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Research Article

Antioxidative changes in *Citrus reticulata* L. induced by drought stress and its effect on root colonization by arbuscular mycorrhizal fungi

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ABSTRACT

The objective of the study is to understand drought induced oxidative stress scavenging mechanism in *Citrus reticulata* in order to understand the interplay between different biochemical components and antioxidants within plant tissue and its effect on arbuscular mycorrhizal fungal diversity and spore density. For this, citrus plants were subjected to water stress by avoiding watering plants for different time periods. Different biochemical and physiological parameters were measured following water stress. Biochemical components such as total sugar and total soluble protein increased along with decline in relative water content and total chlorophyll content during water stress. Accumulation of foliar non enzymatic antioxidants such as carotenoid and ascorbic acid as well as proline were found to be gradually enhanced under water stress. There were significant changes in the activities of antioxidative enzymes such as peroxidase, ascorbate peroxidase, glutathione reductase, catalase, and superoxide dismutase. The stress signals like hydrogen peroxide and malonaldehyde increased under water deficit condition as a response to oxidative damage promoted by lipid peroxidation under elevated free radical formation. Besides, percentage root colonization by arbuscular mycorrhizal fungi and number of spores increased markedly under prolonged drought. The results suggested that plant can withstand drought induced oxidative stress up to certain period, manifested by elevated level of non enzymatic antioxidant content and upregulation of antioxidative enzymes. Increased AMF colonization might also have some role in protecting the plants from drought induced oxidative injury.

Keywords: Drought; *Citrus reticulata*; Reactive oxygen species; Oxidative stress; Non-enzymatic antioxidants; Antioxidative enzymes; Arbuscular mycorrhizal fungi.

1. INTRODUCTION

Citrus or Mandarin is one of the important horticultural crops cultivated in Darjeeling hills, India. Similar to other crops, drought adversely affects plant growth and fruit production of citrus [1]. Exposure of plants to water stress results in production of reactive oxygen species, such as hydrogen peroxide (H_2O_2), superoxide anion radicals ($O^{-}{}^{2}$), hydroxyl radicals (OH), alkoxy radicals

(RO) and singlet oxygen and also increase lipid peroxidation [2] ultimately leading to cell death. Excessive photorespiration during drought induced oxidative stress is the reason behind 70% of total H_2O_2 accumulation [3]. In order to cope up with oxidative stress plant produces different antioxidative enzymes, such peroxidase, ascorbate peroxidase, catalase, glutathione reductase etc. [4]. Non enzymatic antioxidants such as carotenoids and ascorbic acid also accumulate during drought. Accumulation ascorbic acid and H_2O_2 within vacuoles play crucial for ROS scavenging activity in *Arabidopsis thialiana* during water stress [5]. Besides, plants accumulate osmolytes such as proline and total sugar during drought stress which protect plants from dehydration and oxidative injury.

Another kind of stress protective mechanism occurs in nature in association with plant that is plant mycorrhiza. [6]. Arbuscular mycorrhizal fungi (AMF), mainly belonging to the phylum Glomeromycota remain in mutualistic symbiosis between plant roots. AMF colonize around 75% of plant species [7] and help in the uptake of nitrogen and phosphorus, provide tolerance to drought and also help in defense against [8-10]. AM fungi also help to enhance antioxidant activity within plants [11, 12] and also soil water retention capacity [8, 13]. They reduce lipid peroxidation in drought affected plants [14] by induction of different antioxidants, and improving water and mineral uptake from soil [15]. According to Youpensuk et al. [16] *Glomus sensulata* species are commonly found in the citrus rhizosphere. Present study was undertaken to understand interplay between different biochemical parameters and antioxidative signaling during water stress and role of water stress on root colonization and diversity of arbuscular mycorrhiza.

2. MATERIAL AND METHODS

2.1. Experimental design

Two year old *Citrus reticulata* plants, grown in pots were selected for the experiments. Plants were maintained in glass house with proper watering and aeration. Plants were subjected to drought by avoiding watering for 3, 6, 9 days. There were ten replicate pots for each treatment. Leaves from these plants were sampled for different biochemical measurements. In another set of experiments, diversity and population measurement of arbuscular mycorhizal fungi were carried out in drought condition by withholding water of soil. Random plot designing method was followed for the experimental set up in this experiment, where in, two different plots each for control and treatment consisting of 3 columns 10 rows were maintained. In the control plot regular watering was done whereas in the treatment watering was withdrawn to maintain the drought condition for the desired period. Effect of drought on AMF spore density was checked at every 15 day interval till the 45th day when complete wilting of plants was observed.

2.2. Determination methods

2.2.1. Relative water content (RWC)

This was calculated by determining the fresh weight, turgid weight and dry weight of leaf samples under treatment and using formula given by Farooqui et al. [17].

RWC (%) = [(fresh weight - dry weight) / (fully turgid weight - dry weight)] x 100

2.2.2. Extraction and estimation of soluble proteins

Extraction was carried out from leaf tissue in ice cold condition by using 0.05 M sodium phosphate buffer (pH 7.2) following method of Chakraborty et al. [18]. Quantitative estimation of protein was done by the method of Lowry et al. [19] using a standard curve of BSA (Bovine serum albumin).

2.2.3. Extraction and estimation of chlorophyll

Extraction of chlorophyll from the leaves was done according to the method of Harborne [20]. Leaf tissue was homogenized in 80% acetone and filtered in a dark chamber. Filtrate was collected and estimation of chlorophyll was done by measuring the OD of the filtrate at 663 nm and 645 nm respectively in a UV-VIS spectrophotometer and calculated using the formula as given by Arnon [21].

2.2.4. Extraction and estimation of total and reducing sugar

Total and reducing sugar was extracted using Harborne [20] method with minor modifications. Leaf tissue was extracted in 95% of ethanol. Estimation of total sugar was done by Anthrone's method and that of reducing sugar by Somogyi-Nelson method as given by Plummer [22].

2.2.5. Extraction and estimation of proline

Proline content was determined according to the method of Caverzan et al. [23]. Free proline was extracted from 0.5 g of fresh tissue in 10 ml sulphosalicylic acid (3%) and the extract was filtered through Whatman no. 1 filter paper. A known quantity of the filtrate was mixed with 2 ml of acid ninhydrin reagent. The contents were boiled for 1 h in a boiling water bath and cooled rapidly on ice. The color was extracted in 4 ml toluene by vigorous shaking and the organic phase recorded at 520 nm against toluene as blank. Standard curve was prepared for different concentrations of proline.

2.2.6. Extraction and quantification of antioxidative enzymes

For extraction of peroxidase (POX, EC 1.11.1.7), ascorbate peroxidase (APOX, EC 1.11.1.11) and catalase (CAT, EC 1.11.1.6), leaf tissue was crushed in 5 ml of ice cold 50 mM sodium phosphate buffer, pH 7.2 containing 1% (w/v) polyvinylpyrrolidone. Homogenate was centrifuged at 10,000 rpm for 20 minutes at -4°C and supernatant was used for enzyme assays. POX activity was measured spectrophotometrically following the method described by Chakraborty et al. [18] with some modifications, where in the oxidation of O-dianisidine was monitored. In case of APOX, the method described by Asada and Takahashi [24] was followed by determining the quantity of ascorbate oxidized. Catalase activity was assayed by quantifying the amount of H_2O_2 broken down [25].

Extraction of glutathione reductase (GR, EC1. 6.4.2) and superoxide dismutase (SOD, EC1.15.1.1) from leaf tissue was carried out in ice cold 50 mM potassium phosphate buffer, pH 7.6 containing 1% (w/v) polyvinylpyrrolidone. Homogenate was then centrifuged at 10,000 rpm for 20 minutes at -4°C and supernatant was then used as crude enzyme source. GR activity was assayed by using the method described by Lee and Lee [26] by calculating the oxidation of NADPH at 340 nm. SOD activity was assayed following the method of Dhindsa et al. [27] with some minor modifications. One Enzyme Unit was defined as 1 absorbance per min.

2.2.7. Isozyme analysis

Standard PAGE analysis as described by Davis et al. [28] was done for isozyme analysis of peroxidase and staining procedure as described by Reddy and Gasber [29] was followed.

2.2.8. Extraction and quantification of non enzymatic antioxidants

2.2.8.1. Carotenoids

Carotenoids were extracted and estimated following the method described by Lichtenthaler [30]. Leaf tissue was extracted using 100% methanol in the dark and the extract was filtered. Absorbance of the filtrate was immediately noted at multiple wavelengths at 663, 645 and 480 nm in a VIS spectrophotometer.

2.2.8.2. Ascorbate

Ascorbic acid was extracted following the method described by Mukherjee and Choudhuri [31]. Leaves were extracted in 6% TCA under ice cold condition and filtered. Ascorbate was quantified using method as described by Mukherjee and Choudhuri [31]. Reaction mixture consisted of sample, 2% DNPH (in 0.5N HCl) and 1 drop of 10% thiourea (in 70% ethanol). It was kept in boiling water bath for 15 minutes and cooled at 0°C followed after addition of H_2SO_4. Absorbance of the sample was measured at 530 nm in a VIS spectrophotometer. The concentration of ascorbate was calculated from a standard curve plotted with known concentrations of ascorbic acid.

2.2.9. Determination of lipid peroxidation

Malonealdehyde is an end product of lipid peroxidation and its accumulation is an indication of

lipid peroxidation. It was measured by thiobarbiturate reaction where leaf tissue was homogenized in 0.1% (w/v) TCA. Estimation was done by following the method of Heath and Packer [32] by taking absorbance at 600 and 532 nm after and MDA was quantified using extinction coefficient of 155 mM^{-1} cm^{-1}.

2.2.10. H_2O_2 content

H_2O_2 content was measured by method described by Jena and Choudhuri [33]. Leaf tissue was crushed in 50 mm potassium phosphate buffer (pH 6.5), which was then centrifuged at 2,415 g for 25 mins and supernatant was taken for H_2O_2 quantification.

2.2.11. Isolation and identification of AMF sporesand determination of root colonization

Spores of arbuscular mycorrhizal fungi were isolated from rhizosphere soil by wet sieving and decanting method [34]. Approximately 250 g of soil was suspended in 1 L water. Heavier particles were allowed to settle for a few seconds and the liquid was decanted through sieves of decreasing size (BS 60, BS 80, BS 100, BS 150 and BS 200). Mycorrhizal spores extracted from soil sample were observed under dissecting microscope. Spore identification was done based on spore attachment, size, and color of spore using the monograph and the manual of Gardemann and Trappe [35] and Trappe and Schenck [36]. Poly vinyl lacto glycerol (polyvinyl alcohol + lactic acid + Glycerol) was used to prepare semi-permanent slides which affect certain spore characteristics, such as wall characteristics and spore color, spore diameter, attachment present, hyphal wall thickness etc. Isolated spores were stored in Ringer's solution at 4°C. In order to determine the spore population, 100g of soil was suspended in a liter of water. After sieving, the spores obtained were counted. The average spore population and percent root colonization were determined. Spores were identified morphologically by examining their variation in size, colour, wall thickness, shape, wall layers specially germinal wall, coriaceous wall, amorphous wall and beaded wall layers, hyphal branching patterns, the diameter, structure and the staining intensity of hyphae. For root colonization determination, small pieces of roots were cleared using 10% KOH, stained with Trypan Blue and cleared as described by McGonigle et al. [37], following which slides were prepared and observed under light microscope.

2.2.12. Statistical analysis

Data were analysed by using Standard Error and LSD tests at P = 0.05 probability level.

3. RESULTS

Citrus plants showed visible symptoms from 6th day of water stress. Leaves began to turn yellowish from 6th day and on 9th day due to severe water stress leaves became crisp and wilted. Both soil moisture content and relative water content and gradually but significantly decreased with water stress. Compared to control decline in RWC in leaf tissue was 1.118, 1.440, 1.840 folds on 3rd, 6th, and 9th days respectively (Table 1).

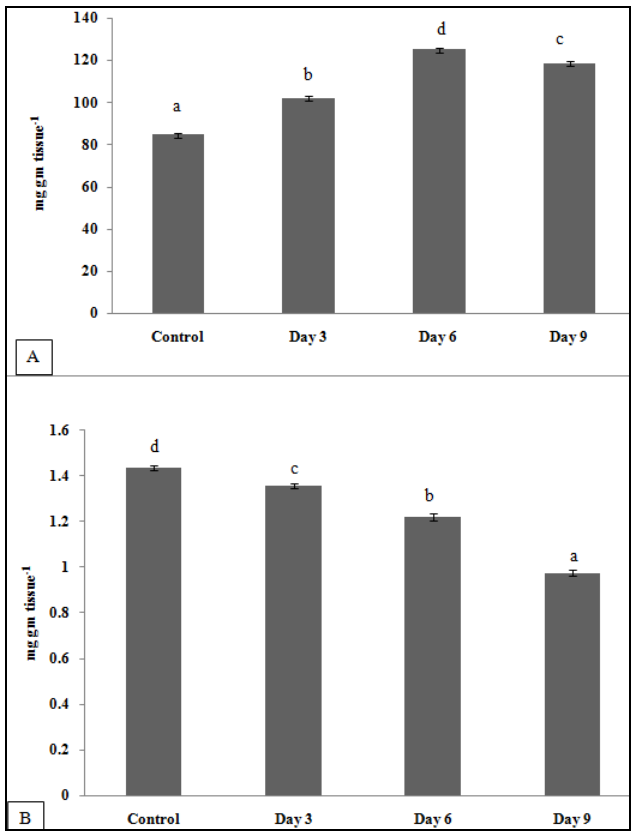

Figure 1. Protein (A) and chlorophyll (B) contents of leaves of citrus subjected to water stress. Different letters over each bar expresses significance at p<0.05 level (LSD test).

Table 1. Effect of different periods of water stress on relative water content of leaves and soil moisture content.

Treatment	Duration of stress (days)	Relative water content (%)	Soil moisture content (%)
Control	0	68.96±0.40[d]	37.14±0.24[d]
Water stress	3	58.40±0.15[c]	27.39±1.17[c]
	6	47.58±0.40[b]	20.17±1.21[b]
	9	37.39±0.36[a]	09.08±0.46[a]

Mean ± standard error; n=10; Means followed by same superscript in each column expresses insignificance at $p<0.05$ level (LSD test).

Table 2. Effect of different periods of water stress on sugar content of leaves.

Treatment	Duration of stress (days)	Total sugar content (mg g tissue^{-1})	Reducing sugar content (mg g tissue^{-1})
Control	0	8.27±0.83[a]	4.59±1.15[a]
Water stress	3	20.66±1.10[c]	8.68±1.15[d]
	6	23.18±0.59[d]	6.82±0.54[bc]
	9	10.44±0.27[b]	6.49±0.92[b]

Mean ± standard error; n=10; Means followed by same superscript in each column expresses insignificance at $p<0.05$ level (LSD test).

3.1. Effect of drought on biochemical components

3.1.1. Protein and chlorophyll

Total protein content showed gradual but statistically significant increase during 3, 6 and 9 days of water stress (Fig. 1). Total chlorophyll content in leaf tissue gradually declined during water stress treatment suggesting adverse affect of drought induced oxidative stress. In leaves of control plants total chlorophyll content was 1.435 mg/g tissue, which reduced to 0.976 mg/g tissue after 9 days of water stress (Fig. 1).

3.1.2. Total sugar and reducing sugar

Total sugar acts as osmolytes and protects the cell from oxidative stress injury. There was sharp and significant increase in total sugar content in treated plants at 3rd and 6th days relative to control plants. In control plants leaf tissue total sugar content was 3.846 mg/g tissue. On 6th days of water stress total sugar content level reached to 23.18 mg/g tissue revealing a 6-fold increase over the normal level. But exposure of plant further to water stress resulted in decline in total sugar content (Table 2). At the 3rd day of water stress reducing sugar content in leaves was enhanced 2-fold in relation to control, following which however, there was a gradual insignificant decrease (Table 2).

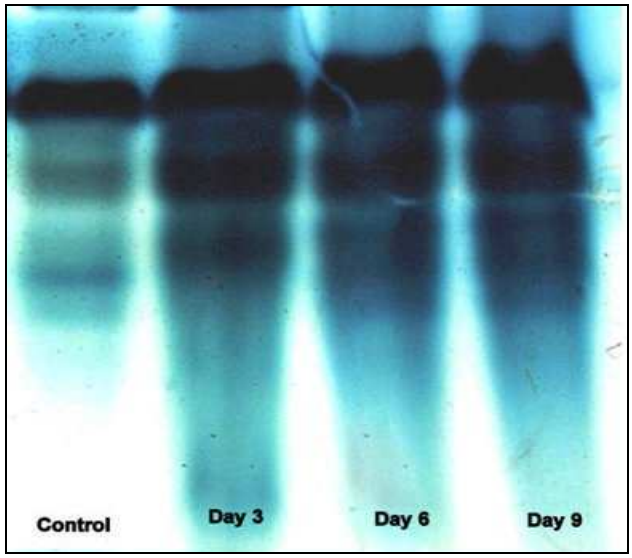

Figure 2. Isozyme analysis of peroxidase extracted from leaves of citrus by PAGE.

3.2. Effect of water stress on antioxidative enzymes

Activities of five important antioxidative enzymes were assayed under control and drought conditions. Among these enzymes, POX activity increased significantly with water stress showing an increase of about 85% on the 9th day of drought in relation to control. In our study, it was observed that activities of CAT, APOX and SOD gradually increased with increase in water stress upto 3rd day of water deficit condition relative to control, after which they declined during 6th and 9th days of stress. In case GR, activity was highest at 6th day with respect to control (Table 3). Peroxidase isozyme patterns of both control and treated plants were analyzed. Peroxidase isozyme profiling shows increase in colour intensity and number of bands at 3rd, 6th and 9th days of drought relative to control (Fig. 2).

Table 3. Activities of antioxidative enzymes of leaves of citrus subjected to different periods of water stress.

Treatment	Duration of stress (days)	Peroxidase activity (ΔA_{460} g tissue^{-1}min^{-1})	Catalase activity (ΔA_{245} g tissue^{-1}min^{-1})	Ascorbate peroxidase activtty (ΔA_{290} g tissue^{-1}min^{-1})	Glutathione reductase activity (µmol NADPH oxidized mg protein^{-1} min^{-1})	Superoxide dismutase activity (Enzyme Units)
Control	0	57.93±1.41^{a}	51.36±1.50^{c}	7.36±1.13^{bc}	2.47±0.11^{b}	33.04±1.80^{c}
Water stress	3	79.86±2.05^{b}	79.37±1.94^{d}	8.90±1.16^{d}	2.90±0.06^{c}	54.80±1.21^{d}
	6	93.04±1.69^{c}	39.86±2.47^{b}	6.29±0.89^{b}	3.35±0.06^{d}	25.65±1.93^{ab}
	9	106.93±1.71^{d}	22.61±1.00^{a}	4.16±0.64^{a}	1.29±0.04^{a}	23.35±1.32^{a}

Mean ± standard error; n =10; Means followed by same superscript in each column expresses insignificance at p<0.05 level (LSD test).

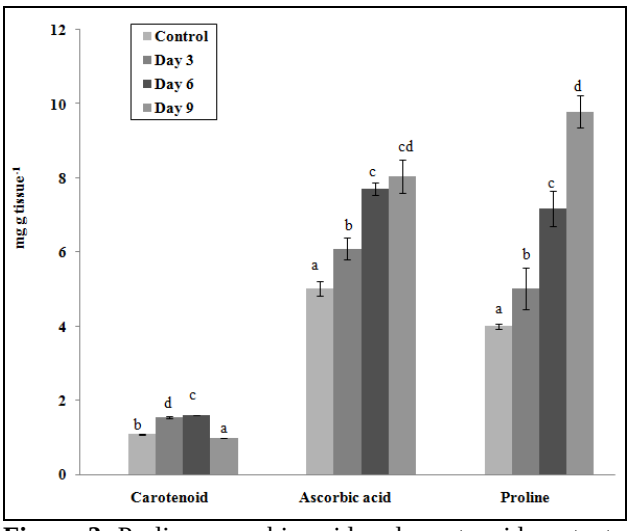

Figure 3. Proline, ascorbic acid and carotenoid contents of leaves of citrus plants subjected to different periods of drought. Different letters over each bar expresses significance at p<0.05 level (LSD test) within each group.

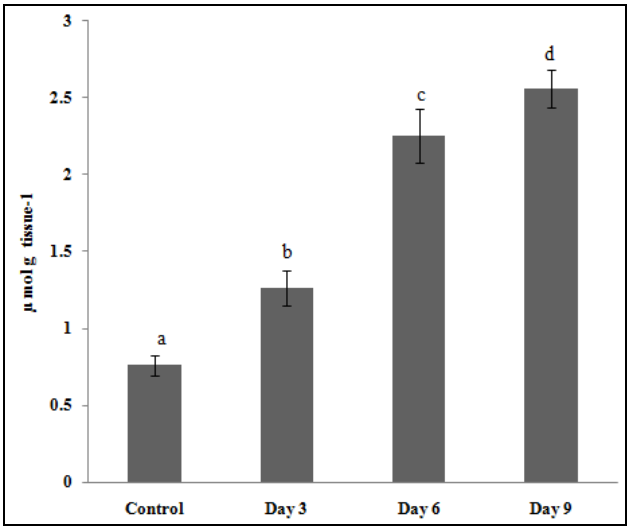

Figure 4. MDA content of leaves of citrus plants under water stress. Different letters over each bar expresses significance at p<0.05 level (LSD test).

