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Original Paper

Effect of phenol on biochemical status of an aquatic fern *Salvinia natans*

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Abstract

Phenols represent an organic group of toxic compounds, disposed of directly or indirectly into water bodies form agricultural and industrial sectors, causing critical impact on aquatic flora and fauna. *Salvinia natans*, an aquatic fern widely identified as a weed and known for its excellent ability to accumulate heavy metals and organic compounds, was selected for this study. In the present study, the ability of *S. natans* to withstand the toxic effects of phenol was assessed in terms of physiological and biochemical changes. Relative water content increased or remained stable up to 150 µM phenol even after 10 days. Accumulation of osmolytes like soluble sugars and proline increased with phenol toxicity, thereby suggesting a resistance mechanism. Increase of activity of antioxidative enzymes up to 100 µM phenol was also observed. Most importantly, there were no visible lesions indicating phenol toxicity on the plant tissues even after 10 days of treatment, which confirmed the phenol stress tolerance of this plant.

Key words: antioxidants, osmolytes, phenol, *Salvinia natans*, weed, water pollution.
Abbreviations: APX, ascorbate peroxidase; CAT, catalase; FW fresh weight; OD, optical density; POX, peroxidase; PVPP, polyvinylpolypyrrolidone; ROS, reactive oxygen species; RWC, relative water content; TW, turgid weight.

Introduction

Water is one of the fundamental components for the existence of life on earth. Any sort of changes in water quality directly influences all life forms. Agricultural and industrial wastes are recognized as major water pollutants, particularly in the form of pesticides, nutrients (phosphorus, sulphur and ammonium), heavy metals (Pb, Hg, Cr, Cu, Cd, and Zn), and various petrochemicals etc. (Hu, Cheng 2013). Low concentrations of these pollutants have effect on ecosystems, which can be treated to a certain extent, but an overload of these pollutants imparts critical and non-restorable damaging effect to ecosystems (Cooper 1993). Among the wide range of water pollutants, phenols play a notable role in polluting water bodies due to their significant presence in agricultural, domestic, industrial wastes, and several organic decaying sources (Anku et al. 2017). Petrochemicals and pharmaceutical industries, photography, textiles, sewage, paper, dye, metal, insecticides, pesticides and explosive production industries also discharge phenolic derivatives into river water (Bruce et al. 1987; Mahamuni, Pandit 2006).

Phenol, also known as carbolic acid (generic name: monohydroxy benzene), belongs to a class of organic compounds containing a hydroxyl group (one or more), generally forms colourless solid crystals at 25 °C, and it is hygroscopic, with a sweet acrid odour, melting temperature of 43 °C and boiling temperature of 182 °C at normal pressure (Bruce et al. 1987). Some common derivatives of phenols, such as chlorophenols, catechol, methoxyphenols, monoterpenes etc., are present in byproducts of petrochemicals and pharmaceutical industries (Michałowicz, Duda 2005). The US Environment Protection Agency has listed phenolic compounds into the priority list of pollutants (Mahamuni, Pandit 2006).

A number of studies have reported the effect of phenolic compounds on aquatic flora and fauna and also strategies for the removal of such harmful and hazardous water polluting compounds. The toxicity of phenolic derivatives has been studied on some macroalgae and spermatophytes like *Chara* sp., *Nitella* sp., *Elodea canadensis, Vallisneria spiralis, Lemna gibba, Lemna obscura*, and *Lemna minor* (Stom, Roth 1981; Einhellig et al. 1985; Ramirez Toro et al. 1988; Christen, Theuer 1996). Macrophytes have been known to actively participate in the conversion of inorganic to organic carbon sources, maintenance of aquatic ecosystems, removal of phosphorous, nitrogen, suspended solids, heavy metals and other trace elements from waste water (Gupta 1980; Dhote 2007). These aquatic macrophytes also control the productivity of zooplankton (Stansfield et al. 1997), aquatic invertebrates by providing diversified habitats and ultimately increase fish production (Pennak 1971; Venugopal, Winfield 1993). Macrophytes are also used as a bio-indicators to detect pollutants, and

in treatment processes to improve water quality (Eullaffroy, Vernet 2003; Rodrigues et al. 2016). Heavy metal biomonitoring has utilized macrophytes such as *Juncus* sp. (for Zn) and *Typha* sp. (for Ni and Cd), and acidification in river water is indicated by some liverworts (Tremp, Kohler 1995; Ladislas et al. 2012).

