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ORIGINAL ARTICLE

Endosulfan induced changes in growth rate, pigment composition and photosynthetic activity of mosquito fern *Azolla microphylla*

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This paper is the first in a series reporting a study on the effects of different concentrations of insecticide, Endosulfan (0-600ppm) was premeditated on 5th day after insecticide exposure with respect to growth rate, pigment composition and photosynthetic activity of *Azolla microphylla* under laboratory conditions which become non-target organism in the rice fields. Endosulfan inhibited the relative growth rate, pigment content and photosynthetic O_2 evolution. Phycocyanin was main target followed by carotenoid and total chlorophyll. Significant increase in pigment, flavonoid and Anthocyanin was noticed after six days of treatment. In contrast to the photosynthetic activity, the rate of respiration in *Azolla microphylla* was increased significantly. Our results show that Endosulfan at normally recommended field rates and intervals are seldom deleterious to the beneficial and Eco friendly *Azolla microphylla* and their activities and thus in turn suppress plant growth and development. Phytotoxity of *Azolla microphylla* can be minimized by restrictions on application, timing, method and rate of application.

Key words: Anthocyanin; Carotenoid; Phytotoxity; Phycocyanin; insecticide

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The water fern *Azolla pinnata*, with tiny roots and short branched stems (rhizomes) bearing alternate leaves floats freely on the surface of water. a cavity formed by enfolding of the adaxial epidermis in the dorsal leaf lobe contains the cyanobiont *anabaena azollae*. Because of the remarkable ability to fix dinitrogen at high rates the *azolla–anabaena* complex has long been used as a green manure in rice cultivation (Tuan and Thuyet 1979, liu 1979). Azolla is a genus of leptosporangiate, an aquatic fern that harbours a heterocyst-forming, nitrogen fixing blue-green alga, *Anabaena Azollae* as a symbiont in the dorsal lobe cavity (Peters and Mayne 1974). Atmospheric nitrogen fixed by the symbiont can fulfill the nitrogen requirement of the association (Peters *et al.* 1980), and supplement the nitrogen needs of rice when Azolla is grown as a green manure. Azolla can fix about 1.1 kg n /ha/day when used as a green manure, and in 30 days, under favourable

environmental condition, about 30 kg n/ha would have been fixed. Apart from Azolla being used as a green manure for rice and other such crops, it significantly improves the soil organic carbon content, thus, sequestering carbon in soils (Ramesh and Chandrasekaran 2004, Raja *et al.* 2012).

It is evident that many insecticides at the recommended field application have had none or accelerating effect on growth of Azolla microphylla but may affect various physiological processes like growth, pigmentation, respiration, nitrogen assimilation, carbon assimilation of *Azolla microphylla*. Pesticides constitute a major anthropogenic addition to natural communities in aquatic communities, a great majority of pesticide impacts are determined from single-species experiments conducted under laboratory conditions. Although this is an essential protocol to rapidly identify the direct impacts of pesticides on organisms, it prevents an assessment of direct and indirect pesticide effects on organisms embedded in their natural ecological contexts. Application of pesticides in the paddy fields has deleterious effects on non-target organisms including Azolla which are photosynthesizing and nitrogen fixing micro-organisms contributing significantly towards soil fertility and crop yield (Raja *et al.* 2012). Endosulfan induced biochemical metabolites like carbohydrates, proteins, amino acids, phenols and enzymes-nitrate reductase, glutamine synthetase and succinate dehydrogenase (Nirmal *et al.* 2011) three cyanobacterial strains were adversely affected by the insecticide doses and inhibition was dose dependent. Reduction in photosynthetic and accessory pigments, metabolites, nitrogen fixing and respiratory enzymes of the test organisms were accompanied with an initial increase in their total protein at lower Organochlorine doses (Nirmal *et al.* 2011). However, the effect of a pesticide varies from one compound to another compound on one organism to another organism. But insecticides particularly Endosulfan induced effect on pigment composition and photosynthetic activity of mosquito fern *Azolla 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 impact of insecticide Endosulfan on pigment and photosynthetic activity in *Azolla microphylla.*

MATERIALS AND METHODS

Plant material Organism 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 free 2/5 strength sterile Hoagland's medium (Peters and Mayne, 1980) and 0.04 mM 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.

