Fluoride toxicity triggered oxidative stress and the activation of antioxidative defence responses in *Spirodela polyrhiza* L. Schleiden

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**ABSTRACT**

The present study was designed to assess the fluoride mediated antioxidant defence responses in *Spirodela polyrhiza*, grown under hydroponic conditions. The plants of *S. polyrhiza* were exposed to different fluoride concentrations (0, 5, 10, 15, 20, 25, 50 ppm) for the time period 24, 72, 120 and 168 h. Fluoride was observed to be readily accumulated by *S. polyrhiza* at all the exposure periods. Increased accumulation of fluoride triggered the oxidative stress that was quite evident from enhanced electrolyte leakage, proline, anthocyanin and phenolic content. To counterbalance the stress, plant responded by the alterations in the activities of antioxidative enzymes (SOD, CAT, APOX, POD, GR), that was also supposed to confer tolerance ability to plant. Moreover, SEM-EDX, CLSM and FT-IR studies were also performed to confirm oxidative damage. Hence, it can be elucidated that this plant can act as an efficient fluoride accumulator and antioxidant defence system minimized fluoride toxicity.

**Introduction**

Inevitable and continuous anthropogenic activities such as industrial emissions, mining, smelting, metal processing, agricultural practices, etc have added a substantial amount of metals and non-metals in the aquatic environment over the past few decades. This resulted in deterioration of the aquatic environment which makes water unfit for aquatic flora and fauna. Released toxic metals and non-metals, whether essential or non-essential ones adversely affect various metabolic activities of living beings, when exceeds the permissible limits in the environment. Fluoride is one of the air, water, and soil contaminant released into the aquatic environment through various anthropogenic activities which include mining, aluminium manufacturing and use of agricultural fertilizers (EPA 1978). Fluoride occurs naturally through weathering of rocks and volcanic ashes (Mackowiak et al. 2003). Exposure of fluoride for a prolonged period, exceeding its permissible limit (>1.5 mg/L) invokes deleterious effects on plants as well as animals. The most relevant inorganic fluorides which led to deleterious impacts on the environment are hydrogen fluoride, sulfur hexafluoride, calcium fluoride, silicofluoride and sodium fluoride (Environment Health Criteria for Fluorides 2002; Sharma and Kaur 2018). Increased level of fluoride can cause fluorosis and is one of the rising global concerns in the world (Msonda et al. 2007).

Notably, among plants, the most visible symptoms of fluoride toxicity are chlorosis, necrosis and marked reduction of growth (Singh et al. 2013). Various physiological and biochemical processes influenced by fluoride toxicity include a decrease in the rate of photosynthesis (Fornasiero 2003), disruption in metabolic activities, increase in foliar lesions (Weinstein and Davison 2004) and changes in plant antioxidative activity (Chakrabarti and Patra 2013). Throughout their life cycle, plants face different abiotic and biotic stress and show response to stress conditions (Cao et al. 2013). Stress conditions stimulated reactive oxygen species (ROS) that alter various cellular activities in the plants which include inhibition of photosynthesis, lipid peroxidation, cell disruption, DNA damage and inhibition of ATP production (Ruley et al. 2004). To cope up with these stress conditions, plants have evolved efficient and complex antioxidative defence mechanisms to reduce or counteract the effects of ROS. These mechanisms include activation of numerous antioxidant enzymes such as SOD, CAT, APOX, POD and GR. Activation of the antioxidant enzyme is required to protect the plant from deleterious effects. All these key enzymes work in coordination. Primary defence role is played by SOD (superoxide dismutase) which dismutates O$_2^-$ to H$_2$O$_2$ and O$_2$. Further accumulation of H$_2$O$_2$ is prevented by the activity of CAT (catalase). Moreover, peroxidases i.e. APOX (ascorbate peroxidase) and POD (guaiacol peroxidase) asldoxetifies H$_2$O$_2$ into H$_2$O via ascorbate-glutathione cycle. Finally, GR (glutathione reductase) catalyzes the reduction of oxidized glutathione disulfide (GSSG) to reduced glutathione (GR) which requires NADPH (Noctor et al. 2002; Gill and Tuteja 2010).

