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Growth, photosynthetic pigments and antioxidant responses of *Azolla filiculoides* to monocrotophos toxicity

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ABSTRACT

The paper deals with the pesticide monocrotophos (0 – 30 ppm) induced changes in physiological and biochemical parameters related to growth and defense system in Azolla filiculoides . Monocrotophos treatment on fresh weight, dry weight, doubling time, chlorophyll, carotenoids, relative growth rate, lipid peroxidation, electrolyte leakage, proline accumulation and activities of superoxide dismutase and peroxidase were analyzed. Growth and photosynthetic pigments, i.e. chlorophyll and carotenoids were adversely affected by monocrotophos treatment and the inhibition was found to be dose dependent. Pesticide treatment with increasing doses accelerated the formation of reactive oxygen species, i.e. O^{-}_{2} and H_2O_2 in cells progressively, whereby an enhanced lipid peroxidation and electrolyte leakage was noticed in A. filiculoides. Proline accumulation also showed a similar trend. As a consequence of reactive oxygen species (ROS) generation in monocrotophos treated plants, the activity of superoxide dismutase (SOD) and peroxidase (POD) were enhanced considerably.

Keywords: *Azolla filiculoides*, monocrotophos, growth, MDA, SOD, POD.

INTRODUTION

The application of insecticide a group of pesticides, in crop fields for selective control of pests in the modern age has led to serious environmental contamination resulting in greater loss of crop productivity and growth of many beneficial microorganisms, phytoplankton's etc. [1]. Though the application of many insecticides are forbidden, the low cost, easy availability, lack of awareness and lax regularity implementation have contributed to the continuous use of the insecticides in tropical and subtropical regions. The removal of these insecticides from soil and aquatic systems has become a difficult problem and as a result of this, they persist in these

ecosystems for a long period of time [2].Water bodies such as ponds, water reservoirs, aquaculture shallow water and paddy fields are highly eutrophic and maintain large standing crops of phytoplankton's and, floating plants and some aquatic fern like *Azolla.* [3]. An *Azolla-Anabaena* association is a favorite biofertilizer of crops, especially in rice paddy fields because of its ability to fix dinitrogen at high rates and low cost. In addition, *Azolla* is a suitable candidate as an animal feed, human food water purifier, medicine, hydrogen fuel, biogas producer, weed controller, suppresser of weeds and reduces ammonia volatilization after chemical nitrogen application and rightly called as "green gold" [4].Though the considerable amount of work on abiotic stress induced inhibitory effect of growth, photosynthetic pigments content and nitrogen metabolism have been done in recent years [5,6,7] but insecticides particularly monocrotophos induced effect on growth ROS generation, antioxidants and lipid peroxidation in *Azolla* particularly *A.filiculoides* are yet to be investigated. Considering the importance of *Azolla* in rice fields, and frequent use of pesticides against pests, the authors set forth the objective of investigating the impact of insecticide monocrotophos on growth, photosynthetic pigments and antioxidant systems, lipid peroxidation and membrane leakage in *Azolla filiculoides.*

EXPERIMENTAL SECTION

Plant material

Azolla filiculoides were collected locally from paddy fields.Plants were washed and cleaned of contamination organisms. The plants were surface sterilized with a solution of mercuric chloride (0.1% for 30 min) and were dipped immediately into a large volume of sterile distilled water. Plants were then transferred into dishes containing combined-N free 2/5 stength sterile Hoagland's medium[8] and 0.04mM ferrous ion as Fe-EDTA, pH 5.6.The cultures were grown at 26 ^{o}C under a 16:8 (light: dark) photoperiod with light from a combination of incandescent and cool white light fluorescent lamps at a photon fluence rate of 95 µmol $m^{-2}s^{-1}$. Fronds were routinely transferred into fresh medium twice a week to maintain plants in a sterile state. Log phase plants were used for experiments.

Growth estimation

Azolla filiculoides plants were rinsed in an aerated iso osmotic solution of sorbitol were blotted dry on filter paper and weighed to represent their fresh weight (FW).Dry weight (DW) was determined by drying the samples in a hot air oven at 90 ^{0}C for 24 h to a constant weight.