3.3. Influence of water stress on non-enzymatic antioxidants and proline

3.3.1. Non-enzymatic antioxidants

Non-enzymatic antioxidants, like carotenoid and ascorbic acid (Fig. 3) increased significantly with increase in days of water stress. In control plants carotenoid content was 0.972 mg/g tissue. Carotenoid content increased 1.59 and 1.64 times at 3th day and 6th days of stress after that it gradually declined. On the contrary ascorbic acid content continued to increase upto 9th day of drought. At 9th day ascorbic acid content was 1.58 fold higher than control plants.

3.3.2. Proline

Proline accumulates in plant tissue as an osmolyte during environmental stress. Our results also showed that proline (Fig. 3) accumulated in water stressed plant tissues and with increase in severity of water stress proline continued to be accumulated in tissues. At 9th day of drought proline content was 146.4% higher than control plant tissues.

3.4. Lipid peroxidation and hydrogen peroxide accumulation

Accumulation of malonaldehyde and hydrogen peroxide are indications of oxidative stress in plant tissues related to formation of reactive oxygen species. In citrus plants both MDA (Fig. 4) and H_2O_2 (Fig. 5) content gradually increased with respect

to control, with the highest accumulation being obtained on the 9th day.

3.5. Effect of water stress on mycorrhizal population and root association

Spore density and diversity of AMF significantly increased during water stressed condition. The AMF spore population was found to be significantly ($p<0.05$) higher in the rhizosphere under drought condition than the control ones (Table 4). Among the AM fungi, *Glomus* was found to be dominant in both control and water stressed soil and further, among the *Glomus* sp., *G. mosseae*, *G. fasciculatum* and *G. aggregatum* were found extensively in both the conditions.

Table 4. Population of AM fungi in water stressed and non-stressed rhizosphere.

No. of days	Control plot	Drought treated plot
	(No of spores/ 100 g of soil)	(No of spores/ 100 g of soil)
0	223 ± 1.15^{a}	226.66 ± 1.15^{a}
15	233 ± 2.88^{c}	384.33 ± 1.36^{b}
30	224 ± 2.30^{ab}	422.66 ± 1.75^{c}
45	235 ± 1.33^{cd}	450.66 ± 1.16^{d}

Mean ± standard error; n=10; Means followed by same superscript in each column expresses insignificance at $p<0.05$ level (LSD test).

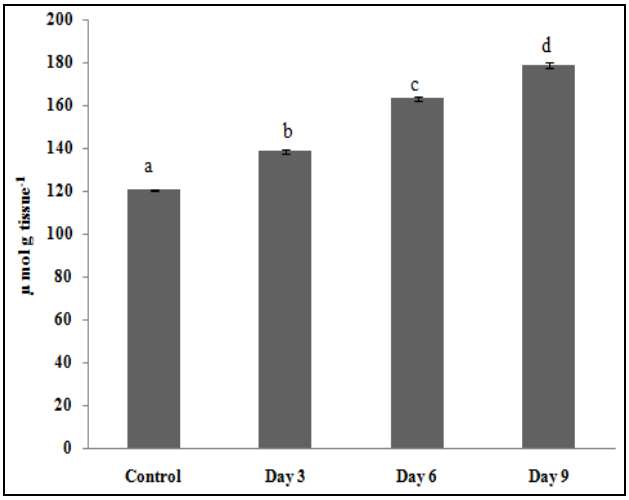

Figure 5. H_2O_2 content of leaves of citrus plants under water stress. Different letters over each bar expresses significance at $p<0.05$ level (LSD test).

Other than *Glomus*, *Gigaspora*, *Scutellospora* and *Acaulospora* were the less common genera found in the soil. *Sclerocystis* was also found to occur in both soils, but was much more common when soil was under stress. Microscopic characteristics of the AMF spores have been outlined in Table 5. Microscopic observations on root association revealed significantly greater association in the roots of plants under water stress in comparison to control soils.

4. DISCUSSION

Crop plants are exposed to several environmental stresses, all affecting plant growth and development, which consequently hamper their productivity. Drought is considered the single most devastating environmental stress, which decreases crop productivity more than any other environmental stress [38]. Exposure of plants to water stress condition decreases production and affect plant growth and development [39, 40]. It has been reported that shoot growth is more affected than root growth by water stress, since continuation of root growth under stress is an adaptive mechanism that facilitates water uptake from deeper soil [41]. Water stress affects relative water content of a plant tissue in many plants [42]. Our result showed that relative water content gradually decreased along with soil moisture content with the days of water stress. It has also been shown that ability to maintain higher RWC is one of the mechanisms of drought tolerance in plants [17, 43, 44]. Besides, RWC acts as a crucial indicator of drought induced oxidative stress in plant leaves [45].

With the onset of drought, the plants respond in several ways in order to achieve certain degree of tolerance. Most of the biochemical constituents are affected to certain degree. Protein content gradually increased initially with the water stress suggesting its probable role in the altered metabolism of the plant. In *Glycine max* drought enhances accumulation of protein [46]. After 6th day of water shortage protein content gradually decreased suggesting probable proteolysis or hydrolysis [47] of protein as the water stress built up. Dehydrin proteins have been shown to be mainly synthesized within plant tissue after exposure to drought. Chlorophyll content showed a decline with water stress. This has

been supported by the work of most previous workers. The decrease in chlorophyll content under drought stress has been considered as a typical symptom of oxidative stress and maybe the result of photo-oxidation and chlorophyll degradation. According to Mafakheri et al. [48] a decrease of total chlorophyll content with drought stress implies a lowered capacity for light harvesting and since the production of ROS is mainly driven by excess energy absorption in the photosynthetic apparatus, this might be avoided by degrading the absorbing pigments.

Table 5. Microscopic characteristics of AMF spores from rhizosphere of *Citrus*.

AMF spore	Colour	Size (µm)	Shape	No. of wall layers	Other characteristics
Glomus constrictum Trappe	Brownish orange to dark brown	160	Globose to sub-globose	1-2	Usually markedly constricted at the base of spore
Glomus mosseae (Nicol. & Gerd.) Gerd. & Trappe	Brown to orange - brown	200	Globose to sub-globose	3	Hyphae double layered
Glomus fasciculatum (Thaxt.) Gerd. & Trappe emend. C. Walker & Koske	Pale yellow to bright brown	70-120	Globose to sub-globose	3	Spores produced directly with one or more subtending hyphae
Glomus aggregatum N.C. Schenck & G.S. Sm. emend. Koske	Pale yellow	200-1800 x 200-1400	Globose to oval	1-2	Sporocarps formed in loose clusters
Gigaspora gigantea (Nicol. & Gerd.) Gerd. & Trappe	Greenish yellow	260-318	Globose to subglobose sometimes ovoid	2	Spores formed from bulbous sporogenous cell
Gigaspora margarita W.N. Becker & I.R. Hall	Yellowish white to sunflower yellow	320-370	Globose to subglobose sometimes ovoid	2	Spores produced singly at the tip of bulbous sporogenous cell
Acaulospora bireticulata F.M. Rothwell & Trappe	Brownish	280-410	Globose	3	Spores borne laterally from neck of sporiferous saccule
Acaulospora spinosa C. Walker & Trappe	Cream to pale orange brown	140-220	Globose to subglobose	2	Layer 2 thickens by formation of pale yellow sublayers followed by synthesis of closely packed rounded spines.
Scutellospora calospora (Koske & C. Walker) C. Walker & F.E. Sanders	Pale yellow with a greenish tint	120-220	Subglobose to oblong, sometimes irregular	2	No presence of subtending hyphae
Scutellospora rubra Stürmer & J.B. Morton	Dark orange brown to red brown at maturity	180	Globose to subglobose	2	No presence of subtending hyphae
Glomus intraradisces N.C. Schenck & G.S. Sm.	Pale-white cream to yellow brown	40-140	Globose to sub-globose	3	Outer two layers only present in young spores, both thin and degrade with spore maturation.

Drought also affected the total sugar content with an initial increase upto 6th day of stress, followed by a decline with further progression. Similar results were also obtained for reducing sugars though their accumulation declined earlier than that of total sugars. In plants, sugars other than being involved as energy sources also play important functions in osmoprotection. Enhanced accumulation of soluble sugars have been correlated to drought tolerance in many plants [49, 50]. Proline is considered to be another major osmoprotectant in plants and its accumulation also significantly increased with periods of drought, which is in conformity of the work of earlier workers [51]. It may also be involved in reducing oxidative damage by scavenging the free radicals [52, 53]. Accumulation of proline in plants under stress is a result of the reciprocal regulation of two pathways: increased expression of proline synthetic enzymes and repressed activity of proline degradation.

Induction of oxidative stress resulting from production of ROS and thus enhancing accumulation of H_2O_2 of O^{2-} in different cellular compartments specially in chloroplast, mitochondria and peroxisome is inevitably associated with water stress causing cellular damage [54, 55]. In order to combat this stress, plants produce an array of antioxidative enzymes which are differentially expressed during different stages of stress and also in tolerant and susceptible genotypes.In the present study it was observed that all the tested antioxidative enzymes CAT, POX, APOX, SOD and GR showed an initial upregulation in activity but increase in period of drought revealed different activities. While POX continued to be upregulated till 9th day, GR activity reduced after 6th day and those of CAT, APOX and SOD after 3 days. Several previous workers have also obtained increased activity of POX during prolonged drought in both tolerant and susceptible [56-58]. While SOD is the first enzyme to be expressed in the antioxidant mechanism, its initial increase contributes to an accumulation of H_2O_2 which is subsequently scavenged by CAT and POX. Both APOX and GR are involved in the ascorbate- glutathione pathway and work in conjunction for scavenging of the radicals [23]. With prolonged drought, plants are no longer able to maintain an elevated level of activity of all the antioxidative enzymes and hence finally succumb to oxidative stress. However, other than the antioxidative enzymes, there are certain small antioxidants such as ascorbate and carotenoids which are also involved in defense against oxidative stress. In our study, ascorbic acid content and carotenoid content gradually increased with severe water shortage. Ascorbic acid, an immunomodulator helps to develop tolerance within plant tissue by influencing signaling between of different phytohormes [59]. Carotenoids helps to protect the plants from oxidative injury during drought by preventing the production of singlet oxygen [60].

The peroxidation of lipids in the cell membrane is one of the most damaging cellular responses observed in response to drought stress [61] and the amount of lipid peroxidation is considered to be one of the determinants which indicate the extremity of stress experienced by the plant. In the present study, MDA content, which is a measure of lipid peroxidation significantly increased during the entire duration of drought, and similar results have also been reported by other workers [53]. H_2O_2 accumulation was also found to be enhanced during drought. The production and degradation of H_2O_2 is regulated by the differential activities of H_2O_2 producing and scavenging enzymes.

AM fungi remain in association with almost all plants [62]. Citrus plants have close association with AM fungi and a number of genera such as different species of *Glomus, Acaulospora, Gigaspora* and *Scutellospora* could be identified in the present study. This result is in conformity with those of earlier workers [63]. Imposition of drought led to higher population of AMF in the rhizosphere as well as greater association in the root. It has been suggested that higher mycorrhizal population may help plants to cope up with dehydration state by some modification in water transport pathways [64]. In our study AM spore density increased significantly with days of drought. In earlier studies, it was reported that AMF could enhance drought and temperature stress tolerance in citrus seedlings [65, 66]. It is probable that extracellular AM hyphae helps plants to increase nutrient concentration and acid phosphatase activity, leading to increased nutrient uptake, better growth and tolerance [66].

5. CONCLUSION

Results clearly indicate that *Citrus reticulata* plants are affected by water stress, which was manifested by decrease in RWC, increase in H_2O_2 and lipid peroxidation, decrease in total chlorophyll content indicating oxidative damage within plant tissue. However, it was evident that the plants upon exposure to water stress, responded by immediate upregulation of activities most of the antioxidative enzymes and small antioxidants, as well as accumulation of osmoprotectants such as proline and soluble sugars. With increase in duration of stress, activities of most of the enzymes declined, with only peroxidase continued to be upregulated indicating its pivotal role in defense against drought. The balance being achieved between the pro-oxidants (stress) and antioxidant machinery quite clearly determines the plant's response. The rhizospheric AMF, the population of which increase significantly during drought also probably contribute to the plant's ability to withstand stress to certain period.

ABBREVIATIONS

RWC: Relative water content, SWC: Soil water content, ROS: Reactive oxygen species, POX: Peroxidase, CAT: Catalase, APOX: Ascorbate peroxidase, SOD: Superoxide dismutase, GR: Glutathione reductase, H_2O_2: Hydrogen peroxidase, MDA: Malonaldehyde, AMF: Arbuscular mycorrhiza.

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AUTHORS' CONTRIBUTION

The biochemical part of work was carried by JS while the mycorrhizal part by AS and both of them were instrumental in writing the paper. UC and BNC were the supervisors who envisaged the work, corrected and revised the paper. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Review Article

Current approaches and problems in malaria vaccine development

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ABSTRACT

The overall objective of this review article is to visualize the current immunological approach in malaria vaccine development which provides a realistic prediction of various disputes against this parasitic disease. Recently, there is no licensed or approved vaccine from food and drug administration (FDA) or world health organization (WHO) against any human parasitic disease. There are number of parasitic diseases especially *Plasmodium falciparum* (malaria), a major cause of infectious mortality, represents a great challenge for parasitic vaccine development for human use. Inspite of extreme research in area of malaria vaccine development, there is no commercial vaccine (except Mosquirix, RTS, S; approved by European regulators, July 2015) available for malaria, parasitic disease. Recently, subunit vaccine constructs (> 20) are being evaluated in clinical trials or in advanced stage of preclinical development. For the last so many years, scientists identified probable target of parasitic disease and turn promising ones into malaria vaccine that can be evaluated.

Keywords: Malaria; *Plasmodium falciparum*; Vaccine; Mortality.

1. INTRODUCTION

Vaccines are one of the most effective modes of treatment available today belong to one of three categories or classification i.e. attenuated microbes, killed microbes or protein subunits [1-3]. On the other hand, conjugate vaccines have been developed against enormous number of encapsulated bacterial pathogens and showed successful reduction in the incidence of various infectious diseases [4, 5]. However, very few licensed subunit vaccines (based on a protein antigen) [6] e.g. hepatitis B surface antigen (HBsAg) and human papilloma virus (HPV) vaccines are available. Now a day, parasitologists trying to develop parasitic vaccine against number of harmful as well as dreadful diseases (e.g. malaria) can hardly ever safely grow and manufacture whole parasites in sufficient numbers to induce immunity [7, 8]. For developing human parasitic vaccine, the task is so difficult but the researchers should try to develop the parasitic vaccine which provides prophylactic as well as therapeutic benefits and also showed potentially preventative benefit against this parasitic disease [8]. There are a number of considerations to be made concerning what strategy a potential vaccine should adopt. The most important strategy for malaria vaccine development should have some criteria i.e. cost-effective, easily admini-

stered and significant reduction in the spread and burden of infectious diseases e.g. malaria. The urgent need for parasitic (i.e. malaria) vaccine against this human parasitic disease is so intense, as mortality and morbidity associated with number of cases of malaria are enormously increasing in Africa [9, 10]. The most devastating effect observed in children's and pregnant women where it does not be able to develop immunity against this parasitic disease [11]. According to physiology or life cycle of infectious diseases, parasites are more complex organisms than bacteria and viruses, with more complicated as well as bewildering structures. Due to these structural variations as well as complexities of parasitic diseases including large number of antigens have been present mainly as proteins and less often from vector systems to try to generate protective immunity, the researchers focused on life cycle stage and antibodies that could potentially elicit an immune response [12, 13].

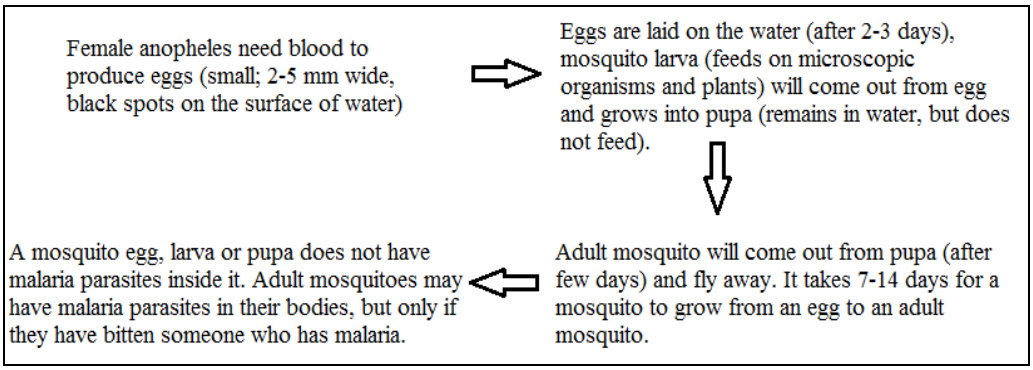

Fig. 1. Life cycle of female *Anopheles*.

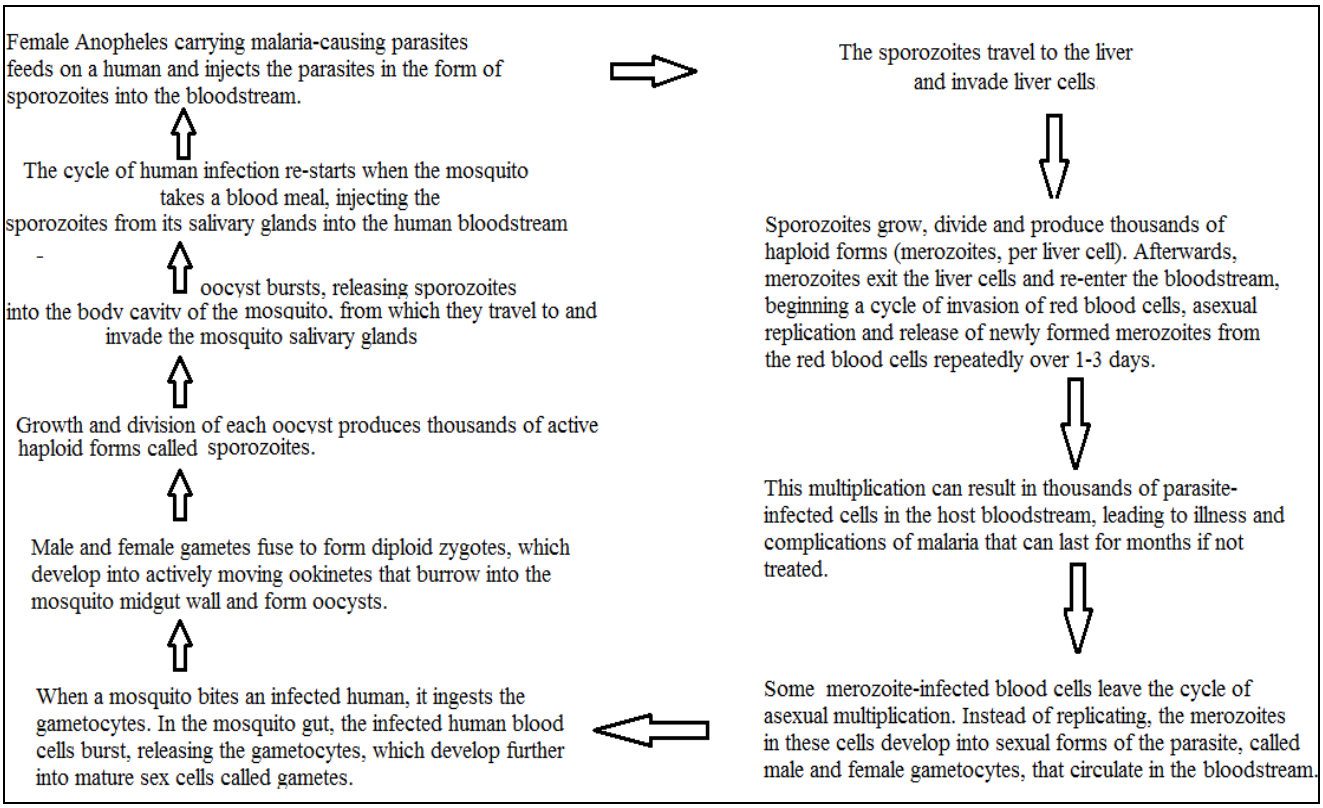

Fig. 2. Life cycle of malaria parasite.

2. MALARIA

Malaria is caused by the *Plasmodium* parasite (spread to humans only through the bites of infected female *Anopheles*). Generally, female anopheles can be recognized very easily by its upturning tail and how to reproduce the female anopheles as shown in Fig. 1. The actual life of the parasite inside the mosquito is a race against time. The maximum life span of the female anopheles mosquito is roughly the same as the time taken for the parasite to go through its growth and development. This period is temporary or short lived in cooler environ-ments and lengthens as the temperature exceeds. Thus the endurance of the parasite depends upon the weather. Once the standard temperature drops below the normal range (15-18°C), the mosquito tends to die before it can transmit malaria. The life cycle of malaria parasite is exceptionally complex (Fig. 2), presenting initial developmental complications. It should be noted that despite the huge number of vaccines available at the current time, there are none that desire to target parasitic infections.

3. ANTIGENIC TARGET OF *PLASMODIUM* SPECIES FOR VACCINE RESEARCH

The various developmental stages involved in the parasitic (*Plasmodium* species) disease represent various opportunities for targeting the diverse range of antigens, thus potentially able to elicit an immune response. As per the life cycle of *Plasmodium falci-parum* (i.e. malaria) is concerned, each develop-mental stage of human parasitic disease could have a vaccine developed [14, 15] specifically to target the antigen.