Salvinia natans is an aquatic macrophyte, considered as one of the worst weeds due to its prolific growth rate and worldwide distribution. This plant has been reported to exhibit excellent capability to accumulate and remove many inorganic and organic compounds from water bodies (Henry-Silva, Camargo 2008; Soares et al. 2008). *Salvinia auriculata* showed sensitivity under cadmium stress and tolerance to moderate salinity, thereby playing the role of an ecological indicator (Gomes et al. 2011; Rodrigues et al. 2016). Accumulation of chromium and aluminium in tissues of *S. natans* induced cellular damage and death, but *S. natans* showed some detoxification capability due to the presence of an antioxidative system and higher H^+/ATPase activity during a stressed condition (Dhir et al. 2009; Mandal et al. 2013).

Extensive research has been done on phenols as water pollutants and its toxicity has been studied, but there is lack of information regarding the physio-biochemical changes in aquatic weeds to phenol exposure. Therefore, the present study was focused on the evaluation of physiochemical reactions of *S. natans* plants subjected to different concentration of phenol. This study therefore provides an insight into the response of *S. natans* to water pollutants like phenols.

Materials and methods

Collection and culture of plant samples

Salvinia natans (L.) All., a free-floating aquatic fern, was chosen as the plant material. The plants were collected from a pond adjacent to the University of North Bengal campus during the May and June, 2018 and maintained in the Departmental water reservoir with optimum nutrient conditions (rock phosphate, magnesium chloride and potassium nitrate in the ratio of 20:3:1 g L^{-1} water) at 25 to 30 °C.

Experimental setup and treatment

Different concentrations of crystalline phenol were prepared in 0.1X Hoagland solution (Hoagland, Arnon 1950). Four different concentrations of phenol solutions (50, 100, 150 and 200 µM) were prepared along with water as a control. Approximately 5 g plant samples were then transferred into each of three replicates for each treatment and kept in ideal growth conditions throughout experiment.

Relative water content

Relative water content (RWC) was estimated according to the protocol of Barr and Weatherley (1962). Fresh leaf and root samples were taken from treatments, and weighted to obtain fresh weight (FW). Samples kept in fully moisture condition were weighed to obtain turgid weight (TW) and then dried at 80 °C to determine the dry weight (DW). RWC was calculated by the following equation:

$$RWC = (FW - DW) / (TW - DW) \times 100 \ (\%).$$

Ascorbate concentration

To extract ascorbate, 6% trichloroacetic acid was used and the filtrate was collected (Mukherjee, Choudhuri 1983). To estimate ascorbate, the spectrophotometric method with reagent 2% dinitrophenyl hydrazine was used. A standard curve of ascorbate was used to calculate concentrations.

Proline concentration

Proline extraction was done by 3% sulfosalicylic acid as described by Bates et al. (1973). The main reagent contained ninhydrin solution and the spectrophotometric absorbance was measured at 520 nm. To quantify proline, a standard curve of L-proline was used.

Total soluble sugar and reducing sugar concentration

The standard protocol of Harborne (1998) was applied to extract total soluble sugar and reducing sugar with 95% ethanol. Total soluble sugar was estimated by anthrone reagent following the protocol given by Plummer (1978). Reducing sugar concentration was quantified by Nelson-Somogyi method described by Marais et al. (1966) and the absorbance was measured in a spectrophotometer at 515 nm using a standard curve of D-glucose.

