Photosynthetic, Biochemical and Enzymatic Investigation of \textit{Azolla microphylla} in Response to an Insecticide-Hexachlorohexahydro-Methano-Benzodioxathiepine-Oxide

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Abstract

A study on the heterocystous, nitrogen fixing Water fern, \textit{Azolla microphylla} was carried out to investigate the effect of an organochlorine insecticide (hexachlorohexahydro-methano benzodioxathiepine-oxide, called as endosulfan) at different concentrations of 0, 50, 100, 200, 400 and 600 ppm on fresh weight, dry weight, photosynthetic pigments, stress metabolites such as ascorbic acid, proteins, and nitrogen metabolism activity like nitrate reductase and nitrate uptake. The inhibition was found to be dose dependent. The insecticide endosulfan showed to be deleteriously affecting the activities in the \textit{Azolla microphylla}. Endosulfan adversely depleted the cellular activities, leading to a marked increase in the Vitamin-C at lower concentration and gradually decreases at higher concentrations. Decrease in protein was clear and activities like nitrate reductase and nitrate uptake also increases up to certain concentration and at higher concentration slightly decreases. Despite of deleterious effects of endosulfan on the \textit{Azolla microphylla}, a unique regenerating ability in presence of the insecticide was observed by the end of five days in the lower doses of insecticide. \textit{Azolla} seems to help sustain the soil nitrogen supply by returning nitrogen to quantities roughly equal to those extracted from the soil by the rice plant.

Key words: \textit{Azolla microphylla}, Endosulfan, Enzymes, Photosynthetic pigments, Stress metabolites, Nitrogen metabolism

Introduction

The increasing use of pesticides in agriculture demands investigation to examine the effect of pesticides on the non-target soil micro-organisms and plants including nitrogen fixing cyanobacteria and their symbionts. A pesticide (herbicides, fungicides and insecticides) adversely affects all aspects of primary and secondary metabolism in crops and animals when applied in agricultural fields. The important factor in using \textit{Azolla} as a biofertilizer for rice crop is its quick decomposition in soil and efficient availability of its nitrogen to rice plant. The quick multiplication rate and rapid decomposing capacity of \textit{Azolla} has become paramount important factor to use as green manure cum biofertilizer in rice field. 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. (Meghraj et al., 1988). 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 (Singh, 1988). 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 (Aida et al., 2006). An Azolla- Anabaena association is a favourite 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 (Wagner, 1973).

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 (Masood et al., 2008) Detrimental effect of pesticides on the growth of aquatic macrophytes are generally known (Seulthorpe, 1967), but still the knowledge of the indirect effect of applied pesticides like monocrotophos on soil micro and macro flora is fragmentally and partly outdated. The influence of pesticides on soil and aquatic algae including Azolla has been a growing concern. Plant exposed to stress shows an increased accumulation of proline (Chris et al., 2006). Studies also showed that organophosphorus insecticides interfere with carbohydrate accumulation in paddy seeds (Gupta and Mishra, 1983). Quinalphos (O, O-diethyl-O-quinoxalin-2-yl phosphorothioate) due to its acaricidal and insecticidal properties is in large scale use in this country. From an annual consumption of 300 metric tons during 1977, the use has risen to 1000 metric tons. But insecticides particularly endosulfan induced effect on growth, enzyme assay and nitrogen metabolism in Azolla particularly A. microphylla 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 Photosynthetic, biochemical and enzymatic investigation of A. microphylla in response to an insecticide – hexachlorohexahydro – methano – benzodioxathiepine - oxide.

**Materials and methods**

**Plant material and growth conditions**

Azolla microphylla, selected for present study, was procured and collected from National Centre for Conservation and Utilization of Blue Green Algae, IARI, New Delhi and cultured in Department of Biological sciences, SHIATS Allahabad. The plants were surface sterilized quickly with a solution of mercuric chloride (0.1% for 30s) followed by dipping the plants into a large volume of sterile distilled water. Washing of the Azolla microphylla with sterile distilled water was repeated several
times. Fronds were then transferred into plastic trays (32×25×6 cm\(^3\)) containing nitrogen free medium. The pH of the medium was adjusted to 7.2. Plastic trays were placed in the Culture Lab, Department of Biological Sciences, SHIATS Allahabad. During the experimental period, average minimum and maximum temperature ranged from 16.7 to 36.8°C, and relative humidity from 55 to 71%. Photosynthetic active radiation (PAR) ranged between 800 -1000 µmol photon m\(^{-2}\) s\(^{-1}\).