- IST initial stage of life cycle, relatively short phase called as pre-erythrocytic or hepatic stage. Only that vaccine at this stage is highly effective as well as protective which have the immuno-potential to protect against those sporozoites invading and possibly inhibiting or eliminating the development of parasites in the hepatocytes (through inducing cytotoxic T-lymphocytes that can destroy the infected hepatocytes). In case, if any infected sporozoites evaded the immune system they would then have the potential to be symptomatic and cause the clinical disease.
- Second phase of the life cycle called as erythro-cytic or blood phase. In this stage, vaccine at least could prevent the multiplication of mero-zoites invasion on red blood cells. For vaccine preparation, this phase is so difficult because of the lack of MHC molecule expression on the surface of erythrocytes.
- Instead numbers of malarial antigens are illus-trate on the surface, only that vaccine is effective which have the capability to raise the antibodies against number of infected antigens present on malaria parasite.
- Another approach is to block or interrupt the process of erythrocyte adherence to blood vessel walls.
- It is thought that this process is explicable for much of the clinical syndrome associated with malarial infection. Therefore a vaccine given to this stage would be more therapeutic and hence administered during clinical episodes to halt further deterioration.
- Finally i.e. last phase of the life cycle that has the potential to be targeted by a vaccine is the sexual stage. In this phase, it does not give any bene-ficial or protective effect to the person in order to prevent the transmission of malaria parasite (preventing the gametocytes from producing multiple sporozoites in the gut wall of the mosquito). This type of transmission blocking vaccine is needed for eliminating the parasitic infections.

This approach related to potential targets for malaria vaccine preparation is essential. Any vac-cine produced would preferably have the ability to be of great therapeutic value as well as preventing further transmission and is likely to consist of a combination of antigens from non-identical phases of the parasite's development.

4. PROGRESS IN THE DEVELOPMENT OF MALARIA VACCINES

In malaria parasite, number of multiple surface antigens is present throughout the life cycle that potentially could act as targets for vaccine preparation. There are number of approaches to target number of diseases i.e. HIV and malaria, the most important are surface expression of the antigen, inhibitory or prohibition effects of specific antibodies on the life cycle and defensive effects

through immunization or passive transfer of antibodies between an immune and a non-immune host [16, 17]. Now a day, majority of research related to malaria parasite has focused only on single strain i.e. *Plasmodium falciparum* (showed higher mortality rate in case of in vitro as well as in vivo studies). There are number of researchers attempted to use the parasitic circumsporozoite protein (dominant surface antigen; initial pre-erythrocytic phase) as vaccine antigen. Actually, the complication incorporated with this circumsporozite protein for vaccine preparation is low efficacy, reactogenicity and low immunogenicity [18]. Another problem is associated with available pharmaceutical vaccine companies include its costs, availability, adverse effects etc. Because of these issues, most of the vaccines may be reduced or eliminated entirely from the market. Recently, GlaxoSmithKline Biologicals introduced new vaccine i.e. Mosquirix which includes a vaccine for hepatitis B, now awaits a review by the World Health Organization (WHO). Hopefully, this malaria vaccine could license the product in the year 2017, according to WHO. For this vaccine, number of clinical trials are already done, this vaccine is effective on children (age 2 to 17 months; efficacy ranging from 26 to 50% in infants and young children), according to the European Medicines Agency (EMA) but these vaccines are ineffective after one year. In addition, there are number of malarial vaccines currently under investigation in clinical trial phases (only few of them as shown below):

- Two new malaria vaccine candidates i.e. Falciparum Merozoite Protein-1 (FMP1, contains a malaria protein) and RTSS (malaria protein in combination with a portion of the commercially available hepatitis B vaccine) combined with an adjuvant (called SBAS2; oil in water emulsion) which helps to stimulate the body's immune system. This vaccine is safe and determined the immune response through various blood tests. Recently, this malaria vaccine candidate is still under Phase I and II trial (clinical trials .gov.identifier: NCT01556945). Hopefully, this vaccine approved in the market and prevents malaria infection.
- Novel malaria vaccine, AdCh63 CS (simian adenovirus encoding *Plasmodium falciparum* liver stage antigen, Circumsporozoite protein). This study is done under Phase 1 trial (clinical trials .gov.identifier: NCT01450280) pertain only for healthy volunteers. The objective of this study is to assess or determined the immune response generated by vaccination with or without MVA CS (modified vaccinia Ankara, Circumsporozoite protein).
- GSK Biologicals (clinical trials .gov.identifier: NCT00307021; Phase 2 trial) is developing a number of malaria vaccine candidates for the routine immunization of infants and children living in malaria-endemic areas. These vaccines provide offer to get better protection against parasitic disease (i.e. Malaria) due to this strain *Plasmodium falciparum*. Candidate vaccines containing the RTS,S antigen would also provide protection against infection with hepatitis B virus (HBV).
- Safety, reactogenicity, immunogenicity and efficacy of GSK Biological's malaria candidate vaccine 257049 (clinical trials .gov.identifier: NCT01857869; Phase 2 trial) administered as standard doses at 0 and 1 months and 1/5th standard dose at 7 months (delayed fractional dose group) and 257049 orchestrate or administered as three standard doses one month apart (0, 1, 2-month group) in healthy malaria-naïve volunteers aged 18-50 years in the sporozoite challenge model.

5. CONSIDERATIONS FOR VACCINE DEVELOPMENT FOR IMPROVED IMMUNE RESPONSE

Plasmodium falciparum is the most prevalent and lethal form of malaria infecting humans in large scale especially clinical cases observed in Asia and Africa, yet the origin and evolutionary history of this important pathogen i.e. *Plasmodium* species always remain controversial [19, 20]. In the course of evolutionary history of western gorillas (*Gorilla gorilla*), comprised parasites that were nearly identical or similar to *Plasmodium falciparum* [20, 21]. These evolutionary findings indicate that *Plasmodium falciparum* is of gorilla origin (not of chimpanzee or ancient human origin) [19, 20]. One of the unique features of *Plasmodium* species has elevated rate of replication, much more higher than that actually want to ensure transmission in the parasite’s life cycle [22]. This enables pharmaceu-

tical treatments that are fruitful in deprecate the reproduction rate but not unsteady it to exert a high selection pressure, thus advocate the development of resistance. The approach of evolutionary change is one of the key necessary deliberations for considering potential vaccine candidates [19, 21]. The development of resistance could cause a consequential curtailment in efficacy of any potential vaccine thus contribute purposeless carefully developed and effective treatment.

Today malaria vaccine is not available because of several antigens present or adjacent on malarial parasites and expressed in several heterologous expression systems like yeast, bacteria, insects and mammalian cells. So, number of researchers focused on designing or synthesizing the new malaria vaccines for human parasitic diseases i.e. vaccines are designed in such a way to reduce the burden of erythrocytes invasion or to kill intraerythrocytic parasites and also aim to prevent the parasite fertilization or inhibiting the early development of parasite. The whole phenomenon called as transmission-blocking immunity and is mediated by specific type of antibodies and other necessary factors that are generally ingested during the blood meal and responsible for inhibiting the parasite development in the mosquito [23, 24]. These antibodies recognize most of the proteins that are expressed on either gametocytes or various parasitic stages that enlarge in the mosquito mid gut and are remembered to be as potential malaria vaccine candidates.

There are two main types of immune response than could be elicited by the parasite i.e. anti-parasitic immunity (ideal vaccine candidate would attempt to generate a more substantial cell-mediated and antibody response on parasite presentation [25, 26]. This would have the benefit of increasing the rate of parasite clearance and also reduced some adverse symptoms which provides the level of consistent future immunity against this parasitic disease) and anti-toxic immunity (suppression of the immune response associated with the production of factors that either induce symptoms or lessen the effect that any noxious by-products [micro-organism presence] have on the development of disease).

6. FUTURE PROSPECTS AND STRATEGIES FOR MALARIA VACCINE DEVELOPMENT

Till date, there are no parasitic vaccines available in the market that provides better protection against this deadly disease i.e. Malaria. The use of live, inactivated or attenuated whole parasites is not reliable and consequently, antigenic particles or subunits/fragments from the parasite are isolated and tested or trial for immunogenicity i.e. the ability to induce an immune response. Recently, researchers studious on the use of sub-unit vaccines, the most recent approaches in the field of sub-unit vaccine development include the use of DNA vaccines as shown in Fig. 3. The efficacy of DNA vaccines can be assessed using an ELISPOT assay. The outcome of this method of testing as well as studying for immune responses is abundantly beneficial when appraise the prospective efficacy of a vaccine candidate and is hoped to enable critical scrutiny of the mechanisms that provide 'partial' protection, thus facilitating immensely understanding of vaccine technology [27, 28].

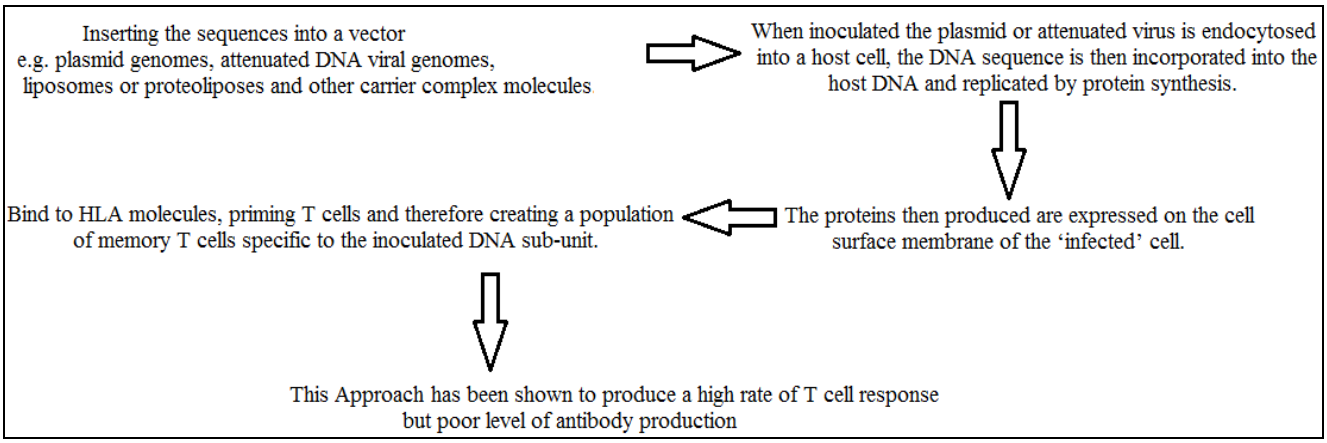

Fig. 3. Use of DNA vaccines.

This approach of potentially allowing the restriction of vaccine candidates to improve the development and the advantage of these modified or improved DNA vaccines over classical attenuated vaccines are innumerable and tried to increased or enlarge in MHC class 1 CD8+ T cell specific responses [29, 30] that potentially as well as beneficially reduce some of the safety concerns associated with vaccine therapy and provide additionally substantial reduction in the price of production cost.

7. CONCLUSION

The ramification of an increasing burden on the public health infrastructure and economic stability of the countries most badly affected is cause for concern, therefore making choice to the currently available treatment options and prevention strategies research priorities. Despite the disease's long history and the reality of a multitude of treatments, around 2 to 3 million lives are still lost or died every year because of the disease with approximately 90% of these in sub-Saharan Africa. An estimated 2 billion people live in areas where malaria is transmitted or communicated, with between 300 and 500 million new infections occurring in this group every year. The development of an efficacious parasitic vaccine for human use has been a great challenge as well as threat for medical science but recent findings observed in clinical trials in order to develop a parasitic vaccine especially for malaria disease which proved to be very useful and developing whole range of vaccines against other harmful parasitic diseases. In addition to this clinical and animal studies have shown that experimental vaccination has some degree of success when using attenuated sporozites.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between AG and SRC. AG anchored the field study, collect the preliminary data. AG and SRC managed the literature searches and produced the initial draft. The final manuscript has been read and approved by both authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Research Article

The protective effects of zinc and vitamin E supplementation against kidney toxicity by lithium in rats

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ABSTRACT

The valuable effects of antioxidants supplementation on lithium-induced nephrotoxicity has not been understood yet. The purpose of this study was to evaluate the renoprotective effect of zinc sulfate (Zn) and/or vitamin E (Vit. E) against lithium chloride (Li)-induced nephrotoxicity in rats. Forty male rats were divided into five groups. The first worked as controls and the other were treated with Li (20 mg/kg daily for 4 weeks). Group I of Li-treated was left without treatment, however, group II, III and IV were treated with Zn (10 mg/kg daily for 4 weeks), Vit. E (10 mg/kg, twice a week for 4 weeks), and the combination of Zn and Vit. E, respectively. Rats were killed for collection of blood and kidneys for biochemical and histological studies. The results showed a significant increase in Li in kidney tissue in all treated groups with Li, however, Zn was only increased in the groups treated with Zn, whereas Cu was similar in all treated and control groups. Plasma levels of creatinine, urea and glucose showed differences among the treated groups. The levels of lipid peroxidation, nitric oxide, glutathione, superoxide dismutase and catalase in renal tissue were significantly increased in Li-treated groups in comparison with the control and ameliorated by treatment with Zn and the combination of Zn and Vit. E. Histological observation showed perivascular edema and interstitial lymphocytic cell reaction in kidney of rats treated with Li, however co-treatment with Zn and/or Vit. E resulted in improvement of the histological changes. In conclusion Li-exposure causes a histological and biochemical changes mediated by oxidative stress and Li accumulation and co-treatment with Zn and/or Vit. E may protect against Li toxicity.

Keywords: Lithium toxicity; Kidney; Vitamin E; Zinc; Oxidative stress; Metal accumulation.

1. INTRODUCTION

Toxic heavy metals in water, air and soil are global problems that are a growing threat to humanity. Renal tubular damage has been known to occur by lithium (Li) toxicity in experimental animals [1]. Treatment by Li is the most effective long-term therapy for bipolar disorder and unipolar depression [2, 3]. Moreover, human exposed to Li by dietary grains and vegetables [4] and as a environmental pollutants by production of metal alloys, ceramics, television screens, color films and batteries [5]. Dysfunction of kidney is often irreversible in spite of Li withdrawal, so early detection

is essential to prevent progression to end stage renal disease [6]. Because, intracellular accumulation of Li in the collecting duct inhibits the glycogen synthase kinase type 3β leading to insensitivity of cells to the actions of aldosterone and vasopressin [7].

Renal diseases are associated with oxidative stress and reduction of nitric oxide (NO), however, it is difficult to determine if this relationship contributes to disease or is a consequence of disease [8]. Study on rats found that small doses of Li (15, 30 mg/kg b.w.) caused oxidative stress in erythrocytes, liver and kidney tissues [9]. Accordingly, administration of antioxidants like N-acetyl cysteine and caffeic acid phenethyl ester, a component of honeybee propolis during Li therapy provides significant protective effect in rat model of Li-induced renal failure [10, 11].

Vitamin E (Vit. E) is an important component of human diet and considered the most effective lipid soluble antioxidant which protects the body's biological systems by preventing lipid peroxidation (LPO) [12]. Zinc (Zn) play important roles in intracellular signaling, cell-mediated immune functions, oxidative stress and inflammation [13]. Because of the health problems induced by many environmental pollutants, much effort has been given in evaluating the relative antioxidant potency of Vit. E and Zn supplementation [13, 14]. Ibrahim et al. [15] found that Zn, Vit. E and their interaction protect brain tissues from Li toxicity with the priority for the combination of Zn and Vit. E.

Regard to the broad way for Li toxicity through medical application and environmental pollutants, the present study was carried out to evaluate the efficacy of Zn and/or Vit. E supplementation on the renal toxicity induced by Li exposure in drinking water.

2. MATERIAL AND METHODS

2.1. Chemicals

Lithium chloride (LiCl), zinc sulphate ($ZnSO_4$), Vit. E (α-tocopherol), superoxide dismutase, epinephrine, thiobarbituric acid (TBA), naphthylethylene diamine dihydrochloride, and 5,5-dithiobis (2-nitrobenzoic acid (DTNB) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were of the highest purity commercially available**.**

2.2. Animals

Forty adult male albino rats (120-150 grams) were purchased from the Animal House of the Faculty of Medicine, Assiut University, Assiut, Egypt. Rats were housed in cages and kept in a room temperature at 25 ± 3^0C with normal 12 h light/12 h dark cycle. They were allowed to acclimatize for one week before the experiments. Rats had free access to water and standard diet.

The experiments were conducted in accordance with ethical guidelines of the Animal Care and Use Committee of Assiut University.

2.3. Animal groups, treatment and collection of samples

Rats were divided into five groups (n = 8). Group I (control) - received normal drinking water, group II - received Li (20 mg/kg b.w.) for 4 weeks in drinking water according to Rana and Parker [16], group III - received Li (20 mg/kg b.w.) and Zn (10 mg/kg b.w.) for 4 weeks in drinking water according to Claverie et al. [17], group IV - received Li (20 mg/kg b.w.) for 4 weeks in drinking water and intraperitoneally injected with Vit. E (100 mg/kg b.w.) twice a week for 4 weeks according to Warren et al. [18], group V - received Li (20 mg/kg b.w.) and Zn (10 mg/kg b.w.) for 4 weeks in drinking water and injected interaperitoneally with Vit. E (100 mg/kg b.w.) twice a week for 4 weeks. After 4 weeks, under anesthesia with diethyl ether, blood was collected in the heparin coated tubes. Plasma specimens were obtained and used for determination of creatinine, urea and glucose. Kidney homogenates were obtained using a tissue homogenizer (IKA Yellow line DI 18 Disperser, Germany). The homogenates (1:10 w/v) were prepared using a 0.1M phosphate buffer (pH 7.4). All homogenates were centrifuged at 6000 rpm for 30 min at 4°C and the supernatants were used for the biochemical assays. Small pieces of kidneys were quickly removed, immersed in 10% formalin, dehydrated and embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin (H&E) and observed by light microscopy according to

Drury and Wallington [19].

2.4. Biochemical measurements

Levels of glucose, uric acid, creatinine in plasma were measured coloimetrically according the manufactured procedures of commercial kits produced by Diamond Diagnostics Egyptian Company for Biotechnology. Total protein content in the supernatant of kidney tissues was measured for calculation the specific enzyme activity by the method of Lowry et al. [20]. Lipid peroxidation products as TBARS were determined according to the method of Ohkawa et al. [21]. Nitric oxide (NO) was measured as nitrite concentration colorimetrically using the method of Ding et al. [22]. Glutathione (GSH) was determined using the method of Beutler et al. [23]. Activity of superoxide dismutase (SOD) was determined according to its ability to inhibit the autoxidation of epinephrine at alkaline medium according to the method of Misra and Fridovich [24]. Activity of catalase (CAT) was determined by the procedure of Gregory and Fridovich [25], basing on its ability to decompose hydrogen peroxide.

Li, Zn and copper (Cu) concentrations in the samples were determined by ICP-MS (Thermo Fisher Scientific (Bremen) GmbH). Standard solutions of multi-elements were prepared from commercial stock standard solutions at concentrations of 100 mg/L deionised water. Working standard solutions were prepared by dilution of stock standard solution with the addition of hydrochloric acid, so that the acid concentration in working standard solutions matched acid concentration in digested solutions.

2.5. Statistical analysis

The statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) for Windows version 16.0 software. All data were represented as mean ± standard error (SE). Data were subjected to one-way analysis of variance (ANOVA) followed by Student's t-test. Statistical probability of $P < 0.05$ was considered to be significant.

3. RESULTS

Plasma levels of creatinine and urea were significantly increased, whereas glucose was significantly decreased in rats exposed to Li compared to control. Co-treatment of rats with Zn and Vit. E resulted in normalization of the plasma levels of creatinine, urea and glucose (Table 1).

The levels of LPO, NO, GSH and the activities of SOD and CAT were significantly increased in kidney tissue of Li treated group in comparison to the control group (Table 2). Co-treatment of rats with Zn or the combination of Zn and Vit. E resulted in normalization of LPO level and the activities of SOD and CAT. However, co-treatment of rats with Vit. E alone failed to normalize LPO, NO, GSH levels as well as SOD activity.

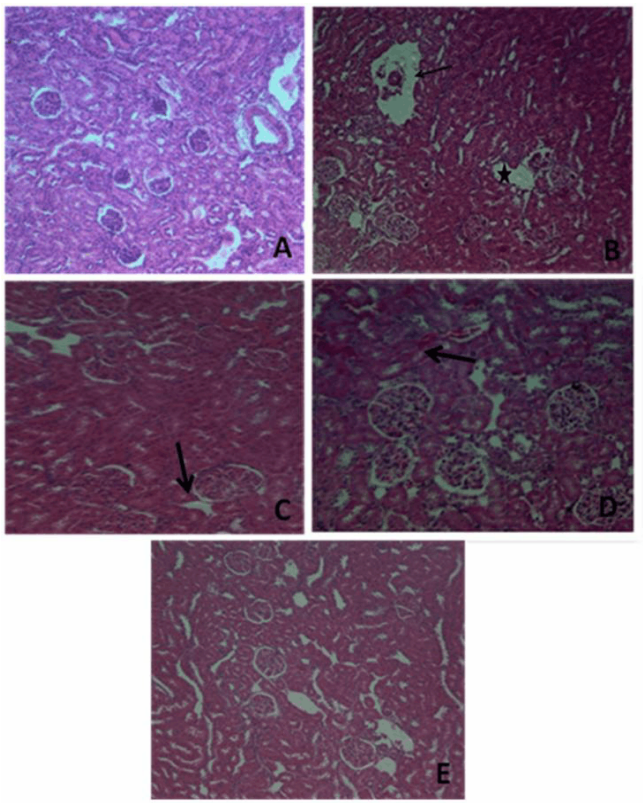

Fig. 1. Photomicrographs of kidney sections from control rat (A), rat exposed to Li showing perivascular edema (arrow) and interstitial lymphocytic cell reaction (star) (B), rat exposed to Li and treated with Zn showing congestion of the glomerular capillaries and edema in the interstatium (arrow) (C), rat exposed to Li and treated with Vit. E showing congestion of the interstitial blood vessels (arrow) (D), and rat exposed to Li and treated with Zn and Vit. E showing slight dilatation of the renal tubules (E) (H&E x 100).

