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EFFECT OF MONOCROTOPHOS ON ELECTROLYTIC LEAKAGE, PROLINE CONTENT AND NITROGEN METABOLISM OF FLOATING PTERIDOPHYTE *AZOLLA MICROPHYLLA*

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The green revolution increased productivity in its initial phase, but excessive application of modern intensive agriculture, e.g., use of chemical fertilizers and pesticides poses a serious threat to the environment and sustainable agriculture. The effects of the green revolution have shifted concern from increasing productivity to sustainability and resource conservation. The goal of this work is to investigate the effect of insecticide monocrotophos on electrolytic leakage, proline content and nitrogen metabolism of small-leaf floating aquatic pteridophyte *Azolla microphylla*, which lives in symbiosis with a nitrogen fixing cyanobacteria, *Anabaena azollae*. Mean root number and root length decreased under monocrotophos as compared to control. At higher concentrations, roots were dillateted, brown and inactive. Electrolytic leakage and proline content increased with increase of monocrotophos concentrations. The nitrogenase activity assayed in the present investigation increased at lower concentrations and decreased at higher concentrations. Exposure of the *Azolla* to different concentrations of monocrotophos decreased the glutamine synthetase activity at all concentrations compared to control. However it was interesting to observe that nitrate reductase activity increased as the concentration of insecticide increases.
Key words: monocrotophos, proline, glutamine synthetase, electrolytic leakage.

Utjecaj monokrotofosa na elektrolitičnu propusnost, sadržaj prolina i metabolizam dušika plutajuće paprati *azolla microphylla*. Zelena revolucija povećava produktivnost u svojoj početnoj fazi, ali prekomjerna primjena suvremene poljoprivrede, npr. korištenje mineralnih gnojiva i pesticida predstavlja ozbiljnu prijetnju za okoliš i održivu poljoprivredu. Učinci zelene revolucije se pomiču od zabrinutosti za povećanu proizvodnju prema održivosti i očuvanju resursa. Cilj ovog rada je istražiti utjecaj insekticida monokrotofosa na elektrolitičku propusnost, sadržaj prolina i metabolizam dušika u sitnolistoj plutajućoj vodenoj paprati *Azolla microphylla*, koja živi u simbiozi s dušik-fiksirajućom cijanobakterijom *Anabaena azollae*. Primjenom ovog sredstva smanjuje se prosječan broj i duljina korijena u odnosu na kontrolu. Pri većim koncentracijama spoja korijeni su proširen, smeđi i nefunkcionalni. Elektrolitička propusnost i sadržaj prolina povećavaju se s povećanjem koncentracije monokrotofosa. U ovom istraživanju vidljivo je da se kod nižih koncentracija primijenjenog insekticida aktivnost nitrogenaze povećava, ali se kod viših koncentracija smanjuje. Izloženost vrste *Azolla* različitim koncentracijama monokrotofosa smanjuje aktivnost glutamin sintetaze kod svih koncentracija u odnosu na kontrolu. Također, zanimljiv je podatak da aktivnost nitrat reduktaze pozitivno korelira s povećanjem koncentracije monokrotofosa.
Ključne riječi: monokrotofos, prolin, glutamin sintetaza, elektrolitička propusnost.

INTRODUCTION

The word *Azolla* is a combination of two Greek words azo (to dry) and allyo (to kill); reflecting the inability of plants to survive dry conditions [1, 2]. Water is a fundamental prerequisite for growth and multiplication of Azolla. The *Azolla–Anabaena azollae* symbiosis is an important N_2-fixing association between eukaryotic fern and prokaryotic cyanobacterium. The *Azolla-Anabaena* association is important agronomically owing to its capacity to fix atmospheric nitrogen at cheaper and faster rates and making it available to crop plants[3].This host–symbiont combination is exploited as biofertilizer for many agricultural crops [4,5].