Chlorophyll and carotenoids estimation

Chloropylls and carotenoids were extracted from fronds with 80% acetone.Chlorophyll and carotenoids were estimated spectrophotometrically according to the method of Litchenthaler and Welburn [9].

Assay of enzymes

POD (EC1.11.1.7) was extracted by homogenizing 50 mg fresh leaves at 4 ^{o}C in 100 mM potassium phosphate buffer (pH 7.0), while SOD (EC 1.15.1.1) was isolated in 100 mM EDTA phosphate buffer (pH 7.8).The homogenate was filtered and centrifuged at 10,000 x g for 15 min and the supernatant obtained was used for enzyme assay. POD, and SOD were determined spectrophotometrically (Systronics India Ltd Model No 117) according to the methods of Gahagen et al.[10] and Giannopolitis and Ries[11] respectively.

Lipid peroxidation estimation

Lipid peroxidation was estimated by measuring the content of 2-thiobarbituric acid-reactive substances in leaf homogenate, prepared in 20% TCA containing 0.5%

2-thiobarbituric acid and heated at 95 ^{o}C for 25 min [12].Melondialdehyde (MDA) content was determined spectrophotometrically at A_{532} and corrected for non specific turbidity at A_{600}.

Proline estimation

Proline concentration in treated and untreated fronds was determined spectrophotometrically by the method of Bates, et al. [13].

Statistical analysis

All the data obtained of *Azolla filiculoides* in terms of growth, chlorophyll, carotenoids SOD, POD, lipid peroxidation, electrolyte leakage and proline accumulation in response to different levels of monocrotophos 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

The present study shows the effect of different concentrations of monocrotophos on the growth,pigments and antioxidants of *A. filiculoides* . All the concentrations of the pesticide reduced the growth of plants as evident from the growth parameters shown in Table 1. Fresh weight and dry weight of pesticide stressed plants were reduced by 17% , 30% ,49% ,50% and 23% ,34% ,47% and 48% respectively at 5,10,15 and 20 ppm concentrations. Plants were highly damaged at the concentration of 30 ppm. Our results are comparable to the observations made by Aida et al. [3] and recently done by El-Shahate et al.[14]. It is found that pesticide has an inhibitory effect on photosynthetic CO_2 assimilation and protein synthesis Battah et al.[15] which could be due to disturbances in nitrogen metabolism and photosynthetic activity or due to an increase in protease activity [16]. Such effect might exert much secondary effect on growth. The result obtained (Table 1.) showed that the doubling time at low concentrations (2.5 and 5 ppm) of monocrotophos was not much affected but at high concentrations it showed a significant increase in DT as compared with control. El-Shahate et al. [14] and Madhaiyan et al. [17] suggested that the application of various fungicide show low toxicity affects on the doubling time of *Azolla* as compared with herbicide or insecticide. Dry matter accumulation per unit dry weight per unit time is presented in Table1. and it is clear that the relative growth rate at 30 ppm monocrotophos nearly 20% reduction has been recorded. Kannaiyan [18] reported that pesticides carbofuron have little effect on the relative growth rate in *A. pinnata* SK-CI. The minor effect of pesticide bensulfuron on the RGR of *Azolla japonica* was recently reported by Aida et al.[3] ; such kind of result was also obtained in metal stressed *Azolla filiculoides* by Khosravi et al.[7].The chlorophyll content of test organism exposed to monocrotophos followed declining trend. Chlorophyll content of plant were reduced by 30%, 36% and 39% at 10, 15, and 20 ppm concentration of the pesticide and the highest damage was observed at 30 ppm. Reduction in chlorophyll content in cyanobacteria and higher plants due to monocrotophos and other pesticide treatment has been reported earlier by Prasad et al.[19]; recently by Mishra et al.[20] and Palanisami et al. [21].Like chlorophyll, carotenoids has also showed the similar response and it was highly damaged at 30 ppm. It is most common believe that the target of monocrotophos is photosynthetic apparatus and pigment synthesis; this pesticide inhibits the biosynthesis or breakdowns the pigment or their precursors as suggested for mung bean and other plants under pesticide stress [22].

