ISSN 2449-8955
5(3) 2015

Volume 5
Number 3
September-December 2015

European Journal of Biological Research

formerly
Journal of Biology and Earth Sciences

MNiSW points 2015: **11**
Index Copernicus 2014: **96.49**

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European Journal of Biological Research

ISSN 2449-8955

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ISSN 2449-8955

European Journal of Biological Research

Research Article

Is the soil K/Na ratio the first defense line against salinity?

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Received: 02 June 2015; **Revised submission:** 25 July 2015; **Accepted:** 28 July 2015

ABSTRACT

The current work carried out to observe the preliminary responses at the first hours of salt stress and to investigate the ability of K^+ and Ca^{2+} as compatible inorganic solutes to overcome the Na^+ injury. Seedlings of broad bean (*Vicia faba*, cv. Giza 40) were translocated to water culture containing 100, 200, 300, 400 and 500 mM NaCl. The flasks divided to two main groups. The first under salinity only and the second group was treated with 5% KCl and 5% $CaCl_2$. Plants were harvested after 6 hours and 24 hour of stress. After 6 hours, the first shock of salt stress, the stressed plants lose their control upon the salt absorption or exclusion. The plants increased their content of sodium. Then sodium concentrations decreased greatly after 24 hours especially in roots. Leaves tended to accumulate K^+ more than roots. The K/Na ratio increased gradually in all the treated plants. The availability of K^+ in the medium increased the value on K/Na ratio in the different organs of the plants treated with K^+ and Ca^{2+} and decreased the accumulated inorganic solutes in the different organs of stressed plants.

Keywords: K/Na ratio, Osmotic adjustment, Salinity, Salt shock, Salt stress, *Vicia faba*.

1. INTRODUCTION

One of the greatest problems in agriculture and crop production is the soil salinity and water deficit. An attempt to meet world food demands accompanied with decline in availability in fresh water has resulted in using water of poor quality for crop irrigation. Considering the need for increasing the crop yield, as well as the decline of good quality irrigation water, crop salt tolerance assessment can be a useful tool [1].

Faba bean is one of the major cool season grain legume crops produced worldwide. Legumes are either sensitive or moderately tolerant to salinity but variability in salinity tolerance among legumes has also been reported. *Vicia faba* (L.) is moderately sensitive to salinity, registering 50% growth reduction at 6.7 dS m^{-1} salinity.

Several researchers have studied the effects of salinity and drought stresses on crop production [2, 3] and the responses of plants to overcome these environmental stresses [4-7].

Salt stress has three fold effects: it reduces water potential, causes ion imbalance or disturbance in ion homeostasis and toxicity [8]. Since salt stress involves both osmotic and ionic stress [9-12]. Salt stress affects all the major processes such as growth, photosynthesis, protein synthesis, energy and lipid metabolism [13, 14].

There are two main mechanisms to overcome the drought and salinity stresses. The first is the quickly response through the re-osmotic adjustment depending upon inorganic solutes [15-19]. The solute particle number is the main reason which causes stress. The second way to overcome stress is depended mainly upon organic solutes [4]. The later way need long time for compound synthesis and transformations to compatible solutes as proline, glycinebetaine, etc.

Accumulation of ions in the leaves under salt stress causes a rapid reduction in net photosynthesis and growth. Excess of Na^+ and Cl^- creates ionic imbalances that may impair the selectivity of root membranes and induce K^+ deficiency. Membranes are vulnerable targets of stress-induced cellular damage and the extent of membrane damage is commonly used as a measure of tolerance to various stresses in plants [20].

Changes in water relations of plants that are stressed by salinity, can be seen in certain studies confirm that, many plants undergo osmotic regulation when they are exposed to salt stress by increasing the negativity of the osmotic potential of the leaf sap [21-25].

The understanding of the first responses under salt stresses may be helpful to put a strategy, which can help stressed plants; to overcome the stress resulted from the irrigation with saline waters. The current work, as one of a series of investigations are carrying to manufacture an osmo-regulator fertilizer, carried out to observe the preliminary responses at the first hours of salt stress. Investigate the ability of K^+ and Ca^{2+} as compatible inorganic solutes to avoid the accumulation of Na^+ and consequently decreasing the injury results from high sodicity.

2. MATERIALS AND METHODS

Seeds of broad bean (*Vicia faba*, cv. Giza 40) were germinated in plastic pots containing sawdust. The seedlings were irrigated with tap water for two weeks. Then the plants were translocated to water culture in conical flasks containing 100, 200, 300, 400 and 500 mM NaCl, five seedlings in each flask. The flasks divided to two main groups. The first contained the previous concentrations of NaCl. Each conical flask of the second group was treated with 5 ml of 5% KCl and 5 ml of 5% $CaCl_2$. The main two groups were subdivided to four groups, with three replicates for each concentration. One group of NaCl treated plants and another of $NaCl_{K+Ca}$ treated plants were harvested after 6 hours of stress. The different organs of the plants were separated and dried in oven at 75 ºC for 24 hours. The other two groups were harvested after 24 hours. The dry roots, stems and leaves samples were grounded into fine powder and the soluble solutes extracted in distilled water according to the method described by El-Sharkawi and Michel [26]. Chloride was determined volumetrically according to Jackson [27]. Sodium and potassium was measured by flame photometry according to Williams and Twine [28]. Calcium and magnesium was determined volumetrically by the versene method as described by Johnson and Ulrich [29]. The ionic fractions; K/Na, Ca/Na were also computed. The data was analyzed in one way ANOVA to determine the least significant difference (LSD) using statistical package for social sciences version 20 (SPSS).

3. RESULTS

After 6 hours of NaCl stress at 100, 200, 300, 400 and 500 mM the plants increased their content of sodium. The accumulation of sodium was higher in roots especially at 100 mM where reached up to 55 mg g^{-1}. There was no gradient inside plants parallel with the external gradient. The sodium concentration decreased greatly after 24 hours especially in roots. At 100 mM NaCl stress the sodium concentration in roots declined from 55 to 6 mg g^{-1}. The availability of K^+ and Ca^{2+} in the medium affected the sodium uptake. The sodium concentration decreased markedly after 6 hours of stress. The lowest Na^+ concentration was recognized after 24 hours in the medium supported by K^+ and Ca^{2+} (Figure 1). Na^+ accumulated in the roots more than the other organs.

As shown in Figure 2 the shoots especially leaves tended to accumulate K^+ more than roots. With time, the plants tend to accumulate more potassium in their tissues to overcome the sodium injury. Therefore, the potassium content was the highest after 24 hours. The availability of K^+ in the external medium decreased the accumulation of potassium inside plants. The lowest K^+ concentration was estimated in the stressed plants, which

treated with Ca^{2+} and K^{+}, after 24 hours. The higher concentration of sodium was estimated in the roots while the potassium in the leaves. Under salt stress, the plants decreased their content of Na^{+} from the range 15-55 mg at the first 6 hours to 5-21 mg after 24 hour.

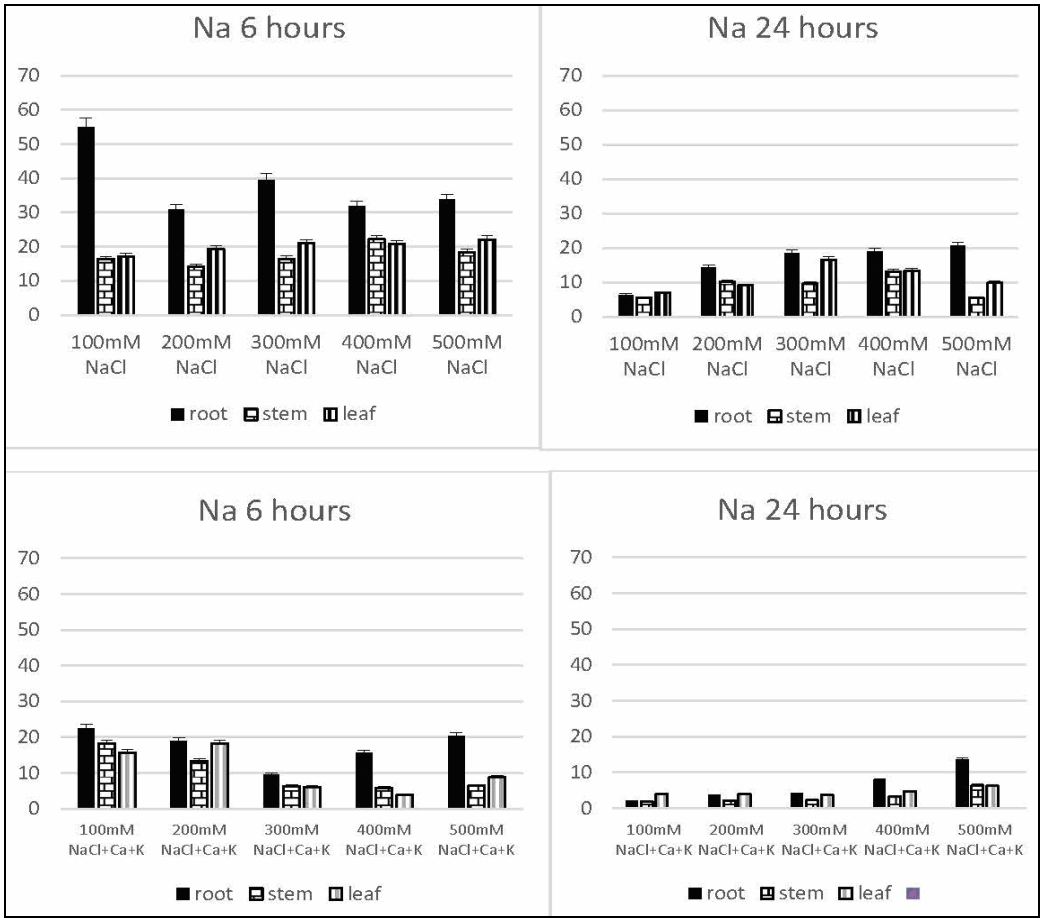

Fig. 1. Sodium concentration mg g^{-1} dry wt inside the different organs of *Vicia faba* plants under different levels of salt stresses and treatments.

The K/Na ratio decreased gradually from the roots to the leaves in all the treated plants (Table 1). It is also noticed that this ratio decreased gradually with salt stress increasing. The availability of K^{+} in the medium increased the value on K/Na ratio in the different organs of the plants treated with K^{+} and Ca^{2+}. After 24 hours, the K/Na ratio increased greatly due to the preference of plants to accumulate potassium or avoid the sodium absorption. It increased between three folds and ten folds compared with the values computed after six hours. The lowest K/Na ratio (0.02) was computed in the plants survived less than 500 mM NaCl stress at the first 6 hours. With time the plants decreased the sodium uptake and increased the absorption of potassium. Therefore, the K/Na ratio increased from 0.02 to 0.08 in the leaves.

At the first 6 hours of salt stress the plants accumulated amounts of calcium and magnesium. The non-treated plants accumulated calcium more than the treated plants with K^{+} and Ca^{2+} in their media (Figure 3). The accumulated Ca^{2+} decreased strongly after 24 hour of salt stress and the plants tended to maintain a constant content of calcium and magnesium.

At the first six hours the plants accumulated more magnesium but the amount of Mg^{2+} decreased with time. Magnesium accumulated more than calcium after 24 hour. There was a tendency of the plants to distribute Mg^{2+} through the plant organs evenly (Figure 4).

Chlorides played the main role as anionic osmoregulator through the gradient of the salt stress. As shown in Figure 5, the roots quickly tended to

accumulate chloride ions to overcome the external salt stress. After 24 hour, the Cl^- transferred to shoots. The non-treated stressed plants accumulated more chlorides than those treated with potassium and calcium. The increase of chloride was paralleled with the external salt stress. The roots had usually the highest concentration of chlorides compared with stems and leaves.

Table 1. The computed K/Na ratio inside the different organs of *Vicia faba* plants under different levels of salt stresses and treatments.

Treatment	K/Na					
	6 hours			24 hours		
	Root	Stem	Leaf	Root	Stem	Leaf
control	0.036	0.09	0.08	0.08	0.11	0.047
100mM NaCl	0.16**	0.14**	0.04**	0.58**	0.46**	0.31**
200mM NaCl	0.20**	0.17**	0.05**	0.37	0.41**	0.13**
300mM NaCl	0.20**	0.20**	0.03**	0.36	0.36**	0.09**
400mM NaCl	0.19**	0.16**	0.04**	0.39	0.31**	0.09**
500mM NaCl	0.13**	0.12	0.02**	0.43*	0.39**	0.08**
100mM NaCl+Ca+K	0.16	0.11	0.09**	0.79**	1.00**	0.52**
200mM NaCl+Ca+K	0.14	0.08	0.07**	0.62**	0.83**	0.34**
300mM NaCl+Ca+K	0.53**	0.49**	0.18**	0.87**	0.98**	0.24**
400mM NaCl+Ca+K	0.62	0.48**	0.14**	0.56	0.69**	0.11**
500mM NaCl+Ca+K	0.56	0.42**	0.10**	0.31*	0.30**	0.07**

**the mean difference is significant at 0.01 level.
*the mean difference is significant at 0.05 level.

Table 2. The computed Ca/Na ratio inside the different organs of *Vicia faba* plants under different levels of salt stresses and treatments.

Treatment	Ca/Na					
	6 hours			24 hours		
	Root	Stem	Leaf	Root	Stem	Leaf
Control	0,067	0.086	0.14	0.15	0.18	0.153
100mM NaCl	0.80*	1.30**	0.20**	0.89**	1.29**	1.07**
200mM NaCl	1.01**	1.21**	0.63**	0.48**	0.55	0.33**
300mM NaCl	0.91**	1.05**	0.49**	0.32	0.52	0.24**
400mM NaCl	0.68**	0.79**	0.25**	0.19	0.38	0.16
500mM NaCl	0.35*	0.36**	0.20**	0.44	0.91**	0.24**.
100mM NaCl+Ca+K	0.80**	0.72**	0.42**	1.82**	2.94**	2.10**
200mM NaCl+Ca+K	0.67**	0.93**	0.36**	1.12**	2.07**	1.69**
300mM NaCl+Ca+K	1.33**	1.34**	0.96**	2.45**	4.89**	1.45**
400mM NaCl+Ca+K	2.33**	1.22**	0.50**	1.24**	1.76**	0.44**
500mM NaCl+Ca+K	0.86**	0.72**	0.23**	0.89**	0.73*	0.42**

**the mean difference is significant at 0.01 level.
*the mean difference is significant at 0.05 level.

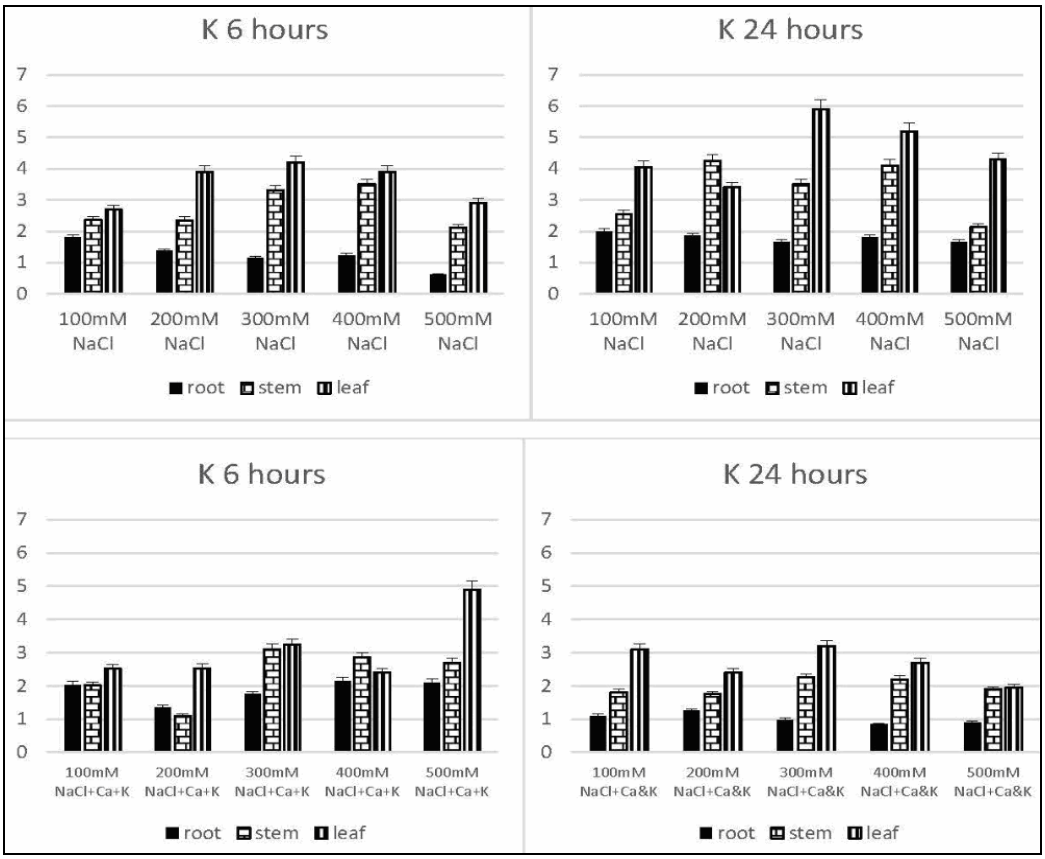

Fig. 2. Potassium concentration mg g^{-1} dry wt inside the different organs of *Vicia faba* plants under different levels of salt stresses and treatments.

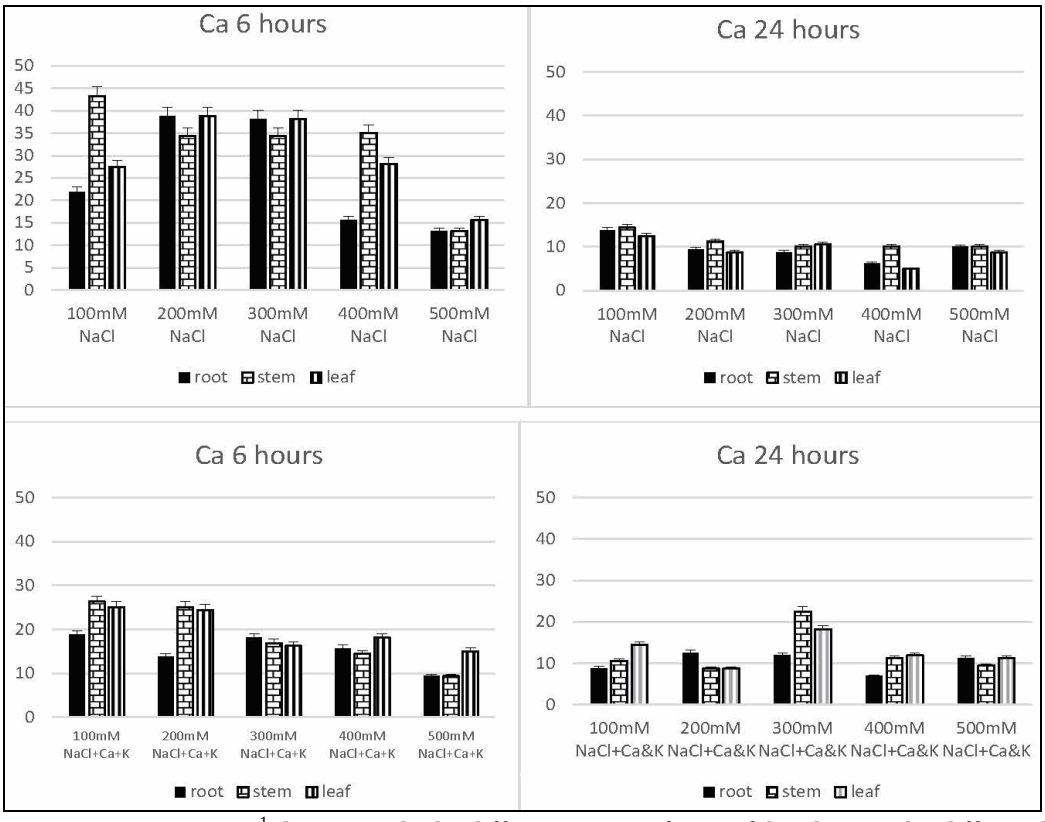

Fig. 3. Calcium concentration mg g^{-1} dry wt inside the different organs of *Vicia faba* plants under different levels of salt stresses and treatments.

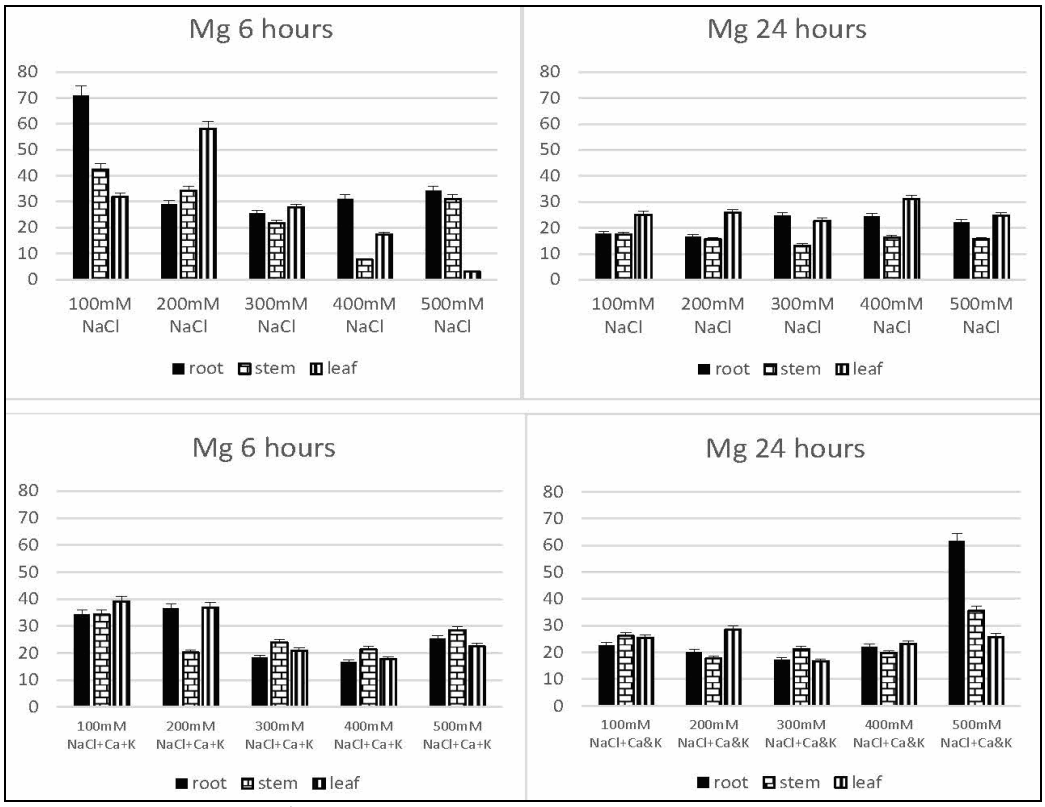

Fig. 4. Magnesium concentration mg g^{-1} dry wt inside the different organs of *Vicia faba* plants under different levels of salt stresses and treatments.