Pesticide Treatment

Pesticide, Endosulfan [hexachloro hexahydro-methanobenzodioxathiepine-Oxide] 35% EC was selected for the treatment. This is widely used Insecticide to control pest of paddy like leaf

hoppers, white flies spider mites etc. in rice fields. LC50 values of the organisms for Endosulfan were determined in terms of quantitative estimation of chlorophyll-a, 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 pesticides were prepared in sterilized double-distilled water and added aseptically to the culture medium to the final concentrations indicated for each treatment.

Estimation of Relative Growth Rate

Relative growth rate is the basic component of growth analysis. RGR is defined at any instant of time as the increase in dry weight per unit dry material present. RGR is expressed in grams per gram per day or µg/g/day. (Subudhi and Watanable, 1981) protocol was followed for estimation of RGR.

Photosynthetic Pigment determination

Azolla fronds were extracted with 90% methanol and its concentration was determined from absorbance at 663 nm using method of Mackinney (1941). Total content of carotenoids (car) was assayed by measuring absorbance of diethyl ether extract at 450 nm (Jensen 1978). Phycocyanin (pc) was extracted in 2.5 mM phosphate buffer (Ph 7.0) after freezing and thawing the amount was determined by method of Bennett and Bogorad (1973).

Estimation of flavonoids and Anthocyanin

The flavonoids and Anthocyanin were determined according to the method of Mirecki and Teramura (1984).The fronds (0.2gm) were weighed and extracted in acidified methanol: HCl (99:1) the homogenate was incubated at 4^0C for 24 hrs. The homogenate was centrifuged for 10 minutes and absorbance of extract was measured at 530 nm and 300nm respectively.

Measurement of photosynthetic O_2 evolution:

Photosynthetic oxygen evolution in treated and untreated Azolla fronds was monitored after 5 days in temperature controlled airtight reaction vessel at 28˚C for 5 min by using a clark type oxygen electrode (Rank Brothers, Cambridge, UK). The Azolla cells were irradiated by projector lamp with 360µmol m^{-2} s^{-1} PAR at the surface of vessel and expressed as µmol O_2 evolved (mg chl a)$^{-1}$ h^{-1} following the method of Fleishhacker and Senger (1978).

Measurement of Respiration rate:

Respiration rate of treated and untreated *Azolla microphylla* cells were determined after 5 days by measuring the oxygen consumption in darkness with the clark type oxygen electrode at 28˚C for 5 minutes (Rank Brothers, Cambridge, UK).

Statistical analysis and figure representation:

Analysis of variance (ANOVA) was computed by SPSS version 11.0 for growth parameters. LSD were tested. Graph illustrations for growth parameters are based on differences in mean values between the control and treated samples.

RESULTS

Relative growth rate

Dry matter accumulation per unit dry weight per unit time is graphically depicted in the form of figures (Fig.1). From the results it is clear that as the concentration increases relative growth rate decreases. At lower concentration there was little effect but at higher concentration the effect was adverse. Increasing level of Endosulfan gradually decreases RGR in comparison to control by 4.01%, 11.06% and 24.3% at 25, 50 and 100ppm

respectively. As the concentration increases from 100ppm to 600ppm, *Azolla microphylla* showed maximum reduction of RGR and decrease was about 37.4 - 81.03% as compared to control. The analysis revealed that the values of Endosulfan were statistically significant at (P≤ 0.01 and P≤ 0.05) Photosynthesis reduction strongly conditions biomass production and growth rates, which are strictly related with crop productivity and yield. (Dias, 2012). Our results could be supported with the result of Arora and Singh (2003), Who had shown less biomass and more doubling time in *Azolla* sp. treated with different concentration of sodium chloride. Recently, Waseem Raja *et al.* (2012) again got same results in *Azolla microphylla* treated with different concentrations of monocrotophos. Reduction in growth may be attributed to inhibition in normal cell division, as reported in barley plant under pretilachlor treatment (Srivastava *et al.* 2008).