The agronomic potential of *Azolla* for growing rice has been recognized nearly globally [6, 7]. *Azolla* has been used alone or in combination with other inorganic nitrogen fertilizers [8, 9]. Proline is a major organic molecule that accumulates in many plants exposed to environmental stresses such as drought and salinity [10, 11]. Under osmotic stress conditions, proline acts as a mediator of osmotic adjustment, as a protector of macromolecules such as proteins and membranes, as a sink for energy, as a scavenger of free radicals, and even as a stress-related signal [12, 13, 10]. A new strategy for increasing rice production, particular in developing countries should be taken in account for programmes to utilize the biological fertilizers which will not only increase the rice productivity, but also improve the long term soil fertility [14]. In these conditions the inoculation of free living cyanobacteria and Azolla in the fields is one of the options. The inoculation increased rice grain yields by an average of 350 kg/ha. When successful, the inoculation is low-cost technology, but its effect is erratic and unpredictable [15].

Monocrotophos is an organo-phosphate insecticide which is systematic in action, penetrates plant tissue rapidly. It controls the broad spectrum of pests including sticking, chewing, boring and spider mites. It has highly fumigant action. Since *Azolla microphylla* is an important plant from an agronomic point of view due to its ability to fix atmospheric nitrogen and pesticides like monocrotophos are also used to check the pest of paddy like leaf hoppers, white flies spider mites etc. so definitely, these pesticides influence the growth, biomass property of *Azolla* [16].

Since *Azolla* occupy an important position in food web, and loss of *Azolla* biomass may seriously affect soil fertility through nitrogen and carbon fixation. Azolla is also utilized as a nitrogen biofertilizer in rice cultivation and insecticides seriously affect the growth of this fern which has been proved from previous investigations, [16, 17, and 18].

However, there is lack of information on the behavior of proline, electrolytic leakage and nitrogen metabolism of *Azolla* plants to different concentrations of monocrotophos toxicity and thus the present investigation has been undertaken.

MATERIALS AND METHODS

Organism and growth conditions

Azolla microphylla was isolated from rice fields near Allahabad and was maintained in the tubs of 35 cm diameter and 12 cm depth. Each tub was filled with 3.5 kg sterilized rice field soil and mixed with single super phosphate of about 300 mg and

water is allowed to stand up to 4 cm above the soil and the tubs were put in open air in the field of Department of Biological Sciences, SHIATS, Allahabad. Each tub was inoculated with 5 gm *Azolla* fronds. After 12 days a thick mass of *Azolla* covered the entire water surface of the tub.

From these tubs *Azolla* fronds were taken out to conduct the lab studies.

Pesticide treatment

Monocrotophos [Dimethyl *(E)*-1-methyl-2-(methylcarbamoyl) vinyl phosphate] 36% SL, Manufactured by Hindustan Pulversing Mills, Industrial Growth Center Samba Jammu (J&K) was selected for the present investigation.

This is widely used insecticide to control the pest of paddy like leaf hoppers, white flies, spider mites etc. Its various concentrations viz. 0, 25, 50, 100, 200, 400, 600, ppm in nutrient medium were prepared for screening experiment.

Estimation of average root length and root number

Average root length and root number were determined by methods of Kurth [19] and Ge-Shi-An [20] respectively.

Estimation of electrolyte leakage

The electrolyte leakage was determined as described by Dionisio-Sese and Tobita method [21]. Fresh *Azolla* plants (200 mg) were cut into pieces of 5 mm length and placed in test tubes containing 10 ml distilled deionized water.

The tubes were incubated in a water bath at 32^0C for 2h and the initial electrical conductivity of the medium (EC_1) was measured. The samples were autoclaved at 121^0C for 20 min to release all electrolytes; cooled to 25^0C and the final electrical conductivity (EC_2) was measured. The electrolyte leakage (EL) was calculated by using the formula:-

$$EL = (EC_1/EC_2) \times 100.$$

Estimation of proline

Proline content in control and treated fronds was estimated by the method of Bates [22]. Fresh *Azolla* fronds (100 mg) were crushed in 3 % (weight/volume) aqueous sulfosalicylic acid, centrifuged at 10,000 g for 10 min and then mixed with 3 % (weight/volume) glacial acetic acid and acid ninhydrin. Samples were heated for 1 h in a water bath at 95 ºC, cooled and extracted with 4 ml toluene by vortexing for 1 min with a test tube mixer. The toluene layer was then separated with the help of a pipette and the absorbance was read at 520 nm using toluene as blank. The amount of proline in sample was obtained by comparing with standard curve.