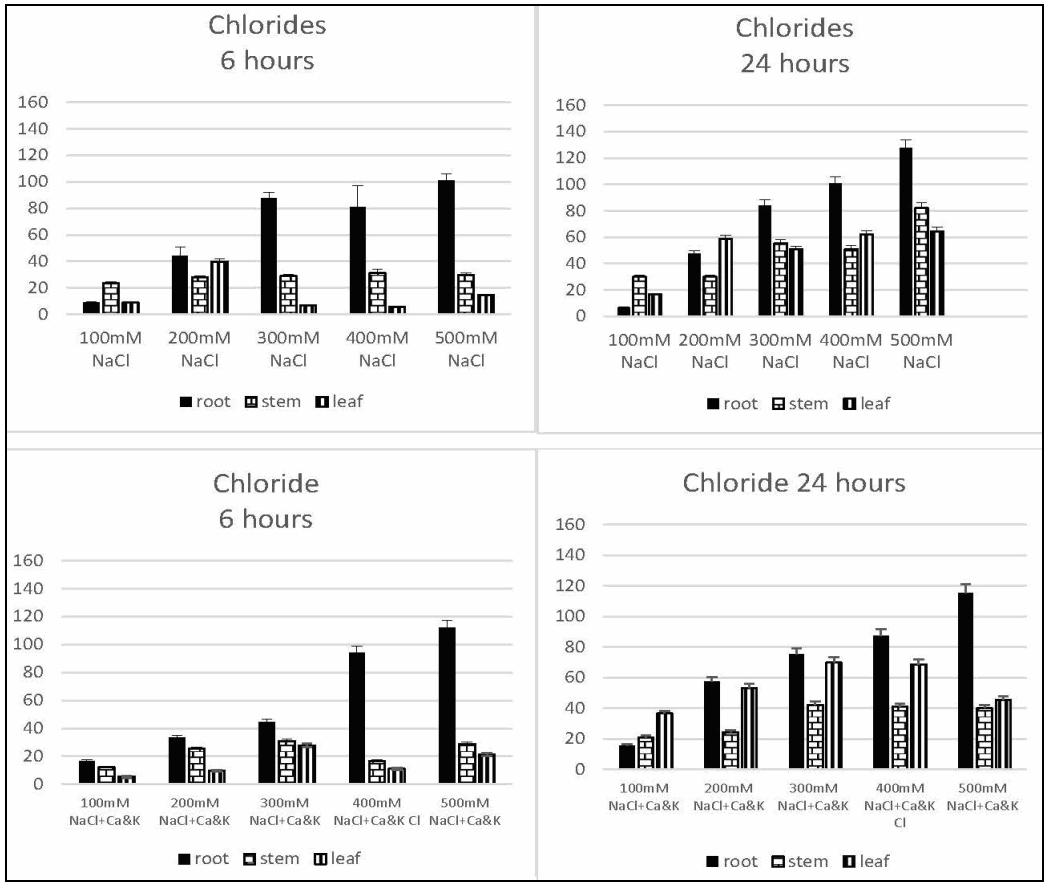

Fig. 5. Chloride concentration mg g^{-1} dry wt inside the different organs of *Vicia faba* plants under different levels of salt stresses and treatments.

The plants accumulated more calcium in the stems to alleviate the sodium toxicity. Therefore, the stems had the highest Ca/Na ratio between the different organs. The availability of calcium in the external medium enhanced the absorption of Ca^{2+} and increased the concentration of calcium ions inside the treated stressed plants (Table 2).

4. DISCUSSION

4.1. The response of plants against external salt stress after six hours

The first response of faba bean to what is so called salt shock was the higher accumulation of sodium at the first 6 hours especially in roots [30]. This salt shock loses the plants their control on solutes absorption. Therefore, the plants accumulated more solutes at the first six hours [31]. The plants tried to accumulate the divalent cations, Ca^{2+} and Mg^{2+} as compatible solutes to avoid the high accumulation of sodium ions [32]. Although the high accumulated amounts of Ca^{2+} and Mg^{2+} at the low concentration of Na^{+}, Ca^{2+} and Mg^{2+} concentrations decreased at higher salt stresses. On the other hand, K^{+} increased gradually with the increase of external stress. The stressed plants increased the Ca^{2+}/ Na^{+} ratio in the stems to avoid sodium injury, while the K^{+}/Na^{+} ratio increased in the leaves [33]. Cl^{-} as a main anion increased in the roots gradually with the increased of external stress [34] and [35]. The addition of calcium and potassium to the external medium decreased the accumulation on inorganic solutes inside different organs.

4.2. The response of plants against external salt stress after 24 hours

With the time, the stressed plants rearranged their defenses against external salt stress. The plants tended to exclude sodium ions. Except K^{+}, both Na^{+} and Ca^{+2} and Mg^{+2} decreased considerably after 24 hours of stress. This reflects the tendency of plants to exclude the high accumulated ions resulted from the first shock of salt stress [36]. The stressed plants started their defense against unfavorable external conditions and preferred to accumulate K^{+} which enhance the protein synthesis. The plants with time depended on organic solutes. The preference of potassium absorption increased the K/Na ratio after 24 hours of stress. The high K/Na ratio is considered as good indicator for salt tolerance [37]. Ca/Na ratio tended to increase in root but decreased in stem and leaves. It is clear that Ca ameliorates the sodium harm effect but K is helpful for biological synthesis [32]. The chloride transported to the the stems and leaves.

4.3. The role of potassium and calcium against external stress

The treatment with 5% K^{+} and 5% Ca^{2+} decreased the absorption of Na^{+} by roots and consequently the accumulation of Na inside plant organs [38]. At the beginning of salt stress, the addition of potassium and calcium decreased the concentration of Na^{+} inside the different organs. this means that the increase in the external K/Na ratio decreased the absorption of Na^{+} [39]. This effect increased with time, therefore the lowest concentration of Na^{+} was estimated after 24 hour in the plants treated with K^{+} and Ca^{+2}. The tendency of plants to accumulate K^{+} under salt stress only was lower when compared with the decrease of Na^{+} concentration inside plants under the effect K^{+} and Ca^{+2} treatment. This explains that the increase of the external K/Na ratio decreases the rate of Na^{+} more than the internal osmotic adjustment and before the Na^{+} exclusion by roots. In the same time, the little increase of potassium enhanced the biological synthesis inside plants. This clear why the plants tended to accumulate K^{+} in the leaves [40]. After the salt shock the plants tended to fix their concentration of the bivalent cations (Ca^{+2} and Mg^{+2}) whether the external medium treated with potassium and calcium or not. The main anion Cl^{-} accumulated regularly paralleled with the increase of salinity especially in the roots.

The greatest salt stress is the most harmful. Therefore, the treatment with K^{+} and Ca^{+2} enhanced the increase of K/Na ratio inside plants at stress above 300 mM NaCl at the first six hours [41]. This encouraged the synthesis of organic solutes quicker than at the lower stresses [42]. After 24 hour, the stressed plants at lower stresses (100 and 200 mM NaCl) increased their K/Na ratio (Table 1). The quick response at higher stresses was necessary to overcome the sodium injury, but the plants at lower

stresses undergo the stress to some time. Therefore, they increased their K/Na ratio after 24 hour of stress. The availability of Ca^{+2} in the external medium was helpful for the increase of Ca/Na ratio inside plant tissues. It is clear that calcium has an ameliorative effect against Na injury. Therefore, the plants increased the Ca/Na ratio after 24 hour in the presence of calcium in the external medium sometimes up to 4.89 (Table 2).

It can be concluded that the first shock of salt stress loses the stressed plant their control upon the salt absorption or exclusion. After 24 hours, the plants rearranged their defenses against salt stress and decreased the ionic concentration inside different plant tissues. The addition of Ca^{+2} and K^{+} improved the salt tolerance and decreased the rate of ionic accumulation considerably at the first moment of salt stress. The external K/Na ratio affected the rate of inorganic solutes absorption.

AUTHORS' CONTRIBUTION

MK: designed the research plan and organized the study, recorded the experimental data, wrote the introduction, results and discussion, revised the manuscript. SH: participated in all the experiments, analyzed the data statistically, wrote the references coordinated the figures and tables. The final manuscript has been read and approved by the authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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ISSN 2449-8955

European Journal of Biological Research

Research Article

Effect of phytocercaricide on certain enzyme activity in parasitic cercaria larva of *Fasciola gigantica*

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Received: 20 May 2015; **Revised submission:** 27 July 2015; **Accepted:** 03 August 2015

ABSTRACT

Fasciolosis is one of the common public health problems worldwide, and cause a great economic loss to us. The life cycle of the *Fasciola* can be interrupted by killing the vector snail or *Fasciola* larva redia/cercaria inside the snail body. Binary combinations of different plant derived cercaricidal were more effective against the cercaria larva of *Fasciola gigantica*. Treatment with binary combination 20% and 60% of 4h LC_{50} of citral (Ci), ferulic acid (Fe), umbelliferone (Um), azadirachtin (Az) and allicin (Al) caused no significant change in the activity of acetylcholinesterase (AChE) in the nervous tissues of exposed *L. acuminata*. Significant inhibition in the AChE activity was noted in whole cercaria larva. AChE activity in the nervous tissues of snail exposed to 60% of 4h LC_{50} of Al+Um was 94.50% of control. AChE and cytochrome oxidase activity was measured to reduced 0.89% and 2.37% of control in whole cercaria larva exposed to 60% of 4h LC_{50} of Az+Al.

Keywords: Binary combination of cercaricides; Cercaria larva; Acetylcholinesterase; Cytochrome oxidase.

1. INTRODUCTION

Fasciolosis is one of the most significant zoonotic diseases caused by *Fasciola gigantica* and *F. hepatica* [1]. *Fasciola* is a digenetic trematode including two hosts; a mammalian host and a molluscan intermediate host [2]. Life cycle of *Fasciola* includes fresh water snails of family Lymnaeidae as an intermediate host [3]. Human infestation of fasciolosis is characterized by fever, eosinophilia, abdominal pain and, exceptionally, acute pancreatitis [4, 5]. In northern India snail *Lymnaea acuminata* is the intermediate host of the *Fasciola* species [6-8]. Human fasciolosis in India has been reported in state of Assam, Bihar, Maharastra, Uttar Pradesh, Arunchal Pradesh and West Bengal [9-13]. The massive use of chemical drugs to control parasite has led to the alarming development of parasite resistance in all livestock species [14]. Natural products are eco-friendly and easily biodegradables less hazed to aquatic environment. Control of snail population below a threshold level

is one of the important methods for effective control of fasciolosis [15, 16]. Use of plant derived larvicides drugs are currently used as the most effective tool for the treatment of fasciolosis. The *Zingiber officinale* (citral), *Ferula asafoetida* (ferulic acid, umbelliferone), *Azadirachta indica* (azadirachtin) and *Allium sativum* (allicin) are the potent larvicides [17]. Sunita et al. [7] has reported that plant products citral, ferulic acid, umbelliferone azadirachtin and allicin are potent larvicide against *Fasciola* larvae. The mechanism of action of their active components causing larval death is not exactly known. The aim of the present study is to evaluate the effect of sub-lethal treatment of different binary combination of citral, ferulic acid, umbelliferone, azadirachtin and allicin on acetylcholinesterase (AChE) and cytochrome oxidase activity in the intermediate host snail *L. acuminata* and cercaria larva of *F. gigantica*.

2. MATERIALS AND METHODS

2.1. Active larvicidal components

Citral, ferulic acid, umbelliferone, azadirachtin were purchased from Sigma chemical Co. (U.S.A). Allicin was prepared by the method of Mohammad and Woodward [18] as modified by Singh and Singh [19].

2.2. Binary Combination of larvicidal component

Binary combinations (1:1 ratio) of citral (Ci), ferulic acid (Fe), umbelliferone (Um), azadirachtin (Az) and allicin (Al) that is Ci+Fe, Ci+Um, Ci+Az, Ci+Al, Fe+Um, Fe+Az, Fe+Al, Az+Um, Az+Al and Al+Um were used for the treatment of snails.

2.3. Animals

2.3.1. In vivo treatment

Adult *L. acuminata* (2.6 ± 0.20 cm in length) were collected locally. Cercarias shedding infected and uninfected snails were separated in two groups. The snails were allowed to acclimatize in laboratory condition for 24h and then treated with sublethal concentration, 20% and 60% 4h LC_{50} of binary combination of active larvicides; Ci+Fe, Ci+Um, Ci+Az, Ci+Al, Fe+Um, Fe+Az, Fe+Al, Az+Um, Az+Al and Al+Um. Selections of these concentrations were based on earlier observation of Sunita et al. [7]. Six Batches were set up for each concentration. Control group (for each batch) contained only dechlorinated tap water without any treatment. The treated animals were removed from the aquaria after 4h and rinsed with water.

2.3.2. AChE activity

Infected snails were exposed to 20% and 60% of 4h LC_{50} of binary combinations (1:1 ratio) of active components. Snails were washed with fresh water and dissected out. Cercaria larva as well as brain of the infected snails was taken for the estimation of acetylcholinesterase (AChE) and cytochrome oxidase. The brain of snails were removed separately and placed on ice cubes, afterwards it was placed on filter paper to remove the adherent water and weight. AChE activity was estimated in treated as well as control group of animals by the method of Ellman et al. [20] as modified by Singh et al. [21]. Snail nervous tissue was homogenized in 50 mg/mL of 0.1M phosphate buffer pH 8.0 for 5 min in an ice bath and centrifuged at 10,00 × g for 30 min at 4^0C. The supernatant was used as an enzyme source 0.05 mL of the supernatant was pipette to a 1.0 mm path length cuvette. To this added 0.1 mL (5×10^{-4}M) of freshly prepared acetyl thiocholine iodide chromomeric agent DTNB reagent 1.45 mL of 0.05 M phosphate buffer pH 8.0 was used. The change in optical density 412 nm was monitored for 3 min at 25^0C. Enzyme activity has been expressed µ mole ʻSH' hydrolysed/ min/ mg protein.

AChE activity in the nervous tissue of withdrawn after 4h snail from treatments were measured in withdrawal snails after 4h exposed of 60% of 4hLC_{50} of binary combination for next 6h fresh water.

2.3.3. Fractionation of cercaria tissue

Cercaria (1×10^6) was cool, concentrated by centrifugation at 4^0C for 2 min at 800 × g. Then 1.0 mL 0.1M phosphate buffer pH 8.0 containing a cocktail of protease inhibitors (10 mM EGTA, 40 µg/mL leupeptin, 20 µg/mL pepstatin,1 mg/mL bacitracin, 20 units/mL aprotinin and 1 mM benza-

midine was added (10 mL), followed by sonication at 50% duty cycle/output control 4 in a bath Ultrasonicssonicator, (Plainview, NY, U.S.A). Sonication was performed for 3×3 min at 4^0C. Each homogenate was used as enzyme source for AChE activity estimation.

2.3.4. Cytochrome oxidase

Activity of cytochrome oxides was measured according to Cooperstein and Lazarow [22] as modified by Singh and Agarwal [23]. 1×10^6 cercaria larva of *Fasciola gigantica* were homogenized in 1.0 mL of 1/30 mol.1 phosphate buffer, pH 7.4 for 5 min (10,000 × g) for 30 min at 4^0C supernatants were used an enzyme source. Enzyme activity at 25^0C was measured in a reduced cytochrome solution (1.7×10^{-5} mol/L) were taken into cuvette and 0.2 ml of cercaria larva homogenate was added. The reactants were mixed by inverting the cuvette several times and absorption at 550 nm was monitored for 3 min. A few grains of potassium ferricyanide were added (to oxidase cytochrome c completely) and the extinction was redetermined. Enzyme activity has been expressed as the average enzyme per min and per mg protein.

2.4. Statistical Analysis

Each result was six time replicated estimation (measurement in six different pool of nervous tissue). The values were expressed as mean ± SE.

Table 1. Inhibition of AChE activity (µmole SH hydrolyzed/mg protein/min) in the nervous tissue of *L. acuminata* exposed to 20% and 60% of 4h LC_{50} of active cercaricides.

Treatment	4h LC_{50} of cercaria		Withdrawal 60% 4h LC_{50}
	Acetylcholinesterase (µmole ´SH´ hydrolyzed/min/mg/protein)		
	20%	60%	
Control	0.91 ± 0.003 (100)		0.91 ± 0.001$^+$ (100)
Ci+Fe	0.90 ± 0.004 (98.91)	0.89 ± 0.003 (97.80)	0.90 ± 0.002$^+$ (98.91)
Ci+Um	0.91 ± 0.001 (100.00)	0.89 ± 0.004 (97.80)	0.90 ± 0.002$^+$ (98.91)
Ci+Az	0.89 ± 0.002 (97.80)	0.87 ± 0.003 (95.60)	0.89 ± 0.001$^+$ (97.80)
Ci+Al	0.88 ± 0.005 (96.70)	0.86 ± 0.002 (94.50)	0.87 ± 0.004$^+$ (95.60)
Fe+Um	0.85 ± 0.002 (95.70)	0.86 ± 0.001 (94.50)	0.88 ± 0.002$^+$ (96.70)
Fe+Az	0.86 ± 0.003 (97.80)	0.86 ± 0.002 (94.50)	0.87 ± 0.003$^+$ (95.60)
Fe+Al	0.89 ± 0.001 (97.80)	0.88 ± 0.003 (96.70)	0.79 ± 0.003$^+$ (86.81)
Az+Um	0.87 ± 0.001 (95.60)	0.86 ± 0.002 (94.50)	0.88 ± 0.001$^+$ (96.70)
Az+Al	0.89 ± 0.003 (97.80)	0.86 ± 0.002 (94.50)	0.88 ± 0.002$^+$ (96.70)
Al+Um	0.87 ± 0.001 (95.60)	0.85 ± 0.002 (93.40)	0.87 ± 0.001$^+$ (95.60)

Each value is mean ± SE of six replicates.

Value is parentheses are per cent change with control taken as 100%.

Concentration (w/v) has been expressed as final concentration in aquarium water.

Significant ($p<0.05$) when‘t’ test was applied in between treated and control group and (+) in between 60% of 4h LC_{50} and withdrawal group.

Table 2. Inhibition of AChE activity (µmole SH hydrolyzed/mg protein/min) and Cytochrome (enzyme activity/min/mg protein) oxidase in the cercaria larva of *F. gigantica* exposed to 20% and 60% of 4h LC_{50} of active cercaricides.

Treatment	4h LC_{50} of cercaria larva			
	Acetylcholinesterase (µmole ´SH´ hydrolyzed/min/mg/protein)		Cytochrome oxidase (enzyme activity/min/mg protein)	
	20%	60%	20%	60%
Control	1.12±0.001 (100)		14.72±0.002 (100)	
Ci+Fe	0.038±0.003* (3.39)	0.036±0.003* (3.21)	0.58±0.002* (3.94)	0.56±0.005* (380)
Ci+Um	0.039±0.001* (3.30)	0.034±0.002* (3.03)	0.56±0.001* (3.80)	0.53±0.006* (3.60)
Ci+Az	0.031±0.002* (2.76)	0.029±0.001* (2.58)	0.54±0.001* (3.66)	0.52±0.003* (3.53)
Ci+Al	0.020±0.004* (1.78)	0.018±0.002* (1.60)	0.57±0.003* (3.87)	0.54±0.001* (3.66)
Fe+Um	0.022±0.003* (1.96)	0.017±0.004* (1.51)	0.52±0.003* (3.53)	0.50±0.004* (3.39)
Fe+Az	0.023±0.004* (2.5)	0.020±0.002* (1.78)	0.51 ±0.002* (3.46)	0.48±0.003* (3.26)
Fe+Al	0.013±0.001* (1.16)	0.011±0.003* (0.98)	0.40±0.004* (2.71)	0.38±0.002* (2.58)
Az+Um	0.016±0.002* (1.42)	0.014±0.001* (1.25)	0.50±0.003* (3.39)	0.49±0.004* (3.32)
Az+Al	0.012±0.001* (1.07)	0.010±0.001* (0.89)	0.38±0.004* (2.58)	0.35±0.001* (2.37)
Al+Um	0.017±0.003* (1.51)	0.015±0.002* (1.33)	0.42±0.002* (2.85)	0.39±0.003* (2.64)

Each value is mean ± SE of six replicates.

Value is parentheses are per cent change with control taken as 100%.

Concentration (w/v) has been expressed as final concentration in aquarium water.

(*) Significant (P<0.05) when‘t’ test was applied in between treated and control group.

Student t-test was applied to determination the significant (p<0.05) different between treated and control animals [24].

3. RESULTS

Treatment with 60% of 4h LC_{50} of different binary combinations of citral, ferulic acid, umbelliferne, azadirachtin and allicin caused significant (P<0.05) inhibition in the activity of acetylcholinesterase in the nervous tissues of *L. acuminata* (Table 1).

In control group, acetylcholinesterase and cytochrome oxidase activity in whole cercaria larvae was 1.12 µmole ´SH´ hydrolyzed/min/mg/protein and 14.72 min/mg protein (Table 2). Treatment with binary combination of 20%and 60% of 4h LC_{50} of larvicides caused significant (p<0.05) inhibition in AChE activity in the cercaria larva. Maximum inhibition up to 2.37% and 0.89% of control in cytochrome oxidase and AChE activity was noted in the whole cercaria exposed to 60% of 4h LC_{50} of Az+Al (Table 2).

4. DISCUSSION

Treatment with 20% and 60% 4h LC_{50} of Ci+Fe, Ci+Um, Ci+Az, Ci+Al, Fe+Um, Fe+Az, Fe+Al, Az+Um, Az+Al and Al+Um caused significant change in AChE activity in nervous tissue of snail *L. acuminata*, as well as in cercaria larva. Acetylcholine has been considered a neurotransmitter in the nervous system of parasitic flatworm [25]. ACh and AChE were shown to be involved in

the motor activity of *Schistosoma mansoni* [26, 27]. Presence of cholinergic system has been established at several developmental stage of the parasite, i.e. in cercaria, schistosomula and adult worms [26, 28].

Although snail treatment with 20% and 60% of 4h LC_{50} of citral, ferulic acid, umbelliferone, azadirachtin and allicin against cercaria larva caused significant change in AChE activity in the snail nervous system, yet their was no mort. It indicates that concentration toxic to cercaria larva is safe to their host snail, as there was no snail death. Cercaria is the infective transmission stage, which leave the first intermediate host snail, in order to infect a second intermediate host cattle or human. This cercaria is short-lived; because they do not feed, conditions encountered will determined the amount of time they stay alive until their energy reserves are used up [29].

Cytochrome oxidase is an important enzyme of electron transport system, responsible for active movement of cercaria larva but is generally absent in adults [30, 31]. Tails have more cytochrome oxidase activity [32] to meet the energy demands of specialized organ. There is a significant decrease in the cytochrome oxidase activity in the treated cercaria. It indicates that along with AChE, inhibition of cytochrome oxidase in cercaria larva is responsible for death of cercaria. Earlier, it has been suggested that if snail population is maintained below a certain threshold level, the incidence of fasciolosis can be reduces [17, 33]. This is very common practice used in campaign to reduce the fasciolosis. Snail is one of the important components of an aquatic ecosystem; it is ideal bio-indicators of healthy aquatic ecosystem. Present study clearly indicate that incidence of fasciolosis can be reduced without killing the snails. These combinations can kill directly the target larva of *F. gigantica* in snail body. Binary combinations are more effective larvicide than their single component treatments [17]. Treatment of snails by binary combination of plant derived larvicidal component is more effective method for control of fasciolosis.

5. CONCLUSION

It can be concluded from the present study that redia and cercaria of *F. gigantica* inside the body of intermediate host snail *L. acuminata* can be killed by different binary combination of active larvicidal component. Mortality of cercaria by the different binary formulations is due to the inhibition of AChE and cytochrome oxidase. Moreover, were no significant change in AChE activity of treated snails. The effective concentration in the binary combinations of each larvicidal component is lower and will be safe to the intermediate host snail and other aquatic animals.