Since, aquatic plants have the ability to tolerate and accumulate heavy metals by different mechanisms (Chua 1998; Maine et al. 2001). Among them, *Lemma minor*, *Lemna gibba*, *Wolffia arrhiza*, and *Azolla pinnata* are known for its potential to remove heavy metals from aquatic ecosystems. Generally, the aquatic plants employed for the purpose of phytoremediation should have enriched biomass, rapid growth rate, and potential to cope with a wide range of environmental conditions. In this context, *Spirodela polyrhiza* has many advantages over *Lemma* species, as first is a perennial aquatic plant with several minute roots, high biomass and large size (0.5–1 cm wide), while second possess single root, low biomass and size not exceeding 5 mm in length (Wang et al. 2014).
Spirodela polyrhiza, which is commonly known as giant duckweed is a free-floating aquatic macrophyte known for its phytoremediation ability for various heavy metals like zinc, arsenic, mercury, lead, etc. Their small size, rapid growth, easy to culture and manage, make them appropriate to be utilized for evaluating the toxicity of various metals, non-metals, and chemicals. Currently, very few reports are available that explain physiological, structural and alterations in antioxidant enzyme activities of S. polyrhiza under fluoride stress. Thus, the present study was undertaken to investigate the fluoride mediated oxidative stress in S. polyrhiza, its uptake and accumulation potential as well as alterations in the behavior of enzymes of the antioxidant defence system. Furthermore, the current study is supported by microscopic observations (SEM and confocal microscopy) to observe stomatal movements, cell viability, and ROS detection to gain more insights into fluoride-induced oxidative burst.

Material and methods

**Sampling site and collection of plant material**

Plant material (S. polyrhiza) was collected from Sewage Treatment Plant (STP) of Guru Nanak Dev University, Amritsar. Plants were thoroughly washed with distilled water to remove dirt or debris prior to the experimentation.

**Chemical preparation**

For the preparation of stock solution (100 ppm) of fluoride 2.21 g of anhydrous NaF was dissolved in 1000 ml of Millipore water. Further, different concentrations of fluoride (5, 10, 15, 20, 25 and 50 ppm) were prepared from a stock solution in 3% Hoagland nutrient medium (Hoagland and Arnon 1950). The Hoagland nutrient medium comprised of macro-nutrients (KNO₃, CaNO₃·2H₂O, Iron, MgSO₄·7H₂O, NH₄NO₃), micronutrients (H₃BO₃, MnCl₂·4H₂O, ZnSO₄·7H₂O, CuSO₄·5H₂O, Na₂MoO₄·2H₂O, Fe-EDTA) and phosphate (K₂HPO₄) mixed in 1000 ml millipore water. All these chemicals were of analytical grade.

**Experimental conditions**

Before giving treatments, plants were acclimatized for a period of one week under laboratory conditions in the seed germinator under controlled conditions (temperature: 25 ± 2°C; light intensity: 115 μmol/m²/s and light/dark cycle: 16/8 h). About 5 g of healthy plants of S. polyrhiza were weighed and kept in Petri plates filled with 100 ml of different fluoride concentrations which were made in Hoagland nutrient medium along with one set of control (only 100 ml Hoagland medium). The experiments were performed in triplicates. Harvesting of plant material was done after 24, 72, 120 and 168 h. Plant material was thoroughly washed, blotted to remove excess water, frozen with liquid nitrogen and then stored in a deep freezer (−80°C). Plant material was then assayed for various enzymatic activities and other parameters.

**Accumulation of fluoride**

Extraction of fluoride content from plant material was carried by using the protocol of Zhou et al. (2012) with slight modifications. Analysis of fluoride was done using ion chromatography (Metrohm Ion Chromatography, Orion-960). Plant samples were dried at 105°C for 25 min and then dried at 80°C for the next 48 h. To determine fluoride content, about 1 g of oven dried plant sample was taken in the crucible and pulverized properly. The plant material was further soaked in 3 ml of 10% Mg(NO₃)₂ (w/v) and 1 ml of 10% NaOH (w/v) for 30 min. The samples were then evaporated and incubated at 200°C in an oven. After carbonization, samples were ashed in a muffle furnace for 6 h at 550°C. Crucibles containing ashed material was finally cooled and rinsed with Millipore water. Rinses of these samples were combined and the total volume was made up to 30 ml. Fluoride content in plant samples was then analyzed using ion chromatography. The accumulation content was reported as mg/kg dry weight of the plant.