Estimation of nitrogenase activity

Dinitrogen is reduced to ammonia by the nitrogenase complex (EC 1.18.2.1), a reaction which is dependent upon reduced ferredoxin and obligatorily coupled to reduction of protons resulting in formation of molecular hydrogen. The determination of nitrogenase activity makes use of the fact that besides N_2, several compounds with a triple bond are reduced by nitrogenase. So nitrogenase reduces acetylene to ethylene.

The acetylene reduction assay of Shapiro and Stadtman [23] has been used. The activity was determined using whole fronds as well as pair of leaves. Assay was performed in triplicate using calibrated stoppered vessel of about 8.5 ml capacity. Fronds with roots (100 mg fresh weight) were taken in each vessel and acetylene concentration was kept at 10%.

Reaction was performed at 25°C and concentration was kept at for 48 h and was estimated by injecting 0.8 ml of 15% (weight/volume) TCA. Ethylene produced in the reaction vessel was analyzed in a gas porapak R column.

Estimation of glutamine synthetase

The γ-glutamine transferase activity of glutamine synthetase is measured by the method of Shapiro and Stadtman [23] by quantifying the amount of γ-glutamyl hydroxamate produced using glutamine as substrate in the presence of ADP and arsenate. One unit of enzyme has been defined as the amount of glutamine synthetase required to catalyze the synthesis of 1μ mol γ –glutamyl hydroxamate per minute under standard transfer assay conditions. Take 2ml of algal suspension. Add 0.25 ml toluene and incubate overnight at 4°C. Centrifuge and discard toluene layer. To the pellet add 0.8 ml extraction buffer (pH 7.0). Also add 1ml of assay mixture and incubate at 37°C for 30 minutes.

Terminate the reaction by adding 2ml of the stop mixture and measure the optical density at 540 nm. The activity is expressed as n mol γ –glutamyl hydroxamate μg proteins^{-1}30 min^{-1}.

Estimation of nitrate reductase

After acquisition, nitrate is reduced to ammonia before being assimilated into amino acid. Reduction of nitrate to ammonia is mediated through two enzyme systems, nitrate reductase (NR) which reduces nitrate (NO_3) to nitrite (NO_2) and nitrite reductase (NIR) which causes further reduction of NO_2 to NH_3. The nitrate reduced activity, in vivo is based upon total nitrite found in a definite volume of culture suspension. The nitrite found is determined by the diazocoupling method of Lowe and Hamilton [24]. For the assay of enzyme, sample is incubated in 5M $NaNO_3$ for 24 hrs. Now 1ml of sample is taken in a test tube and adds 1ml of sulphanilamide and mix well. After 15 minutes, add 1ml of NEDD. Absorbance is taken after 15 minutes at 540 nm and expresses the enzyme activity as μg NO_2 formed per μg protein.

Statistical analysis

All the data of *Azolla microphylla* were obtained in terms of average root length, average root number, electrolyte leakage; proline, nitrogenase activity, nitrate reductase and glutamine synthetase in response to different levels of monocrotophos and were statistically analyzed for their significance. An analysis of variance (ANOVA) was performed using SPSS 10 program.

The significance was tested at 0.05 (5%) level. Values presented in the text indicate mean values± of five replicates.

RESULTS AND DISCUSSION

Effect of monocrotophos on average root number and root length

Average root number decrease by 5.48%, 15.07%, 20.55%, 28.77 at 25 ppm, 50 ppm, 100 ppm and 200 ppm. Maximum reduction of 58.91% was observed at 600 ppm. Roots were physiologically inactive, brown and almost dilapillated.

From observations, it is clear that the root length decreases by 6.92%, 10.95% , 20.16 and 33.06% at 25 ppm, 50 ppm, 100 ppm and 200 ppm respectively.

From 200 ppm onwards, there was continuous decrease in root length, with maximum reduction of 55.7% at 600 ppm.

Data depicting average root number and root length are presented in Fig 1. Pesticides have adverse effect on root length which results in vulnerable effect on plant growth and development [25, 26].

Further the reduction in root length due to monocrotophos could also be explained on the basis of inhibition in the activity of 4-hydroxyphenyl pyruvate deoxygenase (HPPD), and enzyme needed for meristematic tissue as suggested by [25] following insecticide isoxafluote treatment in plant.

