amino acid profiles; Proximate composition; Tryptophan.

J Biol Earth Sci 2013; 3(1): B129-B134

Corresponding author:
C.K.Hindumathy
Department of Biotechnology, V.M.K.V. Engineering College, Vinayaka Missions University, Salem-636 308, Tamil Nadu, India
E-mail: hindumathyck@rediffmail.com

Original Submission: 23 February 2013; Revised Submission: 11 April 2013; Accepted: 12 April 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Fishes are nutritious fooditems and are comparatively less expensive meat source available. Health benefits related to fish consumption are due to the presence of proteins, unsaturated essential fatty acids, minerals and vitamins. The poly unsaturated fatty acids (PUFA), especially ω-3 PUFA in fish are known for their hypocholestermic activity. These nutritional qualities have stimulated increasing demand for fish, worldwide.

Information on biochemical composition of fish finds application in several areas. Today people are more conscious about health foods and fish has got wide acceptance because of its special nutritional qualities. Fish and fishery products finds application as animal feeds. Formulation of such products needs proper data on the biochemical composition. Processing and preservation of fish and fishery products also need correct information on biochemical composition. Information on the biochemical constituents will enable a processing technologist to identify the best possible processing and storage conditions, so that the quality is preserved to the maximum degree.

The four major constituents in the edible portion of fish are water, protein, lipid (fat or oil) and ash (minerals). The analysis of these four basic constituents of fish muscle is often referred to as proximate analysis [1]. Even though data on proximate composition are critical for many applications and investigations on these lines had been carried out from as early as the 1880s [2]. Reliable data on proximate composition of most of the species of fish are difficult to obtain. Stansby [3] had observed that proximate composition was considered to be such an elementary sort of thing that it did not receive due attention from scientist. Even after 40 years, the situation is not different as far as many species of fish are concerned.

But this is not the only or basic reason for the absence of accurate and reliable data on biochemical composition of fish. Fishes are very heterogeneous and highly specialized group evolved through biochemical adaptation and evolution, consisting approximately of 24.000 species, showing extreme variations in size, shape, appearance etc. The habitat and food intake of these species are equally diverse. Some species are exclusively marine while some are confined to fresh water habitats. Some survive in marine as well as fresh water environments. Some marine species migrate to fresh water for spawming. The widely different environmental conditions of temperature, salinity, pressure, availability of food etc, profound influence on the biochemical composition. There may be group specific or even species difference in the biochemical composition. Even within a species, variations occur for individual fish or lots of fish taken at different times or under different conditions [3]. Another type of variation in proximate composition occurs between different parts of the same fish. There is generally an increase in the oil content of the muscle from the tail portion towards the head. Similarly the light and red muscle will vary in the biochemical composition. It is against this background that we have to view the data on the biochemical composition of fish. Data available in literature for the proximate composition of individual species will only indicate the range or average values and these are not usually taken as absolute values.

Today there is an ever increasing awareness about healthy food and fish is finding more acceptance because of its special nutritional qualities. In this context a proper understanding about the biochemical constituents of fish has become a primary requirement for the nutritionists and dieticians. The important health benefits of fish is due to the presence of polyunsaturated fatty acid (PUFA), hypocholestermic activity. But when we consider India, the consumption of these kind of fish are very less, since the nutritional value of such type of fish has not yet studied completely and deeply. Therefore it is necessary to elucidate the major biochemical components like proximate composition, fatty acid composition and cholesterol content. And here we made a comparative study of *Sardinella longiceps*. Hence the main objective of this present study is:

- to estimate amino acid and tryptophan composition
- to determine the proximate composition.

The outcome of this study is expected to help in the elucidation of exact nutritional constituent and the nutritive value available in fishes. Furthermore, it is presumed that when the people become aware of fish essential nutrients, it may result in the increased consumption of these kinds of fish in diet, ultimately escorting them for a

healthy life.

MATERIALS AND METHODS

Sample collection

Specimen selected for the present study were *Sardinella longiceps* collected from Western coastal areas of Kerala, India. Four different forms of same sample were taken for various analyses. They were sardine fresh meat (SFM), sardine cooked meat (SCM), sardine whole extract (SWE) and sardine cooked dried meat (SCDM).

Chemical analysis of samples

Analysis of amino acids except tryptophan was done by standard methods of HPLC. The amino acid tryptophan was analyzed separately due to its tendancy to degrade in acidic medium. Tryptophan was analyzed by standard spectrophotometry method [4].

Proximate analysis

In proximate analysis four major constituents of edible portion of the fish; water, protein, fat and ash was analyzed. Proximate composition was determined as per AOAC [4] method (official analysis for proximate composition documented by Association of Official Agricultural Chemists). The minerals like Na, K and Ca are estimated using flame photometer after dissolving the ash in dilute HCl. The reading is compared against standard solutions of the respective minerals.

RESULTS

Amino acid analysis

The amino acid content in different samples was analyzed. Each amino acid composition in SFM was comparatively more than other samples. Cysteine was totally absent in all the samples. Arginine was absent in SFM but was present in comparable large amount in other three samples. Of all the amino acids, percentage content of valine was maximum in SCM (Fig. 1). The amino acid tryptophan was analyzed separately due to its tendancy to degrade in acidic medium. It was found that tryptophan was present in high level in the SCDM samples (Fig. 2).

Proximate analysis

As a part of proximate composition, moisture content, crude protein content, crude fat content and ash content of the four different samples were analyzed. The moisture content of SFM and SCM was 64.8% and 55.7% respectively. SWE and SCDM had negligible moisture content. The total

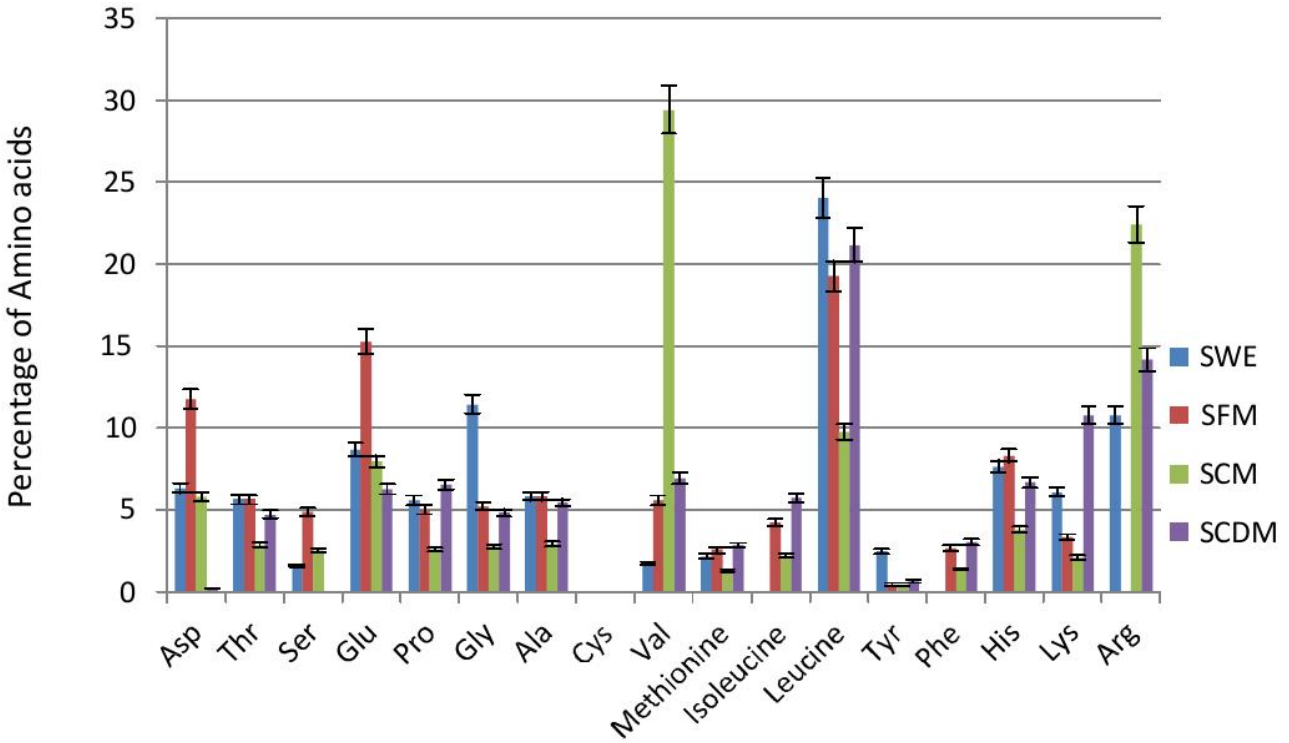


Fig. 1. Analysis of amino acids except tryptophan in SWE, SFM, SCM and SCDM.

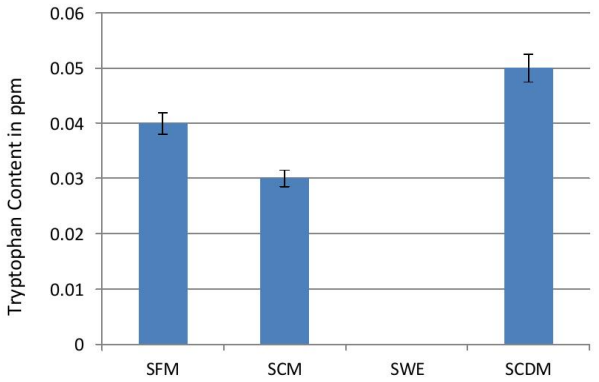


Fig. 2. Tryptophan content in SFM, SWE, SCM, SCDM.

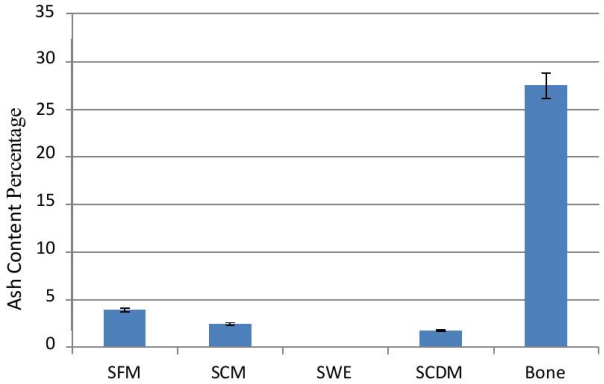


Fig. 3. Ash percentage in SFM, SWE, SCM, SCDM and in bone.

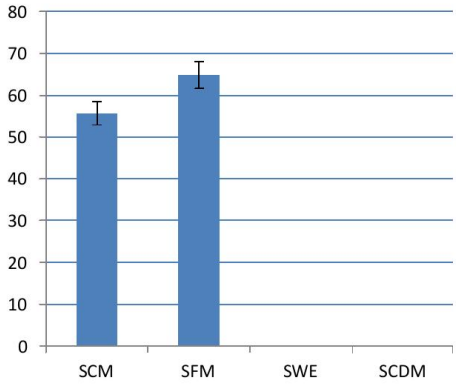


Fig. 4. Moisture content percentage in SCM, SFM, SWE, SCDM.

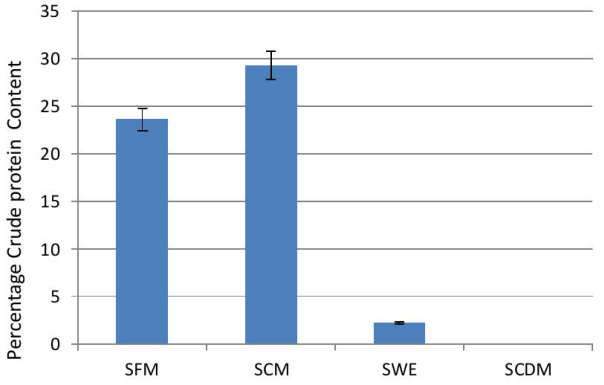


Fig. 5. Percentage of crude protein content in SCM, SFM, SWE, SCDM.

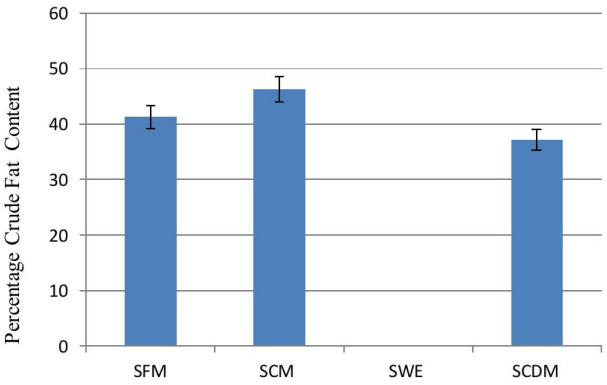


Fig. 6. Percentage of crude fat content in SCM, SFM, SWE, SCDM.

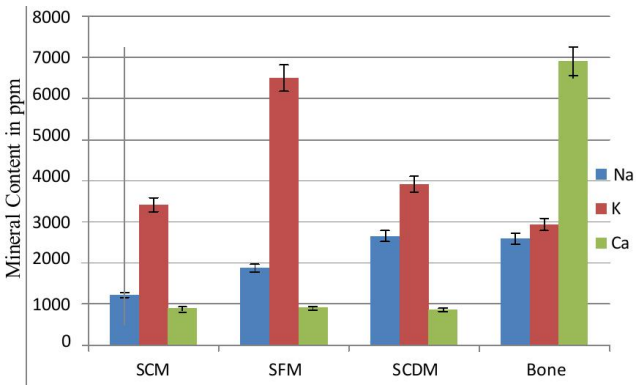


Fig. 7. Na, K and Ca content in SCM, SFM, SWE, SCDM.

crude protein content in SFM was little less than SCM. The crude protein content in SFM was 23.63 % and incase of SDM, it was 29.31%. The crude protein content in SWE was 2.22% and in SCDM, it was negligible. The crude fat content in SFM was estimated as 41.25% and in SCM, it was 46.25%. No trace of crude fat content was seen in SWE. But SCDM showed an appreciable amount of 37.15% crude fat content. Ash content estimation of five different samples including of bone were done. The ash content was maximum in bone with a percentage of 27.48. The ash content in SFM, SCM and SCDM was 3.94%, 2.49% and 1.77% respectively. SWE showed negligible ash content (Fig. 3-6).

Mineral concentration analysis

Sodium, potassium and calcium content was examined in SFM, SCM, SWE, SCDM, bone. Potassium content was found to be maximum in all

the samples except bone, where percentage of calcium was analyzed as maximum. Calcium content in other samples were comparatively less (Fig. 7).

DISCUSSION

The chemical composition of fish varies greatly from one species and one individual to another depending on sex, age, environment and season. Therefore a substantial normal variation is observed for the constituents of fish muscle.

During starvation periods, the fish uses the energy depots in the form of lipids and also may utilize protein, thus depletion of these reserves results in a general diminution of biological condition [5]. This condition will influence the quality of the different fish products and it will condition the yield and process efficiency. Therefore, the knowledge of proximate composition of fishery species has fundamental importance in the application of different technological processes [6-8].

The efficiency of a fish diet is dependent on its balance in relation to the specific requirements of the fish species. Nutrient requirement differences exist between species, particularly in relation to the amino acid composition [9]. Knowledge about the actual amino acid requirements of fishes would be very useful for an appropriate diet formulation and, as a consequence, a better growth performance, yield and higher nutritive value.

The knowledge of the amino acid, minerals and proximate composition for important fish species is desirable, due to the recent dietary and medical emphasis on the role of these nutrients in human health. Despite high demand, commercial value and wide availability of these fish species, there is a need to obtain precise data on their chemical composition because no such data is available for these fish in the study area where these fish are regularly consumed. This study is likely to open new areas of research as the knowledge of biochemical and proximate compositions of fish species is of fundamental importance in the application of different technological processes in fish preservation, processing and product development of high added values.

The ash content obtained in the bones was high. This is due to the high level of chitin strengthened by a high level of calcium metal in the exoskeleton of *M. vollenhovenii* and the shell of *T. fuscatus* [10] and the high ash content is of significance in measuring the mineral content of the species as the amount of ash shows the richness of the food in terms of element composition [11]. The moisture content of SFM and SCM was 64.8% and 55.7% respectively. SWE and SCDM had negligible moisture content [12]. Knowledge of the moisture content of foodstuff serves as a useful index of their keeping qualities and susceptibility to fungi infection and a low moisture content in both species is advantageous in terms of their shelf-life [13].

CONCLUSION

The present work revealed that the *Sardinella longiceps* is a good source of proteins, aminoacids and also significant amount of minerals.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Love RM. 1970. The chemical biology of fishes. Academic Press, New York. pp: 547.
2. Atwater WC. The chemical composition and nutritive value of food fishes and invertebrates. Rep US Commn Fish. 1988; 16: 679-868.
3. Stansby ME. 1962. Proximate composition of fish. In: Fish in Nutrition (Eds. E. Heen, R. Kreuzer) pp. 55-60, Fishing News Books Ltd, London.
4. Official methods of analysis of Association of Analytical Chemists (AOAC). 1990. 15th ed. Washington D.C.
5. Huss HH. Quality and quality changes in fresh fish. FAO Fish Tech Paper. 1995; 348-360.
6. Stansby ME. 1967. Fish oil's. Pabl. Co., New York.
7. Huss HA. Fresh fish quality and quality changes. FAO Fisheries. 1988; 29: 132.
8. Connell JJ. 1975. Control of fish quality. pp.180, Fish News Books ltd, England.
9. Portz L, Cyrino EP. Comparison of the amino acid contents of roe, whole body and muscle tissue and their A/E ratios for largemouth bass *Micropterus salmoides*. Aquacult. Res. 2003; 34: 585-592.
10. Adeyeye EI. Determination of the chemical composition of the nutritionally valuable parts of the male and female crab, *Sudananautes africanus africanus*. Int J Food Sci Nutr. 2002; 53: 189-196.
11. Hanan AT, Madlen MH, Hanaa MS. Residues of some

heavy metals and hormones in freshwater prawn (*Macrobrachium rosenbergii*) and marine shrimps (*Penaeus semisulcatus*) with reference to their nutritive value. World J Zool. 2009; 4(3): 205-215.

12. Omotoso OT. Chemical compositon and nutritive value of the land crab (*Cardisomaarmatum*). Afr J Appl Zool Environ Biol. 2005; 7: 68-72.
13. Bassey SCO, Eteng MU, Eyong EU, Ofem OE, Akunyoung EO, Umoh IB. Comparative nutritional and biochemical evaluation of *Ergeria radiata* (clams) and *Pomecia palludosa* (gastropods). Res J Agric Biol Sci. 2011; 7(1): 98-104.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Ecophysiological studies on three desert plants growing in Wadi Natash, Eastern Desert, Egypt

Suzan A. Sayed, Mohamed A. A. Gadallah, Fawzy M. Salama

Botany Department, Faculty of Science, Assiut University, Assiut, Egypt

ABSTRACT

Physiological adjustments to enhance tolerance or avoidance of drought were studied in three desert plants growing in Wadi Natash (Eastern Desert, Egypt). Studied plants (*Zilla spinosa* (L.) Prantl, *Citrullus colocynthis* (L.) Schrad and *Morettia philaeana* (Dalile) DC.) were collected from four stands. Cell sap osmotic potential, some organic (soluble sugars, total free amino acids, and soluble proteins) and inorganic (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-} and SO_4^{2-}) solute concentration were determined. In addition, cover degree of investigated species (according to the cover-abundance scale of Braun-Blanquet) was also estimated. Substantial osmotic adjustment (up to -1.71 MPa) was observed in *Z. spinosa* collected from stand 4. *Z. spinosa* was dependent on soluble sugars, soluble proteins, free amino acids, SO_4^{2-}, K^{+}, Cl^{-} and Mg^{2+} to readjust their internal osmotic pressure and to improve its water status. It preferred Mg^{2+} more than the two other species. *C. colocynthis* accumulated inorganic solutes more than *Z. spinosa* and less free amino acids. The results suggest that osmotic adjustment was the main water relationship adaptation to cope with drought. Accumulation of soluble sugars, soluble proteins, K^{+}, Cl^{-} and SO_4^{2-} at higher concentration often assist in turgor maintenance and helped to enhance drought tolerance.

Key words: Adjustment; Chlorophyll; Organic solute; Osmotic potential; Soluble sugars.

J Biol Earth Sci 2013; 3(1): B135-B143

Corresponding author:
Suzan A. Sayed
Botany Department, Faculty of Science, Assiut University, Assiut 71516, Egypt
Fax: 0020882342708
Phone: 00201066569682
E-mail: drsuzan1@hotmail.com

Original Submission: 04 March 2013; Revised Submission: 04 May 2013; Accepted: 07 May 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

Abbreviations: a.s.l. = above sea level, Chl = chlorophyll, OP = osmotic potential, SP = soluble proteins, SS = soluble sugars, TAA = total free amino acids, *C. colocynthis* = *Citrullus colocynthis*, *M. philaeana* = *Morettia philaeana*, *Z. spinosa* = *Zilla spinosa*.

INTRODUCTION

Most plants are exposed to water stress due to extreme soil water deficits in arid and semi arid environment. The survival of land plants in such areas relies on the availability of water and their adaptation under stress [1]. Drought resistance is a complex trait involving several interacting properties [2-4].

The adaptation in desert plants is due to their ability to maintain their turgidity and water uptake. The most important mechanism to maintain the plant water potential more negative than the external medium to insure the water uptake is the ability of plants to accumulate the inorganic solutes in high quantities inside their tissues [5-9]. The plants also tend to accumulate the most compatible solutes in cytoplasm to balance the osmotic pressure inside the cells, especially by increasing their content of organic solutes [7].

Wadis are the most widespread ecosystems in the mountainous desert of the world [10]. The wadi system is an extreme case of a temporary inundated ecosystem in which the duration of flooding is shorter than the dry period [11]. Various habitats can be identified in a wadi: channels, bars, banks, etc. [12]. A wadi system embraces all the biotypes and related biocenases found in arid or hyper arid zones [10, 13].

The Egyptian Eastern Desert extends eastwards from the Nile Valley up to Red Sea. It is located on an Eocene calcareous substratum more than 1000 m thick [14], at the junction of the Sahara and Arabian Desert. The origin of wadi courses can be understood in the context of palaeoclimatic history.

Zilla spinosa: blue-green dichotomously branched plant with stiff spinescent branches and soon deciduous fleshy leaves. Plant typically 50-60 cm high with 8-10 mm broad pod. *Z. spinosa* is very widely distributed in the Egyptian deserts. Many glycosides and volatile oil have been isolated from it's aerial parts. *Zilla spinosa* is used by the natives for the treatment (expels) of the kidney stones [15].

Citrullus colocynthis: prostrate, very scabrid herbs with long trailing branches. Young fruits fleshy mottled with dark-green, turning dry and yellow when ripe, extremely bitter in taste. *C. colocynthis* is a medicinal plant with edible seeds. It found wild on the sandy lands. Many glycosides and fixed oil have been isolated from it. Most parts of *C. colocynthis*, especially fruits are used, to treat numerous diseases [16].

Morettia philaeana: a perennial, yellowish green herb. Tomentose, with scabrous little stellate hairs. Stem 20-80 cm long. Leaves oblong-elliptic with short petiole. Flowers white and yellow inside. It is confined to stony and sandy wadis and plains, muddy and moist ground. It is used for grazing [17].

Ecophysiological studies have been powerful in elucidating plant function and identifying traits that are adaptive in specific environmental conditions [18]. Therefore, in this study we focus on some ecophysiological aspects of three of the most common dominant plants in Wadi Natash, Eastern Desert, Egypt to understand the possibility of osmotic adjustment as well as the physiological adaptional traits adopted by these plants to resist drought in desert environment. Accordingly, cell osmotic potential, some organic solutes (soluble sugars, total free amino acids, and soluble proteins) and inorganic solutes (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-} and SO_4^{2-}) were estimated to study their role in adjustment. In addition, cover degree of the species and physicochemical properties of Wadi Natash soil were studied.

MATERIALS AND METHODS

Study area

Wadi Natash is one of the main valleys in the south Eastern Desert (Fig. 1) emanating from the Red Sea Mountains to the west of the watershed i.e. draining generally towards the Nile Valley westward. The upstream side of the wadi lies at latitude 24º 40' N and longitude 34º 30' E at an average altitude of 450 m a.s.l. while reaches south of the elongated hill of Gebel El Nuqura an altitude of 230 m a.s.l. The wadi collects the water of many effluents which join it along its meandering eastern course for about 80 km.

Plant and soil sampling

Zilla spinosa (L.) Prantl, *Citrullus colocynthis* (L.) Schrad and *Morettia philaeana* (Dalile) DC. and the surrounding soil were collected from four stands (20×20 m) in Wadi Natash in the southern part of

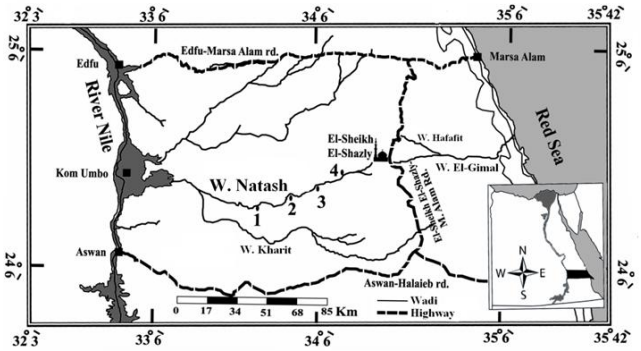


Fig. 1. Location map of sampling stands in Wadi Natash, Eastern Desert, Egypt.

Eastern Desert in January 2011. The species included in this work were selected according to two criteria: coverage and abundance. Samples in triplicate from different studied plants were collected and identified according to Täckholm [19] and Boulos [20].

In each stand the cover degree of investigated species was recorded according to the Braun-Blanquet approach [21].

Sample preparation and measurements

Plant shoots and soil samples were dried in an aerated oven at 70ºC for plants and 105ºC for soils to constant mass and the water content was calculated on dry weight [DW] for soil and on fresh weight [FW] for plants materials.

Different fractions of soil samples were separated by dry sieving methods [22].

Water extracts (1:5 ratio with air dry soil) were prepared to meet the requirement for different determination.

The oven dry plant sample was ground and powdered, to pass a 60 mesh screen and kept in a desiccator. Exactly, 0.5 g powder sample was taken and extracted in 25 ml distilled water by heating at 90ºC in a water bath for two hours and centrifuged at 2000 r.p.m. for 15 minutes.

The osmotic potential of shoot extracts was measured by using 800 Cl Osmometer.

Chlorophyll a and b contents were measured spectrophotometrically [23]. Soluble sugars were determined according to Buysse and Merckx [24].

In the plant extract soluble proteins and total free amino acids were determined according the procedures described by Lowry et al. [25] as well as Lee and Takahashi [26], respectively.

Sodium and potassium were analyzed by flame technique. In this respect flame photometer M7D was used. Chloride content was determined by $AgNO_3$ titration methods as described by Johnson and Ulrich [27]. Calcium and magnesium were determined volumetrically by versene titration method [28]. Sulphate contents were determined by a turbidemetric technique as $BaSO_4$ using barium chloride and acidic sodium chloride solution according to Bardsley and Lancaster [29].

The significant differences between the plants in response to collection site differences were determined by analysis of variance [30].

RESULTS

Soil analysis

Physicochemical characters of Wadi Natash soil were represented in table 1. The pH values fluctuated in the basic range. Generally non significant differences in soil pH due to location changes were noticed. The lowest pH value was recorded at stand 1 and the highest was at stand 3. Electric conductivity values ranged between 24.83 $mScm^{-1}$ at stand 3 and 26.97 $mScm^{-1}$ at stand 4.

The soil texture was sand, clay and silt loam in the four collection stands. The percentages of organic matter were 1.13, 1.15, 1.03 and 1.00% at stand 1, 2, 3 and 4, respectively. Soil water content varied from 0.53% at stand 2 to 2.09 % at stand 4. Altitude of 403 m.a.s.l. was recorded at stand 4 but the lowest altitude (251 m a.s.l.) was at stand 1.

Contents of Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, and SO_4^{2-} in the soil were illustrated in table 1. According their concentrations, the mineral elements were arranged in the following order: $Cl^- > Mg^{2+} > Ca^{2+} > Na^+ > K^+$.

Generally, Na^+ content were higher than those of K^+. The maximum value of Na^+ was recorded at stand 3 but the minimum value was detected at stand 2. Potassium content ranged from 0.022 mg g^{-1} DW at stand 2 to 0.042 mg g^{-1} DW at stand 1.

Calcium contents were lower than those of Mg^{2+} (stand 1 was an exception) and were fluctuated according to the collection stand differences. Their contents ranged from a minimum of 0.133 mg g^{-1} DW at stand 4 to 0.233 mg g^{-1} DW at stand 1. The highest Mg^{2+} content was recorded at stand 2 but the minimum value was noticed at stand 1. The lowest chloride content (0.824 mg g^{-1} DW) was recorded at stand 3 and the highest value (1.269 mg g^{-1} DW) was at stand 4. Sulphate contents ranged from a minimum of 0.35 mg g^{-1} DW at stand 2 to a maximum of 4.90 mg g^{-1} DW at stand 1.

Table 1. Physicochemical properties of soil samples from different stands in Wadi Natash. Data are means of three replicates ± SE.

Parameters	Stand 1	Stand 2	Stand 3	Stand 4
Gravel [%]	0.15±0.01	6.78±0.21	6.78±0.31	6.78±0.28
Coarse sand [%]	2.24±0.12	26.71±1.21	13.72±0.98	22.37±1.34
Fine sand [%]	6.80±0.36	14.91±0.93	7.65±0.52	18.37±0.83
Silt [%]	67.05±1.25	35.38±1.48	52.74±1.93	48.71±1.42
Clay [%]	23.70±0.93	11.22±0.64	20.73±1.11	5.27±0.21
WC [%]	0.68±0.02	0.53±0.01	0.73±0.03	2.09±0.04
OM[%]	1.13±0.12	1.15±0.09	1.03±0.06	1.00±0.03
pH	7.75±0.21	7.93±0.22	8.04±0.19	7.97±0.18
EC [$mScm^{-1}$]	25.17±1.32	25.17±0.93	24.83±0.85	26.97±0.63
K^+	0.042±0.001	0.022±0.002	0.024±0.001	0.029±0.001
Na^+	0.063±0.002	0.050±0.001	0.070±0.000	0.054±0.001
Ca^{2+}	0.233±0.01	0.200±0.01	0.167±0.02	0.133±0.02
Mg^{2+}	0.060±0.01	0.420±0.04	0.320±0.02	0.280±0.02
Cl^-	0.881±0.02	0.940±0.03	0.824±0.02	1.269±0.06
SO_4^{2-}	4.90±0.12	0.35±0.01	0.86±0.02	3.19±0.09

Explanations: WC – water content, OM – organic matter, EC – electric conductivity. Contents of ions were expressed in mg g^{-1} DW.

Table 2. F-values for physicochemical properties of soil samples from different stands in Wadi Natash.

	pH	EC	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	WC
Soil	4.05	4.77	3.02	2.79	2.88	46.02**	27.41**	2.79	17.56**

Explanations: EC – electric conductivity, WC – water content, ** – significant at 1% confidence level.

The effects of collection stand differences on the mineral contents as indicated by F value (table 2) were statistically significant for Mg^{2+}, Cl and soil water content.

Plant analysis

The cover degree estimation for the three studied species (table 3) show that the share of these species varied with stands. *Z. spinosa* and *C. colocynthis* were the dominant plants with high coverage at stands 1-3, but at stand 4 *C. colocynthis* was not recorded.

Chlorophyll a and b contents were higher in *C. colocynthis* than in the two other studied plants (table 4). Chlorophyll a content was varied from 0.27 mg g^{-1} FW of leaves in *Z. spinosa* at stand 2 to 1.35 mg g^{-1} FW in *C. colocynthis* plants grow at stand 1.

Shoot water content (table 4) showed great variation with collection stand difference in *C. colocynthis* and *Z. spinosa* but in *M. philaeana* the change in water content was slight (stand 4 was an exception). In general, plants growing at stand 4 had higher water content than those collected from the other stands. The effects of collection stand changes on the contents of chlorophyll and water status (as indicated by analysis of variance table 5) were statistically significant for the three studied plants.

The studied plants show a clear response in their osmotic potential to their arid environment (table 4). The highest shoot osmotic potential

Table 3. The cover degree of investigated plant species in four stands of Wadi Natash.

Species	Stand 1	Stand 2	Stand 3	Stand 4
Zilla spinosa	4	3	4	3
Morettia philaeana	2	2	1	2
Citrullus colocynthis	3	3	3	-

Table 4. Changes in chlorophyll (Chl a, Chl b) contents (mg g^{-1} FW of leaves), shoot water content (WC, %), osmotic potential (OP, MPa), soluble sugar (SS), soluble protein (SP) and total free amino acid (TAA) contents (mg g^{-1} DW) of *C. colocynthis*, *M. philaeana* and *Z. spinosa* plants. Data are means of three replicates ± SE.

Parameters	Species	Stand 1	Stand 2	Stand 3	Stand 4
Chl a	*Citrullus*	1.35±0.02	0.85±0.02	0.89±0.03	-
	Morettia	1.29±0.03	0.21±0.01	0.47±0.02	0.75±0.02
	Zilla	0.87±0.01	0.27±0.01	0.40±0.01	0.88±0.03
Chl b	*Citrullus*	0.52±0.01	0.17±0.01	0.80±0.02	-
	Morettia	0.39±0.01	0.14±0.01	0.13±0.01	0.12±0.02
	Zilla	0.84±0.02	0.19±0.01	0.23±0.02	0.36±0.03
WC	*Citrullus*	64.63±1.02	76.28±2.01	86.04±2.03	-
	Morettia	76.20±0.93	97. 56±1.93	75.83±2.11	87.31±2.12
	Zilla	62.03±1.11	72.91±1.32	87.25±1.98	93.55±1.99
OP	*Citrullus*	- 1.37±0.04	- 1.20±0.05	- 1.25±0.01	-
	Morettia	- 1.34±0.03	- 1.21±0.01	- 1.23±0.02	- 1.45±0.03
	Zilla	- 1.32±o.o2	- 1.28±0.02	- 1.24±0.03	- 1.71±0.02
SS	*Citrullus*	27.14±2.11	23.27±1.99	68.72±2.32	-
	Morettia	7.83±0.98	13.57±0.85	5.33±0.12	5.80±0.20
	Zilla	23.27±1.25	19.73±0.74	7.99±0.56	11.83±0.32
SP	*Citrullus*	44.21±2.01	36.38±0.99	62.20±2.73	-
	Morettia	30.91±1.98	31.96±1.23	25.90±1.85	37.17±1.05
	Zilla	35.94±2.11	47.11±2.16	50.38±0.96	69.81±2.13
TAA	*Citrullus*	3.18±0.1	2.49±0.09	7.50±0.21	-
	Morettia	8.22±0.25	2.87±0.06	8.15±0.19	17.97±1.04
	Zilla	5.66±0.22	4.08±0.05	8.89±0.23	15.33±0.99

reached about -1.71, -1.45 and -1.37 MPa in *Z. spinosa*, *M. philaeana* and *C. colocynthis*, respectively. The lowest osmotic potential was recorded in *C. colocynthis* at stand 2.

In *M. philaeana* soluble sugar content (table 4) showed limited range of variation in response to location changes (stand 4 was an exception). Mostly non significant differences in soluble sugars were noticed between plants collected from different stands as indicated by analysis of variance (table 5). *C. colocynthis* had higher soluble sugars than the two other species at all study stands.

Soluble proteins contents (table 4) ranged between 25.90 mg g^{-1} DW in *M. philaeana* at stand 3 and 69.81 mg g^{-1} DW in *Z. spinosa* at stand 4. Total free amino acids (table 4) were generally lower than soluble sugars and soluble proteins. Its content fluctuated between 2.49 mg g^{-1} DW in *C. colocynthis* and 17.97 mg g^{-1} DW in *M. philaeana*.

The major elements contents in the studied

Table 5. F-values for chlorophyll (Chl a, Chl b), shoot water content (WC), osmotic potential (OP), soluble sugars (SS), soluble proteins (SP), and total free amino acids (TAA), Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-} in three desert plants (*C. colocynthis*, *M. philaeana* and *Z. spinosa*) sampled from different stands in Wadi Natash.

Parameters	*Zilla spinosa*	*Morettia philaeana*	*Citrullus colocynthis*
Chl a	101.03**	18.32*	73.62**
Chl b	57.78**	138.93**	12.33*
WC	137.32**	68.98**	453.91**
SS	27.16**	4.99	22.82**
SP	23.94**	4.88	6.81
TAA	15.51**	149.19**	46.02**
Na^+	7.38	2.58	250.00**
K^+	19.32**	12.35*	62.89**
Ca^{2+}	22.20**	4.16	264.58**
Mg^{2+}	24.71**	6.35	6.90
Cl^-	199.39**	0.41	0.90
SO_4^{2-}	0.08	64.47**	0.59
OP	12.18*	12.09*	10.28*

Explanations: * - significant at 5% confidence level; ** - significant at 1% confidence level.

Table 6. Contents of Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-} (mg g^{-1} DW) of *Citrullus colocynthis*, *Morettia philaeana* and *Zilla spinosa* plants sampled from different stands in Wadi Natash. Data are means of three replicates ± SE.

Parameters	Species	Stand 1	Stand 2	Stand 3	Stand 4
Na^+	*Citrullus*	1.50±0.02	1.50±0.01	2.00±0.03	-
	Morettia	1.33±0.04	3.17±0.06	1.50±0.01	1.67±0.02
	Zilla	1.00±0.05	2.00±0.04	0.50±0.01	3.00±0.06
K^+	*Citrullus*	23.37±1.34	15.60±0.89	31.23±1.21	-
	Morettia	24.67±1.56	18.12±0.96	20.25±1.03	40.00±2.01
	Zilla	21.80±1.11	22.50±1.12	24.57±0.99	55.80±2.23
Ca^{2+}	*Citrullus*	19.33±1.23	20.00±0.84	6.00±0.12	-
	Morettia	30.33±1.96	23.67±1.02	24.67±2.05	33.00±1.42
	Zilla	14.33±0.68	9.67±0.73	17.67±1.63	40.33±1.98
Mg^{2+}	*Citrullus*	9.60±0.56	9.40±0.62	5.40±0.42	-
	Morettia	13.80±0.98	4.80±0.31	9.00±0.68	13.20±0.69
	Zilla	8.93±0.54	2.60±0.25	7.40±0.37	4.07±0.28
Cl^-	*Citrullus*	38.46±2.03	28.99±1.42	31.95±1.02	-
	Morettia	20.71±1.98	18.34±0.93	21.30±1.11	23.08±1.11
	Zilla	9.47±0.63	23.96±1.50	12.43±0.75	47.90±2.32
SO_4^{2-}	*Citrullus*	85.79±2.35	116.19±3.11	100.02±2.51	-
	Morettia	124.66±3.42	105.60±2.03	137.67±2.86	113.72±1.42
	Zilla	104.23±1.98	100.34±2.15	118.94±2.01	107.43±1.89

plants were illustrated in table 6. Sodium concentration ranged between 0.4 mg g^{-1} DW (in *Z. spinosa* at stand 3) and 3.17 mg g^{-1} DW (in *M. philaeana* at stand 2). Both *Z. spinosa* and *M. philaeana* recorded high accumulation value of K^+. The contents of K^+ fluctuated from 15.6 mg g^{-1} DW in *C. colocynthis* (stand 2) to 55.08 mg g^{-1} DW in *Z. spinosa* at stand 4.

The range of Ca^{2+} (table 6) varied up to six-fold with values between 6.00 mg g^{-1} DW in *C. colocynthis* at stand 3 to 40.33 mg g^{-1} DW in *Z. spinosa* at stand 4. *M. philaeana* and *Z. spinosa* showed high contents of Ca^{2+} than *C. colocynthis*. Magnesium content showed limited range of variation in response to collection stand changes. Mostly non significant differences in Mg^{2+} contents were noticed in *M. philaeana* and *C. colocynthis* plants collected from different stands (table 5). On the contrary, contents of Ca^{2+} were significantly affected by collection stand differences in *Z. spinosa*.

The minimum and the maximum chloride values (table 6) were recorded in *Z. spinosa* collected from stand 1 and 4, respectively. According to the main Cl values of all stands the studied plants can be arranged in the following order:

C. colocynthis > *M. philaeana* > *Z. spinosa*.

Although the SO_4^{2-} contents (table 6) in the soil were small, the studied plants showed high content of SO_4^{2-}. The range of SO_4^{2-} showed great variation with a minimum of 100.02 mg g^{-1} DW in *C. colocynthis* at stand 3 and a maximum of 157.67 mg g^{-1} DW in *M. philaeana* in the same stand.

DISCUSSION

To overcome the external stresses as salinity or water deficiency, the plants tend to readjust their internal osmotic pressure [9]. Osmotic adjustment is considered as one of the most important adaptations of plants to drought, because it allows plants to maintain water absorption, cell turgor and metabolic activity during periods of drought stress, and also enables quick resumption of growth when water becomes available again [4, 31].

The adaptation of the three investigated plants to the arid environment in term of osmotic adjustment was documented in this study. Substantial osmotic adjustment (up to -1.71 MPa) was observed in *Z. spinosa*. To overcome the soil water deficiency, the plants tend to reduce their internal osmotic potentials through accumulation of osmotically active metabolites (e.g. soluble sugars), inorganic solutes (K^+, Ca^{2+}, Mg^{2+}, and Cl^-) and improved water retention properties through the accumulation of soluble proteins (table 4). According to Boscalu et al. [32] the amount of bound water depends on the availability of organic solutes. Organic solutes, especially soluble sugars played more important role in drought adaptation in the xerophytes [33]. In the three studied desert species, SO_4^{2-} ions were accumulated in high concentration compared with Na^+. *Z. spinosa* was dependent mainly on inorganic solutes in their osmotic adjustment. Therefore its contents of K^+, Ca^{2+} and Mg^{2+} were higher than in the other plants. *C. colocynthis* dependent upon Cl^- as anionic osmotical while *M. philaeana* dependent on Ca^{2+}.

Organic solutes known as compatible solutes include amino acids, sugars glycerol and other low molecular weight metabolites, serve a function in cells to lower or balance the osmotic potential of intracellular and extracellular ions in resistance to osmotic stress [34]. Our data showed that *Z. spinosa* and *C. colocynthis* had higher soluble sugars content and chlorophyll content than *M. philaeana*. The higher accumulation of soluble sugars with corresponding higher chlorophyll content means that the increase in soluble sugars was the results of higher photosynthetic activities. The higher soluble sugars concentration may be an adaptive response which involves adjustment of osmotic potential that facilitates the maintenance of favourable water balance [6, 9, 33, 35].

Soluble protein contents in the three studied plants were higher than the total free amino acids. Protein accumulation in leaves and roots is associated with improved drought tolerance [36]. In *C. colocynthis* plants, the total free amino acids content reduced notably under drought. Therefore, amino acids may be not the major osmotic solute in the osmotic adjustment of this species in drought environment. On the contrary, total free amino acid contents were higher in *M. philaeana* and *Z. spinosa* collected from stand 4 than in those collected from other stands. Accumulation of free amino acids under such conditions can be explained by enhancement proteolysis of proteins, inhibition of amino acid incorporation in protein synthesis or both [37]. Accumulation of amino acids under water stress may be actually a part of an adaptive process contributing to osmotic adjustment and has been taken as an index for determining the

drought tolerant potential of many plants [38-40].

According to the specific mechanisms of mineral absorption and utilization, the ability of the study plants to absorb and accumulate ions at different extent appears well documented in this research. In general contents of SO_4^{2-} were higher than the other estimated minerals at all collected stands. K^+ largely accumulated in the shoots of the studied plants collected from different stands (table 6) may be to avoid Na+ toxicity. On the contrary, Na+ contents were lower than Ca^{2+}, Cl^- and SO_4^{2-} contents at all collection stands. *Z. spinosa* preferred Mg^{2+} more than *C. colocynthis* and *M. philaeana*. Chlorides were higher in *M. philaeana* and *Z. spinosa* growing at stand 4 than in those in the other stands. Such variation in mineral accumulation at different stands indicate the ability of the study plants to regulate the uptake and accumulation of the elements from the external source according to their adjustment requirements. This means that ions are important in the generation of osmotic potential in the studied xerophytes plants.

The water status and cell turgidity are the most important feature for plants especially desert plants, to ensure the biological processes. In our study, the maintenance of relatively high water content despite the development of low water potential appears to be a common trait in studied plants. Accumulation of soluble sugars and some mineral ions (tables 4 and 6) and the strong dehydration action of sulphates content on the cell proteins [30] often assist in turgor maintenance.

CONCLUSION

In conclusion, the present study gives a good idea about the physiological behaviour of three of the most common dominant plants in Wadi Natash, Eastern Desert, Egypt. The results indicated that to overcome the external stress in the arid environment the studied plants tend to re-adjust their internal osmotic pressure through accumulation of inorganic and organic solutes. The differences in the concentration of the measured elements are not attributed to the composition of the soil in which the plants grow, but may depend on the interactions of elements or the plant genotype. *Z. spinosa* is the most tolerant plants to drought than the two other species and they are favourable to the conditions of the arid desert.

