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Short-Term Effects of Endosulfan-Induced Toxicity on Accumulation of Antioxidants and Total Phenolic Compounds of *Azolla microphylla*

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ABSTRACT
This study was designed to examine the effects of different concentrations of endosulfan on accumulation of antioxidants and total phenolics, in an aquatic fern *Azolla microphylla* Methods: Azolla microphylla were collected from paddy fields, Department of Biological Sciences, SHIATS, Allahabad. Fronds were cleaned, washed and experiment was carried at an interval of 3 and 7days using 0, 25, 50, 100, 200, 400 and 600 ppm concentrations of endosulfan. The total phenol content was estimated by Folin–Ciocalteu assay and its antioxidant activity was determined by free radical scavenging DPPH assay, Ferric reducing/antioxidant power (FRAP) assay, Phenylalanine ammonia-lyase (PAL) assay and Guaiacol peroxidase (G-POD) assay. The highest total phenolic content (TPC), enzyme and antioxidant activity was observed at 200 ppm and 100 ppm after 3rd and 7th day of incubation respectively. After that there was there was gradual decrease in phenolic content and antioxidant activity. Total phenolic content and antioxidant activity showed increment at lower concentrations however a significant reduction in TPC and antioxidant activity was observed at higher concentrations suggesting a loss in antioxidant power of this potential eco-friendly fern.

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Introduction

Plant exposure to both biotic and abiotic stressors mobilizes or generates, directly or indirectly, diverse signaling molecules and regulates many processes that amplify and specify the physiological response through transcriptional and metabolic changes [1]. *Azolla* is a free floating, ecofriendly, fast growing water fern and also establishes symbiotic relationship with the cyanobiont, *Anabaena azollae* in their leaf cavities. Due to N_2-fixing ability of the cyanobiont, *Azolla* is commonly used as a biofertilizer to improve the fertility of soil and ultimately the agricultural productivity. It is a good replacement of chemical fertilizers and also improves N-balance within few weeks [2]. The inherent nitrogen fixing capacity of indigenous azolla is one of the most important factors aiding in the process of biological nitrogen fixation in paddy field ecosystems [3]. The problems caused by the increased application of insecticides call for multidisciplinary approach, thus incorrect and indiscriminate application of insecticides affects negatively the health of humans, plants and animals [4]. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents [5]. Plants possess many antioxidants, usually classified in two broader categories. They are: enzymatic antioxidants and non-enzymatic antioxidants. The alterations in the activities of antioxidants is observed in the plants exposed to different environmental stresses such as drought, heavy metals, pesticides, ultraviolet radiations etc. Since human activities are increasing the level of pollutants in the environment day by day, it has become an interesting area of research to observe their effects on plant communities (producers of the Ecosystems). The damage to the biological ecosystems may be measured in terms of the morphological and biochemical alterations in primary producers. Insufficient levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells. Numerous studies have been conducted on photosynthetic enzymes, pigments, proteins, and antioxidant compound contents in plants. Endosulfan is a ‘persistent organic pollutant’ (POP), it is persistent in the environment, bioaccumulative, demonstrates long range environmental transport, and causes adverse effects to plant health and the environment. The indiscriminately applying pesticides in pretext of controlling insect pests like yellow stem borer, leaf roller, blue beetle, caterpillar, aphids etc. on paddy crop. Endosulfan applied on paddy crop expected to have adverse effect on growth of *Azolla microphylla* [4]. Though pesticides are applied only on the paddy crop to combat the pests and diseases, the floating fern in paddy field became the non-target victim of such an applied insecticide. The impact of heavy applications of these insecticides has not yet been studied in depth especially on antioxidants and phenolic compounds. Hence accumulation of antioxidants and phenolic compounds in *Azolla microphylla* (Pteridopsida) exposed to endosulfan has been attempted in the present study.

Materials and methods

Plant Material and Growth Conditions

Azolla microphylla were collected locally from paddy fields near Allahabad. 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

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free 2/5 strength sterile Hoagland's medium [6] 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.

Reagents and Working Solutions

All reagents were purchased from Merck or Sigma–Aldrich and were of analytical grade or equivalent. In each set of experiments working solutions were prepared from stock reagents immediately before use. Insecticide, endosulfan 35% EC was selected for the treatment. This is widely used Insecticide to control pest of paddy like leafhoppers, white flies spider mites etc. in rice fields. LC_{50} values of the organisms for endosulfan was determined in terms of quantitative estimation of Chlorophyll content and accordingly, various concentrations 0, 25, 50, 100, 200, 400, 600 ppm in nutrient medium were prepared for screening experiment in all further experiments. Sterile cultures and conditions are maintained throughout the experimental period. Stock solution of the insecticide was prepared in sterilized double distilled water and added aseptically to the culture medium to the final concentrations indicated for each treatment. All activities were determined in *Azolla microphylla* treated with endosulfan for 3 and 7 days incubation period.