**Estimation of antioxidant enzymatic activity**

**Preparation of plant extract**

Frozen plant material (1 g) was ground in liquid nitrogen and homogenized in 3 ml of 0.1 M ice-cold potassium phosphate buffer (pH = 7) using chilled pestle and mortar. Homogenate was then centrifuged at 12000g for 20 min at 4°C. The supernatant was collected and used for the analysis of enzymatic activities. Total soluble protein contents of the enzyme extracts were measured according to the method of the Bradford (1976). Bovine serum albumin (BSA) was used as a protein standard.

**Superoxide dismutase assay** (SOD) (EC 1.15.1.1)

The SOD activity was analyzed by the method given by Kono et al. (1979). The SOD activity was done to determine the ability of the plant to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). Reaction mixture was comprised of 1.8 ml sodium carbonate (50 mM, pH = 10.2), 750 μl NBT (96 μM), 150 μl Triton X-100 150 μl hydroxylamine hydrochloride (20 mM, pH = 6.0) in a cuvette and was added. After 2 min, 20 μl of plant extract was added. Percentage inhibition in the rate of reduction of NBT was recorded at 540 nm absorbance using a spectrophotometer. Specific activity was expressed in μmol min⁻¹ mg⁻¹ protein.

**Catalase assay** (CAT) (EC 1.11.1.6)

The CAT activity was determined by the method given by Aebi (1974). The principle of this assay was to monitor the initial rate of H₂O₂ disappearance. In 3 ml reaction mixture, there were 2640 μl potassium phosphate buffer (100 mM, pH = 7), 300 μl H₂O₂ (150 mM). 40 μl of plant extract was then added in the reaction mixture. The decrease in absorbance was read at 240 nm. The activity of the enzyme was determined by using the extinction coefficient of 3.4 mM⁻¹cm⁻¹. Specific activity was expressed μmol min⁻¹ mg⁻¹ protein.

**Ascorbate peroxidase assay** (APOX) (EC 1.11.1.11)

APOX activity was done by following the protocol of Nakano and Asada (1984). APOX catalyzes H₂O₂ reduction by causing the oxidation of ascorbate. The reaction mixture contained 1.5 ml potassium phosphate buffer (0.1M, pH = 7), 300 μl ascorbate (5 mM), 600 μl H₂O₂ (0.5 mM) and 40 μl
plant extract. The decrease in absorbance was measured at 290 nm. The activity of the enzyme was determined using the extinction coefficient for ascorbate i.e. 2.8 mM cm

\^{-1} \text{mg}^{-1} \text{protein}.

**Guaiacol peroxidase assay (POD) (EC 1.11.1.7)**

POD activity was done as per the method is given by Püttér (1974). The reaction mixture contained, 1.5 ml potassium phosphate buffer (0.1 M, pH = 7), 50 μl guaiacol (20 mM), 300 μl H₂O₂ solution and 40 μl plant extract. Rate of formation of tetra guaiacol was measured at 436 nm and enzymatic activity was calculated using the extinction coefficient of 25.5 mM⁻¹ cm⁻¹. Specific activity was expressed in μmol min

\^{-1} mg^{-1} \text{protein}.

**Glutathione reductase assay (GR) (EC. 1.6.4.2)**

The activity of glutathione reductase was assayed by the protocol given by Carlberg and Mannervik (1985). Reaction mixture was consisted of 1.8 ml potassium phosphate buffer (50 mM, pH = 7.6), 300 μl of EDTA (3 mM), 300 μl NADPH (0.1 mM), 300 μl oxidised glutathione. Finally, 40 μl enzymatic extract was added and absorbance was taken at 340 nm. Enzymatic activity was calculated using the extinction coefficient of 6.22 mM⁻¹ cm⁻¹. Specific activity was expressed in μmol min

\^{-1} mg^{-1} \text{protein}.