Chlorophyll and carotenoid concentration

Extraction of chlorophyll and carotenoids was performed with 100% methanol. Estimation of chlorophyll and carotenoids was made by measuring the absorbance at 470 nm, 652.4 nm and 665.2 nm in a spectrophotometer. Calculation was done by the formula given by Lichtenthaler (1987):

$$\text{Chlorophyll } a = 16.72 \times A_{665.2} - 9.16 \times A_{653.4} \ (\mu g \ mL^{-1});$$

$$\text{Chlorophyll } b = 34.09 \times A_{652.4} - 15.28 \times A_{665.2} \ (\mu g \ mL^{-1});$$

$$\text{Carotenoids} = (1000 \times A_{470} - 1.63 \times \text{Chl } a - 104.96 \times \text{Chl } b) / 221 \ (\mu g \ mL^{-1}).$$

Total phenol and ortho-dihydroxy phenol concentration

For the extraction of phenols, 80% ethanol was used following the protocol of Mahadevan and Sridhar (1982). Total phenol was quantified by using 20% Na_2CO_3 and Folin-Ciocalteau reagent (Bray, Thorpe 1954). Estimation of ortho-dihydroxy phenol was done by the method given by Arnows (1937).

Estimation of proteins and antioxidative enzymes

Quantification of soluble protein was done by Lowry`s method (Lowry et al. 1951). Sodium phosphate buffer (50 mM, pH 7.2) and polyvinylpolypyrrolidone (PVPP) under

ice cold conditions were used to extract protein from plant tissues followed by centrifugation at 10 000 rpm at 4 °C for 15 min. The supernatant obtained was used as the crude extract for measurement. Alkaline-copper tartarate solution and Folin-Ciocalteau reagent was reacted with the protein samples and the absorbance was taken in a UV-VIS spectrophotometer at 660 nm. Quantification was done using a standard curve of bovine serum albumin.

For extraction of antioxidative enzymes, plant samples were crushed in a mortar-pestle in ice cold condition using liquid nitrogen. Sodium phosphate buffer (0.05 M, pH 7.2) was used for ascorbate peroxidase (APX), and 0.05 M sodium phosphate buffer (pH 6.8) for catalase (CAT) and peroxidase (POX) along with PVPP. Additionally, for extraction of APX, buffer also consisted of freshly prepared 1 mM ascorbate. Then the homogenates were centrifuged at 10 000 rpm for 15 min at 4 °C. Supernatants were used as crude enzyme extracts and estimation of protein content was done in each case following Lowry`s method as described earlier.

CAT (EC 1.11.1.6) activity was assayed according to the protocol of Machly and Chance (1954) on the basis of the decomposition of H_2O_2 at 240 nm by the enzyme causing a change in absorbance. The absorbance was noted immediately at specific intervals up to 3 min in a UV-VIS spectrophotometer. The CAT activity was expressed as ΔOD mg^{-1} protein min^{-1}.

POX (EC 1.11.1.7) activity was determined on the basis of the oxidation of o-dianisidine in the presence of H_2O_2 at 460 nm (Chakraborty et al. 1993). The change in absorbance was recorded at specific intervals up to 3 min and expressed as ΔOD mg^{-1} protein min^{-1}.

APX (EC 1.11.1.11) activity was assayed following the method of Asada and Takahashi (1987) on the basis of the decrease in absorbance at 290 nm resulting from the oxidation of ascorbate. Change in absorbance of the reaction mixture consisting of freshly prepared L-ascorbate and H_2O_2, was measured immediately at specific intervals up to 3 min in a UV-VIS spectrophotometer and expressed as ΔOD mg^{-1} protein min^{-1}.

All antioxidative enzyme activities were also calculated by the following equation:

$$\text{Activity} = \Delta\text{OD} \times \text{Dilution factor} \times \text{Final volume} / \text{Amount of protein} \times \text{Time}.$$

Statistical analysis

All the analyses were done in a completely randomized design with three biological replicates. For comparison, groups were subjected to one-way analyses of variance (ANOVA) followed by post hoc analysis using the Duncan's test at $p \leq 0.05$.

Results

Relative water content

The relative water content initially showed a significant increase in 50 and 100 µM phenol treated plants, but a significant decrease was observed for 150 and 200 µM treated plants in comparison to the control after 5 days of treatment at $p < 0.05$ (Table 1). An increase in RWC by 12% was observed at 100 µM concentration after 5 days of treatment. Similarly, an increase of 4% in the case of 50 µM concentration after 10 days of treatment in comparison to the control was observed. At a concentration of 200 µM, significant decrease in the order of 4 and 9% was observed after 5 and 10 days of treatment respectively.