Determination of Total Phenolic Content (TPC) Assay

Phenols were extracted from frozen samples, grounded with a pestel in a mortar and suspended in 0.1 N HCl [7]. Samples were incubated overnight at 4 ◦C in the dark in orbital shaker. The amount of total phenolics was measured by means of Folin − Ciocalteu method [8], using chlorogenic acid (CA) as standard, for which a calibration curve was carried out with solutions of 1, 2, 5, 10, and 20 _g/l of this compound (y = 0.0659x − 0.013, R2 = 0.9997). Results are expressed as micrograms of CA equivalents per g of fresh weight of plants. The data are expressed as mean values ± SD.

Antioxidant Activities

Free radical scavenging (DPPH) assay

Free radical scavenging was determined by using the free radical generator DPPH (2, 2-diphenyl-1- picrylhydrazyl) assay based on slight modification [9]. An aliquot (1 ml) of the serially diluted extract samples was thoroughly mixed and 1 ml of 500 µM DPPH solution was added. The mixture was thoroughly mixed using a vortex and kept in the dark for 30 min. The absorbance, using a spectrophotometer, was then measured at 518 nm against a blank of ethanol without DPPH. Antioxidant activity was calculated by applying known aliquots of Trolox (antioxidant compound) to known concentrations of DPPH solution. Results were expressed as micromoles Trolox equivalents per gram of fresh tissue (µMTroloxg^{-1}FW).

Ferric reducing/antioxidant power (FRAP) assay

A FRAP assay was performed using a modified method of Benzie and Strain [10]. Briefly, a 150 µl aliquot of properly diluted extract was thoroughly mixed with 2850 µl FRAP reagent and incubated at 370C for 4 min. The absorbance was then determined at 593 nm against a blank that was prepared using distilled water. FRAP was freshly prepared by mixing 2.5 ml of a 10 mM 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ) solution in 40 mM HCl with 2.5 ml of 20 mM FeCl3 6H2O and 25 ml of 0.3 M acetate buffer at a pH of 3.6. A calibration curve was prepared using different concentrations of gallic acid (0.016, 0.008, 0.004, 0.002 and 0.001 mg/ml, r2 = 0.997). FRAP values were expressed on a dry weight basis as mg GAE/100 g dry sample.

Enzymatic Activities

Assay of phenylalanine ammonia-lyase (PAL) activity

Activity of PAL was determined in Azolla microphylla by the production of *trans*-cinnamic acid from L-phenylalanine over 1 h at 30^{0}C, and was measured spectrophotometrically by the absorbance change at 290 nm [11]. 1g fresh tissues were homogenized in 10mL acetone and the insoluble residue filtered and dried under vacuum. It was extracted at 4^{0}C by gentle stirring with 50 mL of extraction buffer which contained 38 g/L sodium borate (pH

8.8),0.39g/Lβmercaptoethanol,0.58g/LEDTA(ethylendiami ntetraacetique) and PVPP (polyvinylpolypyrrolidone) at 1 g/100 g of fresh weight. After 1 h of extraction, the solution was filtered through one layer nylon cloth and centrifuged at 20 000 × *g* at 4^{0}C for 15 min and the supernatant collected. The reaction mixture for the assay contained 1.5 mL of sodium borate buffer (pH 8.8), 0.5 mL of supernatant and 1 mL of 2.5 g/L L-phenylalanine added after 10 min of pre-incubation. The activity is expressed as g t-cinnamic acid/h/µg $protein^{-1}$.

Assay of Guaiacol peroxidase (G-POD) activity

Guaiacol peroxidase (G-POD) activity determination was performed according to the method of Forni [12] modified as follows: aliquots (50 mg) of freeze-dried powder were transferred into 5 ml of extraction buffer (0.2 M sodium phosphate buffer containing 1%, w/v insoluble polyvinyl polypyrrolidone (PVPP), 0.1%, w/v endosulfan and protease inhibitor, pH 7.0 and homogenized for 1 h at 4 ^{0}C. This extraction buffer was chosen to prevent protein precipitation by tannins during the extraction. The homogenates were centrifuged for 30 min at 17,000 g at 4 ^{0}C and the supernatants were recovered. These crude extracts were used immediately for spectrophotometric determination of enzymatic activities. Three ml reaction mixture contained sodium phosphate buffer (0.2 M; pH 6.5) 100 µl enzyme extract, 5.3 mM guaiacol, 30 mM H_2O_2, pH 6.5. Increases in absorbance at 470 nm were monitored in a Varian 50 spectrophotometer. Blanks were recorded in the absence of either substrates or enzyme extract, or with boiled crude extracts; in both cases, activity was negligible. The data are expressed as mean values ± SE.