ACKNOWLEDGEMENTS

The authors are indebted to Prof. Dr. Moustafa Youssef of the Geological Department, Faculty of Science, Assiut University for consultations in geological data.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Kramer PJ. 1984. Problems in water relations of plants and cells. In: International Review of cytology. Ed. Kramer PJ. pp. 254-286.
2. Aziz I, Khan A. Proline and water status of some desert shrubs before and after rain. Pak J Bot. 2003; 35: 902-906.
3. Martnez J, Kinet J, Bajji M, Lutts S. NaCl alleviates polyethylene glycol-induced water stress in the halophyte species *Atriplex halimus* L. J Exp Bot. 2005; 62: 2421-2431.
4. Scholz FG, Bucci SJ, Arias N, Meinzer FC, Goldstein G. Osmotic and elastic adjustments in cold desert shrubs differing in rooting depth: coping with drought and subzero temperatures. Oecologia. 2012; 170: 885-897.
5. Kan MA, Ungar IA, Showalter AH. The effect of salinity on growth, water status and ion content of leaf succulent perennial halophytes, *Suaeda fruticosa* (L.) Forssk. J Arid Environ. 2000; 45: 73-84.
6. Gadallah MAA, Sayed SA, Salama FM. Some metabolic aspects of *Zygophyllum coccineum* (L.) growing in different habitats in Eastern Desert, Egypt. Bull Fac Sci, Assiut University. 2001; 30 (I-D): 33-42.
7. Mile O, Meszaros J, Verses SZ, Lakatos G. Ecophysiological study on the salt tolerance of a Pannonia endemism (*Lepidum cressifollum*) in inland saline area. Acta Biol Szeg. 2002; 46: 249-250.
8. Sheded MG, Pulford ID, Hamed AI. Presence of mahor and trace elements in seven medicinal plants growing in the South-Eastern Desert, Egypt. J Arid Environ. 2006; 66: 210-217.
9. Kamel M. Osmotic adjustment in three succulent species of Zygophyllaceae. Afric J Ecol. 2007; 46: 96-104.
10. Fossati J, Pautou G, Paul-Peltier J. Water as resource and disturbance for wadi vegetation in a hyperarid (Wadi Sannur, Eastern Desert, Egypt). J Arid Environ. 1999; 43: 63-77.
11. Evenari M. 1985. Adaptation of plants and animals to the desert environment. In: Hot Desert and Arid Shrublands. Eds. Evenari M, Noy-Meir I, Goodall DW.

Amsterdam, Elsevier.
12. Abdel Rahman AA, Batanouny KH. The water output of the desert vegetation in different microhabitats in Wadi Hoff. J Ecol. 1965; 53: 267-272.
13. Salama FM, Kamel M, El-Tayeh NA, Hammad SA. Vegetation analysis, phenological patterns and chorological affinities in Wadi Qena, Eastern Desert, Egypt. Afric J Ecol. 2012; 50: 193-204.
14. Said R. 1990. The Geology of Egypt Balkema. pp. 734.
15. El-Toumy SA, EL Sharabasy F, Ghanem H, El Kady M, Kassem A. Chemical constituents and pharmacological activities of *Zilla spinosa*. Planta Med. 2011; 77: PM51.
16. Ziyyat A, Legssyur A, Mekhfi H, Dassouli A, Sehrrouchni M, Benjelloun W. Phytotherapy of hypertension and diabetes in oriental morocco. J. Ethnopharmacol. 1997; 58: 45-54.
17. Mahmoud T. 2010. Desert plants of Egypt's Wadi El Gemal National Park. The American University in Cairo Press. pp 163.
18. Ackerly DD, Dudley SA, Sulton SE, Schmitt J, Coleman JS, Linder CR. The evolution of plant ecophysiological traits: recent advances and future directions. Bioscience 2000; 50: 979-995.
19. Täckholm V. 1974. Student Flora of Egypt, 2nd edition: pp. 888. Cairo, University Publishing.
20. Boulos L. 2005. Flora of Egypt. IV vol. pp. 617. Cairo, Al Hadara Publishing.
21. Braun-Blanquet J. 1964. Pflanzensoziologie. pp. 865. Wien, Springer-Verlag.
22. Ryan P J, McKenzie N J, Loughhead A, Ashton L. 1996. New methods for forest soil surveys. In: The role of *Eucalyptus* and other fast growing species. Eds. Eldridge KG, Crowe MP, Olds KM. Csiro Publishing, Collingwood, Victoria.
23. Wellburn AR. The spectral determination of chlorophyll a and b, as well as total caretenoids, using various solvent with spectrophotometers of different solution. J Plant Physiol. 1994; 144: 307-313.
24. Buysse J, Merckx R. An improved colorimetric method to quantify sugar content of plant tissue. J Exp Bot. 1993; 44: 1627-1629.
25. Lowry CH, Rosebrough NJ, Farr AL, Bandall HJ. Protein measurement with the folin phenol reagent. J Biol Chem. 1951; 193: 265-275.
26. Lee YP, Takahashi T. An improved colorimetric determination of amino acids with the use of ninhydrin. Anal Biochem. 1966; 14: 71-77.
27. Johnson C M, Ulrich A. 1959. Analytical methods for use in plant analysis. pp. 766. U.S. Department of Agriculture, California University.
28. Schwarzenbach G, Biederman W. Komplexonex, Erdalklikomplexe van 6- Dioxyazofarbstoffen. Helv Chem Acta. 1948; 31: 678-687.
29. Bardsley CE, Lancaster JD. 1965. Sulfur. In: Methods of Soil Analysis, Part 2. Eds. Black CA, Evans DD, White JL, Jusminger LE, Clark FE. pp. 1102-1116. Madison, Amer Soc Agron.
30. Ostle B. 1963. Statistics in Research. pp. 585. Iowa State University Press.
31. Fahmy G M, Swaf N A. Aspects of leaf senescence in desert plants. 1. Ionic composition, carbohydrates and total protein of green and senescing leaves of four species in zygophyllaceae. J Edu Fac Ain Shams Univ. 1992; 17: 101-116.
32. Boscalu M, Fola O, Scridon S, Liinares J , Vicente O. Osmolyte accumulation in xerophytes as response to environmental stress. UASVM Hort Bull. 2009; 66: 96-102.
33. Paleg LG, Stewart GR, Bradbeer JW. Proline and glycinebetaine influence protein salvation. Plant Physiol. 1984; 75: 974-978.
34. Alkhail MSA, Moftah AE. Adaptation mechanisms of some desert plants grown in central region of Saudi Arabia. Int Res J Agri Sci Soil Sci. 2011; 1(11): 462-470.
35. Chen H, Jiang J. Osmotic adjustment and plant adaptation to environmental changes related to drought and salinity. Environ Rev. 2010; 18: 309-319.
36. Premachandrea GS, Saneoka H, Fujita K, Otaga S. Osmotic adjustment and stomatal response to water deficits in maize. J Exp Bot. 1992; 43: 1451-1456.
37. Merewitz EB, Gianfagna T, Huang B. Protein accumulation in leaves and roots associated with improved drought tolerance in creeping bentgrass expression an ipt gene for cytokinins synthesis. J Exp Bot. 2011; 62: 5311-5333.
38. Duby RS. 1994. Protein synthesis by plants under stressful conditions. In: Handbook of Plant and Crop Stress. Ed. Pessarki M. pp. 277-299. New York. Marcel Decker Inc.
39. Ramanjulu S, Sudhakar MT. Drought tolerance is partly related to amino acid accumulation and ammonia assimilation. A comparative study in two mulberry genotypes differing in drought sensitivity. J Plant Physiol. 1997; 150: 345-350.
40. Gadallah MAA. Effects of water stress, abscisic acid and proline on cotton plants. J Arid Environ. 1995; 30: 315-325.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Role of antioxidant enzymes in amelioration of water deficit and waterlogging stresses on *Vigna sinensis* plants

Abdel E. El-Enany[1], Abdullah D. AL-Anazi[1], Nahla Dief[2], Wafa'a A. Al-Taisan[3]

[1]Department of Biological Sciences, Faculty of Science and Humanities, Shaqra University, Ad-Dawadimi, Saudi Arabia

[2]Botany Department, Faculty of Science, Assiut University, Assiut, Egypt.

[3]Department of Biology, Girls College of Science, University of Dammam, Dammam, Saudi Arabia

ABSTRACT

Global climate change predictions suggest new scenarios with larger arid areas and extreme climatologic events. Thus, it is essential to understand how plants respond to different abiotic stresses in order to improve crop performance. Legume plant *Vigna sinensis* used to understand the physiological responses in comparison to WD and WL. A pot experiment was carried out at climatic greenhouse in three replicates of 75% and 50% water deficit and one fold field capacity (WL). The data revealed that water deficit stress significantly decreased the fresh and dry weights of roots and shoots of tested plants especially at 50% FC and waterlogging. The number of nodules per plant was significantly decreased as a result of imposed to both levels (75% and 50% FC) of WD and WL and sequently nitrogenase activity was significantly decreased as a result of imposed to the WD levels (75% and 50% FC) and WL. The percent of decrease in nitrogenase activity of cowpea plants was 41%, 40% and 37%, respectively. The antioxidant metabolites like phenolic compounds, hydrogen peroxide and ascorbic acids were significantly increased under the both stresses. The activities of some antioxidant enzymes (catalase, ascobate peroxidase and super oxide dismutase) in both stresses were conducted.

Key words: Drought; Flooding; *Vigna sinensis*; Field capacity; Antioxidant enzymes.

J Biol Earth Sci 2013; 3(1): B144-B153

Corresponding author:
Abdel E. El-Enany
Department of Biological Sciences, Faculty of Science and Humanities, Shaqra University,
P.O. Box 1040 Ad-Dawadimi 11911 Saudi Arabia
E-mail: enany203@yahoo.com

Original Submission: 16 April 2013; Revised Submission: 08 May 2013; Accepted: 10 May 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

Abbreviations: APOX: ascorbate peroxidase (EC.1.11.1.11); CAT: catalase (EC.1.11.1.6); SOD: superoxide dismutase (EC 1.15.1.1); POX: peroxidase (EC. 1.11.17); TBA: thiobarbituric acid; WD: Water deficit; WL: Water-logging.

INTRODUCTION

Legumes are the main single source of vegetable protein in human diets and livestock feed, having major impacts on agriculture, environment and health. Due to the legumes capacity of symbiotic nitrogen fixation, these plants are often used to improve soil organic fertility and nitrogen economy [1, 2].

Drought, salinity, extreme temperatures (cold and heat) and oxidative stress are often interrelated; these conditions singularly or in combination induce cellular damage [3, 4]. Prolonged exposure to these abiotic stresses results in altered metabolism and damage to biomolecules [5, 6]. Water deficit is therefore the most important abiotic stress and strategies to sustainable use of water and improve plant drought resistance are urgent and should integrate conventional breeding and biotechnological approaches [7, 8].

Under waterlogged conditions, however, seeds imbibe rapidly, so that seed tissues are destroyed due to the abrupt swelling of the cells, which causes failure of normal germination [9, 10]. In addition, excess water blocks entry of oxygen into the soil, hindering aerobic respiration. When soil moisture is excessive, low oxygen concentrations in the soil inhibit the respiratory activity of germinating seeds, and thereby reduce the speed of germination. During this prolonged germination, seeds are likely to be infected with soil-born diseases, which further reduce the germination percentage. Soil flooding usually reduces plant growth by decreasing the availability of oxygen to roots [11-14]. Deficiency of oxygen, proposed as the main cause of flooding injury, accompanied with accumulation of carbon dioxide, ethylene, manganese, and reduced iron [15], causes premature senescence and leaf chlorosis, necrosis, defoliation, growth cessation, yield losses and death [14].

Antioxidants can control the levels of reactive oxygen species (ROS). ROS are typically produced at low levels during photosynthesis, but ROS concentrations have been shown to increase when plants experience stressful conditions (for a review see [1]). During deep floods, the rate of photosynthesis can be limited by lowered light levels and decreased access to CO_2 [16]. As a result, the photosynthetic electron transport chain (ETC) becomes over-reduced, causing the generation of several ROS, including hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH) and singlet oxygen ($1O_2$) [17]. ROS are also generated by over-reduction of the photosynthetic ETC in response to salt stress, when stomatal closure decreases the CO_2:O_2 ratio in the leaf tissue and inhibits CO_2 fixation [18]. Both water stress conditions (OT and WL) increase the rate of ROS production like superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH). The superoxide radicals and their dismutation product, hydrogen peroxide, can directly attack membrane lipids and also inactivate SH-containing enzymes [17, 19]. The hydroxyl radical, one of the most reactive oxygen species, is responsible for oxygen toxicity in vivo, causing damage to DNA, protein, lipids, and chlorophyll. Plants protect cellular and sub-cellular system from the cytotoxic effects of active oxygen radicals with antioxidative enzymes such as (SOD, CAT, AsA-dep POD and Gua-dep POD) as well as antioxidants of low molecular weight like ascorbic acid, tocopherol, and carotenoids.

The objectives of the present study were to determine the ascertain changes in nodulation and nitrogenase activity during the growth period of legume plant (*Vigna sinensis*) grown under water deficit (moderate and high) and waterlogging stress conditions with an emphasis on detecting changes in the activities of antioxidant substances, antioxidant enzymes such as SOD, CAT, AsA-dep and membrane lipid peroxidation levels (MAD).

MATERIALS AND METHODS

Plant material

Surface sterilized seeds of cowpea (*Vigna unguiculata* L.Walp.) were brought from Faculty of Agriculture, Assiut University, Egypt, as well as to study the interactive effect of different levels of field capacity as water deficit and water logging on growth and metabolic activities of cowpea plants. Experiments were conducted at open green house (from November 2009 to the middle of August 2010). Ten seeds were sown at 0.5 g pot^{-1} at a depth of 1.5 cm. The plastic pots containing 3 Kg clay treated as follow (4 pots were used for each

treatment). The plants were allowed to grow for 4 weeks till harvesting.

Measurements

At the early vegetative stage, after 30 days of planting, fresh plants were separated into shoots and roots. Fresh weights (FW) and DW of shoots and roots were recorded. The pigment fractions (chlorophyll a, chlorophyll b and carotenoids) were estimated using the spectrophotometric method recommended by [20]. Nitrogenase activity was determined on a detached root system in a closed system as described by Lichtenthaler [21]. The drained roots of each treatment were incubated with 5% (v/v) acetylene for 1 h at room temperature. Ethylene produced in the reaction bottle was analyzed by gas chromatograph (Thermo Scientific TRACE GC Ultra equipped with FID detector, Lab of nitrogen fixation in Botany Department, Faculty of Science, Assiut University).

Free and cell wall-bound phenolics were determined according to Kofalvi and Nassuth [22]. Phenolic concentration in the extract was determined from standard curve prepared with gallic acid. The level of lipid peroxidation in plant tissues was determined as 2-thiobarbituric acid (TBA) reactive metabolites, i.e. malondialdehyde (MDA) and the results expressed as µmol MDA/g [23]. The H_2O_2 content of the shoots and roots samples were colorimetrically measured as described by Jena and Choudhuri [24]. The concentration of H_2O_2 was calculated from a standard curve plotted with known concentration of H_2O_2 and expressed as mg/g FW. The ascorbic acid was determined according to Mukherjee and Choudhuri [25]. A standard curve was prepared by different concentrations of ascorbic acid. Proline content was determined according to Bates et al. [26]. Proline concentration was determined using calibration curve of l-proline and expressed as mg/g DW.

Enzyme activity assays

Leaves tissues (0.5 g) were ground to a fine powder in liquid N2 then homogenized in 5 ml of 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM ethylenediamine tetraacetic acid (EDTA) and 0.1g polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 18.000 rpm for 10 min at 4°C and the supernatants were collected and used for assessing the activity of antioxidative enzymes.

Superoxide dismutase (EC 1.15.1.1) activity was determined by the Crosti method [27]. One unit of enzyme activity is defined as 50% inhibition of 6-hydroxydopamine (6-OHDA) auto oxidation under assay conditions.

Ascorbate-dependent peroxidase activity (EC 1.11.1.11) was measured according to Nakano and Asada [28] by monitoring the rate of ascorbate oxidation at 290 nm ($E = 2.8\ mM^{-1}\ cm^{-1}$). The reaction mixture contained 25 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 1 mM H_2O_2, 0.25 mM AsA and the enzyme sample. No change in absorption was found in the absence of AsA in the test medium.

Catalase (EC 1.11.1.6) activity was assayed in a reaction mixture containing 25 mM phosphate buffer (pH 7.0) 10.5 mM H_2O_2 and enzyme in 25 mM phosphate buffer (pH 7.0). The decomposition of H_2O_2 was followed at 240 nm ($E = 39.4\ mM^{-1}\ cm^{-1}$) [29]. Protein concentration in the enzyme extract was determined by the method of Lowry et al. [30].

RESULTS

The effectiveness of water logging and water deficit on shoots and roots growth of cowpea plants presented in Fig. 1. The data revealed that water stress significantly decreased the fresh and dry weights of roots and shoots of tested plants especially at moderate WD level. The total biomass of cowpea plants was significantly decreased under the both stresses (water logging and high water deficit). However, fresh and dry weights of root and shoot of cowpea plants were significantly decreased under high water deficit (50% FC.) more than low water deficit (75% FC.) and flooding. The dry weight of the roots was also higher in flooded plants and this occurs at least in part, because of the higher production of new lateral roots under flooding condition on the sub merged part of shoot under water compare to control plant according to Fig. 2. The stress conditions in the order of reductions in FW and DW of roots and shoots of cowpea plants were high WD (50%) < WL <WD (75%).

The present study demonstrated that, photo-synthetic pigments were significantly affected in plants under water stress as compared to control plants (Fig. 2). Data indicated that Chl a contents of cowpea plant leaves decreased under water stress, especially at flooding stress. Chlorophyll a and b content were significantly raised under both water

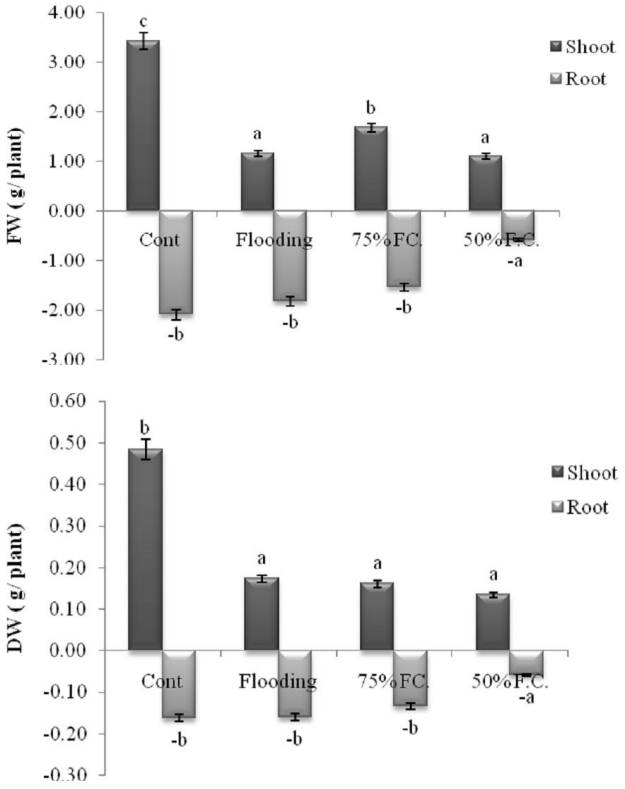


Fig. 1. Reductions in fresh wt (A) of cowpea shoots and roots and dry wt (B) of shoots and roots of cowpea plants under WL, WD, low (75%), high (59%) and control. Data are the average of four replicates. Different letters above the columns indicate significant differences between treatments at P<0.01.

deficit (50% and 75% FC) levels. While, water-logging sharply decreased contents of Chl a and b. The data also showed that, carotenoids content was significantly higher in leaves of cowpea under water deficit and more affected under water logging (Fig. 2). The stress conditions in the order of reductions in pigment fractions contents of cowpea leaves were WL < WD (95%) < WD (50%).

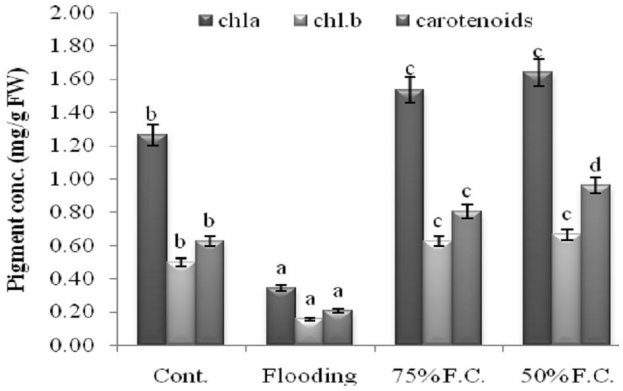


Fig. 2. Photosynthetic pigments content (mg g^{-1} fresh wt.) in cowpea leaves under WL, WD, low (75%), high (59%) and control. Data are the average of four replicates. Different letters above the columns indicate significant differences between treatments at P<0.01.

The data in Table 1 revealed that number of nodules per plant was significantly decreased as a result imposed to the level drought (75% and 50% FC) and water logging. The lowest value of number of nodules of cowpea plants was observed under 50% water deficit. Also, the data in Table 1, revealed that nodule legheamoglobin content was significantly decreased as a result of imposed to the levels of drought (75% and 50% FC), the percentage of decrease in nodule leg hemoglobin content of cowpea plants was 63% and 45%, respectively. The data also showed that, nodule leg hemoglobin content was significantly decreased in cowpea plants under flooding treatment (2 FC) by about 54%. According to the reduction in nodule number and leghaemoglobin content the nitroge-nase activity was significantly decreased as a result imposed to the levels of water deficit (75% and 50% FC) by about 41% and 40% and under flooding by about 37%, respectively.

Table 1. Declines of nodules number (plant $root^{-1}$), leghaemoglobin (mg g^{-1} FW) and nitrogenase activity (mmole g^{-1} FW) of cowpea plants in response to WD (50% + 75%) and WL (2FC).

Treatments	Cowpea plant		
	No. nodules/ plant	Leghaemoglobin	Nitrogenase activity
Cont	19 ± 11^{c}	0.11 ± 0.01^{c}	0.58 ± 0.17^{b}
WD (50 FC)	1.00 ± 0.00^{a}	0.05 ± 0.00^{a}	0.23 ± 0.00^{a}
WD (75 FC)	9.00 ± 1.5^{b}	0.07 ± 0.00^{b}	0.24 ± 0.04^{a}
WL	4.00 ± 1.00^{a}	0.06 ± 0.01^{ab}	0.22 ± 0.11^{a}

*Different letters within the same row indicate significant differences between treatments at P < 0.01.

Table 2. Effect of WD (75% FC and 50% FC) and WL on some non enzymatic antioxidative stress compounds of cowpea plants. Data are the average of four replicates. Different letters within the same row indicate significant differences between treatments at P<0.01.

Parameters \ Treatments		Cowpea plants			
		Cont.	WL	WD (75%)	WD (50%)
Free Phenolic compound mg/g FW	Shoot	0.02±0.00^{a}	0.16±0.02^{b}	0.14±0.01^{b}	0.18±0.01^{b}
	Root	0.05±0.00^{ab}	0.07±0.01^{b}	0.05±0.01^{a}	0.11±0.01^{c}
Bound cell Phenolic compound mg/g FW	Shoot	0.04±0.01^{a}	0.04±0.02^{a}	0.05±0.01^{a}	0.05±0.01^{a}
	Root	0.04±0.00^{d}	0.01±0.00^{a}	0.02±0.00^{b}	0.03±0.00^{c}
MDA mg/g FW	Root	41.2±1.99^{a}	54.44±1.81^{a}	55.35±1.70^{a}	63.7±1.82^{a}
H_2O_2 (mg/g FW)	Shoot	3.38±0.22^{a}	6.08±0.10^{a}	5.27±0.39^{b}	7.56±0.41^{c}
	Root	0.23±0.01^{a}	0.33±0.02^{ab}	0.40±0.02^{ab}	0.51±0.03^{b}
Ascorbic acid mg/g FW	Shoot	1.56±0.08^{a}	2.23±0.2^{b}	1.81±0.05^{a}	2.03±0.06^{b}
Proline mg/g DW	Shoot	1.53±0.03^{a}	1.09±0.08^{a}	2.53±0.42^{b}	3.23±0.39^{b}
	Root	0.68±0.155^{a}	1.25±0.7^{b}	1.41±0.12^{b}	2.01±0.72^{b}

Different antioxidant substances were cited in Table 2. Phenolic compounds are among the most influential and widely distributed as anioxidant products in the plant species, being involved in resistance to different types of stresses. In the present study, effect of water deficit and flooding on phenolic compounds were observed. As can be seen in Table 2, the free phenolic compound in shoots of the tested plant (cowpea) was significantly increased as a result of imposed to the level of water deficit (75% FC and 50% FC) and water logging compared to absolute controls and the conditions were ordered with respect to the increase in free phenolic compounds as flooding > water deficit (5%) > water deficit (75%). Whereas, in roots of cowpea plants the free phenolic compound was significantly increased only under waterlogging condition. Although determination of MDA as an estimation of oxidative damage to lipid membranes is a widely accepted methodology, it is known that carbohydrates and even some amino acids may undergo decomposition and produce MDA as an end-product. Thus, MDA concentration does not always indicate the actual level of lipid peroxidation. In this work, as can seen in Table 2, MDA concentration of the cowpea plant roots was significantly increased. The stress conditions were ordered with respect to increase of MAD in roots as moderate water deficit > low water deficit > waterlogging.

According to the data in Table 2, the production of hydrogen peroxide in shoots was higher than roots especially under stress conditions. H_2O_2 was increased significantly in shoots and roots of cowpea plants under waterlogging and water deficit stresses. The stress conditions were ordered with respect to the level of H_2O_2 production as moderate WD (50% FC) > WL (2FC) > WD (75% FC).

Osmoregulater proline is considered as antioxidant substance and recorded to be accumulated in stressed plants. The interactivate effect of drought and flooding stress treatments on proline metabolism in the leaves and roots of experimental plants are represented in Table 2. The data revealed that, there is a marked and progressive increasing in the production of proline in cowpea plant shoots and roots as the water deficit level increased. Whereas, the level of proline was unaffected with wterlooging in cowpea shoots and significantly raised in roots compared to absolute control plants. The stress conditions ordered according to the level of proline as moderate WD (50% FC) > WD (75% FC) and WL (2 FC). The ascorbate levels in cowpea plant shoots

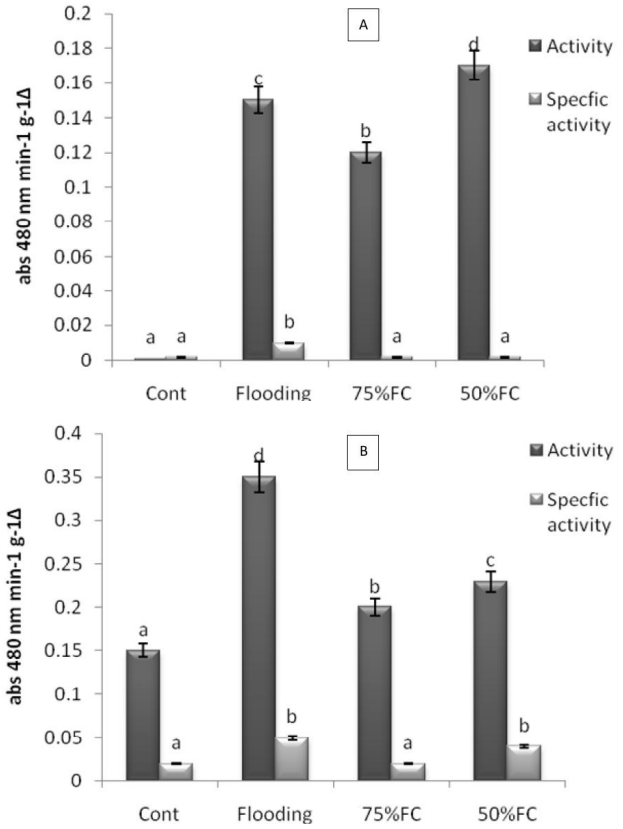


Fig. 3. Variations in SOD activities and specific activities of cowpea plant shoots (A) and roots (B) under WL (2FC) and two levels of WD (75% and 50%). Data are the average of four replicates. Different letters within the same row indicate significant differences between treatments at P<0.01.

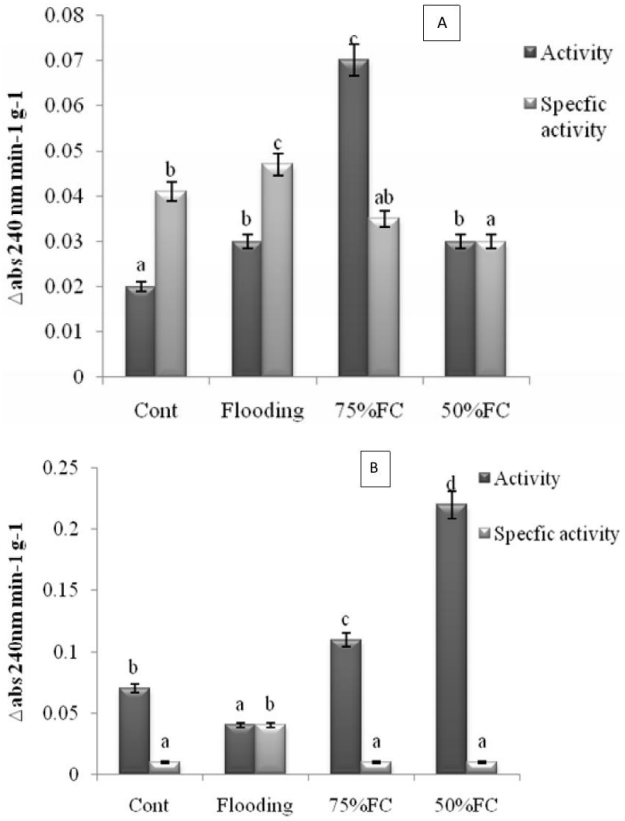


Fig. 4. Variations in CAT activities and specific activities of shoot (A) and root (B) of cowpea plants under WL (2FC) and two levels of WD (75% and 50% FC). Data are the average of four replicates. Different letters within the same row indicate significant differences between treatments at P<0.01.

were determined and cited in Table 2. The level of ascorbate was increased significantly under WL and the moderate level of WD (50% FC).

Activity and specific activity of antioxidant enzymes

Activities of the radical scavenger antioxidant enzymes such as SOD, CAT and AsA-dep POD were investigated in cow plant roots and shoots grown under WD (low and moderate) and WL stress conditions. Fig. 3 shows that SOD activities in cowpea leaves were significantly increased as a result imposed the levels of WD (75% and 50% FC) and WL stresses as compared to absolute control of root and shoots.

The data in Fig. 4 revealed that specific activity of CAT of cowpea shoots under the moderate level of drought (50% FC) was significantly decreased compared to absolute control while CAT activities raised significantly in roots at same level of WD. In roots, CAT activity of cowpea plants was decreased under flooding stress compare to absolute control while CAT activity of cowpea roots was increased under the two levels of WD (75% and 50% FC) as compared to absolute control.

The data in Fig. 5 revealed that APX activity and. the specific activity of APX of cowpea plants increased under the two level drought (75% and 50% FC) compare to absolute control. In roots, the specific activity of APX of cowpea roots mostly unchanged under flooding stress and the two level drought (75% and 50% FC) compared to absolute control. The same trend was observed in roots.

DISCUSSION

Water deficit (WD) and waterlogging (WL) occur in many parts of the world every year. Due to increased climatic variability under global climate change, frequent occurrence of climatic extremes such as heavy rainfalls and reduction in freshwater availability has adversely affected agricultural

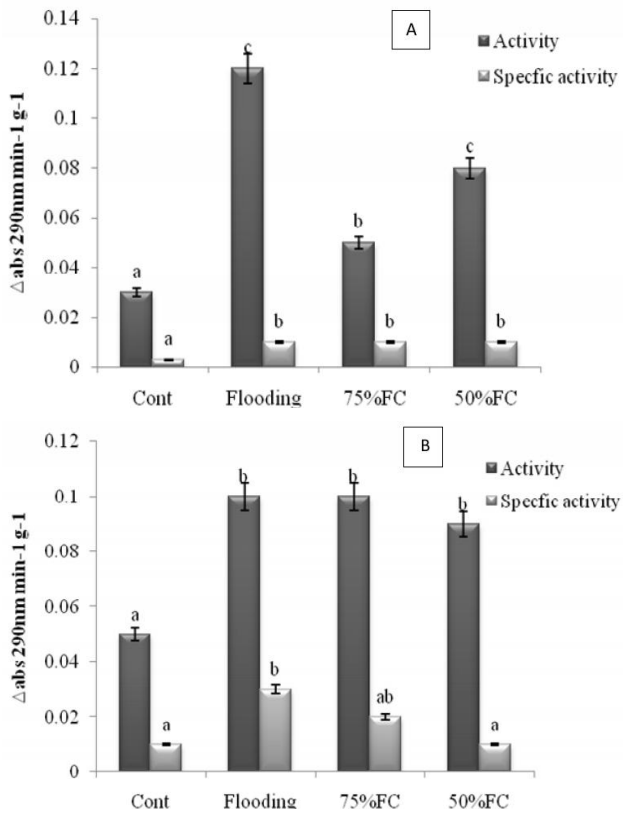


Fig. 5. Variations in AsA-dep POD activities and specific activities in shoots (A) and roots (B) of cowpea plants under WL (2FC) and two levels of WD (75% and 50% FC). Data are the average of four replicates. Different letters within the same row indicate significant differences between treatments at P<0.01.

production and quality [31]. Consequently, WD and WL stresses are increasingly becoming important factors constraining crop yield [1, 32].

One of the most important consequences of the increase in water stress is the oxidative tissue damage in green plants [33, 34]. In this study, cowpea plants grown for 30 days under water deficit induced by reduction in WD (50% and 57%), and those grown for the same amount of time under waterlogging stress (one fold FC). Generally showed significant amounts of reduction in their growth rates represented in fresh and dry weights of roots and shoots. The results also indicated that the elevated levels of the investigated antioxidants and antioxidative enzymes, and the high extent of membrane lipid peroxidation were symptomatic for oxidative stress under both water stress conditions. The present findings revealed that cowpea plants had varying ability to deal with oxidative stress that might govern their differential sensitivity to WD and WL stress status. It seems also clear that cowpea plants respond to water stress by varying in their antioxidant activity (both enzymatic and non-enzymatic), whereas WL induced an over-reduction in CAT activity.

In the our study, there is a marked and progressive increasing in the production of antioxidant compounds e.g phenolic substances, proline and MAD under both abiotic stresses in roots or shoots of cowpea plant (Table 2). The accumulation of phenolic compounds has also been detected in response to drought and a wide array of non-enzymatic antioxidants such as ascorbic acid, flavonoids and phenolic compounds [35]. Plants can decrease their oxygen demands by lowering their respiration rate, increasing the availability of soluble sugars for metabolism and growth, and significantly improving antioxidant protection through the synthesis of phenolic compounds and glutathione [16, 36]. The differential responses of lipid peroxidation and ROS accumulation between shoots and roots may be associated with level of drought stress intensity and different protective mechanisms.

Plants are adapted for minimizing radical damage using their natural defense mechanisms. Thus, the balance between the formation and detoxification of activated oxygen species is critical to cell survival during flooding stress [37, 38]. Within plant cells, SOD constitutes the first level of defense against superoxide radicals. SOD-catalyzed O_2- dismutation renders another reduced oxygen species, hydrogen peroxide (H_2O_2), as a reaction product. This H_2O_2 is removed mainly by the activity of APX and CAT [39-41].

Nakayama et al. [9] and Tian et al. [42] stated that, excess water blocks entry of oxygen into the soil, hindering aerobic respiration. When soil moisture is excessive, low oxygen concentrations in the soil inhibit the respiratory activity of germinating seeds, and thereby reduce the speed of germination. During this prolonged germination, seeds are likely to be infected with soil-born diseases, which further reduces the germination percentage. Reduction of growth mainly depend on nitrgenase activity and fixed nitrogen by root in legume plants. So nitrogenase activity depending on nodule formation and leghaemoglobin content was sharply decreased under both stresses (Table 1). As a leguminous crop, cowpea can fix nitrogen which is an essential element for crop growth. Several studies have shown that drought stress

reduced nitrogen fixation in leguminous plants [43-47]. Also, [47] stated that the reduction in nitrogen fixation under WD and the reduction under drought was similarly.

The present study demonstrated that, photosynthetic pigments were significantly lowered under the both stresses as compared to control plants (Fig. 2). Many researchers found that water stress (WD or WL) induced destruction of chlorophyll [48, 49]. This decrease in chlorophyll directly or indirectly affects the photosynthetic capacity of plants under waterlogged conditions [49]. The loss of Chl during the water stress (WL or WD) stress condition may also related to photo-oxidation resulted in oxidative stress [27]. Carotenoids as well as other antioxidant enzymes correlated positively with water [50-52].

The present results indicated that rapeseed cowpea accumulate higher levels of proline in their shoots and roots under water stress conditions. Overproduction of proline cowpea plants can be conferred an increased osmotolerance. The accumulation of proline in plants in response to water stress is also quite well documented and is considered to play an important role in osmotic adjustment [53]. Proline content of cowpea plants was elevated linearly with increase of water deficit. Proline accumulation in plants might having a scavenger function [55] and act as an osmolyte [54]. Also, Hare et al. [55] and Martínez et al. [56] stated that proline is one of the compatible solutes that accumulate in response to water stress, and the accumulation of these osmolytes represents an important adaptive response to abiotic stress.

CONCLUSION

Our results suggested that the decrease in pigments content confirmed that this represent a sensitive indicator for cowpea stress and the differential responses to both water stresses (WD and WL) status could be related to the differences among the enzymatic antioxidant systems.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Ashraf MA. Waterlogging stress in plants: A review. Afr J Agricult Res. 2012; 7(13): 1976-1981.
2. Das KK, Panda D, Sarkar RK, Reddy JN, Ismail, AM. Submergence tolerance in relation to variable floodwater conditions in rice. Environ. Exp Bot. 2009; 66: 425-434.
3. Ji-Ping G, Dai-Yin C,Hong-Xuan L. Understanding abiotic stress tolerance mechanisms: recent studies on stress response in rice. J Integ Plant Biol. 2007; 49(6): 742-750.
4. Mittler R. Abiotic stress, the field environment and stress combination. Trends Plant Sci. 2006; 11(1): 15-19.
5. Boyer JS. Plant productivity and environment. Science. 1982; 218: 443-448.
6. Araus JL, Slafer GA, Reynolds MP, Royo C. Plant breeding and drought in C3 cereals: What should we breed for? Ann Bot. 2002; 89: 925-940.
7. Chaves MM, Maroco JP, Pereira JS. Understanding plant responses to drought: from genes to the whole plant. Funct Plant Biol. 2003; 30: 239-264.
8. Li H, Syvertsen JP, Stuart RJ, McCoy CW, Schumann AW, Castle WS. Soil and Diaprepes abbreviatus root weevil spatial variability in a poorly drained citrus grove. Soil Sci. 2004; 169: 650-662.
9. Nakayama N, Shimada S, Takahashi M, Kim YH, Arihara J. Effects of waterabsorbing rate of seed on flooding injury in soybean. Jpn J Crop Sci. 2005;74: 325-329.
10. Tian XH, Nakamura T, Kokubun M. The role of seed structure and oxygen responsiveness in pre-germination flooding tolerance of soybean cultivars. Plant Prod Sci. 2005; 8: 157-165.
11. Sorrell BK. Effect of external oxygen demand on radia oxygen loss by *Juncus* roots in titanium citrate solutions. Plant Cell Environ. 1999; 22: 1587-1593.
12. Sorrell BK, Downes MT, Stanger CL. Methanotrophic bacteria and their activity on submerged aquatic macrophytes. Aquat Bot. 2002; 72: 107-119.
13. Voesenek LACJ, Colmer TD, Pierik R, Millenaar FF, Peeters AJ. Plants cope with complete submergence. New Phytol. 2006; 170: 213-226.
14. Bailey-Serres J, Voesenek LACJ. Flooding stress: acclimations and genetic diversity. Annu Rev Plant Biol. 2008; 59: 313-339.
15. Ponnamperuma FN. Effects of flooding on soils. In: Kozlowski TT. (Ed.), Flooding and Plant Growth. Academic Press, Orlando, Florida; 1984, pp: 1-44.
16. Colmer TD, Voesenek LACJ. Flooding tolerance: suites of plant traits in variable environments. Funct Plant Biol. 2009; 36: 665-681.
17. Ahmed S, Nawata E, Hosokawa M, Domae Y, Sakuratani T. Alterations in photosynthesis and some

antioxidant enzymatic activities of mungbean subjected to waterlogging. Plant Sci. 2002; 163: 117-123.
18. Simova-Stoilova L, Demirevskaa K, Kingston-Smith A, Feller U. Involvement of the leaf antioxidant system in the response to soil flooding in two *Trifolium* genotypes differing in their tolerance to waterlogging. Plant Sci. 2012; 183: 43-49.
19. Hossain Z, Lopez-Climent MF-M, Arbona V, Perez-Clemente RM, Gomez-Cadenas A. Modulation of the antioxidant system in citrus under waterlogging and subsequent drainage. J Plant Physiol. 2009; 166: 1391-1404.
20. Lichtenthaler K. Chlorophylls and carotenoids pigments of photosynthetic biomembranes. Meth Enzymol. 1987; 148: 350-382.
21. La Rue TA, Kurz WGW. Estimation of nitrogenase using a calorimetric determination for ethylene. Plant Physical. 1973; 51: 1074-1075.
22. Kofalvi SA, Nassuth A. Influence of wheat streak mosaic virus infction phrenyl propanoid metabolism and the accumulation of phenyl propanoid metabolism and the accumulation of phenolics and lignin in wheat. Physiol Mol Plant Pathol. 1995; 47: 365-377.
23. Mahadevan A, Sridhar R. 1982. Methods of physiological plant pathology. 2 ed. India: Sivakami.
24. Jena S, Choudhuri MA. Glycolate metabolism of three submerged aquatic angiosperms during aging. Aquat Bot. 1981; 12: 345-354.
25. Mukherjee SP, Choudhuri MA. Implications of water stress induced changes in the levels of endogenous ascorbic acid and H_2O_2 in *Vigna* seedlings. Plant Physiol. 1983; 58: 166-170.
26. Bates LS, Waldren RP,Teare ID. Rapid determination of free proline for water-stress studies. Plant Soil. 1973; 39: 205-207.
27. Crosti N, Serviden T, Bajer J, Serra A. Modification of 6-hydroxydopamine technique for the correct determination of superoxide dismutase. J Clin Chem Clin Biochem. 1987; 25: 265-272.
28. Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol.1981; 22: 867-880.
29. Aebi HE. 1983. Catalase. In: Bergmeyer HU (Ed.), Methods of Enzymatic Analysis, vol. 3. Verlag Chemice, Deerfield Beach FL, pp. 273-286.
30. Lowry OD, Roseborough NJ, Farr AL, Rondall RJ. Protein measurement with the folin phenol reagent. J Biol Chem. 1951; 193: 265-257.
31. Candan N, Tarhan L. Tolerance or sensitivity responses of *Mentha pulegium* to osmotic and waterlogging stress in terms of antioxidant defense systems and membrane lipid peroxidation. Environ Exp Bot. 2012; 75: 83-88.
32. Parent C, Berger A, Folzer H, Dat J, Crevecoeur M, Badot P-M, Capelli N. A novel nonsymbiotic hemoglobin from oak: Cellular and tissue specificity of gene expression. New Phytol. 2008; 177: 142-154.
33. Moran JF, Becana M, Iturbe-Ormaetxe I, Frechilla S, Klucas RV, Aparicio-Tejo P. Osmotic induces oxidative stress in pea plants. Planta. 1994; 194: 346-352.
34. Egert M, Tevini M. Influence of osmotic on some physiological parameters symptomatic for oxidative stress in leaves of chives. Environ Exp Bot. 2002; 48: 43-49.
35. Blokhina O, Virolainen E, Fagerstedt KV. Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Bot. 2003; 91: 179-194.
36. Noctor G, Arisi ACM, Jouanin L, Kunert KJ, Rennenberg H, Foyer CH. Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. J Exp Bot. 1998; 49: 623-647.
37. Hsu SY, Hsu ST, Kao CH. The effect of polyethylene glycol on proline accumulation in rice leaves. Biol Plant. 2003; 1: 73-78.
38. Kato-Noguchi H, Morokuma M. Ethanolic fermentation and anoxia tolerance four rice cultivars. J Plant Physiol. 2007; 164: 168-173.
39. Foyer CH, Noctor G. Redox homeostasis and antioxidant signalling: a metabolic link between stress perception and physiological responses. Plant Cell. 2005; 17: 1866-1875.
40. Foyer CH, Noctor G. Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ. 2005; 28: 1056-1071.
41. Kuzaniak E, Sklodowska M. Fungal pathogen-induced changes inthe antioxidant systems of leaf peroxisomes from infected tomato plants. Planta. 2005; 222: 192-200.
42. Tian XH, Nakamura T, Kokubun M. The role of seed structure and oxygen responsiveness in pre-germination flooding tolerance of soybean cultivars. Plant Prod Sci. 2005; 8: 157-165.
43. Hassan OAE, Hall AE. Influences of early and late nitrogen fertilization on yield and nitrogen fixation of cowpea under well-watered and dry field conditions. Field Crops Res. 1987; 15: 229-244.
44. Ramos MLG, Gordon AJ, Minchin FR, Sprent JI, Parsons R. Effect of water stress on nodule physiology and biochemistry of a drought tolerant cultivar of common bean (*Phaseolus vulgaris* L.). Annu Bot. 1999; 83: 57-63.
45. Hungria M, Vargas MAT. Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. Field Crop Res. 2000; 65: 151-164.