Table 1: Effect of monocrotophos on fresh weight, dry weight, doubling time, total chlorophyll, carotenoids and relative growth rate.

Monocrotophos (ppm)	Fresh Weight (gm)	Dry Weight (gm)	Doubling Time (days)	Total Chlorophyll (mg g^{-1} F W)	Carotenoids (mg g^{-1}F W)	RGR μg^{-1}_{1} g^{-1}day^{-}
0	1.5±0.3	0.336±0.1	2.60±1.2	0.544±0.3	0.201±0.1	0.186±0.3.
2.5	1.6±0.5	0.384±0.1	2.77±0.5	0.423±0.1	0.179±0.0	0.190±0.2
	(+1.2)	(+14.28)	(+6.53)	(-22.25)	(-10.12)	(+2)
05	1.3±0.3	0.257±0.1	3.13±1.0	0.394±0.1	0.168±0.1	0.182±0.0
	(-17.7)	(-23.52)	(+20.43)	(-27.58	(-16.25)	(-3)
10	1.1±0.4	0.220±0.1	3.79±1.1	0.377±0.2	0.161±0.0	0.175±0.0
	(-30.08)	(-34.53)	(+45.94)	(-30.70)	(-19.80)	(-6)
15	0.80±0.3	0.176±0.0	6.37±1.5	0.345±0.1	0.151±0.0	0.164±0.0
	(-49.37)	(-47.62)	(+144)	(-36.59)	(-24.85)	(-12)
20	0.78±0.4	0.174±0.0	6.73±1.6	0.329±0.1	0.140±0.0	0.159±0.0
	(-50.64)	(-48.22)	(+158)	(-39.53)	(-30.12)	(-15)
25	0.60±0.3	0.144±0.0	16.43±4.1	0.298±0.2	0.128±0.0	0.154±0.0
	(-62.03)	(-57.15)	(+530)	(-45.23)	(-36.18)	(-18)
30	0.49±0.1	0.107±0.0	30.11±9.5	0.258±0.1	0.113±0.0	0.150±0.0
	(-68.99)	(-68.16)	(+700)	(-47.54)	(-43.61)	(-20)

Values in parenthesis are percent decrease (-) or increase(+) with reference to respective controls. Mean ±SE (n=5). Values with different superscripts are significantly (P< 0.05) different from each other (Analysis of variance).

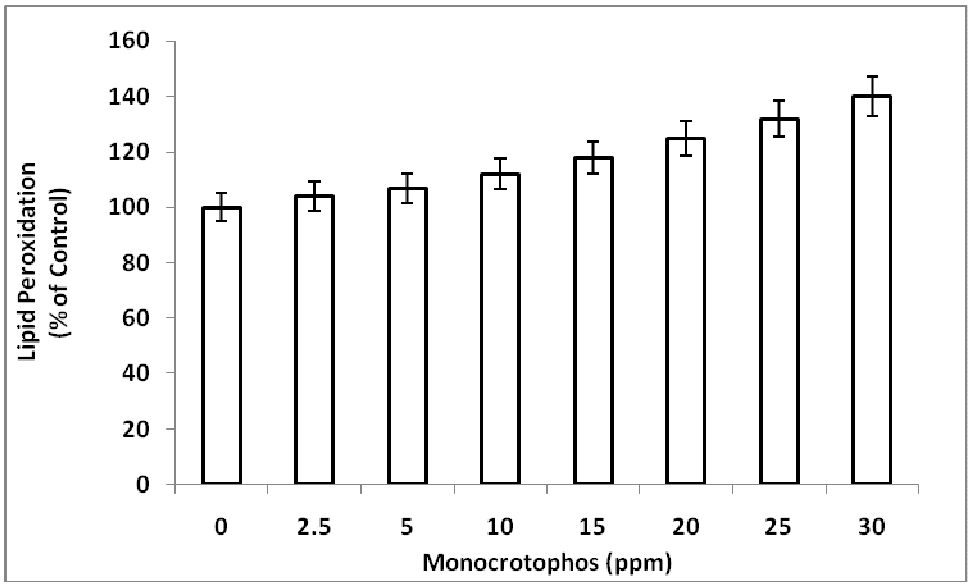

Fig 1: Effect of different concentrations of monocrotophos on lipid peroxidation in *Azolla filiculoides*. Lipid peroxidation in untreated control was 33.25±1.4 [nmol (g FW) $^{-1}$]. Mean± SE.. All the values are significant at P<0.05