Total Chlorophyll

Content of total chlorophyll were reduced by Endosulfan (Fig, 2). Total Chlorophyll was reduced by 4.3%, 9.6% and 21.62% at 25ppm, 50ppm and 100ppm respectively as compared to control. There was a significant reduction in total chlorophyll when concentration of Endosulfan was increased from 100ppm up to 600ppm and generated synergistic reduction in pigment content and decrease in total chlorophyll was 39.11 - 86.12% as compared to control. The analysis revealed that the values of Endosulfan were statistically significant at (P≤ 0.01 and P≤ 0.05). Thus summing up results, Effect of Endosulfan on *Azolla microphylla* is deterimental and there was an inverse relation between concentration and chlorophyll content, thus affecting the plant growth and development. Since plants depend on photosynthesis to assimilate carbon for further growth and overall vigor, photosynthesis impairment has negative consequences in plant biomass production and yield (Dias, 2012) Diverse studies reported that the decline in chlorophyll content might be caused by a reduction in the synthesis of chlorophyll, possibly by increasing chlorophyllase activity by disorderness of chloroplast membrane and by an activation of electron transport in photosystem-I (Leborans *et al.* 1996).

Carotenoid content

Contents of carotenoid in test Azolla microphylla reduced considerably following Endosulfan exposure and decrease was dose dependent (Fig 3). As a result of increased concentration of Endosulfan from 0-600ppm, the contents decrease from 5.02 to 88.33% as compared to control. Compared to total chlorophyll the carotenoid was severely affected. The analysis revealed that the values of Endosulfan were statistically significant at (P≤ 0.01 and P≤ 0.05). Carotenoid protect chlorophyll and photosynthetic membrane from photooxidative damage. Therefore, decline in carotenoid content could have serious consequence on chlorophyll, phycocyanin as well as thylakoid membrane (Prasad *et al.* 2005).

Phycocyanin content

Phycocyanin were severely affected by Endosulfan (Fig, 4). Phycocyanin was reduced by 14.3%, 26.66% and 41.21% at 25ppm, 50ppm and 100ppm respectively as compared to control. There was a significant reduction in phycocyanin when concentration of Endosulfan was increased from 200ppm up to 600ppm and generated synergistic reduction in pigment content and decrease was 66.12 – 91.12% as compared to control. The analysis revealed that the values of Endosulfan

were statistically significant at (P≤ 0.05). Phycocyanin was severely affected which was followed by carotenoid and total chlorophyll. Phycocyanin was more sensitive than chlorophyll and carotenoid which is in consonance with the reports of Tyagi *et al.* (1992), and Sinha and Hader (1998). Strong effect of Endosulfan on phycocyanin may be due its presence on the outer surface of membrane which makes it more prone towards stresses. Our results were also supported by Prasad *et al.* (2005).

Flavonoid and Anthocyanin content

The effect of doses of Endosulfan on *Azolla* fronds resulted in significant ($p < 0.01$) changes in flavonoid and anthocyanin content as insecticide concentrations increased (Fig.5). After 5 days of exposure to Endosulfan the flavonoid content increase by 2.6%, 13.1% and 24.9% at 25, 50 and 100ppm respectively and by 12.1%, 21.11% and 33.61% respectively at same concentration as compared to control. Beyond 100 ppm there was a gradual increase as the concentration increases from 100 to 600ppm. The decrease was about 39.3 to 71.6% in case of flavonoid and 43.96 to 79.22% in case of Anthocyanin. Present results shows that Endosulfan increased Anthocyanin content more than flavonoid content. Increase in flavonoid and anthocyanin contents could also provide protection in plants against oxidative damage. Since these also act as antioxidant metabolite. Bores, *et al.* (1990) reported effective free radical capacity of flavonoids. Boling *et al*. (2001) observed increased tolerance to high light stress in pea and bean plants due to increase in flavonoid content. Brawn (1991) reported that the epidermal layer of oat seedling accumulated large amount of UV absorbing pigment flavonoid and anthocyanin during early development which gave a better protection against UV-B.