The level of Li, Zn, and Cu in the kidney tissues were significantly increased in Li-treated rats and remained in rats co-treated with Zn and/or Vit. E. However, the level of Zn was increased only in rats co-treated with Zn and the combination of Zn and Vit. E and there is no change in the level of Cu among all groups (Table 3).

Histopahological observation of kidney sections from control rat showed normal histological structure of renal cortex, kidney of rat exposed to Li showed perivascular edema and interstitial lymphocytic cell reaction**,** kidney of rat exposed to Li and treated with Zn showed congestion of the glomerular capillaries and edema in the interstatium**,** kidney of rat exposed to Li and treated with Vit. E showed congestion of the interstitial blood vessels**,** and kidney of rat exposed to Li and treated with Zn and Vit. E showed slight dilatation of the renal tubules (Fig. 1).

4. DISCUSSION

It is known that urea and creatinine are renal function markers which increase when the kidney was injured. In the present results elevation of creatinine and urea levels in the plasma of Li treated rats indicates a reduction in glomerular filtration capacity. Moreover, the present reduction in plasma glucose level of rats treated with Li is strongly linked with insufficient ATP generation via glycolysis and TCA cycle [26]. Furthermore, altered level of the glucose and urea in treated rats treated with Li are indicative of hepatic and renal damages [9]. In the present study, treatment with Zn and or Vit. E normalized the levels of glucose, creatinine and urea in plasma. In comparison, treatment with selenium as antioxidant ameliorated the changes in blood parameters induced by exposure of rat to Li [27].

Table 1. Plasma levels of creatinine, urea and glucose in control and treated groups.

Groups / Parameters	Control	Li	Li and Zn	Li and Vit. E	Li, Vit. E and Zn
Creatinine (mg/dl)	0.10 ± 0.004^{a}	0.24 ± 0.03^{b}	0.10 ± 0.01^{a}	0.10 ± 0.01^{a}	0.10 ± 0.01^{a}
Urea (mg/dl)	32.17 ± 0.78^{a}	106.14 ± 1.25^{c}	75.34 ± 6.35^{b}	33.27 ± 2.25^{a}	38.72 ± 2.07^{a}
Glucose (mg/dl)	88.10 ± 3.58^{a}	57.10 ± 1.79^{b}	68.56 ± 3.27^{a}	107.77 ± 8.15^{d}	81.73 ± 2.83^{a}

Letters mean statistically significant difference at $p<0.05$ between different groups.

Table 2. Levels of oxidative stress markers in renal tissues of control and treated groups.

Groups / Parameters	Control	Li	Li and Zn	Li and Vit. E	Li, Vit. E and Zn
LPO (nM of MDA/ mg protein)	1.33 ± 0.26^{a}	9.23 ± 0.96^{c}	2.69 ± 0.40^{ab}	4.46 ± 0.85^{b}	1.16 ± 0.19^{a}
NO (nM of sodium nitrite/mg protein)	28.12 ± 3.02^{a}	137.9 ± 6.76^{d}	55.28 ± 3.09^{b}	112.4 ± 4.78^{c}	39.5 ± 3.49^{ab}
GSH (µg/mg protein)	3.12 ± 0.29^{a}	5.28 ± 1.03^{b}	3.58 ± 0.24^{a}	4.73 ± 1.01^{b}	2.15 ± 0.29^{a}
SOD (U/mg protein)	0.45 ± 0.07^{a}	0.92 ± 0.15^{b}	0.23 ± 0.02^{a}	0.90 ± 0.12^{b}	0.37 ± 0.08^{a}
CAT (U/mg protein)	0.45 ± 0.08^{a}	4.15 ± 0.60^{b}	0.80 ± 0.13^{a}	0.58 ± 0.07^{a}	1.02 ± 0.17^{a}

Letters mean statistically significant difference at $p<0.05$ between different groups.

Table 3. Concentration of Li, Zn, and Cu in kidneys of control and treated groups.

Element	Control	Li	Li and Zn	Li and Vit. E	Li, Zn and Vit. E
Li (µg/gm tissue)	0.00 ± 0.00	23.30 ± 1.10^{c}	18.60 ± 0.90^{c}	21.30 ± 090^{d}	16.10 ± 0.90^{b}
Zn(µg/gm tissue)	0.10 ± 0.00^{a}	0.10 ± 0.00^{a}	6.40 ± 0.30^{c}	0.21 ± 0.01^{b}	7.70 ± 0.50^{d}
Cu(µg/gm tissue)	0.04 ± 0.001^{a}	$0.03\pm .001^{a}$	0.30 ± 0.01^{c}	0.20 ± 0.01^{b}	0.10 ± 0.01^{b}

Letters mean statistically significant difference at $p<0.05$ between different groups.

In the present study, exposure of rats to Li caused a significant increase in LPO, NO and GSH levels and the activities of SOD and CAT in renal tissue. These changes in oxidative stress parameters were confirmed by perivascular edema and interstitial lymphocytic cell reaction. Kiełczykowska et al. [28] found that Li not caused any change in LPO but caused change in the SOD activity. Increased LPO due to induction of polyunsaturated fatty acids peroxidation may be lead to the degradation of phospholipids and cellular deterioration [29]. Catalase is the most important antioxidant for detoxifying excess H_2O_2 to prevent hydroxyl production. The imbalance between SOD and CAT activities could lead to an excessive generation of free radicals. In the present study, the increased in SOD activity in kidneys of Li treated rats support the idea of overproduction of superoxide anion by Li. Accordingly, increased CAT activity is probably related to O_2 dismutation by SOD and H_2O_2 accumulation due to decrease in glutathione peroxidase activity. Then, H_2O_2 react with O_2 to generate OH^- which initiates LPO [30]. The present data showed that Li induced the activities of SOD and CAT in the kidney tissues, however, co-treatment of rats with Zn along Li resulted in decline in the activities of SOD and CAT in comparison with Li treated alone. This is because zinc as structural element of non-mitochondrial form of SOD has antioxidant properties [31-33]. Moreover, Zn is involved in destruction of free radicals through Zn-metallothioneins which may serve as an efficient antagonist in inhibiting LPO to stabilize biomembranes [34, 35] and inhibit NADPH oxidase [13]. Cabre et al. [36] found that Zn supplementation for 12 weeks caused a decrease in lipid peroxidation, together with an increase in metallothionein concentration in alcoholic rats. Also, Zn brings down the elevated levels of SOD, CAT and GPx to within normal limits in rats given ethanol [37]. Antioxidant Vit. E breaks the chain of free radical reactions by allows free radicals to abstract a hydrogen atom from the antioxidant molecule rather than from polyunsaturated fatty acids [38]. Therefore, Vit. E supplementation protects the kidney and testicular tissue against peroxidative damage due to free radical damage in time consuming process [39, 40] and Zn was effective in reversing the oxidative stress induced by Li in the rat brain [15].

In the current study, the level of NO in kidneys of rats treated with Zn and or Vit. E showed a significant decrease with improvement in the histological structure as compared with Li treated rats. Siles et al. [41] and Bashandy [42] found a significant increase in the expression of inducible nitric oxide synthase in the brain of rats treated with Li. The antioxidant effect of Zn and Vit. E may be due to its role in reducing NO because it is mostly involved in regulation of redox status under physiological conditions [43].

The levels of elements in tissues reflect dietary concentrations of these elements [44]. In the present study, Li concentration was increased in kidney tissue of all treated groups, but, Zn was increased in the rats co-treated with Zn or the combination of Zn and Vit. E. Compartmental analysis by Jaeger et al. [46] found that Li was rapidly distributed and accumulated in kidney and liver following ingestion. Moreover, Zn supplementation increased serum Zn levels and decreased oxidative stress [47]. Pharmacologic doses of Li increased Li content in the liver and Cu content of the brain, and showed no effect in liver and kidney. Moreover, it increased Zn content in liver and kidney but did not affect Zn in the brain. Accordingly, Li therapy may induce major changes in the storage of Cu in the brain and Zn in liver and kidney, respectively [48]. The morphological examination of kidney revealed that cellular architecture was disturbed following Li treatment. Similar to our results, several histopathological examination revealed congestive or degenerative lesions, large areas of focal fibrotic lesions and interstitial accumulations of leukocytes, glomerulosclerosis, peritubular infiltration with lymphocytes and mononuclear cells in the kidney of Li treated rats and co-treatment with antioxidant improved those changes [15,49-51].

In conclusion, renal tissues of kidney exposed to Li in drinking water for 4 weeks showed alterations in oxidative stress markers and the concentration of Li with histological changes. Co-treatment of rats with Zn and/or Vit. E ameliorated the oxidative stress and improved the pathological changes in the kidney with the priority for the combination.

AUTHORS' CONTRIBUTIONS

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

TRANSPARENCY DECLARATION

The authors declare that they have no competing interests.

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Research Article

Silver tolerance and silver nanoparticle biosynthesis by *Neoscytalidium novaehollandae* and *Trichoderma inhamatum*

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ABSTRACT

Development of reliable and eco-friendly processes for synthesis of metallic nanoparticles is an important step in the field the applications of nanotechnology. Biological systems provide a useful option to achieve this objective. In this study, Twenty-one silver tolerating fungal species and 3 varieties belonging to 8 genera were collected from 40 soil samples, the most common genus was *Aspergillus* was recovered from 97.5% of the samples on silver supplemented medium, followed by silver tolerance (1 mM and 2 mM) test. The highest two silver tolerant isolates were subjected to rRNA gene sequencing and were confirmed as *Neoscytalidium novaehollandae* and *Trichoderma inhamatum*. The fungal cell filtrates of these two fungi were incubated with $AgNO_3$. The visual observation of brownish color is an indication of silver nanoparticle production. Further characterization was made by UV-visible absorption spectroscopy which showed maximum absorption at 430 and 453 nm for *N. novaehollandae* and *T. inhamatum*, respectively. Transmission Electron Microscope (TEM) revealed the formation of spherical nanoparticles with particles size from 4-20 and 2-15 nm for both fungi, respectively. The synthesized silver nanoparticles were exhibited antibacterial activities against pathogenic some bacterial strains which causes the diseases in human.

Keywords: Silver tolerance; *Trichoderma inhamatum*; *Neoscytalidium novaehollandae*; Silver nanoparticles; Antibacterial activity.

1. INTRODUCTION

Heavy metals are considered the principle cause of environmental pollution [1]. Silver is released at very high amounts to the environment from various industrial activities [2]. Despite the biocidal effects of silver on microorganisms which include blocking the respiratory chain, protein inactivation and errors in DNA transcription processes [3-5]. Many microbial cells have the ability to tolerate stressful situations in presence of toxic metals for their survival [6]. To survive under silver-stress conditions, they develop various mechanisms of resistance including efflux metal ion outside the cell, accumulation, and reduction of the metal ions to a less toxic state, altering metal solubility, altering redox state of metals, extracellular precipitation of metals, and disabling of metal transport function [7-10].

In general fungi have a high tolerance towards metals, and a high wall-binding capacity, as well as intracellular metal uptake capabilities [11]. *Cochliobolus lunatus* and yeast were able to tolerate silver [6, 12]. Fungi play an important role in remediation of toxic silver metal through reduction process. Many fungal species have been harnessed to produce silver nanoparticles. *Fusarium oxysporum*, *Trichoderma harzianum* and *Penicillium nodositutum* were able to synthesize silver nanoparticles extracellularly [13-15]. Nanoparticles synthesized by fungi are more stable with high crystalline degree than other methods [16, 17]. Applications of AgNPs are of great concern in textile fabrics treatment [18], waste water treatment [19], controlling various plant pathogens [20], antitumor [21], pesticide degradation [22], and, killing the human pathogenic bacteria [23]. Antibacterial activity of silver nanoparticles was size dependent. The small size silver nanoparticles mainly attach to the surface of cell membrane and disturb its function [24]. The antibacterial and antiviral behaviors of silver, silver ions, and silver-containing compounds have long been investigated [25, 26].

In this context, the aims of this work were to characterize the silver tolerating fungi from soil in Qena region, study the ability of isolated fungi to silver tolerance, molecular identification of most active strains identify the most potent fungi for silver nanoparticles production and test its activity against pathogenic bacteria.

2. MATERIAL AND METHODS

2.1. Isolation of fungal strains

For isolation of silver tolerant fungi, 40 soil samples were collected from heavy metal contaminated soil samples from industry sites at Qena Governorate. Serial dilutions of the samples were prepared and plated on the glucose Czapek□s agar medium, containing (g/L): glucose, 10; $NaNO_3$, 2; K2HPO4, 1; MgSO4·7H2O, 0.5; KCl, 0.5 and agar, 20 and supplemented by silver nitrate which was added to yield overall Ag^+ ion concentration of 0.5 mM. These plates were then incubated at 28 °C and developing fungal colonies were counted and identified and kept for further silver tolerance tests.

2.2. Silver tolerance

To study the silver tolerance by isolated fungi and determine the highest silver tolerant fungi, 4-mm diameter plugs cut from the margin of actively growing colonies were inoculated centrally to petri plates of 9 cm in diameter containing glucose Czapek□s agar medium supplemented with silver at 1 mM or 2 mM concentration. After incubation at 28 °C, the growth rates were determined by measuring the colony diameter along two perpendicular axes in three replicates for each strain [27].

2.3. Molecular identification of *Neoscytalidium novaehollandae* and *Trichoderma inhamatum*

Based on silver tolerance results *N. novaehollandae* and *T. inhamatum* were chosen to further identification by nuclear ribosomal DNA internal transcribed spacer (ITS) sequencing. For this, fungal genomic DNA was isolated as described by Cenis [28]. ITS region using the primers ITS1 and ITS4 (ITS1: 3'-TCCGTAGGTGAACCTGCGG-5', ITS4: 3'-TCCTCCGCTTATTGATATGC-5'; [29]) were amplified and sequenced. The sequences were submitted to GenBank on the NCBI website (http://www.ncbi.nlm.nih.gov). Sequences obtained in this study were compared with the GenBank database using the BLAST software on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4. Biosynthesis of silver nanoparticles by *N. novaehollandae* and *T. inhamatum*

To prepare the biomass for silver nanoparticles biosynthesis the fungi were grown aerobically in liquid broth containing 0.3% w/v malt extract, 1.0% w/v glucose, 0.3% w/v, yeast extract and 0.5% w/v peptone. The culture flasks were incubated on an orbital shaker at 27 °C and agitated at 150 rpm. The biomass was harvested after 72 h of growth by sieving through a plastic sieve followed by extensive washing with sterile double-distilled water to remove any medium components from the biomass.

Typically 20 g of biomass (wet weight) were soaked in 100 mL sterile double-distilled water for 48 h at 27 °C in an Erlenmeyer flask and agitated as described above. After incubation, the sample was

filtered by Whatman filter paper No. 1. After filtration, a carefully weighed quantity of silver nitrate was added to the Erlenmeyer flask to yield overall Ag^+ ions concentration of 2 mM, and the sample was kept under dark conditions for 96 h.

2.5. Characterization of silver nanoparticles

2.5.1. UV-visible spectral analysis

The detection of AgNPs was primarily carried out by visual observation of color change of the fungal filtrate supplemented with silver nitrate. Further, AgNPs were characterized with the help of dual beam UV-visible spectrophotometer with a resolution of 1 nm in the range of 200-800nm.

2.5.2. TEM analysis of silver nanoparticles

Silver nanoparticles were characterized by transmission electron microscopy (TEM) analysis. TEM samples were prepared by placing a drop of the suspension of silver nanoparticles solution on carbon-coated copper grids and allowing water to evaporate as previously described by El-Sonbaty [21].

2.6. Antibacterial activity

Biosynthesized silver nanoparticles produced by the *N. novaehollandae* and *T. inhamatum*, were tested for antimicrobial activity as described by Srinivasulu et al. [30] using *Enterbacter aerogenes*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Streptococcus pyogenes* bacteria by the agar well-diffusion method. Approximately, 20 ml of Mueller-Hinton nutrient agar medium was poured into sterilized petri-dishes. The bacterial test organisms were grown in nutrient broth for 24 h. A 100 µl nutrient broth culture of each bacterial organism was used to prepare bacterial lawns. Agar wells of 8 mm diameter were prepared with the help of a sterilized stainless steel cork borer. The wells were loaded with either 60 µl of Ag nanoparticles solution, 60 µl of 2 mM silver nitrate or 60 µl of 30 µg ml^{-1} of chloramphenicol as a positive control. The plates were incubated at 37 °C for 24 h and then were examined for the presence of zones of inhibition. The diameter of such zones of inhibition was measured for each organism was recorded and expressed in millimeter unit.

3. RESULTS AND DISSCUSSION

3.1. Isolation of fungal strains

Silver tolerant fungal cultures were isolated from the soil samples collected from Qena Governorate, Egypt. The fungal isolates were characterized on the basis of colony characteristics and microscopic appearance. Twenty-one silver tolerant fungal species and 3 varieties belonging to 8 genera were collected from 40 soil samples (Table 1), the most common genus was *Aspergillus* was recovered from 97.5% of the samples contributing 83.03% of total fungi, the most prevalent species from this genus were: *A. flavus* and *A. niger* emerging in 77.5% and 60% of the samples comprising 42.9% and 9.51% of the total fungi, respectively. *Emercilla* was isolated in moderate frequency of occurrence contributing 47.5% of the samples. The remaining genera were isolated in low and rare frequencies comprising 2.7% of total fungi. The above species and many silver tolerant fungal species were isolated with different numbers and frequencies from various soils in many places around the world [6, 13, 31, 32].

3.2. Silver tolerance

The silver tolerance of all isolated fungal species was tested at concentration 1 mM and 2 mM of $AgNO_3$ on solid medium. All fungal species were grown on two concentration of $AgNO_3$, and the growth diameter of all fungal species was decreased by increasing $AgNO_3$ from 1 mM to 2 mM. The highest growth was achieved by *Trichoderma inhamatum* followed by *Neoscytalidium novaehollandae* and *Mucor hiemalis* (Table 2). This result was in agreement with those obtained by Kieranus [12]. They studied silver tolerance and accumulation in yeast, and found that all tested yeasts strains were able to grow on 1 mM and 2 mM of $AgNO_3$. Also Salunkhe [6] studied silver tolerance in *Cochliobolus lunatus* and found that the dry biomass of *C. lunatus* decreased with the increasing concentration of silver. The silver and other metals tolerance was previously studied by various works around the world [27, 33, 34].

Table 1. Average total count (calculated per g dry soil in every sample), percentage counts (% C, calculated per total fungi), (% F, calculated per 40 samples), number of isolation cases (NCI, out of 40 cases), occurrence remarks (OR) for fungal genera and species recovered from 40 cultivated soil samples on glucose Czapek's agar at 28°C.

Genera and species	ATC	% C	NCI	OR	% F
Aspergillus	59366.3	83.03	39	H	97.5
A. candidus	33.33	0.04	1	R	2.5
A. chevaliere	33.33	0.04	1	R	2.5
A. flavipes	66.66	0.09	1	R	2.5
A. flavus	30666.6	42.9	31	H	77.5
A. flavus var. *columinaris*	33.33	0.04	1	R	2.5
A. niger	6799.92	9.51	24	H	60
A. ochraceus	2566.64	3.6	8	L	20
A. ornatus	33.33	0.04	1	R	2.5
A. sydowii	14699.9	20.55	18	M	45
A. tamari	100	0.14	1	R	2.5
A. terreus	3333.33	4.66	3	R	7.5
A. terrus var. *aureus*	366.66	0.51	3	R	7.5
A. ustus	466.65	0.65	5	L	12.5
A. versicolor	166.66	0.23	1	R	2.5
Botryotrichium piliferons	33.33	0.04	1	R	2.5
Emericella	10200	14.26	19	M	47.5
E. nidulans	9500	13.28	18	M	45
E. nidulans var. *dentata*	700	0.98	3	R	7.5
Mucor hiemalis	1000	1.4	1	R	2.5
Neosyctalidium novaehollandae	33.33	0.04	1	R	2.5
Paecilomyces carnus	33.33	0.04	1	R	2.5
Penicillium	433.32	0.6	5	L	12.5
P. aurantiogresium	333.33	0.46	2	R	5
P. chrysogenum	66.66	0.09	2	R	5
P. duclauxi	33.33	0.04	1	R	2.5
Sterile mycelia	100	0.14	2	R	5
Trichoderma inhamatum	300	0.42	1	R	2.5
Gross total count	71499.8				
Number of gnera	8				
Number of species	21+3 varieties				

H = high occurrence from 20- 40 cases, M = moderate occurrence from 10-19 cases, L = low occurrence from 5-9 cases, R = rare occurrence 1-4 cases.

3.3. Molecular identification of *N. novaehollandae* and *T. inhamatum*

The fungal rRNA sequence generated in this study was deposited in GenBank (accession no. KT006147 and KT006148). The top blast match for this sequence was found to be *N. novaehollandae* and *T. inhamatum* from GenBank with 100 and 99 % maximum sequence similarity for two fungi, respectively and 100% query coverage for both. Sequences from the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA has been commonly used for the identification of fungi [35-39]. Gherbawy and Gashgari [40] identify 31 fungal

strain collected from potato blemishes by blasting sequence result against GenBank.