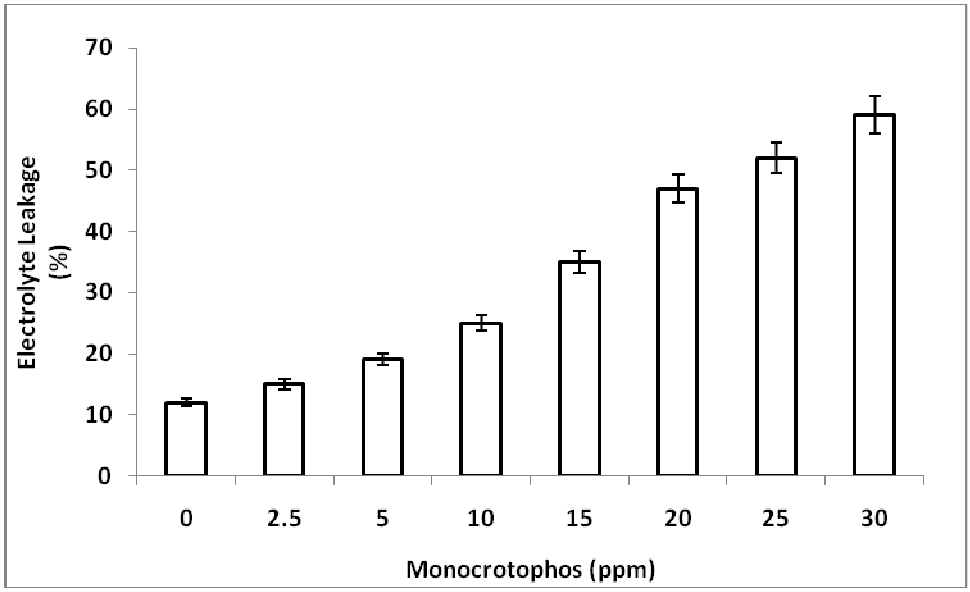

Fig 2: Effect of different concentrations of monocrotophos on electrolyte leakage in *Azolla filiculoides*. All the values are significant at P<0.05

A significant increase in MDA, product of lipid peroxidation in *A. filiculoides* was observed due to monocrotophos (Fig.1). The content of MDA, the end product of lipid peroxidation increased by 4% and 7% at 5 and 10 ppm respectively and it showed an increasing trend continued with rising doses of pesticide. The enhanced lipid peroxidation could be the result of excessive accumulation of reactive oxygen species (ROS) in response to pesticide exposure. The ROS are highly reactive and induce lipid peroxidation, thereby affecting the structural integrity and permeability of cellular membrane [23].Masood et al.[5] also reported increase lipid peroxidation in *A. filiculoides* exposed to UV-B. The stimulated generation of ROS caused increased peroxidation of lipid in the test organism and thus resulted in grater electrolyte leakage (Fig.2). The electrolyte leakage was also found to be dose dependant and it was maximum at 30 ppm. The increased lipid peroxidation and electrolyte leakage due to cell membrane damage confirm our previous finding [24].Recently Sanchez -Viveros et al.[25] also got such kind of result in copper induced *Azolla filiculoides.*