Photosynthetic Oxygen Evolution and Respiration rate

Photosynthetic Oxygen Evolution was decreased by 11.61%, 19.99% and 30.12% following Endosulfan 25ppm, 50ppm and 100ppm respectively, When concentration was increased from 200 to 600ppm, more severe effect on oxygen evolution rate was observed, as it was declined by 46.16 – 78.11% with the tested Endosulfan (Fig.7). The analysis revealed that the values of Endosulfan effects were statistically significant at (P≤ 0.01 and P≤ 0.05). Similar results were obtained by Veveros *et al.* (2010) while working on *Azolla filiculoides* and *Azolla caroliniana* under copper stress. The amount of pigments started decreasing as the concentration of the stress increased. Treatments with higher concentrations of organic compounds showed inhibitory effects on the photosynthetic activity (Sundaram *et al*. 2011).

In contrast to the photosynthetic activity, the rate of respiration in *Azolla microphylla* was increased significantly (Fig.8). Rate of respiration increases by 2.6%, 11.62% and 25.01% at 25 ppm, 50 ppm and 100 ppm respectively in case of Endosulfan as compared to control, There was a significant increment in respiration rate when concentration was increased and reached maximum up to 66.12% at 600 ppm as compared to control. The analysis revealed that the values of Endosulfan effects were statistically significant at (P≤ 0.01 and P≤ 0.05). Our results are supported by Boyer (1982) who observed that salt stress cause inhibition of growth and development, reduction in photosynthetic activity, respiration and protein synthesis in sensitive species. Our results were further supported by Zeeshan and Prasad (2009)

while working on cyanobacterial to UV-B radiations. The increase in dark respiration can be explained by additional energy requirement, metabolic breakdown of the compound, and/or activation of the alternative, cyanide-insensitive, respiration (Dias, 2012)

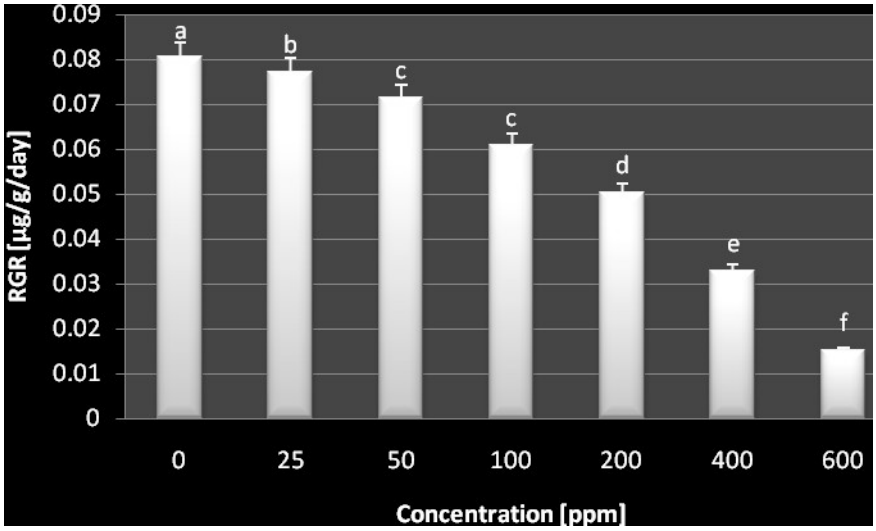

Figure 1. Effect of different concentrations of Endosulfan on Relative growth rate of *Azolla microphylla*. Data are means ± standard error of three replicates. Bars followed by different letters show significant difference at P<0.05 significance level according to Duncan's multiple range test.