3.4. Characterization of silver nanoparticles

3.4.1. Characterization of silver nanoparticles by UV-visible spectroscopy

Change of fungal cell filtrate from pale yellow to brownish after treatment with silver nitrate indicates the formation of AgNPs by tested fungi (Fig. 1). Synthesis of AgNPs exhibits strong absorption in the visible range due to the local surface plasmon resonance. UV-visible absorption spectra for AgNPs prepared from *N. novaehollandae* and *T. inhamatum* using silver nitrate are shown in (Fig. 2). The spectra recorded from the AgNPs solution, it is seen that a blue shift in the onset of absorption is observed in this samples and showed absorbance peak around 430 and 453 nm for *N. novaehollandae* and *T. inhamatum*, respectively, which is specific for the AgNPs (Fig. 2). It is observed; from the spectra of 75 isolates belonging to different 5 species of *Trichoderma* synthesized silver nanoparticles that the silver surface plasmon band occurs at 420 nm [41]. *Aspergillus flavus* when challenged with silver nitrate solution a characteristic surface Plasmon absorption band at 420 nm was observed at 24 h that attained the maximum intensity after 94 h. After 94 h of incubation, no change in intensity at 420 nm was observed indicating complete reduction of silver ions [42]. The ultraviolet-visible spectra of *Aspergillus terreus* and *Fusarium oxysporium* cell filtrates with $AgNO_3$ showed a strong broad peak at 440 nm (SPR band), which indicated the presence of AgNPs [18, 43]. AgNPs produced by *Fusarium oxysporium* and *Neurospora intermedia* exhibit different peaks with different environmental, nutritional factors and $AgNO_3$ concentration [44, 45].

3.4.2. TEM analysis of synthesized AgNPs

TEM analysis was employed to determine the morphology and shape of AgNPs. The representative TEM image has been indicated in Fig. 3. Accordingly for testing strains, the majorities of the AgNPs are relatively uniform in diameter and present in spherical shape. Additionally, TEM images depicted that the particles size synthesized by *N. novaehollandae* are predominantly form 4 to 20 nm but the size of particles produced by *T. inhamatum* were less than 20 nm and mostly in the range of 2-15 nm. The morphology of the nanoparticles synthesized by different species of *Trichoderma* was studied by Devi et al. [41], they found that AgNPs synthesized by different species of *Trichoderma* were found single or aggregated with round and uniform in shape and 6-80 nm in size. On the other hand, the morphology of the nanoparticles synthesized by *Trichoderma reesei* was highly variable and these assemblies were found to be aggregates of silver nanoparticles in the size range 5-50 nm [46].

Table 2. Degree of silver tolerance (calculated as growth diameter in mm) of fungal species tested on 1 mM and 2 mM concentration of $AgNO_3$.

Fungal species	$AgNO_3$ concentration	
	1mM	**2mM**
Aspergillus candidus	14	10
A. chevaliere	7	6
A. flavipes	10	9
A. flavus	13	10
A. flavus var. *columinaris*	18	10
A. niger	16	15
A. ochraceus	6	5
A. ornatus	10	8
A. sydowii	11	10
A. tamarii	15	14
A. terreus	9	8
A. terrus var. *aureus*	12	8
A. ustus	12	10
A. versicolor	7	6
Botryotrichium piliferons	14	10
Emericella nidulans	12	10
E. nidulans var. *dentata*	10	8
Mucor hiemalis	20	16
Neosyctalidium novaehollandae	27	21
Paecilomyces carnus	9	8
Penicillium aurantiogresium	13	10
P. chrysogenum	13	11
P. duclauxi	14	12
Trichoderma inhamatum	59	47

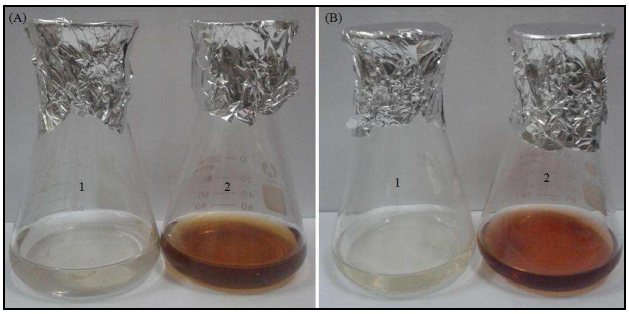

Figure 1. Change of color from pale yellow to brownish, indicating the formation of silver nanoparticles, (A) *N. novaehollandae* cell filtrate (1) without $AgNO_3$ and (2) with $AgNO_3$, (B) *T. inhamatum* cell filtrate (1) without $AgNO_3$ and (2) with $AgNO_3$.

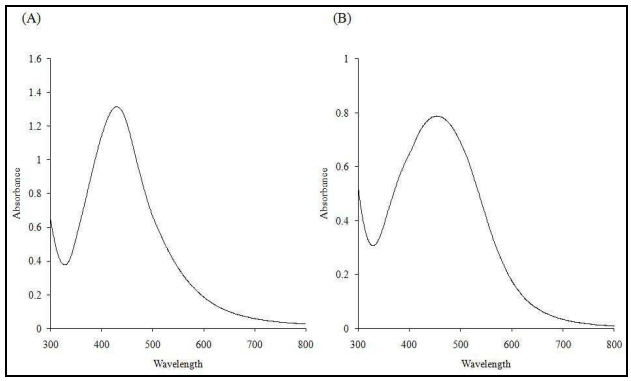

Figure 2. UV-visible spectra of silver nanoparticles synthesized using (A) *N. novaehollandae* and (B) *T. inhamatum*.

Table 3. Antibacterial activity (in diameter in mm) of silver nanoparticles produced by tested fungi against some bacterial strains.

Bacteria	**Chloramphenicol**	**2mM $AgNO_3$**	**AgNPs**	
			N. novaehollandae	*T. inhamatum*
Enterbacter aerogenes	24	13	15	17
Salmonella typhimurium	28	11	14	13
Staphylococcus aureus	25	13	16	13
Streptococcus pyogenes	28	12	13	14

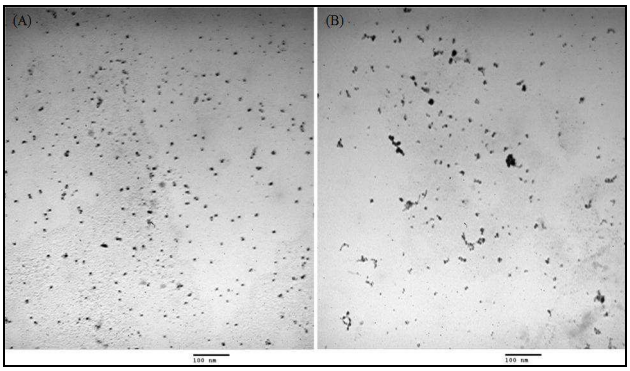

Figure 3. TEM micrograph of silver nanoparticles synthesized using (A) *N. novaehollandae* and (B) *T. inhamatum.*

3.5. Antibacterial activity

The antibacterial activity of biosynthesized AgNPs produced by both *N. novaehollandae* and *T. inhamatum* were investigated against various pathogenic bacteria, like *Enterbacter aerogenes*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Streptococcus pyogenes* using well diffusion technique (Table 3). The result showed variation in diameter of inhibition zone against all the tested bacteria. The highest antimicrobial activity of AgNPs produced by *N. novaehollandae* was observed against *S. aureus* followed by *E. aerogenes*, while in case AgNPs produced by *T. inhamatum* was observed against *E. aerogenes* followed by *S. pyogenes*. These results are in agreement with the previously results obtained by Metuku et al. [47] who studied the effect of silver nanoparticles produced by *Schizophyllum radiatum* on Gram negative and positive bacteria. Their studies showed antimicrobial activities against tested bacteria. A number of possible mechanisms are suggested for the antibacterial activity of AgNPs. Silver ions have been known to bind with the negatively charged bacterial cell wall resulting in the rupture and consequent denaturation of proteins which leads to cell death [48]. The synthesized AgNPs after penetration into the bacteria causes protein denaturation results in inhibition of complete cellular metabolism leading to bacterial cell death [49]. Other proposed mechanisms include the AgNPs with smaller size can act drastically on cell membrane and further interact with DNA and DNA loses its replication ability [24].

4. CONCLUSIONS

Isolates of *N. novaehollandae* and *T. inhamatum* isolated from Egyptian soil samples showed silver tolerance and were able to produce AgNPs with variable size. The produced AgNPs showed significant effect against some pathogenic bacteria.

TRANSPARENCY DECLARATION

The author declares no conflicts of interest.

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Research Article

Growth curve of *Streptococcus oralis*

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ABSTRACT

Difference between the artificial transformation in Gram-negative *E. coli* K-12 (gene cloning with double stranded DNA) and natural transformation in diplococcic Gram-positive *Streptococcus* has been ignored. The artificial transformation, an uptake of recombinant DNA (double stranded only) has been mostly used in gene-cloning experiments with Gram-negative *E. coli* K-12. The exact mechanism of such recombinant DNA uptake by competent *E. coli* K-12 cells still remains unknown. However, the natural transformation in diplococcic *Streptococcus* as initially reported by Dr. Griffith in 1928, has so far been limited within their two types of colonies, smooth and rough. Our data on *S. oralis, S. mutans and S. pneumoniae*, demonstrates that these two colony types are bacterial physiological changes (phenotypes) and therefore the uptake of exogenous donor DNA, double or single stranded, apparently has no role in such a physiological normal growth of bacteria (changes of colony types on solid blood agar medium). The growth curve of *S. oralis* presented in this work clearly demonstrates that there is a pre-competent phase in addition to their competent and post-competent phases of growth.

Keywords: *S. oralis*; growth-curve; pre-competent phase; bio-signaling; penicillin binding protein (PBP).

Importance: The two-component signaling system recently recognized in *S. pneumoniae* (a member of the diplococcic Streptococcal group) and *Mycobacteria TB* plays an important role in the regulation of their growth phases. Complete knowledge of these growth phases is absolutely necessary in developing an alternative but the preventive low-cost therapy since penicillin resistance has already created a crisis in medicine.

1. INTRODUCTION

Griffith's original work published in 1928 has dealt with *S. pneumoniae* smooth and rough colonies and their difference in degree of virulence [1]. It is becoming increasingly clear that Dr Griffith's smooth and rough colonies are their physiological states of growth which have nothing to do with the entry of pneumococcal double-stranded donor DNA fragments as pre-maturely recognized by Avery et al. in 1944 [2]. The tiny DNA fragments of approximately 100 base long (TCA insoluble poly-deoxyribonucleotides) as isolated by early workers can't satisfy the requirement for the minimum size of DNA carrying inheritable genetic character(s) [2-4]. They have made an effort to correlate colony

types and their genetic material DNA. In later years we have visualized the TCA insoluble poly-deoxynucleotides (double stranded DNA fragments) by TEM using appropriate lambda DNA controls. We have not seen fragments of any appreciable size that can fulfill the requirement of the minimum size for carrying inheritable genetic character. In 1953, DNA bio-molecule has been characterized by Watson and Crick and then subsequently molecular biology is born to help us how the genetic information is inscribed in triplet codons of DNA macromolecule or bacterial single chromosome [4-6]. The natural transformation of Gram-positive *S. pneumoniae or S. oralis* differs considerably from the artificial DNA transformation of Gram-negative *E. coli* K-12. In *in vitro* gene cloning experiments with *E. coli* K-12 cloning vectors and ligation of double stranded DNAs appears to be an academic exercise. Briefly, the double stranded DNA fragments derived from different sources are joined to a cloning vector, plasmid or phage DNA that containing the origin of replication (replicons) [7]. Such DNA recombinant technology has distorted the progress of natural transformation in *S. pneumoniae*. The natural transformation of Gram-positive Mitis group bacteria is their growth-curve. Until recently, our knowledge of *S. pneumoniae* growth-curve has been limited only within their two phases of growth, competent phase and the incompetent phase. Difference between the competent diplococcic *Streptococcus* and their incompetent derivatives has been defined as if a fratricidal effect of the competent *S. pneumoniae* destroys their incompetent derivatives [8]. However, these investigators are not aware of the pre-competent phase and therefore their incompetent phase needs further clarification. Both the *S. pneumoniae* and *S. oralis* grow in chains and there is a heterogeneity of population. Until now we are only familiar with the competent and the post-competent phases of *S. pneumoniae* but complete growth-curve reported here includes a pre-competent phase.

The difference between pre-competent and post-competent is that the pre-competent is reversibly incompetent and the post-competent contains also irreversibly incompetent. In pre-competent phase they are just born as spheres, start growing only in size, spherical to oval to attain competent phase. In competent phase they attain an ability to reproduce (reproduction phase, early, mid and late). In this phase, morphologically the bacterial population looks diplococcic (formation of cleavage at the middle of the oval shaped bacteria) but it never means the two individuals. In post-competent phase they show an heterogeneity of their population with thinning of cell wall thickness resulting in the loss of ability to reproduce and respond to their incoming bio-signals (our manuscripts are in preparation). Recently, Berge et al. have shown the recruitment of single stranded nucleotides with 3-prime OH end adhering at the cleavage site of the recipient but there seems to be no entry into the cytoplasm [9]. Our data mostly worked with a close relative of *S. pneumoniae* (low G/C content), *S. oralis* presented in this article to establish how the Mitis group *Streptococcus* initiates its growth-cycle with a pre-competent phase and ends in a long chain containing heterogeneity of all generations.

2. MATERIAL AND METHODS

We have used 2 different strains obtained from ATCC collection: ATCC 6249 (closely related to *S. pneumoniae*) and *S. mutans*: ATCC 25175 (dental pathogen) [10, 11]. Gram-positive *Streptococcus* strains saved in their separate stabs (mother stock): overnight cultures of *S. oralis*, diluted 10,000-fold in a rich broth TSB or BHI by our previously described dilution procedure and grown at 37^0C with or without shaking to saturation. Optical microscopy, Standard Gram-staining technique with crystal violet, and scanning electron microscopy JEOL JSM-7600F have been used [10-12]. Crystal violet solution is always prepared fresh and filtered through a sterile membrane filter disc (0.2 μm). Before starting overnight cultures we have streaked our stock cultures on blood agar medium and the single colony transferred with a sterile tooth picks onto CNA as well as MacConkey-lactose plates [10]. Then the pure colony which grows only on CNA medium is used in our experiments. Gram-negative *E. coli* does not grow on CNA medium, because this medium contains two antibiotics, colistin and nalidixic acid. After shadowing with gold for 10 to 20 seconds, the sample is visualized by a scanning electron microscope, JEOL JSM -7600F at 15 kV.

3. RESULTS

3.1. Growth curve for Mitis group Streptococci: Pre-competent phase, Competent phase, and Post-competent phase

3.1.1. Pre-competent phase

Dr. Griffith's 1928 published note book has clearly identified two types of bacterial colonies, rough (R) and smooth (S) after his clinical sample is grown on blood agar growth media for 24 -72 hours at 37^{o}C [1]. We should not mix up the difference between an individual bacterium as visualized by optical microscopy and their colonies which appear after many generations of multiplication of an individual bacterium on solid blood agar medium. As shown in Fig. 1, the heterogeneity of the bacterial sizes ranging from round (approximately 0.2 μm diameter, light purple) to a full oval size (1.7 μm, purple). However, we have not taken into consideration that the majority (about 70%) are highly reduced in sizes and can't be homogenously stained by the Gram-staining with crystal violet. For the growth curve, we have diluted the overnight culture 10,000 fold in TSB with or without xylitol (2% or more) and grown to saturation. They appear stable in long chains when grown in the presence of xylitol with heterogeneity of color, pink and purple. Our overnight cultures containing this population in chains, are usually ruptured during growth in rich nutrient broth, BHI or TSB by shaking or by the shearing force induced during dilutions in the laboratory buffers. As an outcome, visualization by the standard Gram-staining technique has deprived many of our investigators from seeing this bacterial population in chains of heterogeneity (Fig. 1).

We have also measured the increasing optical density of these bacteria in pre-competent phase but without any increase in colony forming units (cfu) [12]. Until recently, the pre-competent phase has been ignored in the literature. However, increase of the cfu takes place via change of morphological shape: this smooth colony of Dr. Griffith grows from oval to diplococcic and is recognized as the competent phase (early, mid and late log phase) [13]. In order to analyze multiplication of individual bacterial cells we have broken their in-vivo prevailing in chains by the standard dilution procedure usually followed in all academic laboratories. A fraction of bacterial population present in our overnight cultures is also stained pink, not purple and their majority may remain invisible because of their thinning of cell wall thickness or loss of cell wall by being in declining phase.

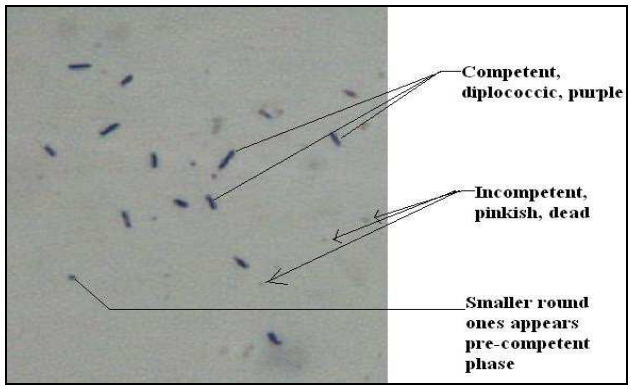

Figure 1. Stationary phase of *S. oralis* diluted 10,000-fold in rich broth, allowed to grow at 37^{o}C and then visualized by optical microscopy after Gram staining (magnification 1000x). Bacterial population maintains heterogeneity of sizes including pre-competent, competent and the incompetent (with thin cell wallspheroplasts and the protoplasts). They show a mixture of purple and pink individuals with heterogeneity of sizes.

3.1.2. Competent-phase: growth in liquid broth and transparent/smooth colonies on blood agar solid medium

This is the early phase of an adult which apparently acquires an ability to secrete pheromone [10]. This is the beginning of competent phase which means a genetic ability to divide or multiply. Cleavage appears at the mid-cell position of the oval shaped bacterium and is introduced in our text books as diplococcic. The diplococcic bacterium is just the shape with a cleavage at the mid-cell position but it never means two bacterial cells. We now summarize that the pre-competent (spherical to oval), competent (oval to diplococcic) and the post-competent all prevail in the same chain. Initiation of reproduction phase (early/mid/late log phase) is the morphological change, the oval becomes diplococcic with a depression at the middle of their oval shape (Fig. 2). Because they grow in a chain, it is not possible to accurately measure their

sizes but the heterogeneity can be stabilized by growing them in the presence of xylitol (2% to higher) as previously visualized by scanning electron microscopy even without the addition any fixing reagents [10]. We are in agreement with Griffith that the bacterial population in chain (rough colony) is conditionally less virulent than their individuals in growth-phase as resulted by the rupture of the same chain under their conditions of growth. We therefore strongly believe that the best remedy is to keep them irreversibly in chains so the possibility of expression of genes responsible for the diseases can be averted. Even the polyvalent vaccine is not a perfect solution because many of these children have not grown their body immune system. The diplococcic shape is an index of reproduction phase of these bacteria which may also make non-coding RNAs used for the differentiation of diseases [14, 15]. Prevention of growth by blocking bacterial genetic ability to receive biological signals should also be a remedy but since these bacteria grow in heterogeneity we must find bacterial family planning to block reproduction but irreversibly. The heterogeneity can be stably contained in their growth chains by growing them in the presence of xylitol (2% or higher) [13].

In support of our observation, we have also observed some small pinkish bacteria in addition to well defined purple colored individuals. The biologically old ones prevail in the same chain with diminishing cell wall thickness and therefore they are poorly stained but stained pink: spherical, small purple coccusis also present and represents the early phase of growth. Based on all data available we strongly believe the competent, transparent and smooth colonies are purple and represent Gram-positive bacterial growth-phase (early, mid or late log phase) but all of them do not develop cleavage simultaneously. Spontaneous breakage of this chain during growth with shaking should show the heterogeneity of sizes and the difference interaction with crystal violet. Gram-staining produces purple, pink or pinkish depending on their cell wall thickness. They all are not incompetent but a fraction which is irreversibly old should satisfy the definition of the incompetent state and thinning of their cell wall may not be always visible because of its poor interaction with crystal violet. Identification of such Gram-stained bacterium by optical microscopy is still necessary to initiate antimicrobial treatment.

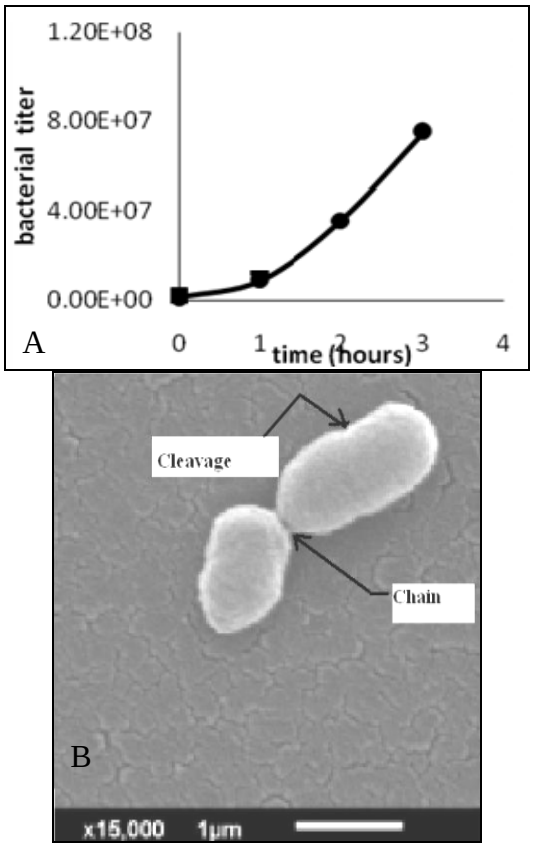

Figure 2. (A) Growth of *S. oralis* in a rich broth without xylitol (growth curve). **(B)** Scanning electron micrograph of two diplococcic bacteria (competent) in a chain (magnification 15000x).