**Determination of anthocyanin pigment, total phenolic content, proline and electrolyte leakage**

Anthocyanin content was assayed by the protocol given by Mancinelli (1984). One gram of fresh plant material was crushed in 3 ml of acidified methanol (99 ml methanol: 1 ml HCl). The extract was centrifuged for 20 min at 12000g at 4°C. The supernatant was collected and incubated at 4°C for 24 h. Absorbance was taken at 530 and 657 nm. The relative amount of anthocyanin was estimated using equation Ab530

\text{OD} = \frac{\text{absorbance at 530 nm}}{\text{absorbance at 657 nm}}

Total phenolic content was determined according to a procedure given by Singleton and Rossi (1965). Five hundred milligrams of plant material was taken and homogenized in methanol using pestle and mortar. The extract was centrifuged and the supernatant was collected. In a test tube, 40 μl of plant extract, 4 ml distilled water and 300 μl Folin–Ciocalteu reagent was added and mixed thoroughly. The reaction mixture was then incubated for 8 min followed by the addition of 600 μl of NaOH solution (2%, w/v) and again incubated at 40°C for 2 h. After cooling, initial electrical conductivity was measured using conductivity meter. Then samples were autoclaved at 121°C for 20 min to release all electrolytes. Samples were then cooled to room temperature and final electrical conductivity was recorded (EC2). Percentage of electrolyte leakage was calculated by following the equation:

\text{Electrolyte leakage (\%) =} \frac{\text{EC1}}{\text{EC2}} \times 100

**Scanning electron microscopic (SEM) studies**

The stomatal studies of plants (control as well as treated) of *S. polyrhiza* were done using scanning electron microscope (Carl Zeiss- EvoLS 10). Leaf samples were overnight fixed in 2.5% glutaraldehyde prepared in 0.1 M potassium phosphate buffer and were then dehydrated using different ethanol series (30%, 50%, 70%, and 90%). Adaxial (ventral) side of leaves was then placed on stubs and coated using silver coater. The surface features of leaves were viewed under a scanning electron microscope at 1.50 KX. The instrument was operated in variable pressure mode (VP mode) and VPSE G3 detector was used. EDX spectra were generated to determine elemental analysis (F⁻) in leaves.

**Confocal laser scanning electron microscopic studies (CLSM)**

The confocal microscope used was a Nikon AiR Laser Scanning confocal microscope (Nikon, Corporations, Japan). For confocal microscopy, the roots of plant samples (control and treated) were washed with distilled water. Roots were then placed in different confocal dyes viz., Propidium iodide (PI), dichlorofluorescein diacetate (H2DCFDA) and monochlorobimane (MGB) for studying the viability of cells, detection of ROS levels and GSH levels respectively occurred during oxidative stress in plants. The He–Ne gas laser was used to excite the electrons at a wavelength of 535 nm for propidium iodide, multilam argon gas laser was used for 2, 7 dichlorofluorescein at 488 nm and for monochlorobimane excitation wavelength of 380 nm was used.

**Fourier transform infrared spectroscopy (FT-IR) analysis**

FTIR spectrometer (Cary 630 FTIR, Agilent technologies) was used as a tool to assess the change in chemical composition and metal binding sites in biological specimens. Plant samples were oven dried for 48 h at 80°C. Samples were crushed in fine powdered form using pestle and mortar. Then powder was mixed with KBr pellets for FTIR analysis were prepared in agate mortars. The absorbance spectra were measured in the range between 400 and 4000 cm

\^{-1}.

**Statistical analysis**

Mean values and standard error were calculated from triplicates for each of the observed parameters. Statistical analysis was performed using two-way ANOVA analysis and Tukey’s honestly significant difference (HSD). Significance of the data was analyzed at 95% confidence levels (p ≤ 0.05).
determine the physiological trends in a plant, principal component analysis (PCA) was performed on nine different physiological parameters (SOD, CAT, APOX, POD and GRanthocyanin, phenols, proline and electrolyte leakage) of S. polyrhiza using SPSS software version 16. Factors loading were classified as ‘strong’, ‘moderate’ and ‘weak’ that corresponds to values of >0.75, 0.75-0.50 and <0.50 respectively (Liu et al. 2003). Moreover, Pearson’s correlation was also carried out to find out the relationship between different antioxidant enzymes (SOD, CAT, APOX, POD and GR) electrolyte leakage, proline, phenols, and anthocyanin content.