**Pesticide treatment**

Widely used insecticide endosulfan (hexachloro hexahydro-methanobenzodiox-athiepine-Oxide) 35% EC was selected for the treatment which was manufactured by Hindustan Pulversing Mills, Industrial Growth Center Samba Jammu. Its various concentrations 0, 25, 50, 100, 200, 400 and 600 ppm in nutrient medium were prepared for screening experiment.

**Growth estimation**

*Azolla microphylla* 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 60°C for 24 h to a constant weight.

**Chlorophyll and Carotenoid estimation**

Chlorophyll and carotenoids were extracted from fronds with 80% acetone. Chlorophyll and carotenoids were estimated spectrophotometrically according to the method of Lichtenthaler and Welburn (1983).

**Protein estimation**

Protein was estimated by method given by (Lowry et al., 1951). The protein content was determined by the standard curve prepared out of the Bovine serum albumin protein and absorbance was measured at 660 nm.

**Ascorbic acid estimation**

Ascorbic acid is an important chemical antioxidant which is responsible for the non-enzymatic scavenging of superoxide radical and hydrogen peroxide, its estimation is based on the formation of pink coloured complex due to the reduction of dinitrophenylhydrazine by ascorbic acid to phenyl hydrazene in acidic medium. It is estimated by the method given by (Mukherjee and Choudhary, 1983) and absorbance is recorded at 530 nm.

**Nitrate uptake estimation**

Nitrate uptake by the *Azolla microphylla* cells were estimated by measuring the depletion of nitrate from external medium spectrophotometrically by brucine-sulphuric acid method of (Nicholas and Nasen, 1957). Nitrate Calibration curve was prepared in the range of 1-100 µm using potassium nitrate as standard solution.

**Nitrate reductase activity (EC 1.6.6.1) estimation**

A known volume of *A. microphylla* was centrifuged (8000 rpm, 5 min) and washed 3-4 times and transferred into culture medium containing 5 mM KNO\(_3\). Samples were withdrawn at regular intervals and formation of nitrite was measured at 540 nm by the diazo-coupling method of (Lowe and Evans, 1964). A calibration curve as
standard solution and the values were calculated accordingly.

Statistical analysis
All the data obtained of *Azolla microphylla* in terms of growth, chlorophyll, carotenoids, protein, ascorbic acid, nitrate reductase activity and ammonia uptake in response to different levels of endosulfan were statistically analysed 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
The present study shows the effect of different concentrations of endosulfan on growth, photosynthetic pigments and nitrogen metabolism of *A. microphylla*. The growth response of the tested *A. microphylla* to insecticide exposure was inhibitory and the effect varied with insecticide dose. All the concentrations of the insecticide reduced the growth parameters shown in table 1. Fresh weight of insecticide stressed plants were reduced by 7.15, 21.43, 28.58 and 47.15% at 50, 100, 200 and 400 ppm concentration respectively and in case of dry weight, the dry weight of the test organism reduced by 14.67, 23.17, 38.42 and 52.79% at 50, 100, 200 and 400 ppm respectively. Plants were highly damaged at the concentration of 600 ppm. Contents of total chlorophyll and carotenoid in test *A. microphylla* reduced considerably following insecticide exposure and decrease was dose dependent (Tab. 1). As a result of increased exposure total chlorophyll content decrease in *A. microphylla* was 3.85, 19.24, 30.77, 44.24 and 61.54% at 50, 100, 200, 400 and 600 ppm respectively and carotenoid content by 9.04, 28.67, 38.95, 56.39 and 68.54% at same concentrations. As compared to total chlorophyll, carotenoid content was severely affected. The test organism was highly damaged at 600 ppm. Thus summing up the results the effect of endosulfan on *A. microphylla* is detrimental and there is an inverse relation between concentration of insecticide on growth and photosynthetic pigments.

Being essential macromolecule of living cells protein play a paramount role in metabolic pathway to understand the effect of endosulfan on *A. microphylla*. Analysis of protein was done after five days of treatment of insecticide (Fig. 1). The concentration of protein was maximum in control showed a considerable decrease with increasing concentration of endosulfan. Protein content reduced by 15, 26 and 48% at 50, 100, and 200 ppm, respectively. Further there was a decrease in protein as concentration increases. The highest decrease was shown at 600 ppm which was about 70%. Ascorbic acid (Vitamin-C) content increases as the concentration of endosulfan increases as depicted in (Fig. 2). The ascorbic acid content increases by 19, 30 and 42% at 50,100 and 200 ppm, respectively. Beyond 200ppm there was a gradual decrease. Thus our result reveals that Vitamin-C content increases up to certain limit and then starts decreasing.