AUTHORS' CONTRIBUTION

All authors are involved in conception and design, drafting the research article, red and approved the final manuscript.

TRANSPARENCY DECLARATION

The authors declare that they have no competing interests.

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ISSN 2449-8955

European Journal of Biological Research

Research Article

Overview on vegetation characteristics of selected populations in the Eastern Desert of Egypt

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Received: 02 June 2015; **Revised submission:** 26 July 2015; **Accepted:** 31 July 2015

ABSTRACT

The current study dealt with the distribution of main plant communities and vegetation grouping in relation to soil characteristics in 22 locations across the Eastern Desert of Egypt. A total of 63 species (20 annuals and 43 perennials) representing 50 genera and 22 different families were recorded. Statistical multivariate analysis divided the vegetation to four groups (A, B, C and D). Group A survives in the south eastern corner of the eastern desert and dominated by several acacias. The percentage of silt and the concentration of magnesium were the factors most affecting the distribution of groups. Group B occupied the middle part of the eastern desert, its vegetation was associated with gravels on soil surface which cover and protect the clay from high temperature and consequently maintain the clay water content. Group C spread from south to north at the west boundaries of the eastern desert. The western boundary lies near the Nile valley where the soil expected amount of moisture and the eastern side near the Red Sea where the air is moist. The high role of phosphate upon the distribution of this group may due to the dependence of plants on the organic solutes to catch the available moisture as bound water to survive the severe desert conditions. Group D occupied the northern part where the temperature is lower and near Lake Naser in the south. This group depends on inorganic solutes to maintain the plant turgidity to remain alive.

Keywords: Egypt, Eastern Desert, Chorology life form, Multivariate analysis, Vegetation.

1. INTRODUCTION

The Eastern Desert of Egypt occupies the area extending from the Nile Valley eastward to the Gulf of Suez and Red Sea which is about 223000 km^2, i.e. 22% of the total area of Egypt. It consists essentially of high back bone of high rugged mountains running parallel to and at a relatively short distance from the coast. These mountains are flanked to the north and west by intensively dissected sedimentary plateau [1]. It is traversed by numerous canyon-like depressions (wadis) running to the Red Sea or to the Nile Valley.

From the early beginnings of the last century, some scholars studied the vegetation of the Eastern Desert. Correlation of soil variables and vegetation

was addressed by several studies of [2-4] that had reported striking gradients referring to soil conditions. The vegetation characteristics are usually affected by the physical and chemical components of the soil. The effect of soil texture, water content, electrical conductivity and inorganic solutes on the vegetation characteristics and species distribution were investigated [4, 5]. The most familiar inorganic solutes in nature are Na^+, K^+, Ca^{2+}, Mg^{2+}, and Cl^- [6, 7]. Other studies dealing with vegetation in relation to different ecological aspect were reported by [3, 8-12].

Salama et al. [7] found an increase in the number of Saharo-Arabian species, high percentages of therophytes and chamaephytes along the deltaic part of Wadi Qena controlled by percentage of magnesium and potassium. Salama et al. [13] found that the Saharo-Arabian element forms were the major component of the floristic structure in the inland wadis of the Eastern Desert because the study area lies within the Saharo-Arabian region. In contrast, the monoregional Sudano-Zambezian element was poorly represented and the distribution of vegetation in their study area was mainly controlled by gravel, pH, SO_4, chlorides, K, Mg, and TSS. Sheded et al. [4] found that the most frequent life forms in the Red Sea coast and the Eastern Desert (from Safaga to south Qusseir) are chamaephytes and the chorological analysis showed that the Saharo-Sindian chorotypes are the dominant members.

Abd El-Ghani et al. [14] studied the vegetation and soil characteristics of *Citrullus colocynthis* with other four common desert climbing plants. *Citrullus colocynthis* was indicated to have a wide geographical range of distribution in Egypt and was linked to a main group with *Zilla spinosa*, *Acacia tortilis* subsp. *tortilis*, and *Pulicaria undulate* with a high presence value (60%). The previous researches were carried in a limited areas, therefore, the current study is carried in wide scale to draw a general view about the main vegetation groups in the eastern desert in relation to soil variables. However, the studied locations were selected in sites where *Citrullus colosynthesis* grows in order to measure its genetic variation and record its associated species (Badr et al. in preparation).

2. MATERIALS AND METHODS

Twenty two locations were selected to study through the years of 2011-2013 in the Eastern Desert of Egypt. The geographical distribution of the selected sites is illustrated in Figure 1.

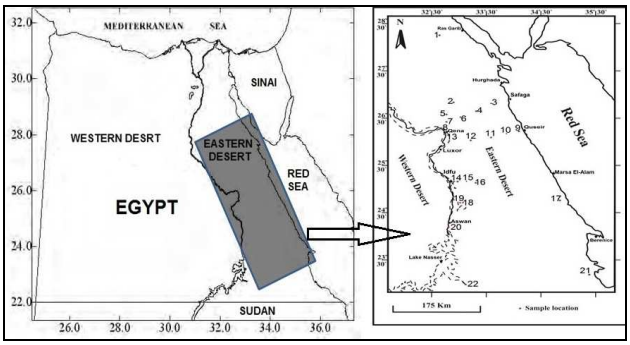

Fig. 1. A map of Egypt illustrating the distribution of the studied locations.

Five quadrats in every location were surveyed. The area of the used quadrats was (25 x 25 m). The recorded taxa were classified according to the life-form system as proposed by Raunkiaer [15]. The presence value of the recorded species was expressed as the number of species occurrence proportionated to the total number of stands. The number of species within each life form category was expressed as a percentage of total number of species in the study area. The different recorded species identified taxonomically according to Cope and Hosni [16], Boulos [17-20] and El-Hadidi and Fayed [21]. Phytogeographical analysis is carried out according to Zohary [22, 23], Abd El-Ghani [24] and Hassan [8]. Numbers of species per genus, and per family were calculated. In order to obtain an effective analysis of the vegetation and related environmental factors, both classification and ordination techniques were employed.

Soil samples were collected from each location at 0-50 cm depth. The samples were brought to the laboratory, air dried, passed through a series of sieves to separate the different soil fractions (coarse sand, fine sand, silt and clay) to determine Soil texture. Soil-water extracts at 1:5 (w/v) were prepared for chemical analysis. The pH values were measured using pH meter (HI 8314), Electrical conductivity (EC) and total dissolved solutes (TDS) was measured using conductivity bridge

(Metter Toledo) according to Jackson [25]. Calcium and magnesium were determined titrimetrically according to Upadhyay and Sharma [26]. Sodium and potassium were determined using the flame photometer technique [25]. Phosphates were determined colourimetrically as phospho-molybdate according to Woods and Mellon [27]. Estimation of chlorides was carried out by titration methods using 0.005 N silver nitrate [28].

2.1. Statistical and vegetation analysis

The soil parameters were analyzed using ANOVA in order to determine significant variation among populations. The correlation coefficient was obtained using SPSS program version 20 [29]. For the vegetation analysis, the two way indicator species analysis TWINISPAN [30] and for ordination, the indirect gradient analysis was undertaken using detrended correspondence analysis DCA [31]. The relationship between plant species variation and environmental variation was assessed using canonical correspondence analysis (CCA). The computer program CANOCO version 4.5 [32] was used for all ordination analyses. In the present study, DCA estimated the compositional gradient in the vegetation data to be larger than 4.0 SD-units for the first axis, thus, Canonical Correspondence Analysis (CCA) is the appropriate ordination method to perform direct gradient analysis [33]. The CCA was performed using 13 environmental variables after exclusion of electric conductivity (EC) and total soluble salts (TSS) due to their high inflation factors. All the default settings were used for CCA, and a Monte Carlo permutation test [34] was used to test for of the eigenvalues of the first canonical axis. Intraset correlations from the CCA were used to assess the importance of the environmental variables.

3. RESULTS AND DISCUSSION

A total of 63 plant species were recorded in the studied sites, these included 43 perennials and 20 annuals and belong to 50 genera from 22 different families (Table 1). The low number of recorded species reflects the severe droughty environmental conditions, especially southward direction but also because the sites were restricted to locations where *Citrullus colosynthesis* grows. The most characteristic families, as shown in Table 1, were Fabaceae (17 species represent 27% of the total number), Asteraceae (7 species, 11%), Zygophyllaceae and Poaceae (6 species, 10% each). The most dominating species were *Zilla spinosa*, *Zygophyllum coccineum* and *Salsola cyclophylla* which had the highest presence values within the examined populations. These results agree with Salama and Fayed [9]; who recognized *Zilla spinosa* and *Salsola imbricata* in one community in Wadi Barramiya. *Zilla spinosa* has also been often recorded in the wadis of the Eastern Desert west of Qusseir province, Wadi Gimal, Wadi Qena, Wadi El-Matuli and Wadi El-Qarn and also in Sinai region, [7, 11, 13, 14, 35].

The recorded species have been attributed to five different life forms (Figure 2). Chamaephytes represented 30.2% (19 species), phanerophytes 28.6% (18 species) and therophytes 22.2% (14 species). The high number of chamaephytes as dwarf plants is suitable life form to resist drought, salinity, sand accumulation and grazing [36].

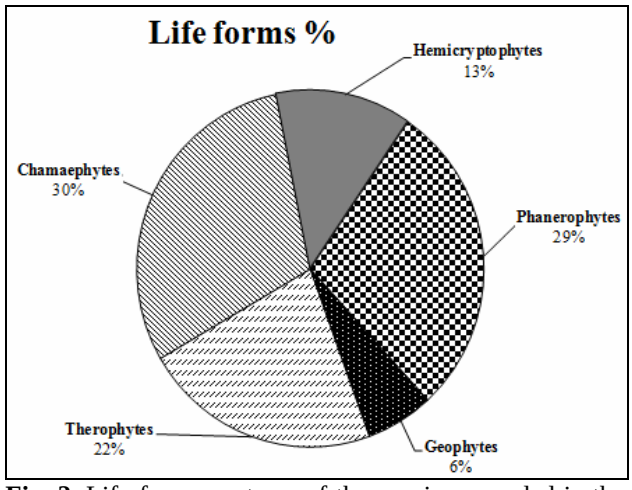

Fig. 2. Life-form spectrum of the species recorded in the 22 examined locations.

Phanerophytes are important elements of semi-desert vegetation because many community and ecosystem processes are regulated by them [37]. In the Eastern Desert of Egypt, Galal and Fahmy [38] indicated the predominance of phanerophytes and chamaephytes over the other life forms in Wadi Gimal in the Eastern Desert of Egypt.

Similarly, high percentages of phanerophytes and chamaephytes were observed by Salama et al. [13] in a study on the Qeft-Quseir Road. Sheded [39] reported that the most frequent life forms in the South Eastern Desert of Egypt are the chamaephytes and therophytes. On the other hand, the low representation of therophytes may be due to the extreme aridity and high salinity [35, 38]. The annual component of vegetation is associated with drought [40], grazing [41] or digging [42] that remove perennials on a small or large scale. These authors are reporting on different types of habitats.

Table 1. Floristic composition, presence value, life forms and chorology of the recorded species.

Species	P (%)	Duration	Life form	Chorology
Family: Fabaceae				
1) *Acacia ehrenbergiana* Hayne	9.1	Per.	Ph.	SSI+SZ
2) *Acacia seyal* Delile	4.5	Per.	Ph.	SA+SZ
3) *Acacia tortilis* (Forssk.) Hayne subsp. *raddiana* (Savi) Brenan	40.9	Per.	Ph.	SSI+SZ
4) *Acacia tortilis* (Forssk.) Hayne subsp. *tortilis*	9.1	Per.	Ph.	SSI+SZ
5) *Alhagi graecorum* Boiss.	13.6	Per.	H.	PAL
6) *Astragalus vogelii* (Webb) Bornm.	22.7	Ann.	Th.	SSI+SZ
7) *Crotalaria aegyptiaca* Benth	4.5	Per.	H.	SZ
8) *Ifloga spicata* (Forssk.) Sch.Bip.	4.5	Ann.	Th.	ME+IT+SSI
9) *Indigofera spiniflora* Boiss.	4.5	Per.	Ph.	SA+SZ
10) *Lotus cytisoides* L.	13.6	Ann.	H.	SSI+SZ
11) *Prosopis juliflora* (Sw.) DC.	4.5	Per.	Ph.	SA
12) *Retama raetam* (Forssk.) Webb and Berthel.	9.1	Per.	Ph.	SA + IT
13) *Senna alexandrina* Mill.	4.5	Per.	Ch.	SSI+SZ
14) *Senna italica* Mill.	4.5	Per.	Ch.	SSI+SZ
15) *Senna holosericea* (Fresen.) Greuter	4.5	Per.	Ch.	SSI+SZ
16) *Senna occidentalis* (L.) Link	4.5	Ann.	Ph.	SZ
17) *Tephrosia purpurea* (L.) Pers subsp. *apollinea* (Delile) Hosni and El-Karemy	4.5	Ann.	Ch.	SSI+SZ
Family: Asteraceae				
18) *Artemisia judaica* L.	4.5	Per.	Ch.	SA
19) *Cotula cinerea* Delile	31.8	Ann.	Th.	SSI
20) *Lactuca serriola* L.	4.5	Ann.	Th.	ME+IT+ES
21) *Launaea nudicaulis* (L.) Hook. f.	4.5	Per.	H.	SA+IT
22) *Pluchea dioscoridis* (L.) DC.	4.5	Per.	Ph.	SSI+SZ
23) *Pulicaria arabica* (L.) Cass.	13.6	Per.	Ch.	ME+IT
24) *Pulicaria undulata* (L.) C. A. Mey.	40.9	Per.	Ch.	SSI+SZ
Family: Poaceae				
25) *Crypsis schoenoides* (L.) Lam.	4.5	Ann.	Th.	COSM
26) *Cymbopogon schoenanthus* (L.) Spreng.	9.1	Per.	G.	SSI+SZ
27) *Cynodon dactylon* (L.) Pers.	9.1	Per.	G.	PAN
28) *Echinochloa colona* (L.) Link	4.5	Ann.	Th.	PAN
29) *Imperata cylindrica* (L.) Raeusch.	4.5	Per.	G.	PAN

Species	P (%)	Duration	Life form	Chorology
30) *Panicum turgidum* Forssk.	4.5	Per.	G.	SSI+SZ+ME+IT
Family: Zygophyllaceae				
31) *Fagonia arabica* L.	27.3	Per.	Ch.	PAN
32) *Fagonia indica* Burm. f.	40.9	Per.	Ch.	SSI+SZ
33) *Fagonia glutinosa* Delile	4.5	Per.	Ch.	SA+IT
34) *Tribulus pentandrus* Forssk.	4.5	Ann.	Th.	SA + SZ
35) *Zygophyllum coccineum* L.	54.5	Per.	Ch.	SSI+SZ
36) *Zygophyllum simplex* L.	27.3	Ann.	Th.	PAL
Family: Asclepiadaceae				
37) *Calotropis procera* (Aiton) W.T. Aiton	22.7	Per.	Ph.	SSI+SZ
38) *Leptadenia arborea (forssk.) schweinf*	4.5	Per.	Ph.	SSI+SZ
39) *Leptadenia pyrotechnica* (Forssk.) Decne.	9.1	Per.	Ph.	SSI+SZ
40) *Pergularia tomentosa* L.	4.5	Per.	Ch.	SSI+SZ
Family: Brassicaceae				
41) *Morettia philaeana* (Delile) DC.	40.9	Ann.	H.	SSI+SZ
42) *Schouwia purpurea* (Forssk.) Schweinf.	22.7	Ann.	Th.	SSI+SZ
43) *Zilla spinosa* (L.) Prantl	81.8	Per.	Ch.	SA+SI
Family: Chenopodiaceae				
44) *Salsola cyclophylla* Baker	45.5	Per.	Ch.	SA
45) *Salsola imbricata* Forssk. subsp. *imbricata*	13.6	Per.	Ch.	SSI+SZ
Family: Resedaceae				
46) *Ochradenus baccatus* Delile	13.6	Per.	Ph.	SSI+SZ
47) *Reseda pruinosa* Delile	4.5	Ann.	Th.	SSI
Family: Solanaceae				
48) *Solanunm nigrum* L.	4.5	Ann.	Ch.	COSM
49) *Solenostemma arghel* (Delile) Hayne	4.5	Per.	Ph.	SSI+SZ
Family: Tamaricaceae				
50) *Tamarix aphylla* (L.) H. Karst.	13.6	Per.	Ph.	SA+SZ+IT
51) *Tamarix nilotica* (Ehrenb.) Bunge	40.9	Per.	Ph.	ME+SA+SZ
Family: Amaranthaceae				
52) *Aerva javanica* (Burm. f.) Juss. ex Schult.	27.3	Per.	Ch.	SSI+SZ
Family: Balanitaceae				
53) *Balanites aegyptiaca* (L.) Delile	4.5	Per.	Ph.	SSI+SZ
Family: Boraginaceae				
54) *Trichodesma africanum* (L.) R. Br.	22.7	Ann.	Ch.	SA + SZ
Family: Caryophyllaceae				
55) *Paronchia argentea* Lam.	4.5	Ann.	Th.	ME
Family: Cleomaceae				
56) *Cleome droserifolia* (Forssk.) Delile	4.5	Per.	H.	SSI+SZ
Family: Cucurbitaceae				
57) *Citrullus colocynthis* (L.) Schard.	100	Per.	H.	ME+ SA+IT
Family: Euphorbiaceae				
58) *Chrozophora oblongifolia* (Delile) A. Juss. ex Spreng.	4.5	Per.	Ch.	ME+IT
Family: Malvaceae				
59) *Malva parviflora* L.	4.5	Ann.	Th.	PAN

Species	P (%)	Duration	Life form	Chorology
Family: Molluginaceae				
60) *Glinus lotoidus* L.	4.5	Ann.	Th.	PAL
Family: Arecaceae				
61) *Phoenix dactylifera* L.	18.2	Per.	Ph.	SSI+SZ
Family: Polygonaceae				
62) *Rumex vesicarius* L.	9.1	Ann.	Th.	ME+SA+SZ
Family: Urticaceae				
63) *Frosskaolea tenacissima* L.	18.2	Per.	H.	SSI+SZ

(Ann.) = Annual, (Per.) = Perennial, (Ph.) = Phanerophytes, (Ch.) = Chamaeophytes, (H.) = Hemicryptophytes, (G.) = Geophytes, (Th.) = Therophytes. **PAL =** Palaeotropic**, PAN =** Pantropic, **COSM =** Cosmopolitan, **SSI** = Saharo-Sindian, **ES** = Euro-Siberian, **SA** = Saharo-Arabian, **ME =** Mediterranean, **SZ =** Sudano-Zambezian, **IT =** Irano-Turanian.

Phytogeographically, Egypt is the meeting point of floristic elements belonging to at least four phytogeographical regions: the African Sudano-Zambezian, the Asiatic Irano-Turanian, the Afro-Asiatic Saharo-Sindian and the Euro-Afro-Asiatic Mediterranean [43]. The chorological analysis of the floristic data revealed that 60% of the species are bi-regional, 13% are mono-regional and 11% pluri-regional (Figure 3). Zohary [44] attributed the dominance of interregional species (bi-, tri- and pluriregionals) over mono-regional ones to the presence of inter-zonal habitats, such as anthropogenic or hydro-, halo- and psammophilous sites. The perennial species (Table 2) represent the skeleton of the vegetation structure, the dominant perennials species were bi-regional [4]. The dominant chorotype element in the studied area was Saharo-Sindian/Sudano-Zambezian. The dominance of this chorotype is due to the presence of Egypt in the middle of Saharo-Sindian region which extends from Morocco to South Iran and Iraq [45] and near the northern boundary of Sudano-Zambezian region [46].

The two-way indicator species analysis (TWINSPAN) classified the stands at the second level into four vegetation groups labeled A-D (Figure 4). These four groups are well separated along the DCA ordination plane of axis 1 and 2, (Figure 5), each sample group comprises a set of stands with greater homogeneity of vegetation, than when compared with other sample groups. Each group is characterized by indicator species, identified by TWINSPAN for each group at each level of hierarchical classification.

It is clear that group A is distributed at the positive end of the first axis of DCA, and group D occupied the negative end, while B and C groups were in the middle of the axis. The soil characteristics for the different detected vegetation groups are summarized in Table 3. The soil of group A (Wadi Hodein, 35°34'14.60"E - 23°2'18.70"N, at an elevation of 53 m asl) was characterized by a high percentage of silt (34.7 ± 0.85%) and high concentration of calcium (0.79 ± 0.3 mg/g) and the lowest percentage of gravels (3.5 ± 0.65%) and fine sand (30.1 ± 1.1%). Also, the concentration of chlorides (0.07 ± 0.02 mg/m), phosphates (0.22 ± 0.16 mg/g) were the lowest. The estimated pH was 7.5 ± 0.08. The indicator species for group A is *Acacia enhrenbergiana* which is dominated in south eastern Egypt [47].

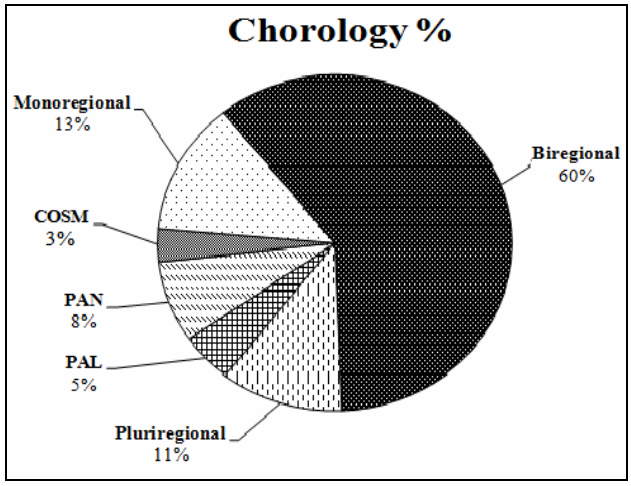

Fig. 3. Chorological analysis of the species recorded in the 22 examined locations.