**Results**

**Fluoride accumulation by S. polyrhiza**

In the present investigation, a significant increase \( (p \leq 0.05) \) in fluoride accumulation in plants of treated S. polyrhiza was observed in dose-duration dependent manner (Table 1). Obtained data revealed that the plants exposed to 50 ppm concentration accumulated the highest amounts of fluoride \( (773.95 \text{ mg/kg dw}) \) after exposure period of 120 h as compared to control followed by 673.9 mg/kg dw at 168 h, 485.65 mg/kg dw at 72 h and 196.3 mg/kg dw at 24 h duration. From the entire investigation, it was evaluated that the experimental plant S. polyrhiza showed significant potentiality with respect to fluoride accumulation.

**Alterations in antioxidant enzymatic activity under fluoride stress**

Fluoride-induced effect on the activities of antioxidative enzymes (SOD, CAT, APOX, POD and GR) is shown in (Figure 1).

**SOD activity**

SOD activity which causes the elimination of superoxide radicals formed during oxidative stress in cells, significantly increased \( (p \leq 0.05) \) with increasing concentrations in the plants of treated S. polyrhiza as compared to control (Figure 1(a)). In the current investigation, the maximum enhanced specific activity of \( (604.3 \mu \text{mol min}^{-1} \text{ mg}^{-1} \text{ protein}) \) was observed at the highest concentration of 50 ppm at the exposure period of 24 h with respect to control. Though with an increase in the time period, SOD activity decreased but with preceding concentrations, it showed an increase which was recorded as 44.2, 207.5, 53.28 \( \mu \text{mol min}^{-1} \text{ mg}^{-1} \) protein at exposure periods of 72, 120 and 168 h respectively at 50 ppm.

**CAT activity**

Catalase plays a crucial role in \( \text{H}_2\text{O}_2 \) scavenging formed in plants during stress by breaking down it into water and oxygen (Mallick and Mohn 2000). CAT activity in S. polyrhiza under fluoride stress in the present study was observed to be increased significantly \( (p \leq 0.05) \) at a high concentration as compared to control (Figure 1(b)). Maximum level in specific activity of CAT was found to be 5.64 \( \mu \text{mol min}^{-1} \text{ mg}^{-1} \) protein at 50 ppm concentration at a very initial exposure period of 24 h, followed by decrease in the activity at 72 h exposure period, with maximum decrease found to be 0.46 \( \mu \text{mol min}^{-1} \text{ mg}^{-1} \) protein. However, at other exposure periods of 120 and 168 h, CAT activity showed a minute increase with increasing fluoride concentrations, with maximum activity recorded as 2.02 and 0.19 \( \mu \text{mol min}^{-1} \text{ mg}^{-1} \) protein respectively.

**APOX activity**

APOX activity is responsible for \( \text{H}_2\text{O}_2 \) scavenging during stress. In present observation, APOX activity increased significantly \( (p \leq 0.05) \) in S. polyrhiza at elevated concentrations, with maximum activity recorded as 45.08 \( \mu \text{mol min}^{-1} \text{ mg}^{-1} \) protein in response to 50 ppm concentration at 24 h exposure period (Figure 1(c)). However, maximum APOX activity of 0.69, 6.72 and 2.09 \( \mu \text{mol min}^{-1} \text{ mg}^{-1} \) protein at 20 ppm concentration was observed for the time period of 72, 120 and 168 h respectively.