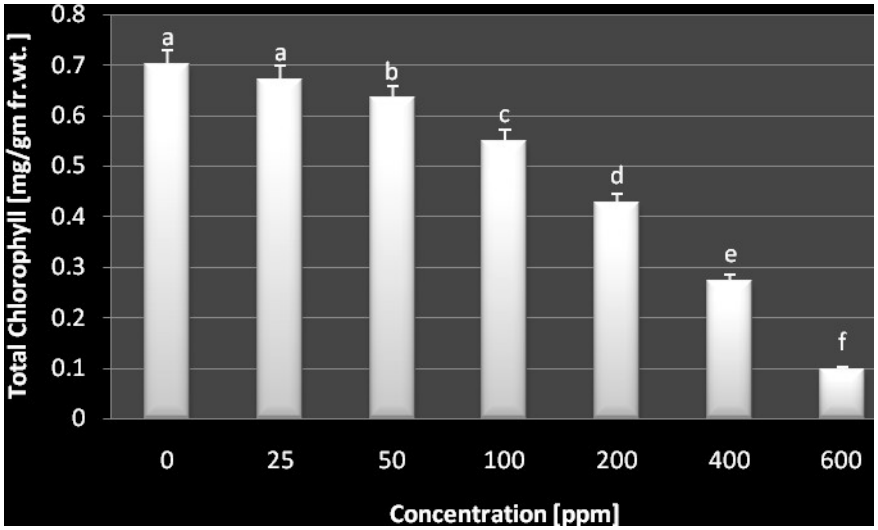

Figure 2. Effect of different concentrations of Endosulfan on total chlorophyll content of *Azolla microphylla*. Data are means ± standard error of three replicates. Bars followed by different letters show significant difference at P<0.05 significance level according to Duncan's multiple range test.

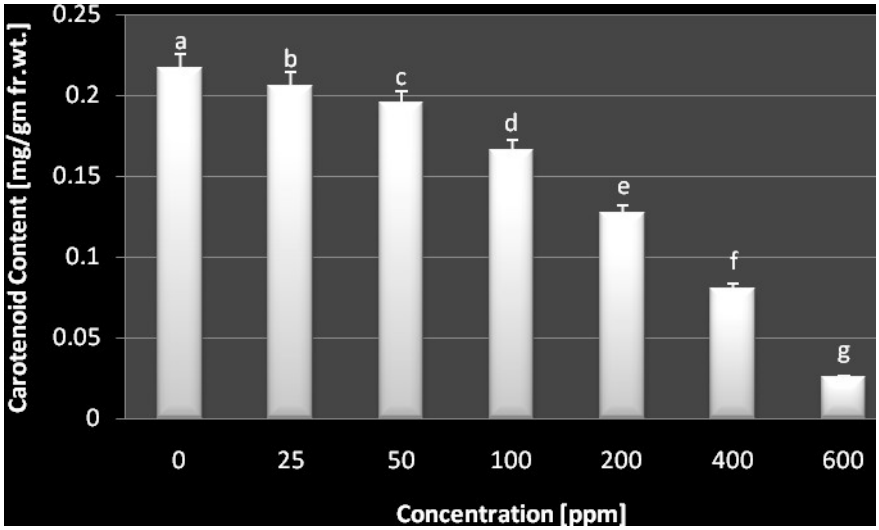

Figure 3. Effect of different concentrations of Endosulfan on carotenoid content of *Azolla microphylla*. Data are means ± standard error of three replicates. Bars followed by different letters show significant difference at P<0.05 significance level according to Duncan's multiple range test.