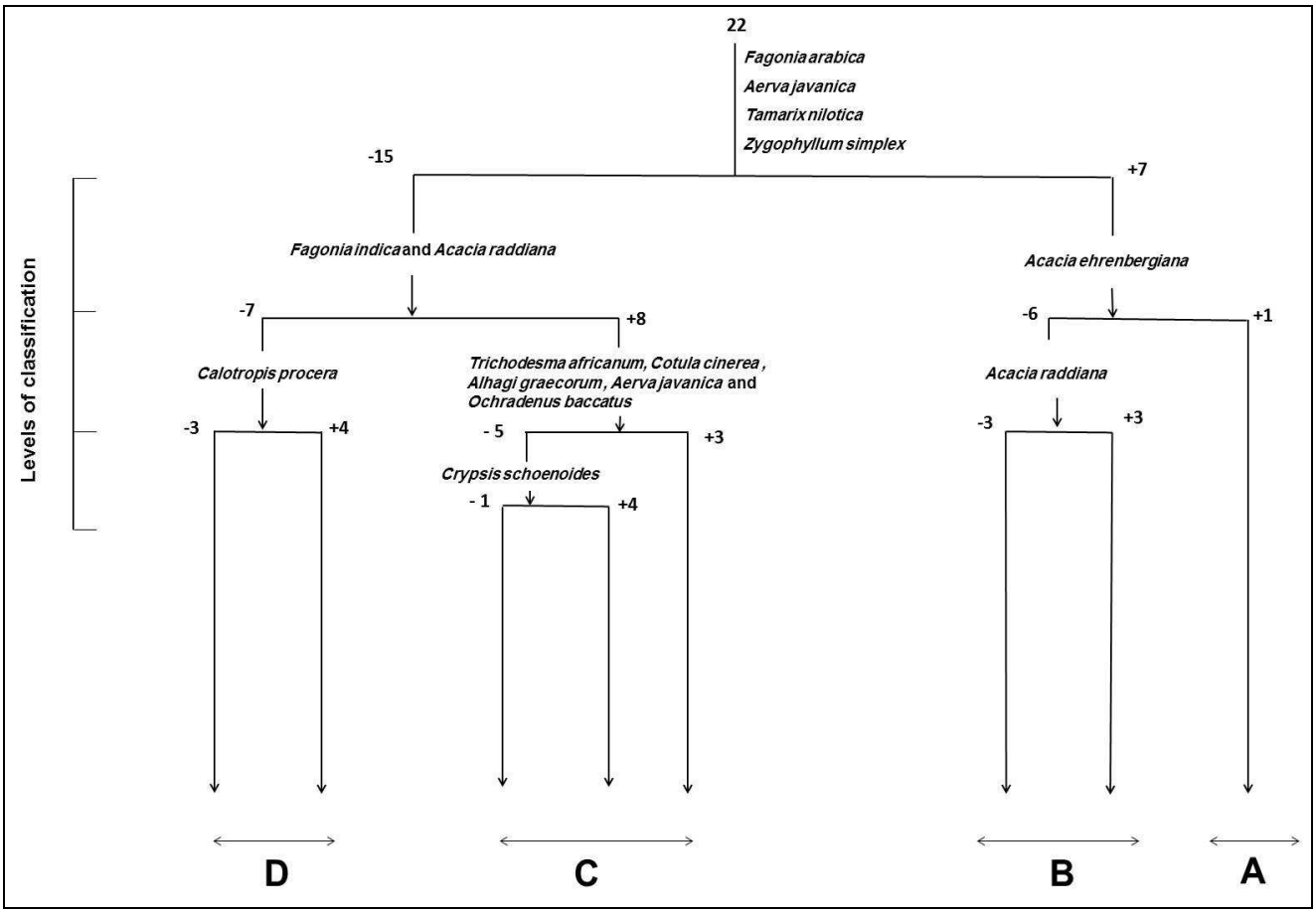

Fig. 4. The dendogram resulting from the TWINSPAN classification of the 22 examined populations in four vegetation groups (A-D).

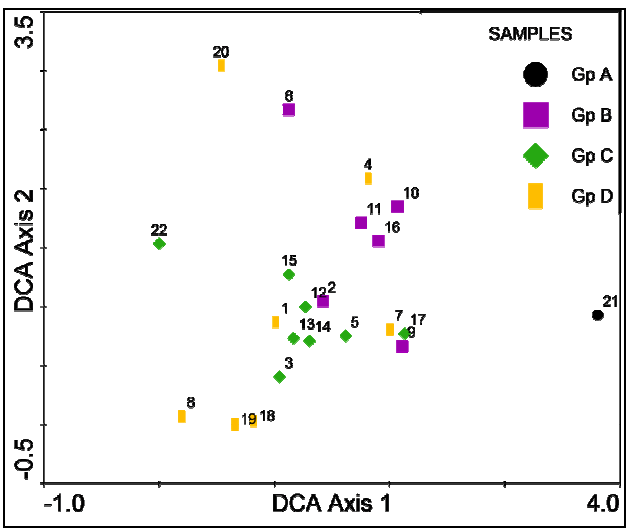

Fig. 5. DCA ordination diagram of the 22 locations on axes 1 and 2 as classified by cluster analysis; A-D are the 4 vegetation groups obtained by TWINSPAN classification.

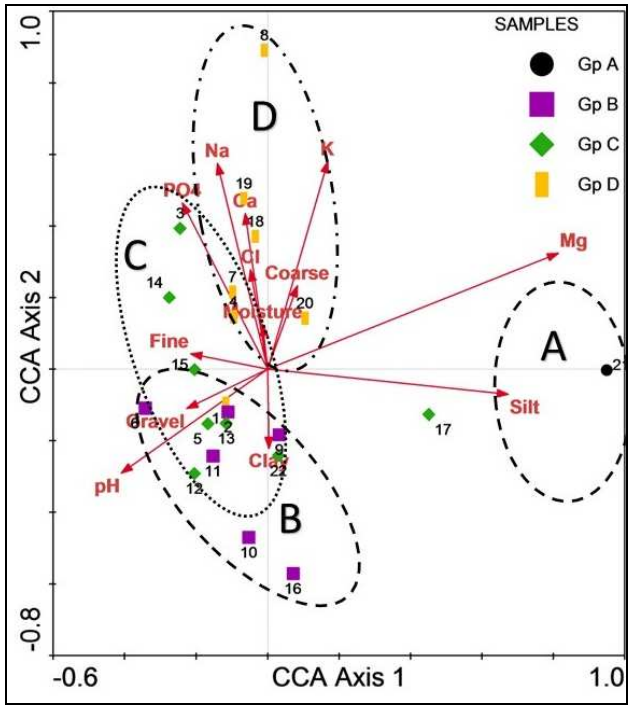

Fig. 6. CCA ordination of the first two axes showing the distribution of the 22 locations with their vegetation groups (A-D) and soil variables.

Table 2. The chorology of the perennial species distributed in the Eastern Desert.

No.	Species	Chorology	A	B	C	D
Mono regional						
1	*Artemisia judaica*	SA		■		
2	*Crotalaria aegyptiaca*	SZ		■		
3	*Prosopis juliflora (Sw.) DC.*	SA	■			
4	*Salsola cyclophylla*	SA		■	■	■
Bi-regional						
5	*Acacia ehrenbergiana*	SSI+SZ	■		■	
6	*Acacia seyal*	SA+SZ			■	
7	*Acacia tortilis raddiana*	SSI+SZ	■	■	■	
8	*Acacia tortilis.tortilis*	SSI+SZ	■		■	
9	*Aerva javanica*	SSI+SZ			■	■
10	*Balanites aegyptiaca*	SSI+SZ			■	
11	*Calotropis procera*	SSI+SZ	■		■	■
12	*Chrozophora oblongifolia*	ME+IT	■		■	
13	*Cleome droserifolia*	SSI+SZ	■	■		
14	*Cymbopogon schoenanthus*	SSI+SZ				■
15	*Fagonia indica*	SSI+SZ	■	■	■	
16	*Fagonia glutinosa*	SA+IT		■		
17	*Frosskaolea tenacissima*	SSI+SZ		■	■	
18	*Indigofera spiniflora*	SA+SZ				■
19	*Launaea nudicaulis*	SA+IT				■
20	*Leptadenia arborea*	SSI+SZ				■
21	*Leptadenia pyrotechnica*	SSI+SZ	■	■		
22	*Ochradenus baccatus*	SSI+SZ			■	■
23	*Pergularia tomentosa*	SSI+SZ		■		
24	*Pluchea dioscoridis*	SSI+SZ				■
25	*Pulicaria arabica*	ME+IT	■	■	■	
26	*Pulicaria undulate*	SSI+SZ		■	■	■
27	*Phoenix dactylifera*	SSI+SZ		■	■	■
28	*Retama raetam*	SA + IT		■	■	
29	*Salsola imbricate*	SSI+SZ	■	■	■	■
30	*Senna alexandrina*	SSI+SZ		■		
31	*Senna italic*	SSI+SZ	■			
32	*Senna holosericea (Fresen.) Greuter*	SSI+SZ	■			
33	*Solenostemma arghel*	SSI+SZ	■			
34	*Zilla spinosa*	SA+SI	■	■	■	■
35	*Zygophyllum coccineum*	SSI+SZ		■	■	■
Pluri-regional						
36	*Citrullus colocynthis*	ME+ SA+IT	■	■	■	■

No.	Species	Chorology	A	B	C	D
37	*Panicum turgidum*	SSI+SZ+ME+IT	■			
38	*Tamarix aphylla*	SA+SZ+IT			■	
39	*Tamarix nilotica*	ME+SA+SZ			■	■
PAN						
40	*Cynodon dactylon*	PAN			■	■
41	*Fagonia arabica*	PAN		■	■	
42	*Imperata cylindrical*	PAN				■
PAL						
43	*Alhagi graecorum*	PAL			■	

PAL = Palaeotropic, **PAN =** Pantropic, **COSM =** Cosmopolitan, **SSI** = Saharo-Sindian, **ES** = Euro-Siberian, **SA** = Saharo-Arabian, **ME =** Mediterranean, **SZ =** Sudano-Zambezian, **IT =** Irano-Turanian.

The percentage of silt and the concentration of magnesium were the factors most affecting the distribution of group A. The correlation between the vegetation groups and the soil variables is indicated on the ordination diagram obtained by CCA in Figure 6. The arrows represent the environmental variables and indicate the direction of maximum change of that variable across the diagram. The length of the arrow is proportional to the rate of change. The strong effect of soil texture and Mg^{2+} content separated group A from the other groups.

Group B soil is characterized by a high percentage of clay (15.0 ± 17.2%) and moisture content (0.47 ± 0.58%). While, it has a low concentrations of TSS (235.7 ± 153.4 mg/l), sodium (1.9 ± 0.69 mg/g), magnesium (0.30 ± 0.17 mg/g) and calcium (0.14 ± 0.05mg/g). As shown in Figure 6, the group B was affected by the soil texture. The vegetation attributed to this group survive in certain place whereas the soil surface is covered by gravels to protect the under layer of fine sand and clay against temperature and the evaporation on their water content. The low water content in these places reflects the strong effect of pH value. Similar findings were reported by Jenny et al. [48] who found that surface sediments of different size classes, water regime, soil salinity and surface deposits were the crucial soil parameters in determining vegetation structure in the arid regions of Wadi Araba in Jordan.

The CCA ordination (Figure 6) indicated that vegetation in group C was affected by the soil texture and chemical characteristics of the soil. It was distributed in the places which have high percentages of gravels (17.5 ± 16.7%) and coarse sand (22.2 ± 18.2 %). Phosphate, calcium and sodium showed a stronger effect than other ions on the vegetation of this group. The vegetation of group C was the most distributed in the eastern desert and constitutes intermediates between groups B and D. Meanwhile, the vegetation in group D was affected by the chemical composition of the soil and the soil moisture. The potassium and sodium ions stronger effect compared with calcium and chloride ions. These data are in agreement with Salama et al. [49] also reported that soil variables particularly EC, Na, Ca, K, Mg, Cl, moisture contents, SO_4, pH and gravels play important roles in the vegetation grouping in the Eastern Desert of Egypt.

The interference between the four groups may reflect its ability to survive under different environmental regimes [14] or/and the heterogeneity and the non-continuity of environmental conditions which formed sparse patches of similar soils.

Depending on the current data, group A survives in the south eastern corner of the eastern desert. This group is dominated by several acacias species which can form a subtropical forest if a sufficient rain is available. Group B occupied the middle part of the eastern desert, its vegetation was associated with gravels on soil surface which cover and protect the clay from high temperature and consequently maintain the clay water content. The group C spreads from south to north at the west boundaries of the eastern desert. The western boundary lies near the Nile valley where the soil had

expected amount of moisture and the eastern side near Red Sea where the air is moist. The high role of phosphate upon the distribution of this group may due to the dependence of plants on the organic solutes to catch the available moisture as bound water to survive the severe desert conditions. Group D occupied the northern part where the temperature is lower and near Lake Naser in the south. This group depends on inorganic solutes to maintain the plant turgidity to remain alive.

Table 3. Mean values ± SD and ANOVA F-values of the soil variables in the 22 locations representing the 4 vegetation groups (A-D) obtained by TWINSPAN classification. *P < 0.05, **P < 0.01.

Character	A	B	C	D	F-ratio
Gravels (%)	3.5 ± 0.65	18.1 ± 19.7	17.5 ± 16.7	13.8 ± 14.2	13.25**
Coarse sand (%)	19.3 ± 1.9	18.7 ± 15.5	22.2 ± 18.2	10.8 ± 13.0	20.21**
Fine sand (%)	30.1 ± 1.1	33.5 ± 25.5	43.6 ± 20.7	65.9 ± 27.0	38.94**
Silt (%)	34.7 ± 0.85	14.8 ± 15.4	8.2 ± 6.7	5.3 ± 3.8	37.60**
Clay (%)	12.5 ± 0.55	15.0 ± 17.2	8.5 ± 7.3	4.2 ± 3.1	4.20**
Moisture content (%)	0.42 ± 0.23	0.47 ± 0.58	0.32 ± 0.23	0.2 ± 0.24	5.20**
pH	7.5 ± 0.08	7.9 ± 0.38	7.9 ± 0.54	8.0 ± 0.41	6.61**
EC (µs)	157.8 ± 43.4	141.8 ± 94.0	635.9 ± 832.2	499.3 ± 587.6	3.06**
TSS(mg/L)	259.8 ± 69.1	235.7 ± 153.4	1056.8 ± 1378.4	823.1 ± 963.6	3.29**
K^+ (mg/g)	1.4 ± 1.0	2.6 ± 1.5	4.4 ± 4.8	11.9 ± 9.4	2.35**
Na^+(mg/g)	3.2 ± 0.55	1.9 ± 0.69	2.9 ± 1.8	3.4 ± 1.9	0.96**
Mg^{++}(mg/g)	0.38 ± 1.03	0.30 ± 0.17	1.0 ± 1.4	0.58 ± 0.5	7.12
Ca^{++}(mg/g)	0.79 ± 0.3	0.14 ± 0.05	0.19 ± 0.2	0.19 ± 0.2	2.12*
Cl^-(mg/g)	0.07 ± 0.02	0.14 ± 0.07	0.43 ± 0.44	0.57 ± 0.8	1.27
PO_4^{-3}(mg/g)	0.22 ± 0.16	0.25 ± 0.14	0.30 ± 0.18	0.29 ± 0.1	1.36

AUTHORS' CONTRIBUTION

MK: Conception and design, Acquisition of data, Writing, Field visit and compilation of data; AB: Supervision, revision and editing. HZ: Field data collection, analysis and interpretation of data. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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ISSN 2449-8955

European Journal
of Biological Research

Research Article

Characterization of lipase enzyme produced by hydrocarbons utilizing fungus *Aspergillus terreus*

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Received: 03 August 2015; **Revised submission:** 11 September 2015; **Accepted:** 18 September 2015

ABSTRACT

Microbial lipase today occupies a place of prominence among biocatalysts owing to their ability to catalyze a wide variety of reactions in aqueous and non aqueous media. In this study, *Aspergillus terreus* showed high lipase production in submerged culture (15.463 ± 0.39 U/mg). The optimum conditions for lipase production by *A. terreus* were pH range 7-9, a temperature of 30-45ºC for a period of 6 days and ionic strength 60 mM NaCl. Lipase production was also detected when *A. terreus* was grown on different hydrocarbons, olive oil or tween 80 but not on sugars indicating that the enzyme is inducible by hydrocarbons as well as oils. The kinetics studies showed that the Km value for *A. terreus* was 8.12 µmol, the optimum pH for lipase activity was 6, the optimum temperature was 30 ± 1ºC and lipase activity could tolerate high levels of ionic strength; however the fungal growth and lipase productivity were very sensitive to increase ionic strength.

Keywords: *Aspergillus terreus*, Enzymes, Lipase, Pollution, Hydrocarbons.

1. INTRODUCTION

Environmental pollution is a worldwide problem and its potential to influence the health of human populations is great [1]. Hydrocarbon pollution is a serious problem in the environment and represents 70% of environmental pollutants [2, 3]. Soil pollution with hydrocarbons causes extensive damage of local environmental system since accumulation of pollutants in animal and plant tissues may cause death or mutations [4]. Hydrocarbon pollution can cause human cancers, including leukemia and increase lead concentration in soil which is especially hazardous for young children causing developmental damage to the brain [5].

The technologists commonly used many techniques for soil remediation from hydrocarbon pollutants; including mechanical burying, evaporation, dispersion, and washing. However, these technologies are expensive and can lead to incomplete decomposition of contaminants. Among the technologies now available and has more advantages to deal with polluted soils are bioremediations; based on the metabolic activity of microorganisms for degradation of hydrocarbons

[6]. The use of fungi in bioremediation have received considerable attention for their potent bioremediation potently by producing enzymes involved in degradation process of wide range of hydrocarbons [7].

Lipase (triacyl glycerol acylhydrolases E.C. 3.1.1.3) is a class of enzymes unique in catalyzing the hydrolysis of fats into fatty acids and glycerol at the water-lipid interface. Lipases produced by fungi are typically extracellular and therefore relatively easy to recover after the fermentation [8].

Many genera as *Penicillium, Rhizopus, Aspergillus* and *Fusarium* have been noted as producers of lipase with desirable properties, which would have potential applications in a number of different areas. Fungal lipase derived mainly from *Aspergillus* sp. [9]. *Aspergillus* spp. are natural 'factories' for the production of enzymes such as cellulases, xylanases, amylases, proteases, and lipases. Owing to their ability for elaborating an array of extracellular enzymes, aspergilli play an important role in the production of industrial enzymes [10].

Lipase play role in oily hydrocarbons biodegradation; Lipase activity has been used as biochemical and biological parameter for testing hydrocarbon degradation. According to its analytical simplicity and rapidity, lipase activity demonstrated as an excellent indicator for monitoring decontamination of hydrocarbon polluted soils [11].

Margesin et al. [12] found that monitoring of soil microbial lipase activity is a valuable indicator of diesel oil biodegradation in freshly contaminated, unfertilized and fertilized soils. Also, Margesin et al. [13] observed a negative relationship between organic pollution and lipase activity in soil; for this reason the measurement of lipase activity may represent a valid tool in monitoring the biodegradation of organic pollutants.

Riffaldi et al. [11] reported that the evaluation of soil biological activities as a monitoring instrument for the decontamination process of diesel-oil contaminated soil was made using measurements of organic carbon content, soil microbial respiration, soil ATP and dehydrogenase, β-glucosidase, lipase enzyme activities. Lipase activity was found to be the most useful parameter for testing hydrocarbon degradation in soil.

2. MATERIALS AND METHODS

2.1. Microorganism

The fungus used in this study was *A. terreus*, isolated from kerosene polluted soil from El-Minya governorate, Egypt at 28 ± 1°C. The fungus was maintained in Potato Dextrose Agar medium (PDA).

2.2. Screening for extracellular lipase production

Modified basal salt medium (BSM) containing (g/l): NH_4NO_3, 1; K_2HPO4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.025; $CaCL_2$, 0.025; $FeSO_4 \cdot 7H_2O$, 0.015; $ZnSO_4 \cdot 7H_2O$, 0.005, distilled water, 1000 ml and pH adjust to 7.0. After sterilization of medium chloramphenicol, 250 mg and 1% (v/v) sterilized tween 80 were added [14, 15]. One ml of the spore suspension was transferred to 30 ml of pervious medium. Inoculated flasks were incubated at 30 ± 1˚C under shaking (150 rpm) for 6 days, and then filtrate was centrifuged under cooling at 10000 x g for 30 min at 4˚C, and the supernatants was subsequently collected for lipase activity determination. Lipase activity was determined by using p-nitrophenylpalmitate (pNPP) as described by Licia et al. [16].

2.3. Lipase specific activity

Lipase specific activity was defined as the number of lipase units per mg extracellular proteins. Concentration of the extracellular protein in the crude enzyme was measured as described by Lowery et al. [17].

2.4. Optimization for lipase production

Various parameters including pH, incubation temperature, incubation period, ionic strength and carbon source were tested to obtain the optimum production / activity of lipase by the selected strain.

2.5. Effect of initial pH

The fungal isolate was grown on screening medium at different initial pH ranging from 2 to 12. The initial pH was adjusted by 0.1 M HCl or 0.1 M NaOH. After 6 days the fungal mass

was harvested and yield of lipase produced was determined.

2.6. Effect of incubation temperature

The effect of incubation temperature on lipase activity was studied in a range of 10 to 45˚C. The fungal isolate was grown on screening medium for 6 days. Subsequently fungal mass harvested and yield of lipase produced was determined.

2.7. Effect of incubation period

The effect of incubation period on lipase activity was studied over range of 2 to 12 days. The fungal isolate has been grown on screening medium. Subsequently fungal mass harvested and yield of lipase produced was determined.

2.8. Effect of ionic strength

The effect of ionic strength on lipase activity was studied at different salt concentrations in a range of 0.0 to 1 M NaCl. Fungal isolates were grown on screening medium for 6 days then fungal mass harvested and yield of lipase produced was determined.

2.9. Effect of carbon source

Different carbon sources were examined to determine their effect on lipase production namely, dextrose, sucrose, cellulose, tween 80, hexane, toluene, olive oil and crude oil. Fungal isolates were grown on BSM supplemented with the carbon source and fungal mass harvested and yield of lipase produced was determined.

2.10. Kinetics of extracellular lipase

2.10.1. The K_m value of lipase

For determination of the (Michaels constant) value of lipase, different pNPP concentrations in reaction mixture (3 ml) were used. The reaction mixture at each pNPP concentration contained the same amount of lipase (1 ml) and 0.5 ml phosphate buffer pH 7, 100 mM. The reaction was left for 45 min at 30 ˚C and lipase activity was determined as mentioned above. The K_m value of lipase was determined using Lineweaver-Bulk plots of various pNPP concentrations against lipase activity in each assay in the reaction mixture.

2.10.2. pH-dependency of lipase activity

The pH-dependency of lipase activity was examined in a pH range from 3-9.5. Different buffer systems were used in accordance with the respective pH range: 100 mM Glycine-HCl buffer for pH range 3-5, 100 mM phosphate buffer for pH range 6-7 and 100 mM Tris-HCl buffer for pH range 8-9.5.

2.10.3. Temperature dependency of lipase activity

The optimal temperature for lipase activity was determined in a temperature range 10-90˚C.

2.10.4. Ionic strength dependency

The optimal ionic strength for lipase activity was determined at different NaCl concentrations of 0.0 to 2 M NaCl in reaction mixture.

2.10.5. Thermostability of lipase activity

Enzyme solution was reincubated in 100 mM phosphate buffer (pH=7.0) for 60 min at different temperatures covering the range of 50-90˚C. The enzyme activity was then determined at 30˚C.

3. RESULTS

3.1. Screening for extracellular lipase production

Aspergillus terreus isolated from kerosene polluted soil from El-Minya governorate, Egypt showed high lipase production (15.463 ± 0.39 U/ml) and the high lipase specific activity (142.74 ± 0.95 U/mg protein), the high yield (463.9 ± 11.98 U/30 ml culture) and thus, it was used for further optimization and kinetic studies.

3.2. Optimization of lipase production

Initial pH, the current study showed that extracellular lipase has been produced by *Asper-*

gillus terreus in all pH values except extreme acidic pH (pH 2) which show a complete inhibition in fungal growth and lipase production. Generally, fungus was able to grow in the pH range from 4 to 12 and produced higher amount of lipase enzyme in alkaline medium than acidic medium. *Aspergillus terreus* showed an optimum pH range 7-9 for lipase production (Fig. 1).