Ascorbate and proline concentration

After 5 days of phenol treatment the ascorbate concentration significantly decreased for all treatments of plants when compared to the control (Table 2). Maximum decrease of 44% was observed in 150 µM and minimum decrease of 27% was observed in both 50 and 100 µM phenol treatment compared to the control. After 10 days of treatment ascorbate concentration significantly increased for all phenol treatments when compared to the control at $p < 0.05$ (Table 2). Highest ascorbate accumulation (by 72%) was observed in 100 µM of phenol concentration compared to the control. After 10 days of treatment, however, accumulation of ascorbate in the plants significantly decreased in comparison to that at 5 days.

Highest increase in proline concentration was observed at 200 µM phenol concentration (21% increase), after 5 days (Table 2). After 10 days, accumulation of proline significantly increased by 78% and 59% respectively in 50 and 100 µM treated plants, but a significant decrease was observed at higher phenol concentrations in comparison to the control. Also, proline concentration was found to significantly increase along with increasing phenol

Table 1. Effect of different concentrations of phenol on relative water content and protein concentration. Values represent mean ± SD. Different letters in the column indicates significant differences in comparison between treatments determined by one-way ANOVA and post hoc analysis by Duncan's test at $p < 0.05$

Phenol (µM)	Relative water content (%)		Protein (mg g^{-1} FW)	
	Day 5	Day 10	Day 5	Day 10
0	85.87 ± 2.55 B	85.05 ± 1.04 b	0.676 ± 0.01 C	0.500 ± 0.030 b
50	90.00 ± 1.30 C	88.17 ± 1.13 b	1.115 ± 0.010 E	0.700 ± 0.017 c
100	96.09 ± 0.94 D	86.17 ± 0.83 b	0.595 ± 0.010 B	0.805 ± 0.010 d
150	85.33 ± 1.78 AB	87.93 ± 2.65 b	0.420 ± 0.014 A	0.350 ± 0.018 a
200	82.46 ± 0.99 A	78.23 ± 1.99 a	0.760 ± 0.022 D	0.360 ± 0.020 a

Table 2. Effect of different concentrations of phenol on ascorbate and proline concentration. Values represent mean ± SD. Different letters in the column indicates significant differences in comparison between treatments determined by one-way ANOVA and post hoc analysis by Duncan's test at $p < 0.05$

Phenol (μM)	Ascorbate (mg g^{-1} FW)		Proline (mg g^{-1} FW)	
	Day 5	Day 10	Day 5	Day 10
0	1.50 ± 0.07 C	0.55 ± 0.02 a	0.224 ± 0.010 B	0.304 ± 0.005 c
50	1.10 ± 0.04 B	0.80 ± 0.07 bc	0.192 ± 0.008 A	0.544 ± 0.009 e
100	1.10 ± 0.02 B	0.95 ± 0.08 d	0.224 ± 0.011 B	0.484 ± 0.006 d
150	0.85 ± 0.05 A	0.90 ± 0.04 cd	0.192 ± 0.009 A	0.242 ± 0.006 a
200	1.35 ± 0.07 C	0.70 ± 0.04 b	0.272 ± 0.012 C	0.272 ± 0.013 b

concentration when the plants were treated for longer duration (10 days) at 150 μM.

Total soluble sugar and reducing sugar concentration

After 5 days of treatment, the total soluble sugar concentration increased by 18% in both 100 and 150 μM phenol treated plants, which was significant at $p < 0.05$ (Table 3). However, significant decrease in total soluble sugar concentration by 13% in both 50 μM and 200 μM treatment after 5 days was observed when compared to control plants. After 10 days of treatment, significant increase in soluble sugar concentration by 18% in 100 μM and 16% in 150 μM phenol treated plants was recorded.