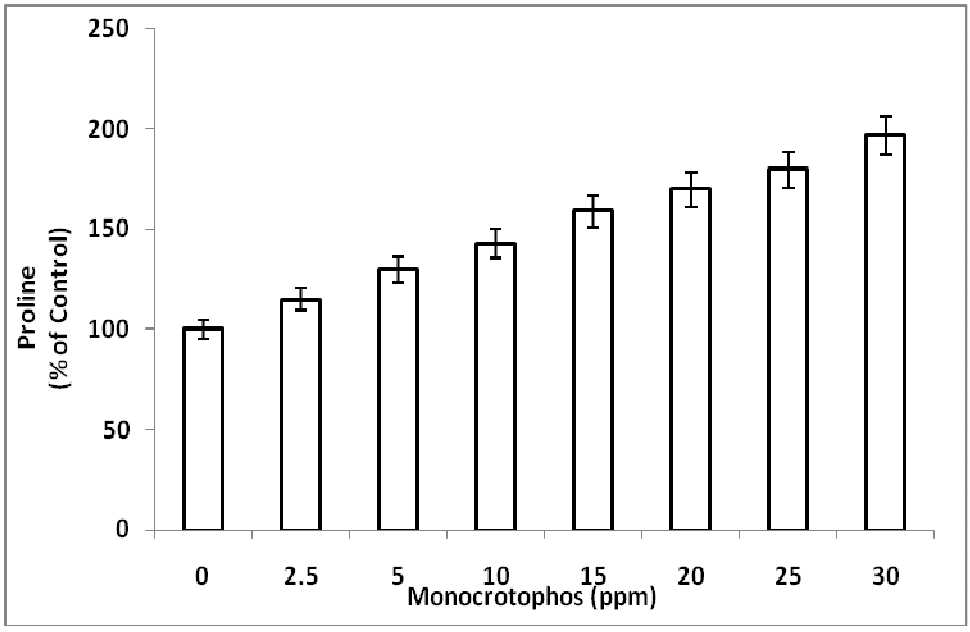

Fig 3: Effect of different concentrations of monocrotophos on proline in *Azolla Filiculoides.* Proline in untreated control was 6 [µg g^{-1} FW]. Mean± SE. All the values are significant at P<0.05

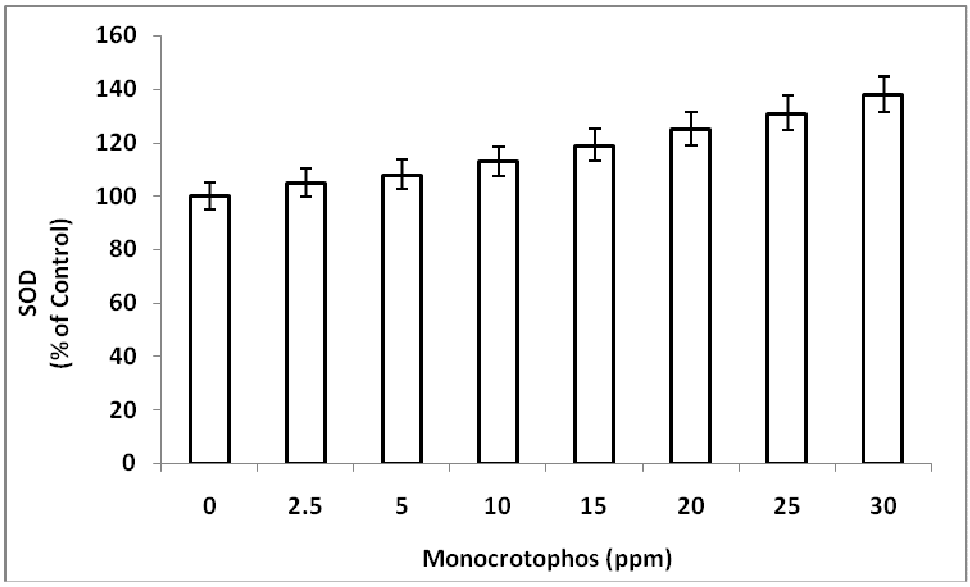

Fig 4: Effect of different concentrations of monocrotophos on superoxide dismutase (SOD) in *Azolla filiculoides.* SOD in untreated control was 5.2±0.9 [Unit (g FW) $^{-1}$]. Mean± SE. All the values are significant at P<0.05