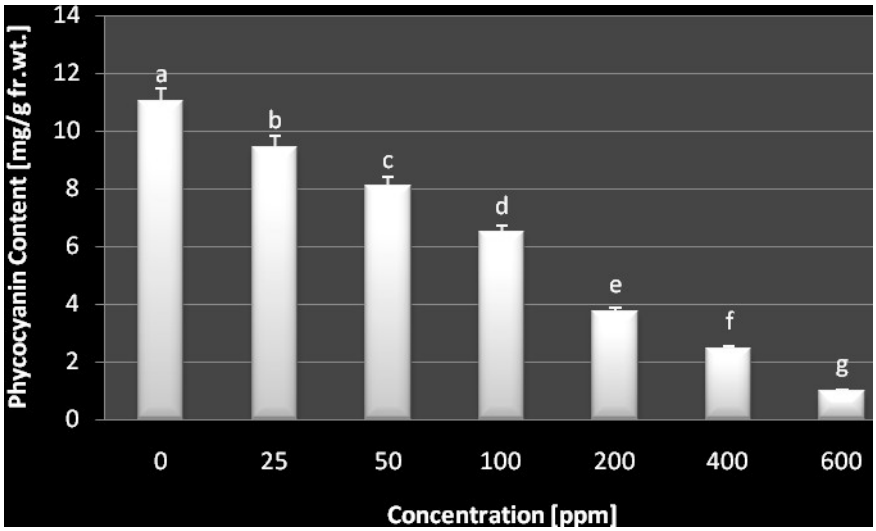

Figure 4. Effect of different concentrations of Endosulfan on Phycocyanin content of *Azolla microphylla.* Data are means ± standard error of three replicates. Bars followed by different letters show significant difference at P<0.05 significance level according to Duncan's multiple range test.

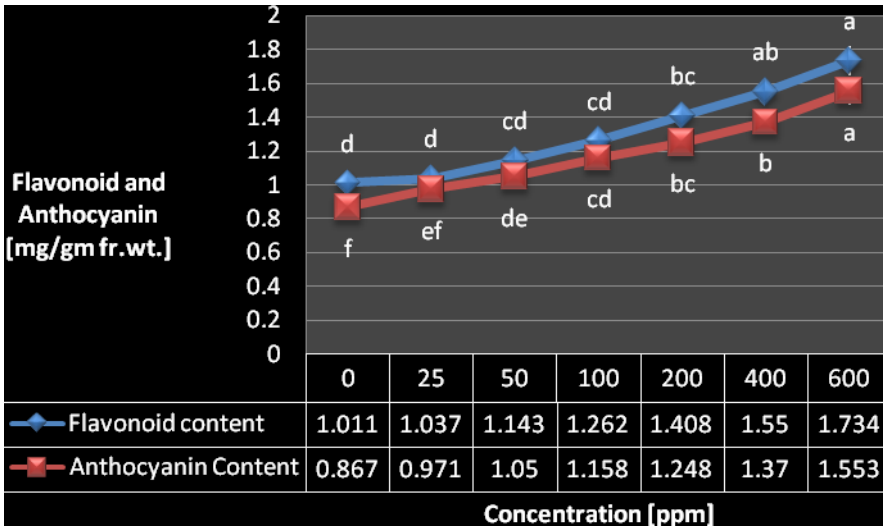

Figure 5. Effect of different concentrations of Endosulfan on Flavonoid and Anthocyanin content of *Azolla microphylla*. Data are means ± standard error of three replicates. Bars followed by different letters show significant difference at P<0.05 significance level according to Duncan's multiple range test.