Reducing sugar concentration decreased (no statistical significance) in all the phenol treated plants after 5 days of treatment (Table 3). Highest decrease in reducing sugar concentration by 17% in 50 μM phenol treated plants was observed after 5 days of treatment. Reducing sugar concentration after 10 days significantly decreased at $p < 0.05$ in all the treatments except at 50 μM concentration. Highest decrease in reducing sugar concentration was observed in the 200 μM treatment (58%) when compared to control plants.

Chlorophyll and carotenoid concentration

Amount of chlorophyll *a* significantly decreased in 50, 100 and 150 μM and increased (by 12%) in 200 μM phenol treated plants after 5 days (Table 4). Chlorophyll *b* concentration on the other hand significantly decreased at $p > 0.05$ in all plants; maximum reduction of 35% was observed in 200 μM phenol treated plants after 5 days. Carotenoid concentration significantly increased by 40% in 100 μM and decreased at 50 and 150 μM phenol concentrations compared to the control after 5 days of treatment.

After 10 days of treatment, chlorophyll *a* concentration significantly increased by 3.5% in 50 μM and 21% in 100 μM treatments (Table 4). Similarly, chlorophyll *b* concentration increased significantly by 2% in 50 μM and 23% in 100 μM phenol treated plants after 10 days. After

Table 3. Effect of different concentrations of phenol on total soluble sugar and reducing sugar concentration. Values represent mean ± SD. Different letters in the column indicates significant differences in comparison between treatments determined by one-way ANOVA and post hoc analysis by Duncan's test at $p < 0.05$

Phenol (μM)	Total soluble sugar (mg g^{-1} FW)		Reducing sugar (mg g^{-1} FW)	
	Day 5	Day 10	Day 5	Day 10
0	1.60 ± 0.13 AB	1.60 ± 0.11 b	1.50 ± 0.14 A	1.05 ± 0.14 bc
50	1.40 ± 0.09 A	1.40 ± 0.12 a	1.25 ± 0.19 A	1.10 ± 0.12 c
100	1.90 ± 0.13 B	1.90 ± 0.14 c	1.35 ± 0.26 A	0.80 ± 0.11 b
150	1.90 ± 0.11 B	1.85 ± 0.14 c	1.45 ± 0.21 A	0.85 ± 0.14 bc
200	1.40 ± 0.12 A	1.38 ± 0.12 a	1.30 ± 0.15 A	0.45 ± 0.15 a

Table 4. Effect of different concentrations of phenol on chlorophyll *a*, chlorophyll *b* and carotenoid concentration.Values represent mean ± SD. Different letters in the column indicates significant differences in comparison between treatments determined by one-way ANOVA and post hoc analysis by Duncan's test at $p < 0.05$

Phenol (μM)	Chlorophyll *a* (mg g^{-1} FW)		Chlorophyll *b* (mg g^{-1} FW)		Carotenoids (mg g^{-1} FW)	
	Day 5	Day 10	Day 5	Day 10	Day 5	Day 10
0	0.342 ± 0.014 D	0.512 ± 0.016 b	0.208 ± 0.020 C	0.278 ± 0.060 a	0.063 ± 0.007 A	0.087 ± 0.046 a
50	0.322 ± 0.012 C	0.531 ± 0.002 b	0.183 ± 0.013 AB	0.283 ± 0.022 a	0.043 ± 0.007 A	0.109 ± 0.007 a
100	0.292 ± 0.007 B	0.648 ± 0.025 c	0.170 ± 0.040 AB	0.361 ± 0.035 b	0.105 ± 0.023 B	0.124 ± 0.020 a
150	0.268 ± 0.012 A	0.497 ± 0.021 b	0.140 ± 0.025 A	0.270 ± 0.022 a	0.051 ± 0.013 A	0.091 ± 0.021 a
200	0.382 ± 0.009 E	0.440 ± 0.019 a	0.137 ± 0.020 A	0.242 ± 0.043 a	0.068 ± 0.001 A	0.097 ± 0.003 a

Table 5. Effect of different concentrations of phenol on total soluble sugar and reducing sugar concentration. Values represent mean ± SD. Different letters in the column indicates significant differences in comparison between treatments determined by one-way ANOVA and post hoc analysis by Duncan's test at $p < 0.05$