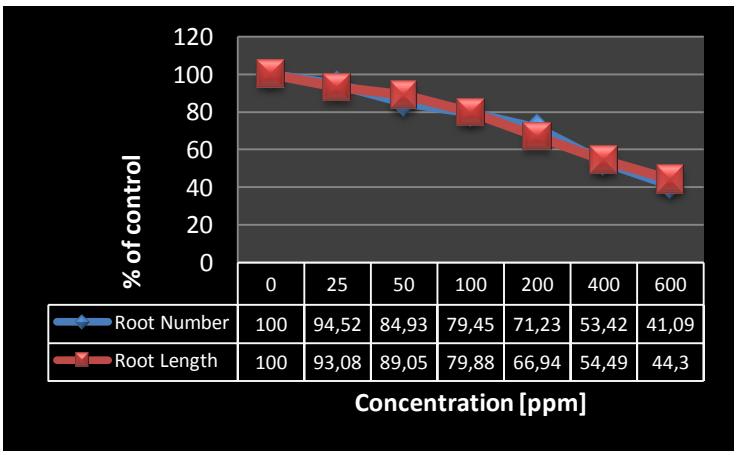

Figure 1. Effect of different concentrations of Monocrotophos on Root number and Root length of *Azolla microphylla.* Root number and root length in untreated control was 7.3 and 2.257 cm, respectively. Values are mean ±SE of five replicates (P<0.05)

Slika 1. Utjecaj različitih koncentracija Monokrotofosa na broj i duljinu korijena vodene paprati *Azolla microphylla*. U netretiranoj kontroli broj i dužina korijena bili su 7.3 i 2.257 cm. Sve vrijednosti su izražene kao srednja vrijednost ± SE pet replika (P <0,05)

Effect of monocrotophos on electrolyte leakage of *azolla microphylla*

Membrane stability is the widely used criterion to assess the damage due to pesticide induced stress. The percentage of electrolyte leakage is graphically depicted in Fig 2. Electrolyte leakage was found to increase significantly with increasing concentration of monocrotophos and maximum leakage was observed at 600 ppm, about 3fold as that of control. Thus, enhanced lipid peroxidation leads to increase electrolyte leakage, due to cell membrane damage [27]. It was presumed that the extent of membrane damage was so severe in such species where electrolyte leakage was highest (in percentage) under stress condition. Low level of electrolyte leakage and MDA content at low doses of pesticides (in ppm) may be one of the reasons for the observed tolerance of *Azolla microphylla*.

Recently similar results were observed by [17] following monocrotophos stress on *Azolla filiculoides*.

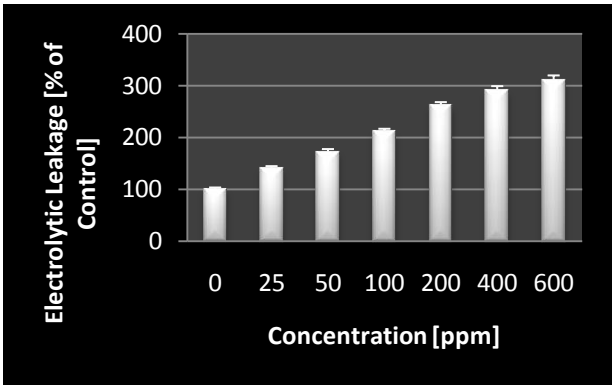

Figure 2. Effect of different concentrations of Monocrotophos on electrolytic leakage (electrolyte leakage in untreated control was 12%). Values are mean ±SE of five replicates (P<0.05)
Slika 2. Utjecaj različitih koncentracija Monokrotofosa na elektrolitičku propusnost (elektrolitička propusnost u netretiranoj kontroli bilo je 12%). Sve vrijednosti su izražene kao srednja vrijednost ± SE pet replika (P <0,05)