3.1.3. The post-competent phase (stationary or latent phase in liquid broth and opaque or rough colonies in blood agar growth medium

The post-competent phase contains their total population with all heterogeneity of their shapes and sizes.The post component phase culture, equivalent to R colony on blood agar (solid medium) shows their prevailing in heterogeneity. One such chain of *S. oralis* is presented in Fig. 3A. In the same field of electron micrograph, the diplococcic individuals in a pair and the old in a short chain are also seen. They are dissociated from the main chain probably during sample preparation. The old have lost the cell wall thickness by the loss of peptidoglycan layers and therefore they look like spheroplasts with diminishing sizes. Size heterogeneity of the entire population has been clearly visualized by SEM

when the same sample is subjected to shearing forces as shown in Fig. 3B. We conclude that the pre-competent phase has not yet been recognized but the competent-phase grown on blood agar has been recognized as transparent or smooth colonies. However, the post competent phase contains heterogeneity of all growth phases but in a single chain.

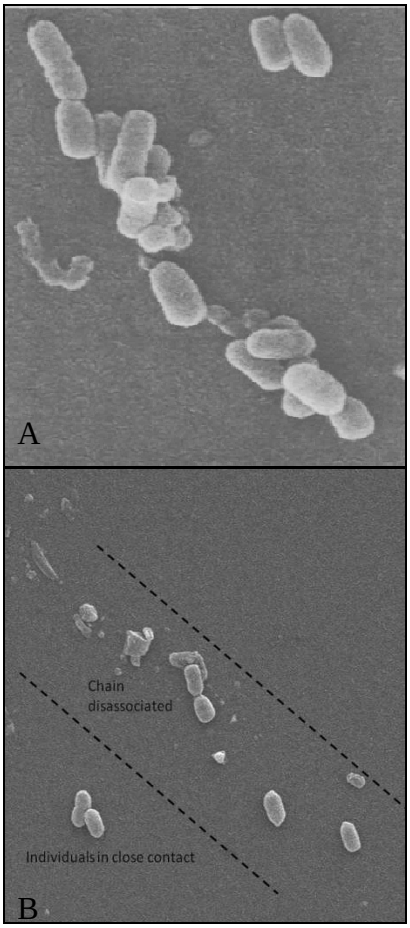

Figure 3. (A) *S. oralis* rough colony as visualized by scanning electron microscopy (SEM) at a magnification of 3000x. In this preparation physical stress is avoided by the presence of 6.5% sucrose. The heterogeneous members of the rough colony prevailing in a long chain with clusters. Individual members in a pair (above this long chain) and the old population in a short chain (below the long chain) appear due to random breakage during sample preparation. **(B)** Size heterogeneity of the *S. oralis* (rough colony) when the clusters in chains are broken by vigorous shaking.

4. DISCUSSION

In agreement with Dr. Griffith we like to re-emphasize that the smooth and rough colonies of diplococcic streptococcus are the two physiological states of growth, adult to old in natural transformation but not any change in genotypes which usually mean chromosomal alterations.

In this work we have discovered an additional phase of growth, termed pre-competent phase is added before competent and post-competent phase. In the absence of such complete knowledge of growth curve the Gram-positive Streptococcal genetics has not flourished. Even now some of the investigators are trying to justify an entry of donor DNA segments (single) in such natural transformation of *S. pneumoniae,* incompetent (pre-mature) to competent (child bearing ability, reproduction) and competent to post competent (mixture of competent and irreversibly old).

Major question remains about the role of donor DNA in such a natural growth cycle of the mitis group Streptococci? To the contrary Berge et al. have shown the presence of 3'hydroxyl end of the donor DNA at the mid-cell position (cleavage site) [9]! If bacterial cell division begins at the cleavage site, then it seems logical that the replicating chromosome remains attached to the replicating bacterial cell membrane and thus justifies the formation of mesosome [9, 16]. Unification of such fragmentary evidence leads us to one conclusion that the chromosome replication and segregation of the replicated chromosomes into their daughters are probably simultaneous events that takes place in the cleavage. In Gram-positive diplococcic *S. oralis* the initiation site of its replicating chromosome (approximately, 1900 Kb) has not yet been confirmed. In *S. pneumoniae*, Drs Firshein and Gelman have separated distinct DNA-protein-phospholipid subcomplexes and also the presence of DNA polymerase III activity [16].

Finally, our interpretation is that our miracle drug is used not to induce mutants but to select mutants who are evolved by mis-match repair due to increased infidelity of the *Streptococci* DNA replication. However, co-ordination of these genetic eventsis regulated by their two-component signaling system. In order to stop them from growing, only option left in our medicine is to help these bacteria to go for birth control by interfering in their bio-communication by the combined use of xylitol and fluoride [13, 17]. This is a part of the two- or one component signal transduction.

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AUTHORS' CONTRIBUTION

SP: has done most of the experiments, prepared the first draft of the manuscript; AP: has been involve in all medical related questions and shared some writing assigments; BC: helped prepare the final manuscript. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Research Article

Effect of mareb crude oil, dispersed oil and dispersant (OSD) on filtration rates of the clams *Tivela ponderosa* under laboratory conditions

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ABSTRACT

This study includes laboratory experiments of filter-feeding activity of bivalves *Tivela ponderosa*. The filtration rate of bivalves *Tivela ponderosa* was controlled hourly at 5^{th} and 10^{th} day (every two and six hours). The bivalve *Tivela ponderosa* is exposed to 0.5 ml, 1.0 ml and 1.5 ml/l of three chemicals test (Crude oil, Crude oil with Dispersant and Dispersant OSD) in addition to liter of seawater through experimental time. Results of the study showed that the Dispersant had a less effect than Crude oil or a mixture of oil with dispersant. A mixture of oil with dispersant is more effect on filter-feeding activity of *Tivela ponderosa*. There are no mortalities during the early 5 days for the three different chemicals test. Bivalves are usually increased in her activity during the first 2 hours and decreased during the next 6 hours of the experiments. The filtration rate of exposed clams was reduced in comparison with the control especially in the presence of polluted chemicals. The condition index of the exposed clams significantly reduced with respect to the control index, which indicated that the treated clams had an uncomfortable condition with low food intake and high energy demand to counteract the toxic effect of toxic chemicals.

Keywords: Filtration rates; Condition Index; Crude oil; Dispersant; *Tivela ponderosa* clams.

1. INTRODUCTION

A number of studies was investigated the clearance rate of marine mussels inhabiting different coastal waters of the world have been published [1-5]. The filtration rate in bivalve molluscs get impaired by pollutants. Therefore, these rates have been widely accepted as a toxicity index. Rate of filtration has been suggested as a reliable sub-lethal toxicity index [3, 5] for filter-feeding bivalves and being a non-destructive method can be carried out with ease. Effects of toxicants like hydrocarbon and chlorinated hydrocarbons on clearance rate of marine mussels have been worked out by D'silva, Reddy and Menon, and Widdows [6-8]. Environmental factors such as salinity, oxygen tension etc., are also known to influence the filtration rate [9]. Recently, several of the physiology researchers have suggested that behaviour of aquatic organisms, which can be collectively termed bio-indicators, can be used as an effective means of improving aquatic environmental monitoring strategies [10-13]. These physiological indices are being the integration of several relatively inexpensive tests should provide a better

assessment of stress from exposure to complex mixtures of xenobiotics in the environment than measurement of any single variable and, they may also serve as early-warning signals of population or community effects [14]. A recent development of environmental bioassays is the biomarker approach which provide early distress signals of exposure to bioavailable contaminants [15, 16]. However, the simultaneous measurement of these cellular responses with organismal responses has been widely recommended for the accurate prediction of changes in the population and community structure [17, 18].

The aim of this study was using bivalves (*Tivela ponderosa*) as bioindicator to determine the effect of toxic chemicals Mareb Crude oil, oil with Dispersant and Dispersant OSD (Oil Spill Dispersant) on physiological processes of the bivalves such as filtration (filter-feeding), and also to study the condition index of the exposed clams.

2. MATERIAL AND METHODS

Bivalves were collected from Abyan Coast in Aden Governorate. They were collected by hand during the spring low tides in the evening times to avoid higher temperatures and were then kept in open canvas sacs containing wet sand to minimize frictions, desiccation and then transported to the laboratory immediately. They were protected from agitation during the transportation. Bivalve were cleaned by gentle rubbing in clean seawater to remove the clogged sediment and mucus and kept in aquaria of uniform size, 40 cm long, 25 cm wide 20 cm height, each aquaria containing clean and filtered seawater. Clams of uniform size of (47 ± 1) mm long were used in the study to avoid susceptible size-based variations in response to the test chemicals of (Crude oil, Crude oil with Dispersant and Dispersant). At the end of the acclimatization time - 4 days, all clams are in a good condition to tolerate the experimental different conditions. There should be less than 2% mortality during acclimatization [19]. In the present study neutral red method [20-23] was followed to assess the filtration rate of bivalves *Tivela ponderosa* after exposure ten days to various sublethal three chemicals test are Crude oil, Crude oil with Dispersant and Dispersant (0.5 ml, 1.0 ml and 1.5 ml/l). 10 mg of neutral red (BHD Ltd.) was dissolved in minimum quantity of distilled water and diluted with seawater to get a concentration of 1.0 mg/l in the test containers. 400 ml of the prepared neutral red solution was added to each glass beaker. Then four clams from each treated group and control group were transferred carefully to the solutions and kept for 15 minutes to acclimatize. The filtration rate was monitored at hourly intervals for five days and ten days (every two and six hours). 10 ml of the solution was withdrawn at the end of each hour acidified and then the concentration of the neutral red solutions were determined by reading the optical density at 530 nm on a spectrophotometer. The above mentioned method of filtration measurement was followed. The filtration rate was calculated by the equation of Coughlan [21].

$$\text{Filtration rate (m)} = \frac{M}{nt} \log_e \frac{Co}{Ct}$$

Where: m - Filtration rate in ml/min., M - Volume of test solution in ml, n - Number of animals in the test vessels, Co - Dye concentration in initial sample), Ct - Dye concentration in final sample, and t - Time between dye samples in minutes).

After termination of the experiment, the soft tissues were shucked and dried at 80° C to a constant weight in a hot air oven.

A condition index relating dry tissue weight to shell length was calculated by:

$$\text{Condition index} = \frac{\text{Dry Weigh (g)}}{\text{Shell length (mm)}} \times 100$$

The soft tissues were shucked off the shells, weighted then dried as above and reweighted to get the dry tissue weights. Then the dry/wet tissue weight ratio in percentage was calculated.

$$\text{Dry/Wet weight ratio} = \frac{\text{Dry Weight}}{\text{Wet weight}} \times 100$$

3. RESULTS

3.1. Filtration rates

The filtration rates of bivalves *Tivela ponderosa* which were transferred to chemicals test-free seawater after exposure to test chemicals solutions (Crude oil, Crude with Dispersant and Dispersant) for 5 and 10 days are depicted in Tables 1, 2 and Fig. 1 - for 5 days, and depicted in Tables 3, 4 and

Fig. 2 - for 10 days. When the clams exposed to chemicals test showed variation of filtration rate in relation to the control. The average rate of filtration during 5 days decreased slightly in case of the clams exposed to the low test chemicals concentrations (0.5 ml/l) and when the clams exposed to medium and high test chemicals concentrations (0.1 ml/l) and (1.5 ml/l) showed decreased the rate of filtration compared with the control. And finally the filtration rate in high concentrations reached to the lowest rate within 5 to 10 days for each test chemicals.

3.2. Condition Index

The observed values of condition index are the ratio percentage between the dry weight (g) and shell length (mm), and are given in Tables 5, 6 and Fig. 2, which indicate a reduction in condition index of the clams exposed to Mareb Crude oil, Crude oil with Dispersant and Dispersant respectively for 5 and 10 days. The condition index of the control clams was 2.927 after 5 days, and 2.751 after 10 days. Where decreasing condition index of the clams exposed to different concentrations Mareb Crude oil during 5 and 10 days, in the low concentration (0.5 ml/l) to 2.587 and 2.446, the medium concentration (0.1 ml/l) were 2.452 and 2.238, the high concentration (0.1 ml/l) were 2.396 and 2.198, and for Mareb Crude oil with Dispersant were 2.673 and 2.428, 2.527 and 2.328, 2.270 and 2.095, for Dispersant were 2.738 and 2.210, 2.379 and 2.114, 2.584 and 2.487, respectively.

Table 1. The filtration rate (ml min^{-1} g^{-1} dry weight) of bivalves exposed to test chemical concentrations for five days (after two hours) values are mean ± S.D for 2 determination.

Concentrations (ml/l)	Exposure time of bivalve to test chemicals for five days (after two hours)		
	Mareb Crude Oil Mean ± S.D	Mareb Crude Oil with Dispersant Mean ± S.D	Dispersant OSD Mean ± S.D
control	0.283 ± 0.005	0.283 ± 0.005	0.283 ± 0.005
0.5	0.283 ± 0.023	0.283 ± 0.023	0.189 ± 0.005
1.0	0.224 ± 0.055	0.177 ± 0.022	0.100 ± 0.006
1.5	0.166 ± 0.022	0.138 ± 0.014	0.073 ± 0.014

Table 2. The filtration rate (ml min^{-1} g^{-1} dry weight) of bivalves exposed to test chemical concentrations for five days (after six hours) values are mean ± S.D for 2 determination.

Concentrations (ml/l)	Exposure time bivalve to test chemicals for five days (after six hours)		
	Mareb Crude Oil Mean ± S.D	Mareb Crude Oil with Dispersant Mean ± S.D	Dispersant OSD Mean ± S.D
control	0.113 ± 0.005	0.113 ± 0.005	0.113 ± 0.005
0.5	0.108 ± 0.005	0.129 ± 0.001	0.104 ± 0.005
1.0	0.093 ± 0.004	0.115 ± 0.008	0.072 ± 0.008
1.5	0.076 ± 0.008	0.051 ± 0.002	0.041 ± 0.005

Table 3. The filtration rate (ml min^{-1} g^{-1} dry weight) of bivalves exposed to test chemical concentrations for ten days (after two hours) values are mean ± S.D.

Concentrations (ml/l)	Exposure time of bivalve to test chemical concentrations for ten days (after two hours)		
	Mareb Crude Oil Mean ± S.D	Mareb Crude Oil with Dispersant Mean ± S.D	Dispersant OSD Mean ± S.D
control	0.425 ± 0.021	0.425 ± 0.021	0.425 ± 0.021
0.5	0.381 ± 0.021	0.406 ± 0.014	0.406 ± 0.014
1.0	1.285 ± 0.015	0.331 ± 0.005	0.331 ± 0.005
1.5	0.061 ± 0.071	0.276 ± 0.021	0.276 ± 0.021

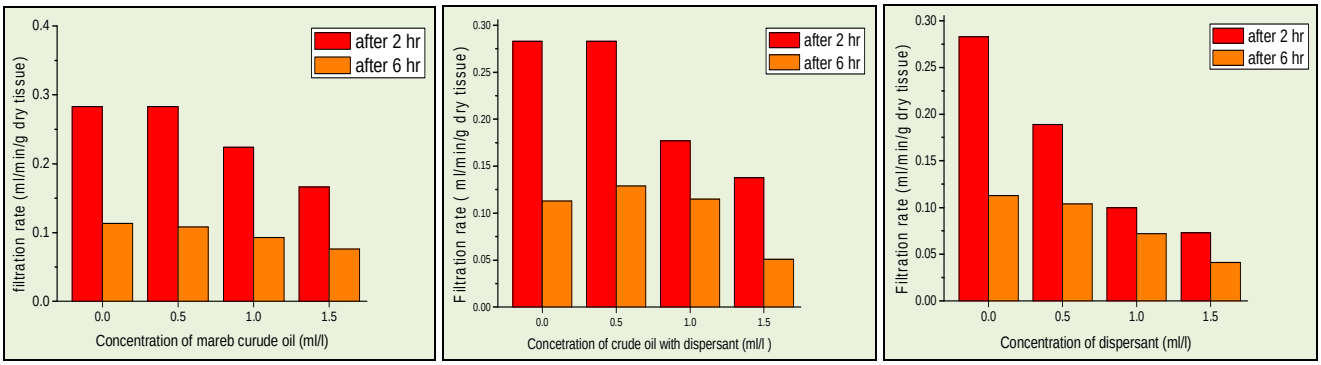

Figure 1. Filtration rate of *Tivela ponderosa* after 5 days exposed to test chemical concentrations (after two and six hours).

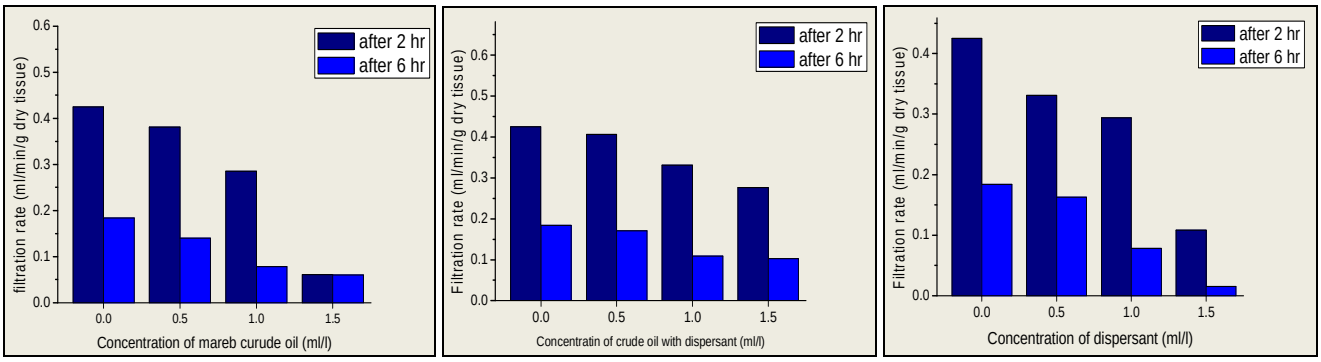

Figure 2. Filtration rate by *Tivela ponderosa* after 10 days exposed to test chemical concentrations (after two and six hours).

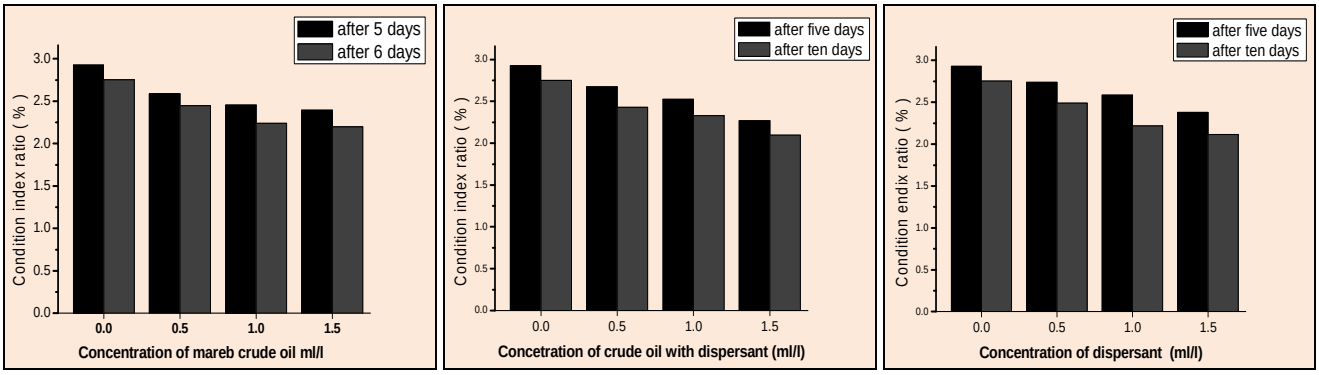

Figure 3. Effect of test chemical concentrations on condition index of *Tivela ponderosa* after five and ten days exposure.

Table 4. The filtration rate (ml min^{-1} g^{-1} dry weight) of bivalves exposed to test chemical concentrations for ten days (after six hours) values are mean ± S.D for 2 determination.

Concentrations (ml/l)	**Exposure time of bivalve to test chemical concentrations for ten days (after six hours)**		
	Mareb Crude Oil	**Mareb Crude Oil with Dispersant**	**Dispersant OSD**
	Mean ± S.D	**Mean ± S.D**	**Mean ± S.D**
control	0.184 ± 0.005	0.184 ± 0.005	0.184 ± 0.005
0.5	0.140 ± 0.005	0.171 ± 0.027	0.163 ± 0.004
1.0	0.078 ± 0.014	0.109 ± 0.023	0.078 ± 0.014
1.5	0.060 ± 0.006	0.103 ± 0.014	0.015 ± 0.007

Table 5. *Tivela ponderosa*: condition index as a function of test chemical concentrations for five days.

Concentrations (ml/l)	Condition index for *Tivela ponderosa*		
	Mareb Crude Oil Mean ± S.D	Mareb Crude Oil with Dispersant Mean ± S.D	Dispersant OSD Mean ± S.D
control	2.927 ± 0.134	2.927 ± 0.134	2.927 ± 0.134
0.5	2.587 ± 0.326	2.673 ± 0.071	2.738 ± 0.260
1.0	2.452 ± 0.289	2.527 ± 0.372	2.379 ± 0.206
1.5	2.396 ± 0.182	2.270 ± 0.064	2.584 ± 0.018

Table 6. *Tivela ponderosa*: condition index as a function of test chemical concentrations for ten days.