**POD activity**

POD also acts on \( \text{H}_2\text{O}_2 \) and is the member of large peroxidase family. As depicted in (Figure 1(d)), likewise other enzymatic activity, a significant increase \( (p \leq 0.05) \) in POD activity was also observed at all the concentrations in the present investigation over the control. Maximum POD activity of 2.77 \( \mu \text{mol min}^{-1} \text{ mg}^{-1} \) protein was exhibited at the highest concentration of 50 ppm during the initial exposure period of 24 h. However, increased POD activity of 0.77, 0.67 and 0.30 \( \mu \text{mol min}^{-1} \text{ mg}^{-1} \) protein was also observed at the exposure period of 72, 120 and 168 h respectively.

**Table 1. Fluoride accumulation (mg/kg dw) in fronds of Spirodela polyrhiza at different concentrations and exposure periods.**

| Concentrations (ppm) | 24       | 72       | 120      | 168      |
|----------------------|----------|----------|----------|----------|
| Control (mg/kg dw)   |          |          |          |          |
| 5                    | 65.1 ± 0.51 | 64.6 ± 8.10 | 80.78 ± 10.45 | 67.6 ± 1.47 |
| 10                   | 63.1 ± 0.26 | 95.8 ± 11.48 | 145.4 ± 18.61 | 185.1 ± 2.77 |
| 15                   | 75.3 ± 5.63 | 136.6 ± 17.55 | 177.6 ± 21.34 | 216.6 ± 0.35 |
| 20                   | 59.8 ± 0.26 | 174.6 ± 24.13 | 237.1 ± 31.28 | 329.7 ± 6.40 |
| 25                   | 105.7 ± 4.77 | 220.1 ± 27.16 | 317.9 ± 41.65 | 294.1 ± 8.22 |
| 50                   | 196.3 ± 6.70 | 485.6 ± 0.95 | 773.9 ± 2.05 | 673.9 ± 10.82 |

Two-way ANOVA

\[ F \text{ ratio (6, 56) } 220.88^* \]

\[ F \text{ ratio (6, 56) } 566.98^* \]

\[ F \text{ ratio (18, 56) } 38.13^* \]

HSD value 79.53

Data shown are Mean ± S.E of triplicate values \((n = 3)\), two way ANOVA, Tukey’s HSD. *Significant at \( p \leq 0.05 \).

ND = not detected.
GR is required for balancing glutathione levels in the cells (Gomes-Junior et al., 2006). GR activity also showed the similar increasing trend as other enzymes showed in our present work at all the concentrations and the increase in activity was found to be significant \( p \leq 0.05 \) as compared to control (Figure 1(e)). Maximum GR activity was observed to be 17.4 and 0.57 µmol min\(^{-1}\) mg\(^{-1}\) protein at high concentration of 50 ppm for 24 h and 72 h respectively. However, at the exposure period of 120 and 168 h, the maximum specific activity was recorded as 0.97 and 1.60 µmol min\(^{-1}\) mg\(^{-1}\) protein at 25 and 20 ppm concentration of fluoride respectively.

**GR activity**

Anthocyanin play a key role as an antioxidant by protecting the plant against ROS. In the present study, fluoride stress significantly \( p \leq 0.05 \) enhanced the content of anthocyanin pigment at high concentrations of fluoride at all the exposure periods (Table 2). Maximum accumulation of 50.0, 59.7, 14.2 and 18.18% was found at 50 ppm for 24, 72, 120 and 168 h exposure period as compared to their respective controls. Increased anthocyanin pigment is also considered as an indicator of stress.

**Fluoride-induced accumulation of anthocyanin, proline, phenolic contents and increased electrolyte leakage (%)**

Figure 1. Effect of fluoride on activities of antioxidant enzymes (a) SOD activity (b) CAT activity (c) APOX activity (d) POD activity (e) GR activity. Data is shown as Mean ± S.E of triplicate values \( (n = 3) \), two way ANOVA, Tukey’s HSD. *Significant at \( p \leq 0.05 \).
Table 2. Effect of fluoride on anthocyanin (OD/gfw), phenolic content (mg/gfw) proline (µmoles/gfw) and electrolyte leakage (%) of Spirodela polyrhiza at different concentrations and exposure periods.