46. Pimratch S, Jogloy S, Vorasoot N, Toomsan B, Kesmala T, Patanothai A, Holbrook CC. Effects of drought on characters related to nitrogen fixation in peanut. Asian J Plant Sci. 2010; 9: 402-413.
47. Pimratch S, Jogloy S, Vorasoot N, Toomsan Kesmala T, Patanothai A, Holbrook CC. Effect of drought stress on traits related to N_2 fixation in eleven peanut (*Arachis hypogaea* L.) genotypes differing in degrees of resistance to drought. Asian J Plant Sci. 2008; 7: 334-342.
48. Jackson P, Roberston M, Cooper M, Hammer G. The role of physiological understanding in plant breeding; from a breeding perspective. Field Crop Res. 1996; 49: 11-37.
49. Ashraf MA, Ahmad MSA, Ashraf M, Al-Qurainy F, Ashraf MY. Alleviation of waterlogging stress in upland cotton (*Gossypium hirsutum* L.) by exogenous application of potassium in soil and as a foliar spray. Crop Pasture Sci. 2011; 62: 25-38.
50. Salama S, Trivedi S, Busheva M, Arafa AA, Garab G, Erdei L. Effects of NaCl salinity on growth, cation accumulation, chloroplast structure and function in wheat cultivars differing in salt tolerance. J Plant Physiol. 1994; 14: 241-247.
51. Abdeshahian M, Nabipour M, Meskarbashee M. Chlorophyll fluorescence as criterion for the diagnosis salt stress in wheat (*Triticum aestivum*) plants. Int J Chem Biol Eng. 2010; 4: 184-186.
52. Saleem M, Ashraf M, Akram NA. Salt NaCl-induced modulation in some key physio-biochemical attributes in okra (*Abelmoschus esculentus* L.). J Agron Crop Sci. 2011; 197: 202-213.
53. Wright GC, Nageswara Rao RC, Farquhar GD. Water-use efficiency and carbon isotope discrimination in peanut under water deficit conditions. Crop Sci. 1994; 34: 92-97.
54. Parida AK, Das AB. Salt tolerance and salinity effects on plants. A review. Ecotox Environ Saf. 2005; 60: 324-349.
55. Hare PD, Cress WA, Van Staden J. Proline synthesis and degradation: a model system for elucidating stress-related signal transduction. J Exp Bot. 1999; 50: 413-434.
56. Martínez JP, Silva H, Ledent JF, Pinto M. Effect of drought stress on the osmotic adjustment, cell wall elasticity and cell volume of six cultivars of common bean (*Phaseolus vulgaris* L.). Eur J Agron. 2007; 26: 30-38.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

BIOLOGY ORIGINAL ARTICLE

Effect of papain and piperonyl butoxide/MGK-264 on the reproduction/development of *Lymnaea acuminata*

Farhat Hanif, Dinesh K. Singh

Malacology Laboratory, Department of Zoology, DDU Gorakhpur University, Gorakhpur, India

ABSTRACT

The effects of sub-lethal treatments (20% and 60% of 24h LC_{50}) of papain and its binary combination (1:5) with piperonyl butoxide (PB)/MGK-264 on the reproduction and development of *Lymnaea acuminata* has been studied. There was a significant decrease in the fecundity, hatchability and survival of the young snails. Treatment with 60% of 24h LC_{50} of papain + PB caused complete arrest of fecundity. No hatching was noted in eggs laid by snails exposed to 60% of 24h LC_{50} of papain + MGK-264. Development could not proceed beyond the veliger stage due to the death of the embryo. The developmental arrest was noticed in many egg strings leading to the high rate of mortality and thereby reduction in hatchability of the eggs. Growth rate of the survived snails was also very slow and sexual maturity could not be attained even in three months.

Key words: Fascioliosis; Papain; Synergist; Reproduction; Development; *Lymnaea acuminata*.

J Biol Earth Sci 2013; 3(1): B154-B163

Corresponding author:
Dinesh K. Singh
Malacology Laboratory, Department of Zoology,
DDU Gorakhpur University, Gorakhpur,
273009, U.P., India.
Tel: +91-551-2202187(O);
9454211574 (Mobile).
E-mail: dksingh_gpu@yahoo.co.in

Original Submission: 04 April 2013; Revised Submission: 10 May 2013; Accepted: 14 May 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Fasciolosis is a worldwide zoonotic disease caused by a trematode parasite of the genus *Fasciola* that infects over 17 million people causing significant morbidity and mortality [1, 2]. Human infection causes serious hepatic pathological consequences [3]. Two major species, *F. hepatica* Linnaeus, 1758 and *F. gigantica* Cobbold, 1855 are the causative agents of fasciolosis in ruminants and in humans worldwide. *F. gigantica* causes outbreaks in tropical areas of southern Asia, Southeast Asia, and Africa. Infection is most prevalent in regions with intensive sheep and cattle production [4]. The snail *Lymnaea acuminata* is the intermediate host of the *F. gigantica* [5]. This snail breeds year round and lays eggs on the lower surface of the aquatic plants. Treatment of *Fasciola* requires high or multiple doses of drugs with frequent side effects [6], therefore snail control is considered not only complementary but essential in *Fasciola* control. It is regarded as a rapid and efficient method for reducing or eliminating transmission and is among the methods of choice to bring these diseases under an adequate control through the removal of a vital link in the life cycle of the parasite [7]. The use of synthetic molluscicides poses hazardous environmental effects and toxicities to non-target organisms, even man. This has generated the need to search novel natural molluscicidal compounds from plants as possible alternatives to synthetic products [8]. Treatment with plant derived molluscicides is in agreement with ecological and economic considerations. It has been previously reported that papain, active molluscicidal component present in *Caraica papaya* is a potent molluscicide [9]. Besides killing the harmful snails, if a molluscicide possesses a property to reduce its reproductive capacity even at sub-lethal doses, it is considered to be more advantageous in snail control programmes [10, 11]. In the present study, the effects of sub-lethal (20% and 60% of 24h LC_{50}) exposure of papain alone and in binary combination (1:5) with piperonyl butoxide and MGK-264 on the reproduction and development of snail *L. acuminata* were examined.

MATERIALS AND METHODS

Pure compound

Papain (Cysteine protease) was purchased from Sigma Chemical Co., USA.

Synergists

The synergist piperonyl butoxide [α {2-(2-butoxy ethoxy) ethoxy} - 4, 5-methylene dioxy-2-propyl toluene] and MGK-264 (N-octyl bicyclo-heptene dicarboximide) were supplied by Mc Laughlin Gormley King Co., USA.

Fecundity experiment

Sexually mature *L. acuminata* of average size (2.25 ± 0.30 cm in length) were collected locally from ponds, pools and lakes of Gorakhpur district. These snails were kept in glass aquaria containing dechlorinated tap water for 72h to undergo acclimatization. The snail *Lymnaea acuminata* lays eggs in the form of elongated gelatinous capsules (egg masses or egg strings) on the lower surface of leaves of aquatic vegetation. These egg masses may have 2-4 rows of eggs, with number of eggs ranging from 5 upto 200 or sometimes even more. Groups of 20 snails kept in glass jars containing 3 litres of dechlorinated tap water and lotus leaves (egg laying surface) were exposed separately to sub-lethal concentration (20% and 60% of 24h LC_{50}) of papain and its binary combination (1:5) with synergist piperonyl butoxide (PB) and MGK-264 (Table 1). The total number of egg masses and eggs laid by the groups of snails were counted every 24h to 96h.

As it is difficult to identify the mother snails for a

Table 1. Sublethal concentration of papain alone and in binary combination with synergist PB and MGK-264 against snail *L. acuminata*.

Treatment	24h LC_{50} (mg/l)	20% of 24h LC_{50} (mg/l)	60% of 24h LC_{50} (mg/l)
Papain	16.63	3.32	9.97
Papain + PB	7.69	1.53	4.61
Papain + MGK	9.15	1.83	5.49

particular spawn, capsules containing eggs from each treated groups were separated carefully with the help of scalpel from the lower surface of lotus leaves and incubated at 30°C in covered petridishes containing the same concentration as given to adult snails. The development of embryos was observed under microscope upto their hatching and the duration of each stage and hatching period was noted. As the eggs were transparent and surrounded by mucus string, changes taking place upto the time of hatching could be observed directly. The egg capsules besides conveniently permitting observations of growing embryos also act as a diffusion barrier and prevent passage of large molecules.

Hatchability was studied with the eggs laid after 24h to 96h exposure. Dead embryos (lacks embryonic movement and become opaque) were removed to avoid any contamination. Immediately after hatching the miniature snails were transferred to freshwater. These snails were reared by feeding them on cabbage, carrot, lotus leaves and their survival was observed.

Statistical analysis

A student t–test was applied to determine the significant ($P < 0.05$) differences between treated and control animals. Product moment correlation coefficient was applied between exposure time and fecundity/survival of snails [12].

RESULTS

L. acuminata is oviparous. The eggs laid are encapsulated in a protective jelly strand known as egg mass or egg strand, within which the eggs are arranged longitudinally in 2-4 rows. The eggs are isolecithal and oval in shape with the zygote located eccentrically within it. The eggs are transparent so the embryonic development can be observed under the microscope directly. The entire embryonic development and the life cycle of *L. acuminata* until hatching takes place inside single egg and can be divided into four stages (Table 2). The young snails escaped from the egg by first rasping through the egg membrane. Perforation of the membrane was aided by the snail periodically thrusting its foot against the wall. After hatching, the empty egg membrane collapsed. Outside the egg, the snail moved freely in the jelly of the egg capsule and escaped out through a hole made by itself or through an opening made by another snail previously.

In the control groups, the eggs were of equal size and equidistant from each other while in the treated groups, size of the egg masses was

Table 2. Characteristic features of embryonic stages of development and juveniles in *L. acuminata*.

Embryonic stage		Characteristic features
Morula		Embryo is brownish solid, almost opaque, no motions.
Trocophore		Beginning with slow jerking movements, developing mouth, velum and head vesicles, later on uninterrupted rotation of embryo.
Veliger	Early Veliger	Embryo started elongation, curved foot muscle, development of shell gland through the extension of velum.
	Late Veliger	Demarcation of head-foot region from visceral mass, the embryo becomes transparent, pair of tentacles with eyes at their bases, heart beat could be observed, periodic contraction movements of visceral mass towards head-foot region.
Hippo		Complete metamorphosis to young snail, embryo fully developed, eyes and heart beat could be seen clearly, shell and foot clearly separated, mantle fold could be identified.
Juveniles		Possesses all the structures found in adult individuals, thin transparent shell covered the mantle, foot and tentacles well developed.

comparatively small and decreased number of eggs of variable size. Normally, each egg contains one embryo but can sometimes contain more than one (polyembryony). In the present study 2 -3 embryos were observed in some eggs.

The data on the morphological observations i.e. fecundity, different developmental periods, hatchability and survivality of young snails in *L. acuminata* was recorded.

A significant reduction in fecundity was noted in *L. acuminata*, exposed to papain and its binary combination with synergist PB and MGK-264 (Table 3). No egg laying was noted in the snails exposed to 60% of 24h LC_{50} of papain + PB after 24, 48, 72 and 96h. Treatment with 20% of 24h LC_{50} of papain + PB and 20% and 60% of 24h LC_{50} of papain + MGK caused no egg laying after 72h. After 96h exposure, no egg laying was noticed in any of the treatments. A decrease in the number of egg masses and its size was observed with the increase in exposure time and the number of eggs per egg masses was also reduced. Effect of these treatments could also be seen on the duration of different stages of development, hatching time and

Table 3. Effect of papain 20% and 60% alone and in binary combination with synergist PB and MGK-264 on the fecundity of snail *L. acuminata*.

Name of Molluscicide	Treatment (mg/l)	Fecundity after 24h (No. of eggs of 20 snails)	Fecundity after 48h (No. of eggs of 20 snails)	Fecundity after 72h (No. of eggs of 20 snails)	Fecundity after 96h (No. of eggs of 20 snails)
	Control	245.66±1.17	193.66±0.91	185.00±1.26	188.33±0.55
Papain	20% of 24h LC_{50}	+159.00±0.72*	118.66±0.75*	73.33±1.11*	-
	60% of 24h LC_{50}	+126.66±0.55*	57.33±1.28*	16.66±0.55*	-
Papain + PB	20% of 24h LC_{50}	+96.00±0.63*	18.33±0.91*	-	-
	60% of 24h LC_{50}	-	-	-	-
Papain + MGK	20% of 24h LC_{50}	+117.33±0.75*	12.33±0.55*	-	-
	60% of 24h LC_{50}	+26.66±0.75*	5.00±0.22*	-	-

Each value is mean ± SE of six replicates, each replicate represents the egg laid by a group of 20 snail

(-) means - No egg laying was observed

* P < 0.05 significant when student's t test was applied to treated and control groups

+ There was a significant (p < 0.05) negative correlation in between exposure period and fecundity of *L. acuminata*

hatchability of the snails (Table 4, 5). The duration of the larval stages in treated groups was increased in comparison to control i.e. the embryos in treated groups experienced delay in development compared to the untreated controls. In the controls morula stage lasted for 1 day, trocophore 1-2 days, veliger 2 days and hippo 2-3 days while in treated snails morula stage extended upto 2-3 days, trocophore 2-3 days, veliger 2-5 days and hippo 3-5 days. The hatching time was prolonged in the treated groups (9-16 days) with respect to the control group (7-9) days. Eggs exposed to 60% of papain + MGK failed to hatch due to the death of the embryo after the veliger stage.

After hatching the treated miniature snails were found attached to the walls of the container. Their shell was very thin and tentacles were smaller in comparison to the control groups. Also their movement was slow than the control snails. Survival of these snails was observed for about few months and the survivality progressively decreased with time (Table 6). There was a significant ($P < 0.05$) negative correlation between exposure period and survival of the young snails hatched from the eggs laid by treated snails. Maximum decrease in survivality was noticed in the snails exposed to 20% of 24h LC_{50} of papain + PB (100% mortality in about 90 days) followed by papain + MGK. The growth rate was also very slow and the survived snails could not attain sexual maturity even in 3 months.

DISCUSSION

Sublethal exposures (20% and 60% of 24h LC_{50}) of papain alone and in combination with synergist PB and MGK-264 significantly reduced the reproductive capacity, survivality, prolonged the hatching time and had an adverse effect on the development of the snail *L. acuminata*. Earlier, Rao and Singh [11] and Singh et al. [13] have reported that *Azadirachta indica*, *Cedrus deodara*, *Allium sativum*, *Nerium indicum*, *Annona squamosa* and *Argemone mexicana* significantly reduced the

Table 4. Effect of papain 20% and 60% alone and in binary combination with synergist PB and MGK-264 on the duration of embryonic stages and hatching time of snail *L. acuminata*.

S.N.	Treatment	Stages	20% (Duration in days)	60% (Duration in days)	Hatching Time (In Days) 20%	60%
1.	Control	Morula	1day		7-9	
		Trocophore	1-2 days			
		Veliger	2 days			
		Hippo	2-3 days			
2.	Papain	Morula	1 day	2 days	9-12	10-13
		Trocophore	2-3 days	2-3 days		
		Veliger	2-4 days	3-4 days		
		Hippo	3-4 days	3-4 days		
3.	Papain+PB	Morula	2-3 days	No egg laying	12-16	—
		Trocophore	2-3 days			
		Veliger	3-5 days			
		Hippo	3-5 days			
4.	Papain+MGK	Morula	2 days	2-3 days	11-14	No Hatching
		Trocophore	2-3 days	2-3days		
		Veliger	3-4 days	2-4 days		
		Hippo	3-5 days	Death		

reproductive capacity of *Achtina fulica* and *L. acuminata*.

PB/MGK-264 synergized the effect of papain so there was more reduction in fecundity by their combination rather than the single treatment. This is probably due to reduced detoxification of the active molluscicidal component by mixed function oxidases (MFO), as PB/MGK-264 are inhibitors of these [14]. Consequently, as the titre of the active component is high at target site, it will be more effective in reducing the reproduction in snail. The CDCs (caudodorsal cells) located in the brain of snail *L. acuminata* releases the ovulation hormone [15]. Thus, papain an active molluscicidal component of *Carica papaya* [9] may possibly affect the CDC thereby, reducing the release of ovulation hormone which in turn resulted in a decrease in the fecundity of the snail. It has been earlier reported that papain caused a significant inhibition of the acetylcholinesterase (AChE), acid phosphatase

Table 5. Effect of papain 20% and 60% alone and in binary combination with synergist PB and MGK-264 on the % hatchability of snail *L. acuminata*.

Name of Molluscicide	Treatment (mg/l)	% Hatchability (24h Treated eggs)	% Hatchability (48h Treated eggs)	% Hatchability (72h Treated eggs)	% Hatchability (96h Treated eggs)	Total % Hatchability
Control		100	100	100	100	100
Papain	20% of 24h LC_{50}	78.18±0.57*	69.92±0.65*	33.55±1.12*	-	66.07±0.61
	60% of 24h LC_{50}	76.03±0.76*	55.62±1.65*	17.73±1.62*	-	65.39±0.87
Papain + PB	20% of 24h LC_{50}	75.00±0.25*	12.63±0.57*	-	-	65.03±0.41
	60% of 24h LC_{50}	-	-	-	-	-
Papain + MGK	20% of 24h LC_{50}	74.11±0.88*	40.29±1.25*	-	-	70.91±0.83
	60% of 24h LC_{50}	No Hatching	No Hatching	-	-	-

(-) means - No hatchability

* P < 0.05 significant when student's t test was applied to treated and control groups

Table 6. Effect of papain 20% and 60% alone and in binary combination with synergist PB and MGK-264 on the % survival of snail *L. acuminata*.

Name of Molluscicide	Treatment (mg/l)	Survival (24h after hatching) %	Survival (48h after hatching) %	Survival (72h after hatching) %	Survival (96h after hatching) %	Survival (15 days after hatching) %	Survival (30 days after hatching) %	Survival (45 days after hatching) %	Survival (60 days after hatching) %	Survival (75 days after hatching) %	Survival (90 days after hatching) %
	Control	100	100	100	100	100	100	100	100	100	100
Papain	20% of 24h LC_{50}	+88.96±0.42 *	78.92±0.65 *	71.15±0.53 *	53.03±0.27 *	31.61±0.15 *	16.52±0.08 *	8.01±0.35 *	5.30±0.15 *	5.15±0.22 *	4.58±0.16 *
	60% of 24h LC_{50}	+87.57±0.13 *	82.25±0.26 *	68.51±0.35 *	51.79±0.20 *	32.00±0.25 *	18.74±0.23 *	8.86±0.31 *	6.81±0.35 *	5.05±0.35 *	4.02±0.67 *
Papain + PB	20% of 24h LC_{50}	+87.44±0.19 *	70.84±0.19 *	50.66±0.23 *	42.60±0.21 *	37.65±0.22 *	15.68±0.21 *	7.61±0.24 *	4.92±0.26 *	2.27±0.24 *	-
	60% of 24h LC_{50}	-	-	-	-	-	-	-	-	-	-
Papain + MGK	20% of 24h LC_{50}	+88.75±0.19 *	79.72±0.19 *	66.68±0.27 *	54.00±0.28 *	32.60±0.12 *	16.29±0.12 *	7.23±0.08 *	5.05±0.12 *	4.66±0.37 *	2.31±0.33 *
	60% of 24h LC_{50}	-	-	-	-	-	-	-	-	-	-

(-) means - No survival

* $P < 0.05$ significant when student's t test was applied to treated and control groups

+ There was a significant ($p < 0.05$) negative correlation in between exposure period and survival of *L. acuminata*

(ACP) and alkaline phosphatase (ALP) activity in the nervous tissue of *L. acuminata*. It may be possible that papain affect the gonadal tissue of the treated snails directly or indirectly through the neurohormones. The upsetting of glycemic homeostasis is an indication of intoxication [16]. The detoxification processes consume high amounts of energy, resulting in exhaustion of the carbohydrate deposits, thus leading to the reduction of fecundity that would otherwise be used for reproductive activity [17]. Singh and Singh [18] also demonstrated a reduction in fecundity in *L. acuminata*, possibly as a result of diminished proteins, amino acids, DNA and RNA in the snail's ovotestis.

The embryonic stages exhibited developmental arrest in the egg capsules resulting into the death of embryo. This observation goes in agreement with Bhide et al [19] who reported development arrest in most of the egg capsules due to deviation in protein fractions resulting into high percentage of mortality and low percentage of hatchability in nuvan treated groups of *Lymnaea stagnalis*. Besides this teratogenesis and deformities in larval stages were also observed in most of the egg capsules which could be correlated with the depletion of most of the protein fractions [19]. There was a significant reduction in the hatchability of the eggs and survival of the miniature snails treated with papain and its combination with PB/MGK-264. It seems that the reduction in hatchability of *L. acuminata* may be due to the interference with embryonic growth and development of the snails. Bhide et al [19] demonstrated that one of the potent causes of low percentage of hatchability of young snails from their corresponding egg capsules was the phenomenon of polyembryony in nuvan treated egg masses which showed the high rate of mortality due to the lack of metabolites for their progressive development in comparison to control groups. The colour of the egg capsules in the control group was dark cream but in treated groups changed to white [20].

Time-dependent reduction in the survival of hatched miniature snail, even after transfer to fresh water snail indicates that chemicals received either from the mother snail or in the eggs are lethal to hatched juvenile snails. Ferreira et al [21] assessed the effect of caffeine and thymol on the survival and growth of young *Subulina octona* of different ages and demonstrated reduced survival. The shell of the treated snails was thin as compared to the control. ALP which is inhibited by papain, plays a critical role in protein synthesis [22] and shell formation [23]. Thus it can be said that inhibition of ALP by papain resulted into the thinning of the shell of snail. Another possible reason for this may be decalcification [24], as observed by De Schamphelaere [25] according to which there was lower calcium in the haemolymph of snail *L. stagnalis* exposed to cobalt.

The growth rate was also very slow and even after about three months maturity could not be attained. The time required for attainment of sexual maturity in case of *L. acuminata* at 25-28°C is about 46-52 days [26, 27]. According to De Schamphelaere [25], reduced growth could potentially be explained by impaired feeding activity of the snails as a consequence of Co exposure. Feeding inhibition is a well-known response of aquatic organisms to chemicals exposure [28, 29]. Grosell et al. [30] suggested that the inhibition of Ca uptake by metals could potentially impair snail growth if Ca influx would become limiting for growth of the shell, which consists almost entirely of $CaCO_3$ [31]. The reduction in growth of treated snails may be due to interference of the molluscicides with the physiological activities of the snails [32]. As the time to reach maturity of the offspring of exposed adults was longer, it implies shorter reproductive life and thus smaller populations in the future.

CONCLUSION

From the present study it is clear that PB/MGK-264 synergizes the efficacy of papain and gives significant control of reproduction of the snails even at sub-lethal exposure. These treatments are not only effective in killing the snails but also possess a capability of making them sterile. Besides this it also kills the eggs, causes death of the embryo during developmental stages thereby inhibiting its hatching and increases the mortality of the hatched miniature snails. The use of the natural products in combination with PB/MGK-264 would have an added advantage against aquatic snails as it would be non toxic to the non target animals and cause only short term environmental toxicity, if any.

ACKNOWLEDGEMENT

One of the authors Farhat Hanif is thankful to

University Grants Commission (UGC), New Delhi for financial assistance [Maulana Azad National Fellowship: F1-17.1/2011/MANF-MUS-UTT-4320/ (SA-III/Website)].

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Mas-Coma S, Bargues MD, Valero MA. Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol. 2005; 35(11-12): 1255-1278.
2. WHO. 2006. Report of the WHO informal meeting on use of triclabendazole in fascioliasis control. 17-18 October, WHO headquarter, Geneva, Switzerland.
3. Soliman FM. Epidemiological review of human and animal fascioliasis in Egypt. J Infect Dev Countr. 2008; 2(3): 182-189.
4. WHO. 2007. Report of the WHO Informal Meeting on use of triclabendazole in fascioliasis control. WHO/CDS/NTD/PCT/2007.1
5. Agarwal RA, Singh DK. Harmful gastropods and their control. Acta Hydrochim Hydrobiol. 1988; 16(2): 113-138.
6. Abdul-Samie RE, Soliman EO, El-Nemr H, Masou A. Study of Il 1b, Il 4, Il 5 and Ige before and after Mirazid therapy in children with intestinal schistosomiasis and fascsioliasis. N Y Sci J. 2010; 3(12): 116-122.
7. Mello-Silva CC, Vasconcellos MC, Pinheiro J, Rodrigus MLA. Physiological changes in *Biomphalaria glabrata* (Say), (Pulonata: Planorbidae) caused by sublethal concentrations of the latex of *Euphorbia splendens* var. hisloppii N.E.B. (Euphorbiaceae). Mem Inst Oswaldo Cruz. 2006; 101(1): 3-8.
8. Luna JS, Santos AF, Lima MRF, Omena MC, Mendonca FAC, Bieber LW, et al. A study of the larvicidal and molluscicidal activities of some medicinal plants from northeast Brazil. J Ethnopharmacol. 2005; 97: 199-206.
9. Jaiswal P, Singh DK. Molluscicidal activity of *Carica papaya* and *Areca catechu* against the freshwater snail *Lymnaea acuminata*. Vet Parasitol. 2008; 152(3-4): 264-270.
10. Marston A, Hostettmann K. Plant molluscicides. Phytochem. 1985; 24(4): 639-652.
11. Rao IG, Singh DK. Effect of single and binary combinations of plant-derived molluscicides on reproduction and survival of the snail *Achatina fulica*. Arch Environ Contam Toxicol. 2000; 39(4): 486-493.
12. Sokal RR, Rohlf FJ. 1973. Introduction to Biostatistics. pp. 271-273. San Francisco, Calif USA, WH Freeman and Co.
13. Singh P, Singh VK, Singh DK. Effect of binary combination of some plant-derived molluscicides with MGK-264 or piperonyl butoxide on the reproduction of the snail *Lymnaea acuminata*. Pest Manag Sci. 2005; 61(2): 204-208.
14. Matsumura F. 1985. Toxicology of Insecticides. 2nd ed. New York, Plenum Press.
15. Roubos EW, Boer HH, Schot LPC. Peptidergic neurons and the control of neuroendocrine activity in the fresh water snail *Lymnaea stagnalis*. Proc Int Sym Neurosec. 1981; 8: 119-127.
16. Mello-Silva CC, Vilar MM, Vasconcellos MC, Pinheiro J, Rodrigues MLA. Carbohydrate metabolism alterations in *Biomphalaria glabrata* infected with *Schistosoma mansoni* and exposed to *Euphorbia splendens* var. hislopii latex. Mem Inst Oswaldo Cruz. 2010; 105(4): 1-4.
17. Silva L, Souza B, de Almeida Bessa EC, Pinheiro J. Effect of successive applications of the sublethal concentration of *Solanum paniculatum* in *Subulina octona* (Subulinidae). J Nat Prod. 2012; 5: 157-167.
18. Singh A, Singh DK. Effect of herbal molluscicides and their combinations on the reproduction of the snail *Lymnaea acuminata*. Arch Environ Contam Toxicol. 2004; 46(4): 470-477.
19. Bhide M, Gupta P, Khan MA, Dubey U, Thakur P, Nema P, et al. Morphological and biochemical studies on the different developmental stages of a fresh water snail, *Lymnaea stagnalis* (Lymnaeidae) after treatment with some pesticides. J Environ Biol. 2006; 27(2): 359-366.
20. Bhide M. Effect of nuvan, methyl parathion and thimet on the mortality, behaviour and reproduction performance of freshwater mollusk, *Lymnaea stagnalis*. J Environ Biol. 1998; 19(4): 325-332.
21. Ferreira P, Soares GLG, D'avila S, Bessa ECA. The influence of caffeine and thymol on the survival, growth and reproduction of *Subulina octona* (Bruguiere, 1789) (Mollusca, Subulinidae). Braz Arch Biol Technol. 2009; 52(4): 945-952.
22. Pilo B, Asnani MV, Shah RV. Studies on wound healing and repair in pigeon liver. III. Histochemical studies on acid and alkaline phosphatase activity during the processes. J Anim Morphol Physiol. 1972; 19(2): 205-212.
23. Timmermans LPM. Studies on shell formation in molluscs. Neth J Zool. 1969; 19: 417-523.
24. Bhide M. Effect of thiourea on the development of *Lymnaea stagnalis*. Sym on Man. Biol Devel Environ. 1986; 46: 42.
25. De Schamphelaere KAC, Koene JM, Heijerick DG, Janssen CR. Reduction of growth and haemolymph Ca levels in the freshwater snail *Lymnaea stagnalis* chronically exposed to cobalt. Ecotox Environ Safe.

2008; 71(1): 65-70.
26. Taha TC. Effect of temperature on the growth, life cycle and fecundity of the freshwater snails *Lymnaea* (*Radix*) *luteola* and *Lymnaea* (*Radix*) *acuminata* (Mollusca: Gastropoda). Philipp J Sci. 1992; 121(4): 419-434.
27. Jahan MS, Rahman MR. Breeding biology of fresh water snail *Lymnaea* (*Radix*) *acuminata* Lamarck (Gastropoda: Lymnaeidae). Univ J Zool Rajshahi Univ. 1996; 14 and 15: 47-54.
28. Allen Y, Calow P, Baird DJ. A mechanistic model of contaminant-induced feeding inhibition in *Daphnia magna*. Environ Toxicol Chem. 1995; 14(9): 1625-1630.
29. Crichton CA, Conrad AU, Baird DJ. Assessing stream grazer response to stress: a post-exposure feeding bioassay using the freshwater snail *Lymnaea peregra* (Muller). Bull Environ Contam Toxicol. 2004; 72(3): 564-570.
30. Grosell M, Gerdes RM, Brix KV. Chronic toxicity of lead to three freshwater invertebrates - *Brachionus calyciflorus*, *Chironomus tentans*, and *Lymnaea stagnalis*. Environ Toxicol Chem. 2006; 25(1): 97–104.
31. Van Der Borght O, Van Puymbroeck S. Calcium metabolism in a freshwater mollusc: quantitative importance of water and food as supply for calcium during growth. Nature. 1966; 210: 791-793.
32. Bakry FA, Sakran AMA, Ismael NMM. Molluscicidal effect of fungicide, herbicide and plant extract on some biological and physiological parameters of *Biomphalaria alexandrina*. J Egypt Soc Parasitol. 2002; 32(3): 821-835.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

MEDICINE REVIEW

Współczesna farmakoterapia astmy oskrzelowej
Contemporary pharmacotherapy of asthma

Joanna Szkaradkiewicz

Apteka Zakopiańska, Poznań, Poland

STRESZCZENIE

Astma oskrzelowa jest chorobą powszechną a jej występowanie zwiększa się na całym świecie, zarówno w krajach rozwiniętych, jak i rozwijających się. Dostępne są jednak skuteczne leki, ograniczające możliwość rozwoju zaostrzenia choroby. Najważniejszymi lekami pozostają wziewne glikokortykosteroidy, najlepiej stosowane w terapii skojarzonej z LABA, a także w postaci preparatów złożonych. Inna grupa leków - leki antyleukotrienowe potwierdziły zasadność ich stosowania w terapii łączonej z wziewnymi glikokortykosteroidami. Stosuje się również leki biologiczne. W procesie farmakoterapii ważną rolę odgrywa też edukacja pacjenta i kontrola czynników środowiskowych.

ABSTRACT

Asthma is a common disease and its incidence increases around the world, in both developed and developing countries. There are, however, effective medicines, limiting the possibility of the development of exacerbations. The most important drugs are inhaled glucocorticosteroids, preferably used in combination therapy with LABA and also in the form of combination preparations. Another group of drugs - antileucotrienes confirmed the validity of their use in combination therapy with inhaled glucocorticosteroids. Shall also apply biologic drugs. In the process of pharmacotherapy an important role also plays education of a patient and monitoring of environmental factors.

Słowa kluczowe: Astma oskrzelowa; Glikokortykosteroidy; Adrenomimetyki; Farmakoterapia.
Key words: Asthma; Glucocorticosteroids; Adrenomimetics; Pharmacotherapy.

J Biol Earth Sci 2013; 3(1): M1-M13

Corresponding author:
Joanna Szkaradkiewicz
Apteka Zakopiańska
ul. Zakopiańska 66, 60-467 Poznań, Poland
E-mail: asiaszk@op.pl

Original Submission: 20 October 2012; Revised Submission: 15 December 2012; Accepted: 21 December 2012

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

Wykaz skrótów:
ESCAP - Epidemiologia Chorób Alergicznych w Polsce
WAO - Światowa Organizacja Alergii
WHO - Światowa Organizacja Zdrowia
GINA - (ang. Global Initiative For Asthma) Światowa Inicjatywa Zwalczania Astmy
GKS - glikokortykosteroidy
wGKS - wziewne glikokortykosteroidy
SABA - (ang. short-acting β2 agonists) krótko-działające β2-adrenomimetyki
LABA - (ang. long-acting β2 agonists) długo-działające β2-adrenomimetyki
RABA - (ang. rapid acting β2 agonists) szybko-działający β2-adrenomimetyk
LT - receptory-receptory leukotrienowe
EMEA - Europejska Agencja Leków
FDA - Amerykańska Agencja ds. Żywności i Leków

WPROWADZENIE

Astma oskrzelowa jest przewlekłą chorobą o podłożu zapalnym. Zapalenie indukuje napady duszności, kaszlu i uczucia ciężkości w klatce piersiowej. W wyniku zapalenia dochodzi do różnych zmian strukturalnych czyli przebudowy (remodelingu) w obrębie całego drzewa oskrzelowego, również małych oskrzeli (< 2mm). Zmiany te obejmują: włóknienie pozanabłonkowe, naciek z komórek zapalnych, zwiększenie objętości mięśniówki gładkiej ścian dróg oddechowych oraz obrzęk. Wymienione zmiany powodują nadreaktywność i obturację oskrzeli prowadząc do niewydolności oddechowej [1].

Astma oskrzelowa może występować u dzieci i dorosłych w każdym wieku. Według ekspertów Światowej Organizacji Alergii (WAO) na astmę choruje 300 mln osób na całym świecie (dane z Białej Księgi 2011-2012) [2]. W Polsce choruje około 4 mln ludzi z czego ponad połowie nie postawiono diagnozy (dane z badania ESCAP). Z danych szacunkowych WHO wynika, że co roku na świecie dochodzi do 250.000 zgonów z powodu astmy, którym można byłoby zapobiec. Zbyt rzadkie rozpoznawanie i niekiedy nieodpowiednie leczenie astmy powoduje, że ta przewlekła choroba dolnych dróg oddechowych stanowi poważny problem zdrowia publicznego [3]. Czynniki ryzyka przedstawiono w Tabeli 1.

PRZEGLĄD

Strategia leczenia astmy

Strategia leczenia astmy oparta jest na:
1) leczeniu farmakologicznym
2) kontroli czynników środowiskowych i unikaniu alergenów
3) ciągłej edukacji pacjenta i współpracy z lekarzem i farmaceutą
4) immunoterapii (odczulaniu).

Podstawą leczenia astmy jest farmakoterapia, ponieważ jej zastosowanie nie tylko przynosi kontrolę objawów choroby i jest koniecznością w stanie astmatycznym, ale także poprawia jakość życia chorych. Światowa Inicjatywa Zwalczania Astmy (GINA) w 2006r. wyodrębniła dwie, stosowane w leczeniu astmy oskrzelowej, następujące grupy leków: leki ratunkowe oraz leki kontrolujące astmę [4, 5].

Leki ratunkowe działają szybko, odwracając

Tabela 1. Czynniki ryzyka astmy

Czynniki ryzyka astmy	
niezależne	zależne
▪ Nadreaktywność oskrzeli ▪ Wytwarzanie nadmiernej ilości przeciwciał IgE ▪ Atopia ▪ Rasa czarna ▪ Płeć żeńska u dorosłych i męska u dzieci	▪ Alergeny (pyłki roztocza kurzu domowego, sierść i naskórek zwierząt, grzyby pleśniowe) ▪ Czynniki drażniące (zanieczyszczenie środowiska, dym, formaldehyd) ▪ Zakażenia dróg oddechowych: bakteryjne i wirusowe - rinowirusy RV, syncytialne wirusy oddechowe (RSV), wirusy grypy i paragrypy ▪ Alergiczny nieżyt nosa ANN ▪ Dym tytoniowy (palenie czynne i bierne) ▪ Dieta bogata w: konserwanty (benzoesan i glutaminian sodu), barwniki azowe, substancje zapachowe i smakowe ▪ Leki (NLPZ, inhibitory ACE, β-blokery) ▪ Status społeczny i ekonomiczny

skurcz oskrzeli i łagodząc objawy duszności. Zalecane jest stosować je doraźnie, do przerywania napadu duszności. Do tej grupy zaliczamy:

- krótko-działające β2-mimetyki (wziewne i doustne),
- wziewne leki przeciwcholinergiczne,
- krótko-działająca teofilina.

Leki kontrolujące astmę to leki zapobiegające napadom duszności. Są przyjmowane codziennie jako długofalowa podstawa leczenia. Pozwalają utrzymać chorobę pod kliniczną kontrolą głównie dzięki ich przeciwzapalnym właściwościom. Do tej grupy należą:

- wziewne i ogólnoustrojowe glikokortykosteriody,
- leki przeciwleukotrienowe,
- długo-działające β2-mimetyki w połączeniu z wziewnymi GKS,
- teofilina o przedłużonym uwalnianiu,
- kromony,
- omalizumab.

Obecnie najskuteczniejszymi lekami przeciwzapalnymi spośród leków kontrolujących astmę są wziewne GKS. Stanowią one leki pierwszego rzutu stosowane w leczeniu przewlekłej astmy i są rekomendowane w zaleceniach GINA. Badania dowodzą, że leki te poprawiają czynność płuc, kontrolują stan zapalny, zmniejszają nadreaktywność dróg oddechowych, poprawiają jakość życia, zapobiegają zaostrzeniom i zmniejszają ich ciężkość oraz obniżają śmiertelność [6].

W Polsce zarejestrowane są następujące wziewne GKS: beklometazon (np. CORTARE), mometazon (np. ASMANEX TWISTHALER), budezonid (np. PULMICORT, NEBBUD, MIFLONIDE, BUDIAIR) flutykazon (np. FLIXOTIDE) i cyklezonid (ALVESCO).

Wziewne GKS różnią się siłą działania i biodostępnością jednak ma to niewielkie znaczenie, ponieważ w praktyce klinicznej stosowane są dawki ekwiwalentne. Największą skuteczność wziewnych GKS osiąga się po zastosowaniu stosunkowo niskich dawek równoważnym 400 μg budezonidu na dobę (Tabela 2) [7].

Spośród wszystkich wziewnych GKS lekiem, który ma najlepszy profil bezpieczeństwa jest cyklezonid, będący prolekiem. Pod wpływem endogennych esteraz płucnych cyklezonid jest aktywowany do czynnego metabolitu desisobutyrylu cyklezonidu (des-CIC), który wywołuje silny efekt przeciwzapalny. Lek zatem charakteryzuje unikalna wybiórczość narządowa, a w konsekwencji brak objawów miejscowych ze strony górnych dróg oddechowych (kandydoza jamy ustnej i gardła, dysfonia, chrypka, kaszel z podrażnienia). Cyklezonid silnie i prawie całkowicie wiąże się z białkami osocza (99% leku) stąd też nie przenika do innych tkanek i nie wywołuje efektów systemowych [8, 9].

U większości pacjentów z astmą obok leczenia przeciwzapalnego wGKS konieczne jest zastosowanie rozkurczowych leków β2-adrenergicznych. Są to podstawowe leki bronchodilatacyjne pozwalające zachować drożność dróg oddechowych. Ze względu na różnice w czasie działania β2-mimetyki można podzielić na krótko-działające (short-acting β2 agonists - SABA): fenoterol, salbutamol, terbutalina oraz długo-działające (long-acting β2 agonists - LABA): salmeterol, bambuterol per os. W grupie LABA, którą cechuje długi i wolny czas działania, można wyróżnić formoterol mający szybki początek

Tabela 2. Dzienne dawki ekwiwalentne oszacowane dla wybranych wziewnych GKS u dorosłych chorych na astmę

Nazwa Leku	Niska dawka dzienna (μg)	Średnia dawka dzienna (μg)	Wysoka dawka dzienna (μg)
Budezonid	200-400	>400-800	>800-1600
Propionian flutikazonu	100-250	>250-500	>500-1000
Dipropionian beklometazonu CFC	200-500	>500-1000	>1000-2000
Dipropionian beklometazonu HFA	100-250	>250-500	>500-1000
Cyklezonid	80-160	>160-320	>320-1280
Mometazon	200	≥400	≥800

działania, jest to tzw. rapid acting β2 agonists RABA.

Leki β2-adrenergiczne (Tabela 3) zmniejszają uwalnianie mediatorów z komórek układu immunologicznego dzięki czemu zwalniają szybkość procesu zapalnego w dolnych drogach oddechowych. Leki te hamują przepuszczalność naczyń płucnych i obwodowych, stymulują ruchy rzęsek i pobudzają wydzielanie surfaktantu poprawiając klirens śluzowo-rzęskowy w oskrzelach. Wziewne leki β2-adrenergiczne powodują mniej ogólnoustrojowych działań niepożądanych takich jak stymulacja sercowo-naczyniowa, drżenie mięśniowe czy hipokaliemia w porównaniu do β-mimetyków doustnych. Jednak LABA nie powinny być podawane w monoterapii ponieważ leki te nie hamują procesu zapalnego w oskrzelach [10].

Przewlekłe stosowanie zarówno krótko- jak i długo-działajacych β2-mimetyków wywołuje tolerancję na lek. Jest ona spowodowana zmniejszeniem gęstości receptorów β2 na powierzchni komórek w płucach, jest to tzw. proces down regulation. Ponadto, długotrwała monoterapia LABA niesie z sobą ryzyko ciężkich zaostrzeń astmy oraz zgonów. W związku z tymi faktami LABA są stosowane wyłącznie w terapii skojarzonej z odpowiednią dawką wGKS, co jest obecnie standardem w nowoczesnej farmakoterapii astmy.

Skojarzenie wziewnego GKS z LABA ujawniło synergizm względem działania obu leków. Glikokortykosteroidy, poprzez działanie na poziomie genetycznym, korzystnie wpływają na liczbę receptorów β2 w płucach, zapobiegając tym samym zjawisku down regulation. Natomiast β2-mimetyki potęgują przeciwzapalne działanie wGKS, co pozwala na zmniejszenie ich dawek i zwiększenie bezpieczeństwa terapii przy jednoczesnym uzyskaniu tego samego efektu klinicznego.

Leczenie skojarzone polepsza funkcję płuc, zmniejsza objawy astmy, zmniejsza zużycie leków doraźnych i redukuje liczbę zaostrzeń. U większości pacjentów stosujących wGKS plus LABA kliniczna kontrola astmy jest osiągana szybciej i przy niższej dawce steroidu. Wysoka skuteczność terapii skojarzonej spowodowała pojawienie się preparatów złożonych, dostarczających w jednej inhalacji glikokortykosteroid i β-mimetyk jednocześnie. Liczne badania potwierdziły, że podanie LABA i wGKS w jednym inhalatorze jest, co najmniej równie skuteczne, jak podanie ich w oddzielnych inhalatorach. Dopuszczonymi do obrotu w Polsce są następujące połączenia wGKS z LABA: propionian flutikazonu + salmeterol, dipropionian beklometazonu + fumaran formoterolu, budezonid + formoterol (Tabela 4) [11].

Połączenie synergistycznie działających wziewnego GKS i LABA w jednym inhalatorze daje efekt addycyjny, który związany jest z lepszą infiltracją wGKS do tkanki płucnej oraz intensywniejszym efektem rozkurczowym β2-agonisty. Współdziałanie obu leków przekłada się

Tabela 3. Zestawienie wziewnych leków β2-adrenergicznych i ich przykładowych preparatów handlowych, postaci, dawek oraz dawkowania

Nazwa chemiczna	Nazwa handlowa	Postać i dawki	Dawkowanie
Fenoterol (SABA)	Berotec N	aerozol pMDI 100mg	1-2 inhalacje x 1-3dz. max. do 8 inhalacji/dz.
Salbutamol (SABA)	Ventolin Salbutamol Hasco	aerozol pMDI100µg dysk DPI 200µg płyn 0.1%, 0.2% do nebulizacji syrop 2mg/5ml	1-2 inhalacje x dz. 1x1, max. 1x4 2,5 mg x1, max. do 4x 4mg (10ml) 3-4xdz.
Salmeterol (LABA)	Pulmoterol Serevent	aerozol DPI 50µg aerozol pMDI 25µg dysk DPI 50µg	2x1 inhalacja max. 2x2 inhalacje 2x2 inhalacje max. 2x4 inhalacje 2x1 inhalacja
Formoterol (RABA)	Atimos Diffumax Easyhaler Foradil Forastmin Oxis Turbohaler	aerozol pMDI 12µg aerozol DPI 12µg j.w. j.w. aerozol DPI 4,5µg aerozol DPI 9µg	2x1 inhalacja max.2x2 inhalacje j.w. j.w. j.w. 1-2 x 1-2 inhalacje j.w.

więc istotnie na zmniejszenie stanu zapalnego w drogach oddechowych [10, 12].