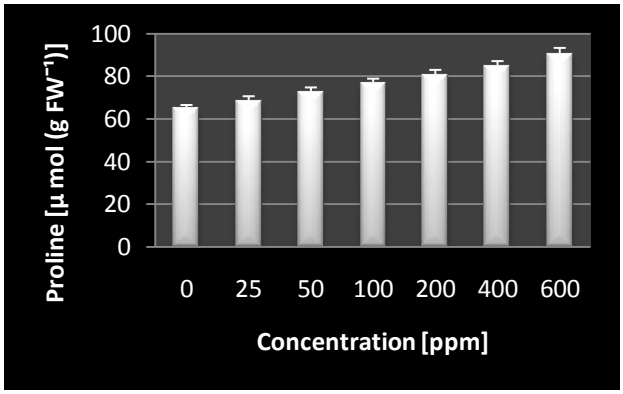

Figure 3. Effect of different concentrations of Monocrotophos on proline content of *Azolla microphylla.* Values are mean ±SE of five replicates (P<0.05)
Slika 3. Utjecaj različitih koncentracija Monokrotofosa na sadržaj prolina u vrste *Azolla microphylla.* Sve vrijednosti su izražene kao srednja vrijednost ± SE pet replika (P <0,05)

Effect of monocrotophos on organic osmolyte (proline) of a*zolla microphylla*

Proline is an important non-enzymatic antioxidant compound. Due to insecticide treatment the level of proline accumulation had significantly increased in *Azolla* fronds. Following six days incubation in insecticide treatment the intracellular proline content was highest (40.32%) at 600 ppm [Fig 3]. Similarly, increase in proline accumulation under UV-B stress has also been reported in *Azolla pinnata* and *Azolla filiculoides* by [28], although the mechanism of accumulation of proline in plant or plant parts exposed to stress is still unknown. It is suspected that decrease in the activity of the respiratory electron transport system leads to the accumulation of NADH and H^+ [29]. Proline accumulation might be an adaptive mechanism for reducing the level of accumulated NADH, and the acidity is used for synthesizing each molecule of proline from glutamic acid. Enhanced level of proline may have also conferred the capacity to detoxify active oxygen species efficiently in *A. microphylla*. Hyper accumulation of proline in plants is linked with detoxification against stress induced oxidative stress [30].

Effect of monocrotophos on nitrogenase activity of a*zolla microphylla*

The nitrogenase activity assayed in the present investigation is graphically depicted in Fig 4. From the findings, it is clear that as the concentration of monocrotophos increases, the nitrogenase activity also increases by 9%, 17% and 29%, at 25ppm, 50ppm and 100ppm respectively and then an inconsistent decrease was obtained after 100 ppm. Thus we put forth that percent nitrogen and nitrogen yield were significantly affected by monocrotophos. A similar trend was reported by [31] that the insecticide carbofuran (furadan) significantly increased dinitrogenase activity of *A. pinnata*. Moreover, it was increased by application of a low concentration of lindan [32]. On the other hand, [33] found that the herbicide saturn reduced the growth and dinitrogenase activity of *A. pinnata*. [34] reported that addition of pesticides to the growth media substantially reduced the dinitrogenase activity of pure cultures of *G. Diazotrophicus* and recently [35] also showed similar results.

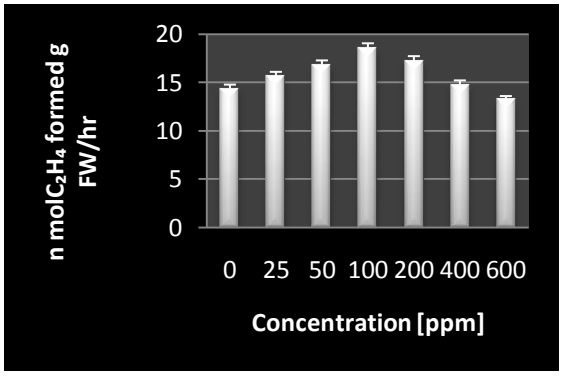

Figure 4. Effect of different concentrations of Monocrotophos on nitrogenase activity of *Azolla microphylla.* Values are mean ±SE of five replicates (P<0.05)
Slika 4. Utjecaj različitih koncentracija Monokrotofosa na aktivnost nitrogenaze vodene paprati *Azolla microphylla*. Sve vrijednosti su izražene kao srednja vrijednost ± SE pet replika (P <0,05)

Effect of monocrotophos on enzymes of nitrogen assimilation of *azolla microphylla*

Fig. 5 and 6 shows the effect of monocrotophos on nitrate reductase and glutamine synthetase activity of *Azolla microphylla*. Both these enzymes behaved differently in response to monocrotophos. Exposure to different concentrations of monocrotophos, decreased the glutamine synthetase activity by 4%, 12% and 21% at 25 ppm, 50 ppm, 100 ppm respectively and further there was gradual decrease as the concentration increases.