Phenol (μM)	Total phenol (mg g^{-1} FW)		o-Dihydroxy phenol (mg g^{-1} FW)	
	Day 5	Day 10	Day 5	Day 10
0	4.00 ± 0.43 A	9.00 ± 0.17 a	0.95 ± 0.02 A	2.65 ± 0.06 c
50	9.00 ± 0.26 C	10.00 ± 0.31 a	2.35 ± 0.04 D	2.85 ± 0.04 d
100	8.50 ± 0.36 C	9.00 ± 0.52 a	2.60 ± 0.08 E	2.60 ± 0.11 c
150	13.50 ± 0.36 D	9.50 ± 0.17 a	2.20 ± 0.04 C	2.35 ± 0.03 b
200	7.00 ± 0.45 B	9.50 ± 0.26 a	2.00 ± 0.11 B	2.20 ± 0.10 a

10 days of treatment carotenoid concentration increased in all treatment and the highest increase (with no statistical significance), by 30%, was in 100 μM phenol treated plants.

Total phenol content and o-dihydroxy phenol concentration

Total phenolic concentration significantly increased for all four treatments, compared to control plants, after 5 days (Table 5). Highest accumulation of total phenolics was 337% in the 150 μM phenol treatment. Similarly, after 10 days of treatment, total phenolic concentration increased in three treatments (50, 150 and 200 μM). The highest increase of 11% was observed in 50 μM phenol treated plants after 10 days of treatment.

After 5 days of treatment, o-dihydroxy phenolic concentration was also observed to be significantly higher in all the four treatment plants after 5 days (Table 5). Maximum accumulation of o-dihydroxy phenol, by 173%, was observed in the 100 μM phenol treatment. However, after 10 days of treatment, o-dihydroxy phenolic concentration was found to decrease in all the treatments, except in the 50 μM treatment (significant increase of 7%). Highest decrease in o-dihydroxy phenolic concentration by 17% was observed in 200 μM treated plants after 10 days of treatment.

Total soluble protein and antioxidative enzyme activity

Protein concentration increased significantly by 64% in 50 μM and 12% in 200 μM phenol treated plants in comparison to control plants after 5 days of treatment (Table 1). However, in the case of 100 μM and 150 μM phenol concentration, protein concentration significantly decreased at $p < 0.05$, by 12% and 38%, respectively. After 10 days of treatment, protein concentration significantly increased by 40% in 50 μM and 60% in 100 μM concentrations in comparison to the control. Protein concentration in general decreased in all treatments after 10 days of treatment when compared to that of 5 day treated plants.

CAT activity was lower in all treatments except 150 μM (31% enhancement) concentration after 5 days of phenol treatment (Table 6). Maximum reduction of 58% in CAT activity was observed in the 50 μM phenol treatment after 5 days. However, after 10 days of treatment, significant change was observed only at 150 μM (16% increase). After phenol treatment, CAT activity significantly increased at 10 days compared to 5 day treated plants.

After 5 days of treatment, POX activity was found to be significantly higher by 41%, 173% and 13% in 100, 150 and 200 μM phenol treated plants respectively (Table 6). However, a significant reduction (by 62%) in POX activity was found in 50 μM phenol treatments after 5 days of treatment compared to the control. After 10 days of treatment, maximum increase up to 43% was observed with no statistical significance at $p < 0.05$ in the case of 50 μM phenol treated plants compared to control plants (Table 6).

APX activity was found to be increased significantly at $p < 0.05$ by 14% and 38% respectively in 100 and 150 μM phenol treated plants after 5 days (Table 6). Significant reduction (by 50% and 20%) of in APX activity was

Table 6. Effect of different concentrations of phenol on antioxidant enzyme activity. CAT, catalase; POX, peroxidase; APX, ascorbate peroxidase. Values represent mean ± SD. Different letters in the column indicates significant differences in comparison between treatments determined by one-way ANOVA and post hoc analysis by Duncan's test at $p < 0.05$