Leczenie astmy preparatami złożonymi ma wiele zalet. Przede wszystkim zmniejszenie liczby inhalatorów i uproszczenie schematu dawkowania jest wygodniejsze dla pacjentów i sprawia, że chorzy chętniej stosują się do zaleceń lekarzy. Prawidłowo stosowane preparaty dają lepsze rezultaty w leczeniu, a w efekcie lepszą kontrolę astmy i zmniejszenie częstości zaostrzeń, co z kolei ma znaczący wpływ na poprawę jakości życia pacjentów z astmą. Terapia preparatami skojarzonymi zwiększa istotnie bezpieczeństwo pacjentów. Po pierwsze, z uwagi na niższą dawkę wziewnego steroidu, a po drugie terapia wGKS + LABA w jednym inhalatorze wyklucza ewentualność stosowania β2-agonisty w monoterapii, co nierzadko ma miejsce u chorych stosujących osobne inhalatory.

Ważną kwestię w terapii astmy stanowią koszty finansowe leczenia, zarówno bezpośrednie jak i pośrednie (Tabela 5). Terapia preparatami złożonymi może skutecznie je obniżyć. Wszystkie zarejestrowane w Polsce przeciwastmatyczne leki złożone są refundowane i w porównaniu do leczenia skojarzonego są tańsze, co daje większe prawdopodobieństwo, że pacjent wykupi lek w aptece [11, 13]. Co więcej, zmniejszenie liczby zaostrzeń, redukcja stosowanych leków doraźnych przez chorego, zmniejszenie liczby hospitalizacji nie pozostają bez znaczenia. Koszty pośrednie związane z nieobecnością w pracy i przed-

Tabela 4. Zestawienie preparatów złożonych, zawierających wGKS i LABA, zarejestrowanych w Polsce

Kombinacja preparatu złożonego GKS+LABA	Nazwa handlowa	Postać	Dawki (μg) GKS/LABA	Dawkowanie	Wskazanie
propionian flutikazonu + salmeterol	Seretide 50 Seretide 125 Seretide 250	aerozol pMDI	50/25 125/25 250/25	1 inhalacja x 2	Leczenie podtrzymujące
	Seretide Dysk 100 Seretide Dysk 250 Seretide Dysk 500	dysk DPI	100/50 250/50 500/50	2 inhalacje x 2	Leczenie Podtrzymujące
dipropionian beklometazonu + fumaran formoterolu	Fostex	aerozol pMDI	100/6	1-2 inhalacje x 2	Leczenie Podtrzymujące
budesonid + formoterol	Symbicort Turbohaler	aerozol DPI	80/4,5 160/4,5 320/9	1-2 inhalacje x 2	Leczenie podtrzymujące i doraźne

Tabela 5. Porównanie bezpośrednich kosztów pacjenta stosującego flutikazon i salmeterol (n.p. Obwieszczenia Ministra Zdrowia z dnia 26 pazdziernika 2012r. w sprawie wykazu refundowanych leków,środków spożywczych specjalnego przeznaczenia żywieniowego oraz wyrobów medycznych na dzień 1 listopada 2012r.)

Leki skojarzone	Cena dla pacjenta (PLN)	Leki złożone	Cena dla pacjenta (PLN)
Flixotide 50μg po 120dawek aerozol	29,28	Seretide 25/50 po 120 dawek aerozol	3,20
Serevent 25μg po 120 dawek aerozol	25,42		
Flixotide 125μg po 120dawek aerozol	56,44	Seretide 25/125 po 120 dawek aerozol	19,63
Serevent 25μg po 120 dawek aerozol	25,42		
Flixotide 250μg po 120dawek aerozol	66,18	Seretide 25/250 po 120 dawek aerozol	13,49
Serevent 25μg po 120 dawek aerozol	25,42		

wczesnymi zgonami, także ulegają zmniejszeniu.

Korzyści wynikające ze stosowania preparatów złożonych sprawiają, że terapia astmy poprzez łączne podawanie wGKS i LABA we wspólnym inhalatorze jest całkowicie zasadna, jednak nie u wszystkich pacjentów znajduje ona zastosowanie. Wówczas alternatywą dla leczenia glikokortykosteroidami są leki antyleukotrienowe, mające właściwości przeciwzapalne i przeciwalergiczne [14]. Choć leki te, w porównaniu do wGKS charakteryzują się słabszym działaniem przeciwzapalnym, to nawet przy długotrwałym stosowaniu wykazują lepszy od steroidów profil bezpieczeństwa. Leki antyleukotrienowe w mniejszym stopniu od LABA hamują skurcz oskrzeli, ale znacząco zmniejszają liczbę eozynofilów w drogach oddechowych co powoduje redukcję częstości nawracających obturacji. Leki te poprawiają czynność płuc, obniżają liczbę zaostrzeń, istotnie zmniejszają objawy nocne i dzienne oraz redukują zużycie leków doraźnych u chorych we wszystkich grupach wiekowych. Ta grupa leków znajduje zastosowanie w monoterapii u pacjentów z przewlekłą astmą łagodną jako alternatywa do wGKS, w astmie przewlekłej umiarkowanej jako lek dodatkowy do terapii wGKS i LABA aby poprawić kontrolę astmy bez konieczności zwiększania dawki steroidów wziewnych, w astmie aspirynowej, w astmie z towarzyszącym alergicznym nieżytem nosa i astmie z atopowym zapaleniem skóry. Leki antyleukotrienowe są szczególnie polecane u dzieci zapobiegawczo w astmie epizodycznej i przewlekłej o lekkim przebiegu oraz w stanach bronchospastycznych spowodowanych zakażeniami wirusowymi oraz w astmie wysiłkowej [15, 16].

Do leków antyleukotrienowych zaliczamy inhibitory 5-lipooksygenazy leukotrienów, której jedynym przedstawicielem jest zileuton nie dostępny w Polsce z uwagi na znaczną hepatotoksyczność oraz antagonistów receptorów leukotrienowych. Najbardziej znanym związkiem blokujący receptor dla leukotrienów cysteinylowych CysLT1 jest montelukast, w Polsce dostępny jest jego oryginał oraz wiele leków generycznych [17].

Obok wysokiego profilu bezpieczeństwa montelukastu do jego zalet można zaliczyć także postać leku, która niewątpliwie ma znaczenie. W przypadku wGKS pacjenci nierzadko mają problemy z właściwą techniką inhalacyjną. Montelukast (Tabela 6) jest dostępny w komfortowej, dostosowanej do wieku pacjentów postaci, a mianowicie: dla dzieci od 6. miesiąca jest to postać granulatu, dla starszych dzieci są to tabletki do rozgryzania i żucia, a dla dorosłych i młodzieży tabletki powlekane. Schemat dawko-

Tabela 6. Przykłady preparatów zawierających montelukast w dawce 10 mg

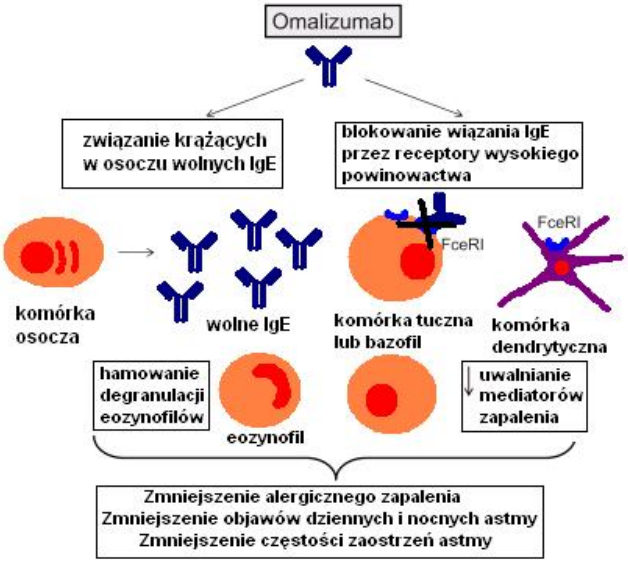
Nazwa handlowa	Producent	Kraj pochodzenia	Refundacja (odpłatność)	Cena dla pacjenta
Singulair	Merck	Wielka Brytania	brak	ok. 30zł (cena umowna)
Astmodil	Polfarmex S.A.	Polska	30%	8,69
Drimon	Teva Polska	Polska	30%	27,79
Monkasta	Krka	Słowenia	30%	13,91
Montelak	Medres s.r.o.	Czechy	30%	30,68
Milukante	Adamed	Polska	30%	14,50
Montelukast Sandor	Sandoz GmbH	Austria	30%	17,96
Montelukast Bluefish	Bluefish	Szwecja	30%	8,21
Montessan	Apotex Europe	Holandia	30%	23,74
Promonta	Nycomed Pharma	Polska	30%	8,72
Symlukast	SymPhar	Polska	30%	8,69
Vizendo	GlaxoSmithKline	Wielka Brytania	30%	24,90

wania tego leku jest także dogodny dla chorych (Tabela 7).

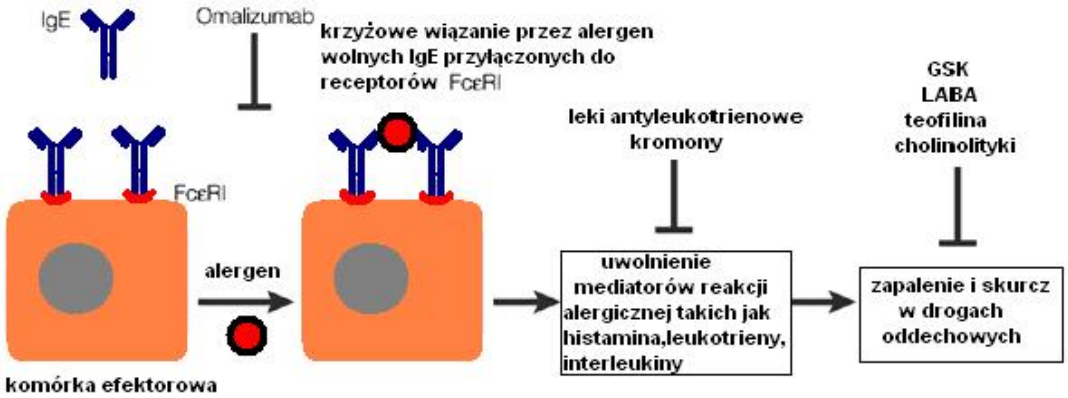
Przyjmowanie montelukastu należy kontynuować zarówno wtedy, gdy objawy astmy są pod kontrolą, a także w przypadku nagłego zaostrzenia choroby. U pacjentów, u których zastosowano już terapię wGKS zmiana leczenia na antagonistów LT-receptorów może zwiększać ryzyko utraty kontroli choroby. W takim przypadku można zastosować leczenie skojarzone wGKS z lekiem antyleukotrienowym, co potęguje efekt przeciwzapalny bez konieczności zwiększania dawki wGKS [17, 18].

U pacjentów z ciężką przewlekłą niekontrolowaną astmą alergiczną, u których pomimo wdrożenia terapii dwulekowej a nawet kilku lekowej, nie uzyskano kontroli choroby należy rozważyć zastosowanie terapii anty-IgE. Terapia anty-IgE to terapia za pomocą leku biologicznego-omalizumabu, który zmniejsza liczbę wolnych cząsteczek IgE w osoczu [19, 20].

Omalizumab to rekombinowane humanizowane monoklonalne przeciwciało anty-IgE, które hamuje kaskadę zapalenia alergicznego w drogach oddechowych i tym sposobem zmniejsza objawy kliniczne astmy. Mechanizm działania leku polega na wybiórczym wiązaniu się jego cząsteczki z krążącymi w osoczu IgE, co zapobiega przyłączaniu się wolnych cząsteczek IgE do receptorów wysokiego powinowactwa FcεRI na komórkach efektorowych (bazofilach, komórkach tucznych i dendrytycznych) (Ryc. 1, 2). W konsekwencji nie dochodzi do degranulacji komórek układu immunologicznego i nie następuje uwolnienie mediatorów zapalenia do krwi. Omalizumab (Xolair, Novartis) został zaaprobowany przez FDA, EMEA, a także dostępny jest w Polsce i od 1 listopada 2012 refundowany w ramach programu lekowego dla chorych na ciężką astmę alergiczną. Lek ten można zastosować jedynie u pacjentów, u których astma została wywołana przez immunoglobulinę E, a więc w leczeniu IgE zależnej astmy oskrzelowej opornej na leczenie [19]. Preparat można podawać osobom dorosłym, młodzieży oraz dzieciom w wieku od 6. roku życia (omalizumab refundowany jest u pacjentów powyżej 12 roku życia). Omalizumab podawany jest podskórnie przez lekarza lub pielęgniarkę po uprzednim ustaleniu przez lekarza dawki leku. Właściwą dawkę leku oraz częstość jej podawania ustala się na podstawie wyjściowego stężenia IgE oznaczonego przed rozpoczęciem leczenia oraz masy ciała pacjenta. Pacjent otrzymuje każdorazowo od 1 do 4 wstrzyknięć leku, które są podawane co dwa lub co cztery tygodnie. Omalizumab przeznaczony jest do



Ryc. 1. Schemat mechanizmu działania leku omalizumab (na podstawie [21])



Ryc. 2. Punkty uchwytu działania leków przeciwastmatycznych (na podstawie [22])

Tabela 7. Schemat dawkowania montelukastu u różnych grup wiekowych pacjentów na podstawie preparatów Singulair 10, Singulair 5, Singulair 4 oraz SingulairMini

Wiek pacjenta	Dawka	Postać leku	Dawkowanie	Uwagi
Dorośli i młodzież od 15 lat	10 mg	tabletki powlekane	1x1 wieczorem	Niezależnie od posiłku
Dzieci od 6-14 lat	5 mg	tabletki do rozgryzania i żucia	1x1 wieczorem	Tabletki przyjmować 1h przed lub 2h po posiłku
Dzieci 2-5 lat	4 mg	tabletki do rozgryzania i żucia	1x1 wieczorem	Tabletki przyjmować 1h przed lub 2h po posiłku
Dzieci od 6. miesiąca do 5 lat	4 mg	granulat	1x1 wieczorem	Niezależnie od posiłku; lek można podać bezpośrednio doustnie lub po wymieszaniu z łyżką miękkiego pokarmu i podać bezpośrednio po przygotowaniu; granulatu nie należy rozpuszczać w płynie, można go popić płynem

długotrwałej terapii, gdyż pełny efekt leczenia u chorych uzyskuje się dopiero po 12 do 16 tygodniach terapii anty-IgE. Lek ten nie jest wskazany w leczeniu nagłych zaostrzeń choroby bądź stanu astmatycznego. Istotne jest kontynuowanie przez pacjenta dotychczasowego leczenia kortyko-steroidami ogólnoustrojowymi lub wziewnymi. Możliwe jest jednak, pod ścisłą kontrolą lekarza, stopniowe zmniejszanie dawek steroidów, a nawet całkowite odstawienie doustnych GKS. Terapia omalizumabem jest dobrze tolerowana przez chorych i posiada zadowalający profil bezpieczeństwa. Większość działań niepożądanych jakie mogą wystąpić mają charakter łagodny lub umiarkowany i związane są z reakcją w miejscu iniekcji (rumień, świąd, wysypka, pokrzywka, obrzęk, ocieplenie). Omalizumab jest lekiem biologicznym i podczas jego stosowania może dojść do indukcji chorób z autoagresji, może wystąpić choroba posurowicza czy reakcje anafilaktyczne, co jednak zaobserwowano bardzo rzadko [23]. Lek ten poprawia kontrolę astmy poprzez redukcję liczby zaostrzeń i hospitalizacji, zmniejsza objawy choroby i wpływa na poprawę jakości życia [19].

Do leków kontrolujących przebieg astmy należą także kromony, które wykazują jednak słabe działanie przeciwzapalne i charakteryzują się niską skutecznością kliniczną. Zastosowanie kromoglikanów (kromoglikan diodowy Cromoxal) jest zatem ograniczone i sprowadza się do zapobiegania astmie oskrzelowej o podłożu alergicznym, jako leczenie alternatywne, jednak nie rekomendowane w monoterapii.

Podobnie sytuacja wygląda w przypadku preparatów teofiliny w postaci o przedłużonym uwalnianiu (Euphyllin CR Retard, Euphyllin Long, Theospirex retard 100 i 300, Theovent 100 i 300). Teofilina ma słabe właściwości przeciwzapalne i niską skuteczność jako lek kontrolujący przebieg choroby natomiast jej stosowanie związane jest z wysokim ryzykiem wystąpienia objawów ubocznych od arytmii serca do zgonów włącznie. Jest ona stosowana wyłącznie jako lek dodatkowy, u pacjentów, u których nie uzyskano kontroli po zastosowaniu LABA i dużych dawek GKS. Niestety efektywność terapii wGKS plus teofilina jest mniej skuteczna od połączenia wGKS plus LABA [5, 18].

Z drugiej strony teofilina i jej pochodne (aminofilina) jako leki ratunkowe są ważnymi preparatami w leczeniu stanu astmatycznego [1]. Ze względu na wąski indeks terapeutyczny (10-20 μg/ml to stężenie terapeutyczne natomiast > 20 μg/ml pojawiają się objawy przedawkowania), objawy niepożądane zagrażające życiu i duże różnice osobnicze w eliminacji teofiliny zaleca się monitorowanie jej stężeń we krwi.

Do bronchodilatacyjnych leków doraźnie stosowanych w astmie należą także cholinolityki (bromek ipratropium ATROVENT - płyn do nebulizacji, ATROVENT N - aerozol wziewny). Ipratropium jest alternatywnym lekiem rozkurczowym dla pacjentów, u których po zastosowaniu LABA wystąpiły takie działania niepożądane jak tachykardia, arytmia czy drżenia mięśniowe. Z powodu addycyjnych właściwości w zapobieganiu napadom duszności w astmie

oskrzelowej bromek ipatropium może być stosowany łącznie z fenoterolem. Takie połączenie w postaci preparatu złożonego jest dostępne w Polsce (BERODUAL - płyn do nebulizacji, BERODUAL N - aerozol wziewny).

Immunoterapia

Zadaniem immunoterapii jest wywołanie tolerancji i podwyższenie progu wrażliwości na określone alergeny. U chorych na astmę oskrzelową, którzy są uczuleni na alergeny roztoczy, pyłków traw i drzew, sierści zwierząt stopniowe podawanie, w odpowiednich odstępach czasu, wzrastających dawek alergenu powoduje korzystne zmiany odpowiedzi immunologicznej [26].

Standardowym sposobem tego leczenia jest immunoterapia podskórna. Swoista immunoterapia daje lepsze efekty u osób młodych, z jeszcze nie zaawansowanym procesem zapalnym choroby [18]. Leczenie odczulające powinno być stosowane przez 3 do 5 lat, natomiast korzystny efekt utrzymuję się wiele lat. Przeciwwskazaniem do zastosowania immunoterapii jest astma ciężka.

Edukacja pacjenta

Współczesna farmakoterapia daje olbrzymie możliwości w leczeniu chorób przewlekłych takich jak astma (różne połączenia leków, monitorowanie różnych dawek, nowe leki i nowe metody leczenia), jednak jej efekty będą zależeć także od jakości współpracy na linii lekarz-pacjent-farmaceuta. W większości populacji na świecie brakuje odpowiedniej edukacji i świadomości przewlekłej choroby, jaką jest astma oskrzelowa. Właściwe szkolenie i edukacja chorych i ich rodzin jest fundamentem scalającym efektywne leczenie tej choroby. Współpraca pacjenta z lekarzem rodzinnym, pulmonologiem i farmaceutą może zaowocować znaczną poprawą kontroli astmy, a tym samym jakości życia chorego. Oswojenie pacjenta z chorobą leży w sugestii zarówno lekarza jak i farmaceuty. Zadaniem obu jest wyjaśnianie wszelkich niejasności dotyczących terapii i stosowanych leków. Dużo częściej bywa, jednak że pacjenci trapieni wątpliwościami szukają pomocy w aptece. Stąd, tak istotne jest odpowiednie przygotowanie merytoryczne farmaceutów i realizowanie planu opieki farmaceutycznej.

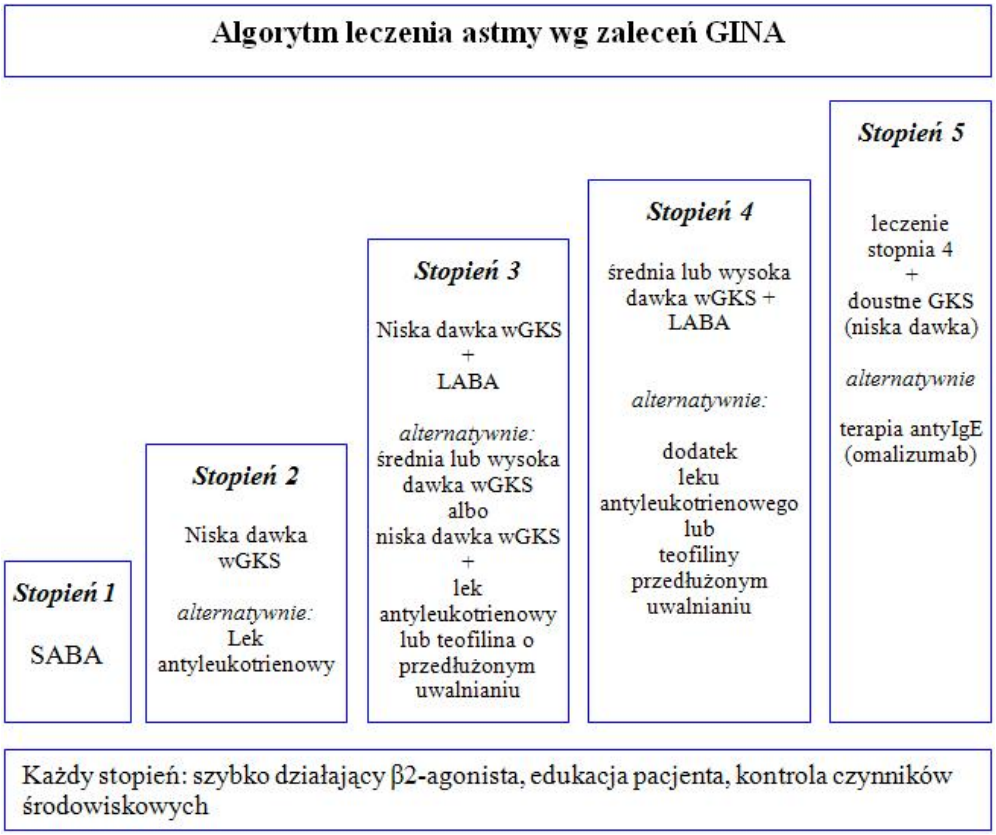
Wdrażanie edukacji prozdrowotnej i motywowanie pacjentów do odpowiednich działań prewencyjnych m. in. unikania kontaktu z alergenem, zachęcanie rozpoczęcia terapii antynikotynowej, wyjaśnianie istotnej roli szczepień przeciw grypie, które powinny być wykonywane co roku najlepiej wczesną jesienią, jest domeną farmaceuty. W aptece pacjent powinien być poinformowany o właściwej terapii inhalacyjnej, a mianowicie kolejności stosowanych leków wziewnych i konieczności przepłukania jamy ustnej po aplikacji, a także na tyle, ile to możliwe zademonstrowanie pacjentowi sposobu korzystania z inhalatorów. Farmaceuta może też uświadomić pacjentowi potrzebę rehabilitacji ogólnej i oddechowej. Takie działania, w połączeniu z opieką lekarską mogą przełożyć się na dobrą kontrolę przebiegu choroby i komfort pacjentów.

Efektywność leczenia astmy oskrzelowej

Chorzy na astmę stanowią grupę pacjentów wymagających zintegrowanej strategii leczenia i opieki lekarza rodzinnego, pulmonologa oraz

Tabela 8. Klasyfikacja astmy według stopni kontroli zgodna z wytycznymi GINA

Cecha	Astma kontrolowana	Astma częściowo kontrolowana	Astma Niekontrolowana
Objawy w ciągu dnia	Nie występują (≤2/tydzień)	>2/tydzień	≥3 kryteria astmy częściowo kontrolowanej obecne w którymkolwiek tyg.
Ograniczona aktywność fizyczna	Nie występuje	Jakiekolwiek ograniczenie	
Objawy nocne (przebudzenia)	Nie występują	Jakiekolwiek ograniczenie	
Konieczność stosowania leków doraźnych	Brak (≤2/tydzień)	>2/tydzień	
Czynność płuc (PEF lub FEV1)	Prawidłowa	<80% wartości prawidłowej	
Zaostrzenia	Nie występują	≥1/rok	1 w tygodniu

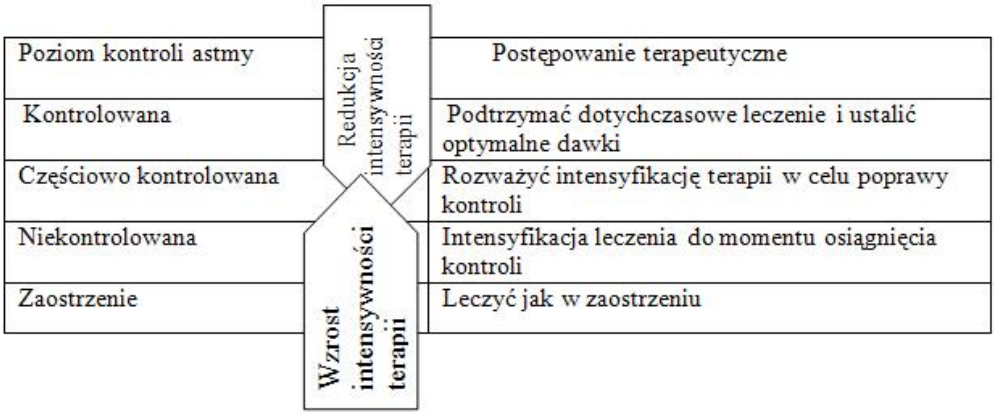


Ryc. 3. Stopnie intensywności terapii w astmie wg GINA (2011-2012)

farmaceuty. Samo leczenie astmy powinno być skoncentrowane na opanowaniu objawów choroby u pacjentów i zmniejszeniu niedogodności związanych z terapią czyli m. in. ilość stosowanych leków, działania niepożądane tych leków, łatwość i umiejętność odpowiedniego zastosowania leku wziewnego. Takie postępowanie znacząco może wpływać na poprawę jakości życia pacjenta, leczenie będzie zatem efektywne. Miernikiem takiego efektywnego leczenia astmy jest jej kontrola. Według zaleceń programu Światowej Inicjatywy Zwalczania Astmy (GINA) aktualna jej klasyfikacja jest zdeterminowana przez stopień kontroli choroby, a nie stopień jej ciężkości (Tabela 8) [5, 24].

Gdy astma nie jest kontrolowana może bardzo ograniczać aktywność życiową chorego, a nawet prowadzić do przedwczesnego zgonu. Stąd celem leczenia astmy jest osiągnięcie i utrzymanie całkowitej kontroli choroby. Efektem uzyskania w pełni kontrolowanej astmy jest zmniejszenie ilości ataków choroby w ciągu dnia i nocy, zmniejszenie częstotliwości stosowania leków doraźnych i uzyskanie prawidłowych wartości spirometrycznych. Zatem pełna kontrola choroby istotnie wpływa na poprawę jakości życia chorych na astmę. Współczesna farmakoterapia astmy ma także ograniczać ewentualne przyszłe ryzyko związane z: zaostrzeniem choroby, przyspieszonym pogorszeniem funkcji płuc, objawami ubocznymi zastosowanych w terapii leków.

W celu osiągnięcia kontroli u pacjentów zaleca się stosowanie 5-stopniowego algorytmu leczenia astmy opracowanego przez ekspertów GINA (Ryc. 3). Na każdym stopniu leczenia ważna jest edukacja pacjentów i kontrola czynników środowiskowych. Na każdym stopniu terapii można zastosować leczenie preferowane lub alternatywne w zależności od odpowiedzi chorego na leczenie przeciwastmatyczne. Stopnie od 1 do 5, prezentujące stopnie nasilenia choroby (od łagodnej poprzez umiarkowaną, średnio umiarkowaną do ciężkiej), jednocześnie wskazują na rosnącą intensywność leczenia. Pierwszy stopień leczenia sprowadza się jedynie do zastosowania w razie potrzeby leku ratunkowego. U większości pacjentów rekomen-dowany jest szybko działający β2-mimetyk wziewny (wSABA). Ewentualnie można zastosować lek antycholinergiczny lub doustnego krótko działającego β2-agonistę bądź krótko działającą teofilinę, jednak te leki cechuje powolniejszy początek działania i większe ryzyko pojawienia się



Ryc. 4. Modyfikacje terapii przeciwastmatycznej w zależności od stopnia kontroli

działań ubocznych. Począwszy od 2 stopnia terapii do 5 włącza się do kuracji lek kontrolujący przebieg astmy, który stosowany jest przez pacjentów regularnie obok stosowanych doraźnie leków ratunkowych.

Według zaleceń GINA najważniejszymi lekami kontrolującymi są wziewne GKS, które są lekami pierwszego rzutu. Na drugim stopniu znajdują się więc właśnie wGKS, rekomendowane w niskiej dawce jako inicjujące leczenie przeciwzapalne u chorych w każdym wieku. Jako leki drugiego wyboru mogą być zastosowane leki antyleukotrienowe, szczególnie u pacjentów z towarzyszącym alergicznym nieżytem nosa, pacjentów nie tolerujących ubocznych efektów GKS (takich jak utrzymująca się chrypka) lub pacjentów ze steroidofobią. Inne opcje leczenia są dostępne, jednak nie rekomendowane jako inicjujące leki kontrolne lub leki pierwszego rzutu (teofilina o przedłużonym uwalnianiu, kromony) [5].

Według wskazań GINA, chorzy na 3 stopniu rozpoczynają terapię skojarzoną: niska dawka wGKS plus wziewny LABA, która może być zastosowana w postaci wspólnego inhalatora (preparaty złożone) bądź w osobnych inhalatorach. Niska dawka wziewnego steroidu jest zazwyczaj wystarczająca do osiągnięcia kontroli ze względu na addycyjne działanie zastosowanego połączenia. Inną opcją, rekomendowaną szczególnie dla dzieci, jest zwiększenie dawki wGKS i zastosowanie go w monoterapii do czasu osiągnięcia kontroli astmy. Dobre rezultaty daje też skojarzone leczenie wGKS w niskiej dawce plus lek antyleukotrienowy. Jako alternatywne leczenie można rozważyć zastosowanie wGKS w niskiej dawce z teofiliną o przedłużonym uwalnianiu.

U pacjentów, u których zastosowana terapia nie przyniosła oczekiwanych rezultatów należy wdrożyć leczenie na 4 stopniu. Zazwyczaj zwiększenie dawki wGKS do średniej lub wysokiej, w połączeniu z LABA jest optymalnym preferowanym rozwiązaniem. Ewentualnie jako lek dodatkowy do tego schematu leczenia można zastosować lek antyleukotrienowy lub teofilinę o przedłużonym uwalnianiu.

Leczenie na 5 stopniu dotyczy pacjentów z ciężką do leczenia postacią astmy, u których ogranicza ona znacznie jakość życia. W tym przypadku do preferowanej terapii poziomu 4, dodatkowo stosowane są doustne glikokortykosteroidy, ewentualnie u osób z astmą alergiczną można rozważyć zastosowanie terapii anty-IgE.

Istotnym zaleceniem wg raportu GINA jest zredukowanie intensywności leczenia po uzyskaniu kontroli astmy (Ryc. 4) [25]. Jest to bardzo ważne ze względu na dobranie odpowiedniej terapii u danego pacjenta i zredukowanie dawek do minimalnych, potrzebnych do utrzymania kontroli choroby i ograniczenia do minimum ryzyka wystąpienia działań niepożądanych. Po 3 miesiącach od uzyskania kontroli choroby, pod warunkiem, że w tym czasie nie było oznak jej utraty, można rozpocząć próbę redukcji dawek, kierując się następującymi przesłankami:

- jeśli astma jest kontrolowana poprzez stosowanie wGKS w niskiej dawce to należy zmienić jego dawkowanie na 1 x dziennie
- jeśli astma jest kontrolowana poprzez stosowanie wGKS w średniej lub wysokiej dawce to należy obniżać dawkę o 50% co 3 miesiące do minimalnej dawki gwarantującej kontrolę
- jeśli astma jest kontrolowana poprzez stosowanie terapii skojarzonej wGKS + LABA to

należy w pierwszej kolejności obniżać o 50% dawkę wGKS co 3 miesiące do niskiej gwarantującej kontrolę, po czym można spróbować odstawić LABA

- jeśli astma jest kontrolowana poprzez stosowanie wGKS w połączeniu z innym niż LABA lekiem kontrolującym, to podobnie należy w pierwszej kolejności obniżać o 50% dawkę wGKS co 3 miesiące do niskiej gwarantującej kontrolę, a nastepnie podjąć próbę odstawienia drugiego leku kontrolującego
- wg zaleceń GINA istnieje możliwość odstawienia na próbę leków kontrolujących astmę, jeżeli przez co najmniej rok choroba jest całkowicie kontrolowana na najniższej dawce leku kontrolującego i nie ma objawów astmy.

W momencie utraty kontroli należy jak najszybciej zintensyfikować leczenie, co przy pojawieniu się pierwszych objawów może uchronić chorego przed zaostrzeniem astmy.

PODSUMOWANIE

Występowanie astmy zwiększa się na całym świecie, zarówno w krajach rozwiniętych, jak i rozwijających się, stąd właśnie jest to duży problem zdrowia publicznego. Dostępne są jednak skuteczne leki ograniczające możliwość rozwoju zaostrzenia choroby. Najważniejszymi lekami pozostają wziewne GKS, najlepiej stosowane w terapii skojarzonej z LABA, a także w postaci preparatów złożonych wygodnych dla pacjentów. Stosunkowo jeszcze nowa grupa leków, jakimi są leki antyleukotrieniwe potwierdziły zasadność ich stosowania w terapii łączonej z wGKS. Obiecująco wygląda też zastosowanie leków biologicznych w terapii astmy oskrzelowej, gdyż omalizumab ma dobry profil bezpieczeństwa i wysoką skuteczność. Współczesna farmakoterapia astmy jest procesem cyklicznym, w którym powtarzają się 3 elementy leczenia: ocena stopnia kontroli choroby, osiągnięcie i utrzymanie kontroli przebiegu astmy. W procesie tym ważną rolę odgrywa też edukacja pacjenta i kontrola czynników środowiskowych.

DEKLARACJA

Autorka deklaruje nie występowanie konfliktu interesów.

PIŚMIENNICTWO

1. Chazan R. Postępowanie w stanie astmatycznym. Alergia Astma Immunol. 1997; 2(2): 87-96.
2. Biała Księga Alergii Światowej Organizacji Alergii 2011-2012. www.wao.org.
3. www.who.org
4. Global Strategy for Asthma Management and Prevention. Global Initiative for Asthma (GINA). 2011. www.ginaasthma.org
5. Kupryś-Lipińska I, Kuna P. Farmakoterapia astmy i alergicznego nieżytu nosa zgodnie z najnowszymi wytycznymi GINA i ARIA. Ter Alergol. 2012; 4(271): 24-29.
6. Chazan R. Blaski i cienie stosowania glikokortykoterapii. Ter Pneumonol. 2010; 10 (231): 83-88.
7. Grzanka A, Jarząb J. Problemy związane z określaniem równoważności dawek wziewnych glikokortykosteroidów. Pol Merk Lek. 2010; 28(164): 134-137.
8. Agencja Ochrony Technologii Medycznych. 2011. Alvesco (cyklezonid) w leczeniu astmy oskrzelowej. Raport w sprawie oceny świadczenia opieki zdrowotnej. Warszawa.
9. Brzostek D, Jarmuszczak-Walczyńska A, Zaniewska D. Zamiana steroidu wziewnego na cyklezonid, jako sposób na przywrócenie kontroli astmy oskrzelowej i poprawę tolerancji leczenia. Terapia W gabinecie lekarza rodzinnego. 2012; 9(2/278): 24-27.
10. Chazan R. Leczenie skojarzone GKS/LABA - nowe trendy w leczeniu chorych na astmę i przewlekłą obturacyją chorobę płuc. Pol Merk Lek. 2010; 28(166): 251.
11. www.mz.gov.pl
12. Kupryś-Lipińska I, Kuna P. Jak wybrać optymalny lek kontrolujący w astmie oskrzelowej - czy wyniki badań pragmatycznych wpływają na naszą codzienną praktykę? Ter Alergol. 2012; 4(271): 9-18.
13. www.urpl.gov.pl
14. Janiec W. (red.). 2010. Kompendium farmakologii. PZWL, Warszawa.
15. Chądzyńska J, Krenke K, Kulus M. Postępowanie w astmie oskrzelowej u dzieci w świetle zaleceń ICON. Ter Pneumonol. 2012; 11-12(280-281): 35-40.
16. Panaszek B. Czynniki wpływające na skuteczność leczenia inhalacyjnego astmy oskrzelowej i POChP u pacjentów w podeszłym wieku. Ter Pneumonol. 2012; 11-12 (280-281): 42-49.
17. Kuziemski K. Nowe Spojrzenie na montelukast - lek antyleukotrienowy. Forum Med Rodz. 2009; 3(5): 380-387.
18. O'Byrne PM. Światowe wytyczne postepowania w astmie - stan obecny i perspektywy. Med Prakt. 2010/12. www.mp.pl

19. Kupryś-Lipińska I, Kuna P. Omalizumab, rekombinowane humanizowane monoklinalne przeciwciało anty-IgE – nowe kierunki badań nad zastosowaniem klinicznym leku. Pneumonol Alergol Pol. 2009; 77: 43-51.
20. Mazurek J. Astma ciężka - terapia anty-IgE. Ter Pneumonol. 2010; 10(231): 89-88.
21. Pelaia G, Gallelli L, Renda T, Romeo P, Busceti MT, Grembiale RD, Maselli R, Marsico SA, Vatrella A. Update on optimal use of omalizumab in management of asthma. J Asthma Allergy. 2011; 4: 49-59. doi: 10.2147/JAA.S14520.
22. Ames SA, Gleeson CD, Kirkpatrick P. Omalizumab. Nature Rev Drug Discov. 2004; 3: 199-200. doi:10.1038/nrd1334.
23. Brzostek D, Kokot M, Pawliczak R. Astma niekontrolowana – jaki sposób postępowania wybrać? Terapia W gabinecie lekarza rodzinnego. 2012; 9(2/278): 19-22.
24. Przybyłowski T. Metody diagnostyczne w monitorowaniu i kontroli astmy. Ter Pneumonol. 2010; 10(231): 73-81.
25. Kupryś-Lipińska I, Kuna P. Jak stopniowo redukować leczenie u chorych z dobrze kontrolowaną astmą? Ter Alergol. 2012; 4(271): 19-22.
26. Jurkiewicz D. Przewlekły alergiczny nieżyt nosa. Przewodnik Lekarza. 2003; 1(49): 24-37.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

MEDICINE REVIEW

Microbiology of chronic periodontitis

Anna K. Szkaradkiewicz[1], Tomasz M. Karpiński[2]

[1]University of Medical Sciences in Poznań, Department of Conservative Dentistry and Periodontology, Poznań, Poland

[2]University of Medical Sciences in Poznań, Department of Medical Microbiology, Poznań, Poland

ABSTRACT

Chronic periodontitis is an oral infection that results into destruction involving the gums, cementum, periodontium and alveolar process bone. The major etiology of periodontitis is bacterial plaque, which harbors a variety of pathogenic bacteria known as periopathogens or periodontopathogens. The most important periopathogens are anaerobic bacteria: *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Treponema denticola*, *Fusobacterium nucleatum* and the relative anaerobe *Aggregatibacter actinomycetemcomitans*. Periopathogens can affect the immune system cells, stimulating them to produce inflammatory mediators. The development of inflammatory lesions in chronic periodontitis may restrict oral lactobacilli.

Key words: Chronic periodontitis; Periopathogens; *Porphyromonas gingivalis*; *Prevotella intermedia*; *Aggregatibacter actinomycetemcomitans*.

J Biol Earth Sci 2013; 3(1): M14-M20

Corresponding author:
M.D., Ph.D. Anna K. Szkaradkiewicz
University of Medical Sciences in Poznań
Department of Conservative Dentistry and Periodontology
Bukowska 70, str., 60-812 Poznań, Poland
e-mail: aniaszk@op.pl

Original Submission: 21 January 2013; Revised Submission: 17 February 2013; Accepted: 19 February 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Chronic periodontitis is an oral infection that results into destruction involving the gums, cementum, periodontium and alveolar process bone. Chronic periodontitis is characterized by moderate or severe clinical course, and can occur in different age groups, but most commonly in adults. The incidence and severity of periodontitis increase with age [1]. The main clinical features of chronic periodontitis are: loss of connective tissue attachment, alveolar bone loss, the presence of periodontal pockets and gingival inflammation. Additional symptoms of this disease are: the occurrence of supra- and subgingival calculus, swelling or gum recession, bleeding, furcation exposure, mobility of the teeth, a bad taste, and halitosis [2-5].

Severity of periodontitis is determined on the basis of the following criteria:

1) in moderate periodontitis:
- Gingival Index GI > 0
- Sulcus Bleeding Index SBI > 0
- Clinical Attachment Loss CAL: 3-4 mm
- at least 2 teeth with depth of pockets PPD > 4 mm

2) in severe periodontitis:
- Gingival Index GI > 0
- Sulcus Bleeding Index SBI > 0
- Clinical Attachment Loss CAL > 5 mm
- pockets with a depth > 5 mm [6-8].

REVIEW

The main etiological agent of chronic periodontitis is bacterial plaque. It may cause damage to host tissues directly or via pro-inflammatory mediators. Plaque bacteria have harmful effects on fibroblasts, epithelial and endothelial cells and extracellular matrix components. They can also affect the immune system cells, stimulating them to produce inflammatory mediators. Patients with periodontitis display higher concentrations of various cytokines including TNF-a, IL-1b, IL-6, IL-17, IL-23, and matrix metalloproteinase (MMP)-8 and MMP-9 in their GCF. Consequently, analysis of GCF samples may provide valuable information regarding the pathophysiologic processes associated with periodontitis [9-18].

The major etiology of periodontitis is bacterial plaque, which harbors a variety of pathogenic bacteria known as periopathogens or periodontopathogens. The most important periopathogens are anaerobic bacteria: *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Treponema denticola*, *Fusobacterium nucleatum* and the relative anaerobe *Aggregatibacter actinomycetemcomitans*. These organisms express a number of potential virulence factors and induce host inflammatory mediators, eventually leading to connective tissue breakdown and alveolar bone resorption [19-23].

In a recent studies, using 16S ribosomal cloning and sequencing, it was demonstrated that in periodontitis may occur many bacterial genera both culturable and unculturable. Associated with periodontitis are several genera, many of them uncultivated, including *Peptostreptococcus* and *Filifactor*. In periodontitis are elevated amounts of the genera *Megasphaera* and *Desulfobulbus*, and several species or phylotypes of *Campylobacter*, *Selenomonas*, *Deferribacteres*, *Dialister*, *Catonella*, *Tannerella*, *Streptococcus*, *Atopobium*, *Eubacterium* and *Treponema* [24-26].

Currently, based on the research of Socransky team stands out specific groups of bacteria (called complexes) with particular importance in the pathogenesis of periodontitis. *Porphyromonas gingivalis* creates with the species: *Tannerella forsythia* and *Treponema denticola* so called red complex, which appears to be associated with disease symptoms in adult periodontitis. The second important group of bacteria forms orange complex, comprising 13 species, including *Fusobacterium nucleatum* and *Prevotella intermedia*. Orange complex bacteria are an essential link in allowing the colonization of periodontal tissue by a red complex. A separate group of bacteria forms green complex, which includes species: *Capnocytophaga sputigena*, *C. gingivalis*, and *Eikenella corrodens*. These species are also associated with disease symptoms in adult periodontitis, but with a milder clinical course in contrast to the red complex [27-29]. There is co-occurrence of green group and yellow complex, mainly consisting of the oral streptococci, group "Mitis" (*S. mitis*, *S. oralis*, *S. gordonii*, *S. sanguinis*) and connected to this complex *Parvimonas micra* belonging to dominant periopathogens [30].