However, it was interesting to observe that nitrate reductase activity increased by 9.77%, 26.17%, 45.67% and 73.33% at 25 ppm, 50 ppm, 100 ppm and 200 ppm respectively. Beyond 200 ppm there was a decrease in nitrate reductase activity. Previous studies had shown that saturn has an inhibitory effect on photosynthetic CO_2 assimilation [36] and inhibited protein synthesis [37], which could be due to disturbances in nitrogen metabolism and photosynthetic activity [36] or due to an increase in protease activity [38]. Such effects might exert many secondary effects on growth of *Azolla* species [35, 39].

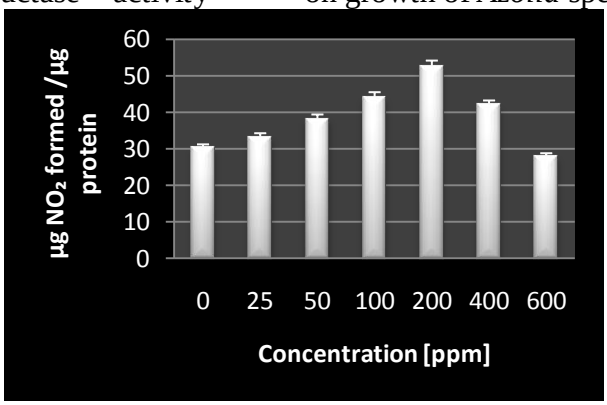

Figure 5. Effect of different concentrations of Monocrotophos on nitrate reductase activity of *Azolla microphylla.* Values are mean ±SE of five replicates (P<0.05)
Slika 5. Utjecaj različitih koncentracija Monokrotofosa na aktivnost nitrat reduktaze u vrste *Azolla microphylla.* Sve vrijednosti su izražene kao srednja vrijednost ± SE pet replika (P <0,05)

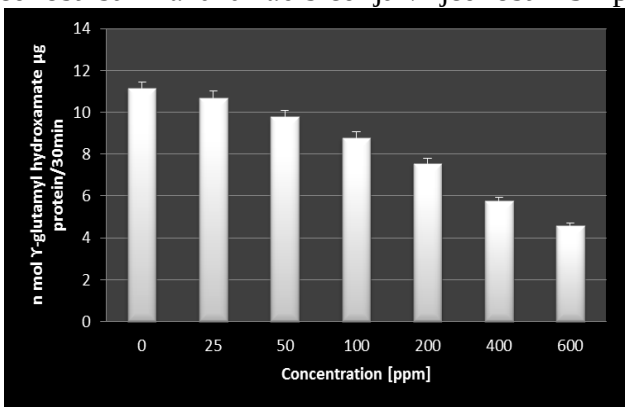

Figure 6. Effect of different concentrations of Monocrotophos on glutamine synthetase activity of *Azolla microphylla.* Values are mean ±SE of five replicates (P<0.01)
Slika 6. Utjecaj različitih koncentracija Monokrotofosa na aktivnost glutamin sintetaze vodene paprati, *Azolla microphylla.* Sve vrijednosti su izražene kao srednja vrijednost ± SE pet replika (P <0,01)

CONCLUSION

Azolla is a pioneer in areas disturbed by man or animals, where its rapid growth rate and nitrogen fixing ability give it a competitive edge. It was obvious from the results obtained throughout this experiment that *Azolla microphylla* showed higher tolerance to monocrotophos at lower concentration, as compared with higher concentrations.

The levels of electrolytic leakage and proline significantly enhanced the resistance of *Azolla microphylla* towards monocrotophos stress. It is suggested for future that with integrated research, the present problem of *Azolla* should be solved jointly by efforts of biologists to improve *Azolla* plants capable of growing in low water, fixing more amount of nitrogen and increasing resistant to pesticides. The multiple use of Azolla as biofertilizer, green manure, compost and feed for animals will improve its economic benefits in future.

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