Incubation temperature, results of the effect of different incubation temperatures on production of lipase are shown in Fig. 2. The fungus was able to grow in all temperature degrees ranged from 10 to 45 ± 1ºC but with different amounts. The optimum temperature for lipase production was 45 ± 1ºC.

Incubation period, lipase production by *Aspergillus terreus* increased with growth up to the start of the stationary phase and was optimum after 6 days. Subsequently, the lipase production decrease sharply at late stages of the stationary growth phase (Fig. 3).

Ionic strength, the ionic strength dependency of lipase production was studied using NaCl. Generally, high concentrations of NaCl (< 600 mM NaCl) caused a complete inhibition of fungal growth and lipase production. Both fungal growth and lipase production decreased by increasing the ionic strength indicating that both are likely linked. *Aspergillus terreus* produced higher amount of lipase at low ionic strength (< 60 mM NaCl) followed by a clear decline in activity with increasing NaCl concentration in culture media (Fig. 4).

Effect of carbon sources, different carbon sources were tested for their ability to support lipase production by *Aspergillus terreus* (Fig. 5). No lipase activity was detected in *A. terreus* cultures containing dextrose, sucrose and cellulose as a carbon source, while lipase produced only in cultures containing hydrocarbons, olive oil or tween 80 as a carbon source indicating that the enzyme is inducible by hydrocarbons as well as oils. In the present investigation the optimum conditions for lipase production by *Aspergillus terreus* were pH range 7-9, a temperature of 30-45ºC for a period of 6 days and ionic strength 60 mM NaCl. Both fungal growth and lipase production decreased by increasing the ionic strength indicating that both are likely linked.

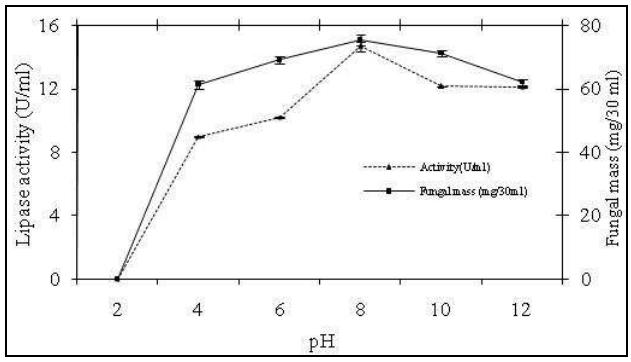

Fig. 1. Effect of intial pH on lipase production of *Aspergillus terreus.*

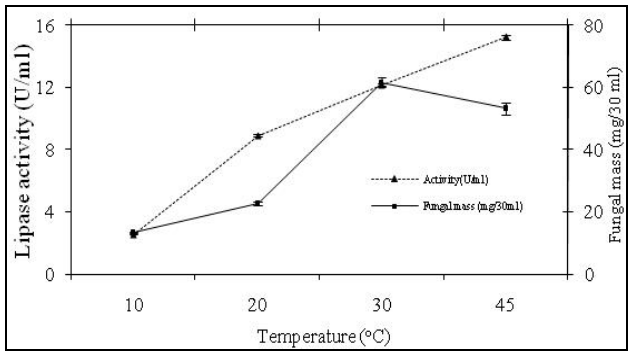

Fig. 2. Effect of temperature on lipase production of *Aspergillus terreus.*

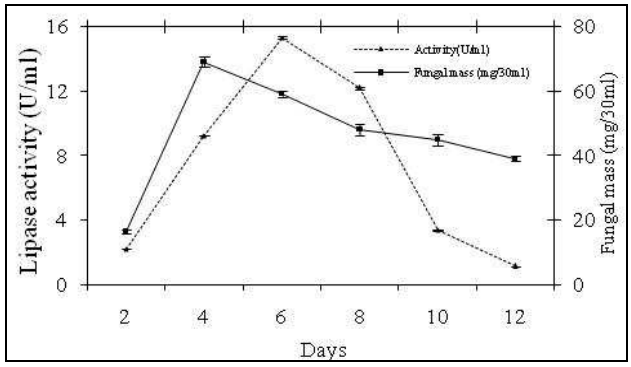

Fig. 3. Effect of incubation period on lipase production of *Aspergillus terreus.*

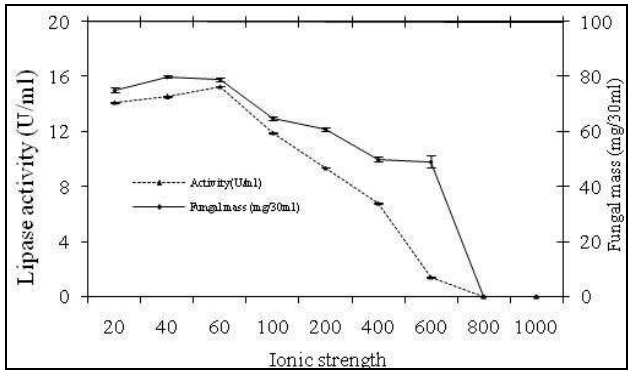

Fig. 4. Effect of ionic strength on lipase production of *Aspergillus terreus.*

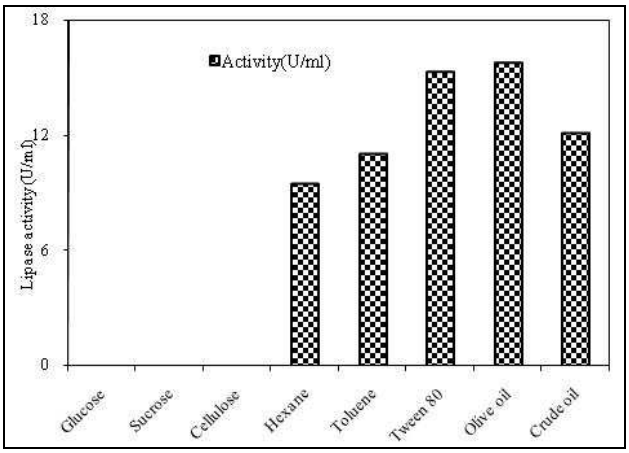

Fig. 5. Effect of carbon source on lipase production by *Aspergillus terreus.*

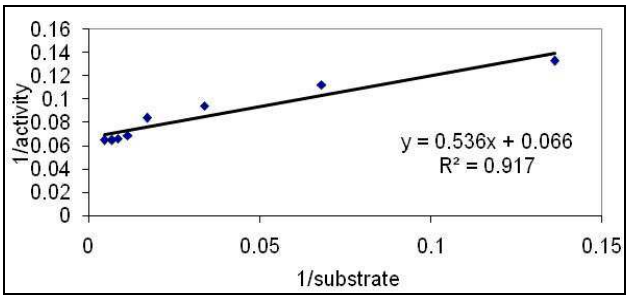

Fig. 6. Lineweaver-Burk plot of extracellular lipase of *Aspergillus terreus.*

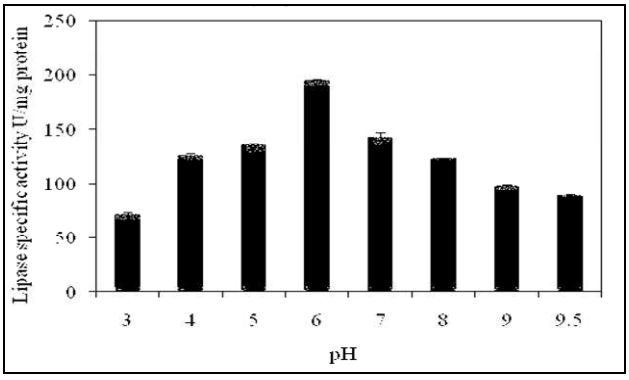

Fig. 7. Effect of pH on lipase activity of *Aspergillus terreus.*

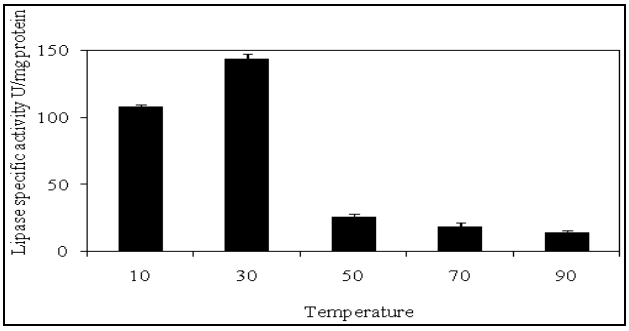

Fig. 8. Effect of temperature on lipase activity of *Aspergillus terreus.*

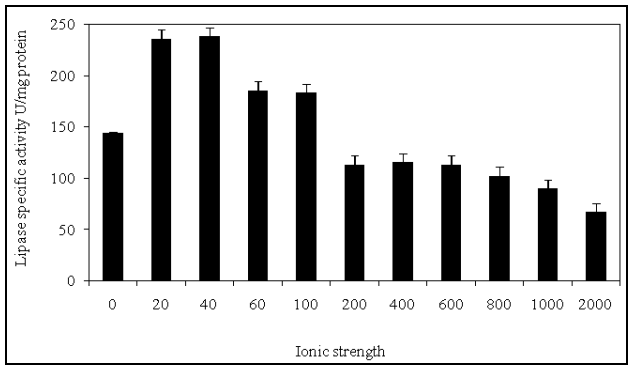

Fig. 9. Effect of ionic strength on lipase activity of *Aspergillus terreus.*

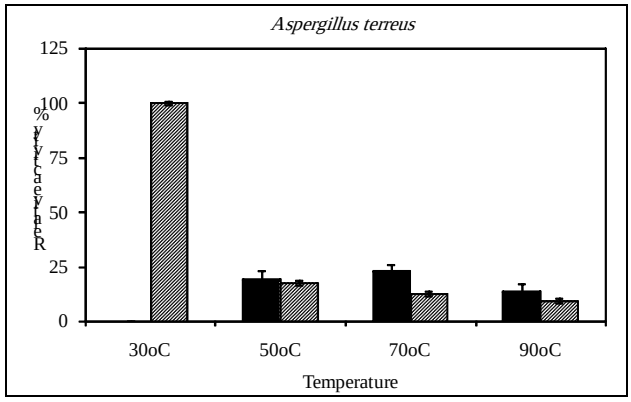

Fig. 10. Thermostability of lipases from *Aspergillus terreus.*

3.3. Kinetics of extracellular lipase

The K_m values of lipase for *A. terreus* was 8.12 µmol pNPP, respectively. The V_{max} for *A. terreus* was 15.5 µmol pNPP/ min. (Fig. 6). The optimum pH for lipase activity by *A. terreus* was 6 (Fig. 7). The optimum temperature for lipase activity by *A. terreus* was 30 ± 1ºC as shown in Fig. 8. The optimum ionic strentgh for lipase activity by *A. terreus* was 20-40 mM NaCl. Lipase activity decreased at ionic strength higher than 100 mM NaCl (Fig. 9). Lipase activity could tolerate high levels of ionic strength; however the fungal growth and lipase productivity were very sensitive to the increase in ionic strength. The optimum temperature for *A. terreus* was 30 ± 1ºC. Lipase of *Aspergillus terreus* could slightly tolerate high temperature degrees (Fig. 10).

4. DISCUSSION

In the current study *Aspergillus terreus* isolated from kerosene polluted soil from El-Minya governorate showed the high lipase production (15.463 ± 0.39 U/ml) and high lipase specific activity (142.74 ± 0.95 U/mg protein), the high yield (463.9 ± 11.98 U/30 ml culture). In agree with our results Ghosh et al. [19] reported that *Aspergillus terreus* is one of the main lipase producing fungi. Riffaldi et al. [11] reported that lipase enzyme activity has been used as biochemical and biological parameter for hydrocarbon degradation and demonstrated as an excellent indicator for monitoring decontamination of hydrocarbon polluted soils. Chuks Ugochukwu et al. [18] reported that lipase activities of *Aspergillus*, *Candida* and *Fusarium* isolated from soil contaminated with crude oil can be used as indicator for microbial degradation of crude oil.

Lipase production was detected when *A. terreus* was grown on hydrocarbons, olive oil or tween 80 but not on sugars indicating that enzyme is inducible by hydrocarbons as well as oils. Pokorny et al. [20] showed that the addition of olive oil in concentrations between 0.5% and 2% increased the lipase production by *Aspergillus niger* to a maximum at 72h.

Remarkably lipase activity in the filtrate was detectable very early in the incubation period (2 days). The maximum lipase activity was obtained after 6 days of incubation, while maximum cell mass was detected at 4 days. Cihangir and Sarikaya [21] indicated that four days of incubation of *Aspergillus* sp. was optimum for its lipase activity, while biomass production increased after three days.

Its worth to mention that the extreme acidic pH made a complete inhibition of fungal growth and it has been observed that with increasing pH value enzyme production increase until get its maximum activity ay pH 8. In agreement of our results Ohnish et al. [22] reported that the production of lipase in supernatant increase with increasing pH up to value of 8.3 which has been observed during lipase production by *Aspergillus oryzae*.

The K_m and V_{max} values for *Aspergillus terreus* were crude extracellular lipase were evaluated from a Lineweaver-Burk plot and found to be 8.12 µmol pNPP and 15.5 µmol pNPP/min, respectively. Shu et al. [23] reported that the values of K_m and V_{max} of a lipase from *Aspergillus niger* calculated from the Lineweaver-Burk plot using pNPP as hydrolysis substrate were 7.37 mmol/L and 25.91 µmol/(min × mg), respectively.

The lipase enzyme by *A. terreus* showed an optimum pH for lipase activity 6. In consistence many microbial lipases have their optimum activity at a pH range of 7-9 as reported by Zhang et al. [24]. Prabhakar et al. [25] mentiend that the optimum pH for lipase activity of *Rhizopus* isolated from oil contaminated soils was pH 6.0.

The optimum ionic strength for lipase activity by *A. terreus* was 20-40 mM NaCl. Lipase activity could tolerate high levels of ionic strength; however the fungal growth and lipase productivity was very sensitive to the increase in ionic strength. Lipase activity decreased at ionic strength higher than 100 mM NaCl [26].

The optimum temperature for the fungs was 30 ± 1ºC. In agreement with our results Prabhakar et al. [25] mentiend that the optimum temperature for lipase activity of *Rhizopus* isolated from oil contaminated soils was 30ºC. Yadav et al. [27] reported that *Aspergillus terreus* lipase showed excellent temperature tolerance (15-90 °C) and was highly thermostable, retaining 100% activity at 60°C for 24 h.

5. CONCLUSION

The results presented in this study suggested that *Aspergillus terreus* isolated from Kerosene polluted soils can be potentially used in lipase production as well as in bioremediation of polluted soils specially oily polluted soils. Accordingly, the researchers seeking higher lipase producing fungi can be advised to isolate them from hydrocarbon polluted soils.

AUTHORS' CONTRIBUTION

GAM: Designed, recorded the experimental data and wrote the research. MMMK: Participate in research design, revision and editing. FMM: Participate in research design, revision and editing. MMKB: Supervision, revision and editing. The final manuscript has been read and approved by all authors.

ACKNOWLEDGMENTS

The authors are very grateful for the insightful and helpful comments, constructive suggestions and careful corrections made by the Editor and the anonymous Referees for further improvements of this manuscript.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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ISSN 2449-8955

European Journal of Biological Research

Review Article

Dichlorvos toxicity on fish - a review

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Received: 21 May 2015; **Revised submission:** 18 September 2015; **Accepted:** 24 September 2015

ABSTRACT

Pesticides are a very important group of environmental pollutants used in intensive agriculture practices for protection against diseases and pests in order to increase total yield of crops with minimum investment of labour and efforts. Pesticide exposure may also fatal to many non-target organisms like fish where it hampers its health through impairment of metabolism, occasionally leading to the death of the fish. Among the diverse group of pesticides, dichlorvos (2,2-dichlorovinyl dimethyl phosphate), an organophosphate compound, is commonly used against a wide range of pests in agricultural field, farm animals and man, and as an anthelminthic also. Dichlorvos has elicited worldwide concern as it induces many significant changes in fish biology out of which neurotoxicity, behavioural change, alteration in biochemical parameters, bioaccumulation in tissues, undesired developmental changes, chromosomal changes, histopathological changes etc. are a few in the account of dichlorvos toxicity, This study is a review of toxicological effects of dichlorvos in fish.

Keywords: Dichlorvos, Toxicity, $LC_{50,}$ Fish.

1. INTRODUCTION

Pesticide as defined by United Nations Environment Programme [1] is any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest. Amongst others, organophosphorus pesticides (OPs) are the most commonly used pesticides in the world due to their rapid degradation ability [2]. Unfortunately, OPs harm beyond their target and can contribute severe, long term population effects on terrestrial and aquatic non-target species, predominantly vertebrates [3].

Dichlorvos [2,2-dichlorovinyl dimethyl phosphate (IUPAC), 2,2-dichloroethenyl dimethyl-phosphate (CAS)] was first registered for use in 1948 [2]. It has a molecular formula $C_4H_7C_{l2}O_4P$ and molecular weight of 220.98 with CAS registry number 62-73-7. Dichlorvos or formulations containing dichlorvos appear under trade names of "Vapona", "Nuvan", "Nogos", "Cossman's Fly-Cake", "Phoracide", "Herkol", "Alco Fly Fighter Insect Spray", "Lethalaire Bantam 8", "Lethalaire F-83", "Real-Kill Fly and Mosquito Killer", "Kill-Fly Resin Strip", "Misect", "Atgard V", "De-Pester Insect Strip", "Vaponex", "Vaponicide", "Vaporette Bar", "Dedevap", "No-Pest Strip" [4]

Das [5] cited Binukumar and Gill [6] that dichlorvos is one of the most commonly used organophosphate pesticides in developing countries. World Health Organization classified dichlorvos as a Class IB, 'highly hazardous' chemical [7]. Acceptable data on dichlorvos indicates that it is very highly toxic to freshwater fish (LC_{50} = 183 ppb for most sensitive species), moderately toxic to estuarine/marine fish (EC_{50} = 7350 ppb for the one species tested). Chronic studies established NOAEL values of 5.2 ppb (rainbow trout), 960 ppb (sheepshead minnow), 0.0058 ppb (daphnid) and 1.48 ppb (mysid shrimp) [2].

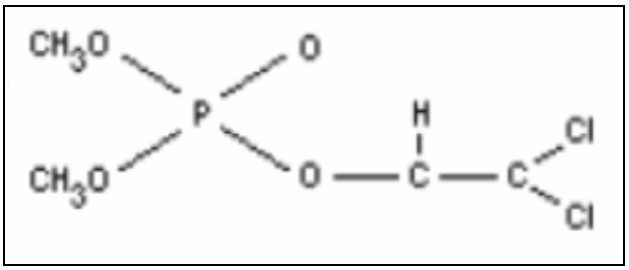

Fig. 1. Structural formula of dichlorvos (2,2-dichlorovinyl dimethyl phosphate).

Dichlorvos is used to control a wide range of mite and insect pests of plants, farm animals and man, and as an anthelminthic also. On plants it is used as a pre-harvest treatment for fruits, vegetables, rice and field crops. It is also used in the form of aerosols and impregnated resin strips on stored products and in food processing industries and similar locations and in automatic dispensers. Many countries add it to stored grain as a dust, spray or emulsion. On livestock, usually it is used as sprays, aerosols and as anthelminthic pellets for oral dosing of poultry, pigs and horses. Dichlorvos is used extensively in hospitals, granaries and transport and food processing units and in aeroplane for disinfection during flight [8].

DDVP is registered to control insect pests in agricultural sites, commercial, institutional and industrial sites; in and around homes; and on pets. DDVP is also used in greenhouses; mushroom houses; storage areas for bulk, packaged and bagged raw and processed agricultural commodities; food manufacturing/processing plants; animal premises; and non-food areas of food-handling units. It is also registered for direct dermal pour-on treatment of cattle and poultry. DDVP is not registered for direct use on any field grown commodities [2].

DDVP exposure in the form of swallowing, inhalation, and absorption via dermal route is reported as poisonous [9]. Even though dichlorvos is reported to be a contact and stomach insecticide for variety of crop pests, it is also toxic to fish and other aquatic organisms [10]. It is extremely toxic pesticides to aquatic organisms and hampers fish health through impairment of metabolism sometimes leading to death [5]. Present study is an effort to review the toxicological effects of dichlorvos in fish.

2. ACUTE TOXICITY OF ACTIVE CONSTITUENT AND FORMULATIONS TO FISH

The acute toxic effects of dichlorvos in different species fish were studied by different researchers. Results of various studies indicating the acute toxicity of dichlorvos (active constituent and various formulations) to fish are summarized in Table 1. The 96-h LC_{50} values of dichlorvos in fish were found between 0.004-11.6 mg/L [11].

According to the report of WHO **[**12], dichlorvos is highly toxic (LC_{50} in the range 0.1-1 mg/L) to moderately toxic (LC_{50} in the range 1 to 10 mg/L) to fish, with a few reports indicating slight toxicity (LC_{50} in the range 10-100 mg/L). The range in acute toxicity (LC_{50}) of dichlorvos to fish from these studies was ~0.2 mg/L to >40 mg/L, with the lowest value being 0.122 mg/L for larvae of the herring.

Lewallen and Wilder [13] reported that dichlorvos (evidently active constituent added in acetone) was not lethal to either 1 week old or 1 month old fry of rainbow trout at 1 mg/L, but caused 100% lethality at 10 mg/L. In a paper describing Japanese aquatic toxicity testing methods, Nishiuchi in 1974 indicated that the 48 h LC_{50} of dichlorvos to carp is in the range 0.5-10 mg/L. The 96 day LC_{50} values in freshwater fish, fathead minnows, carp, Japanese killifish, guppy, bluegill, and trout, ranged from 0.17 to 11.1 mg/L [14].

Table 1: LC_{50} value of dichlorvos pesticide of different technical grade for different species - reports seen by researcher.

Sl No	Test Species	Duration of exposure	LC_{50} value/range	References
1.	*Heteropneustes fossilis*	96 h	6.45 mg/L	Ahmad and Gautam, 2014 [15]
2.	*Cyprinus carpio*	96 h	2.51 mg/L	Günde and Yerli, 2012 [16]
3.	*Poecilia reticulata*	96 h	1.84 mg/L	
4.	*Labeo rohita*	96 h	42.66ppm	Bhat et al., 2012 [17]
5.	*Cirrhinus mrigala*	96 h	9.1 ppm	Velmurugan et al., 2009 [18]
6.	Sheephead minnow	96 h	7.5 ppm	Jones & Davis, 1994 [19]
7.	*Salvelinus namaycush*	96 h	0.18 ppm	Mayer & Ellersieck 1986 [20]
8.	*Clarias batrachus*	96 h	8.9 mg/L	Verma et al., 1983 [21]
9.	*Saccobranchus fossilis*	96 h	6.6 mg/L	Verma et al., 1982 [22]
10.	*Cyprinus carpio*	96 h	0.34 mg/L	Verma et al., 1981 [23]
11.	*Ophiopcephalus punctatus*	96 h	2.3 mg/L	Verma et al., 1981 [24]
12.	*Mystus vittatus*	96 h	0.5 mg/L	Verma et al., 1980, 1981 [25,24]
13.	*Gambusia affinis*	96 h	5.3 mg/L	Johnson and Finley, 1980 [26]
14.	*Pimephales promelas*	96 h	12 mg/L	
15.	*Lepomis macrochirus*	96 h	0.9 mg/L	
16.	*Salvelinus namaycush*	96 h	0.2 mg/L	
17.	*Lepomis macrochirus*	96 h	0.48 mg/L	Kenaga, 1979 [27]
18.	Spot	96 h	0.55 mg/L	
19.	*Tilapia mossambica*	96 h	1.4 - 1.9 mg/L	Spehar et al., 1981 cited Rath and Misra, 1979 [28, 29]
20.	*Cyprinus carpio*	48 h	0.5-10 mg/L	Nishiuchi, 1974 [30]
21.	Bluegill sunfish	48 h	0.70 mg/L	Pimentel, 1971 cited earlier reports [3]
22.	Bluegill sunfish	24 h	1 mg/L	
23.	*Rasbora heteromorpha*	24 h	10 mg/L	
24.	Rainbow trout	24 h	0.5 mg/L	Anon, 1968 [31]
25.	Bluegill sunfish	24 h	1.0 mg/L	

3. CHRONIC TOXICITY OF ACTIVE CONSTITUENT AND FORMULATIONS TO FISH

Verma et al. [21] reported an estimated Maximum Acceptable Toxicant Concentration (MATC) for dichlorvos to carp of 16-20 µg/L, based on the total weight of live fish at 60 days divided by the original number of exposed fry (evidently with exposure continuing under similar conditions of solution renewal).