Porphyromonas gingivalis

Porphyromonas gingivalis (Fig. 1) is a clinically

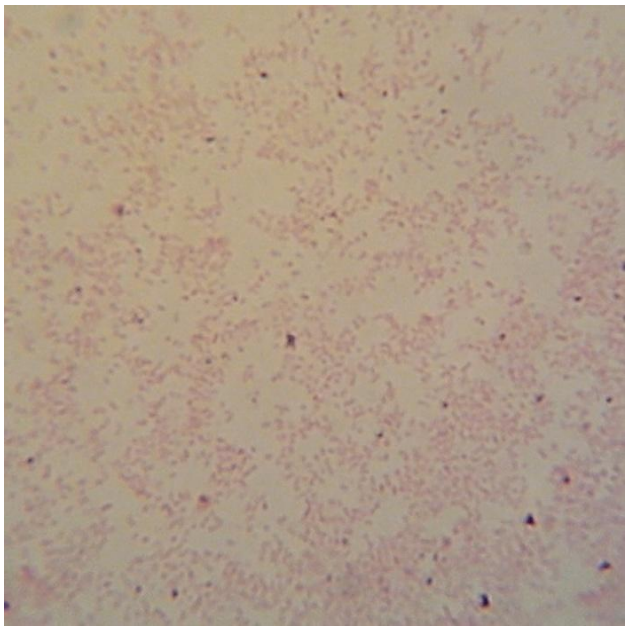


Fig. 1. *Porphyromonas gingivalis* in Gram staining.

important species observed as more than 40% of patients with periodontitis. [31] Strains of *P. gingivalis* have fimbriae with numerous adhesins which ensure adherence of the bacteria to the periodontal tissues and allow co-aggregation with other species, and also induce pro-inflammatory cytokine response. Different proteinases, particularly cysteine proteinases (gingipains), are considered the prominent virulence factor of the pathogen. These proteinases are responsible for the high proteolytic activity of the bacterium. On the basis of substrate specificity gingipains were divided into arginine-specific called arg-gingipains (RgpA and RgpB) and lysine-specific KGP, known as lys-gingipain [32-35]. Gingipains have a strong influence on the immune system by regulating the activity of cytokines [36, 37].

P. gingivalis strongly induces the production of pro-inflammatory cytokines IL-1, IL-6, IL-8 and TNF-alpha by neutrophils, monocytes and macrophages. It has been shown that arg-gingipain destroys C3 related with opsonization, so that *P. gingivalis* is resistant to phagocytosis by neutrophils. In turn, lys-gingipain degrades C5, releasing C5a component and therefore stimulates inflammation. Proteases damage also the extracellular matrix proteins [36, 38, 39]. The fermentation end products of *P. gingivalis*, such as acetic acid, propionic acid and butyric acid, and volatile sulfur compounds produced in large quantities can affect cytotoxic the host cells. Most *P. gingivalis* strains are able to produce capsular polysaccharides. This results in reduced binding of *P. gingivalis* to PMN and inhibited phagocytosis. Lipopolysaccharide (LPS) present in the cell wall plays the role of the antigen and activates cytokines [40, 41].

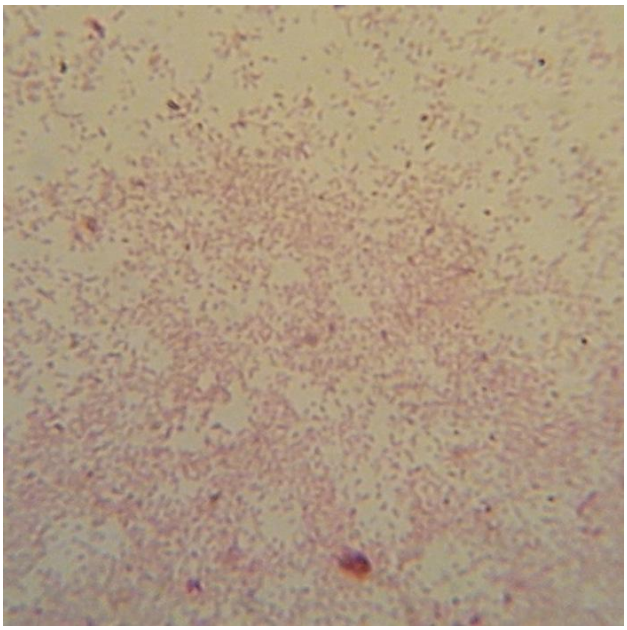


Fig. 2. *Prevotella intermedia* in Gram staining.

Prevotella intermedia

Prevotella intermedia (Fig. 2) is characterized by hemolytic activity. Proteases produced by *Prevotella* are capable of destroying a number of proteins including collagen and fibronectin. Has been shown that proteases possess trypsin-like properties characteristic for cysteine proteinases and are capable of antibodies damaging, in particular IgG and fibrinogen, what reduces the effectiveness of the host immune and inflammatory defense [42, 43]. LPS produced by *Prevotella* may participate in the periodontal destruction and alveolar bone loss through the osteoclastogenesis stimulation, as well as reduce bone formation [44].

Aggregatibacter actinomycetemcomitans

Aggregatibacter actinomycetemcomitans (Fig. 3) is now considered to be the dominant etiologic factor in the early beginning periodontal disease, and may be associated with the red complex [45]. Strains of this species are relatively anaerobic, capnophilic bacteria with microcapsule and bunch of fimbriae (BF) with the ability to adhere to the periodontal tissues and autoaggregation.

Strains of *A. actinomycetemcomitans* produce leukotoxin (LtxA), which is cytotoxic to neutrophils

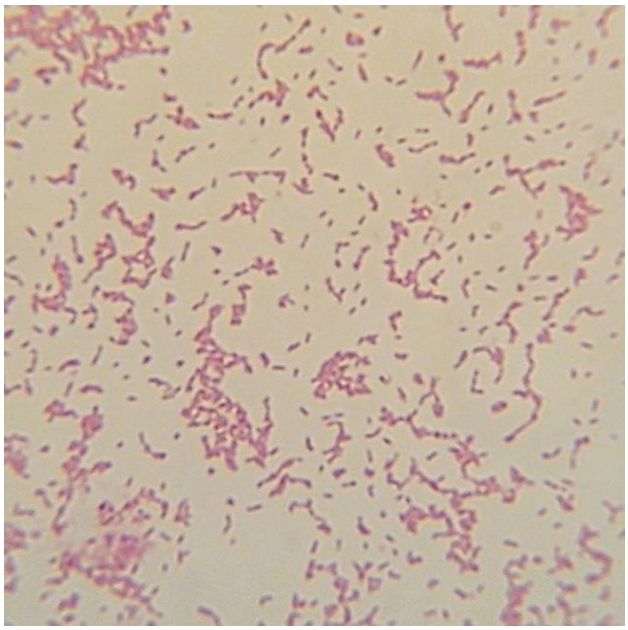


Fig. 3. *Aggregatibacter actinomycetemcomitans* in Gram staining.

and monocytes/ macrophages. LtxA toxin damages cell membranes and by apoptosis causes lysis of polymorpho-nuclear leukocytes, monocytes and T cells [46-48]. The second major toxin of *A. actinomycetemcomitans* is CDT toxin (cytolethal distending toxin) which, by blocking the cell cycle in G2 phase in T cells induces apoptosis in these cells. Cells infected by *A. actinomycetemcomitans* undergo apoptosis, which may be the cause of the development of periodontal disease [49, 50]. Lipopolysaccharide (LPS) of *A. actinomycetemcomitans* can induce tolerant response in macrophages which secrete TNF-alpha, IL-1beta and methylproteinase-9 enzyme that degrades tissue. This process may play an important role in the modulation of the host inflammatory response and progression of periodontitis [51].

Tannerella forsythia

Tannerella forsythia have over the outer membrane an additional protective structure - S surface layer formed by regularly arranged two protein subunits. Experimental studies show that the layer S of *T. forsythia* strains may provide their adherence to host cells, and invasiveness. At the same time, an important factors for their pathogenicity are produced enzymes: trypsin-like protease (PrtH), and glycosidases. The end products of the fermentation of *T. forsythia*, such as acetic acid, propionic acid and butyric acid can affect cytotoxic on host cells [52, 53].

Treponema denticola

Strains of the *Treponema denticola* species belong to the spiral bacteria, characterized by an active movement. [54] These spirochetes have the ability to migrate and penetrate into undamaged periodontal tissue. At the same time, an important virulence factors are produced enzymes: trypsin, chymotrypsin, esterase and alkaline phosphatase, allowing synergistic effects of *T. denticola* strains with the other two species of red complex [55, 56].

Fusobacterium nucleatum

Fusobacterium nucleatum produces DNase, an enzyme that degrades DNA. *F. nucleatum* generates also butyric acid, metabolic end products, and irritates the fibroblast of the gum, leading to necrotic lesions, abscesses and periodontal disease [57]. By scavenging oxygen and oxidative free radicals from dental plaque, *F. nucleatum* helps to maintain and support the conditions for major anaerobic periodontal pathogens [58].

Recently it has been demonstrated that different strains of *Lactobacillus* spp. (including *L. reuteri*, *L. acidophilus*, *L. brevis*) may be of importance in prevention against progress of chronic periodontitis [59-65]. Oral lactobacilli may restrict development of inflammatory lesions in chronic periodontitis. It has been shown that H_2O_2-producing oral lactobacilli may prevent against progress of chronic periodontitis, most probably restricting secretory activity of Th17 cells and growth of periodontopathogens [16].

CONCLUSIONS

Periodontitis as a chronic oral infection can lead to rapid destruction of periodontal tissues. On the development of the disease have an impact many bacteria, in particular anaerobic bacteria which act on fibroblasts, epithelial and endothelial cells and extracellular matrix components. They can also affect the immune cells, stimulating them to produce inflammatory mediators.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Albandar JM, Rams TE. Global epidemiology of periodontal diseases: an overview. Periodontol 2000. 2002; 29: 7-10.
2. Dentino A, Lee S, Mailhot J, Hefti AF. Principles of periodontology. Periodontol 2000. 2013; 61(1): 16-53.
3. Mombelli A. Clinical parameters: biological validity and clinical utility. Periodontol 2000. 2005; 39: 30-39.
4. Pihlström BL, Michalowicz BS, Johnson NW. Periodontal diseases. Lancet. 2005; 366: 1809-1820.
5. Koczorowski R, Karpiński TM, Hofman J. A study of the relationship between halitosis and periodontal diseases [in Polish]. Dental Forum. 2004; 30(1): 51-56.
6. Armitage GC. Classifying periodontal diseases – a long-standing dilemma. Periodontol 2000. 2002; 30: 9-23.
7. World Health Organization. Oral Health Surveys: basic methods. 4th ed. 1997: 26-39. Geneva, WHO.
8. Górska R. The new guidelines of the American Academy of Periodontology [in Polish]. Dent Med Probl. 2012; 49(1): 47-51.
9. Duarte PM, de Oliveira MC, Tambeli CH, Parada CA, Casati MZ, Nociti FH Jr. Overexpression of interleukin-1beta and interleukin-6 may play an important role in periodontal breakdown in type 2 diabetic patients. J Periodontal Res. 2007; 42: 377-381.
10. Ebersole JL. Humoral immune responses in gingival crevice fluid: local and systemic implications. Periodontol 2000. 2003; 31: 135-166.
11. Kardesler L, Buduneli N, Cetinkalp S, Lappin D, Kinane DF. Gingival crevicular fluid IL-6, tPA, PAI-2, albumin levels following initial periodontal treatment in chronic periodontitis patients with or without type 2 diabetes. Inflamm Res. 2011; 60: 143-151.
12. Kardesler L, Biyikoglu B, Cetinkalp S, Pitkala M, Sorsa T, Buduneli N. Crevicular fluid matrix metalloproteinase-8, -13, and TIMP-1 levels in type 2 diabetics. Oral Dis. 2010; 16: 476-481.
13. Konopka T, Ziętek M, Radwan-Oczko M. The role of interleukins in the pathogenesis of periodontitis [in Polish]. Mag Stom. 1995; 5(6): 24-30.
14. Lamster IB, Novak MJ. Host mediators in gingival crevicular fluid: implications for the pathogenesis of periodontal disease. Crit Rev Oral Biol Med. 1992; 3(1/2):31-60.
15. Madianos PN, Bobetsis YA, Kinane DF. Generation of inflammatory stimuli: how bacteria set up inflammatory responses in the gingival. J Clin Periodontol. 2005; 32(suppl.6): 57-71.
16. Szkaradkiewicz AK, Karpiński TM, Zeidler A, Wyganowska-Świątkowska M, Szkaradkiewicz A. Protective effect of oral lactobacilli in pathogenesis of chronic periodontitis. J Physiol Pharmacol. 2011; 62: 685-689.
17. Vernal R, Dutzan N, Chaparro A, Puente J, Antonieta Valenzuela M, Gamonal J. Levels of interleukin-17 in gingival crevicular fluid and in supernatants of cellular cultures of gingival tissue from patients with chronic periodontitis. J Clin Periodontol. 2005; 32: 383-389.
18. Zaremba ML. Periodontal diseases and the increase of the inflammatory response [in Polish]. Czas Stomatol. 2009; 62: 531-548.
19. Colombo AV, Silva CM, Haffajee A, Colombo AP. Identification of oral bacteria associated with crevicular epithelial cells from chronic periodontitis lesions. J Med Microbiol. 2006; 55: 609-615.
20. Kebschull M, Papapanou PN. Periodontal microbial complexes associated with specific cell and tissue responses. J Clin Periodontol. 2011; 38(Suppl.11): 17-27.
21. Sbordone L, DiGenio M, Bortolaia C. Bacterial virulence in the etiology of periodontal diseases. Minerva Stomatol. 2000; 49: 485-500.
22. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol. 1998; 25: 134-144.
23. Socransky SS, Haffajee AD. Periodontal microbial ecology. Periodontol 2000. 2005; 38: 135-187.
24. Kumar PS, Griffen AL, Moeschberger ML, Leys EJ. Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis. J Clin Microbiol. 2005; 43(8): 3944-3955.
25. Sizova MV, Hohmann T, Hazen A, Paster BJ, Halem SR, Murphy CM, et al. New approaches for isolation of previously uncultivated oral bacteria. Appl Environ Microbiol. 2012; 78(1): 194-203.
26. Wade WG. The oral microbiome in health and disease. Pharmacol Res. 2013; 69(1): 137-143.
27. Haffajee AD, Socransky SS, Patel MR, Song X. Microbial complexes in supragingival plaque. Oral Microbiol Immunol. 2008; 23: 196-205.
28. Haffajee AD, Socransky SS. Introduction to microbial aspects of periodontal biofilm communities, development and treatment. Periodontol 2000. 2006; 42:7-12.
29. Haffajee AD, Teles RP, Socransky SS. The effect of periodontal therapy on the composition of the subgingival microbiota. Periodontol 2000. 2006; 42: 219-258.
30. Tindall BJ, Euzéby JP. Proposal of *Parvimonas* gen. nov. and *Quatrionicoccus* gen. nov. as replacements for the illegitimate, prokaryotic, generic names *Micromonas* Murdoch and Shah 2000 and *Quadricoccus* Maszenan et al. 2002, respectively. Int J Syst Evol Microbiol. 2006; 56: 2711-2730.
31. Cutler CW, Kalmar JR, Genco CA. Pathogenic strategies of the oral anaerobe, *Porphyromonas*

gingivalis. Trends Microbiol. 1995; 3: 45-51.

32. Curtis MA, Thickett A, Slaney JM, Rangarajan M, Aduse-Opoku J, Shepherd P, et al. Variable carbohydrate modifications to the catalytic chains of the RgpA and RgpB proteases of *Porphyromonas gingivalis* W50. Infect Immun. 1999; 67: 3816-3823.
33. Imamura T. The role of gingipains in the pathogenesis of periodontal disease. J Periodontol. 2003; 74: 111-118.
34. Lamont RJ, Jenkinson HF. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. Microbiol Mol Biol Rev. 1998; 62(4): 1244-1263.
35. Potempa J, Pike RN. Corruption of innate immunity by bacterial proteases. J Innate Immun. 2009; 1: 70-87.
36. Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of *Porphyromonas gingivalis*. Periodontol 2000. 1999; 20: 168-238.
37. Stathopoulou PG, Benakanakere MR, Galicia JC, Kinane DF. The host cytokine response to *Porphyromonas gingivalis* is modified by gingipains. Oral Microbiol Immunol. 2009; 24: 11-17.
38. Andrian E, Grenier D, Rouabhia M. In vitro models of tissue penetration and destruction by *Porphyromonas gingivalis*. Infect Immun. 2004; 72(8): 4689-4698.
39. Holt SC, Ebersole JL. *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: 'the red complex', a prototype polybacterial pathogenic consortium in periodontitis. Periodontol 2000. 2005; 38:72-122.
40. Al-Shibani N, Windsor LJ. Effects of *Porphyromonas gingivalis* on human gingival fibroblasts from healthy and inflamed tissues. J Periodont Res. 2008; 43: 465-470.
41. Jain S, Darveau RP. Contribution of *Porphyromonas gingivalis* lipopolysachharide to periodontitis. Periodontol 2000. 2010; 54(1): 53-70.
42. Takahashi N, Sato T. Dipeptide utilization by the periodontal pathogens *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens* and *Fusobacterium nucleatum*. Oral Microbiol Immunol. 2002; 17: 50-54.
43. Mallorquí-Fernández N, Manandhar SP, Mallorquí-Fernández G, Usón I, Wawrzonek K, Kantyka T, et al. A new autocatalytic activation mechanism for cysteine proteases revealed by *Prevotella intermedia* interpain A. J Biol Chem. 2008; 283(5): 2871-2882.
44. Chung YH, Chang EJ, Kim SJ, Kim HH, Kim HM, Lee SB, Ko JS. Lipopolysaccharide from *Prevotella nigrescens* stimulates osteoclastogenesis in cocultures of bone marrow mononuclear cells and primary osteoblasts. J Periodontol Res. 2006; 41: 288-296.
45. Fine DH, Markowitz K, Furgang D, Velliyagounder K. *Aggregatibacter actinomycetemcomitans* as an early colonizer of oral tissues: epithelium as a reservoir? J Clin Microbiol. 2010; 48: 4464-4473.
46. Henderson B, Ward JM, Ready D. *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*: a triple A periodontopathogen? Periodontol 2000. 2010; 54(1): 78-105.
47. Henderson B, Wilson M, Sharp L, Ward JM. *Actinobacillus actinomycetemcomitans*. J Med Microbiol. 2002; 51: 1013-1020.
48. Kachlany SC. *Aggregatibacter actinomycetemcomitans* leukotoxin: from threat to therapy. J Dent Res. 2010; 89(6): 561-570.
49. Saiki K, Gomi T, Konishi K. Deletion and purification studies to elucidate the structure of the *Actinobacillus actinomycetemcomitans* cytolethal distending toxin. J Biochem. 2004; 136: 335-342.
50. Sugai M, Kawamoto T, Péres SY, Ueno Y, Komatsuzawa H, Fujiwara T, et al. The cell cycle-specific growth-inhibitory factor produced by *Actinobacillus actinomycetemcomitans* is a cytolethal distending toxin. Infect Immun. 1998; 66(10): 5008-5019.
51. Fives-Taylor PM, Meyer DH, Mintz KP, Brissette C. Virulence factors of *Actinobacillus actinomycetemcomitans*. Periodontol 2000. 1999; 20: 136–167.
52. Tanner AC, Izard J. *Tannerella forsythia*, a periodontal pathogen entering the genomic era. Periodontol 2000. 2006; 42: 88-113.
53. van Winkelhoff AJ, Loos BG, van der Reijden WA, van der Velden U. *Porphyromonas gingivalis*, *Bacteroides forsythus* and other putative periodontal pathogens in subjects with and without periodontal destruction. J Clin Periodontol. 2002; 29: 1023-1028.
54. Seshadri R, Myers GS, Tettelin H, Eisen JA, Heidelberg JF, Dodson RJ, et al. Comparison of the genome of the oral pathogen *Treponema denticola* with other spirochete genomes. Proc Natl Acad Sci USA. 2004; 101: 5646-5651.
55. Dashper SG, Seers CA, Tan KH, Reynolds EC. Virulence factors of the oral spirochete *Treponema denticola*. J Dent Res. 2011; 90: 691-703.
56. Sela MN. Role of *Treponema denticola* in periodontal diseases. Crit Rev Oral Biol Med. 2001; 12: 399-413.
57. Diaz PI, Zilm PS, Rogers AH. *Fusobacterium nucleatum* supports the growth of *Porphyromonas gingivalis* in oxygenated and carbon-dioxide-depleted environments. Microbiology. 2002; 148: 467-472.
58. Periasamy S, Kolenbrander PE. *Aggregatibacter actinomycetemcomitans* builds mutualistic biofilm communities with *Fusobacterium nucleatum* and *Veillonella* species in saliva. Infect Immun. 2009; 77: 3542-3551.
59. Andrzejewska E, Szkaradkiewicz AK. Antagonistic effect of *Lactobacillus acidophilus* to selected

periodontopathogens [in Polish]. XXVII Congress of the Polish Society of Microbiologists. September 5-8, 2012; Lublin, Poland. Scientific Materials.

60. Riccia DN, Bizzini F, Perilli MG, Polimeni A, Trinchieri V, Amicosante G, Cifone MG. Anti-inflammatory effects of *Lactobacillus brevis* (CD2) on periodontal disease. Oral Dis. 2007; 13(4): 376-385.
61. Iniesta M, Herrera D, Montero E, Zurbriggen M, Matos AR, Marín MJ, et al. Probiotic effects of orally administered *Lactobacillus reuteri*-containing tablets on the subgingival and salivary microbiota in patients with gingivitis. A randomized trial. J Clin Periodontol. 2012; 39: 736-744.
62. Kang MS, Oh JS, Lee HC, Lim HS, Lee SW, Yang KH, et al. Inhibitory effect of *Lactobacillus reuteri* on periodontopathic and cariogenic bacteria. J Microbiol. 2011; 49(2): 193-199.
63. Teanpaisan R, Piwat S, Dahlèn G. Inhibitory effect of oral *Lactobacillus* against oral pathogens. Lett Appl Microbiol. 2011; 53: 452-459.
64. Szkaradkiewicz AK, Stopa J. *Lactobacillus* spp. of oral cavity microflora in chronic periodontitis. Pol J Environ Stud. 2008; 17: 236-242.
65. Szkaradkiewicz AK, Tukiendorf B, Stopa J. Oral lactobacilli and periodontitis. Clin Microbiol Infect. 2006; 12: 0371.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

MEDICINE REVIEW

Microbiology of dental caries

Tomasz M. Karpiński[1], Anna K. Szkaradkiewicz[2]

[1]University of Medical Sciences in Poznań, Department of Medical Microbiology, Poznań, Poland

[2]University of Medical Sciences in Poznań, Department of Conservative Dentistry and Periodontology, Poznań, Poland

ABSTRACT

Dental caries is one of the most prevalent chronic diseases of people worldwide. The disease process may involve enamel, dentin and cement, causing decalcification of these tissues and disintegration of the organic substances. It is believed that bacteria of the species *Streptococcus mutans* is the main factor that initiates caries, and the bacteria of the genus *Lactobacillus* are important in further caries development, especially in the dentin. Caries can also be caused by other bacteria, including members of the mitis, anginosus and salivarius groups of streptococci, *Enterococcus faecalis*, *Actinomyces naeslundii*, *A. viscosus*, *Rothia dentocariosa*, *Propionibacterium, Prevotella*, *Veillonella*, *Bifidobacterium* and *Scardovia*.

Key words: Dental caries; Dental plaque; *Streptococcus mutans*; *Streptococcus sobrinus; Lactobacillus*.

J Biol Earth Sci 2013; 3(1): M21-M24

Corresponding author:
Tomasz M. Karpiński
University of Medical Sciences in Poznań
Department of Medical Microbiology
Wieniawskiego 3, str., 61-712 Poznań, Poland
e-mail: tkarpin@interia.pl

Original Submission: 09 February 2013; Revised Submission: 06 March 2013; Accepted: 08 March 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Dental caries is a multifactorial, chronic bacterial disease, that causes demineralization and destruction of the hard tissues, usually by production of acid by bacterial fermentation of the food debris accumulated on the tooth surface. Today, caries remain one of the most common diseases of people worldwide. Individuals are susceptible to this disease throughout their lifetime. Worldwide, approximately 36% of the population have dental caries in their permanent teeth. In baby teeth it affects about 9% of the population [1]. Risk of caries includes physical, biological, environmental, behavioural and lifestyle-related factors [2].

REVIEW

The essence of the teeth carious process is local demineralization of enamel, expressing in degradation of hydroxyapatites. This process is initiated within bacterial biofilm - dental plaque, that covers a tooth surface. Caries lesions develop where oral biofilms are allowed to mature and remain on teeth for long periods. On environmental acidification of the tooth, affects not only the number and species of bacteria, but also the release rate, viscosity and buffer capacity of saliva, the presence of fluoride in enamel and plaque, type of diet and the frequency of sugars consumption. Lowering the pH of the plaque below the "critical value" (5.5 to hydroxyapatite, 4.5 to fluoroapatite, 6.7 to cement) causes dissolution of calcium phosphates included in the hydroxyapatite and initializes loss of the tooth mineral substances [2, 3]. Over time the caries process may involve enamel, dentin and cement, causing decalcification of these tissues and disintegration of the organic substances. Caries progression can give rise to infection of the tooth pulp, which can spread to the supporting tissues and the jaws [3].

About 700 different bacteria species have been identified from the human oral microbiome [4]. In the pathogenesis of dental caries an important role play cariogenic bacteria, i.e. oral streptococci, especially of group mutans and lactic acid bacteria (*Lactobacillus* spp.). It is believed that bacteria of the species *Streptococcus mutans* is the main factor that initiates caries and very important factor of enamel decay. The bacteria of the genus *Lactobacillus* are important in further caries development, especially in the dentin. Mutans streptococci and lactobacilli are characterized by the ability to grow in an acid environment and the property of rapid metabolism of sugars supplied in the diet to organic acids, including lactic acid [5-7]. The microbial community from dentinal lesions is diverse and contains many facultatively- and obligately-anaerobic bacteria belonging to the genera *Actinomyces*, *Bifidobacterium*, *Eubacterium*, *Lactobacillus*, *Parvimonas* and *Rothia*. Streptococci are recovered less frequently [4]. Caries can also be caused by other bacteria, including members of the mitis, anginosus and salivarius groups of streptococci, *Propionibacterium, Enterococcus faecalis* and *Scardovia* [8-10].

In molecular studies using 16S rRNA analysis has been demonstrated, that the predominant microbes in deep caries lesions were *S. mutans* and genus *Lactobacillus* but also included the genera *Prevotella*, *Selenomonas*, *Dialister*, *Fusobacterium*, *Bifidobacterium* and *Pseudoramibacter* [11, 12]. In studies of Becker et al. [13] and Aas et al. [14], they reported that genera associated with dental caries in both primary and permanent dentitions are *Streptococcus* including *S. mutans*, *S. sanguinis* and non-*S. mutans* streptococci, *Veillonella*, *Actinomyces*, *Bifidobacterium*, *Lactobacillus*, *Propionibacterium*, and *Atopobium*. In other study, has been shown that in plaque significantly associated with dental caries are the genera of *Streptococcus*, *Veillonella*, *Actinomyces*, *Granulicatella*, *Leptotrichia* and *Thiomonas* [15].

The development of the caries lesion is conditioned by the carbohydrates supply frequency, mainly sucrose and glucose from food, sometimes impact of cariogenic factors, as well as the tooth surface susceptibility. Recent studies confirm participation of the genetic factors in the pathogenesis of dental caries [16, 17].

Mutans streptococci

Mutans streptococci are the most cariogenic pathogens as they are highly acidogenic, producing short-chain acids which dissolve hard tissues of teeth. They metabolize sucrose to synthesize insoluble extracellular polysaccharides, which enhance their adherence to the tooth surface and encourage biofilm formation. The reactions are catalyzed by three isozymes of glucosyltransferases

[18]. The most important mutans streptococci isolated from tooth caries samples are *S. mutans* and *S. sobrinus*. *S. mutans* is more cariogenic than *S. sobrinus* because specific cell-surface proteins, which aid in its primary attachment to the tooth. *S. sobrinus* lacks such proteins [19].

Streptococcus mutans

S. mutans is able to metabolise a number of sugars and glycosides such as glucose, fructose, sucrose, lactose, galactose, mannose, cellobiose, glucosides, trehalose, maltose and a previously unrecognised, group of sugar-alcohols. In the presence of extracellular glucose and sucrose, *S. mutans* synthesizes intracellular glycogen-like polysaccharides (IPSs) [20]. *S. mutans* produces also mutacins (bacteriocins), what is considered to be an important factor in the colonization and establishment of *S. mutans* in the dental biofilm [21].

Streptococcus sobrinus

S. sobrinus has been implicated in caries development particularly in instances where caries development appears to be independent of *S. mutans*. It is interesting that *S. sobrinus* displays higher acid production and acid tolerance compared to *S. mutans* [19, 22].

Lactobacilli

Among the *Lactobacillus* rods in the oral cavity occur: *L. acidophilus*, *L. casei*, *L. fermentum*, *L. delbrueckii*, *L. plantarum*, *L. jensenii*, *L. brevis*, *L. salivarius* and *L. gasseri*. Lactobacilli are divided into two main groups:

- homofermentative which in the fermentation process of glucose produce mainly lactic acid, e.g. *Lactobacillus casei*, *Lactobacillus acidophilus*,
- heterofermentative which in addition to lactic acid produce acetate, ethanol and carbon dioxide, e.g. *Lactobacillus fermentum* [23, 24].

Lactobacilli are isolated from deep caries lesions but rarely just before the development of dental caries and in the early tooth decay. It is believed that they are pioneering microorganisms in the caries progress, especially in dentin [6]. Studies have shown that Lactobacilli are a dominant part of the flora inhabiting the deep cavities, and their number correlates with the amount of carbohydrates [25-27].

Recently it is known that lactobacilli demonstrate also antagonistic action against periodontopathogens such as *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia* and *Porphyromonas gingivalis*, inhibiting their growth [28-30]. This action consists in the production of acids mainly lactic acid, lowering the pH of the environment, release of hydrogen peroxide [31, 32] and bacteriocins [33-35]. Thus, *Lactobacillus* in addition to a cariogenic activity also play a key role in maintaining the microecological balance in the mouth and gastrointestinal tract.

CONCLUSIONS

Dental caries is one of the most prevalent diseases in the world, that causes demineralization and destruction of the hard tissues of teeth. In the development of caries a major role play bacteria of the *Streptococcus* and *Lactobacillus* genera which are acidogenic and aciduric. At the same time, recent studies show that in the dental caries development may also affect other bacteria.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Vos T, Flaxman AD, Naghavi M, Lozano R, Michaud C, Ezzati M, et al. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet. 2012; 380: 2163-2196.
2. Selwitz RH, Ismail A, Pitts NB. Dental caries. Lancet. 2007; 369: 51-59.
3. Featherstone JD. The continuum of dental caries – evidence for a dynamic disease process. J Dent Res. 2004; 83(C): C39-C42.
4. Marsch PD, Martin MV. 2009. Oral Microbiology. 5th ed. Elsevier, pp. 222.
5. Byun R, Nadkarni MA, Chhour KL, Martin FE, Jacques NA, Hunter N. Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries. J Clin Microbiol. 2004; 42: 3128-3136.
6. Smith SI, Aweh AJ, Coker AO, Savage KO, Abosede DA, Oyedeji KS. Lactobacilli in human dental caries and saliva. Microbios. 2001; 105(411): 77-85.
7. Marsh PD. Microbiologic aspects of dental plaque and dental caries. Dent Clin North Am. 1999; 43(4): 599-614.
8. Tanzer JM, Livingston J, Thompson AM. The microbiology of primary dental caries in humans. J Dent Educ. 2001; 65(10): 1028-1037.

9. Thomas RZ, Zijnge V, Ciçek A, de Soet JJ, Harmsen HJ, Huysmans MC. Shifts in the microbial population in relation to in situ caries progression. Caries Res. 2012; 46(5): 427-431.
10. Wade WG. The oral microbiome in health and disease. Pharmacol Res. 2013; 69(1): 137-143.
11. Munson MA, Banerjee A, Watson TF, Wade WG. Molecular analysis of the microflora associated with dental caries. J Clin Microbiol. 2004; 42(7): 3023-3029.
12. Chhour KL, Nadkarni MA, Byun R, Martin FE, Jacques NA, Hunter N. Molecular analysis of microbial diversity in advanced caries. J Clin Microbiol. 2005; 43(2): 843-849.
13. Becker MR, Paster BJ, Leys EJ, Moeschberger ML, Kenyon SG, Galvin JL, et al. Molecular analysis of bacterial species associated with childhood caries. J Clin Microbiol. 2002; 40: 1001-1009.
14. Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, Dewhirst FE, et al. Bacteria of dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol. 2008; 46: 1407-1417.
15. Ling Z, Kong J, Jia P, Wei C, Wang Y, Pan Z, et al. Analysis of oral microbiota in children with dental caries by PCR-DGGE and barcoded pyrosequencing. Microb Ecol. 2010; 60: 677-690.
16. Bretz WA, Corby PM, Schork NJ, Robinson MT, Coelho M, Costa S, et al. Longitudinal analysis of heritability for dental caries traits. J Dent Res. 2005; 84(11): 1047-1051.
17. Hassell TM. Genetic influences in caries and periodontal diseases. Crit Rev Oral Biol Med. 1995; 6(4): 319-342.
18. Islam B, Khan SN, Khan AU. Dental caries: from infection to prevention. Med Sci Monit. 2007; 13(11): RA196-203.
19. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev. 1986; 50(4): 353-380.
20. Peterson SN, Snesrud E, Schork NJ, Bretz WA. Dental caries pathogenicity: a genomic and metagenomic perspective. Int Dent J. 2011; 61(Suppl 1): 11-22.
21. Merritt J, Qi F. The mutacins of *Streptococcus mutans*: regulation and ecology. Mol Oral Microbiol. 2012; 27(2): 57-69.
22. Kolenbrander PE. Oral microbial communities: biofilms, interactions, and genetic systems. Annu Rev Microbiol. 2000; 54: 413-437.
23. Claesson MJ, van Sinderen D, O'Toole PW. The genus *Lactobacillus* – a genomic basis for understanding its diversity. FEMS Microbiol Lett. 2007; 269: 22-28.
24. Klein G, Pack A, Bonaparte C, Reuter G. Taxonomy and physiology of probiotic lactic acid bacteria. Int J Food Microbiol. 1998; 41: 103-125.
25. Caufield PW, Li Y, Dasanayake A, Saxena D. Diversity of lactobacilli in the oral cavities of young women with dental caries. Caries Res. 2007; 41(1): 2-8.
26. Stecksén-Blicks C. Salivary counts of lactobacilli and *Streptococcus mutans* in caries prediction. Scand J Dent Res. 1985; 93: 204-212.
27. van Houte J, Lopman J, Kent R. The predominant cultivable flora of sound and carious human root surfaces. J Dent Res. 1994; 73(11): 1727-1734.
28. Köll-Klais P, Mandar R, Leibur E, Marcotte H, Hammarstrom L, Mikelsaar M. Oral lactobacilli in chronic periodontitis and periodontal health: species composition and antimicrobial activity. Oral Microbiol Immunol. 2005; 20(6): 354-361.
29. Andrzejewska E, Szkaradkiewicz AK. 2012. Antagonistic effect of *Lactobacillus acidophilus* to selected periodontopathogens [in Polish]. XXVII Congress of the Polish Society of Microbiologists. September 5-8, 2012, Lublin, Poland. Abstracts: P-X-381.
30. Szkaradkiewicz AK, Karpiński TM, Zeidler A, Wyganowska-Świątkowska M, Szkaradkiewicz A. Protective effect of oral lactobacilli in pathogenesis of chronic periodontitis. J Physiol Pharmacol. 2011; 62: 685-689.
31. Ryan CS, Kleinberg I. Bacteria in human mouths involved in the production and utilization of hydrogen peroxide. Arch Oral Biol. 1995; 40(8): 753-763.
32. Lin MY, Yen CL. Antioxidative ability of lactic acid bacteria. J Agric Food Chem. 1999; 47: 1460-1466.
33. Klaenhammer TR. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev. 1993; 12: 39-86.
34. Strahinic I, Busarcevic M, Pavlica D, Milasin J, Golic N, Topisirovic L. Molecular and biochemical characterizations of human oral lactobacilli as putative probiotic candidates. Oral Microbiol Immunol. 2007; 22: 111-117.
35. Słońska A, Klimuszko D. Bacteriocins of probiotic rods of the *Lactobacillus* genus [in Polish]. Post Mikrobiol. 2010; 40(2): 87-96.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

MEDICINE REVIEW

The biological and medical significance of poisonous animals

Hossam El-Din M. Omar

Zoology Department, Faculty of Science, Assiut University, Egypt

ABSTRACT

Animal kingdom possesses numerous poisonous species that produce venoms (actively delivered) or toxins (passively delivered). Poisonous animals are found in most classes of the Animal Kingdom and in most habitats, both terrestrial and marine. Poisonous animals have a significant health problem for populations in the world and are neglected environmental diseases of the rural tropics. Poisonous animals include a variety of animal species; sea snakes, stinging fish, jellyfish, corals, cone shells, blue-ringed octopuses, sea urchins, snakes (elapids, vipers, and rattlesnakes), scorpion, spiders, bee, wasp and ant. Poisonous animal are rich sources of toxins that often target-with high potency and variable specificity. Animal toxins have made a significant contribution to enhancing knowledge in human physiology and pharmacology. Information on the nature and mechanism of action of these toxins has enabled a more scientific approach to the treatment of their intoxications. This paper reviews the knowledge about the various aspects related to the name, habitat, biological and medical importance of poisonous animals of different major animal phyla. In addition, this review will discuss the mechanism of venoms or toxins toxicity and therapeutic uses of particular fractions of venoms or toxins from different sources.

Key words: Poisonous animals; Scorpion; Snake; Spider; Jelly fish; Octopus; Venoms; Neurotoxin; Conotoxin; Tetrodotoxin; Phospholipase A.

J Biol Earth Sci 2013; 3(1): M25-M41

Corresponding author:
Hossam El-Din M. Omar
Zoology Department, Faculty of Science, Assiut University, 71516, Assiut, Egypt
E-mail: hossameldin.mo@gmail.com

Original Submission: 10 March 2013; Revised Submission: 17 April 2013; Accepted: 19 April 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Animal venoms and toxins are now recognized as major sources of bioactive molecules that may be tomorrow's new drug leads. Venom is a secretion produced in a specialized gland in one animal and delivered to a target animal through the infliction of a wound. This secretion must contain molecules that disrupt normal physiological processes [1]. Venoms may be used to kill prey and/or to defend the delivering organism against attack by predators. Venoms are complex mixtures of pharmacologically highly active substances and can cause a wide range of symptoms [2].

The venom which contains mucopolysaccharides, hyaluronidase, phospholipase, serotonin, histamine, enzyme inhibitors, and protein is namely neurotoxic peptide (Table 1) [3, 4]. The neurotoxic peptides are responsible for the symptoms that present during envenomation by interacting with ion channels and have the potential to cause massive damage to nervous system of both vertebrates and invertebrates [5]. Pre-synaptic neuromuscular junction neurotoxins act at the neuromuscular junction, damaging the terminal axon followed by cessation of all neurotransmitter release and irreversible paralysis. Post-synaptic toxins act by reversible binding to the acetylcholine receptor on the skeletal muscle end plate. Tetrodotoxin found in saliva of the Australian blue ringed octopus and the flesh of puffer fish causes rapid and reversible paralysis of skeletal muscle by blocking nerve transmission through action on the sodium channels of axons. A variety of potassium channel blocking toxins exist in the venoms of some scorpion and cone shell. Some snake venoms contain myolysins which caused myolysis of skeletal muscles, however, cardiac effects are prominent in envenoming by scorpions, jellyfish and cone shells.

Some venoms contain true anticoagulants components that directly inhibit portions of the clotting cascade resulting in prolonged clotting times (Table 2). The viperid zinc metalloproteinases causes capillary leakage resulting in haemorrhagic necrosis. Renal damage may follow envenoming by a wide range of venomous animals as a secondary effect of venom induced hypotension. Some snakes e.g vipers, pit vipers, cobras commonly cause major local tissue injury, as a result of cytolytic phospholipase A2 toxins. A few spiders cause local necrosis as the most prominent feature of envenomnation [6, 7].

Venom allergens cause immunostimulation of body tissues and show strong T cell responses in hypersensitive patients and signify the production of allergen specific IgE antibodies and generate anaphylactic reactions. Generally, venom toxins make fast release of certain chemicals i.e. serotonins, kinins, prostaglandins and leukotrienes that results in visible clinical symptoms related to paralysis, inflammation, swelling and itching [8].

Table 1. Major venomous animal groups commonly associated with neurotoxic paralysis [9].

Type of animal	Examples	Type of neurotoxin
Elapid snakes	Kraits	Pre- and postsynaptic
	Coral snakes	Postsynaptic
	Mambas	Dendrotoxins and fasciculins
	King cobra	Postsynaptic
	Selected Australian snakes; tiger snakes, taipans, rough scaled snake, death adders, copperheads	Pre- and postsynaptic
	Sea snakes	Postsynaptic
Viperid snakes	Mohave rattlesnake	Presynaptic
	Neotropical rattlesnakes	Presynaptic
	Sri Lankan Russell's viper	Postsynaptic
Ticks	Paralysis ticks, *Ixodes* and *Dermacentor* spp.	Presynaptic
Cone shells	Variety of *Conus* spp.	Conotoxins
Octopusses	Blue ringed octopuses, *Hapalochlaena* spp.	Tetrodotoxin

REVIEW

1. Phylum Protozoa

Dinoflagellates, unicellular marine protozoons, produce some of the largest and most complex polyketides identified to date. The biological activities of these molecules are quite diverse [11].

2. Phylum Porifera

Sponges are simple multicellular animals, living mainly in shallow coastal and fresh waters around the world. They either attach to rock, seaweed or a hard-shelled animal or burrow into calcareous shells or rock. Most sponges are harmless to humans, while a few venomous species exist that have tiny spicules made of silicon can penetrate the skin and deliver venom. The effect of picking up a venomous sponge is not immediate but some time after the contact the area of skin touched becomes red and swelled with severe pain for days or even weeks. Moreover, the external surface of some species of sponges has small perforations from which chemical substances or crinitoxins are extruded. Three species including the red-beard sponge (*Micronia prolifera*), fire sponge (*Tedania ignis*) and poison-bun sponge (*Fibulila* sp.) causes dermatitis [12].

The majority of sponge glycosides were isolated from sponges belonging to species of orders Astrophorida and Poecilosclerida. The distribution and biological activities of these compounds suggest their parallel origin and evolution as defensive agents in several taxa of the class. Chemically, these compounds are very diverse and quite different in the structures of the both aglycone and carbohydrate moieties, when compared with other glycosides of marine or terrestrial origin. Sponge glycosides demonstrate antiprotozoal, antifungal and antitumor activities [13]. Moreover, marine sponges produce vast range of antitumor, antiviral, antiinflammatory, immunosuppressive, antibiotic, and other bioactive molecules that can

Table 2. Major venomous animal groups expected to cause primary coagulopathy [10].

Type of animal	Examples	Type of venom action
Colubrid snakes	Boomslang, vine snake Yamakagashi, red necked keelback	Procoagulant
Elapid snakes	- Selected Australian snakes; tiger snakes, rough scaled snake, taipans, brown snakes, broad headed snakes - Selected Australian snakes; mulga snakes, Collett's snake, black snakes, Papuan black Snake	- Procoagulant - Anticoagulant
Viperid snakes	- Saw scaled or carpet vipers - Gaboon vipers and puff adders - Russell's vipers - Malayan pit viper - North American rattlesnakes - North American copperheads - South American pit vipers (selected *Bothrops* spp.) - Asian green pit vipers (selected *Trimeresurus* spp.) - EuroAsian vipers (selected *Vipera* spp.)	- Procoagulant, disintegrins, haemorrhagins - Procoagulant, antiplatelet, disintegrins, haemorrhagins - Procoagulant, haemorrhagins - Procoagulant, antiplatelet, haemorrhagins - Procoagulant, fibrinolytic, antiplatelet, disintegrins, haemorrhagins - Procoagulant, anticoagulant, fibrinolytic, disintegrins - Procoagulant, Anticoagulant, fibrinolytic, disintegrins, haemorrhagins - Anticoagulant, fibrinolytic, antiplatelet, haemorrhagins - Procoagulant, disintegrins, haemorrhagins,
Insects	Latin American caterpillars, *Lonomia* spp.	Procoagulant

affect the pathogenesis of many human diseases. The relationship between the chemical structures of the secondary metabolites from sponges and the diseases they affect is not clear. However, different components affect the targeted disease through microtubule stabilization or interaction with DNA to combat tumors. Also, most bioactive metabolites from sponges are inhibitors of certain enzymes, which often mediate or produce mediators of intracellular or intercellular messengers that are involved in the pathogenesis of a disease. The potency of sponge-derived medicines lies in the fact that each of these thousands of metabolites and their derivatives has its own specific dose-related inhibitory effect, efficacy, and potential side effects [14].

Halichondrin B was initially purified from the sponge *Halichondria okadai* in Japan and has shown in vivo activity in melanoma and leukaemia models. It can also be obtained from the deep-water sponge *Lissodendoryx*, which is found in New Zealand. This compound is also active in various human tumour-cell models in vitro and in vivo and appears to interfere with microtubule function [15]. New bisindole alkaloids of the topsentin and hamacanthin classes have been isolated from the Mediterranean sponge *Rhaphisia lacazei* showed significant antiproliferative activity against a series of human cell lines in vitro [16]. Moreover, initial studies on sesquiterpenes, parahigginols, and parahigginic acid isolated from a Taiwanese marine sponge *Parahigginsia* sp. revealed that these compounds were cytotoxic against tumour cells [17].