Statistical analysis

Data were subjected to analysis of variance, and mean comparisons were made using Duncan's new multiple range test. Statistical analyses were carried out using the SPSS statistical software (SPSS, Inc., Chicago, IL).

Results and discussion

Total phenolic content (tpc)

Table1 shows the total phenolic content of *Azolla microphylla* at different days due to endosulfan stress. The highest level of phenolic content (3.46 mg/g FW) was found at 200ppm after 3 days of stress, while the lowest content (2.19 mg/g FW) was found at 600ppm which was siginificantly less than the control. The decline level of total phenolic compound after 200ppm indicated the lowering of its antioxidant activity. Highest phenolic content (3.1mg/g FW) was found at100ppm after 7 days of endosulfan stress, while lowest (1.2 mg/g FW) was found at 600ppm. The decline level of total phenolic compound started after 100ppm to 600ppm .These results suggested that biosynthesis and accumulation of secondary metabolites increases up to certain concentration then it gradually decreases. Phenolic compounds are known to have

antioxidant properties for plants including Azolla ferns, established under either biotic or abiotic stressful conditions [13, 14], thus conferring better tolerance, fitness, and growth as it was observed in this study for *Azolla microphylla* at low endosulfan concentrations. Nevertheless, the negative effects of high concentrations of endosulfan on the growth of azolla may be related to impaired synthesis of phenolics and other antioxidant compounds by which, the aquatic fern was not able to alleviate the toxicity and the oxidative stress

Values followed by different alphabets shows significant difference at (P< 0.01) from control. Values represent the mean ± SD according to Duncan's Multiple Range Test.

Antioxidant activities

DPPH free radical scavenging activity

Azolla microphylla membranes have a high content of polyunsaturated fatty acids, which are easily oxidised to form lipid peroxides. Free radical scavenging activity was determined depending on both endosulfan concentration and incubation time. As shown in table 2, the levels of DPPH in control conditions increases significantly up to 200ppm after 3 days (P < 0.01) and after 200ppm it decreased suddenly in concentration dependent manner. After 7 days of incubation time, DPPH activity increases up to 100ppm and after 100ppm it decreases in dose dependent manner. At 600ppm (after 7 days) DPPH activity was noticed half than the control values. DPPH is a free radical compound that has been widely used to determine the free radical scavenging capacity of various samples [15, 16] because of its stability (in radical form), simplicity and fast assay [17]. DPPH-radical scavenging activity is a measure of non-enzymatic antioxidant activity. DPPH is stable nitrogen centered free radical which can be effectively scavenged by antioxidants [18, 19]. Hence it has been widely used to test the ability of compounds as free radical scavengers or hydrogen donors [20] and to evaluate the antioxidant activity of plant extracts relative to other methods [21].

Values followed by different alphabets shows significant difference at (P< 0.01) from control. Values represent the mean ± SD according to Duncan's Multiple Range Test.

Ferric Reducing/Antioxidant Power (FRAP) Activity

The antioxidant potential of *Azolla microphylla (*depending on endosulfan concentration and incubation time) was estimated from its ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) complex. As shown in table 3, the levels of FRAP increased significantly up to 200ppm after 3 days (P < 0.01) with respect to control and but after 200ppm a gradual decrease in FRAP activity was observed. In case of 7 days incubation time, FRAP activity showed increment up to 100ppm and after 100ppm it decreases in a dose dependent manner. At 600ppm FRAP activity was less than the control values. Our result suggests that antioxidant power of *Azolla microphylla* was lost as the concentration of endosulfan increases. The inhibition of FRAP above 100 and 200ppm concentrations observed for *Azolla microphylla*, could be an effect on molecular disruption of the enzyme. Such concentrations of endosulfan could cause oxidation of the membranes and the imbalance of enzymatic molecular structure, resulting in a decrease in activity. Faced with these concentrations of insecticide the antioxidant mechanism would be surpassed and would not be effective in protecting the cells from the destructive action of free radicals responsible of cellular oxidative stress. This will take cells to death as observed in present study. Lower FRAP values of at higher concentrations could be due to the presence of compounds not reactive towards FRAP [22]. This may be explained from the basic concept that antioxidants are reducing agents because of their ability to donate a single electron or hydrogen atom for reduction. However, not all reducing agents are antioxidants [23].The antioxidant activities of phenolic compounds are mainly due to their redox properties, including free radical scavenging, hydrogen donating and singlet oxygen quenching [24]. Pulido *et al.,* [25] reported similar results on the reducing capacity of polyphenols, as determined by a FRAP assay. This seems to depend on the degree of hydroxylation and extent of conjugation of the phenolic compounds. However, the FRAP test cannot detect compounds which act by radical quenching (H^+ transfer), particularly thiols and proteins [26].