Phenol (μM)	CAT (ΔOD min^{-1} mg^{-1} protein)		POX (ΔOD min^{-1} mg^{-1} protein)		APX (ΔOD min^{-1} mg^{-1} protein)	
	Day 5	Day 10	Day 5	Day 10	Day 5	Day 10
0	0.00041 ± 0.0001 A	0.00055 ± 0.0001 ab	0.0074 ± 0.0027 AB	0.0035 ± 0.0010 a	0.0026 ± 0.0007 AB	0.0040 ± 0.0016 bc
50	0.00017 ± 0.0001 A	0.00032 ± 0.0001 a	0.0028 ± 0.0008 A	0.0050 ± 0.0027 a	0.0013 ± 0.0004 A	0.0021 ± 0.0008 b
100	0.00034 ± 0.0001 A	0.00025 ± 0.0001 a	0.0118 ± 0.0023 B	0.0025 ± 0.0010 a	0.0023 ± 0.0008 AB	0.0019 ± 0.0007 a
150	0.00054 ± 0.0005 A	0.00064 ± 0.0004 b	0.0202 ± 0.0062 C	0.0043 ± 0.0016 a	0.0036 ± 0.0023 C	0.0043 ± 0.0016 c
200	0.00028 ± 0.0001 A	0.00042 ± 0.0002 ab	0.0083 ± 0.0027 AB	0.0035 ± 0.0013 a	0.0021 ± 0.0008 AB	0.0049 ± 0.0013 c

also observed at 50 and 200 μM phenol concentrations, respectively, after 5 days. After 10 days of treatment, APX activity was found to be significantly increased by 7% in 150 and 21% in 200 μM treated plants (Table 6). Also, APX activity was found to be significantly higher after 10 days of treatment when compared to that of 5 day treated plants.

Discussion

Phenol phytotoxicity considerably affects plant growth. A previous study on duckweed (*Lemna gibba*) showed negative effects on the morphology and vegetative growth at 0.08 mM and 0.54 mM concentrations of phenols (Barber et al. 1995; Sharma et al. 1997). However, in *S. natans*, no effects in terms of morphological changes were observed, which implies that morphology alone cannot establish the degree of tolerance, for which other physiological and biochemical parameters are required to be taken into consideration.

Ascorbate, an essential metabolite, plays a crucial role in cellular functions, and thus plants tend to accumulate it during stress in both photosynthetic and non-photosynthetic tissues. Ascorbate functions as both primary and secondary antioxidants, it eliminates ROS, plays a role in photoprotection, and preserves activity of enzymes (Noctor, Foyer 1998). At higher concentration of phenol (150 μM), accumulation of ascorbate in *S. natans* significantly increased after 10 days of treatment, suggesting its protective response to phenol stress. This type of increase in ascorbate concentration has been observed in cases of heavy metal stress in bean seedlings (up to 14, 15, 17 and 19% increase for Pb, Cu, Cd and Hg stress, respectively), pointing towards a similar mechanism of combating phenol stress in *S. natans* (Kirbag Zengin, Munzuroglu 2005).

It has been emphasized that proline act as an excellent osmolyte under a wide range of abiotic and environmental stresses, and in addition functions as a signaling molecule, metal chelator and as an antioxidative defense molecule (Abdul Jaleel et al. 2007; Misra, Gupta 2005; Yang et al. 2009). In the present study, proline accumulation at higher phenol concentrations was observed after 5 days of treatment, indicating the activation of a stress-responsive mechanism in *S. natans*. This is in agreement with results from another study where the exposure of soybean plants to bisphenol-A induced increased accumulation of proline in roots (Zhang et al. (2016).

Accumulation of soluble sugars and reducing sugars under stressed conditions also function as a solute, which helps in water influx and maintaining cellular turgidity, improving cellular osmolarity under stressed conditions (Hare et al. 1998). Total soluble sugar concentration was also found to increase at higher phenol concentration (≥ 100 μM) after 5 days of exposure. However, with increasing phenol concentration, reducing sugar concentration decreased, indicating the reduced metabolism of the sugars in response to phenol stress.