WHO [7] cited various reports of investigation by Verma et al. [22, 24, 32, 33] based on studies with African catfish exposed to sub-lethal concentrations of dichlorvos (45, 90 µg/L) for 30 days. Effects were observed on blood chemistry and levels of various enzymes and from the results a 'No Observed Adverse Effect Concentration' of 30 µg/L was derived. However the results obtained was not clear at the two doses mentioned above.

4. NEUROTOXIC EFFECTS

Dichlorvos exposure inhibits acetylcholinesterase activity in brain, plasma, and red blood cells; acetylcholinesterase is an enzyme that is important for neurological function [2, 34].

The major mechanism for the action of dichlorvos is inhibition of acetylcholinesterase (AChE), leading to an increase in the level of acetylcholine in the synaptic cleft and hence producing both nicotinic and muscarinic signs and symptoms of intoxication in the peripheral and central nervous system like nausea, vomiting, lacrimation, salivation, bradicardia, miosis and finally death may occur due to respiratory failure [6, 35].

Inhibition of AChE induces alteration in the swimming behavior, shaking palsy, spasms and other undesirable effects [36]. Disorders in AChE activity can also weaken feeding, identification, avoidance and escaping from predators, spatial orientation of the species, and reproductive behavior [37].

5. BIOACCUMULATION

Dichlorvos does not significantly bioaccumulate *in* fish [38]. In a 168 hour bioaccumulation study with carp, bioconcentration factor (BCF) of dichlorvos was less than 0.5 [39].

6. BEHAVIORAL TOXICITY

Günde, and Yerli [16] calculated and found 96 h acute LC_{50} values (95 % confidence limits) of technical dichlorvos, dissolved in acetone, using a static bioassay system to guppy and carp as 1.84 mg/L and 2.51 mg/L respectively. Their study revealed that the behavioral changes in guppy started 30 min after dosing. The 0.5 mg/L (lowest) concentration had similar behavior with the control group. Fish group exposed to 1 mg/L showed less general activity with occasional loss of equilibrium. Loss of equilibrium was reported to be more frequent in the 3 mg/L concentration. The 5 mg/L concentration group stayed motionless close to the water surface and later fell to the aquarium bottom in an uncontrolled manner. The highest concentration of 8 mg/L reported high intensities in responses like the loss of equilibrium, hanging vertically in water, quick gill movement, inconsistent swimming, sudden swimming motion in a spiral fashion, after long periods of motionlessness lying down on the aquarium bottom and suddenly starting to move. According to their observation, the behavioral changes in carp started 1 h after dosing. The lowest concentration had similar behavior with the control group. One group of fish exposed to 2 mg/L showed less general activity while other group exposed with 3 mg/L stayed motionless near to the water surface and later fell to the aquarium bottom in an uncontrolled manner. The highest concentration group showed the loss of equilibrium, hanging vertically in water, after long periods of motionlessness lying down on the aquarium bottom and suddenly starting to move. The control groups showed normal behavior during the test period [16].

Bhat et al. [17] reported the 96h LC_{50} value of dichlorvos on *Labeo rohita* as 42.66 ppm. The LC_{50} concentration for 96h was calculated by probit analysis method of Finney's [40]. After exposure to the pesticides, the *Labeo rohita* showed behavioral changes, they aggregated at one corner of aquarium, irregular, erratic and darting swimming movements and loss of equilibrium. They slowly became lethargic, hyper excited, restless and secreted excess mucus all over their bodies. The fish exhibited peculiar behavior of trying to leap out from the pesticide medium which can be viewed as an escaping phenomenon. They often spiral rolled at intervals and finally the fishes sank to bottom with their least operculum movements and died with their mouth opened.

7. DEVELOPMENTAL EFFECTS

In a study carried out by Sisman [41], examined effects of the pesticide dichlorvos on embryonic development of zebrafish. Developmental abnormalities have been observed in embryos and larvae, such as no blood flow, cardiac edema, delayed hatching, and vertebra malformations.

Mir et al. [42] had studied the effects of sublethal concentrations of the organophosphate pesticide dichlorvos (Neon) (0.65 mg/l, 0.90 mg/l and 1.17 mg/l) on the gonadosomatic index of the fish, *Cyprinus carpio communis*.. The study revealed that the Ganadosomatic index was decreased with the increase in concentration, whereas it was increased with increase in exposure at all concentrations. The researchers had noticed that the reduction in GSI values was the maximum at the highest concentrations of the pesticide in series. Ovaries of the dichlorvos treated fish showed

histomorphological disorders. Furthermore, the reduced GSI was found directly proportional to the pesticide concentration and duration of exposure.

8. ALTERATIONS IN BIOCHEMICAL PARAMETERS

Ahmad and Gautam [15] photmetrically investigated effects of various concentration of Nuvan (2,2 dichlorovinyl dimethyl phosphate) exposure on days 7, 15, 30 and 60 for many serum biochemical parameters in the freshwater teleost fish, *Heteropneustes fossilis.* The technical grade insecticide Nuvan selected for the study was the trademark of AMVAC Corporation of India. The 96 h LC_{50} value was estimated by using the log-dose probit regression line method. And LC_{50} for 96 h of Nuvan to *H. fossilis* was calculated as 6.45 mg/L. On the basis of LC_{50} value they determined sub-lethal concentrations of 0.26 mg/L, 0.32 mg/L, and 0.43 Mg/L which are 1/15, 1/20, 1/15 of LC_{50} respectively. Various biochemical parameters such as Serum Protein, Serum Albumin, Serum Creatinine, Serum Bilirubin and Serum Urea have been studied as diagnostic tools. Results indicated that Serum total Protein and Serum Albumin decreased significantly whereas Serum Creatinine, Bilirubin and Serum Urea increased with increase in Nuvan concentration and time of exposure when compared with control group which is free from treatment. It was discussed by the authors that depletion of the total protein in the study may be due to inhibition of RNA sysnthesis disturbing the protein synthesis. They suggested that the fall of Serum Albumin in the treated fish can be attributed to the liver damage. Creatinine increase was correlated with the impact of the harmful pesticide on the kidney. Increased urea in the experimental fish was reported to be due to the inability of the damaged kidney to filter the urea up to the normal levels.

Lakshmanan et al. [43] had treated *Oreochromis mossambicus* (Peters) with sub lethal doses of 0.00375 ppm, 0.0075 ppm and 0.015 ppm of dichlorvos with an exposure period of 7th, 14th and 21st days. The researchers had reported significant decrease in the liver, kidney and muscle protein content with increase in exposure period and they have suggested that depletion of tissue total proteins after 7 days exposure period may be due to increased proteolysis thereby contributing to the availability of free amino acids that may be fed to the tricarboxylic acid (TCA) cycle and further possible utilization of its products for metabolic process. The results observed in this investigation suggested that the test parameters could be used as potential biomarkers for monitoring residual pesticides present in aquatic environments. But the researchers felt that more detailed laboratory study is required for application of these findings. The researchers also felt the need of further investigation to make it clear that whether these pesticide-induced responses in fish were related to the level of stress hormones (especially catecholamines and cortisol), enzymatic kinetics, and molecular mechanisms or not.

For the entire study, Lakshmanan et al. had adopted Colorimetric micro method [44] for the quantitative estimation of tissue glycogen. Total protein content in the tissues was estimated by the method of Lowry et al. [45]. For the estimation of tissue albumin Biuret method [46] was adopted.

In an investigation carried out by Koul et al. [47], Renewal Toxic Test Method [48] was used to find out the LC_{50} concentration of dichlorvos (DDVP, 20% EC) on the fish *Channa gachua* (HAM.). Fishes were exposed to sub-lethal concentration (0.012 mg/l) for 16, 24 and 48 hours in acute toxicity studies and 15, 30 and 45 days in chronic toxicity studies. Control fishes were maintained under identical conditions without pesticide in the medium. In this study, the researchers had observed that the exposure of sub-lethal concentration of dichlorvos led to the decrease in the level of plasma glucose, total protein, and lipid peroxidation while a significant increase was observed in the levels of cholesterol, alkaline phosphatase in plasma, triglyceride in plasma, serum bilirubin, serum creatinine, SGPT and SGOT.

9. HISTOPATHOLOGICAL CHANGES

Velmurugan et al. [18] carried out a study on the gills and liver tissues of Cirrhinus mrigala exposed to dichlorvos to assess the histopathological effects through light microscopy.

Commercial grade dichlorvos (76% EC) (Hindustan Ciba Geigy Ltd., Mumbai, India) was

used in this study. Renewal toxic test methods (APHA, 1995 [49]) were done to find out the 96-h LC_{50} concentration, which was 9.1 ppm for the fish. The fishes were divided into three groups and placed in separate glass aquaria.Fifteen fishes were used for each group. Groups I and II fishes were exposed to sublethal concentrations of 0.91 ppm and 1.82 ppm of dichlorvos respectively for 10 days with parallel untreated control group (Group III). These concentrations were chosen because they were lower than the lethal concentrations for *C. mrigala*.

No histopathological changes were observed in the gill of the control fish. The most common changes in *C. mrigala* for both 0.91 ppm and 1.82 ppm concentrations of dichlorvos were hyperplasia, desquamation, and necrosis of epithelial, epithelial lifting, oedema, lamellar fusion, collapsed secondary lamellae and curling of secondary lamellae. At exposure to dichlorvos at 0.91 ppm, the gills of experimental fish showed also severe aneurism in the secondary lamellae with the rupture of the pillar cells.

No histopathological changes were observed in the liver of the control fish. In the liver tissues of fish exposed to dichlorvos concentrations of 0.91 ppm and 1.82 ppm, cloudy swelling of hepatocytes, congestion, vacuolar degeneration, karyolysis, karyohexis, dilation of sinusoids and nuclear hypertrophy were seen.

10. EFFECTS ON CHROMOSOME

Exposure of *Channa punctatus* to dichlorvos concentration of 0.01 ppm caused chromosomal aberrations in the form of centromeric gaps, chromatid gaps, chromatid breaks, sub-chromatid breaks, attenuation, extra fragments, pycnosis, and stubbed arms in kidney cells after exposure periods of 24, 48, 72 and 96 h [36].

Interestingly, there was an inverse relationship between duration of exposure and aberration frequency. Longer exposures to dichlorvos were associated with lower frequencies of aberrations. The toxicity of dichlorvos has also been related to alterations in DNA replication, which causes mutations [50] and cellular hyper proliferation as a result of local irritation [51-53].

11. CONCLUSION

An ocean of research has been done to study the effect of pesticide on living organisms. Presently it is well known fact to all that pesticides possess greater risk to higher vertebrates as it accumulates in higher concentration in the tissue of organisms occupying the topmost niche of the food chain owing to the property of biological magnification. Unfortunately, pesticides are being used in an alarming rate for the sake of earning a handsome amount from the crop with low labour and effort by the people to invest it again to treat an ailment resulting from pesticide effect! Time has already conceded to think this issue again as many international bodies already made it clear about the harmful effects of the pesticide through their published reports and also suggested many alternatives to combat with the problem of pest in agricultural field. In spite of spending a huge amount of fund in the research work every year to find the deleterious changes in tissue of organisms due to pesticide exposure, it is better to have utilized this fund for the search of better way of pesticide control which will restore environmental balance.

AUTHORS' CONTRIBUTION

Both authors contibuted equally to this work. The final manuscript has been read and approved by both authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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ISSN 2449-8955

European Journal of Biological Research

Research Article

Geographic variation in phytochemical constituents and allelopathic potential of *Pinus halepensis* barks

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Received: 21 August 2015; **Revised submission:** 27 September 2015; **Accepted:** 01 October 2015

ABSTRACT

Aqueous extracts (10, 20, 30 and 40 g/L) of *Pinus halepensis* barks, collected from Bizerte (B), Tabarka (T), Seliana (S) and Oueslatia (O) to elucidate the influence of ecological sites on allelopathic potential. Aqueous barks extracts from (S) and (O) have revealed a higher rate of phenolic acids than those from (T) and (B), respectively 13.23, 13.8, 11.63 and 10.37 mg/mL. Alep pin barks were analyzed using HPLC/UV for the identification and quantification of the phenolic compounds, among which in particular the catechin acetate, the gallic acid, the rutine hydrate, luteolin 7 glucoside and the cinnamic acid. In fact, the aqueous extract of barks from (S) revealed a highest level, respectively 2.61, 1.74, 1.61, 1.36, and 1.21 mg/mL. The *Pinus halepensis* barks was analyzed by GC and GC-MS. As a result, 29 compounds were identified representing 89% made up basically by β-caryophyllene, α-humulene. As for allelopathic activity, aqueous extracts of barks significantly delayed germination, reduced its rate and affected the seedling growth mainly the (S) and (O) extracts. The root growth of the two targets has shown a high sensibility compared to the shoot lengths. Pot cultures were conducted by the incorporation of barks powder (50 and 100 g/kg) or the irrigation with their aqueous extracts at 20 and 40 g/L. *Pinus halepensis* barks and its extracts have shown a high herbicide potent, particularly the one collected from (S) and (O), may be favorably used for incorporating in agricultural systems for sustainable weed management.

Keywords: Allelopathic potential, Barks, Phenolic acids, Phytochemical content, *Pinus halepensis*.

1. INTRODUCTION

Conifer forests are allelochimical-producing, and have a strong allelopathic potential [1]. *Pinus halepensis* Mill is one of the major conifers in Algeria, Morocco and Tunisia covering approximately 1.3 million hectares, one of the principal essences given the zone it covers [2]. Continually

expanding, heliophilous, invasive, and rich in secondary metabolites, *Pinus halepensis* could influence the secondary succession because of its great allelopathic potential [2-5]. Indeed this potential is influenced by the abiotic factors such as the high temperatures, hydrous stress, light, soil characteristics (pH, the structure and the state of the nutrients, texture, the presence of contaminants), altitude and the latitude [6-9]. This allelopathic potential depends on abiotic factors such as the edaphic microclimate, the intensity and the duration of rainfall [10]. These secondary metabolites are of great importance for the relations between the plant and its environment [11].

The bark of pine was a bothersome residue for the wood industry, abundantly available and cheap [12, 13], rich in polyphenols, phenolic acids fatty, aliphatic, and resinic acids [14-16]. Those secondary metabolites show an important ecological role in the allelopathic processes [5].

Many plants use chemical interactions, such as allelopathy [8], a principal factor in the management, implementation and growth of plants [17]. They have a negative impact on the surrounding plants under natural conditions [18], like in the agrosystems [19]. Many plant-derived compounds, [20] have herbicide effects without causing damage to the environment [21]. The use of secondary metabolites could be effective in the management of weeds [22]. Indeed the improvement of the agricultural output depends partly on weeding [23]. The development of natural pesticides would make it possible to decrease the use of chemical pesticides [24] and their negative impact on the environment [25].

We conducted the work to evaluate the herbicide potential of the pine barks of Alep and to explore the influence of the ecological sites Bizerte (B), Tabarka (T), Seliana (S) and Oueslatia (O) on the production of allelochemicals.

2. Materials and methods

2.1. Sampling Sites

The barks of *Pinus halepensis* were randomly collected from 20 trees in a 10×10 m^2 area in the Tunisian pine forests of Bizerte, Tabarka, Seliana, and Oueslatia, in January 2012. The samples were dried in a ventilated and lit place. Forty grams of each dried and grinded biomass was tempered in 1 L distilled water at ambient temperature for 24 h. The extracts were filtered through a paper filter (Whatman N°1) 3-5 times and saved at 4 °C in the dark until use [22].

2.2. Climatic data

The climatic data displayed in Table 1; were provided by the weather services (The Tunisian National Institute of Meterology).

Table 1. Climatic data of the four stations of sampling (According to the National institute of Meteorology).

Climatic data		**Bizerte**	**Tabarka**	**Seliana**	**Oueslatia**
Rainfall		450-1500 mm/an	450-1500 mm/an	150-450 mm/an	100-400 mm/an
Altitude		21m	5 m	560 m	654 m
Location		37°14'N 9°45'E	36°56'N 8°46'E	35°57'N 9°28'E	35°52'N 9°30'E
Temp (°C)	Max(August)	30.3	34.7	36.3	31.6
	Min (December)	8.9	7.8	7.2	8

2.3. Bioassays with aqueous extracts

Barks aqueous extracts were prepared by soaking 40 g of dried biomass for 24 h in 1L of sterilized distilled water, diluted to give 10, 20 and 30 g/L [6]. They were tested on *Raphanus sativus* L. (radish) and *Triticum aestivum* L. (wheat), used as model plants in the studies on the allelopathy at the laboratory. Target seeds were surface sterilized with 0.525 g/L sodium hypochlorite for 15 min, then rinsed four times with deionized water, imbibed in it at 22 °C for 12 h and carefully blotted using a

folded paper towel [26]. Twenty imbibed seeds of target species were separately placed on filter papers in Petri dishes, 5 mL of each extract per treatment. Seeds irrigated with distilled water were used as controls. The seeds were germinated in a growth chamber with 400 µmol photons $m^{-2}s^{-1}$ photosynthetically active radiation (PAR) at 22/24 °C for 14/10 h light and dark periods, respectively [6].

2.4. Phytochemical screening

2.4.1. Total phenolic content (TPC) determination

TPC in the extracts were estimated by a colorimetric assay based on the procedures described by Paras and Hardeep; Reis et al. [27, 28]. Basically, 1 ml of sample was mixed with Folin-Ciocalteu reagent (5 mL, previously diluted with water 1:10, v/v) and sodium carbonate (75 g/L, 4 mL). The tubes were mixed in vortex for 15 s and kept aside for 30 min at 40°C for color development. Absorbance was measured at 765 nm (Analytikjena spectrophotometer; Jena, Germany). TPC was expressed as mg gallic acid equivalent /g dry matter (mg GAE/g dw) using gallic acid calibration curve ($R^2 = 0.985$).

2.4.2. Total flavonoid content (TFd) determination

TFd were determined according to the method of Zhishen et al. [29] with some modifications. The extract (250 µl) was mixed with 1.25 mL of distilled water and 75 µl of a 5% $NaNO_2$ solution. After 5 min, 150 µl of 10% $AlCl_3 \cdot H_2O$ solution was added. After 6 min, 500 µl of 1 M NaOH and 275 µl of distilled water were added to prepare the mixture. The solution was mixed well and the absorbance was read at 510 nm. (+)-Catechin was used as standard and the results were expressed as mg of (+)-catechin equivalents (CE) per g of the dry matter.

2.4.3. Condensed tannins content (TPA) determination

TPA was determined according to the method of Julkunen-Titto [30]. An aliquot (50 µL) of each extract or standard solution was mixed with 1.5 mL of 4% vanillin (prepared with methanol) and then 750 µL of concentrated HCl were added. The well mixed solution was incubated at ambient temperature in the dark for 20 min. The absorbance against blank was read at 500 nm. The results were expressed as mg of (+)-catechin equivalents (CE) per g of the dry matter.

2.4.4. Determination of o-diphenols

1 ml of a solution of HCl (0.5 N), 1 ml of a solution of a mixture of $NaNO_2$ (10 g) and $NaMoO_4 \cdot 2H_2O$ (10 g) in 100 ml H_2O, and finally 1 ml of a solution of NaOH (1 N) were added to 100 µl of each aqueous extract. After 30 min, o-diphenols were read at 500 nm. The o-diphenols were expressed on a dry weight basis as mg tyrosol equivalents per g of the dry matter [31].

2.4.5. Identification of phenolic compounds (HPLC/UV) in the extracts

The presence and amount of phenolic compounds in the extracts were studied by reversed phase HPLC analysis using a binary gradient elution. The phenolic compounds analysis was carried out by Usingan Agilent Technologies 1100 series liquid chromatography (HPLC, Palo Alto, CA) coupled with an UV-vis multiwavelength detector. The separation was carried out on a 250 mm × 8 mm, particle size 5 µm Eurospher-100C_{18} reversed phase column at ambient temperature. The mobile phase consisted of acetonitrile (solvent A) and water with 0.2% sulphuric acid (solvent B). The flow rate was kept at 0.8 ml min^{-1}. The gradient program was as follows: 15% A/85% B, 0-12 min; 40% A/60% B, 12-14 min; 60% A/40% B, 14-18 min; 80%A/20% B, 18-20 min; 90% A/10% B, 20-24 min; 100% A, 24-28 min. The injection volume was 20 µl, and peaks were monitored at 280 nm. Samples were filtered through a 0.45 µm membrane filter before injection. Peaks were identified by congruent retention times compared with standards.

2.4.6. Volatile compound analyses

Supelco (Bellefonte, PA, USA) SPME devices coated with polydimethylsiloxane (PDMS, 100 µm) were used to sample the headspace of two date seeds inserted into a 10-mL glass vial and allowed

to equilibrate for 30 min. After the equilibration time, the fibre was exposed to the headspace for 50 min at room temperature. Once sampling was finished, the fibre was withdrawn into the needles and transferred to the injection port of the GC-MS system. GC-EIMS analyses were performed with a Varian (Palo Alto, CA, USA) CP 3800 gas chromatograph equipped with a DB-5 capillary column (30 m x 0.25 mm x 0.25 µm; Agilent, Santa Clara, CA, USA) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions were as follows: injector and transfer line temperatures were 250 and 240 ^{o}C, respectively; oven temperature was programmed from 60 to 240 ^{o}C at 3 ^{o}C min^{-1}; carrier gas was helium at 1 mL min^{-1}; splitless injection. The identification of the constituents was based on a comparison of their retention times with those of authentic samples (Collection of volatile compounds purchased from Sigma-Aldrich Italia and ⁄ or Carlo Erba Italia as pure compounds or analytical kits; except for the two 2-tridecenes that have been identified by mean of their mass spectral data), comparing their linear retention indices (LRI) relative to a series of n-hydrocarbons, and on computer matching against commercial (NIST 98 and Adams) and homemade library mass spectra, and MS literature data [32, 33]. Moreover, the molecular weights of all the substances identified were confirmed by GC-CIMS, using methanol as ionizing gas. Results were expressed as relative percentages obtained by peak area normalization [34].

2.5. Effect of the aqueous extracts

2.5.1. Effect on germination

Germination was given including the number of seeds germinated at 24 hour intervals for 6 days. The length of the roots and the air parts of young seedlings of target species were measured 7 days after sowing [6]. The data were transformed into percentage of control for the analysis. The index of germination GI was calculated by using the following formula [35].