Values followed by different alphabets shows significant difference at (P< 0.01) from control. Values represent the mean ± SD according to Duncan's Multiple Range Test.

Enzymatic Activities

Phenylalanine ammonia-lyase (PAL) Activity

Endosulfan effect on PAL activity was different for different days (Table 4). After 3 days of exposure, enzyme activity increased in a concentration dependent manner, reaching a maximum at 200 ppm (3.89) and after 200ppm it decreases. The values decreased and were negatively related to the concentrations of endosulfan. Whereas in case of 7 days treatment, the highest PAL activity was observed at 100ppm and after that it decreased gradually. Minimum PAL activity was observed at 600 ppm (1.01) which was significantly less than control. In this study, we found that PAL activities were stimulated by the insecticide at lower concentrations and reverse was found at higher concentration. PAL is the key enzyme in the shikimic acid pathway, the end products from the phenylpropanoid biosynthetic pathway, as substrates for oxidation. PAL activity and phenolics production were positively related. These responses were again detected mostly within the 3rd day, when the beginning of surfactant taken up was detected. At higher concentrations of endosulfan the enzymatic activity decreased at the end of experiment, because of the toxicity of endosulfan accumulated in the plants [27].

Values followed by different alphabets shows significant difference at (P< 0.01) from control. Values represent the mean ± SD according to Duncan's Multiple Range Test.

Guaiacol Peroxidase (G-POD) Activity

The activity of G-POD in treated plants progressively increased with increasing concentrations as compared to control (Table 5). The effect of endosulfan doses on *Azolla* fronds resulted in significant (p < 0.001) changes in guaiacol peroxidase activity as concentrations increased. After 3 days of exposure, activity increases up to 200ppm and but as expected it also showed a sudden decrement and reaches minimum (0.301) at 600 ppm. In case of 7 days incubation time, activity increases up to 100ppm and after 100ppm it decreases and reaches minimum (0.253) at 600 ppm which was significantly less than control. The activity of G-POD varies considerably depending upon plant species and stress conditions [28]. All the lower concentrations of endosulfan used here stimulated and enhanced the G-POD activity. Such activity of GPOD may eventually reduce the cell metabolic damage by simultaneous induction during plant growth [29]. In the present result, pattern of increase of G-POD during period of endosulfan stress suggests that possibly G-POD is playing a role as an important scavenger of H_2O_2 and confirm the expression of endosulfan toxicity or induced oxidative stress. Higher peroxidase activities in water plants have been related to the tolerance to the pollutants [30, 31], although controversy exists in *Azolla microphylla* treated with

xenobiotic, e.g. pesticides, G-POD activity was not stimulated at higher concentrations [32, 33] or transient induction was detected [34]. These findings are in agreement with the literature which shows that G-POD upregulation is strongly induced at the beginning of an event, and slowly decrease within time [35].

Values followed by different alphabets shows significant difference at (P< 0.01) from control. Values represent the mean ± SD according to Duncan's Multiple Range Test.

On the basis of above results, it can be concluded that the extract of *Azolla microphylla* is capable of scavenging a wide range of synthetic and naturally occurring free radicals. It is evident from the present study that the *Azolla microphylla* could be utilized as a good natural source of antioxidants and a possible food supplement. The data may contribute to a rational basis for the use of antioxidant rich extracts in the therapy of diseases related to oxidative stress. The phyto-constituents quantified in the present study have a great value in human health care system. Phenols have been reported as an active, quenching of oxygen-derived free radicals by donating hydrogen atom or an electron to the free radicals [36]. Antioxidant models showed a low to moderate antioxidant activity. Total antioxidant capacity and reducing power assay along with different free radical scavenging methods helps us in determining the overall antioxidant potential of a plant. In human body free radicals such as hydroxyl radical and hydrogen peroxide get bind to DNA nucleotides thus, causing damage to various biological systems which may result in carcinogenesis, mutagenesis, and cytotoxicity. Therefore, plants rich in phenolic, antioxidants and flavonoid are considered as a potential antioxidant agent as they neutralizes the free radicals via donation of hydrogen atom, quenching of oxygen and by chelation of metals thus, minimizing oxidative stress [37, 38]. Physicochemical standards, obtained from the present study, will provide a proper identity and authenticity of *Azolla microphylla* which may help in maintaining its quality and purity and will prevent it from damage due to insecticides.