Chlorophyll *a* and *b* concentration of *S. natans* plants decreased after 5 days of phenol exposure. Similar decrease in the concentration of chlorophyll *a* and *b* concentration was reported in *S. natans* on exposure to glyphosate and copper (Liu et al. 2019). However, in the current study, when the plants were exposed to phenol for longer duration, chlorophyll a and b concentration increased in plants treated by up to 100 μM phenol, indicating a higher tolerance level. Also, several studies have reported the reduction of chlorophyll *a*/*b* ratio due to environmental stresses. Decrease in chlorophyll *a*/*b* ratio in mung bean plants subjected to heavy metal stress was reported (Tewari et al. 2002). However, in our study it was observed that the chlorophyll *a*/*b* ratio increased after 5 days of treatment with increasing phenol concentrations, which remained unchanged after 10 days of treatment. Also, it has been acknowledged that carotenoid concentration tends to decrease in response to environmental stresses, for instance in case of *Vicia faba* and *Zea mays* plants subjected to salinity stress (Gadallah 1999; Singh et al. 2008). Under increasing phenol concentration, we also observed that the carotenoid concentration decreased after 5 days of phenol stress, but after 10 days of treatment carotenoid concentration increased up to 100 μM concentration compared to the control, indicating relative tolerance to phenol stress. Also, accumulation of phenol and o-dihydroxy phenol significantly increased for all phenol treatments after 5 days and almost remained the same after 10 days of treatment.

Protein concentration generally increased due to moderate phenol treatment after 5 days and 10 days, but was found to decrease at higher phenol concentration. Previous studies on another aquatic plant, *Spirodela polyrrhiza*, exposed to arsenic and cadmium stress have shown a similar trend in protein concentration (Seth et al. 2007).

Under biotic and abiotic stress, plants produce an excessive amount of reactive oxygen species (ROS), which is involved in DNA damage and cytotoxicity. During stress condition a complex antioxidant system protects cellular organelles by scavenging ROS in plants (Noctor, Foyer 1998). The stress tolerance of any plant depends on the improved activities of its antioxidant enzymes, irrespective of the biosynthesis of proteins. In this study, we evaluated the activities of CAT, POX and APX in *S. natans* plants exposed to phenol stress. Among the antioxidative enzymes, CAT is considered as one of most important H_2O_2 scavenging enzymes (de Azevedo Neto et al. 2005). In this study, with increasing phenol concentrations, activity of CAT increased at 150 μM concentration and was lower at highest concentration of phenol after 5 and 10 days of treatments. Similar findings were reported in *Solanum lycopersicum* subjected to nonylphenol exposure (Jiang et al. 2019). Enhanced CAT activity is associated with the reduction of ROS content in celsl, thereby providing cellular

stability (Esfandiari et al. 2007). In our study, POX activity mainly increased at higher phenol treatments (≥ 150 μM) after 5 days. Similar fluctuations in POX activity was reported in a study involving the effect of heavy metal stress in *Kandelia candel* and *Bruguiera gymnorrhiza* (Zhang et al. 2007). Similarly, APX activity was found to be increased at higher phenol concentration after 5 days of treatment. Also, comparatively higher APX activity was observed after 10 days of treatment. Similar observations have been reported in *S. natans* plants after exposure to glyphosate and copper (Liu et al. 2019). The increase of activity of antioxidative enzymes like CAT, POX and APX in *S. natans* could therefore be correlated with the better management of phenol stress contributing to the scavenging of ROS.

Conclusion

Salvinia natans is a free floating aquatic fern growing proliferously in water bodies. In the present study, the plant showed a high degree of stress tolerance to varying degree of stress imparted by phenol. The resistance of the plant could be initially suggested from the morphological status of the plant, which almost remain unaltered after 10 days of exposure to phenol stress. RWC content of the plant also showed low decline at high concentrations. Accumulation of phenol and soluble sugars at the initial stages of phenol exposure and the increase in the activities of antioxidative enzymes in plant tissues could be correlated to the unaltered morphological status of the plants subjected to phenol stress. Therefore, we can conclude that the plant is comparatively tolerant to phenol stress and further studies at the molecular level is essential for elucidating the mechanism of the stress tolerance in detail.

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