$GI= (N_1) x1 + (N_2 - N_1) x (1/2) + (N_3 - N_2) x (1/3) + •••$

Where N_1, N_2, N_3,…, N_n: percentage of germinated seeds observed after 1,2,3,…, N days. This index represents the delay in the germination induced by the extract [36]. The percentage of germination inhibition was determined according to the formula:

[% germination inhibition] = [% germination control - % germination extract]

The percentage of inhibition/stimulation was calculated under the terms of the formula of Chung et al. [37]:

[Inhibition (-) /Stimulation (+)] = [(Extracted - Control) /Control] x 100.

2.5.2. Effect on the growth

The effect of the aqueous extracts on the growth was estimated by measuring the length of the root and the principal stem 7 days after germination. The results were expressed as a percentage of the control. The percentages of inhibition or stimulation induced by the various extracts were calculated [37].

2.6. Pot culture assay

2.6.1. Powder incorporation in soil

The vegetal powder of the barks, taken from various sites was incorporated in soil sample to the proportions of 50 and 100 g/kg. The soil without powder was used as a control. The mixtures were placed in 10 cm diameter plastic pots, each containing 250 g [38]. The experiment was undertaken under a greenhouse. The length of the roots and the principal stem were measured at the end of day 20 of culture. The treatments were randomly laid out in a device with three repetitions and the data were transformed into a percentage of the control for analysis [6].

2.6.2. Irrigation with the aqueous extracts

The target plants were sown in pots of 10 cm in diameter filled with the same soil type. The pots were irrigated with the aqueous extracts prepared from the various types of biomass of pine of Alep at two concentrations (20 and 40g/l). The added volume was of 10ml and the ground was humidified each time it desiccates. The treatments were randomly laid out in a device with three repetitions and the data were transformed into percentage of the control for analysis [6].

2.7. Statistical analysis

The biological tests in the laboratory and the greenhouse were carried out with three repetitions and five times for the phytochemical analyses. All the data were reported on average ± standard deviation using SPSS 18 program. An ANOVA of LSD post hoc test was carried out with the same software in order to analyze the differences between the treatments. The Pearson correlation between the essays of the different sites having the same concentration was made for each species and each concentration. The averages were separated on the level of probability 0.05.

3. Results

3.1. Phytochemical screening

The contents of the *Pinus halepensis* barks collected from the four ecological sites Bizerte (B), Tabarka (T), Seliana (S) and Oueslatia (O) in total polyphenols (TPC), O-diphenols, flavonoids (TFd) and in condensed tannins (TPA), revealed significant differences depending on the origin of the biomass (Table 2). This production of allelochemicals is partially due to genetic factors and is partly determined by environmental conditions [39]. It partly accounts for the high TPC contents in the barks from (S) and (O) compared with those of (B) and (T), which are respectively 75.34 and 71.46 mg GAE/g dw (Table 2). Flavonoids can play a significant role in the protection of the plants against the UV-A and UV-B [40]. Their production varies according to the plant geographical site [41]. The highest content was recorded in the barks of the Seliana forest with 36.44 CEQ/g dw, an average of 28.79 CEQ/g dw for The three other aqueous extract. Covelo et al. [39] showed that the content of tannins, in the pine forests, strongly depends on the availability of light. Indeed, the biomass of source (O) and (S) presents the highest content, respectively 6.79 and 5.85 CEmg/g dw, however (B) and (T) aqueous extract revealed a less rate of TPA, an average of 5.29 CEmg/g dw. This dissimilarity could be explained by the effect of the climatic factors [42].

3.2. Identification of phenolic compounds (HPLC/UV) in the barks extracts

Phenolic acids (caffeic, ferulic and cinnamic acids), polyphenols, tannins, flavonols (quercetin) are inhibitors of germination [43]. The effect of the phenolics compounds on germination is related to the regulation of endogenous auxine, the permeability of the seed tegument and the procurement of oxygen to the embryo [44].

In this study, HPLC showed many phenolic acids in the aqueous extracts of the barks of *Pinus halepensis* (Table 3). Elevated levels of phenolic acids is related to the mechanisms of defense of the plant against a microorganism attack [45], involved in resistance to various types of stress [46]. Indeed, the aqueous extracts of *Pinus halepensis* bark from (S) and (O) revealed higher rate of phenolic acids than those from (T) and (B), respectively of 13.23, 13.8, 11.63 and 10.37 mg/ml; this may be explained by the low rainfall and high temperatures of the two harvesting site (S) and (O). In fact, the aqueous extracts from (S) and (O) revealed higher levels of gallic and cinnamic acids, catechine acetate, rutine hydrate and Luteolin 7 glucoside, compared to those from (B) and (T) this may be explained by the low rainfall and high temperatures of the two harvesting site Seliana and Oueslatia (Table 3). In fact, the aqueous extracts of barks from (S) revealed higher levels of gallic acid 1,74 mg/mL, cinnamic acid 1,21 mg/mL, catechin acetate 2,61 mg/mL rutine hydrate 1,6 mg/mL and luteolin 7 glucoside 1,36 mg/ml (Table 3). Such flavonoids have strong allelopathic potent [47]. This may explain the high potential inhibitory of aqueous extracts of the barks collected from (S) and (O) on the germination and growth of target plants.

3.3. Chemical composition

Twenty-nine compounds were identified (Table 4), accounting for 94, 6-99, 7% of the aroma extract. The biomass of *Pinus halepensis* accumulate aroma compounds differently according to the geographic area: barks from Seliana and Oueslatia produced a higher numbers of monoterpene hydrocarbons, 10.5% (Table 5).

The major constituents of the volatile fraction from Oueslatia were *β*-caryophyllene (66.3%),

α-humulene (7.3%) (Table 4). However, barks from Tabarka showed a low percentage of β-caryophyllene (58.4%), α-humulene (1.4%) (Table 4). These compounds have been reported to have herbicidal activities [48]. Wang et al. [49] showed that (*E*)-caryophyllene at the dose of 3 mg/L significantly inhibited the germination rates and seedling growth of *Brassica campestris* and *Raphanus sativus*. Singh have demonstrated that exposure of seedling to α-pinene act to inhibited seedling growth causing oxidative damage in root tissue [50]. Barks from Oueslatia was characterized by the highest amount of sesquiterpene hydrocarbons (84.9%), made up by β-caryophyllene and α-humulene, and lowest amount of hydrocarbons monoterpenes (10.5%) (Table 4).

However, barks collected from Bizerte and Tabarka showed a low percentage, an average of (58.5%) for β-caryophyllene, (8.85%) for α-humulene (Table 4).

3.4. Effect of the aqueous extracts of *Pinus halepensis* barks on germination

In Table 6, the percentage of inhibitions obtained in the presence of the aqueous extracts of barks from the four sites. A more or less similar effect was recorded for the seeds of radish and wheat. It was noted that the inhibition, induced by the aqueous extracts of the barks on the germination of radish and wheat, increased with the augmentation of concentration of these extracts. At 10 g/L of the aqueous extract, recorded inhibitions of the germination of the seeds of radish were of 11.7% (B), 10% (T), 21.7% (S) and 16.7% (O). However at 30 g/L, the herbicide effect of the aqueous extracts was more announced and the reductions were of 20% (B), 18.35% (T), 28.35% (S) and 33.35% (O) (table 6).Several studies have shown that the inhibition degree increases with the augmentation of concentrations of the extract [22]. For all the concentrations, the site effect was shown; the inhibition of germination was more important for the aqueous extracts from (S) and (O), while the weakest reduction was recorded in the presence of the aqueous extract of the barks from (T). At 40 g/L, the seeds of wheat had an almost similar sensitivity towards the aqueous extracts of the barks of Bizerte, Tabarka and Oueslatia, with an average inhibition of 28.9%, but Seliana aqueous extract induced an inhibition of germination of 35% (Table 6). The richness of TPC, TFd, TPA and O-diphenols of the aqueous extract from (S) could explain the effect observed. Indeed, Bais et al. announced that the flavonoids have allelopathic effects [51]. The qualitative differences of these compounds in the extracts could contribute to different phytotoxicity rates [21].

At 40 g/L, the germination indexes recorded in the presence of the four extracts were similar for wheat and for radish with respective averages of 55.52 and 55.77; 64.08 and 56.96 at 30 g/L (Table 7). The aqueous extracts of the barks do not affect only the rate of germination, but also the extension of germination over longer periods. Similar observations were noted by Tiger et al. [52]. The presences of allelochemicals involve a delay of germination by disturbing mitochondrial breathing and metabolic enzymes implied in glycolysis and oxidative pentose phosphate pathway (OPPP) [21, 22, 53]. In addition, allelochemicals disturb peroxidase, alpha-amylase activities, cellular division and differentiation and the metabolism of phytohormones [54].

3.5. Effect of the aqueous extracts of the *Pinus halepensis* barks on the growth

3.5.1. On root growth

The lengths of the air parts or the roots are parameters usually used for the determination of allelopathic effects on the development of plants [52]. The results show a very significant effect of the aqueous extracts of the barks from B, T, S and O, even at weak concentrations, essentially in the presence of Seliana extract (Fig. 1).

The reduction of the root growth of wheat seedlings, at 10 g/L, ranged between 75 and 85% and between 95 and 99% at the strongest concentration, 40 g/L (Fig. 2). The growth of the roots in the presence of the aqueous extracts of the barks from the four sites, showed high inhibitions proportional to the concentrations (Fig. 2). Similar results were reported by Ladhari et al. [22]. Radish was shown to be more sensitive to the aqueous extracts of the barks of *Pinus halepensis* (Fig. 3).

Table 2. The total polyphenols (TPC), O-diphenols, Flavonoids, condensed tannins (TPA) content in the barks of *P. halepensis* collected from the forests of Bizerte (B), Tabarka (T), Seliana (S) and Oueslatia (O).

Site of sampling	Bizerte (B)	Tabarka (T)	Seliana (S)	Oueslatia (O)
TPC (mg GAE/g dw)	$71.76^{b**} \pm 1.05$	$68.64^{a**} \pm 2.03$	$75.34^{c**} \pm 2.04$	$71.46^{b**} \pm 1.09$
o-diphenols (mg eq tyrosol/g dw)	$4.75^{a} \pm 1.33$	$5.01^{a**} \pm 0.30$	$5.37^{a} \pm 0.37$	$6.11^{a} \pm 0.51$
flavonoids ((CEQ) /g dw)	$29.97^{a**} \pm 2.04$	$26.69^{a**} \pm 3.04$	$36.44^{b} \pm 0.44$	$29.71^{a} \pm 1.12$
TPA (CE mg /g dw)	$5.55^{ab**} \pm 0.95$	$5.04^{a**} \pm 1.05$	$5.85^{ab**} \pm 0.54$	$6.79^{b**} \pm 0.25$

All analyses are the average of three measurements ± standard deviation. The averages with the same letters in a column are not significantly different with P <0.05. ** indicates a significant Pearson correlation at the level 0.01 between TPC, o-diphenols, Flavonoids and the TPA of the barks of the same site.

Table 3. Phenolic acids contents (mg/mL) in the barks aqueous extracts of *Pinus halepensis.*

Compounds (mg/mL)	Barks from Bizerte	Barks from Tabarka	Barks from Seliana	Barks from Oueslatia
Gallic acid	$1.71^{b**} \pm 0,1$	-	$1.74^{b**} \pm 0.26$	$1.53^{b} \pm 0.43$
Catechin acetate	-	-	$2.61^{b} \pm 0.3$	$1.31^{c} \pm 0.69$
Catechine hydrate	$1.28^{b**} \pm 0.2$	$2.82^{c**} \pm 0.82$	-	-
Resorcinol	-	-	-	0.6
Chlogénic acid	-	0.91	-	-
Syringic acid	-	$1.67^{b} \pm 0.07$	-	$1.7^{b} \pm 0.4$
Hydroxy phenylacetate	-	-	2.09	-
Catechol	1.23	-	-	-
Rutine hydrate	-	-	$1.61^{b*} \pm 0.61$	$1.23^{b*} \pm 0.44$
Verbascoside	$1.12^{b**} \pm 0.07$	$1.45^{b**} \pm 0.31$	-	-
Luteolin 7 glucoside	$1.31^{a} \pm 0.63$	$1.45^{a**} \pm 0.1$	$1.36^{a} \pm 0.46$	$1.7^{a**} \pm 0.11$
Neringenin	-	$0.76^{b**} \pm 0.4$	-	$1.21^{c**} \pm 0.21$
Apegenin 7 glucoside	$2.73^{c**} \pm 0.94$	-	1.01	$1.31^{b**} \pm 0.31$
Fereulic acid	-	-	1.84	-
m-Coumaric acid	-	$1.54^{c} \pm 0.5$	-	$0.89^{b} \pm 0.2$
Phenylacetate	$0.15^{a**} \pm 0.1$	-	-	$0.61^{b**} \pm 0.4$
Resveratrol	0.13	-	-	-
Luteolin	$0.11^{b} \pm 0.06$	$0.07^{b} \pm 0.01$	-	-
Pinoresinol	$0.1^{a**} \pm 0.05$	$0.12^{a**} \pm 0.03$	$0.52^{a} \pm 0.48$	$0.31^{a} \pm 0.1$
Naphtoresorcinol	-	-	0.2	-
Cinamic acide	$0.65^{a**} \pm 0.3$	$0.5^{a**} \pm 0.29$	$1.21^{b**} \pm 0.21$	$1.34^{b**} \pm 0.43$
Apigenin	-	0.71	-	-
2,4.D Pestanal	-	0.15	-	-
Flavon	$0.21^{a**} \pm 0.01$	$0.02^{b**} \pm 0.02$	$0.05^{b**} \pm 0.03$	$0.06^{b**} \pm 0.04$
Total (mg/mL)	10.37	11.63	13.23	13.8

All analyses are the average of three measurements ± standard deviation. The averages with the same letters in a column are not significantly different at P <0.05. * indicates a significant Pearson correlation o at the level 0.05 and ** at the level 0.01 between compounds of the barks collected from the forests of Bizerte (B), Tabarka (T), Seliana (S) and Oueslatia (O).

Table 4. Composition of volatiles obtained from Barks of *Pinus halepensis* according to their different geographical origin (B.B: Barks from Bizerte; B.T: Barks from Tabarka; B.S: Barks from Seliana; B.O: Barks from Oueslatia) (L.R.I: Linear Index Retention).

Compound	L.R.I.	Barks from Bizerte	Barks from Tabarka	Barks from Seliana	Barks from Oueslatia
α-pinene	941	1.6	2.1	2.1	2.4
β-pinene	982	-	0.4	0.2	0.7
myrcene	993	1.2	0.8	1.3	1.8
δ-3-carene	1013	-	-	0.4	0.5
p-cymene	1028	0.4	0.5	0.4	0.4
limonene	1032	2.2	1.8	2	2.9
terpinolene	1090	1.2	1.1	1.1	1.8
linalool	1101	0.6	0.5	0.4	0.3
nonanal	1104	1.9	0.8	1.7	0.5
phenyl ethyl alcohol	1141	-	0.5	-	0.2
camphor	1145	0.6	0.4	0.2	0.2
4-terpineol	1178	0.8	0.9	0.9	0.8
isobornyl acetate	1287	-	0.4	0.3	0.4
α-cubebene	1353	1.3	1.4	1.1	0.8
β-copaene	1430	3.6	3.5	3.2	2.6
β-caryophyllene	1419	59.3	58.4	61.7	66.3
β-ylangene	1422	1.6	1.4	1.4	0.9
α-humulene	1455	8.4	9.3	10.4	7.3
(E)-β-farnesene	1459	-	-	0.3	0.3
alloaromadendrene	1462	-	-	0.3	0.2
γ-muurolene	1478	0.5	0.7	0.5	0.3
valencene	1493	1.6	1	1.4	0.5
α-muurolene	1501	1.7	2.9	1.4	0.9
δ-cadinene	1524	1.1	1.7	0.9	0.5
caryophyllene oxide	1582	1.2	3.6	3.3	1.9
humulene epoxide II	1607	0	0.7	0.5	0.2
γ-muurolene	1478	0.5	0.7	0.5	0.3
valencene	1493	1.6	1	0.4	0.5
α-muurolene	1501	1.7	1.9	1.4	0.9
Total identified		99.6	98.4	99.7	97.3

Table 5. Chemical composition groups of barks of *Pinus halepensis*

	Barks from Bizerte	Barks from Tabarka	Barks from Seliana	Barks from Oueslatia
Monoterpene hydrocarbons (%)	6.6	6.7	7.5	10.5
Oxygenated monoterpenes (%)	3.9	3	3.5	2.2
Sesquiterpene hydrocarbons (%)	82.9	83.9	84.9	82.3
Oxygenated sesquiterpenes (%)	1.2	4.3	3.8	2.1
Others (%)	0	0.5	0	0.2
Total (%)	94.6	98.4	99.7	97.3

Table 6. Summary table of the % of inhibition of germination of *Triticum aestivum* and *Raphanus sativus* in the presence of the aqueous extracts of the barks of *P. halepensis* from the four sources Bizerte (B), Tabarka (T), Seliana (S) and Oueslatia (O).

Site of sampling		% inhibitions of germination			
		Bizerte	**Tabarka**	**Seliana**	**Oueslatia**
wheat	10 g/L	$11.7^{a**} \pm 1.02$	$10^{a**} \pm 1.81$	$21.7^{a**} \pm 0.87$	$16.7^{a**} \pm 0,95$
	20 g/L	$23.35^{a} \pm 2.39$	$23.45^{c**} \pm 0.43$	$30^{b**} \pm 2.93$	$28.34^{b} \pm 1.76$
	30 g/L	$20^{a*} \pm 1.6$	$18.35^{b*} \pm 0.97$	$28.35^{b} \pm 1.35$	$33.35^{c} \pm 3.36$
	40 g/L	$30^{b} \pm 2.9$	$28.35^{d} \pm 2.25$	$35^{c} \pm 2.31$	$28.34^{b} \pm 1.36$
radish	10 g/L	$11.67^{a**} \pm 0.67$	$10^{a**} \pm 0.78$	$16.7^{a**} \pm 2.34$	$13.35^{a**} \pm 0.35$
	20 g/L	$16.34^{b**} \pm 0.91$	$14.35^{b**} \pm 2.35$	$20^{b} \pm 1.43$	$18^{b} \pm 0.61$
	30 g/L	$23.35^{c} \pm 3.35$	$22.35^{c} \pm 0.42$	$26.7^{c**} \pm 0.72$	$28.34^{c**} \pm 0.83$
	40 g/L	$30^{d*} \pm 0.49$	$28.7^{d*} \pm 0.46$	$30^{d**} \pm 0.35$	$29.34^{c**} \pm 2.76$

All analyses are the average of three measurements ± standard deviation. The averages with the same letters in a column are not significantly different with P <0.05. * indicates a significant Pearson correlation at the level 0.05 and ** at the level 0.01between tests having the same concentrations of each target species.

Table 7. Summary table of the indices of germination of *Triticum aestivum* and *Raphanus sativus* in the presence of the aqueous extracts of the barks of *P. halepensis* from the four sources Bizerte (B), Tabarka (T), Seliana (S) and Oueslatia (O).

Site of sampling		Indices of germination (GI)			
		Bizerte	**Tabarka**	**Seliana**	**Oueslatia**
wheat	10 g/L	$74.53^{c} \pm 3.1$	$80.61^{c**} \pm 3$	$58.75^{b} \pm 3.04$	$65.55^{b**} \pm 2.04$
	20 g/L	$64.21^{b} \pm 2$	$66.27^{b**} \pm 2.7$	$60.27^{b} \pm 1.09$	$54.56^{a**} \pm 4.36$
	30 g/L	$67.49^{b} \pm 3.51$	$66.7^{b} \pm 1.07$	$59.85^{b} \pm 4.08$	$62.3^{b} \pm 0.7$
	40 g/L	$54.44^{a} \pm 4$	$58.6^{a} \pm 0.95$	$52.48^{a**} \pm 2.02$	$56.59^{a**} \pm 0.84$
radish	10 g/L	$81.93^{d**} \pm 1.05$	$75.56^{c} \pm 5.07$	$73.65^{c**} \pm 2.04$	$73.35^{a**} \pm 3.77$
	20 g/L	$52.88^{c} \pm 3.01$	$61.98^{b**} \pm 0.27$	$66.25^{b**} \pm 0.09$	$66.45^{a**} \pm 2.96$
	30 g/L	$61.66^{b**} \pm 2.05$	$54^{a**} \pm 5$	$55^{a**} \pm 2.04$	$57.2^{a**} \pm 1.63$
	40 g/L	$55.33^{a**} \pm 3.05$	$57.41^{a*} \pm 4.01$	$55.43^{a**} \pm 1.94$	$56.92^{b*} \pm 0.94$

All analyses are the average of three measurements ± standard deviation. The averages with the same letters in a column are not significantly different with P <0.05. * indicates a significant Pearson correlation at the level 0.05 and ** at the level 0.01between tests having the same concentrations of each target species.

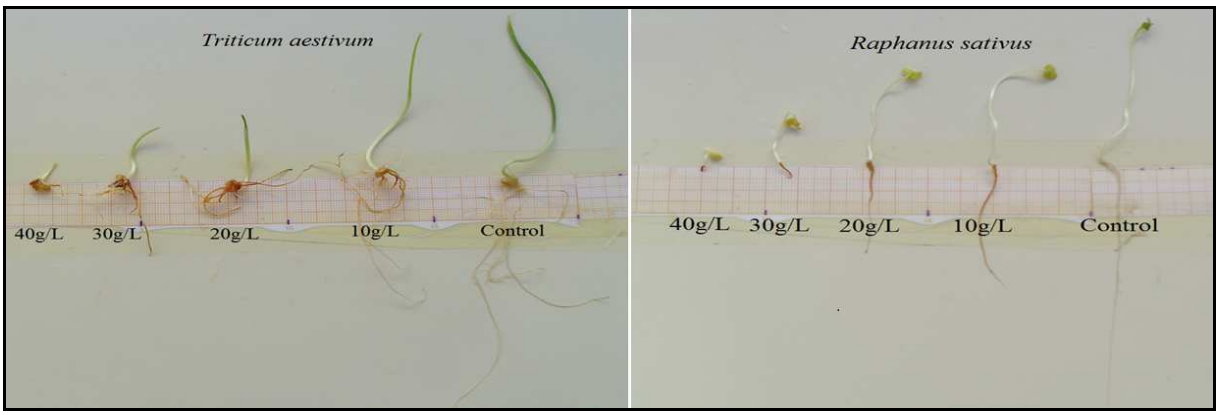

Figure 1. Effect of aqueous extract of *Pinus halepensis* barks from Seliana on the growth of *Triticum aestivum* and *Raphanus sativus.*

Indeed, Prati and Bossdorf indicated that the degree of allelopathic interference is specific to the species and can even vary within the same species [55]. At 10 g/L, the barks aqueous extract caused an inhibition ranging between 87 and 92% (Fig. 3). At 40 g/L, inhibitions exceeded 94% and reached 97% in the presence of the aqueous extract from (S). In all tests, the site effect was elucidated and a higher toxicity rate was attributed to the extracts from (S) and (O) (fig.1). These extracts are richer in TPC, TPA, TFd and o-diphenols. The allelopathic effect is due mainly to phenolic compounds [56].