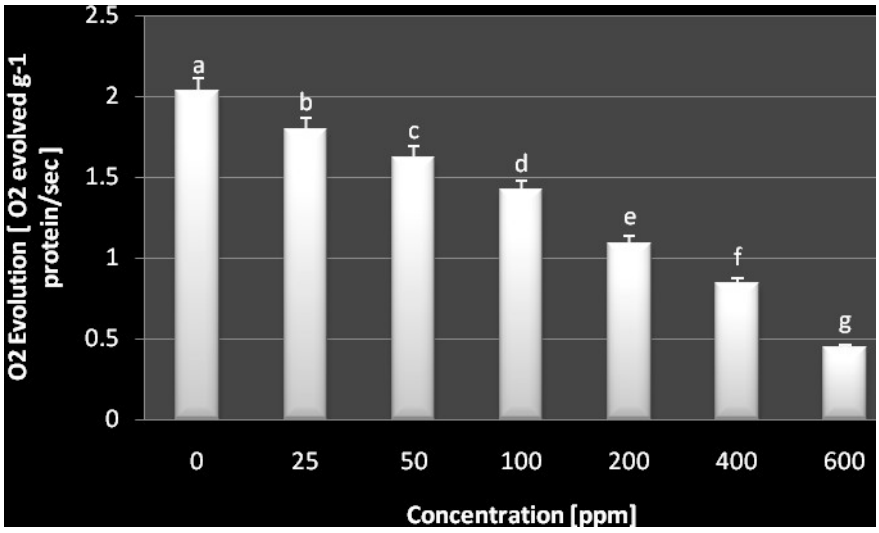

Figure 6. Effect of different concentrations of Endosulfan on Flavonoid and Anthocyanin content of *Azolla microphylla*. Data are means ± standard error of three replicates. Bars followed by different letters show significant difference at P<0.05 significance level according to Duncan's multiple range test.

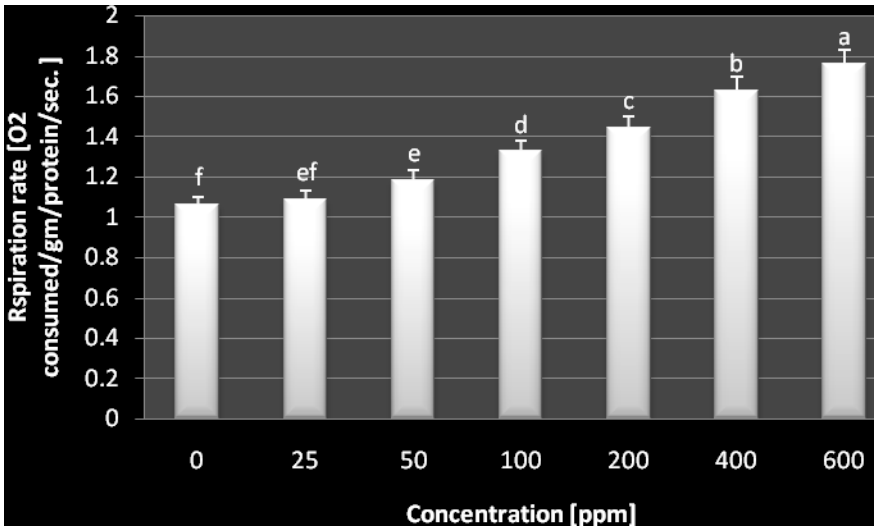

Figure 7. Effect of different concentrations of Endosulfan on respiration rate of *Azolla microphylla*. Data are means ± standard error of three replicates. Bars followed by different letters show significant difference at P<0.05 significance level according to Duncan's multiple range test.

CONCLUSION

The literature on the effects of pesticides on Azolla microphylla from rice fields is abundant but dominated by laboratory experiments whose results cannot be extrapolated to field conditions. Pesticides at normally recommended field rates and intervals are seldom deleterious to the beneficial and Eco friendly *Azolla microphylla* and their activities. Our experiments show that Endosulfan affect *Azolla microphylla* and caused significant reduction in growth rate, contents of photosynthetic pigments and photosynthetic O_2 evolution. In contrast to this respiration rate, flavonoids and Anthocyanin activities were enhanced, thus Azolla microphylla could be used as an early indicator of pesticide pollution. Additionally, the chlorophyll and photosynthetic activity tests may be used as physiological indicators' to understand in part, the main mode of action of insecticides on the photosynthetic apparatus of *Azolla* ferns. Thus, future investigation on the subject should be considered in order to produce more reliable data to identify insecticide photosynthetic targets and build a comprehensive model of the physiological response of plant exposed to insecticides.

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