An attempt was made to study the effects of various concentrations of endosulfan on nitrogen metabolism of test
Table 1. Effect of endosulfan on fresh weight, dry weight, total chlorophyll and carotenoid content of Azolla microphylla. Values in parenthesis are percent decrease (-) with reference to respective controls. Mean ±SE (n=3). Values are significantly (P<0.05) different from each other (Analysis of variance).

<table>
<thead>
<tr>
<th>Endosulfan (ppm)</th>
<th>Fresh Weight (gm)</th>
<th>Dry Weight (gm)</th>
<th>Total Chlorophyll (mg/gm f. wt.)</th>
<th>Carotenoids (mg/gm f.wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.4±0.3</td>
<td>0.341±0.1</td>
<td>0.52±0.4</td>
<td>0.321±0.1</td>
</tr>
<tr>
<td>50</td>
<td>1.3±0.3</td>
<td>0.291±0.1</td>
<td>0.50±0.3</td>
<td>0.292±0.2</td>
</tr>
<tr>
<td></td>
<td>(-7.15)</td>
<td>(-14.67)</td>
<td>(-3.85)</td>
<td>(-9.04)</td>
</tr>
<tr>
<td>100</td>
<td>1.1±0.4</td>
<td>0.262±0.1</td>
<td>0.42±0.1</td>
<td>0.229±0.1</td>
</tr>
<tr>
<td></td>
<td>(-21.43)</td>
<td>(-23.17)</td>
<td>(-19.24)</td>
<td>(-28.67)</td>
</tr>
<tr>
<td>200</td>
<td>1.0±0.4</td>
<td>0.211±0.1</td>
<td>0.36±0.2</td>
<td>0.196±0.3</td>
</tr>
<tr>
<td></td>
<td>(-28.58)</td>
<td>(-38.77)</td>
<td>(-30.77)</td>
<td>(-38.95)</td>
</tr>
<tr>
<td>400</td>
<td>0.74±0.4</td>
<td>0.161±0.0</td>
<td>0.23±0.2</td>
<td>0.140±0.0</td>
</tr>
<tr>
<td></td>
<td>(-47.15)</td>
<td>(-52.79)</td>
<td>(-44.24)</td>
<td>(-56.39)</td>
</tr>
<tr>
<td>600</td>
<td>0.46±0.1</td>
<td>0.109±0.1</td>
<td>0.20±0.1</td>
<td>0.101±0.0</td>
</tr>
<tr>
<td></td>
<td>(-67.15)</td>
<td>(-68.04)</td>
<td>(-61.54)</td>
<td>(-68.54)</td>
</tr>
</tbody>
</table>

Figure 1. Effect of different concentrations of endosulfan on Protein content in Azolla microphylla. Protein content in untreated control was 0.998±0.4 mg/gm fresh weight. Mean± SE... All the values are significant at P<0.05.
Figure 2. Effect of different concentrations of endosulfan on Ascorbic acid content in *Azolla microphylla*. Ascorbic acid content in untreated control was 24.7±1.3 µg gm⁻¹ fresh weight. Mean± SE. All the values are significant at P<0.05.

Figure 3. Effect of different concentrations of endosulfan on Nitrate uptake content in *Azolla microphylla*. Nitrate uptake content in untreated control was 9.01±1.7 µ mol mg⁻¹ Protein h⁻¹ Mean± SE. All the values are significant at P<0.05.
organism (Figs. 3-4). Nitrate uptake by A. microphylla after five days of treatment was clear that as the concentration of endosulfan increases, the nitrate uptake also increases by 10, 20%, at 50 and 100 ppm. However concentration beyond 100 ppm there was a slight decrease in nitrate uptake. The highest decrease was shown at 600 ppm which was about 14%. Also a case of nitrate reductase activity the concentration of endosulfan increases the activity by 18, 32% at 50 and 100 ppm. But beyond there was also a slight decrease in nitrate reductase activity. Thus, summing up the results, at higher concentration the nitrate uptake declined. The accumulation increases at lower concentration of pesticides; this could be due to more demand of host organism for nitrogen. Thus, we put forth that present nitrogen and nitrogen yield were significantly affected by endosulfan but nitrate reduction activity was affected at higher concentration of endosulfan.