Conflict of Interest Statement

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Table 1. Total phenolic content of *Azolla microphylla* treated with different concentrations of Endosulfan stages.

endosulfan (ppm)	total phenolic content (mg/g fw)	
	AFTER 3 DAYS	AFTER 7 DAYS
0	2.24 ± 0.11^{D}	2.28 ± 0.02^{E}
25	2.46 ± 0.01^{C}	2.56 ± 0.06^{D}
50	2.82 ± 0.00^{B}	2.97 ± 0.61^{C}
100	3.10 ± 0.61^{A}	3.21 ± 1.01^{B}
200	2.32 ± 0.54^{CD}	3.46 ± 0.88^{A}
400	1.81 ± 0.04^{E}	2.43 ± 0.11^{DE}
600	1.20 ± 0.24^{F}	2.19 ± 0.05^{F}

Values followed by different alphabets shows significant difference at (P< 0.01) from control. Values represent the mean ± SD according to Duncan's Multiple Range Test.

Table 2. Free radical scavenging activities from DPPH assay in *Azolla microphylla* treated with different concentrations of Endosulfan.

Endosulfan (ppm)	DPPH Activity (μM Trolox g^{-1} FW)	
	After 3 Days	After 7 Days
0	10.361 ± 0.23^{ef}	10.217 ± 0.33^{e}
25	12.983 ± 0.37^{d}	11.532 ± 0.41^{d}
50	14.232 ± 0.15^{bc}	13.912 ± 0.41^{ab}
100	14.431 ± 0.39^{b}	14.126 ± 0.34^{a}
200	15.526 ± 0.29^{a}	13.512 ± 0.38^{c}
400	10.523 ± 0.25^{e}	9.931 ± 0.27^{ef}
600	10.001 ± 0.17^{g}	6.126 ± 0.40^{g}

Values followed by different alphabets shows significant difference at (P< 0.01) from control. Values represent the mean ± SD according to Duncan's Multiple Range Test.

Table 3. Ferric reducing/antioxidant power (FRAP) in *Azolla microphylla* treated with different concentrations of Endosulfan.

Endosulfan (ppm)	FRAP (mg GAE/100g)	
	After 3 Days	After 7 Days
0	0.058 ± 0.03^{e}	0.050 ± 0.07^{e}
25	0.080 ± 0.01^{c}	0.071 ± 0.11^{d}
50	0.091 ± 0.02^{b}	0.086 ± 0.04^{b}
100	0.099 ± 0.02^{ab}	0.095 ± 0.02^{a}
200	0.103 ± 0.01^{a}	0.081 ± 0.03^{bc}
400	0.069 ± 0.04^{d}	0.041 ± 0.06^{f}
600	0.041 ± 0.06^{f}	0.028 ± 0.05^{g}

Table 4. Phenylalanine ammonia-lyase (PAL) activity of *Azolla microphylla* treated with different concentrations of Endosulfan

Endosulfan (ppm)	PAL Activity (µg t-cinnamic acid/ hr × mg protein^{-1})	
	After 3 Days	After 7 Days
0	2.313 ± 0.23^{ef}	2.126 ± 1.12^{e}
25	.810 ± 0.17^{d}	2.521 ± 1.01^{d}
50	3.111 ± 0.25^{c}	2.983 ± 2.03^{c}
100	3.732 ± 0.15^{ab}	3.660 ± 4.34^{a}
200	3.891 ± 20.11^{a}	3.310 ± 2.01^{b}
400	2.416 ± 0.09^{e}	2.031 ± 1.12^{ef}
600	2.101 ± 0.14^{g}	1.011 ± 1.01^{g}

Table 5. Guaiacol peroxidase (G-POD) activity of *Azolla microphylla* treated with different concentrations of Endosulfan

Endosulfan (ppm)	G-POD Activity (Unit/g Lyophilized)	
	After 3 Days	After 7 Days
0	0.313 ± 0.11^{f}	0.321 ± 0.02^{de}
25	0.456 ± 0.01^{d}	0.337 ± 0.06^{d}
50	0.503 ± 0.00^{c}	0.425 ± 0.01^{b}
100	0.610 ± 0.01^{b}	0.526 ± 0.01^{a}
200	0.688 ± 0.04^{a}	0.401 ± 0.08^{bc}

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