3. Phylum Cnidaria

Cnidarians are relatively simple radially symmetrical body. Their body cavity has a single opening surrounded commonly by tentacles equipped with special cells known as cnidocytes. Cnidarians are separated into four groups: the Hydrozoa (plume-like hydroids, medusae and Siphonophora), Scyphozoa (free-swimming jellyfish), Cubozoa (box-shaped medusae) and Anthozoa (hard and soft corals and anemones). Hydroids and jellyfish possess nematocysts, on the other hand sea anemones and true corals have spirocysts with adhesive cnidae threads [18, 19].

Cnidarian venoms are contained in the nematocysts secreted by the Golgi apparatus of nematoblasts, the cells specialized for this function. The nematocysts contain a tightly spiralized and differently shaped thread, which according to the species, is provided with spines and with a basal enlarged portion known as the 'shaft'; after mechanical or chemical stimulation, the thread is averted, injecting the venom contained into the capsule [20].

Nematocyst in the tentacles of the sea anemone contains a coiled hollow filament containing a potent toxin affecting voltage gated Na^+ and K^+ channels, acid-sensing ion channels, actinoporins and protease inhibitors [21-23]. Actinoporins are highly toxic to fish and crustaceans, which may be the natural prey of sea anemones. In most cases, a sting by the nematocysts causes local inflammations, pain and edema [24]. Toxin from sea anemone is potently inhibits T-lymphocyte proliferation in models of certain autoimmune diseases [25]. All species of jellyfish in the Mediterranean are relatively harmless; however, *Pelagia noctiluca*, *Chrysaora hysoscella* and *Rhopilema nomadica* are considered the most venomous [26]. The delayed jellyfish envenomation syndrome with serious multiple organ dysfunction or systemic damages developed after jellyfish stings may be attributed to the synergy of cytotoxicity, vasoconstriction effect and other specific target organ toxicities of jellyfish venom [27].

Some bioactive substances were discovered in cnidarians, such as prostaglandins (PGA2) in the gorgonian *Plaxaura homomalla* [28], Palytoxin local anaesthetic and vasoconstrictive agent and induce ion currents in mouse neuroblastoma cells discovered in the zoanthid *Palythoa toxica* [29, 30]. Cytolytic and antitumoral prostanoid compounds from the Anthozoan *Clavularia viridis* were shown to inhibit the growth of HL-60 leukemic cells [31]. Moreover, the incidence and growth of tumors induced by N-Ethyl-N-Nitrosourea were affected by the crude venom of the scyphozoan *Cassiopea xamachana* [32] and the growth of Ehrlich ascites tumors grafted in mice was inhibited by crude extracts of jellyfish and soft corals [33]. Also, equinatoxin extracted from *Actinia equina* showed antitumoral activity on cultured cells [34].

4. Phylum Arthropoda

Basically according to Koehler and Diclaro [35] venomous arthropods produce venoms that can be classified as; venoms that produce blisters (e.g., blister beetles, certain stinging caterpillars, millipedes), venoms that attack the central nervous

system (e.g., black and brown widow spiders, bark scorpions, certain ticks, Hymenoptera, wheel bugs), venoms that destroy tissue, or cytolytic and hemolytic toxins (e.g., Hymenoptera, fire ants, ground scorpions, mites, chiggers, wheel bugs, brown recluse spider), venoms that prevent blood from clotting, or hemorrhagic toxins (e.g., lice, fleas, ticks, mites, true bugs, biting flies).

4a. Class Insects

Venomous insects are known from the orders Lepidoptera, Hemiptera, and Hymenoptera [36]. The method of delivery may be active, such as the sting apparatus of Hymenoptera (bees and wasps), and the mouthparts of Hemiptera (stylets), or passive such as the modified setae in some lepidopteran larvae (caterpillars). Hymenopterans are insects that inject venom with a stinging apparatus connected to venom glands in the terminal part of the abdomen. Some species of ants lack a sting and instead spray their venom. Honeybees and wasps are widely and numerously distributed in cold and tropical climates; therefore, most humans experience multiple stings during a lifetime. Single stings are dangerous for people who are allergic to the venom. Direct toxic effects, as opposed to allergic reactions account for 15% of all deaths caused by hymenopteran stings [37].

Insect venom is a poisonous substance that contains a complex mixture of certain proteins, enzymes, small peptides, certain inorganic elements and acids. These venom components are responsible for multiple pharmacological effects in different organisms. These venoms act at cellular level and break the normal barrier to leak out molecules across the cell membrane and form ion channels by attaching themselves to the membrane surface. Insect venom toxins elevate the level of blood sugar, lactate, glucagon and cortisol and cause massive destruction of erythrocytes and nerve cells. In addition, insect venom possess highly potent short peptides act on ion channels of excitable cells and inhibit the activity of important metabolic enzymes. Melittin is a short peptide that shows cytotoxicity and cause intravascular hemolysis of erythrocytes, leucocytes, platelets and vascular endothelium. It is highly basic peptide that inserts itself into the phospholipid bi-layer of cell membranes [8].

Venom secreted from the salivary glands of ticks during the blood meal is absorbed by the host and systemically distributed. Paralysis results from the ixovotoxin, very similar to botulinum toxin due to inhibition of the acetylcholine release at the neuromuscular junction and autonomic ganglia [38, 39]. Both ixovotoxin and botulinum toxin demonstrate temperature dependence in rat models and shows increased muscular twitching activity as the temperature is reduced [40]. The antimicrobial venom peptides of honey bee *Apis mellifera* are present on the cuticle of adult bees and on the nest wax. It has been suggested that these substances act as a social antiseptic device. Venom functions are well beyond the classical stereotype of defense against predators, and the different nesting biology of these species may be related to the use of the venom in a social immunity context [41]. Bumblebee venom contains a variety of components, including bombolitin, phospholipase A2, serine proteases, and serine protease inhibitors. A bumblebee (*Bombus terrestris*) venom serine protease inhibitor that acts as a plasmin inhibitor was consists of a 58-amino acid mature peptide that displays features consistent with snake venom Kunitz-type inhibitors, including six conserved cysteine residues and a P1 site [42]. *Solenopsis* fire ants are native to the Americas, with most of the species occurring in lower regions of South America [43]. Fire ant venom includes more than 95% piperidinic alkaloids and less than 5% aqueous fraction of allergenic proteins [44, 45].

4b. Class Arachnida

From the venom of arachnids (scorpion and spiders) several hundred peptides have been isolated and characterized, most of which are relatively short peptides that interfere with cellular communication and impair proper function. Cloning genes extracted from the venomous glands of arachnids is revealed thousands of novel sequences [46]. Spiders employ venom jaws that are connected to venom glands to catch prey and for use in self defense. Most spiders either have venom jaws that are too small to penetrate human skin or their venom is too weak to produce substantial envenoming. Spider bites may go unnoticed until clinical signs and symptoms develop. Systemic neurotoxic envenoming is caused by widow spiders (*Latrodectus* sp.), wandering spiders (*Phoneutria* sp.), and funnel web spiders (*Atrax* sp. and *Hadronyche* sp.) are resembles envenoming from scorpion stings. The

clinical course of envenoming by these spiders is also predominantly triggered by catecholamine release [3, 47, 48]. Over a period of more than 300 million years, spiders have evolved an extensive library of bioactive peptides. Moreover, in contrast with man-made combinatorial peptide libraries, spider-venom peptides have been pre-optimized for high affinity and selectivity against a diverse range of molecular targets. It is therefore not surprising that numerous spider-venom peptides have been characterized that potently and selectively modulate the activity of a diverse range of therapeutic targets [49].

Spider venoms are complex mixtures of neurotoxic peptides, proteins and low molecular mass organic molecules. Their neurotoxic activity is due to the interaction of the venom components with cellular receptors in particular ion channels. Spider venoms have proven to be a rich source of highly specific peptide ligands for selected subtypes of potassium, sodium and calcium channels, and these toxins have been used to elucidate the structure and physiological roles of the channels in excitable and non-excitable cells [50-52].

The Brazilian tarantula *Acanthoscurria paulensis* venom induced many behavioral and physiological changes in mice. An inotropic effect produced on frog heart is probably due to the low molecular mass compounds present in the more hydrophilic fractions of venom that may act either by inducting the release of acetylcholine from parasympathetic terminals or by directly acting as a cholinergic agonist [53].

Loxoscelism is a set of signs and symptoms caused by the bite of spiders of the genus *Loxosceles* [54]. *Loxosceles* (Araneae, Sicariidae) can be found in temperate and tropical regions of America, Oceania, Asia, Africa and Europe [55, 56]. This genus represents a public health problem in Brazil, mainly in South and Southeast regions, with more than 3000 cases reported annually by the Ministry of Health [52]. Usually, the clinical manifestations of loxoscelism are characterized by necroulcerative dermatitis at the site of the bite. However the envenoming can also cause systemic effects leading to acute renal failure, which may be lethal [52, 57, 58]. Locally, lesions caused by loxosceles venom present edema, hemorrhage, inflammation with dominance of neutrophils, rhabdomyolisis, damage to the vessels wall, thrombosis, and dermonecrosis [59, 60]. Recently, by using a cDNA library and transcriptome analysis, a novel expression profile has been elaborated for *Loxosceles intermedia* gland venom. This recently developed profile has allowed the identification of additional toxins as components of the venom, including insecticidal peptides similar to knottins, astacin-like metalloproteases, venom allergen, a translationally controlled tumor protein family member, serine protease inhibitors, and neurotoxins similar to Magi 3 [61, 62]. In addition, the biotechnological use of *Loxosceles* toxins could provide information related to the tridimensional structure of identified toxins, through crystallography and X-ray diffraction and/or nuclear magnetic resonance for soluble toxins [63], from such data, synthetic ligands, analogs, or inhibitors could be designed for biotechnological purposes [64].

Scorpions are found in all the world causes problems in tropical and subtropical regions. Travelers are stung when they accidentally squeeze scorpions that are hiding in beds, luggage, shoes, and clothing [65]. Scorpions are actually very beneficial to ecosystems because they eat insects, spiders, centipedes and even other scorpions. In turn, they provide an important food source for large centipedes, tarantulas, snakes, some lizards, birds, bats, and other small mammals. Scorpion venom varies from species to species, but generally consists of different mixtures of neurotoxins [66].

Scorpion toxins are classified according to their structure, mode of action, and binding site on different channels or channel subtypes [67]. The long chain toxins affecting sodium channels have been subdivided primarily into two major subtypes, α- and β-toxins [68]. The α-toxins bind to receptor site 3 of the voltage-gated Na^+ channels of vertebrates in a membrane-dependent manner [69]. The major effects of α-toxins induce a prolongation of the action potential of nerves and muscles by fast inactivation of sodium channels receptor affinity dependent upon membrane potential [67, 70]. The β-toxins are isolated from American scorpions, bind to receptor site 4 on vertebrate Na^+ channels and producing a shift to amore negative membrane potential [71, 72]. So, Scorpion β-toxins have been used as pharmacological tools in the study of voltage-activated Na^+ channels [51].

Systemic envenoming is caused by members of the genera *Centruroides*; *Tityus*; *Androctonus*, *Buthus*, *Leiurus*, *Nebo*; *Hemiscorpius*; *Parabuthus*; and *Mesobuthus* [2]. Local envenoming causes

pain, erythema, and swelling. Systemic envenoming usually develops in two phases: a cholinergic phase involving vomiting, sweating, hypersalivation, priapism, bradycardia, and arterial hypotension, followed by an adrenergic phase involving arterial hypertension, tachycardia, and cardiac failure. Cranial nerves and neuromuscular junctions and respiratory organs may be affected [73]. The mediators affecting inflammatory processes may be released after scorpion envenomation including kinins, ecosanoids, platelet activating factor, nitric oxide, and cytokines [74]. A total of 74 fractions were separated from the Urodacidae scorpions, the most widely distributed in Australia, allowing the identification of approximately 274 different molecular masses with molecular weights varying from 287 to 43.437 Da. The most abundant peptides were those from 1 kDa and 4–5 kDa representing antimicrobial peptides and putative potassium channel toxins, respectively. The transcriptome analysis of the venom glands of the same scorpion species, resulting cDNA library 172 expressed sequence tags [75].

5. Phylum Molusca

The most important venomous mollusks are from the Gastropoda and Cephalopoda classes. Gastropoda (genus *Conus*) contain mollusks able to envenom prey and occasionally even Man. Octopus vulgaris is a common marine animal that can be found in nearly all tropical and semitropical waters around the world. *Octopus vulgaris* bite resulting in an ulcerative lesion with slow wound healing owing to *P. oryzihabitans* infection [76]. The blue-ringed octopus (*Hapalochlaena maculata* and *Hapalochlaena lunulata*) inoculates maculotoxin from their saliva glands through their horny beak. Recently maculotoxin was demonstrated as identical to tetrodotoxin [77]. Tetrodotoxin is a potent neurotoxin that blocks axonal sodium channels and provokes a muscular paralysis similar to that observed in accidents with *Conus* shells including fatal respiratory arrest [78]. Mollusks of the genus *Conus* present a venomous apparatus composed of radulae, a chitin structure linked to glands, which injects potent neurotoxic peptides, conotoxins, causing serious human envenomation that is associate with the blockage of certain receptors and muscular paralysis [79]. The venom from any one *Conus* species contains a large number of peptides. Every conotoxin serves as a highly specific ligand, each with a particular molecular target. Binding of the peptide ligand to its target leads to a biologically relevant change in physiological function [80]. Currently, conotoxins are a valuable tool of scientific research due to the intense pharmacological activity presented by the peptides. One of the drugs in clinical tests is ziconotide which is a peptide that blocks the neuronal calcium canals with excellent effect in the treatment of chronic and severe painful processes [81]. Moreover, conotoxins from different super-families were commonly found to have similar distributions. A new conotoxin, PCCSKLHDNSCCGL was sequenced [87]. Conotoxins composed mostly of 100–250 disulfide-bridged peptides are synthesized in the epithelial cells of the venom duct, then secreted in discrete parts of the same duct, a convoluted gland often several centimeters long [83]. Conotoxins bind to receptors such as voltage- and ligand-gated ion channels, G-protein-coupled receptors, and neurotransmitter transporters in the muscular and nervous system [84].

Conotoxins provide a vast library of peptides with unique abilities to discriminate among types and subtypes of ion channels in a manner that is unmatched by the typical small molecule drugs which dominate the pharmaceutical industry. In addition, cone venom peptides are small and inherently stable, making them ideal leads for peptide therapeutics, especially ion channel therapeutics. The high structural resolution now obtained with modern NMR spectroscopy and X-ray crystallography provides emerging opportunities to use conotoxins as templates for the design of smaller peptidomimetics that incorporate the selectivity and potency of conotoxins. Because of its selectivity and potency, w-conotoxin MVIIA (Ziconotide) is being developed as a drug for the treatment of chronic pain. With improvement in methods of delivering peptides, it is anticipated that conopeptides can be modified for effective oral delivery [85]. The purified peptide from crassispirids venom of marine gastropods has 29 amino acid residues long, with the sequence: GSCGLPCHEN-RRCGWACYCDDGICKPLRV [86].

6. Phylum Echinodermata

Generally, some species of echinodermata are poisonous some of them are venomous. The venomous species inject their toxin into the victim through spines or other similar structures. The

poisonous species contain poison within their tissues which affect the victim when consumed. The venomous species can be consumed after being cooked, but poisonous species should never be consumed as the poison will not be inactivated even by the high temperature of cooking also [87]. *Globiferous pedicellariae* or *Sphaerechinus granularis* are venomous defensive appendages consisting of a stalk bearing a head made of three movable jaws. Each jaw is supported by a calcareous valve ending with a terminal grooved tooth. The venom apparatus is located in each jaw and consists of a venom gland surrounded by a muscular envelope and terminating in a duct which completely encircles the terminal tooth of the valve. In mature pedicellariae the venom is stored in intracellular vacuoles of highly differentiated cells. Upon contraction of the muscular envelope the venom is released via a holocrine mechanism and infiltrates the predator's tissues through the wound inflicted by the three calcareous teeth of the valves [88].

7. Phylum Vertebrata

7a. Class Fishes

Venomous fish stings are a common environment hazard worldwide. Venomous fish carry venom gland bearing fin rays for self defense. The venom glands are located mainly in the dorsal fins, but they can be found in the ventral and anal fins as in scorpion fish, lion fish, and stone fish or in the dorsal and pectoral fin as in catfish-mostly freshwater species. Stingrays have one or more serrated stings located on their whip-like tails. Fresh water stingrays are found in rivers and lakes in South America and Africa. Weever fish of the Mediterranean and Eastern Atlantic coastal waters and toad fish possess venomous stings on their gill covers and in the dorsal fins [19, 47]. Stings from venomous fish cause agonizing pain and mechanical injury that destroys tissue. In rare instances, deeply penetrating stings can affect large blood vessels and major nerves. Some species of venomous fish can cause systemic envenomination [6, 47, 89]. Venoms from stonefish (genus *Synanceja*) have marked effects on the cardiovascular and neuromuscular systems, vascular perpeability and exhibit haemolytic and hyaluronidase activity [4]. Venomous fish are often involved in human accidents and symptoms of envenomation include local (intense pain and swelling) and systemic effects (cardiovascular and neurological disorders). Stonefish antivenom evoked an immune cross-reactive response with scorpionfish venom and is efficient in neutralizing the most prominent toxic effects of scorpionfish venom. This is in accordance with the hypothesis that venomous fish belonging to different genera or inhabiting different regions may share venom compounds with similar antigenic properties [90]. However the only commercially available antivenom is against the Indo-Pacific stonefish *Synanceja trachynis*. The venom similarities between Indo-Pacific and Atlantic venomous fish, suggested that the scorpionfish *Scorpaena plumieri* venom compound responsible for the inflammatory and cardiovascular effects [91].

Lionfish, members of the genera *Pterois*, *Parapterois* and *Dendrochirus* have venomous glandular tissues in dorsal, pelvic and anal spines. The lionfish toxins have been shown to cross-react with the stonefish toxins by neutralization tests using the commercial stonefish antivenom. Two species of *Pterois* lionfish (*P. antennata* and *P. volitans*) contain a 75 kDa protein cross-reacting with neoverrucotoxin. Then, the amino acid sequences of the *P. antennata* and *P. volitans* toxins were successfully determined by cDNA cloning using primers designed from the highly conserved sequences of the stonefish toxins. Remarkably, either a-subunits (699 amino acid residues) or b-subunits (698 amino acid residues) of the *P. antennata* and *P. volitans* toxins share as high as 99% sequence identity with each other. Furthermore, both a- and b-subunits of the lionfish toxins exhibit high sequence identity with each other and also with the b-subunits of the stonefish toxins [92].

7b. Class Amphibia

The amphibian skin contain various bioactive molecules that possess potential therapeutic activities like antibacterial, antifungal, antiprotozoal, antidiabetic, antineoplastic, alagesic and sleep inducing properties [93]. Frog skins alone have 24 different structural groups of 500 alkaloids [94]. The glanular skin gland secretions include amines, peptides, proteins, steroids, water soluble alkaloids and lipid-soluble alkaloids. The peptides include bradykinin, sauvagine, physaelaemin, caerulein, bombesin, dermorphins and adenoregulin [95]. Magainins are class of chemicals secreted by frogs

that are vasoactive peptides and has antibiotic properties that protect the skin [96], it work against bacterial and fungal pathogens by attacking their membranes, forming pores in the pathogens membrane that kill the organism [97]. The bufadienolides which are biosynthesized from cholesterol in the toads' diet are five times more deadly than the cardenolides. In vitro and animal models studies suggested that bufadienolides could be used clinically in place of, or in combination with cardenolides to improve treatment of congestive heart failure [98].

Chemical defense in a dendrobatid poison frog is dependent on geographic location and habitat type. A total of 232 alkaloids, representing 21 structural classes were detected in skin extracts from the dendrobatid poison frog *Oophaga pumilio* [99]. Lipid-soluble alkaloids that discovered in amphibian skin includes steroidal samandarines from salamanders, the batrachotoxins, histrionicotoxins, gephyrotoxins, and epibatidine from neotropical poison frogs, the pumiliotoxins, allopumiliotoxins, homopumiliotoxins, and decahydroquinolines from certain genera of anurans, and the pseudophrynamines from one genus of Australian frogs [100]. Bufadienolides are cardioactive steroids responsible for the anti-inflammatory actions of toad venom [101]. Venom of Bufo marinus toad contains a Na^+, K^+-ATPase inhibitor with potent vasoconstrictor activity [102].

Several studies describing the biochemical characterization of the components from the skin secretion of *Phyllomedusa* genus have allowed the identification of biologically active peptides that are very similar to the mammalian hormones, neuropeptides, as well as the broad-spectrum cytolytic antimicrobial peptides [103]. The antimicrobial peptides are grouped in seven families namely dermaseptins, phylloseptins, plasticins, dermatoxins, phylloxins, hyposins, and orphan peptides [104]. Some of these peptides; dermaseptins, phylloseptins, hyposins, and the bradykinin-related peptides were isolated and characterized from *Phyllomedusa hypochondrialis* skin secretion [96, 103-105]. The activity against grampositive and gram-negative bacteria, yeast and fungi were reported for dermaseptins [106], while antibacterial activity and antiparasitic activity against *Trypanosoma cruzi* were demonstrated for phylloseptins [107].

7c. Class Reptilia

No single characteristic distinguishes a poisonous snake from a harmless one except the presence of poison fangs and glands. The proteroglypha have in front of the upper jaw permanent erect fangs (fixed fangs), however, the solenoglypha have erectile fangs that can rise to an erect position (folded fangs). The fixed-fang snakes usually have neurotoxic venoms and the folded-fang snakes usually have hemotoxic venoms. However, the most poisonous snakes have both neurotoxic and hemotoxic venom. Of about 2000 different species of snakes, only 300 are venomous. Venomous snakes are found in the families Colubridae, Elapidae, Hydrophidae, Viperidae and Crotalidae. Over 95% of the dry weight of most venoms is polypeptide which includes enzymes, toxins and small peptides, each class being capable of modulating the physiological response of envenomed animals. More than 20 enzymes have been detected in snake venom and 12 are found in the majority of venoms. Hyaluronidase is present in all snake venoms facilitating the distribution of other venom components throughout the tissues of the prey [108].

Snake venoms are among the best pharmacologically characterized natural toxins chiefly because of their deleterious effects on humans. The most common classes of snake venom enzymes include phospholipase A2, phosphodiesterase, phosphomonoesterase, L-amino acid oxidase, specific endopeptidases, and nonspecific endopeptidases [109]. Snake venoms comprise a complex pool of proteins (more than 90% of the dry weight), organic compounds with low molecular mass and inorganic compounds [110-111]. Their quantitative and qualitative composition may vary according to factors such as snake species, age, seasonal period and diet [112]. There are 13 species of rattlesnakes in Arizona are most active from March through October and shelter in abandoned burrows of other animals, brush/ woodpiles, and rock crevices. They generally eat small mammals (rodents), lizards, and birds. Rattlesnakes use camouflage as a defense mechanism and to help them catch prey. While camouflage makes it difficult for people to see and avoid them, their audible rattle provides an exceptional warning most of the time [66]. *B. jararacussu*, *B. brazili* and *B. atrox* venoms at concentrations up to 5 mg/mL were able to induce

breakage in the DNA of human lymphocytes [113]. Hump-nosed pit vipers of genus *Hypnale* are the commonest cause of snake bite in Sri Lanka [114]. Metallo-and serine proteases have been identified in several colubrid venoms, and phospholipase A2 is a more frequent component than has been previously recognized. Venom phosphodiesterase, acetylcholinesterase and prothrombin activator activities occur in some venoms. Postsynaptic neurotoxins and myotoxins have been partially characterized for venoms from several species [115]. Moreover, the venom of the family Viperidae, including the saw-scaled viper, is rich in serine proteinases and metalloproteinases, which affect the nervous system, complementary system, blood coagulation, platelet aggregation and blood pressure [116]. Novel hyaluronidase CcHaseII (33 kDa) of the most dangerous Egyptian horned viper *Cerastes cerastes* (Cc) is purified. The spreading property of the purified enzyme promoted distribution of other venom components and generalized tissue destruction. The importance of venom hyaluronidase as a therapeutic target and identification of nontoxic inhibitors of the enzyme could play an important role in the efficient management of snakebite [117].

Myotoxins play a major role in the pathogenesis of the envenomations caused by snake bites in large parts of the world where this is a very relevant public health problem. They are basic proteins that can be classified into three main groups belonging to structurally distinct protein families: the 'small' myotoxins, the cardiotoxins and the PLA2 myotoxins [118]. The pathology caused by cardiotoxins and PLA2 myotoxins develops rapidly and it is associated with marked damage to the sarcolemma, whereas pathology associated with 'small' myotoxins has a more delayed onset and sarcolemma damage is not apparent [119]. Among fast acting myotoxins, cobra cardiotoxins, they cause severe tissue necrosis and systolic heart arrest in snakebite victims. Lys49-PLA2 myotoxins, an important component of various viperid snake venoms, they cause severe myonecrosis. So, these toxins are used as tools to study skeletal muscle repair and regeneration, a process that can be very limited after snakebites [120].

Snake venoms are a rich source of molecules act via the adhesion molecules. The benefits of these molecules for the treatment of certain diseases are: a shorter half-life, reversible inhibition, easier to control a problem and very low immunogenicity. For example, the antihypertensive drug captopril, modelled from the venom of the Brazilian arrowhead viper (*Bothrops jaracusa*); the anticoagulant Integrilin (eptifibatide), a heptapeptide derived from a protein found in the venom of the American southeastern pygmy rattlesnake (*Sistrurus miliarius barbouri*); Ancrod, a compound isolated from the venom of the Malaysian pit viper (*Agkistrodon rhodostoma*) for use in the treatment of heparin-induced thrombocytopenia and stroke and alfimeprase, a novel fibrinolytic metallo-proteinase for thrombolysis derived from venom of southern copperhead snake (*Agkistrodon contortrix contortrix*) (Table 3).

Table 3. Drugs derived from snake venom [121].

Name	Snake	Target and function of treatment
Capoten ® (Captropil)	*Bothrops jaracusa*	Angiotensin converted enzyme (ACE) inhibitor/ high blood pressure
Integrilin ® (Eptifibatide)	*Sisturus miliarus barbouri*	Platelet aggregation inhibitor/acute coronary syndrome
Aggrastat ® (tirofiban)	*Echis carinatus*	GPIIb-IIIa inhibitor/myocardial infarct, refractory ischemia
Exanta	*Cobra*	Thrombin inhibitor/arterial fibrillation and blood
Alfimeprase	*Agkistrodon contortrix contortrix*	Thrombolytic/ Acute ischemic stroke, acute peripheral arterial occlusion
Ancrod ® (viprinex)	*Agkistrodon rhodostoma*	Fibrinogen inhibitor/ stroke
hemocoagulase	*Bothrops atrox*	Thrombin-like effect and thromboplastin activity/ prevention and treatment of haemorrhage

The boldly colored *Gila monster* is a venomous reptile can deliver a painful bite if handled. *Gila monster* move slowly and feed on animals that cannot easily escape, such as young rodents and the eggs of other reptiles and ground-nesting birds. They are most active in the spring and take refuge underground during hot weather. Fortunately, *Gila monster* attempt to bite humans only as a defensive measure when they feel threatened and always prefer escape to defense. *Gila monster* is a venomous reptile found in the lower elevations of southern and western Arizona. They can deliver a painful bite if handled. *Gila monster* are most active in the spring and take refuge underground during hot weather [66]. Sea snakes are mainly a hazard to fishermen in the subtropics and tropics who are bitten when emptying fishing nets or when wading in muddy waters. Rhabdomyolysis is the main feature of envenomination by sea snakes. Early clinical signs are muscular pain and tenderness followed by placid paralysis and renal failure [2].

7d. Class Aves

Homobatrachotoxin was reported in 1992 to occur in the skins and feathers of three passerine bird species in the genus *Pitohui* (family Pachycephalidae) endemic to New Guinea and considered toxic by New Guineans [122].

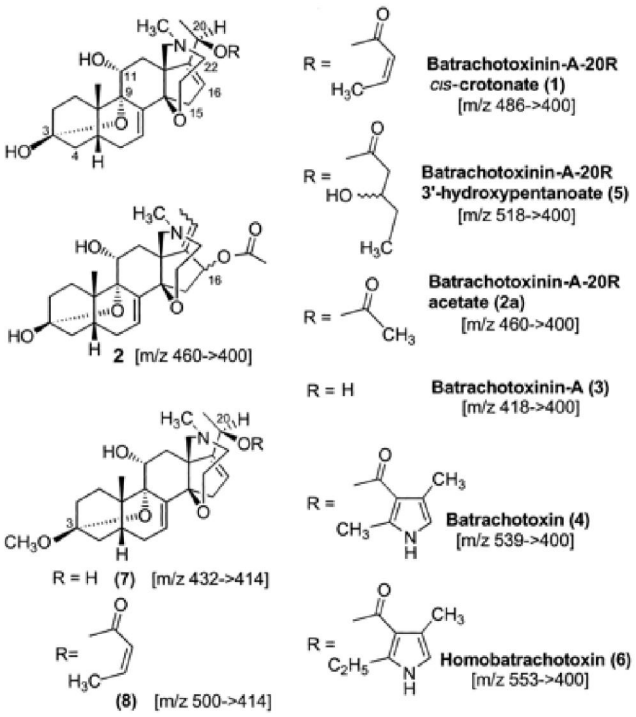


Fig. 1. Batrachotoxins found in feathers and/or skins of New Guinean passerine birds [124].

Homobatrachotoxin is a member of a group of neurotoxic steroidal alkaloids, collectively called batrachotoxins that stabilize the open form of voltage-gated sodium channels in nerve and muscle membranes [123]. Dumbacher et al. [124] were detected and measured batrachotoxins by using HPLC-mass spectrometry, in five species of New Guinean birds of the genus *Pitohui* as well as a species of a second toxic bird *Ifrita kowaldi*.

The alkaloids, identified in feathers and skin, were batrachotoxinin-A cis-crotonate (1), batrachotoxinin-A and an isomer (3 and 3a, respectively), batrachotoxin (4), batrachotoxinin-A 3′-hydroxypentanoate (5), homobatrachotoxin (6), and mono- and dihydroxylated derivatives of homobatrachotoxin. The highest levels of batrachotoxins were generally present in the contour feathers of belly, breast, or legs in *Pitohui dichrous*, *Pitohui kirhocephalus*, and *Ifrita kowaldi*. Lesser amounts are found in head, back, tail, and wing feathers. Batrachotoxin (4) and homobatrachotoxin (6) were found only in feathers and not in skin. The levels of batrachotoxins varied widely for different populations of Pitohui and Ifrita, a result compatible with the hypothesis that these birds are sequestering toxins from a dietary source (Fig. 1).

Feathers constitute the first line of defense against consumers in birds. Many predators such as raptors and carnivores, pluck them from carcasses before commencing to feed. Thus, it is not surprising from a functional standpoint that feathers serve as a repository of defensive chemicals. Dumbacher et al. [124] suggested that batrachotoxins might be transferred from feathers onto eggs or nest materials, thus affording protection against nest-raiding vertebrates. Breast or belly contour feathers contained the highest toxin concentrations and might rub off onto eggs or be deposited in the nest to provide protection from predators such as snakes, rodents or other birds; particularly any predator that might normally eat an egg whole.

Batrachotoxins found in *Pitohui* or *Ifrita* feathers may repel or kill lice or other parasites [125]. In addition, batrachotoxin-laden dander or feather pieces shed from the birds may impart these nonvolatile toxins to other organisms including humans. A survey of birds for batrachotoxins and related compounds also is illustrated, particularly in light of the discovery of alkaloids in the red warbler (*Ergaticus ruber*) from Mexico [126].

7e. Class Mammals

The occurrence of venom in mammals has long been considered of minor importance. Mammalian venoms form a heterogeneous group having different compositions and modes of action and are present in three classes of mammals, Insectivora, Monotremata, and Chiroptera. A fourth order, Primates, is proposed to have venomous representatives [127]. The taxonomically complex group Insectivora holds most of the venomous mammals. With the exception of vampire bats, these are the only mammals so far observed to produce toxic saliva. The American shorttailed shrew (*Blarina brevicauda*), the Hispaniolan solenodon (*Solenodon paradoxus*), the European water shrew (*Neomys fodiens*) and the Mediterranean water shrew (*Neomys anomalus*) provided the most definite evidences for salivary venom [128]. European folk-tales focused on the effects of shrew bites upon cattle and horses, describing the affected animals as paralyzed and deprived of feeling, hinting to systemic effects [129]. The purification of the toxic component of the *B. brevicauda* saliva, blarina toxin was achieved by Kita et al. [130] as a glycosylated protein composed of 253 amino acids with a kallikrein-like protease activity. This toxin cleaves kininogens producing kinins, including bradykinin, an inflammation mediator which increases vascular permeability and lowers blood pressure.

The platypus (*Ornithorhynchus anatinus*) is thought to be the only venomous Monotremata representative. When gland secretion of platypus was injected subcutaneously into a rabbit it produced localized swelling and tenderness. However, intravenously injection into a rabbits it caused a rapid fall in blood pressure and respiratory distress were observed, being followed by death [131]. The *O. anatinus* venom is a complex mixture of 19 different fractions. The peptide fractions include C-type natriuretic peptides, defensin-like peptides, nerve growth factors, isomerases, hyaluronidase, protease, and uncharacterized proteins [132, 133]. Two distinct classes of anticoagulants are found in the saliva of vampire bats i.e. plasminogen activators and inhibitors of proteinases [134]. Plasminogen activators act producing localized proteolysis in tissue remodeling, wound healing and neuronal plasticity [135]. The venom of a loris reaches the target animal by means of prolonged bites. It was originally thought that the animal's salivawas responsible for the observed symptoms, causing anaphylaxis on already sensitized individuals [136]. Alterman [137] established a connection between this habit and the painful loris bites, proposing that toxins from the brachial gland exudate would be responsible for the observable effects on humans. The brachial gland exudate is a complex mixture, comprising volatile low molecular weight metabolites and non-volatile high molecular weight protein fractions [138]. An unidentified steroid and polypeptides generated by mixing brachial gland exudate and saliva were suggested to be the active toxic component of the venom [139].

Table 4. Summary of venomous mammals and their venoms [127].

Mammalian Order	Venomous representatives	Venom source	Known toxic components	Most common effects
Insectivora	Shrews and solenodons	Salivary gland	Blarina toxin	Breathing disturbance, paralysis and convulsions
Monotremata	Platypus	Crural gland	C-type natrituretic peptides, defensin-like peptides, nerve growth factor	Acute pain and swelling
Chiroptera	Vampire bats	Salivary gland	Plasminogen activators, Draculin is a glycoprotein found in the saliva of vampire bats	Prolonged bleeding
Primates	Slow and pygmy slow lorises	Brachial gland	BGE protein (is a heterodimeric protein with 17.6 kDa)	Allergic reactions

CONCLUSION

Very many poisonous animals are medically important. Envenomation causes multiple disorders of which neurotoxicity is sometimes the major problem. Some of the natural toxins acting on the cardiovascular system are very potent and highly specific for some receptors in cardiac tissue. Although some molecules of venoms possess high receptors specificity, they are used as therapeutic drugs. More studies on their structure-function may provide useful information for synthesis of smaller analogues with lower toxicity. So, interventions by scientists and clinicians have made it possible to use the venom proteins as potential drugs for multiple disorders or for drug design.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. Fry BG, Vidal N, Norman JA, Vonk FJ, Scheib H, Ramjan SF, et al. Early evolution of the venom system in lizards and snakes. Nature. 2006; 439: 584-588.
2. Junghanss T, Bodio M. Medically important venomous animals: biology, prevention, first aid, and clinical management. Clin Infect Dis. 2006; 43(10): 1309-1317.
3. Müller GJ. Black and brown widow spider bites in South Africa, a series of 45 cases. S Afr Med J. 1993; 83:399-405.
4. Gwee MCE, Gopalakrishnakone P, Yuen R, Khoo HE, Low KSY. A review of stonefish venoms and toxins. Pharmacol Therap. 1994; 64(3): 509-528.
5. Mebs D. Scorpions and snakes, such as cobras, mambas and vipers made the African continent famous for venomous animals. Bull Soc Pathol Exot. 2002; 95 (3): 31.
6. Meier J, White J. 1995. Handbook of clinical toxicology of animal venoms and poisons. Boca Raton, FL, CRC Press.
7. Williamson JA, Fenner PJ, Burnett JW, Rifkin JF (eds.). 1996. Venomous and poisonous marine animals: a medical and biological handbook. Sydney: University of New South Wales Press.
8. Upadhyay RK, Ahmad S. Allergic and toxic responses of insect venom and it's immunotherapy. J Pharm Res. 2010; 3(12): 3123-3128.
9. White J, Dart RC. 2007. Snakebite: A Brief Medical Guide. Stirling.
10. White J. 2010. Venomous animals: clinical toxicology. Molecular, Clinical and Environmental Toxicology. Volume 2: Clinical Toxicology. pp: 233-291.
11. Rein KS, Borrone J. Polyketides from dinoflagellates: origins, pharmacology and biosynthesis. Comp Biochem Physiol B Biochem Mol Biol. 1999; 124: 117-131.
12. Isbister GK, John NA, Hooper JNA. Clinical effects of stings by sponges of the genus *Tedania* and a review of sponge stings worldwide. Toxicon. 2005; 46: 782-785.
13. Kalinin VI, Ivanchina NV, Krasokhin VB, Makarieva TN, Stonik VA. Glycosides from marine sponges (*Porifera*, Demospongiae): Structures, taxonomical distribution, biological activities and biological roles. Mar Drugs. 2012; 10: 1671-1710.
14. Sipkema D, Franssen MCR, Osinga R, Tramper J, Wijffels RH. Marine sponges as pharmacy. Marine Biotechnol. 2005; 7: 142-167.
15. Kitagawa I, Kobayashi M. Antitumor marine natural products. Gan To Kagaku Ryoho. 1990; 17: 322-329.
16. Casapullo A, Bifulco G, Bruno I, Riccio R. New bisindole alkaloids of the topsentin and hamacanthin classes from the Mediterranean marine sponge *Rhaphisia lacazei*. J Nat Prod. 2000; 63: 447-451.
17. Chen CY, Shen YC, Chen YJ. Bioactive sesquiterpenes from a Taiwanese marine sponge *Parahigginsia* sp. J Nat Prod. 1999; 62: 573-576.
18. Russell FE. 1965. Marine toxins and venomous and poisonous marine animals. In: Russell FS, ed. Advances in marine biology. Vol. 3. London, Academic Press.
19. Halstead BW. 1988. Poisonous and venomous marine animals of the world. 2nd ed. New Jersey: Darwin Press.
20. Watson GM, Wood RL. 2008. Colloquium on terminology. In: The Biology of Nematocysts.
21. Belmonte G., Menestrina G, Pederzolli C, Krizaj I, Gubensek F, Turk T, Macek P. Primary and secondary structure of a pore-forming toxin from the sea anemone, *Actinia equina* L., and its association with lipid vesicles. Biochim. Biophys. Acta. 1994; 1192: 197-204.
22. Diochot S, Loret E, Bruhn T, Beress, L, Lazdunski M. APETx1, a new toxin from the sea anemone *Anthopleura elegantissima*, blocks voltage-gated human ether-a-go-go-related gene potassium channels. Mol Pharmacol. 2003; 64: 59-69.
23. Diochot S, Baron A, Rash LD, Deval E, Escoubas P, Scarzello S, et al. A new sea anemone peptide, APETx2, inhibits ASIC3, a major acid-sensitive channel in sensory neurons. Embo J. 2004; 23: 1516-1525.
24. Bosmans F, Tytgat J. Sea anemone venom as a source of insecticidal peptides acting on voltage-

gated Na^+ channels. Toxicon. 2007; 49: 550-560.
25. Turk T, William R, Kem WR. The phylum Cnidaria and investigations of its toxins and venoms until 1990. Toxicon. 2009; 54: 1031-1037.
26. Mariottini GL, Pane L. Mediterranean Jellyfish Venoms: A Review on Scyphomedusae. Mar. Drugs. 2010; 8(4): 1122-1152.
27. Wang B, Zhang L, Zheng J, Wang O, Wang T, Lu J, et al. Multiple organ dysfunction: A delayed envenomation syndrome caused by tentacle extract from the jellyfish *Cyanea capillata*. Toxicon. 2013; 61: 54-61.
28. Weinheimer AJ, Spraggins RL. The occurrence of two new prostaglandin derivatives (15-epi-PGA2 and its acetate, methyl ester) in the Gorgonian *Plaxaura homomalla*. chemistry of coelenterates. XV. Tetrahedron Lett. 1989; 10: 5185-5188.
29. Moore RE, Scheuer PJ. Palytoxin: a new marine toxin from a Coelenterate. Science. 1971; 172: 495-498.
30. Rouzaire-Dubois B, Dubois J. Characterization of palytoxin-induced channels in mouse neuroblastoma cells. Toxicon. 1990; 28: 1147-1158.
31. Honda A, Yamamoto Y, Mori Y, Yamada Y, Kikuchi H. Antileukemic effect of coral-prostanoids clavulones from the stolonifer *Clavularia viridis* on human myeloid leukemia (HL-60) cells. Biochem Biophys Res Commun. 1985; 130: 515-523.
32. Orduña-Novoa K, Segura-Puertas L, Sánchez-Rodríguez J, Meléndez A, Nava-Ruíz C, Rembao D, et al. Possible antitumoral effect of the crude venom of *Cassiopea xamachana* (Cnidaria: Scyphozoa) on tumors of the central nervous system induced by N-Ethyl-N-Nitrosourea (ENU) in rats. Proc West Pharmacol Soc. 2003; 46: 85-87.
33. Tabrah FL, Kashiwagi M, Norton TR. Antitumor activity in mice of four coelenterate extracts. Int J Clin Pharmacol. 1976; 5: 420-422.
34. Giraldi T, Ferlan I, Romeo D. Antitumour activity of equinatoxin. Chem Biol Interact. 1976; 13: 199-203.
35. Koehler PG, Diclaro JW. 2012. Stinging or Venomous Insects and Related Pests. http://edis.ifas.ufl.edu.
36. Blum MS. 1981. Chemical defenses in arthropods. Academic Press. New York. pp. 562.
37. Müller UR. 1990. Insect sting allergy. Stuttgart: Gustav Fischer.
38. Murnaghan MF. Site and mechanism of tick paralysis. Science. 1960; 131: 418-419.
39. Grattan-Smith PJ, Morris JG, Johnston HM. Clinical and neurophysiological features of tick paralysis. Brain. 1997; 120: 1975-1987.
40. Cooper BJ, Spence I. Temperature-dependent inhibition of evoked acetylcholine release in tick paralysis. Nature. 1976; 263: 693-695.
41. Baracchi D, Francese S, Turillazzi S. Beyond the antipredatory defence: Honey bee venom function as a component of social immunity. Toxicon. 2011; 58: 550-557.
42. Qiu Y, Lee KS, Choo YM, Kong D, Yoon HJ, Jin BR. Molecular cloning and antifibrinolytic activity of a serine protease inhibitor from bumblebee (*Bombus terrestris*) venom. Toxicon. 2013; 63: 1-6.
43. Tschinkel WR. 2006. The Fire Ants. Belknap/Harvard University Press, Cambridge, Mass, USA.
44. Hoffman DR, Smith AM, Schmidt M, Moffitt JE, Guralnick M. Allergens in Hymenoptera venom. XXII. Comparison of venoms from two species of imported fire ants, *Solenopsis invicta* and *richteri*. J Allergy Clin Immunol. 1990; 85: 988-996.
45. Hoffman DR, Sakell RH, Schmidt M Sol i 1, the phospholipase allergen of imported fire ant venom. J Allergy Clin Immunol. 2005; 115: 611-616.
46. Quintero-Hernández V, Ortiz E, Rendón-Anaya M, Schwartz EF, Becerril B, Corzo G, Possani LD. Scorpion and spider venom peptides: Gene cloning and peptide expression. Toxicon. 2011; 58: 644-663.
47. Sutherland SK, Tibballs J. 2001. Australian animal toxins: the creatures, their toxins and care of the poisoned patient. 2nd ed. Melbourne: Oxford University Press.
48. Warrell DA. 2003. Injuries, envenoming, poisoning, and allergic reactions caused by animals. In: Warrell DA, Cox TM, Firth JD, eds. Oxford Textbook of Medicine. 4th ed. Vol 1. Oxford: Oxford University Press, pp: 923-946.
49. Saez NJ, Senff S, Jensen JE, Er, SY, Herzig V, Rash LD, King GF. Spider-Venom peptides as therapeutics. Toxins. 2010; 2: 2851-2871.
50. Escoubas P, Diochot S, Corzo G. Structure and pharmacology of spider venom neurotoxins. Biochimie. 2000; 82: 893-907.
51. Escoubas P, De Weille JR, Lecov A; Diochot S, Waldmann R, Champigny G, et al. Isolation of a tarantula toxin specific for a class of protonated Na^+ channels. J Biol Chem. 2000; 275: 25116.
52. Hogan CJ, Barbaro KC, Winkel K. Loxoscelism: old obstacles, new directions. Ann Emerg Med. 2004; 44: 608-624.
53. Frangež R, Grandič M, Vrecl M. 2012. Cardiovascular pathophysiologyproduced by natural toxins and their possible therapeutic implications. Cardiotoxicity of oncologic treatments, Fiuza M (Ed.), Publisher InTech, pp. 1-16.
54 Da Silva PH, Da Silveira RB, Appel MH, Mangili OC, Gremski W, Veiga SS. Brown spiders and loxoscelism. Toxicon. 2004; 44: 693-709.
55. Swanson DL, Vetter RS. Loxoscelism. Clin Dermatol. 2006; 24(3): 213-221.
56. Souza AL, Málaque CM, Sztajnbok J, Romano CC, Duarte AJ, Seguro AC. Loxosceles venom-induced