3.5.2. On the air parts growth

At 10 g/L, the recorded reductions in the growth of the air parts of wheat, in the presence of the aqueous extracts of the barks of *Pinus halepensis*, were respectively of 6%, 4%, 20% and 14% for the extracts of the barks from Bizerte, Tabarka, Seliana and Oueslatia (Fig. 2). At 40 g/L, inhibitions of the seedlings growth ranged between 70% and 82%. Once again, the air parts of radish were more vulnerable, compared to those of wheat and inhibitions exceeded 40% at the weakest concentration (Fig. 2). It reached 59% in the presence of the extract from (S). At the highest concentration, the reductions of the air parts were between 91% and 97%. The roots of the two target species (wheat and radish) were more affected compared to the shoots (fig.1). Indeed, during the absorption of water, a low amount of the solution is available for the stem cells and the leaves [6], that's why they are less affected than the roots. The allelochemicals in the aqueous extracts reduce the length of the seedlings by the inhibition of the cellular division and elongation, acting on the expression and the synthesis of the DNA and the RNA [57, 58]. The aqueous extracts of the barks from (S) and (O) were most toxic on the air parts which can be partly explained by their richness in phenolic compounds compared to those from (B) and (T). Indeed the production and release of allelochemicals depend on temperature and rainfall [6]. These allelochemicals act on the meristematic cells by the reduction in lengthening [59].

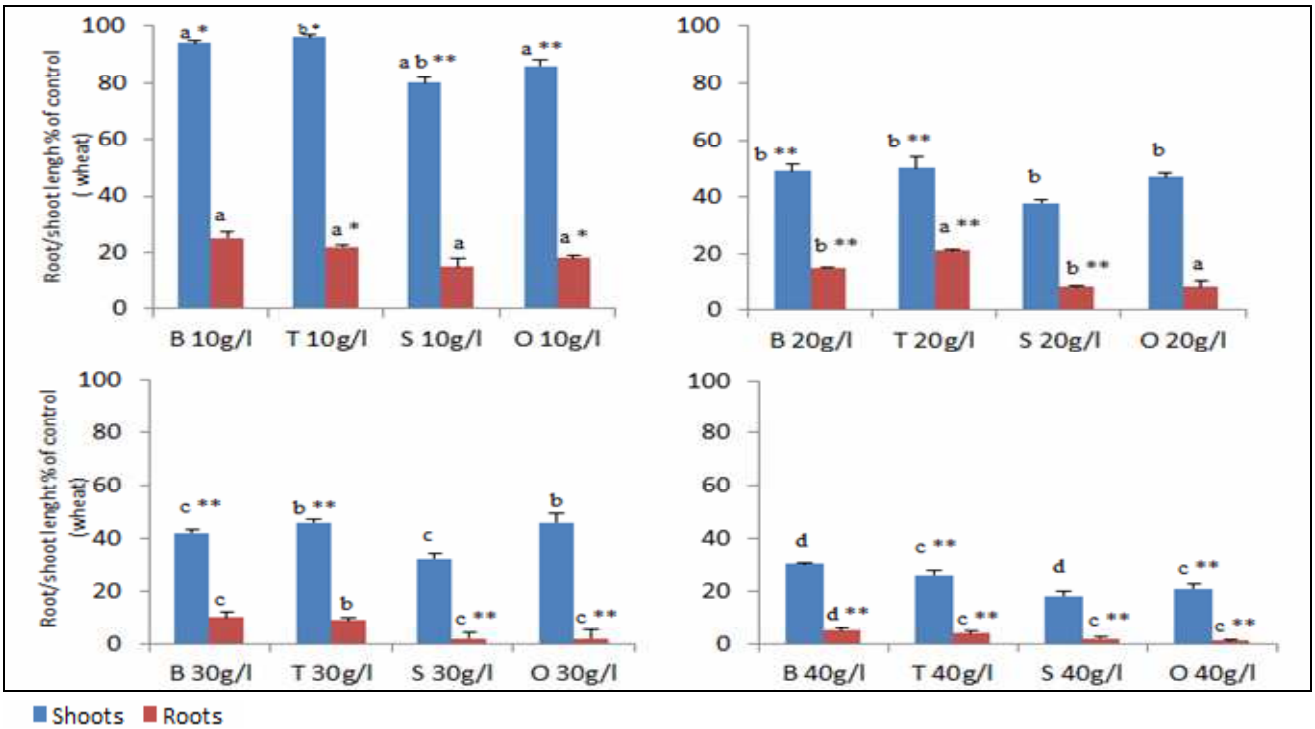

Figure 2. Summary table of the effect of the aqueous extracts of barks of *Pinus halepensis* from the four sources B, T, S and O on the growth of the roots and of the air parts of the seedlings of *Triticum aestivum*.

All analyses are the average of three measurements ± standard deviation. The averages with the same letters in a column are not significantly different with P <0, 05. * indicates a significant Pearson correlation at the level 0.05 and ** at the level 0.01between tests having tests the same concentrations of each target species.

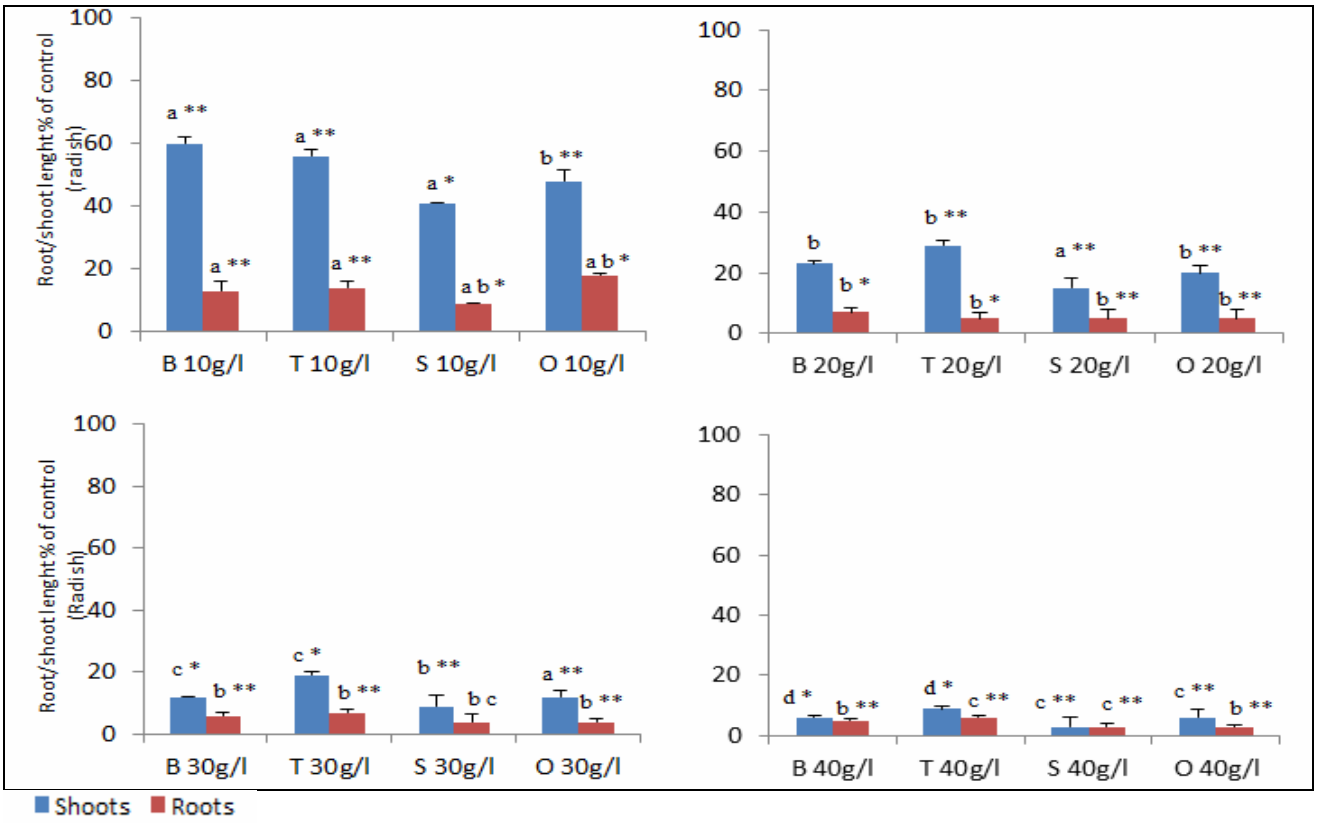

Figure 3. Summary table of the effect of the aqueous extracts of barks of *Pinus halepensis* from the four sources B, T, S and O on the growth of the roots and of the air parts of the seedlings of *Raphanus sativus*.
All analyses are the average of three measurements ± standard deviation. The averages with the same letters in a column are not significantly different with P <0, 05. * indicates a significant Pearson correlation at the level 0.05 and ** at the level 0.01between tests having tests the same concentrations of each target species.

3.6. Activity in soil

3.6.1. Effects of the irrigation with aqueous extracts from P. halepensis barks on seedlings growth

Aqueous extracts of the *P. halepensis* barks, from (B, T, S, and O), were prepared at two concentrations (20 and 40 g/L), and were used to irrigate the pots where the two target plants wheat and radish were cultivated. In fact, Omezzine et *al*. used cultures in pots in order to show the effects of aqueous extracts to show reproducibility of results under natural conditions and to evaluate the biological activity of allelochemical compounds released by the vegetal residues [6]. In our work, the irrigation with the barks aqueous extract involved a very marked reduction in the growth of the roots of wheat, especially at 40 g/L (Fig. 4). Inhibition increased proportionally with the augmentation of concentration of the extract. Indeed, the extracts of the barks at 20 g/L caused reductions in wheat roots growth ranging from 75.9% to 85.5% (Table 7). For the radish roots, which are more sensitive to the extracts, inhibitions were comprised between 94.5% and 99.2%, which proves a different behavior of the roots according to the species targets in the presence of the allelochemicals with a strong sensitivity of radish (table 7). The extract coming from Seliana was the most toxic and that of Tabarka was the least. At 40 g/L there was an almost total stop of radish root growth in the presence of the extracts from the four sites. At this concentration, the ecological site effect appeared in wheat and the extracts from (S) and (O) proved their higher toxic power higher. The respective length reduction of the roots of wheat and radish were of 95.1% and 92.4% (table 7). Compared to that of the roots, the growth of the shoots of wheat and radish were less affected by the irrigation with the aqueous extracts of the *Pinus halepensis* barks. At 20 g/L, the reduction did not exceed 24.6%, in the presence of the aqueous

extract from S and growth inhibition (10.8%) was induced by the extract from (B). At 40 g/L, the site effect of the aqueous extracts of the barks showed that the one prepared from the biomass of Seliana was most toxic on the growth with inhibition of 62.6%. An average of 51.5% was recorded for the extracts from the other sources. At the same concentration, the reduction of the growth of the radish shoots for the extract from (S) was of 84.7% compared to the average of 73.1% of the aqueous extracts of the barks from Bizerte, Tabarka and Oueslatia (Table 7). Our results are in agreement with those shown by Omezzine et al and Seal et al. [6, 60].

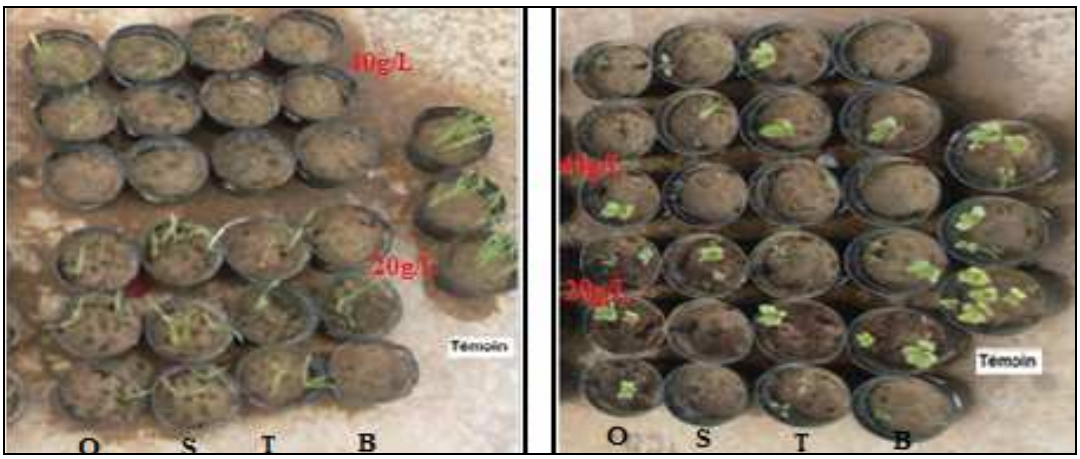

Figure 4. Culture of *Triticum aestivum* and *Raphanus sativus* on soil irrigated with aqueous extract of barks of *P. halepensis* from the four sites (B, T, S and O), 20 days after incorporation.

Table 7. Summary table of the effect of irrigation with aqueous extracts of the barks of *Pinus halepensis*, from the four sources, Bizerte, Tabarka, Seliana and Oueslatia incorporated in the soil at 20 g/L and 40 g/L, on the growth of the roots and the air parts of the seedlings of *Triticum aestivum* and *Raphanus sativus.*

Aqueous Extract (g/L)	% inhibitions of roots growth				% inhibitions of shoots growth			
	Bizerte	**Tabarka**	**Seliana**	**Oueslatia**	**Bizerte**	**Tabarka**	**Seliana**	**Oueslatia**
	Triticum aestivum							
20	75.9^{c**} ±2.4	78.7^{c**} ±1.5	85.5^{c**} ±1.4	82.1^{c**} ±1.2	10.8^{a*} ±0.4	16.4^{a*} ±1.2	24.6^{a**} ±1.3	20.9^{a**} ±0.9
40	85.3^{d*} ±2.9	79.7^{c*} ±0.9	95.1^{d*} ±0.9	92.4^{d} ±0.5	51.5^{b*} ±1.2	50.1^{b*} ±1.1	62.6^{b**} ±2.6	53.2^{b**} ±0.2
	Raphanus sativus							
20	96.6^{c**} ±2.4	94.5^{c} ±1.5	99.2^{c**} ±1.4	96.6^{c**} ±1.1	38.6^{a} ±0.4	44.5^{a**} ±1.2	58.9^{a**} ±1.3	38.6^{a**} ±0.9
40	97.3^{d} ±2.9	97.7^{c**} ±0.9	99.8^{d**} ±1.0	97.9^{d**} ±0.5	74.4^{b} ±1.2	70.9^{b**} ±1.1	84.7^{b*} ±2.6	74.4^{b**} ±0.2

All the analyses are the average of three measurements ± standard deviation. The averages with the same letters in a column are not significantly different with P <0.05. *indicates a significant Pearson correlation at level 0.05 and ** at level 0.01 between tests having t the same concentrations of each target species

3.6.2. Effect of the incorporation of P. halepensis bark powder in the soil on the growth of the target plants

The powder of the *P. halepensis* barks of the four sources (B, T, S, and O) was mixed with a soil sample in two amounts (50 g/kg and 100 g/kg) in order to see whether the effects recorded in bioassays are reproducible in experiments in pots. The same target plants were retained (wheat and radish).

The results related to the effect of the incorporation of the powder in the soil, on the growth of wheat and radish showed that the biomass

of the pine of Alep is very toxic (Table 8) with reductions ranging from 98.8% to 100% in the presence of the powders from B, T, S and O. The effects of the four types of biomasses are comparable for the two target plants and the sensitivity of the air parts was similar to that of the roots. The richness of the *Pinus halepensis* barks powder in polyphenols, flavonoids and allelochemical substances, explains the strong herbicide power of this biomass. Our results are in agreement with those reported in bibliography. Once in the ground, allelochemicals interfere with the neighbouring plants [61, 62] act in the stage the pre- and post-emergence of seedlings, and on the bank of seeds [11].

Table 8. Summary table of the effect of the powder of the barks of *Pinus halepensis* from the four sources, Bizerte, Tabarka, Seliana and Oueslatia incorporated in the soil at 50g/kg and 100 g/kg, on the growth of the roots and the air parts of the seedlings of *Triticum aestivum* and *Raphanus sativus*.

Dose (g/kg)	**Bizerte**	**Tabarka**	**Seliana**	**Oueslatia**	**Bizerte**	**Tabarka**	**Seliana**	**Oueslatia**
	% inhibitions of the shoots growth wheat				**% inhibitions of the roots growth wheat**			
50	99.9^{a}*±0,1	99.7^{a**}±0,2	99.8^{a**}±0.2	99.9^{a**}±0.2	99.8^{a*}±0.6	99.6^{a*}±0.0	99.7^{a*}±0.4	99.7^{a*}±0.4
100	100^{a}	99.5^{a**}±0,3	100^{a**}	100^{a**}	100^{b**}	99.6^{a}±0.18	100^{b}	100^{b}
	% inhibitions of the shoots growth radish				**% inhibitions of the roots growth radish**			
50	99.7^{a**}±0.3	100^{b**}	100^{a}	98.9^{a**}±0.0	99.8^{a*}± 0.8	100^{b}	100^{b}	98.8^{a*}± 0.7
100	100^{a}	100^{b}	100^{a**}	100^{a**}	100^{b}	100^{b}	100^{b}	100^{b}

All the analyses are the average of three measurements ± standard deviation. The averages with the same letters in a column are not significantly different with P <0, 05. ** At the level 0.01 between tests having the same concentrations of each target species.

4. Conclusion

The objective of this study was to evaluate the allelopathic potential of the *Pinus halepensis* barks collected from the pine forests of Bizerte (B), Tabarka (T), Seliana (S) and Oueslatia (O). Indeed the two littoral sites (B) and (T) are characterized by a rainfall higher than 1100 mm/year, whereas the two other continental sites (S) and (O) receive only 400 mm/year. The average temperatures of Bizerte and Tabarka are of 11.7°C in winter and 24.6°C in summer whereas for the two other sites they are of 12.3°C and 28°C. The aqueous extract of the *Pinus halepensis* barks from (O), prepared at a concentration of 40 g/L, caused an inhibition of 33.35% of the radish seeds germination. At the same concentration the aqueous extract from (S) induced a reduction of 30% of the germination of the wheat seeds. The ecological site showed a high toxicity effect of the continental extracts (S and O) compared to aqueous extracts of the littoral sites (B and T). Aqueous extracts of the *Pinus halepensis barks* from (S) and (O) have a higher toxicity level and are richer in TPC, TPA, and TFd and in o-diphenols than those from (B) and (T). Phenols, the derivatives of the benzoic and cinnamic acids, flavonoids and tannins are substances having an allelopathic activity [63]. However these chemical products are not toxic for the donor plant [57]. Pine bark is rich in phenolic compounds [64]. The main tannin structures found in maritime pine bark are catechin/epicatechin, epigallocatechin and epicatechin gallate [65]. Indeed, several species of pine showed a strong allelopathic potential [1]. The results showed a very high allelopathic potential in the aqueous extracts of the barks of the pine of Alep from (O) inhibiting the root growth of wheat by up to 99% at a concentration of 40 g/L and of 97% for the roots of radish. The inhibitions induced by the aqueous extracts of the barks on the germination of radish and wheat increased with the increasing concentrations. The root growth is an excellent indicator of the phytotoxic effect of allelochemicals [6, 52]. Exposed directly to the aqueous extract, rich in allelochemicals, the root cells are more affected [21]. A higher permeability of the roots to

allelochemicals was demonstrated when compared to that of the air parts [66]. That's because they are the first to absorb environmental allelochemicals [67]. The air parts of the two target species (wheat and radish) were less affected, compared to the roots. At 40 g/L growth inhibitions of the air parts of wheat ranged between 70% and 82%, with a maximum reduction in the presence of the aqueous extract from (S). The same extract at the same concentration was the most harmful for the growth of radish with an inhibition of 97%. A wealth of rutin hydrate and luteolin 7 glucoside for aqueous extracts from (S) and (O), the average concentration was respectively 1.42, 1.53 mg/mL. Results of previous studies showed that the length reduction of the air parts and the roots was directly related to the action of allelochemicals, which is a proof of their effect [11]. This reduction can be attributed to the reduced rate of cellular division and the elongation of the cells due to the presence of allelochemicals in the aqueous extracts [22] which act on cellular differentiation, the absorption of ions and water, breathing, photosynthesis, enzymatic function, and the transduction of the signal as well as the form of the genes [54]. A strong inhibition of seedling growth by aqueous extracts from (S) and (O) may be due to the wealth of cinnamic and gallic acids. The average is respectively 1.63 and 1.27 mg/mL. The cultures in pots irrigated with the aqueous extracts of the *Pinus halepensis* barks from B, T, S and O, significantly inhibited the growth of the seedlings of Wheat and Radish. Cultures in pots were opted in order to demonstrate the effects which could be reproduced under natural conditions [68] and to evaluate the biological activity of allelochemical compounds released by the vegetal residues [21]. At 20 g/L, the aqueous extracts of the barks (S) induced inhibitions of the Wheat roots by up to 85.5%, it was of 96.6% for those of Radish in the presence of the extract from (O) compared to the controls. At the same concentration, the air parts were less affected by the irrigation with the aqueous extracts of the barks of the Alep pine. These results corroborate with those of Omezzine et al. [21] which proved that the roots are more sensitive than the air parts with a much higher phytotoxicity when the concentration increases. The high toxicity of the aqueous extract of the barks collected from the Oueslatia can be explained by the fact that the effectiveness of allelopathic compounds in the soil is very dependent on the biotic and abiotic conditions [69]. The results relating to the effect of the incorporation of the powder on the soil, on the growth of Wheat and Radish showed that the biomass of the Alep pine is very toxic with reductions ranging between 98.8% et 100% in the presence of the powders from B, T, S and O.

The richness of the *Pinus halepensis* barks residues in polyphenols and flavonoids, allelochemical substances, explains the strong herbicide power of this biomass. In fact our results are in agreement with those reported in the bibliography. Once in the soil, the allelochemicals interfere with the neighboring plants [64, 70] acting on the pre and post-emergence stages, and on the bank of seeds [11, 71]. Similar results showed that the incorporation of the residues in the soil or the irrigation with aqueous extracts of *Inula crithmoides* L. of *Pine Wollemi*, fruit peels of the coffee [6, 11, 60] led to the inhibition of the growth of several plant species. Compared with in vitro results, the allelopathic tests in vivo, in the soil, were less toxic and the growth of the target species was less affected. The soil micro-organisms can also play a part in the allelochemical released in the ground [69, 72]. The major constituents of the volatile fraction of green needles collected from Oueslatia were β-caryophyllene, α-humulene, compounds have been reported to have herbicidal activities, by reductions of the growth of the shoots and roots. Allelochemicals causes several damages [73, 74].

Our results showed the strong herbicide power of the aqueous extracts *in vitro*, which was proven by the test *in vivo* by the irrigation with the aqueous extracts or the incorporation in the soil of the vegetal powder which could be used like an organic herbicide.

Indeed, as a reaction to the increase in the resistance of weeds to the pesticides of synthesis [63], there has been a growing interest in the last decades in compounds having allelopathic properties [52] which can lead to the discovery of natural weed herbicides which do not damage to the environment [20, 75] but are effective against weeds that have become resistant to many synthetic herbicides [23].

ACKNOWLEDGEMENT

This research project was supported by a grant from the Research Laboratory "Bioressources: Integrative Biology and Valorisation", Higher Institute of Biotechnology of Monastir, Tunisia.

AUTHORS' CONTRIBUTION

RT: Acquisition of data, writing, analysis and interpretation of data of the manuscript. HC: Administrative, technical support. FG: GC-MS analyzes. AL: Administrative support. Ahmed NH: revision of the manuscript. All authors read and approved the final of the manuscript.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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