| Exposure period (h) | Concentration (ppm) | Anthocyanin (OD/gfw) | Phenolic content (mg/gfw) | Proline (µmoles/gfw) | Electrolyte leakage (%) |
|---------------------|----------------------|----------------------|--------------------------|---------------------|------------------------|
| 24                  | Control              | 0.34 ± 0.015         | 0.14 ± 0.02              | 41.73 ± 0.51        | 6.29 ± 0.49            |
|                     | 5                    | 0.44 ± 0.011         | 0.14 ± 0.06              | 49.61 ± 0.48        | 5.95 ± 0.33            |
|                     | 10                   | 0.40 ± 0.032         | 0.15 ± 0.02              | 49.65 ± 6.60        | 6.25 ± 0.05            |
|                     | 15                   | 0.32 ± 0.005         | 0.25 ± 0.01              | 62.89 ± 8.49        | 7.53 ± 0.48            |
|                     | 20                   | 0.18 ± 0.004         | 0.26 ± 0.01              | 72.88 ± 5.14        | 10.17 ± 0.10           |
|                     | 25                   | 0.32 ± 0.002         | 0.25 ± 0.01              | 74.71 ± 4.69        | 6.24 ± 0.22            |
|                     | 50                   | 0.51 ± 0.001         | 0.30 ± 0.08              | 56.57 ± 7.06        | 7.78 ± 0.36            |
| 72                  | Control              | 1.64 ± 0.312         | 0.04 ± 0.02              | 35.49 ± 4.85        | 10.08 ± 0.31           |
|                     | 5                    | 2.22 ± 0.225         | 0.08 ± 0.01              | 28.09 ± 9.70        | 7.27 ± 0.10            |
|                     | 10                   | 2.30 ± 0.082         | 0.11 ± 0.01              | 35.83 ± 18.75       | 10.64 ± 0.18           |
|                     | 15                   | 2.44 ± 0.064         | 0.17 ± 0.006             | 68.78 ± 9.54        | 8.99 ± 0.30            |
|                     | 20                   | 2.54 ± 0.018         | 0.18 ± 0.03              | 70.08 ± 1.61        | 12.63 ± 0.19           |
|                     | 25                   | 2.55 ± 0.019         | 0.28 ± 0.01              | 73.88 ± 3.33        | 12.50 ± 0.35           |
|                     | 50                   | 2.62 ± 0.037         | 0.37 ± 0.02              | 72.05 ± 9.49        | 16.32 ± 0.55           |
| 120                 | Control              | 0.63 ± 0.025         | 0.07 ± 0.01              | 30.09 ± 4.95        | 13.47 ± 2.91           |
|                     | 5                    | 0.60 ± 0.013         | 0.15 ± 0.01              | 42.69 ± 0.16        | 14.07 ± 3.07           |
|                     | 10                   | 0.61 ± 0.024         | 0.23 ± 0.03              | 43.15 ± 2.19        | 13.20 ± 2.79           |
|                     | 15                   | 0.53 ± 0.002         | 0.34 ± 0.02              | 50.48 ± 12.6        | 14.43 ± 2.72           |
|                     | 20                   | 0.55 ± 0.004         | 0.43 ± 0.02              | 75.17 ± 7.86        | 17.74 ± 2.33           |
|                     | 25                   | 0.68 ± 0.010         | 0.43 ± 0.031             | 82.54 ± 3.30        | 16.01 ± 1.57           |
|                     | 50                   | 0.72 ± 0.018         | 0.50 ± 0.02              | 86.58 ± 3.52        | 17.35 ± 0.80           |
| 168                 | Control              | 0.44 ± 0.127         | 0.11 ± 0.04              | 47.36 ± 4.22        | 21.48 ± 2.95           |
|                     | 5                    | 0.45 ± 0.015         | 0.27 ± 0.13              | 52.81 ± 2.11        | 32.99 ± 5.23           |
|                     | 10                   | 0.50 ± 0.019         | 0.30 ± 0.10              | 61.84 ± 0.61        | 30.36 ± 1.39           |
|                     | 15                   | 0.40 ± 0.006         | 0.34 ± 0.01              | 55.84 ± 7.97        | 29.17 ± 2.57           |
|                     | 20                   | 0.48 ± 0.012         | 0.29 ± 0.14              | 73.24 ± 1.99        | 27.88 ± 3.65           |
|                     | 25                   | 0.49 ± 0.011         | 0.48 ± 0.02              | 70.86 ± 2.61        | 25.52 ± 4.97           |
|                     | 50                   | 0.52 ± 0.008         | 0.51 ± 0.01              | 72.83 ± 1.04        | 32.49 ± 5.48           |