cytokine activation, hemolysis, and acute kidney injury. Toxicon. 2008; 51: 151-156.
57. Málaque CMS, Castro-Valencia JE, Cardoso JLC, França FOS, Bárbaro KC, Fan HW. Clinical and epidemiological features of definitive and presumed loxoscelism in São Paulo, Brazil. Rev Inst Med Trop São Paulo. 2002; 44(3): 139-143.
58. Abdulkader RCRM, Barbaro KC, Barros EJG, Burdmann EA. Nephrotoxicity of insect and spider in Latin America. Sem Nephrol. 2008; 28: 373-382.
59. Ospedal KZ, Appel MH, Neto JF, Mangili OC, Veiga SS, Gremski W. Histopathological findings in rabbits after experimental acute exposure to the *Loxosceles intermedia* (Brown spider) venom. Int J Exp Pathol. 2002; 83: 287-294.
60. Pereira NB, Kalapothakis E, Campos LP, Vasconcelos AC, Moro L. Apoptose na patogenia do Loxoscelismo cutâneo experimental em coelhos: abordagens morfológica e imunoistoquímica. Rev Bras Toxicol. 2010; 23(1): 136.
61. Corzo G, Gilles N, Satake H, Villegas E, Dai L, Nakajima T, Haupt J. Primary structures of the major peptide toxins from the venom of the spider Macrothele gigas that bind to sites 3 and 4 in the sodium channel. FEBS Lett. 2003; 547: 43-50.
62. Gremski LH, da Silveira RB, Chaim OM, Probst CM, Ferrer VP, Nowatzki J, et al. A novel expression profile of the *Loxosceles intermedia* spider venomous gland revealed by transcriptome analysis. Mol Biosyst. 2010; 6: 2403-2416.
63. Biley P, White J. Venom as a source of useful biologically active molecules. Emerg Med. 2001; 13: 28-36.
64. Chaim OM, Trevisan-Silva D, Chaves-Moreira D, Wille ACM, Ferrer VP, Matsubara FH, et al. Brown spider (*Loxosceles* genus) venom toxins: tools for biological purposes. Toxicon. 2011; 3: 309-344.
65. Keegan HL. 1980. Scorpions of medical importance. Jackson, MS: University Press of Mississippi.
66. Dolan C, Mannan B. 2009. Venomous Wildlife. Arizona cooperative extension. College Agriculture and Life Sciences. The University of Arizona.
67. Possani LD, Merino E, Corona M, Bolivar F, Becerril B. Peptides and genes coding for scorpion toxins that affect ion-channels. Biochimie. 2000; 82: 861-868.
68. Jover E, Couraud F, Rochat H. Two types of scorpion neurotoxins characterized by their binding to two separate receptor sites on rat brain synaptosomes. Biochem Biophys Res Commun. 1980; 95: 1607-1614.
69. Catterall WA. Cellular and molecular biology of voltage gated sodium channels. Physiol Rev. 1992; 72: S15-S48.
70. Possani LD, Becerril B, Delepierre M, Tytgat J. Scorpion toxins specific for Na^+-channels. Eur J Biochem. 1999; 264: 287-300.
71. Cestele S, Catterall WA. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. Biochimie. 2000; 82: 883-892.
72. Shichor I, Zlotkin E, Ilan N. Domain 2 of *Drosophila para* voltage-gated sodium channel confers insect properties to a rat brain channel. J Neurosci. 2002; 22: 4364-4371.
73. Curry SC, Vance MV, Ryan PJ, Kunkel DB, Northey WT. Envenomation by the scorpion *Centruroides sculpturatus*. J Toxicol Clin Toxicol. 1983; 21: 417-449.
74. Petricevich VL. Scorpion venom and the inflammatory response. Mediators Inflamm. 2010: 1-16.
75. Luna-Ramírez K, Quintero-Hernández V, Vargas-Jaimes L, Batista CVF, Kenneth D, Winkel KD, Possani LD. Characterization of the venom from the Australian scorpion *Urodacus yaschenkoi*: Molecular mass analysis of components, cDNA sequences and peptides with antimicrobial activity. Toxicon. 2012; 63: 44-54.
76. Aigner BA, Ollert M, Seifert F, Ring J, Phil D, Plotz SG. *Pseudomonas oryzihabitans* cutaneous ulceration from *Octopus vulgaris* bite. A case report and review of the literature. Arch Dermatol. 2011; 147(8): 963-966.
77. Sheumack DD, Hoeden MEH, Spence I, Quinin RJ. Maculotoxin: a neurotoxin from the venom glands of the octopus *Hapalochlaena maculosa* identified a tetrodotoxin. Science. 1978; 199: 188-189.
78. Walker DG. Survival after severe envenomation by the blue-ringed octopus (*Hapalochlaena maculosa*). Med J Aust. 1983; 2: 663-665.
79. Junior VH, Neto JB, Cobo VJ. Venomous mollusks: the risks of human accidents by *Conus* snails (Gastropoda: Conidae) in Brazil. Rev Soc Brasil Med Trop. 2006; 39(5): 498-500.
80. Olivera BM, Cruz LJ. Conotoxins, in retrospect. Toxicon. 2001; 39: 7-14.
81. Miljanich GP. Ziconotide: neuronal calcium channel blocker for treating severe chronic pain. Curr Med Chem. 2004; 11: 3029-3040.
82. Dobson R, Collodoro M, Gilles N, Turtoi A, De Pauw E, Quinton L. Secretion and maturation of conotoxins in the venom ducts of *Conus textile*. Toxicon. 2012; 60: 1370-1379.
83. Tayo LL, Lu B, Cruz LJ, Yates JR. Proteomic analysis provides insights on venom processing in *Conus textile*. J Proteome Res. 2010; 9(5): 2292-2301.
84. Moller C, Mari F. 9.3 KDa components of the injected venom of *Conus purpurascens* define a new five-disulfide conotoxin framework. Biopolymers. 2011; 96: 158-165.
85. Adamsa DJ, Alewoodb PF, Craikb DJ, Drinkwaterc

RG, Lewisa RJ. Conotoxins and their potential pharmaceutical applications. Drug Develop Res. 1999; 46(3-4): 219-234.
86. Cabang AB, Imperial JS, Gajewiak J, Watkins M, Corneli PS, Olivera BM, Concepcion GP. Characterization of a venom peptide from a crassispirid gastropod. Toxicon. 2011; 58: 672-680.
87. James DB. 2010. Marine poisonous echinoderms. Fishing Chimes, vol. 30, No. 1.
88. Ghyoot M, Dubbois P, Janqoux M. The venom apparatus of the globiferous pedicellariae of the toxopneustid *Sphaerechinus granularis* (Echinodermata, Echinoida): Fine structure and mechanism of venom discharge. Zoomorphol. 1996; 114: 73-82.
89. Fenner PJ, Williamson JA, Skinner RA. Fatal and non-fatal stingray envenomation. Med J Aust. 1989; 151: 621-625.
90. Church JE, Hodgson WC. The pharmacological activity of fish venoms. Toxicon. 2002; 40: 1083-1093.
91. Gomes HL, Menezes TN, Carnielli JBT, Andrich F, Evangelista KS, Chávez-Olórtegui C, et al. Stonefish antivenom neutralises the inflammatory and cardiovascular effects induced by scorpionfish *Scorpaena plumieri* venom. Toxicon. 2011; 57: 992-999.
92. Kiriake A, Shiomi K. Some properties and cDNA cloning of proteinaceous toxins from two species of lionfish (*Pterois antennata* and *Pterois volitans*). Toxicon. 2011; 58: 494-501.
93. Gomes A, Giri B, Soha A, Mshra R. Bioactive molecules from amphiabian skin: Their biological activities with reference to therapeutic potentials for possible drug development. Indian J Exp Biol. 2007; 45: 579-593.
94. Millius S. 2004. Frogs Borrow poison for skin from ants. Science News, vol. 165, No 19.
95. Chen Orr DF, Bjourson AJ, McClean S, Rourke MO, Hirst DG, Rao Shaw C. Novel bradykinins and their precursor cDNAs from European yellow-bellied toad (*Bombina variegata*) skin. Eur J Biochem. 2002; 269: 4693-4700.
96. Tempone AG, Melhem O, Prado FO, Motoie G, Hiramoto RM, Turk T, Keb WR. The phylum cnidria and investigations of its toxins and venom until 1990. Toxicon. 2009; 54: 1031-1037.
97. Arnusch CJ, Branderhorst H, de Kruijff B, Liskamp RMJ, Breukink E, Pieters RJ. Enhanced membrane pore formation by multimeric/oligomeric antimicrobial peptides. Biochem. 2007; 46: 13437-13442.
98. Barrueto F, Jr, Kirrane BM, Cotter BW, Hoffman RS, Nelson LS. Cardioactive steroid poisoning: a comparison of plant- and animal-derived compounds. J Med Toxicol. 2006; 2: 152-155.
99. Saporito RA, Donnelly MA, Jain P, Garraffo HM, Spande TF, Daly JW. Spatial and temporal patterns of alkaloids variation in the poison frog *Oohpaga pumilio* in Costa Rica and Panama over 30 years. Toxicon. 2007; 50: 757-778.
100. Daly JW, Spande TF, Garraffo HM. Alkaloids from amphibian skin: a tabulation of over eight-hundred compounds. Nat Prod. 2005; 10: 1556-1575.
101. Ma H, Hiang J, Zhang J, Zhou J, Ding A, Ly G, et al. Protective effect of taurine on cardiotoxicity of the bufadienolides derived from toad (*Bufo bufo gargarizans* Canto) venom in guinea-pigs in vivo and in vitro. Toxicol Mech Methods. 2012; 22: 1-8.
102. Bagrov AY, Roukoyatkina NI, Pinaev AG, Dmitrieva RT, Fedorova OV. Effects of two endogenous Na^+, K^+-ATP-ase inhibirors, marinobufagenin and ovabain, on isolated rat aorta. Eur J Pharmacol. 1995; 274: 151-158.
103. Conceição K, Konno K, Richardson M, Antoniazzi MM, Jared C, Daffre S, et al. Isolation and biochemical characterization of peptides presenting antimicrobial activity from the skin of *Phyllomedusa hypochondrialis*. Peptides. 2006; 12: 3092-3099.
104. Amiche M, Ladram A, Nicolas P. A consistent nomenclature of antimicrobial peptides isolated from frogs of the subfamily Phyllomedusinae. Peptides. 2008; 29: 2074-2082.
105. Brand GD, Krause FC, Silva LP, Leite JR, Melo JA. Bradykinin-related peptides from *Phyllomedusa hypochondrialis*. Peptides. 2006; 9: 2137-2146.
106. Mor A, Nicolas P. Isolation and structure of novel defensive peptides from frog skin. Eur J Biochem. 1994; 219: 145-154.
107. Leite JR, Silva LP, Rodrigues MIS, Prates MV, Brand GD, Lacava BM, et al. Phylloseptins: a novel class of anti-acterial and anti-protozoan peptides from the *Phyllomedusa* genus. Peptides. 2005; 4, 565-573.
108. Karallibdde L. Animal toxins. Brit J Anaesth. 1995; 74: 319-327.
109. Iwanaga S, Suzuki T. 1979. Enzymes in snake venom. In: Handbook of Experimental Pharmacology. Lee CY (Ed.) New York, Springer-Verlag.
110. Bieber AL. 1979. Metal and nonprotein constituents in snake venoms. In: Handbook of Experimental Pharmacology. Lee CY (Ed.) New York, Springer-Verlag.
111. Ramos OHP, Selistre-de-Araujo HS. Snake venom metalloproteases-structure and function of catalytic and disintegrin domains B. Compar Biochem Physiol. 2006; Part C 142: 328-346.
112. Daltry JC, Wüster W, Thorpe RS. Diet and snake venom evolution. Nature. 1996; 379: 537-540.
113. Marcussi S, Stábeli RG, Santos-Filho NA, Menaldo DL, Silva Pereira LL, Zuliani JB, et al. Genotoxic effect of *Bothrops* snake venoms and isolated toxins on human lymphocyte DNA. Toxicon. 2013; 65: 9-14.
114. Maduwage K, Isbister GK, Silva A, Bowatta S,

Mendis S, Gawarammana I. Epidemiology and clinical effects of hump-nosed pit viper (Genus: *Hypnale*) envenoming in Sri Lanka. Toxicon. 2013; 61: 11-15.

115. Mackessy SP. Biochemistry and pharmacology of colubrid snake. J Toxicol Toxin Rev. 2002; 21(1&2): 43-83.
116. Salmanizadeh H, Babaie M; Zolfagharian H. In vivo evaluation of homeostatic effects of *Echis carinatus* snake venom in Iran. J Venom Anim Toxins Incl Trop Dis. 2013; 19: 3-8.
117. Wahby AF, El-Sayed ME, EL-Mezayen HA, Salama WH, Abdel-Aty AM, Fahmy AS. Egyptian horned viper *Cerastes cerastes* venom hyaluronidase: Purification, partial characterization and evidence for its action as a spreading factor. Toxicon. 2012; 60: 1380-1389.
118. Lomonte B, Angulo Y, Calderón L. An overview of lysine-49 phospholipase A2 myotoxins from crotalid snake venoms and their structural determinants of myotoxic action. Toxicon. 2003; 42: 885-901.
119. Fletcher JE, Hubert M, Wieland SJ, Gong QH, Jiang MS. Similarities and differences in mechanisms of cardiotoxins, melittin and other myotoxins. Toxicon. 1996; 34: 1301-1311.
120. Tonello F, Simonato M, Aita A, Pizzo P, Fernández J, Lomonte B, Gutiérrez JM, Montecucco C. A Lys49-PLA2 myotoxin of *Bothrops asper* triggers a rapid death of macrophages that involves autocrine purinergic receptor signaling. Cell Death Dis. 2002; 3: e343.
121. Sarray S, Luis J, El Ayeb M, Marrakchi N. 2013. Snake venom peptides: Promising molecules with anti-tumor effects. In: Bioactive Food Peptides in Health and Disease. InTech.
122. Dumbacher JP, Beehler BM, Spande TF, Garraffo HM, Daly JW. Homobatrachotoxin in the genus *Pitohui*: chemical defense in birds? Science. 1992; 258: 799-801.
123. Albuquerque EX, Daly JW, Witkop B. Batrachotoxin: chemistry and pharmacology. Science. 1971; 172(3987): 995-1002.
124. Dumbacher JP, Spande TF, Daly JW. Batrachotoxin alkaloids from passerine birds: A second toxic bird genus (*Ifrita kowaldi*) from New Guinea. Proc Natl Acad Sci USA. 2000; 97: 12970-12975.
125. Dumbacher JP. Evolution of toxicity in pitohuis: I. Effects of homobatrachotoxin on chewing lice (order Phthiraptera). Auk. 1999; 116: 957-963.
126. Escalante P, Emanuel V, Daly JW. Alkaloids in extracts of feathers of the red warbler. J Ornithol. 1994; 135: 410.
127. Ligabue-Braun R, Verli H, Regina C. Venomous mammals: A review. Toxicon. 2012; 59: 680-695.
128. Pournelle GH. 1968. Classification, biology, and description of the venom apparatus of insectivores of the genera *Solenodon*, *Neomys*, and *Blarina*. In: Bücherl W, Buckley EE, Deulofeu V. (Eds.), Venomous Animals and Their Venoms. Academic Press, New York, pp. 31-42.
129. Dufton MJ. Venomous mammals. Pharmacol Ther. 1992; 53: 199-215.
130. Kita M, Nakamura Y, Okumura Y, Ohdachi SD, Oba Y, Yoshikuni M, et al. Blarina toxin, a mammalian lethal venom from the short-tailed shrew *Blarina brevicauda*: isolation and characterization. Proc Natl Acad Sci USA. 2004; 101: 7542-7547.
131. Kellaway CH, LeMessurier DH. The venom of the platypus (*Ornithorhynchus anatinus*). Aust Exp Biol Med Sci. 1935; 13: 205-221.
132. Kourie JL. A component of platypus (*Ornithorhynchus anatinus*) venom forms slow-kinetics cation channels. J Membr Biol. 1999; 172: 37-45.
133. Whittington C, Belov K. Platypus venom: a review. Aust Mammal. 2007; 29: 57-62.
134. Basanova AV, Baskova IP, Zavalova LL. Vascular-platelet and plasma hemostasis regulators from bloodsucking animals. Biochem. 2002; 67: 143-150.
135. Schleuning WD. Vampire bat plasminogen activator DSPA-alpha-1 (desmoteplase): a thrombolytic drug optimized by natural selection. Haemostasis. 2001; 31: 118-122.
136. Wilde H. Anaphylactic shock following bite by a 'Slow loris', *Nycticebus coucang*. Am J Trop Med Hyg. 1972; 21: 592-594.
137. Alterman L. Isolation of toxins from brachial gland exudates from *Nycticebus coucang*. Am J Phys Anthropol. 1990; 81: 87.
138. Hagey LR, Fry BG, Fitch-Snyder H. 2007. Talking defensively: a dual use for the brachial gland exudate of slow and pygmy lorises. In: Gurski S, Nekaris KA. (Eds.). Primate Anti-predatory Strategies. vol. 2. Springer, New York, pp. 253-272.
139. Alterman L, Hale ME. Comparisons of toxins from brachial gland exudates of *Nycticebus coucang* and *N. pygmaeus*. Am J Phys Anthropol. 1991; 12: 43.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

MEDICINE REVIEW

Probiotics and prebiotics

Anna K. Szkaradkiewicz[1], Tomasz M. Karpiński[2]

[1]Poznań University of Medical Sciences, Department of Conservative Dentistry and Periodontology, Poznań, Poland

[2]Poznań University of Medical Sciences, Department of Medical Microbiology, Poznań, Poland

ABSTRACT

Probiotics are live microorganisms which, when administered in the appropriate dosage have a positive effect on the host health. Probiotic strains must have established origin, the so-called GRAS status (generally recognized as safe) and confirmed by tests safety of use. This strain should demonstrates its capability of survival, metabolize, adhesion to the intestinal mucosa and transient colonization of the gastrointestinal tract. The key importance have also antagonistic properties against potentially pathogenic species. To microorganisms having probiotic activity belong bacteria of the genera *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Streptococcus*, *Enterococcus*, and the yeasts *Saccharomyces boulardii*. A prebiotic is a non-digestible food ingredient that selectively stimulates the growth and activity of selected probiotic strains. Synbiotic is the food product comprising in its composition, both probiotic and prebiotic.

Key words: Probiotics; Prebiotics; Synbiotics; *Lactobacillus*; *Bifidobacterium*; *Saccharomyces boulardii*.

J Biol Earth Sci 2013; 3(1): M42-M47

Corresponding author:
Anna K. Szkaradkiewicz
Poznań University of Medical Sciences,
Department of Conservative Dentistry and Periodontology,
Bukowska 70, str., 60-812 Poznań, Poland
e-mail: aniaszk@op.pl

Original Submission: 19 March 2013; Revised Submission: 26 April 2013; Accepted: 30 April 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

INTRODUCTION

Probiotics, according to FAO/WHO definition, are live microorganisms which when administered in adequate amount confer a health benefit to the host [1]. Prebiotics are selectively fermented ingredients that result in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health [2].

REVIEW

The recent history of probiotics began in the early 1900s, when Elie Metchnikoff hypothesized that the longevity of peasants was linked to their consumption of fermented milk products containing lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus delbruekii* subsp. *bulgaricus*). He suggested that these beneficial bacteria decreased intestinal pH, and suppressed harmful bacteria [3].

Probiotics are considered as safe (GRAS status: generally recognized as safe). Probiotics must meet several conditions:

- must not lose its properties during storage,
- must be normally present in the human intestine,
- must be able to survive in GI tract and colonize the intestine cells,
- must have beneficial effects for human health,
- must have antagonism against pathogenic micro-organisms,
- must not demonstrate side-effects [1, 4].

Currently, as probiotics are used selected bacterial strains derived from the natural human intestinal flora, particularly: *Lactobacillus acidophilus* (Fig. 1), *Lactobacillus casei* (GG, Shirota, DN-114 001), *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Bifidus essensis*, *Streptococcus salivarius* ssp. *thermophilus*. The *Escherichia coli* strain Nissle 1917 is one of the examples of a non-LAB probiotic. In addition to the bacteria as probiotics are also used strains of yeast *Saccharomyces boulardii* [5, 6].

Mode of action of probiotics has not been well understood. It was found that probiotic bacteria can stimulate host defense mechanisms. Probiotics act on the humoral and cellular response, stimulating the immune system [7, 8]. They affect not only the stability of the intestinal microflora, but also to facilitate the digestion [9]. Probiotic bacteria are also involved in the inhibition of hypersensitivity reactions to food antigens [10]. Effect of probiotics is also associated with the modulation of the immune response by stimulating the synthesis of immunoglobulins and cytokines. Numerous studies have shown the ability of *Lactobacillus* species to macrophage activation and to increase their ability to phagocytosis [11-13]. Probiotic bacteria soften inflammatory reactions caused by bacteria, fungi (mainly *Candida*), and viruses, and at the same time enhance the immune responses [14].

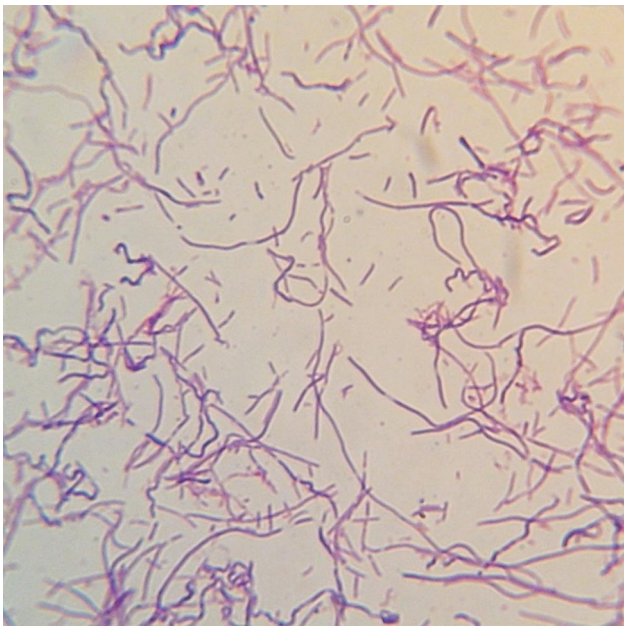


Fig. 1. *Lactobacillus acidophilus* in Gram staining.

Various probiotic strains, including *Lactobacillus reuteri* ATCC 55730, *L. rhamnosus* GG, *L. casei* DN-114 001 shorten duration of acute infectious diarrhea in children, travelers diarrhea and diarrhea caused by *Clostridium difficile* infection. *Bifidobacterium animalis* DN173010, *B. lactis* Bb-12 and *B. bifidum* are most commonly used in the maintenance of homeostasis of intestinal microflora and reducing of intestinal passage [15-18]. *Lactobacillus* strains by reducing the activity of β-glucuronidase, nitroreductase and hydrolases have anti-cancer effect. Also affect the relief of symptoms in Crohn's disease and ulcerative colitis [19, 20].

Saccharomyces boulardii is a non-pathogenic strain of yeast, demonstrating features of a probiotic. It is not susceptible to digestion or hydrochloric acid. *S. boulardii* inhibits the growth of many pathogenic microorganismsfor at reduces the severity of infection, has effect on binding of

bacterial toxins with the intestinal receptors, and acts immunoprotective. It is used in the prevention of post-antibiotic diarrhea, diarrhea in travelers, diarrhea caused by *Clostridium difficile* and inflammatory bowel diseases [6, 21-23]. Potential effects of probiotics are shown in Table 1.

The antimicrobial activity of lactic acid bacteria consists in the production of acids mainly lactic acid, lowering the pH of the environment, release of hydrogen peroxide [28, 29], bacteriocins [30-32] and biosurfactant [33]. Lactobacilli constitute about 1% of the normal oral cavity microflora. The bacteria of the *Lactobacillus* genus possess immunoregulatory properties and modulate inflammatory reaction, representing an important element of local antimicrobial resistance [34]. Lactobacilli may be important in preventing the progression of chronic periodontal disease, interacting by antagonism toward periopathogens [35-38]. Servin showed that lactic acid bacteria which produce hydrogen peroxide stimulate the production of cytokines by inflammatory cells [39]. Other data indicate the important role of bacteriocins secreted by *Lactobacillus* spp. in modifying the composition of the oral cavity microflora [1, 40]. *Lactobacillus reuteri* is one of the already well-known species of probiotic bacteria with documented action in many bacterial infections [42-48]. Reuterin, produced by *L. reuteri*, shows a broad spectrum of antibacterial activity, and acts to suppress the production of pro-inflammatory cytokines. Reuterin blocks adherence and prevents the colonization of pathogens [49, 50]. Lactobacilli may therefore be one of the factors regulating and modifying the occurrence and the number of periopathogens.

The term of probiotic is closely related to two other concepts: prebiotic and synbiotic. A prebiotic is a non-digestible food ingredient that selectively stimulates the growth and activity of selected intestinal strains can thus affect a favorable change in the balance of intestinal microflora. Prebiotics are predominantly dietary fibers, particularly soluble, also called "colonic food", consisting of specific carbohydrates. To substances selectively stimulating selected bacterial strains include: inulin, oligofructose, lactulose, fructooligosaccharides (FOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS) [25, 51]. Prebiotics are present in many edible plants such as chicory, onions, leeks, garlic, asparagus, wheat, bananas, oats, soybeans. Many commercial prebiotics are obtained from vegetable raw materials or produced by enzymatic way through the hydrolysis of complex polysaccharides or the trans-glycosylation of mono- or disaccharides [51, 52].

Table 1. Potential effects of probiotics [1, 24-27].

Improvement and stabilization of gut microbiota composition
Improvement of intestinal functions
Aid in lactose digestion
Effects on mineral absorption
They allow the biosynthesis of vitamin K and vitamins of group B
Reduction of risk of intestinal inflammations and infections
Protective effect against acute diarrhea, including travelers diarrhea and post-antibiotic diarrhea
Treatment *Clostridium difficile* diarrhoea
Initiation and regulation/modulation of immune functions
Treatment and prevention of allergies
Treatment of juvenile reumatoid arthritis
Reduction of risk of obesity, type 2 diabetes, metabolic syndrome, etc.
Hypocholesterolaemic and cardioprotective effects
Reduction of risk of colon cancer
Prevention of urogenital infections
Prevention and treatment of gastritis caused by *Helicobacter pylori*

Synbiotic is a food product consisting in the composition of both: the probiotic and prebiotic [25]. In 1995 Gibson and Roberfroid defined synbiotic as "a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare" [12].

Probiotics are considered as safe drugs, but in particular cases *Lactobacillus* spp. that colonize mucous membrane of the mouth and throat can cause bacteremia and severe opportunistic infections [53, 54].

CONCLUSIONS

Probiotics are live, non-pathogenic micro-organisms, which include lactic acid bacteria and the yeast Saccharomyces boulardii. They have health benefits for the host, and their action is multidirectional. Probiotic strains should demonstrate capability of survival, metabolize, adhesion to the intestinal mucosa, transient colonization of the gastrointestinal tract, and antagonistic properties against potentially pathogenic species. Prebiotics have the properties to supporting the probiotics, stimulate the growth and activity of selected probiotic strains.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

REFERENCES

1. FAO/WHO. 2006. Probiotics in food. Health and nutritional properties and guidelines for evaluation. FAO Food and Nutrition Paper 85, pp. 50.
2. ISAPP. 2008. 6th Meeting of the International Scientific Association of Probiotics and Prebiotics, London, Ontario.
3. Gordon S. Elie Metchnikoff: father of natural immunity. Eur J Immunol. 2008; 38(12): 3257-3264.
4. EFSA. Opinion of the Scientific Committee on Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA J. 2007; 587: 8-16.
5. World Gastroenterology Organisation Global Guideline. 2011. Probiotics and prebiotics. October 2011, pp: 28.
6. Trafalska E, Grzybowska K. Probiotics – alternative to antibiotics? [in Polish]. Wiad Lek. 2004; 57(9–10): 491–498.
7. Górska S, Jarząb A, Gamian A. Probiotic bacteria in the human digestive tract as a factor stimulating the immune system [in Polish]. Postepy Hig Med Dosw. 2009; 63: 653-667.
8. Górska-Frączek S, Gamian A. The probiotic bacteria and their effect on immune system cells [in Polish]. Zakażenia. 2013; 1: 12-17.
9. Ross RP, Mills S, Hill C. Specific metabolite production by gut microbiota as a basis for probiotic function. Int Dairy J. 2010; 20: 269-276.
10. Lee JY, Mazmanian SK. Has the microbiota played a critical role in the evolution of the adaptive immune system? Science. 2010; 330: 1768-1773.
11. Schiffrin EJ, Rochat F, Link-Amster H, Aeschlimann JM, Donnet-Hughes A. Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. J Dairy Sci. 1995; 78: 491-497.
12. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr. 1995; 125: 1401-1412.
13. Matsuguchi T, Takagi A, Matsuzaki T, Nagaoka M, Ishikawa K, Yokokura T, Yoshikai Y. Lipoteichoic acids from *Lactobacillus* strains elicit strong tumor necrosis factor alpha-inducing activities in macrophages through toll-like receptor 2. Clin Diagn Labor Immunol. 2003; 10(2): 259-266.
14. Iannitti T, Palmieri B. Therapeutical use of probiotic formulations in clinical practice. Clin Nutr. 2010; 29(6):701-725.
15. Vasiljevic T, Shah NP. Probiotics – from Metchnikoff to bioactives. Int Dairy J. 2008; 18: 714-728.
16. Salazar-Lindo E, Miranda-Langschwager P, Campos-Sanchez M, Chea-Woo E, Sack RB. *Lactobacillus casei* strain GG in the treatment of infants with acute watery diarrhea: a randomized, double-blind, placebo controlled clinical trial. BMC Pediatr. 2004; 4: 18, doi: 10.1186/1471-2431-4-18.
17. Sazawal S, Hiremath G, Dhingra U, Malik P, Deb S, Black RE. Efficacy of probiotics in prevention of acute diarrhoea: a meta-analysis of masked, randomised, placebo-controlled trials. Lancet Infect Dis. 2006; 6: 374-382.
18. Szajewska H, Skórka A, Ruszczyński M, Gieruszczak-Białek D. Meta-analysis: *Lactobacillus* GG for treating acute diarrhoea in children. Aliment Pharmacol Ther. 2007; 25: 871-881.
19. Meijer BJ, Dieleman LA. Probiotics in the treatment of human inflammatory bowel diseases: update 2011. J Clin Gastroenterol. 2011; 45 Suppl: S139-144.
20. Cain AM, Karpa KD. Clinical utility of probiotics in inflammatory bowel disease. Altern Ther Health Med.

2011; 17(1): 72-79.
21. Surawicz CM, Elmer GW, Speelman P, McFarland LV, Chinn J, van Belle G. Prevention of antibiotic-associated diarrhoea by *Saccharomyces boulardii*. Am J Gastroenterol. 1989; 96: 981-988.
22. Kollaritsch HH, Kemnser P, Wiederman G, Scheiner O. Prevention of traveller's diarrhoea; comparison of different non-antibiotic preparations. Travel Med Int. 1989; 7: 9-17.
23. Guslandi M, Mezzi G, Sorghi M. *Saccharomyces boulardii* in maintenance treatment of Crohn's disease. Dig Dis Sci. 2000; 45: 1462-1464.
24. Sanders ME. Probiotics. Food Technol. 1999; 53(11): 67-77.
25. Binns N. 2013. Probiotics, prebiotics and the gut microbiota. International Life Sciences Institute, pp. 32.
26. Danysz A. 2002. Bacteria which treat: probiosis in the prevention and treatment of certain diseases of the gastrointestinal tract [in Polish]. Krotex-Poland, pp. 37.
27. Andrzejewska E, Szkaradkiewicz A. Antagonistic effect of *Lactobacillus acidophilus* against clinical isolates of *Helicobacter pylori* [in Polish]. Med Dosw Microbiol 2007; 59: 59-64.
28. Ryan CS, Kleinberg I. Bacteria in human mouths involved in the production and utilization of hydrogen peroxide. Arch Oral Biol. 1995; 40(8): 753-763.
29. Lin MY, Yen CL. Antioxidative ability of lactic acid bacteria. J Agric Food Chem. 1999; 47: 1460-1466.
30. Klaenhammer TR. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev. 1993; 12: 39-86.
31. Strahinic I, Busarcevic M, Pavlica D, Milasin J, Golic N, Topisirovic L. Molecular and biochemical characterizations of human oral lactobacilli as putative probiotic candidates. Oral Microbiol Immunol. 2007; 22: 111-117.
32. Słońska A, Klimuszko D. Bacteriocins of probiotic rods of the *Lactobacillus* genus [in Polish]. Post Mikrobiol. 2010; 40(2): 87-96.
33. Silva M, Jacobus NV, Deneke C, Gorbach SL. Antimicrobial substance from a human *Lactobacillus* strain. Antimicrob Agents Chemother. 1987; 31(8): 1231-1233.
34. Foligne B, Nutten S, Grangette C, Dennin V, Goudercourt D, Poiret S, et al. Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria. World J Gastroenterol. 2007; 13(2): 236-243.
35. Köll-Klais P, Mandar R, Leibur E, Marcotte H, Hammarstrom L, Mikelsaar M. Oral lactobacilli in chronic periodontitis and periodontal health: species composition and antimicrobial activity. Oral Microbiol Immunol. 2005; 20(6): 354-361.
36. Szkaradkiewicz AK, Stopa J. *Lactobacillus* spp. of oral cavity microflora in chronic periodontitis. Pol J Environ Stud. 2008; 17(6A): 236-242.
37. Szkaradkiewicz AK, Karpiński TM, Zeidler A, Wyganowska-Świątkowska M, Szkaradkiewicz A. Protective effect of oral lactobacilli in pathogenesis of chronic periodontitis. J Physiol Pharmacol. 2011; 62: 685-689.
38. Andrzejewska E, Szkaradkiewicz AK. Antagonistic effect of *Lactobacillus acidophilus* to selected periodontopathogens. XXVII Congress of the Polish Society of Microbiologists. September 5-8, 2012; Lublin, Poland. Scientific Materials.
39. Servin AL. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol Rev. 2004; 28: 405-440.
40. Eijsink VG, Axelsson L, Diep DB, Havarstein LS, Holo H, Nes IF. Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. Antonie van Leeuwenhoek. 2002; 81(1-4): 639-654.
41. Garneau S, Martin NI, Vederas JC. Two-peptide bacteriocins produced by lactic acid bacteria. Biochimie. 2002; 84: 577-592.
42. Cadieux P, Wind A, Sommer P, Schaefer L, Crowley K, Britton RA, Reid G. Evaluation of reuterin production in urogenital probiotic *Lactobacillus reuteri* RC-14. Appl Environ Microbiol. 2008; 74(15): 4645-4649.
43. Efrati C, Nicolini G, Cannaviello C, O'Sed NP, Valabrega S. *Helicobacter pylori* eradication: sequential therapy and *Lactobacillus reuteri* supplementation. World J Gastroenterol. 2012; 18(43): 6250-6254.
44. Francavilla R, Lionetti E, Castellaneta SP, Magista AM, Maurogiovanni G, Bucci N, et al. Inhibition of *Helicobacter pylori* infection in humans by *Lactobacillus reuteri* ATCC 55730 and effect on eradication therapy: a pilot study. Helicobacter. 2008; 13(2): 127-134.
45. Hunter C, Dimaguila MA, Gal P, Wimmer JE Jr, Ransom JL, Carlos RQ, et al. Effect of routine probiotic, *Lactobacillus reuteri* DSM 17938, use on rates of necrotizing enterocolitis in neonates with birthweight < 1000 grams: a sequential analysis. BMC Pediatr. 2012; 12(142): 1-6.
46. Liu Y, Fatheree NY, Mangalat N, Rhoads JM. Human-derived probiotic *Lactobacillus reuteri* strains differentially reduce intestinal inflammation. Am J Physiol Gastrointest Liver Physiol. 2010; 299: G1087-G1096.
47. Prince T, McBain AJ, O'Neill CA. *Lactobacillus reuteri* protects epidermal keratinocytes from *Staphylococcus aureus*-induced cell death by competitive exlusion. Appl Environ Microbiol. 2012; 78(15): 5119-

5126.

48. Szajewska H, Gyrczuk E, Horvath A. *Lactobacillus reuteri* DSM 17938 for the management of infantile colic in breastfed infants: a randomized, double-blind, placebo-controlled trial. J Pediatr. 2013; 162(2): 257-262.
49. Jones SE, Versalovic J. Probiotic *Lactobacillus reuteri* biofilms produce antimicrobial and anti-inflammatory factors. BMC Microbiol. 2009; 9: 35.
50. Ma D, Forsythe P, Bienenstock J. Live *Lactobacillus reuteri* is essential for the inhibitory effect on tumor necrosis factor alpha-induced interleukin-8 expression. Infect Immunol. 2004; 72: 5308-5322.
51 Chow J. Probiotics and prebiotics: A brief overview. J Ren Nutr. 2002; 12(2): 76-86.
52. Kelly G. Inulin-type prebiotics – a review: part 1. Altern Med Rev. 2008; 13(4): 315-329.
53. Muszyński Z, Mirska I, Matuska K. Rods of *Lactobacillus* genus – factor of opportunistic infections in children [in Polish]. Przegl Epidemiol. 2007; 61: 79-84.
54. Kochan P, Chmielarczyk A, Szymaniak L, Brykczynski M, Galant K, Zych A, et al. *Lactobacillus rhamnosus* administration causes sepsis in a cardiosurgical patient – is the time right to revise probiotic safety guidelines? Clin Microbiol Infect. 2011; 17(10): 1589-1592.

ISSN: 2084-3577

TMKARPIŃSKI PUBLISHER

Journal of Biology and Earth Sciences

EARTH SCIENCES ORIGINAL ARTICLE

Wstępna charakterystyka skał żyłowych występujących w Dolinie Małaja Biełaja w Chibinach
Preliminary characterization of the mineral veins occurring in the Malaya Belaya Valley in the Khibiny

Miłosz A. Huber

Department of Geology and Protection of Lithosphere, Maria Curie-Skłodowska University, Lublin, Poland

STRESZCZENIE

Praca dotyczy identyfikacji skał żyłowych z centralnej części Masywu Chibińskiego, będącego intruzją centralną alkalicznych skał, powstałych w późnym dewonie jako produkt wzmożonego magmatyzmu typu kontynentalnego związanego z plama gorąca, która w tym rejonie przyczyniła się do wielu podobnych alkalicznych intruzji. Zidentyfikowano głównie mikrosjenity, tinguaity oraz melteigity. Skały te poddano badaniom petrologicznym (mikroskopia optyczna, XRD). Identyfikacja skał żyłowych jest szczególnie istotna w kontekście określenia procesów związanych z krystalizacja resztkową oraz iniekcji niewielkich porcji magmy które musiały mieć miejsce podczas stygnięcia intruzji budującej obecnie Masyw Chibiński. Szczególnie interesujące są skały melteigitowe powstałe jako produkt mieszania się pierwotnych magmowych utworów z otaczającymi skałami alkalicznymi co uwidacznia się choćby w składzie mineralnym tych skał i korozją minerałów związanych z zasadowym środowiskiem powstawania.

ABSTRACT

The focus of this study is identification of mineral veins from central part of Khibina massive, which are late Devonian alkaline intrusion. Khibina Massive are product of continental magmatism connecting with a hot spot, which are present in this area, making a lot of the same intrusion. It was identificated a microsyenite, tinguaite and melteigite mostly. From these rocks was collected a few of samples, which made a petrology observation (optical microscopy, XRD analysis). These rocks are strongly important in to description a final crystallization and injection of a small addition of magmas, which was present in the Khibina Massive. Most interested are melteigite rocks which are a product of mixing a primary magma with alkaline fluids, which is visible in mineral composition in these rocks and secondary processes.

Słowa kluczowe: Chibiny; Małaja Biełaja; petrografia; skały żyłowe.
Key words: Khibiny; Malaya Bielaya; petrography; mineral veins.

J Biol Earth Sci 2013; 3(1): E1-E11

Corresponding author:
Miłosz A. Huber
Department of Geology and Protection of Lithosphere, Institute of Earth Science, Maria Curie-Skłodowska University, 20-718 Lublin, Al. Kraśnicka 2cd, Poland,
e-mail: mhuber@biotop.umcs.lublin.pl

Original Submission: 29 November 2012; Revised Submission: 12 January 2013; Accepted: 15 January 2013

http://www.journals.tmkarpinski.com/index.php/jbes or http://jbes.strefa.pl
e-mail: jbes@interia.eu

WSTĘP

Masyw Chibiński znajduje się w środkowej części płw. Kola i jest to wczesnopaleozoiczna intruzja skał wysokoalkalicznych [1-3]. W zachodniej części Masywu Chibińskiego znajduje się dolina Małaja Biełaja (Fig. 1).

Jest to dolina o charakterze U-kształtnym z wieloma formami postglacjalnymi (cyrki lodowcowe, moreny itp.), biorąc swój początek w środkowej części masywu chibińskiego na stokach góry Pietierlusa oraz Wschodnia Pietierlusa które stanowią kulminację pasma Razwumczorr (Fig. 2a, b). W dolinie tej odsłaniają się przeważnie masywne alkaliczne sjenity [4-6]. W strefach tektonicznych znajdują się liczne żyły, które ogólnie można zaliczyć do mikrosjenitów, ijolitów i urtytów oraz trachitów i alkalicznych oliwinowych gabr [5, 7-9].

MATERIAŁ I METODY

Skały do badań pobrano w latach 1999-2012 a następnie poddano szeregowi analiz. Wykonano badania makroskopowe in situ oraz mikroskopowe za pomocą mikroskopu optycznego Leica oraz mikroskopu elektronowego Hitachi SU6600 z przystawką EDS, który znajduje się w Pracowni Mikroskopii Optycznej i Elektronowej Zakładu Geologii i Ochrony Litosfery UMCS. Skały te poddano też analizom katodoluminescencyjnym, dzięki uprzejmości dr hab. A. Gałuszki w Zakładzie Geochemii i Ochrony środowiska Uniwersytetu Jana Kochanowskiego w Kielcach.

Wykaz prób znajduje się w tabeli 1. W tym rejonie odsłaniają się masywne sjenity alkaliczne o barwie zielonkawej, strukturze granonematokrystalicznej, teksturze zbitej, bezładnej, rzadziej kierunkowej (Fig. 3a). Tło skały (Fig. 3b,c) budują autromorficzne kryształy nefelinu (do 25% obj.), ortoklazu (do 24% obj.) i apatytu (do 15% obj.), pomiędzy którymi znajdują się igły egirynu - akmitu (do 21% obj.), oraz ksenomorficzne kryształy eudialitu (często z licznymi wrostkami tych ostatnich –do 15% obj.), plagioklazów (kwaśne odmiany do 10% obj.). W skałach tych podrzędnie występują też w ilości do 15% obj: ilmenit, tytanit, magnetyt, arfvedsonit, riebeckit, astrofyllit, aenigmatyt, lorenzenit i inne.

Masywne sjenity – chibinity tną liczne żyły mineralne. Analizowane próbki to głównie żyłowe sjenity, ale występują też melanosjenity przechodzące do ijolitu i urtytu, nefelinitu (zwłaszcza w strefie ciał rudnych, gdzie tworzą skupienia tytanitowo–nefelinowe). Żyły te posiadają skomplikowaną budowę wskazując na wypełnienia tektonicznych uskoków i rozłamów, które były wielokrotnie uruchamiana i na nowo wypełniana

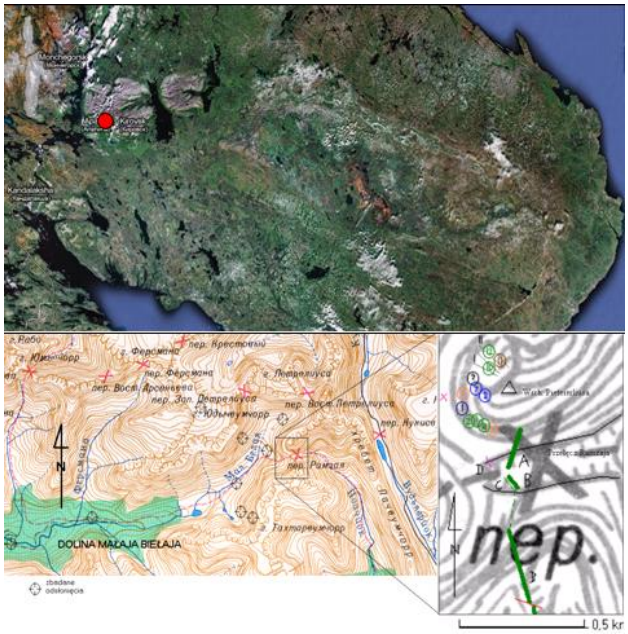


Fig. 1. Plan sytuacyjny terenu badań wraz z zaznaczeniem miejsca poboru prób.