Discussion
Heavy use of pesticides reduces the growth of plants higher as well as lower plants. Several physiological and biochemical mechanisms are involved in response of Azolla to pesticide stress. Reduction in fresh weight and dry weight was clear after five days of incubation at different concentration in ppm of endosulfan. Our results are comparable to the observations made by (Aida et al., 2006) and recently done by (El-Shahate et al., 2011). It is found that pesticide has an inhibitory effect on photosynthetic CO₂ assimilation and protein synthesis (Battah et al., 2001) which could be due to disturbances in nitrogen
metabolism and photosynthetic activity or due to increase in protease activity (Kaushik and Venkataraman, 1983) such effects might exert much secondary effect on growth. (Kalita, 1997) has demonstrated that high concentration of melathion inhibit the growth of *Azolla pinnata*. The reduction in dry weight and fresh weight by endosulfan might be due to chemical which effects, the tissue binding process in *Azolla* at higher concentrations. This may also be caused by the disturbance with Hill reaction and electron transport system in photosynthesis as has been observed in spinach due to application of an insecticide methyl parathion (Moreland and Novitzky, 1984). The reduced growth in response to higher concentration of melathion may result from reduction in protein and DNA content (Sengupta et al., 1986).

The damaging effect of endosulfan on photosynthetic pigments of *A. microphylla* was noticed after five days of treatment. Deleterious effects were observed on both the photosynthetic pigments, chlorophyll was more affected than carotenoids. The pesticides are known to inhibit chlorophyll biosynthesis particularly by inhibiting δ-aminolevulinicacid dehydrogenase and protochlorophyllide reductase (Dubey, 1997). Under stress conditions carotenoid pigments are less affected than chlorophyll resulting in a low chlorophyll/carotenoid ratio, and results are obtained in *Azolla* fronds treated with pesticides and are in consequences with earlier findings (Prasad et al., 2005). Since carotenoids are less affected, it also act as an antioxidant metabolite (Chris et al., 2006; Dai et al., 2006), it protects chlorophyll and photosynthetic membrane from oxidative damage, therefore decline in carotenoids could have serious consequences on chlorophyll as well as thylakoid membrane which may lead to reduction in photosynthetic capability of *A. microphylla*. Like chlorophyll, protein of the *A. microphylla* was also inhibited by enhanced doses of pesticides which are in agreement with results of earlier work of (Holst et al., 1982). Malaga and (Malliga and Subramanian, 1989) also reported inhibition in the protein content of *Azolla* fronds following different doses of pesticides and it could be co-related with reduced photosynthetic activity, nitrogen metabolism and nucleic acid damage under pesticide stress. (Reddy et al., 2004). Recently (Prasad and Zeeshan, 2004) have shown reduction in growth (Protein) of cyanobacterium *Plectonema boryanum* under monocrotophos stress which is in agreement with our findings.

A significant increase in ascorbic acid (Vitamin C) content in fronds of *Azolla microphylla* was observed in response to pesticide stress. Ascorbic acid an important antioxidant, which react not only with H₂O₂ but also with O₂, OH and lipid hydroperoxidases (Reddy et al., 2004). Diazole-treatment increased the ascorbic acid content in tomato seedlings (Senaratna et al., 1988) Ascorbate can function as a terminal antioxidant because of the redox potential of ascorbic acid/monodehydro ascorbate (MDA) pair (+280 nm) is lower than that of most of the bioradicals (Scandalios et al., 1997), however very little is known about the regulation of ascorbic acid biosynthesis in higher plants. A high level of endogenous ascorbate is essential to effectively maintain the antioxidant system
that protects plants from oxidative damage due to the biotic and abiotic stress (Shigeoka et al., 2002).

The nitrate reductase activity was increased significantly at 100 ppm of both the pesticide concentration. According to Singh and Mahapatra (1998), nitrate reductase activity was mostly determined by nitrate flux into metabolic pool, so it may be possible that there is an indirect effect of salinity on nitrate reductase activity through modification in nitrate uptake. Expression and activity of nitrate reductase was affected by nitrate under pesticide stress and nitrate is reported to have protective role for nitrate reductase enzyme against action of proteases and/inhibitors besides inducing the synthesis of nitrate reductase protein through nitrate reductase gene expression. 100 ppm concentration of both the pesticide stress increased the nitrate uptake and nitrate reductase activity possibly because of high demand of nitrogen during stress condition (Holst et al., 1982; Suseela, 2001).

Conclusion
The environmental hazards of pesticides would be intensified far greater than expected in the soils already contaminated with pesticide which in turn affect the productivity of Azolla plants under field conditions (Raja et al., 2012). In a review on Azolla calls Azolla “a green gold mine”. The results demonstrated differential response in terms of growth, chlorophyll, carotenoids, Proteins, Ascorbic acid content, and Nitrogen metabolism activities of Azolla microphylla in response to endosulfan. The strong inhibitory effect on the growth and photosynthetic pigments could be correlated with endosulfan induced inhibition. In contrast to this, Proteins, Ascorbic acid and nitrogen metabolism 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 endosulfan 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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References