Concentrations (ml/l)	Condition index for *Tivela ponderosa*		
	Mareb Crude Oil Mean ± S.D	Mareb Crude Oil with Dispersant Mean ± S.D	Dispersant OSD Mean ± S.D
control	2.751 ± 0.232	2.751 ± 0.232	2.751 ± 0.232
0.5	2.446 ± 0.312	2.428 ± 0.074	2.21 ± 0.0817
1.0	2.238 ± 0.565	2.328 ± 0.419	2.114 ± 0.323
1.5	2.198 ± 0.203	2.095 ± 0.541	2.487 ± 0.403

Table 7. Dry-wet weight ratio as a function of test chemical concentrations five days. Values are means ± S.D, n=4.

Concentrations (ml/l)	Dry-wet Weight Ratio for *Tivela ponderosa*		
	Mareb Crude Oil Mean ± S.D	Mareb Crude Oil with Dispersant Mean ± S.D	Dispersant OSD Mean ± S.D
0.0 (control)	25.11 ± 0.44	25.11 ± 0.44	25.11 ± 0.44
0.5	24.34 ± 0.36	23.52 ± 0.16	24.76 ± 0.20
1.0	22.89 ± 0.06	22.50 ± 0.71	23.73 ± 0.37
1.5	21.93 ± 0.44	21.79 ± 0.22	23.08 ± 0.11

Table 8. Dry-wet weight ratio as a function of test chemical concentrations exposure ten days. Values are means ± S.D, n=4.

Concentrations (ml/l)	Dry-wet Weight Ratio for *Tivela ponderosa*		
	Mareb Crude Oil Mean ± S.D	Mareb Crude Oil with Dispersant Mean ± S.D	Dispersant OSD Mean ± S.D
0.0 (control)	25.08 ± 0.07	25.08 ± 0.07	25.08 ± 0.07
0.5	24.24 ± 0.35	23.07 ± 0.70	23.81 ± 0.23
1.0	22.06 ± 0.06	21.73 ± 0.31	22.94 ± 0.73
1.5	21.12 ± 0.07	20.81 ± 0.97	22.23 ± 0.25

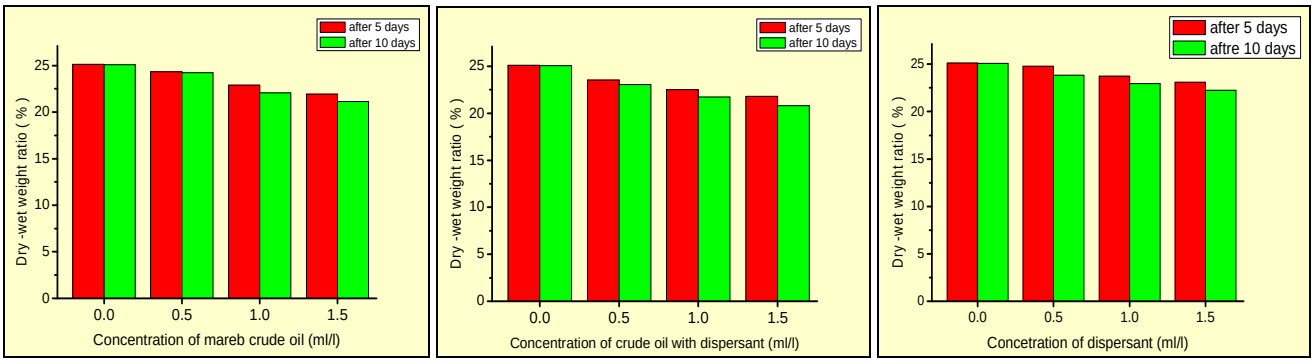

Figure 4. Effect of test chemical concentrations on dry-wet weight ratio of *Tivela ponderosa* after (five-ten days).

3.3. Dry-Wet Weight Ratio

The data of the ratio between dry and wet weights of the control and exposed clams during 5 and 10 days are shown in Tables 7, 8 and Fig. 3. The decreased ratio was more or less inversely proportional to the degree for each test chemicals concentrations compared to the control. The ratio were 25.11 and 25.08 in the control and in the exposed clams to Crude oil, Crude oil with Dispersant and Dispersant respectively compared to the control were 24.34, 24.24; 22.89, 22.06; 21.93, 21.12 and 23.52, 23.07; 22.50, 21.73; 21.79, 20.81 and 24.76, 23.81; 23.73, 22.94; 23.08, 22.23.

4. DISCUSSION

4.1. The filtration rate

The effect of pollutants on filtration states that measurements of filtration rate gives a reliable indication of toxic effects. There are many endogenous and exogenous factors which may be expected to influence the effect of pollutants on the filtration rate of clam [3].

The present investigation showed that the filtration rate of *Tivela ponderosa* exposed to test chemicals reduction in the rate of filtration in all the concentrations with respect to the control. The reduction of the filtration rate in the presence of test chemicals concentrations can be explained to be the influence of test chemicals on the chemoreceptors of the clams which affect the activity of the lateral cilia of ctenidia filaments by reducing the water intake through the inhalant siphon. According to Foster-Smith [24] the filtration rate of bivalves is directly dependent on the activity of the lateral cilia of the gill filaments with changes in the size of ostia and inhalant and exhalent siphons or apertures affecting the resistance to flow through the gills. Furthermore the general narcotization of the clams in the high phenanthrene concentrations might be a possible cause for the lowest filtration rate in those organisms as it was evident in both the conditions. Another possibility for the reduction of filtration rate may be an overloading of the gills with the dye particles trapped by the mucus which resulted in minimizing the gill porosity and hence slowing the flowing rate. Similar explanation was given by Jorgensen [25] who explained the low filtration due to overloading of the gills with microorganisms which reduced the rate of filtration. These results are supported by Eapen [26] who recorded a decrease in filtration rate of *Anadara granosa* which exposed to naphthalene for 96 hours, also a slight increase of filtration rate in clams were exposed to the lowest naphthalene concentration when transferred to naphthalene-free seawater which corresponds with the present results.

Table 4 showed a reduction in filtration timewise in the medium and high concentrations (1.0 ml/l, 1.5 ml/l), that the filtration rate was maximum in the first hour then decreased subsequently until it became minimum in the fifth hour of the experiment. Similar findings were recorded by Durve [20] in his studies on the filtration rate of *Meretrix casta* in various salinities. Axiak and George [27] were studied the gill functions and cilia activities of the clam, *Venus verrucosa* exposed to petroleum hydrocarbons, and reported a reduced velocity of metachronal waves which indicates a reduced frequency of lateral cilia beating with a resultant decrease in pumping rates. In addition the activities of terminal and sensory cilia were enhanced and mucus production increased. Similarly, Johnson [28] reported a change in cilia behavior due to exposure of petroleum products.

4.2. Condition Index

This study has shown that both the condition index and the ratio of dry-wet weight tissues in *Tivela ponderosa* were significantly altered by the exposure to phenanthrene. The condition index parameter is another tool to interpret the growth rate of the animal and the actual energy balance indicating protein, carbohydrate and lipid catabolism to counteract the stressful conditions of pollution. The present results showed a gradual decrease of condition index as the test chemicals concentrations increased. This condition was also observed by other authors like Roesijidi and Anderson [29] in *Macoma inquinata* and Stekoll et al. [30] in *Macoma ballhica*. Both the studies related the decrease in condition index to the negative energy balance which indicates energy utilization rather than storage. Grauby and Spliid [31] recorded a highly significant negative correlation with PAH in the

common mussel. According to Anderson [32] the time factor is necessary in obtaining a good result in condition index.

The dry-wet weight ration also showed significant variation from the control with the decrease in values corresponding to the increase in phenanthrene concentration. The faster decrease in the dry weight in relation to the wet tissue weight may be explained by the loss of dry weight for using their energy reserves due to phenanthrene exposure. This state probably occurred because of various factors such as decreasing in feeding accompanied by the increase in metabolic rate and reduction in filtering rates. These results are in agreement with the conclusion recorded by Sophia and Balasubramanian [33] who studied the clam *Meretrix casta* exposed to various Crude and fuel oils whereby they lost dry weight faster than wet weight. Stekoll et al. [30] reported similar results.

AUTHORS' CONTRIBUTION

NAH and ASD have done the practical work; NAH wrote the research; EAS analized the results; NAAS supervised and helped in results analysis. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Research Article

Feeding of bait containing attractant and sublethal dose of different molluscicides and the reproduction of snail *Indoplanorbis exustus*

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ABSTRACT

The snail control is one of the important methods in the campaign to reduce the incidence of fasciolosis. In order to achieve this objective, the method of bait formulation containing an attractant and molluscicide is an appropriate approach to lure the snail to the molluscicide. The present study was to observe the effects of sublethal (60% of 24h LC_{50}) feeding of active molluscicide ferulic acid, umbelliferone, eugenol and limonene in bait containing attractant carbohydrate (starch, maltose) on the, fecundity, hatchability and survival of snail *Indoplanorbis exustus*. Bait containing attractant carbohydrate and molluscicides significantly reduced the reproductive capacity of the snail *Indoplanorbis exustus*. Maximum reduction in fecundity (50.64% of control) was observed in snail fed to starch + eugenol. In withdrawal group significant recovery was noted in all bait fed snails (90.71% of control). The hatching period of egg laid by treated group was prolonged from 11 to 19 days with respect to 10 to 13 days in control snails. Percent hatchability as well as survivability of young snail were significantly reduced in bait fed snails.

Keywords: Carbohydrate; Bait formulation; Molluscicides; Reproduction; *Indoplanorbis exustus.*

1. INTRODUCTION

Fasciolosis is a global zoonotic disease [1]. There is a very high incidence of fasciolosis in the cattle population of the eastern region of the Uttar Pradesh [2]. Liver fluke species of the genus *Fasciola* is the causative agent of this disease [3, 4]. The snail *Indoplanorbis exustus* is acknowledged intermediate host of these fluke [5, 6]. At the optimum temperature of 30 ^{0}C each snail can lay up to 800 eggs [7]. One of the possible approaches to eradicate or control this problem is to interrupt the life cycle of the parasitic trematodes by eliminating the snail. A number of chemically diverse plant molluscicide have been isolated and identified [8]. However, the natural products in general have an advantage over synthetic products, because natural products have ecofriendly, biodegradable and hence are less likely to accumulate in the environment. Previously, it has been reported by us that ferulic acid, umbelliferone (*Ferula asafoetida*), eugenol (*Syzygium aromaticum*) and limonene (*Carum carvi*) were potent molluscicide [9]. The use of bait contai-

ning snail attractant and molluscicides is a good technique for the species specific control of snails. This method has ecological and toxicological advantage over the use of conventional pesticide [10-13]. Attractant carbohydrate starch and maltose are very effective against harmful snail *I. exustus* [12]. In the present study effect of feeding bait formulations containing sublethal (60% of 24h LC_{50}) doses of molluscicides on the reproduction of the snail *I. exustus* were studied.

2. MATERIAL AND METHODS

2.1. Test animals

The adult snails *Indoplanorbis exustus* (0.85 ± 0.37 cm in shell length) were collected from local ponds, lakes and low lying submerged fields of Gorakhpur and were used as test animals. The snails were acclimatized for 72h in laboratory conditions in a glass aquarium containing dechlorinated tap water (temp. 22-24 ^{0}C; pH 7.3-7.4; dissolve oxygen 6.6-7.3 ppm; free carbon dioxide 5.3-6.4 ppm; bicarbonate alkalinity 103-106 ppm). Twenty experimental snails were kept in glass aquaria containing 3L of dechlorinated tap water at 24 to 26 ^{0}C. *I. exustus* laid their eggs on the lower surface of leaves of the aquatic plants in the form of elongated gelatinous capsules containing 10-600 eggs.

2.2. Pure chemical compounds

Agar-agar, carbohydrate (starch and maltose) and active molluscicide ferulic acid, umbelliferone, eugenol and limonene were used in the bait formulations. The pure active components ferulic acid (4-Hydroxy-3-methoxycinnamic), umbelliferone (7-Hydroxy coumarin; 7-hydroxy-2H-1-benzopyran -2-on), eugenol (2-Methoxy-4-(2-propenyl) phenol) and limonene (R)-4-Isopropenyl-1-methyl-1-cyclohexene) were purchased from Sigma Chemical Co. (USA).

2.3. Bait formulations with active molluscicidal components

Formulations of bait containing carbohydrate (starch and maltose 10 mM) and sub-lethal (60% of 24h LC_{50}) molluscicides ferulic acid, umbelliferone, eugenol and limonene were prepared in 100 ml of 2% agar solution by the method of Madsen [14] and modified by the Kumar et al. [12]. Concentrations of carbohydrate/molluscicides were based on the earlier reports of Tiwari and Singh [15, 16] and Kumar et al. [12]. These solutions were spread at a uniform thickness of 5 mm. After cooling the bait containing sublethal molluscicides were cut out a corer measuring 5 mm in diameter. Six replicates were prepared for each concentration. Control group of snails were fed with bait without molluscicide.

2.4. Experimental procedure

The experimental procedure was performed by the method by Tiwari and Singh [15, 16]. The experimental equipments consist of a clean glass aquarium having a diameter of 30 cm. Each aquarium was divided into four concentric zones; Zone 3 (Central zone), 2, 1 (Middle zone) and zone 0 (Outer zone) had diameters of 13, 18, 24 and 30 cm, respectively. A small annular elevation of 9 mm height and 2.4 cm diameter was made in the centre of aquarium (Zone 3). Zone 0, zone 1, zone 2 and zone 3 had an area of 254.35, 197.82, 121.68 and 132.6 cm^2, respectively on the periphery of aquarium (Fig. 1). The aquaria were then filled with 3L of dechlorinated tap water to a height of 8 mm and maintained at 26 ± 1 ^{0}C. At the start of the experiment twenty snails of uniform size were placed on the circumference of zone 0. Simultaneously, one of the prepared bait of different active component (molluscicides) was added on the small annular elevation in the center (Zone 3). Six sets of experiments have been designed with twenty snails in each replicate.

2.5. Treatments

Snails were fed to bait containing sublethal (60% of 24h LC_{50}) concentration of ferulic acid/ umbelliferone/eugenol/limonene and carbohydrate starch/maltose as attractant and their effect on the reproduction was studied by the method of Kumar et al. [12]. Groups of 20 snails in 3L water were fed to sublethal concentrations (60% of 24h LC_{50}) of different combinations of molluscicides with carbohydrate.

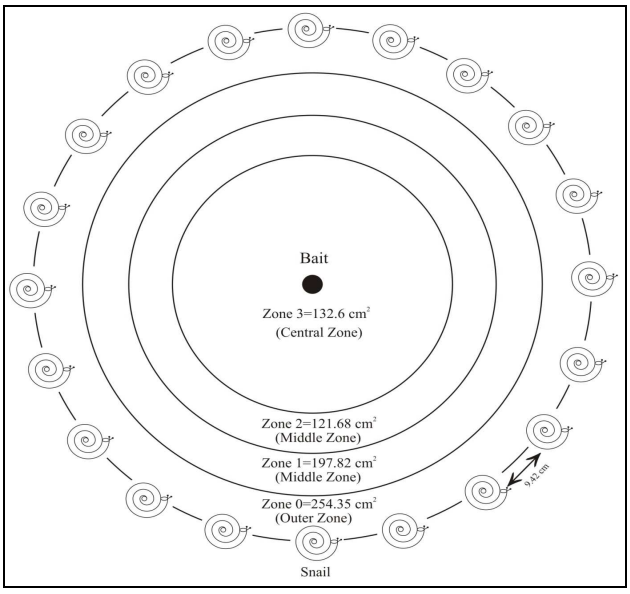

Figure 1. Experimental design of the aquarium for the feeding of bait containing attractant and sublethal dose (60% of 24h LC_{50}) of different molluscicide on the reproduction of snail *I. exustus*. Diameter of Zone 3, Zone 2, Zone 1 and Zone 0 were 13, 18, 24 and 30 cm, respectively. Bait was placed in the center of Zone 3, whereas 20 marked snails were placed at periphery of Zone 0. The distance between the two snails was 9.42 cm.

2.6. Fecundity, hatchability and survivability of snail

These experiments were performed according to the method of Kumar et al. [17]. The total number of eggs laid by bait fed and control snails were counted after every 24h for 96h. Since it is difficult to detect the mother snails for particular spawn, capsules containing eggs from each feeded group were incubated at 30 ^{0}C in covered petridishes. The development of embryos at regular intervals was observed under binocular microscope until hatching. Per cent hatching was studied only with eggs laid after the 24h feeding period. Dead embryo was removed to avoid any contamination. Survival of young snails was observed up to 72h. Snails were transferred to fresh water after a 96h feeding period to observe the effect of the bait after withdrawal.

2.7. Statistical analysis

Each experiment was replicated at least 6 times. Values were expressed as Mean ± SE. Students 't' test was applied to determine the significant ($P<0.05$) difference between bait feeded and control group of the animals. Product moment correlation coefficient was applied in between exposure time and different values of fecundity/ survival of hatched snails [18].

3. RESULTS

In control groups of 20 snails laid 230-240 eggs/day. There was a significant ($p<0.05$) reduction in the fecundity of snail *I. exustus* feeding to 60% of LC_{50}/24h of ferulic acid, umbelliferone, eugenol and limonene with carbohydrate (starch/maltose) as a snail attractants in bait formulations (Fig. 2). No egg lying after 96h feeding was observed in snails fed to 60% of 24h LC_{50} of bait formulations of starch + ferulic acid /eugenol or maltose + ferulic acid. The hatching period was prolonged in treated group (11-19 days) with respect to control group (10-13 days) (Fig. 3). Withdrawal of snail after 96h feeding of baits for next 72h in fresh water caused a significant ($P<0.05$) recovery in the fecundity of snails with respect to their corresponding treatment (Fig. 2).

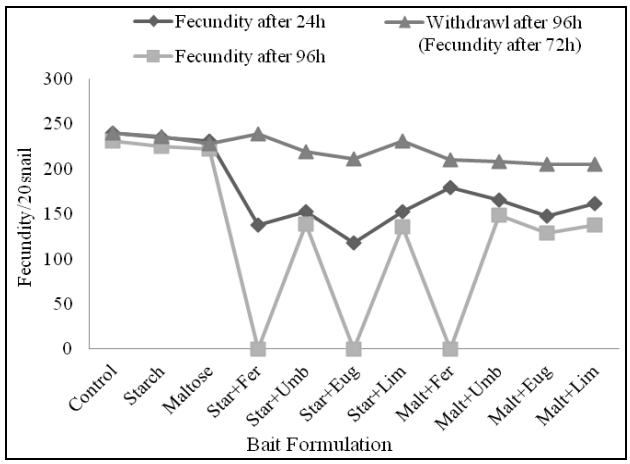

Figure 2. Effect of sublethal feeding (60% of 24h LC_{50}) of molluscicides ferulic acid/umbelliferone/eugenol/limonene and carbohydrate (starch/maltose) as attractant in bait formulation on the fecundity after 96h of the snail *Indoplanorbis exustus*.

Each value is mean ± SE of six replicates. Each replicates represents the egg laid by the group of 20 snails. Student "t" test was applied to treated and control groups. Product moment correlation coefficient showed that there was significant ($P<0.05$) negative correlation in between the exposure period and fecundity of snail *Indoplanorbis exustus*. Abbreviations: Star=Starch, Malt=Maltose, Fer=ferulic acid, Umb=umbellifelone, Eug=eugenol, Lim=limonene.

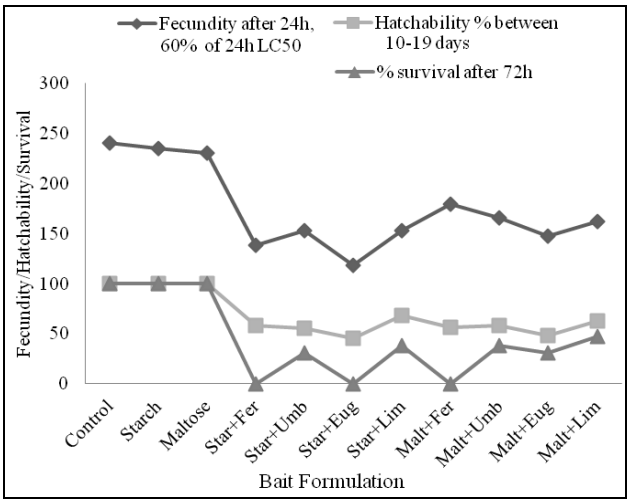

Figure 3. Effect of sublethal feeding (60% of 24h LC_{50}) of molluscicides ferulic acid/umbelliferone/eugenol/limonene and carbohydrate (starch/maltose) as attractant in bait formulation on the fecundity, % hatchability and % survival after 72h of the snail *Indoplanorbis exustus*.

Each value is mean ± SE of six replicates. Each replicates represent the egg laid by the group of 20 snails. Significant ($P<0.05$) when student "t" test was applied to treated and control groups. Product moment correlation coefficient showed that there was significant ($P<0.05$) negative correlation in between survival period and survival of the snail *Indoplanorbis exustus*.

Non survival after 72h was noted in young snails fed with 60% of 24h LC_{50} of starch + ferulic acid/eugenol and 60% of 24h LC_{50} of maltose + ferulic acid (Fig. 3). There was a significant ($P<0.05$) negative correlation between the feeding time and survival of young snails hatched from eggs laid by snail feeded to 60% of 24h LC_{50} of different formulations of bait (Fig. 3).