Fig. 2a. Dolina Małaja Biełaja.

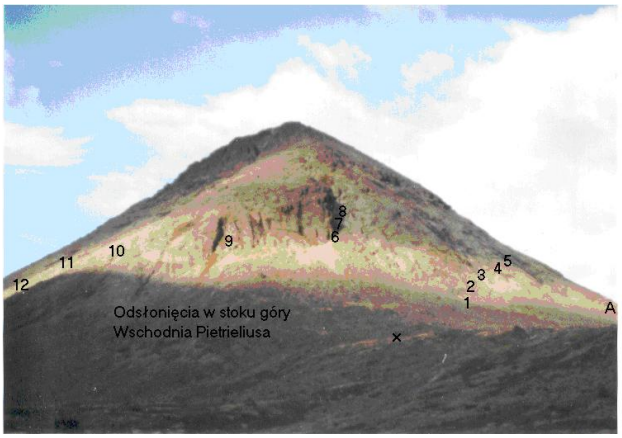


Fig. 2b. Góra Wostoczna Pietierlusa.

Tabela 1. Wykaz splanimetrowanych próbek skał (skały żyłowe zaznaczono kursywą).

Lokalizacja	Nr próby	Skład określony planimetrycznie [%obj.]					Nazwa skały
		Ne	K-sk	Pl	Ciemne	Inne	
Dol. Małaja Biełaja	20CH99	0	29	63	8	0	Alkaliczny sjenit
	21CH99	10	49	27	4	10(ap)	*Foidowy trachit*
	22CH99	21	0	0	79	0	*Foifdowy piroksenit*
	30CH99	9	28	10	52	0	*Melteigit*
	17CH99	0	26	16	58	0	*Melteigit*
Cyrk Lodowcowy w Dol. Małaja Biełaja	15CH99	0	10	23	67	0	Sjenit
	13CH99	0	8	53	32	4(ap)4(zr)	Alkaliczny melasjenit
	28CH99	27	23	0	41	7(ap)2(zr)	Foidowy sjneit (Chibinit)
	29CH99	5	6	4	67	18(ap)	Foidowy sjenit
	23CH99	10	26	16	43	5(ap)	*Tefrytowy fonolit*
	16CH99	0	45	0	55	0	Sjnit z tlenkami Fe
Odsłonięcia w zboczu góry Zachodnia Pietierlusa	07CH02	25	24	5	30	15(ap)	Foidowy sjenit (Chibinit)
	08aCH02	3	7	4	69	7(ol)10(ap)	*Ne-Ol alk. klinopiroksenit*
	10CH02	20	30	0	35	7(ap)8(zr)	*Fiodowy sjenit (Chibinit)*
	11CH02-II wyp.	10	12	0	71	8(ap)	*Ijolit*
	11CH02-I wyp.	7	41	9	39	4(ap)	*Fiodowy sjenit (Chibinit)*
	13CH02	24	10	0	37	17(ap)12(zr)	*Fiodowy sjenit (Chibinit)*
	14CH02	17	14	0	58	2(ap)9(zr)	*Ijolit*
	15CH02	2	55	0	34	9(ap)	*Fiodowy mikrosjenit*
	16CH02-II wyp.	13	16	0	71	0	*Fiodowy sjenit (Chibinit)*
	16CH02-I wyp.	39	0	0	55	7(ap)	*Ijolit*
	17CH02	3	25	0	72	0	*Fiodowy sjenit (Chibinit)*
	18CH02	15	15	9	55	6(ap)	*Melteigit*
	19CH02-I wyp.	13	57	0	27	4(ap)	*Fiodowy sjenit (Chibinit)*
	19CH02-II wyp.	6	18	0	52	24(ap)	*Fiodowy sjenit (Chibinit)*
	20CH02	1	38	0	55	6(ap)	*Fiodowy sjenit (Chibinit)*
	21CH02	1	12	0	84	4(ap)	*Ijolit*
	22CH02-I wyp.	3	20	3	28	48(zr)	*Fiodowy sjenit (Chibinit)*
	22CH02-II wyp.	0	91	0	5	5(ap)	*Fiodowy sjenit (Chibinit)*
	23CH02	5	22	1	72	0	*Mikrosjneit -tinguait*
	24CH02	11	20	0	45	11(ap)13(zr)	*Fiodowy sjenit (Chibinit)*

Fig. 3a. Makrofotografia typowych sjenitów – chibinitów.

Fig. 3b. Jeden z przykładów pegmatytu w Chibinach.

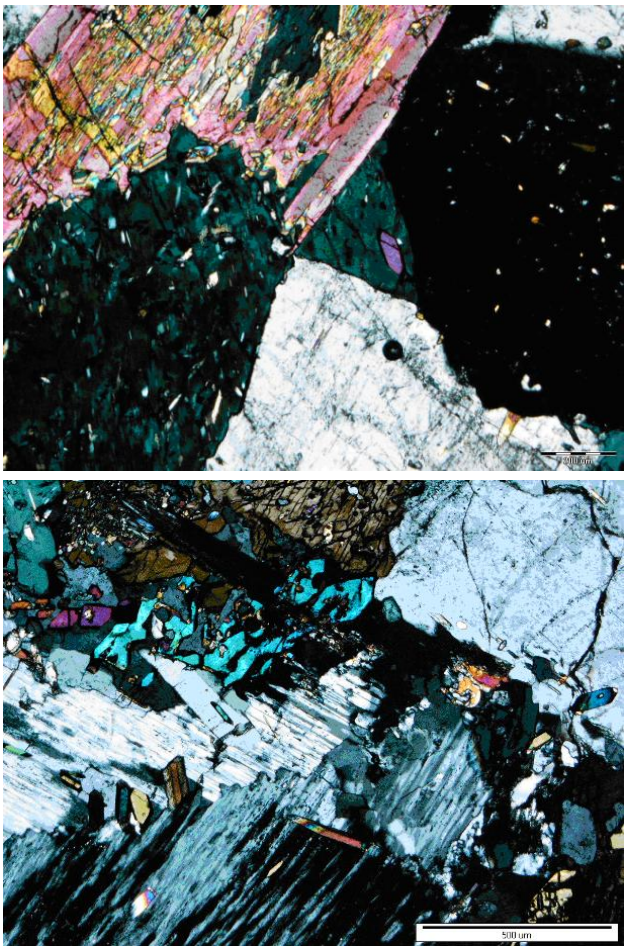


Fig. 3c. Mikrofotografie (w świetle przechodzącym spolaryzowanym, nikole skrzyżowane) typowych sjenitów z terenu badań.

nową substancja mineralną, rzadziej brekcja starszych skał z otoczenia (Fig. 4).

WYNIKI

Utwory zostały zbadane za pomocą mikroskopu polaryzacyjnego w świetle przechodzącym i odbitym. Analiza tych skał pozwoliła wydzielić kilka odmian petrologicznych żył (Tabela 1):

- żyłowe sjenity i mikrosjenity (próbki 20CH99, 15CH99, 13CH99, 28CH99, 29CH99, 16CH99, 07CH02, 10CH02, 11CH02a, 13CH02, 15CH02, 16CH02b, 17CH02, 19CH02a, 20CH02, 22CH02, 24CH02, 01aCH03),
- ijolity (11CH02b, 14CH02, 16CH02a, 21CH02),
- tefrytowe fonolity (23CH99) i tinguaity (21CH99, 23CH02) oraz
- melteigity (30CH99, 17CH99, 08aCH02, 18CH02) i piroksenity (22CH99).

Żyłowe sjenity i ijolity

Sjenity żyłowe stanowią najliczniejszą grupę skał. Tworzą zarówno żyły o charakterze ostrych kontaktów jak i (częściej) takie, które posiadają nieostre granice. Posiadają zwykle charakter częściowo skonsolidowany ze skałą otoczenia, są odkształcone plastycznie, wykazują budinaż. Zwykle są to mikrosjenity (ijolity) o podwyższonym udziale minerałów melanokratycznych (do 70% obj.), bogate są w minerały rudne, głównie z grupy tlenków żelaza i tytanu z domieszkami siarczków (w tym siarczków miedzi). Takie bardziej melanokratyczne odmiany sjenitów często spotykane są w powtórnych wypełnieniach żył. Mikrosjenity najczęściej posiadają struktury grano-nematokrystaliczne. Tekstury masywne, choć zdarzają się ilinijne, podkreślone przez kryształy mikroklinu a także zonalnych eudialitów i egirynów (Fig. 5a). Tło skały stanowią tabliczkowe kryształy ortoklazy (Fig. 5b, c), rzadziej mikroklinu (do 55% obj.), oraz egirynu (do 53% obj.) i arfvedsonitu (do

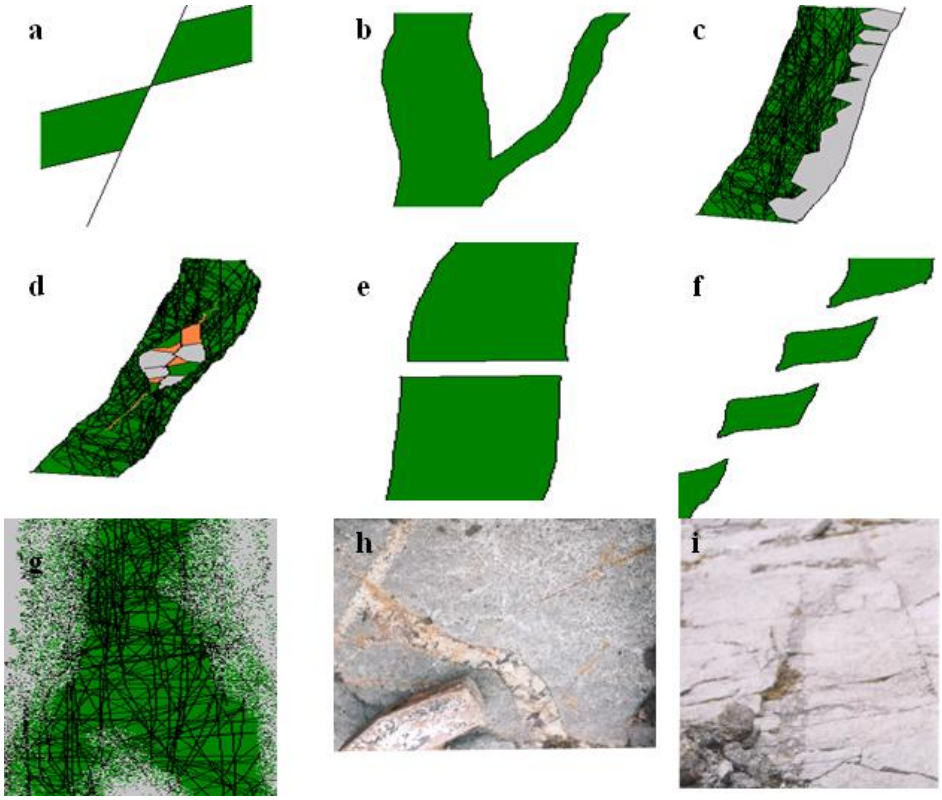


Fig. 4. Typy żył spotykane w terenia badań: a) o ostrym kontakcie zuskokowane, b) z odnogami, c) kilkuetapowe, d) z gniazdami pegmatytowymi, e) złuskowane, f) z budinażem, g) o nieostrych granicach, h) poprzecinane –różnowiekowe z propagacją jonów w strefie otoczenia (najczęściej K+), i) żyły wykorzystujące starszy system spękań.

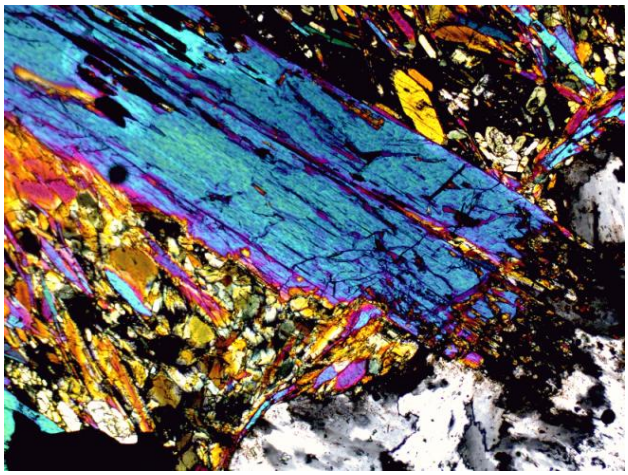
24% obj.), pomiędzy którymi znajdują się apatyt (do 24% obj., Fig. 6a), nefelin (do 39% obj, Fig. 5d), eudialit (do 48% obj.), plagioklazy kwaśne (do 9% obj.), minerały rudne. Ortoklaz i plagioklazy wykazują zbliźniaczenia typu albitowego. Augity wykazują zastępowania, które często obejmują minerały z grupy augitu, i augitu egirynowego postępując ku egirynizacji wraz z krystalizacją augitu. W niektórych próbkach występuje uralityzacja augitu. Obok egirynu licznie występuje riebeckit (Fig. 5e), arfvedsonit, aenigmatyt (Fig. 5f, g). Eudialit wykazuje często zonalną budowę charakteryzującą się strefami anomalnego ściemniania światła (Fig. 5h). Kryształy te na ogół są wykształcone euhedralnie tworząc pojedyncze osobniki lub zrosty.

W skale widoczne są liczne kryształy robaczkowo wykształconego ilmenitu. Są to pospinelowe struktury rozpadowe (Fig. 5i, j). Magnetyt ulega procesom wtórnym przechodząc w maghemit który wykazuje ślady wietrzenia objawiające się występowaniem i hematytu.

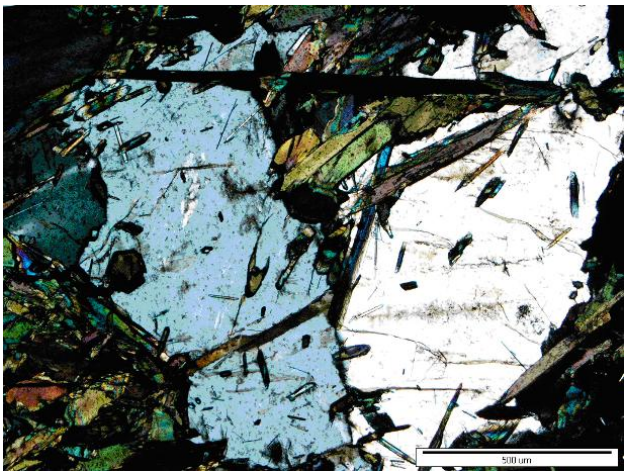
Trachity i tinguaity

Trachity to skały barwy szarej lub szaroróżowej z fenokryształami skalenia potasowego (Fig. 6b). Posiadają one strukturę porfirową, teksturę zbitą, bezładną. Fenokryształy tworzy ortoklaz (49% obj.) dochodzący do 1 cm, zwykle zbliźniaczony zgodnie z prawem albitowym. Obok skalenia pojawiają się osobniki augitu o charakterze prakryształów z widoczną strukturą sitową, wypełnioną przez minerały tła (4%). Tło skały tworzą drobne kryształy ortoklazu, współwystępujące z egirynem, akmitem, niekiedy augitem (Fig. 6b). Żyły tego typu rzadziej występują w centralnej części Chibin, częściej będąc spotykane w niższych partiach doliny Małaja Biełaja.

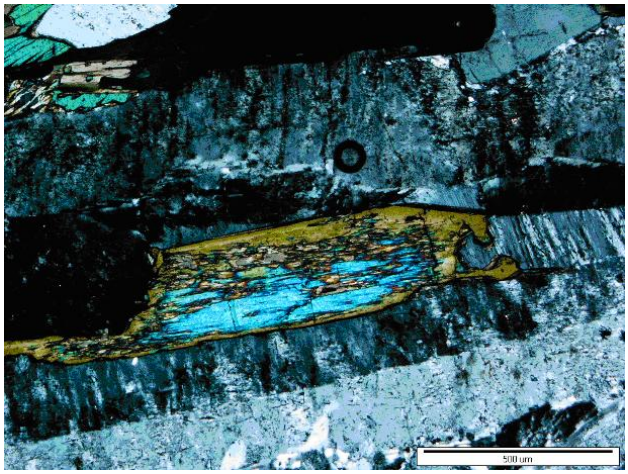
Tinguaity charakteryzują się barwą zieloną strukturą nematoblastyczną, teksturą spilśniona, promienistą, zbitą (Fig. 7f). Tło skały stanowią promieniste skupienia egirynu i astrofyllitu (do 45% obj.). W interstycjach tych minerałów występują ortoklaz (22%), nefelin (5%) oraz tytanit (do 3% obj). W skale licznie występują tez magnetyt i ilmenit (do 4 % obj.).Wykształcone są w postaci wąskich lamelek towarzyszących egirynom. Utwory te spotyka się najczęściej w centralnej części Chibin.



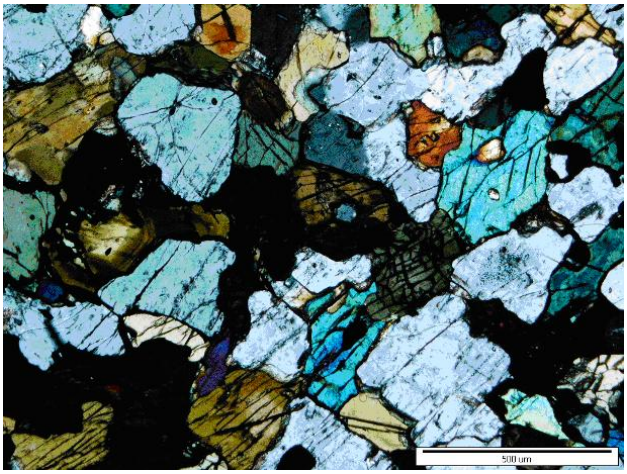
5a. Obraz w świetle spolaryzowanym (PL) przedstawiający zonalnego egirynu-augitu (próbka 01aCH03).



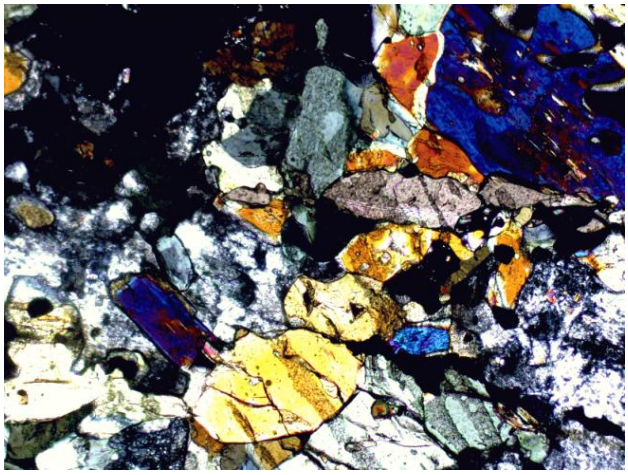
5b. Egiryn na tle ortoklazu (PL, próbka 14CH02).



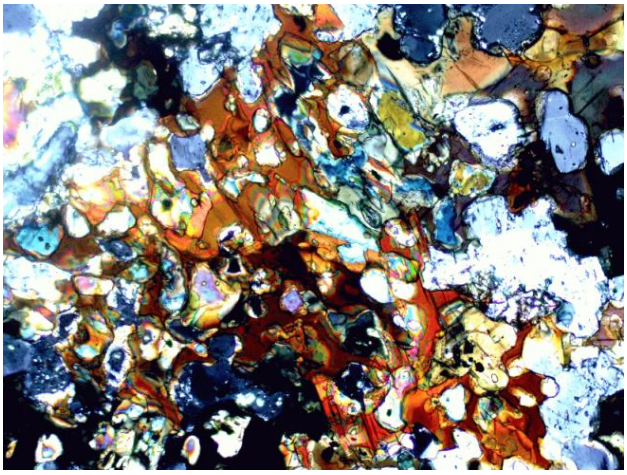
5c. Egiryn-augit na tle linijnej struktury podkreślonej przez zbliźniaczone ortoklazy (PL, próbka 19CH02).



5d. Kryształy egirynu oraz riebeckitu na tle plagioklazów oraz nefelinu (PL, próbka 17CH02).

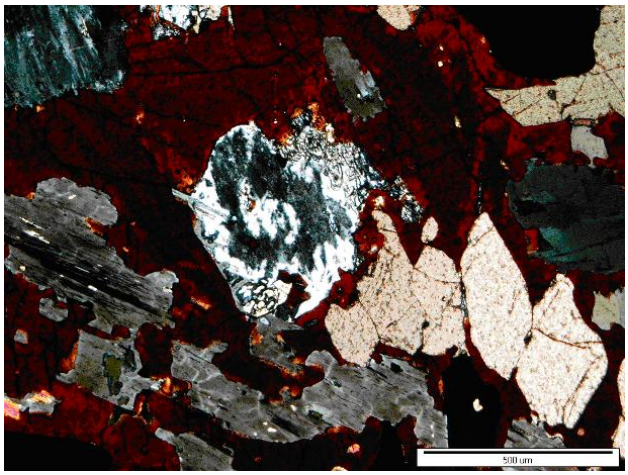


5e. Riebeckiry, tytanity oraz enigmatyty na tle apatytu w żyłowym sjenicie (PL, 15Ch03).

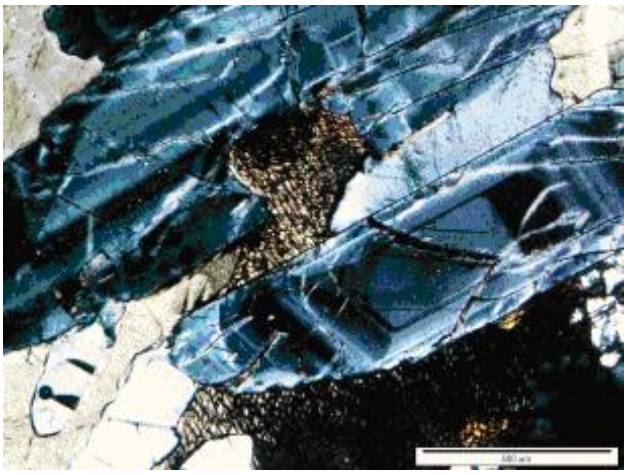


5f. Enigmatyt oraz arfvedsonit na tle jasnych apatytów w typowym żyłowym sjenicie (PL, 23CH03).

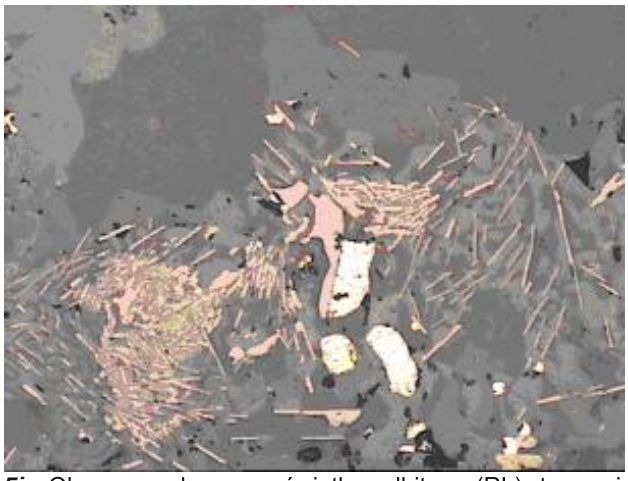
Fig. 5. Mikrofotografie typowych sjenitów i ijolitów, uzyskane za pomocą mikroskopu polaryzacyjnego.



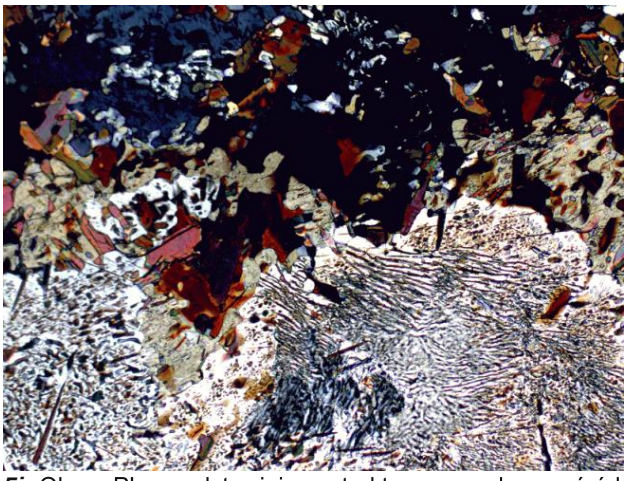
5g. Mikrofotografia enigmatytu oraz tytanitu (PL, próbka 24CH02).



5h. Zonalne eudiality oraz astrofyllit (PL, próbka 19CH02).



5i. Obraz uzyskany w świetle odbitym (RL) typowej struktury rozpadowej, pospinelowej z ilmenitem oraz siarczkiem (pirytem) w tle (próbka 11CH02).



5j. Obraz PL przedstawiający struktury rozpadowe wśród ulegających korozji piroksenów i wydzielających się tlenków żelaza i tytanu (próbka 02CH03).

Fig. 5. cd. - Mikrofotografie typowych sjenitów i ijolitów, uzyskane za pomocą mikroskopu polaryzacyjnego.

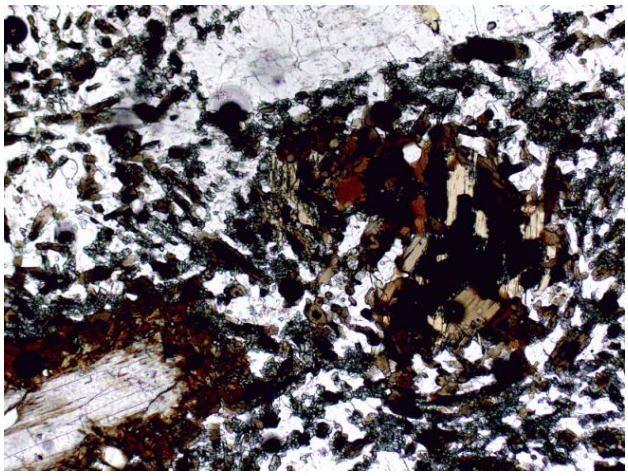
Żyłowe pegmatyty i pegmatyty gniazdowe

Pegmatyty gniazdowe (dochodzące do 0,5 m) występują w postaci soczew w masywnych sjenitach. Zbudowane są głównie z tabliczek nefelinu, plagioklazów i apatytu (Fig. 3b, 6d). Pomiędzy nimi znajdują się minerały ciemne takie jak egiryn, rzadziej astrofyllit. Kryształy te są wykształcone w postaci palisadowej, tworząc druzowate wypełnienie. W interstycjach egirynu i plagioklazu znajdują się eudialit i ilmenit jako minerały anhedralne (ksenomorficzne).

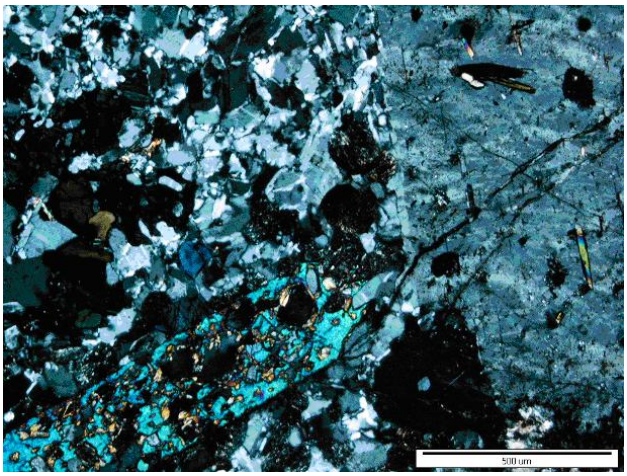
Pegmatyty żyłowe zwykle wykształcone są w postaci wielkokrystalicznych odmian sjenitu z dużymi, dochodzącymi do kilkunastu cm kryształami ortoklazu lub mikroklinu, eudialitu i egirynu. W skałach tych często dochodzi do wyraźnego dominowania jednego z minerałów nad innymi. Zwykle dominują minerały alkaliczne (skalenie – mikroklin lub ortoklaz, rzadziej apatyt, nefelin, egiryn). Zdarzają się odmiany o złożonej budowie zbudowane z palisadowych minerałów wchodzących w skład sjenitów (eudiality, nefeliny i egiryny) pierwszej generacji na której krystalizuje wielkokrystaliczny mikroklin drugiej generacji (Fig. 8b).

Melteigity i piroksenity

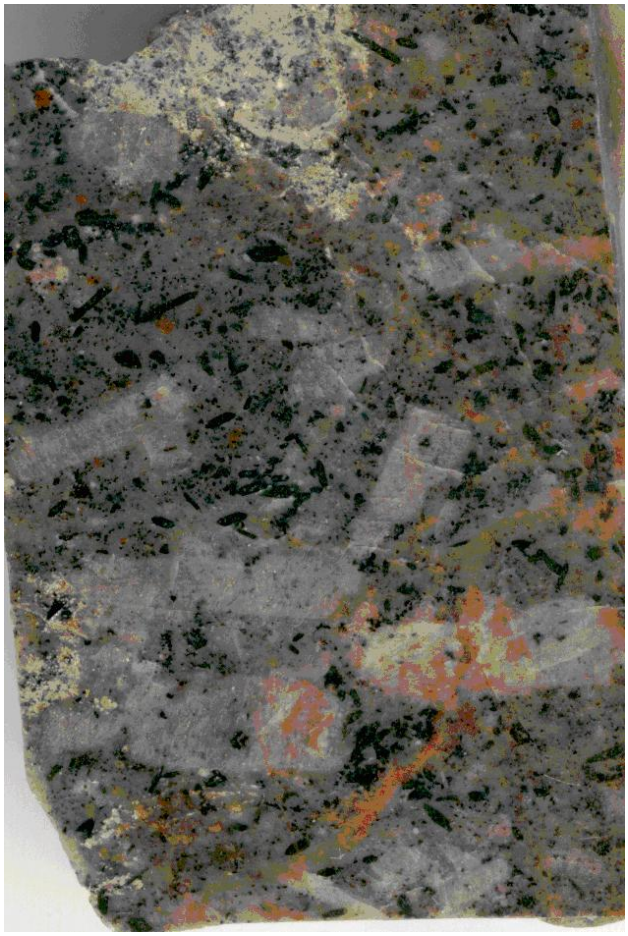
Melteigity to żylowe skały ostrym kontakcie ze skałami otoczenia. Są to utwory o strukturach grano-lepidonematokrystalicznych, teksturach masywnych, poikilitowych, porfirowatych. Minerały tworzące fenokrysztay to augit (do 24% obj.), i



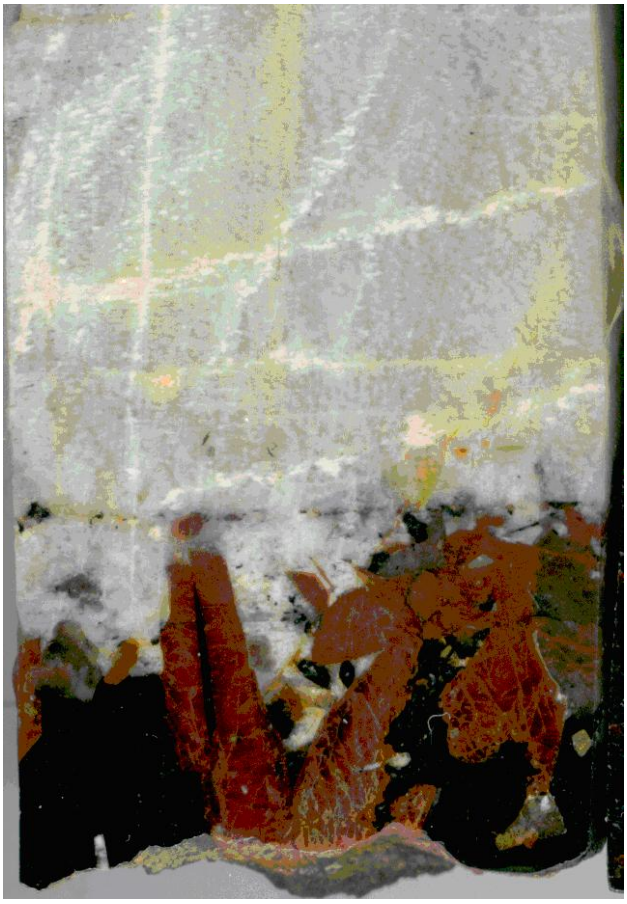
6a. Pirokseny z obwódkami reakcyjnymi oraz tlenki żelaza na tle masy skaleniowej w trachicie (PL, 30CH99).



6b. Dwie generacje skaleni w trachicie (PL, 21CH99).

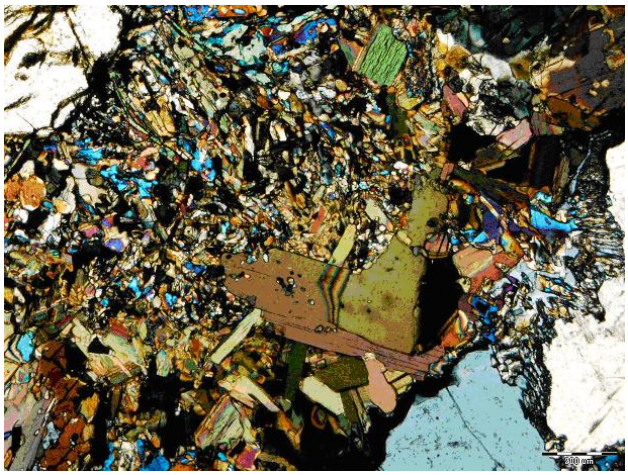


6c. Makrofotografia typowego trachitu (21CH99).

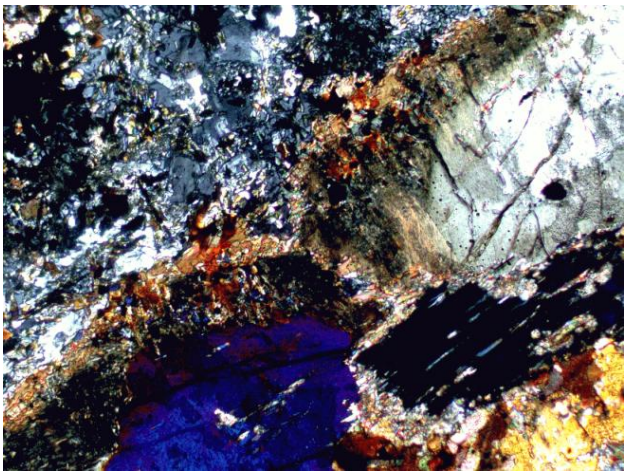


6d. Makrofotografia typowego pegmatytu.

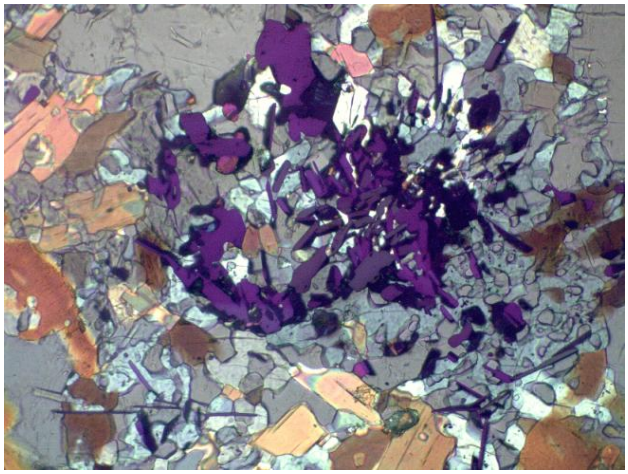
Fig. 6. Mikrofotografie typowych trachitów oraz pegmatytu.



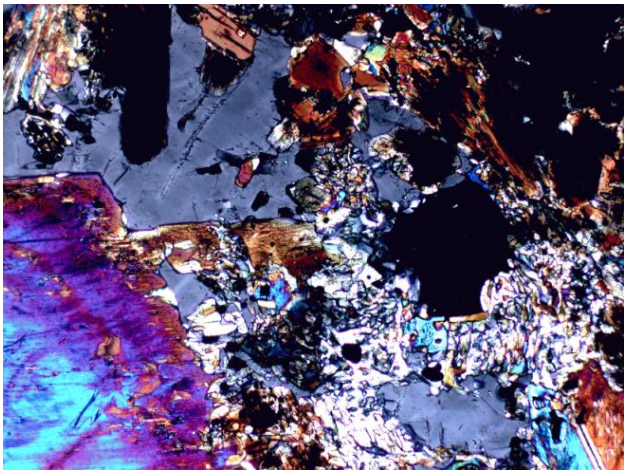
7a. Typowa korona reakcyjna na około oliwinu (PL, 08CH02).



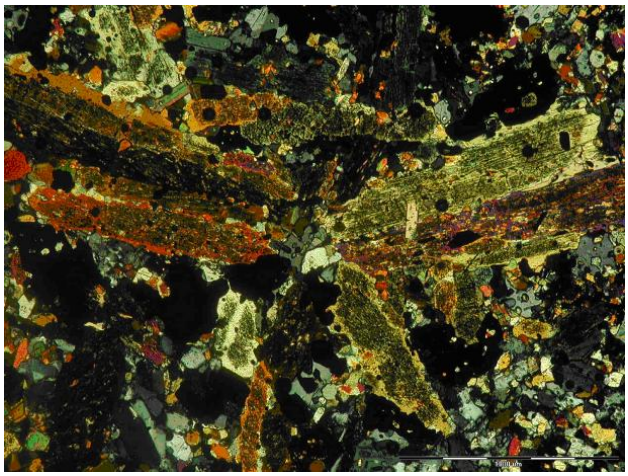
7b. Typowe korony reakcyjne na tle piroksenów w melteigicie (PL, 17Ch03).



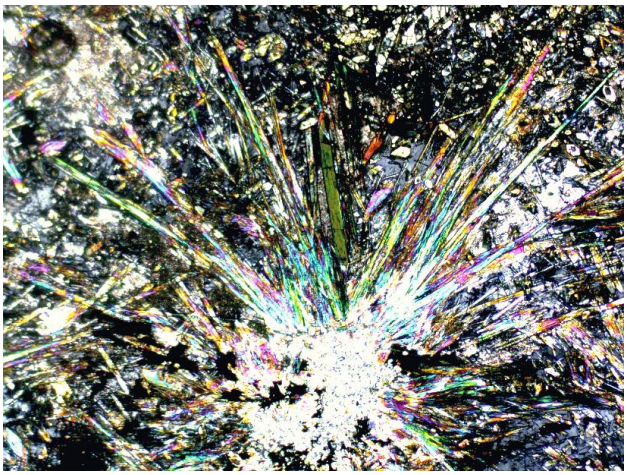
7c. Obraz w świetle odbitym (RL) przedstawiający ilmenity na tle piroksenów i wtórnego flogopitu (próbka 03CH03).



7d. Zonalny egiryn, apatyt oraz arfvedsonit i ilmenity w melteigicie (PL, próbka 09aCH03).



7e. Typowe promieniste skupienie augitu w piroksenicie (PL, 22CH99).



7f. Egirynowe słońca w tinguaicie (PL, 20CH03).

Fig. 7. Mikrofotografie typowych melteigitów i tinguaitu.

migdały zbudowane zwykle z oliwinu z obwódką korozyjną – łyszczykową (do 7% obj., Fig. 7a). Obwódka tak składa się z wysokożelazistego biotytu i flogopitu i otacza niekiey tę fenokryształy augitu (Fig. 7b, c). W tle skały występuje apatyt (do 10% obj., Fig. 7d), nefelin (do 15% obj.), oraz ortoklaz (do 15% obj.) i plagioklazy (do 9% obj.). Rzadziej spotyka się skały wykształcone w postaci piroksenitów z występującymi w nich tzw. piroksenowymi (augitowymi) słońcami (do 79% obj., Fig. 7e). W melteigitach pirokseny charakteryzują się niekiedy strukturami promienistymi np. słońca augitowe. Częstym zjawiskiem jest przejście augitu w augit egirynowy i egiryn. W skałach tych dostrzeżono robaczkowaty ilmenit który stanowi rozpadową struktuę pospinelową. W skałach tych dostrzeżono także chalkopiryt a także heikukit i kubanit. W innych przypadkach dostrzezono także strefy rozpadowe pirytu i chalkopirytu do tiosiarczanów. Żyły melteigitowe częściej występują w centralnych strefach Chibin, podczas gdy piroksenitowe w peryferyjnych.

WNIOSKI

Opisane powyżej skały żyłowe z Chibin wyraźnie związane są z kilkuetapowym procesem wnikania materiału magmowego w już skonsolidowane skały Chibin. Najprawdopodobniej wynikało to z samego faktu krystalizacji plutonu który przyczynił się do powstania masywu Chibińskiego, gdzie po skrystalizowaniu właściwych sjenitów doszło do migracji tzw. roztworów resztkowych, niekiedy o charakterze niedopasowanym o czym świadczą znajdowane w tych skałach liczne rzadkie minerały. W trakcie krystalizacji skał żyłowych dochodziło do zmian fizykochemicznych, które przyczyniły się do tonalności augitu, eudialitu i innych minerałów.

Odmiennym typem skał są melteigity, które wyraźnie posiadają skład minerałów różniący się od otoczenia. Prawdopodobnie powstały w wyniku iniekcji drobnych porcji magmy o charakterze bardziej pierwotnym, zasadowym lub wiążą się z dyferencjacją ogniska magmowego i późniejszą wędrówką ku górze resztkowych derywatów o charakterze zasadowym, gdzie stawały się one metastabilne, pochłaniając po drodze dużą ilość materiału alkalicznego (Fig. 7) [10]. Świadczą o tym minerały cumulusu które następnie w wyniku migracji wzdłuż tektonicznych nieciągłości uległy korozji kosztem powstania nowej generacji która obecnie stanowi większość tła skalnego. Pegmatyty gniazdowe z egirynem, apatytem, eudialitem i nefelinem stanowią produkt krystalizacji resztkowej związanej z nierównomiernym stygnięciem skał budujących masyw Chibiński. Obok tego rodzaju skał spotyka się też liczne ksenolity o różnym charakterze, typowe dla intruzji magmowej.

PODZIĘKOWANIA

Za pomoc w badaniach oraz udostępnienie sprzętu serdecznie dziękuję Pani dr hab. A. Gałuszce z Zakładu Geochemii i Ochrony Środowiska Uniwersytetu Jana Kochanowskiego w Kielcach.

Niniejsze badania zrealizowano dzięki grantowi Uniwersytetu im. Marii Curii-Skłodowskiej w Lublinie, dla „Młodych Naukowców" nr: MPK 12-1000-D11.

DEKLARACJA

Autor deklaruje nie występowanie konfliktu interesów.

PIŚMIENNICTWO

1. Boruckiy BE. 1989. Minerały skałotwórcze wysoko-alkalicznych kompleksów. [In Russian], wyd. Nauka, pp. 214.
2. Mitrofanov AF. 2000. Geological characteristics of Kola peninsula. Russian Academy of Science, Apatity, pp. 166.
3. Pozhylienko WI. et al. 2002. Geologia rudnych rejonów Murmańskiego okręgu. [In Russian], Apatity , pp. 360.
4. Arzamastsev A.A. 1994. Unique Paleozoic Intrusions of the Kola Peninsula. Apatity: Kola Scientific Centre: 79.
5. Kogarko LN, Kononova VA, Orlova MP, Woolley AR. 1995. Alkaline Rocks and Carbonatites of the World. Part 2: Former USSR. London: Chapman & Hall; p. 226.
6. Kramm U, Kogarko LN, Kononova VA, Vartiainen H. The Kola alkaline province of the CIS and Finland: precise Rb–Sr ages define 380–360 Ma age range for all magmatism. Lithos. 1993; 30: 33-44.
7. Kukharenko AA, Orlova MP, Bulakh AG, Bagdasarov EA, Rimskaya-Korsakova OM, Nefedov EI, et al. 1965. The Caledonian Complex of Ultrabasic Alkaline Rocks and Carbonatites of the Kola Peninsula and North Karelia [In Russian]. Moscow: Nedra; p. 772.
8. Huber M, Hałas S, Sikorska M. Evolution of prehnite-

albite-calcite veins in metamorphic rocks from the Lapland Granulite Belt (Kandalaksha region of Kola Peninsula). Geologija. 2007; 57: 1-7.

9. Hałas S, Huber M, Piestrzynski A. Petrology of gabbroides and isotope signature of sulfide mineralization from Fedorov-Pansky layered mafic intrusion, Kola Peninsula, Russia. Geochronometria. 2009; 33: 19-22.
10. Arzamastsev A, Yakovenchuk V, Pakhomovsky Y, Ivanyuk G. 2008. The Khibina and Lovozero alkaline massifs: Geology and unique mineralization. 33 IGC excursion materials.