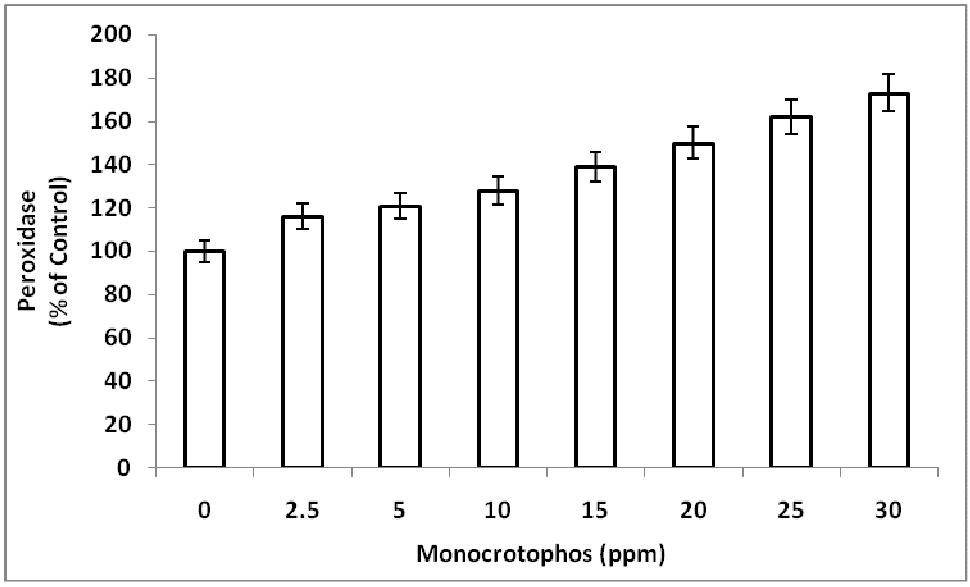

Fig 5: Effect of different concentrations of monocrotophos on peroxidase (POD) in *Azolla filiculoides.* POD in untreated control was 39.12±2.7 [n mol (g F W) $^{-1}$ min^{-1}]. Mean± SE. All the values are significant at P<0.05

Following monocrotophos treatment the proline level (Fig.3) in *Azolla* increased by 19% ,35% , and 49% at 2.5, 5 and 10 ppm and the highest level was found at 30 ppm i.e. 97%. Multiple defense systems that include both enzymatic and non enzymatic compounds have been reported in *Azolla filiuloides* against UV-B damage Masood et al.[5]. Enhancement in proline level may conferred the capacity to detoxify reactive oxygen species efficiently. This agrees well with the earlier observations that proline plays a significant role in detoxification of ROS [26]. However, an earlier observation by Chris et al.[27] showed that NaCl pre-treatment resulted in reduced lipid peroxidation, electrolyte leakage and cellular H_2O_2 levels in in *Cylindrospermum* sp. exposed to UV-B due to cellular proline production induced by NaCl pre-treatment.

The total SOD activity of *A. filiculoides* exposed to monocrotophos increased steadily. SOD activity of *Azolla* plants increased by 5%, and 8%, at 5 and 10 ppm and it was highest at 30 ppm concentration (Fig.4) .A similar trend was also observed in the POD activity [Fig.5] .Recent evidence has shown that ROS, especially H_2O_2 and O^-_2, are involved in cellular signaling processes as secondary messengers to induce a number of genes and enzymes such as POD and SOD [28], which invoke reactive oxygen species in stressed organisms. Thus the increased level of O^-_2 and H_2O_2 triggered the activity of several antioxidant enzymes such as superoxide dismutase, peroxidase in *Azolla filiculoides* at all the concentrations of monocrotophos tested. Prasad et al. [23] observed enhanced activity of SOD and POD in *Plectonema boryanum* exposed to insecticide endosulfan. Changes in the level of antioxidants molecules are signals of plant tolerance/adaptation to stress condition.

CONCLUSION

The results demonstrated response in terms of growth, chlorophyll, carotenoids, proline accumulation, electrolyte leakage, lipid peroxidation, SOD and POD activities of *Azolla filiculoides* in response to monocrotophos. The strong inhibitory effect on the growth and photosynthetic pigments could be correlated with monocrotophos induced inhibition. In contrast to this, lipid peroxidation, electrolyte leakage, SOD and POD activities were enhanced. These observations serve as baseline data for the evaluation and quantification of *Azolla* genotypes towards increasing the usefulness of *Azolla Anabaena* association as biofertilizer. Elucidation of physiological and biochemical response to monocrotophos is important in this organism because

the application of pesticide in crop fields is increasing and potential use of *Azolla* as a biofertilizer in such environment needs to be investigated in detail especially at molecular level .

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