4. DISCUSSION

Present study clearly demonstrates that the *I. exustus* were attracted by the bait pellets. *I. exustus* showed a behavioral response towards the different combination of carbohydrate and molluscicides. It has been reported that gastropods detect the carbohydrates as indicator of their food [12, 16, 19, 20]. The greater attraction of the snail *Lymnaea acuminata* and *I. exustus* to starch and maltose in the snail attractant pellets is possibly due to fact that in nature starch is the major carbohydrate stored in aquatic plant and maltose is released by some epiphytic algae where snail are found [12, 14, 21]. The result section clearly indicates that sublethal feeding of bait containing molluscicide (60% of 24h LC_{50} and attractant) and attractant starch/maltose significantly reduced the reproductive capacity of snail *I. exustus*. Bait formulations with 60% of 24h LC_{50} of starch + ferulic acid/starch + eugenol/maltose + ferulic acid significantly ($P<0.05$) reduced the fecundity of *I. exustus* within 72h.

Direct releases of plant derived molluscicide (ferulic acid, umbelliferone, eugenol and limonene) in aquatic environment have dose dependent influence on the fecundity of snails *Lymnaea acuminata* [22]. A number of plant products have been effectively used for control of snail reproduction [23-26]. Kumar et al., [17] has reported the reproduction of *Lymnaea acuminata* fed to bait containing binary combination of amino acids with molluscicide. Maximum reduction in the fecundity was observed in bait containing 60% of 24h LC_{50} of ferulic acid + valine + aspartic acid. Kumar et al., [12] have reported the bait containing 60 % of 96h LC_{50} of eugenol with starch + histidine, starch + methionine; respectively inhibit alkaline phosphatase (ALP) (20 % of control) and acetylcholinesterase (AChE) (49.49 % of control) activity in the nervous tissue of *L. acuminata*. Although, the 0.01 % concentration of eugenol caused a significant decrease in the fecundity of potato tuber moth (*Phthorimaea aperculla*) and decreased the percentage of egg hatchability [27]. The significant inhibition of certain enzymes in the nervous tissue of snail by bait containing starch + eugenol and starch + ferulic acid may be the cause of the reduction in hatchability and survival of young snail *I. exustus*. In the present study the mode of entry of active molluscicides into the snail *Indoplanorbis exustus* body through the digestive system, this may affect the caudodarsal cells (CDSs) in brain and ultimately decrease the release of the ovulation hormone that resulted a decrease in the fecundity of bait feeded snails. Roubos et al., [28] has reported that the caudodarsal cell is responsible for the fecundity of snail *Lymnaea*. Kumar et al., [29, 30] reported that there was a depletion of amino acid/protein and nucleic acid levels in the ovotestis of *L. acuminata.* Alkaline phosphatase play a critical role in protein synthesis [31], shell formation [32] and other secretary activity [33] and its inhibition may result in the reduction of protein level in gastropods [34].

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

Therefore, the transfer of mother snails to fresh water for the next 72h after 96h bait feeding all the feeded snails leads to a significant recovery in the fecundity. Thus, recovery of the effects would be an added advantage in their use against aquatic target snails as they would cause only short-lived effects. The bait formulations technique with sublethal molluscicides is a new concept for the control of snail population. This technique can be used to make a significant sterility in snails as well as in development of embryonic stages without releasing more active molluscicide in aquatic environment.

AUTHORS' CONTRIBUTION

All authors are involved in conception, design and drafting of this research article. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Research Article

Winter wheat yield and soil physical properties responses to different tillage and irrigation

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ABSTRACT

Adopting a better tillage system not only improves the soil properties and crop productivity but also improves water use efficiency. A field experiment was conducted over 2 years in Norman E Borlaug Crop Research Centre of the Govind Ballabh Pant University of Agriculture and Technology, Pantnagar region of Uttarakhand, India to determine the effect of tillage systems and irrigation treatments on soil physical properties and yield of winter wheat (*Triticum aestivum* L.). Plots having conventional tillage along with frequent irrigation showed higher values of saturated hydraulic conductivity while zero tillage gaves the higher values of bulk density, mean weight diameter, plant available water capacity, effective porosity and soil organic carbon. Wheat yield was not significantly affected by tillage practices. There was a significant increase in wheat yield in the plots under I4 over I1 and water use efficiency between irrigation treatments was also significantly different. Hence, for wheat crop in a sandy loam soil of the Uttarakhand, farmers may adopt CT with four irrigations to achieve maximum yield. This information is important for farmers from the point of view of increasing production and profitability.

Keywords: Tillage; Irrigation; Yield; Soil physical properties; Water use efficiency.

1. INTRODUCTION

Wheat (*Triticum aestivum* L.), second most important cereal crop in India, with an average annual production of 80 Mt and contribute approximately 11.79 percent in world's wheat production. Wheat is the crop which is sown and harvested every month in different parts of the world [1]. One of the main constraints in wheat production is improvement of production and profitability. In any cropping system, to increase the production there is need to improve the input use efficiency. So, to overcome this, producers started to adopt different resource conserving practices like zero tillage, surface seeding and manipulations in numbers of irrigations [2].

Tillage and irrigation can be considered as most critical management practices, as both affect the production potential of crop and soil hydro-physical properties [3]. Big issue under rainfed farming in sub-temperate regions of the Indian Himalayas is irrigation [4]. Role of irrigation is quite clear as; soil moisture deficits at critical crop growth stages can affect the wheat yield. There is correlation between tillage intensity and soil

moisture regime. Hatfield and Stewart [5] reported that conservation tillage is helpful in maintaining soil moisture in comparison to intensive tillage operation. Tillage plays importnat role in changing initial state of soil which modify whole environment, like bulk density and porosity which affects the infiltration rate of soil [6]. Bhattachariya [4] and Li [7] already reported that, change in bulk density depends on the intensity of tillage systems. Different tillage systems produce different results like zero tillage (ZT) promotes SOC sequestration [8], improved soil aggregates [9] and better pore size allocation [10] while conventional tillage (CT) usually increases available water capacity and infiltration rate and decreases runoff [11]. Field experiments with zero tillage in wheat at several locations shown encouraging results [12, 13], while Cavalieri [14], reported low wheat yield under zero tillage due to formation of compact layer below the plough layer. On the other side, conventional tillage (CT) in specific ecosystems may improve some key soil properties compared to those under NT [15].

Hence, implementation of proper tillage practice in wheat system is most important for better resource conservation and in optimizing crop production. Even though the mean rainfall during wheat growing season are 37 and 191.7 mm, during 2011-2012 and 2012-2013, respectively (Table 1).

Table 1. Total rainfall received during the wheat growing season (Oct-Apr) over the years (2011-2013) at Pantnagar, Uttarakhand, India.

Years	Rainfall (mm)
2011-2012	37.0
2012-2013	191.7
Mean (2011-2013)	233

As soil moisture is available due to rain, but there is an urgent need to organize the irrigation scheduling during the crop period. Scanty literature is there regarding to the subject, how different tillage and irrigation schedules affect yield performance of wheat and soil properties. Moreover, we found no report on the scheduling of irrigation in this region under different tillage practices. Therefore, this investigation was undertaken to study the effect of tillage practices on soil physical properties, water use and grain yield of wheat crop under irrigation at critical growth stages of wheat in a sandy loam soil of the Uttarakhand.

2. MATERIAL AND METHODS

2.1. Experimental site

The experiment was conducted at the Norman E Borlaug Crop Research Centre of the Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand. The experimental site was situated at $28^0$30'N latitude, $79^0$30'E longitude and 243.8 m altitude. The soil (0-15 cm deep) of the experimental field had a pH of 6.4, bulk density of 1.41 Mg m^{-3}, SOC of 0.73 %, available P of 14 kg ha^{-1} and available K of 145 kg ha^{-1}. The subsurface soil (15-30 cm deep) had a bulk density of 1.44 Mg m^{-3}.

2.2. Experimental design and treatments

The experiment was laid out in a two factorial randomized block design with tillage operation:

1. Zero tillage (ZT): removing weeds & previous crop residues, minimum soil disturbance was achieved with line openers for sowing,
2. Conventional tillage (CT): disc harrow were used two times up to 30 cm followed by manual land leveling in each plot and
3. Deep tillage (DT): done by plowing the field by Mold bold plough up to 45 cm followed by land leveling as first factor and application of five irrigations as second factor: I_1: crown root initiation (CRI), I_2: CRI + late tillering (LT), I_3: CRI + LT + late joining (LJ), I_4: CRI + LT + LJ + flowering (F), and I_5: CRI + LT + LJ + F + milking (M), were applied at critical growth stage. These treatments were repeated in the same plots during both the years. Each treatment was replicated three times and plot size was of 20.0 m^2.

2.3. Crop management

Shallow furrows at 3 to 5 cm depth were opened at 23 cm distance using tractor driven furrow opener and sowing of wheat (UP-2565) was done manually. The furrows were covered immediately manually. The fertilizer dose was 120:60:40

(N:P:K) kg ha^{-1}. Full rate of P as single super-phosphate, K as muriate of potash were applied at the time of sowing and, N was applied in 3 splits (1/2 at the time of sowing, 1/4 at crown root initiation stage and remaining 1/4 at pre-ear head emergence) using urea. For controlling the pre-emergence weeds Pendimethiline 3.3 liter per ha was applied on the date of sowing followed by two hand weeding at about one and two month after sowing with the help of khurpi. Crop was irrigated according to the irrigation schedules in the different treatments. Grain yield of crop was determined during both the cropping seasons.

2.4. Soil sampling and analysis

Triplicate undisturbed soil cores (15 cm high and 7.6 cm diameter) were obtained from 0-15 to 15-30 cm soil depths after the wheat harvest from each plot. Soil parameters, such as soil organic C [16], bulk density, saturated hydraulic conductivity [17] and effective porosity [18] were determined. Water retention was determined at water potentials of -30 and -1500 kPa using pressure plate apparatus. Water retention multiplied by the soil depth gives plant available water capacity.

For wheat yield estimation, crop was harvested from 2 m^2 area in the center of plot with the help of sickles and left there for sun-drying. After drying, biological and grain yield were determined. The water use efficiency (WUE) was computed as: WUE (kg ha^{-1} mm^{-1}) = grain yield (kg ha^{-1})/total water use (mm).

2.5. Statistical analysis

The laboratory and experimental data were analyzed by using standard procedure for a two factorial randomized block design with the help of computer applying analysis of variance (ANOVA) technique [19]. The differences among treatments were compared by applying F test of significance at 5% level of significance or probability. Tillage and irrigation system means were separated using the two factorial randomized block design at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Soil bulk density

Soil bulk density determined after the crop was higher in the ZT and I_1 plots than the other plots (Table 2) and significantly affected by tillage practices. However, application of water had considerable effect on soil bulk density. There was very little variation in bulk density values between the tilled and irrigated plots. High soil bulk value indicates less disturbance of soil under ZT plots, which creates low porosity compared to tilled plots. These results are in congruence with the studies of Bajpai and Tripathi [20] and Bhattachariya [4]. They also reported higher value of soil bulk density under ZT at the soil surface compared with tilled soil.

3.2. Saturated hydraulic conductivity (Ksat)

Saturated hydraulic conductivity values under DT and I_5 plots were higher than the other plots (Table 2). The interaction effects of tillage and irrigation treatments were non-significant in the 0-15 and 15-30 cm soil layer. This is mainly due to tillage, there is reorganization of pores in tilled plots which increases the permeability of soil and degree of increment depends on the tillage depth [4, 21].

3.3. Mean weight diameter

Mean weight diameter (MWD) of soil particles decreases significantly under DT plots in both soil layer (Tables 2) and the similar effect of the different irrigation treatments was found, I_5 plots have high MWD values. There was non-significant interaction effect of tillage and irrigation treatments. The significant effects of tillage methods on MWD were mainly tillage-induced, as it affects SOC content and soil microbial activity. Lal [9] reported that zero tilled plots have higher SOC content which imparts better aggregation in soil while under DT aggregate size is less due to oxidation of exposed soil organic matter [22].

3.4. Plant available water capacity (PAWC)

Water retention capacity decreases with more tillage operations, so the PAWC, found higher in ZT plots (Tables 2) while, due to number of irrigations, there was major improvement in PAWC in both soil depths. This was mainly attributed due to the presence of higher amounts of organic matter and increased bulk density values in ZT plots, so the water retention was also higher. Similar findings were reported by Azooz [23] and Bhattachariya [4], that due to under ZT plots can hold on more soil moisture.

Table 2. Effect of tillage and irrigation on soil physical properties and soil organic C at wheat harvest (after 2 years) in surface 0-15 cm (a) and sub-surface 15-30 cm (b) layer.

Treatments	Bulk density (Mg m^{-3})		Saturated hydraulic conductivity (unit)		Mean weight diameter (mm)		Plant available water capacity (cm/15 cm soil layer)	
	a	b	a	b	a	b	a	b
Tillage (T)								
ZT	1.43	1.45	21.3	17.1	0.74	0.63	2.63	2.26
CT	1.39	1.41	21.8	17.6	0.71	0.62	2.36	1.96
DT	1.36	1.38	22.3	18.3	0.58	0.52	2.11	1.78
(P=0.05)	0.14	0.97	0.31	0.32	0.11	0.16	0.20	0.15
Irrigation (I)								
I_1	1.42	1.45	19.8	15.7	0.60	0.52	2.22	1.77
I_2	1.40	1.42	20.7	16.5	0.64	0.55	2.28	1.93
I_3	1.39	1.41	21.7	17.6	0.68	0.60	2.38	2.02
I_4	1.38	1.40	23.0	18.8	0.72	0.63	2.44	2.10
I_5	1.37	1.39	23.9	19.8	0.75	0.65	2.51	2.19
(P=0.05)	0.12	0.12	0.40	0.41	0.14	0.21	0.26	0.20
T X I (P=0.05)	NS	NS	NS	NS	NS	NS	0.46	0.35

NS indicates not significant. ZT indicates zero tillage; CT indicates conventional tillage and DT indicates deep tillage. I_1, I_2, I_3, I_4 and I_5 indicate I_1: crown root initiation (CRI), I_2: CRI + late tillering (LT), I_3: CRI + LT + late joining (LJ), I_4: CRI + LT + LJ + flowering (F), and I_5: CRI + LT + LJ + F + milking (M), respectively.

Table 3. Effect of tillage and irrigation on effective porosity and soil organic C at wheat harvest (after 2 years).

Treatments	Effective porosity (m^3 m^{-3})	Soil organic carbon (%)
Tillage		
ZT	0.187	0.76
CT	0.174	0.74
DT	0.162	0.70
(P=0.05)	0.11	0.39
Irrigation		
I_1	0.164	0.73
I_2	0.170	0.74
I_3	0.175	0.70
I_4	0.179	0.74
I_5	0.184	0.75
(P=0.05)	0.14	NS
T X I (P=0.05)	NS	NS

NS indicates not significant. ZT indicates zero tillage and CT indicates conventional tillage and DT indicates deep tillage. I_1, I_2, I_3, I_4 and I_5 indicate I_1: crown root initiation (CRI), I_2: CRI + late tillering (LT), I_3: CRI + LT + late joining (LJ), I_4: CRI + LT + LJ + flowering (F), and I_5: CRI + LT + LJ + F + milking (M), respectively.

Table 4. Effect of tillage practices and irrigation levels on grain yield and water use efficiency of wheat.

Treatments	2011-2012					2012-2013				
	Grain yield (kg/ha)	Irrigation (mm)	Rainfall (mm)	Water use (mm)	Water use efficiency (kg/ha/mm)	Grain yield (kg/ha)	Irrigation (mm)	Rainfall (mm)	Water use (mm)	Water use efficiency (kg/ha/mm)
Tillage										
ZT	2946	300	37	337	15.8	3583	300	191.7	491.7	9.9
CT	3438	300	37	337	18.2	3933	300	191.7	491.7	11.0
DT	3340	300	37	337	18.2	3708	300	191.7	491.7	10.5
(P=0.05)	222.52				1.34	168.92				0.46
Irrigation										
I_1	2911	60	37	97	30.0	3430	60	191.7	251.7	13.6
I_2	3180	120	37	157	20.2	3569	120	191.7	311.7	11.4
I_3	3027	180	37	217	13.9	3736	180	191.7	371.7	10.0
I_4	3486	240	37	277	12.5	4173	240	191.7	431.7	9.6
I_5	3602	300	37	337	10.6	3798	300	191.7	491.7	7.7
(P=0.05)	287.27				1.73	256.00				0.59
TXI (P=0.05)	NS				NS	377.73				1.39

NS indicates not significant. ZT indicates zero tillage, CT indicates conventional tillage and DT indicates deep tillage. I_1, I_2, I_3, I_4 and I_5 indicate I_1: crown root initiation (CRI), I_2: CRI + late tillering (LT), I_3: CRI + LT + late joining (LJ), I_4: CRI + LT + LJ + flowering (F), and I_5: CRI + LT + LJ + F + milking (M), respectively.

3.5. Effective porosity

Although the effective porosity was significantly different under tilled plots, under ZT plots inclination in porosity in 0-15 cm soil layer was observed when compared with other plots (Table 3). Irrigation treatments had also noticeable effect on the porosity of soil. ZT management practice reduces number of large pores and increases small pores, due to this high effective porosity are maintained. Pagliai [24] reported that pores, i.e. macro as well as micro, are important to maintain the water holding capacity, good soil structure and soil-plant-water relationships.

3.6. Soil organic C (SOC)

Soil organic C contents in 0-15 cm soil depth were higher under zero tillage (ZT) than other ones, while irrigation and the interaction effects of tillage and irrigation produces non-significant ($P < 0.05$) effect (Table 3). According to Kay and Vanden Bygaart [25] less disintegration of the soil matrix under ZT plots slows down the SOC mineralization and imparts higher concentration of SOC. Tillage accelerates the decomposition and C mineralization rate of organic matter [26]. Similarly, Koga and Tsuji [27], Presley [28] and Mukherjee and Lal [29] also reported that SOC is significantly higher in ZT plots due to less soil interruption in comparison to tilled ones.

3.7. Wheat yield

Wheat yield did not affect significantly due to reduction in tillage operation during both the cropping seasons as there was only 491.0 kg ha^{-1} and 350.0 kg ha^{-1}wheat yield decline under ZT in comparison to CT (Table 4). Irrigation treatments also showed significant yield differences during both the years of the study. Analysis of the experimental data, wheat yield was not significantly different under different tillage practices, but less cost of cultivation and early sowing of crop under ZT is the main benefit [21]. During the 2011-2012, wheat yields were significantly affected by different levels of irrigation, with higher yield in the plots under I_4 over I_1 and I_5 over I_1. During 2012-2013, results again showed the similar trend as recorded in 2011-2012. Plots under I_4 had statistically similar grain yield as the plots under I_1. Thus, our results during 2011-2013 indicated that the cost of culti-

vation may be reduced under I_4 plots by saving one-irrigation because there was not too much difference in yield between I_4 and I_5 irrigation plots. The reason for achieving the higher yield under conventional tillage and deep tillage is proper field preparation, lesser weed competition and enrichment of the nutrient in the plough zone, where roots of the crop had sufficient nutrient and water availability to gain or optimizing the yield target. Similar results were also reported by Singh [30] and Gangwar [31] they observed that among the different tillage levels, conventional tillage recorded the highest grain yield of wheat followed by RT and ZT. Increased yield with conventional tillage over zero tillage in wheat was also reported by several workers [32, 33]. The lower yield in zero tillage plots was mainly due to heavy crop/weed competition. Higher grain yield (1830 kg ha^{-1}) and straw yield (2820 kg ha^{-1}) of wheat (cv. Sonalika), was reported when irrigations were applied five times at crown root initiation, tillering, jointing, flowering and dough stages by Cheema [34]. Singh and Patel [35] reported that stress at tillering, flowering and grain filling stages reduced both grain yield and plant biomass in wheat. Singh and Patel [35] also reported mean grain yield of 458 kg ha^{-1}, when plots were irrigated before sowing only (BS), 1328 kg ha^{-1} when irrigation was applied at BS + crown root initiation (CRI) and 1826 kg ha^{-1} when full irrigations were applied as per the requirement of the crop.

3.8. Water use efficiency (WUE) of wheat

CT showed higher value of water use about 13.8% in 2011-12 and 10.0% in 2012-2013 (Table 4) as compared to ZT plots. WUE values were also similar, because there was no significant difference in yield among the tillage treatments. Under different irrigation treatments, WUE values differ significantly because water use values increases frequently with the number of irrigations. Similar findings were reported by Bhattacharya [10] who conducted an experiment on sandy clay loam soil in Almora and observed that WUE was highest in more tilled plot in comparison to zero tillage plots and decreases with increase in the number of irrigation. Deshmukh [36] also found that the highest WUE (8.64 kg ha^{-1} mm^{-1}) when one irrigation was applied at crown root initiation stage. Lower values for WUE were recorded when irrigation frequency increased from one to five (7.65 kg ha^{-1} mm^{-1}).

4. CONCLUSION

The study has revealed that it is possible that under ZT there was more SOC content over CT and DT. More tillage operation will cause more disruption of soil and its physical properties in the surface soil layer. Also, there were no significant differences in yield and WUE of wheat due to different tillage practices. The superiority of ZT is also due to less loss of water via leaching due to better rearrangement of pore size classes for faster water transmission under saturated conditions. There was a significant increase in wheat yield in the plots where four irrigations were applied over one-irrigation or over two irrigations. Soil properties (SOC, bulk density and saturated hydraulic conductivity) measured after harvest of crop during both the cropping seasons did not significantly affected by irrigation treatments. Hence, for wheat crop in a sandy loam soil of Uttarakhand, farmers may adopt CT with four irrigations for achieving maximum yield and they can save the cost of one irrigation. If there was water stress for irrigation, ZT may be adopted due to improve SOC status, soil physical properties, for saving machine, labor and irrigation water cost. However, ZT practice may be also be adopted with proper chemical weed management practices.

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AUTHORS' CONTRIBUTION

GM and HSK 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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