Two-way ANOVA

\[ F_{\text{ratio (3, 56)}} = 1436^* \]
\[ F_{\text{ratio (6, 56)}} = 5.78^* \]
\[ F_{\text{ratio (18, 56)}} = 6.37^* \]
\[ \text{HSD value} = 0.35 \]

Data shown are Mean ± S.E of triplicate values (n = 3), two way ANOVA, Tukey’s HSD. *Significant at p ≤ 0.05.
Present investigation also showed a significant increase ($p \leq 0.05$) in phenolic content in dose-duration dependent manner when compared to control (Table 2) with maximum phenolic content was found to be 0.30, 0.37, 0.50 and 0.51 mg/g fresh weight at 50 ppm for 24, 72, 120 and 168 h exposure period.

Proline helps in maintaining the osmotic potential of plants and the results revealed that there was an increase in proline content in S. polyrhiza under fluoride stress with increasing concentrations but the increase was not statistically significant with respect to control. Maximum proline content was found to be 86.5 µmol/gfw at the highest concentration of 50 ppm at 120 h, while minimum content of 28.095 µmol/gfw at low concentration of 5 ppm at exposure period of 72 h was observed in fluoride-treated plant (Table 2). Notably, the maximum increase of 187% was observed in the plants exposed to 50 ppm fluoride for 120 h in this present investigation with respect to control.

Increase in percentage of electrolyte leakage in S. polyrhiza under fluoride stress was observed in concentrations and duration-dependent manner, although the increase was not found statistically significant ($p \leq 0.05$) as compared to control. Maximum leakage of 32.99 and 32.49% of electrolyte was observed at 5 and 50 ppm respectively for the time period of 168 h, while minimum leakage of 5.95% electrolyte was observed at low concentration of 5 ppm at 24 h (Table 2).

**Scanning electron microscopic observations**

SEM studies revealed significant morphological changes in leaf surface of fluoride-treated S. polyrhiza including stomatal movements. The control sample showed a high number of open stomata, while fluoride treated leaf samples showed closed stomata as imaged by SEM (Figure 2). In order to confirm the accumulation of $F^-$ ion, EDX-ray microanalysis was performed. It showed a characteristic peak of fluoride in treated plants which implied that plants accumulated $F^-$ content at high concentration of 50 ppm for 168 h, while control plant showed no peak of fluoride. SEM-EDX spectra of control and fluoride-treated leaf surface are shown in (Figure 2) from where it was evident that exposure to 50 ppm fluoride for 168 h resulted in less number of open stomata and loss of cell shape as compared to control. S. polyrhiza responded to fluoride stress by stomata opening and closing.

**Confocal microscopic observations**

For detection of cell viability, reactive oxygen species (ROS), glutathione levels (GSH) during oxidative stress in $F^-$ treated plants, fluorescent dyes (PI, H2DCFDA, and MCB) were used and images were examined by using confocal microscopy (Figure 3). PI helped in differentiation between healthy and affected cell as it is unable to penetrate the membrane of live cells (Lecoeur 2002) PI dye when fused with DNA appeared as red fluorescent dots and can be visualized. Additionally, visualization of ROS generation during $F^-$ induced oxidative stress was well demonstrated by staining the roots with H2DCFDA. Notably, being non-fluorescent, this particular dye upon oxidation by hydrogen peroxide gets converted into 2,7'-dichlorofluorescein (DCF) which is green fluorescent. Stressed plant, thus showed highly green fluorescence as compared to unstressed one. MCB was used to monitor changes in GSH levels in $F^-$ treated root cells. The present investigation revealed crossing of PI, H2DCFDA and MCB dye to fluoride treated root cells of S. polyrhiza. PI-stained roots of S. polyrhiza showed red fluorescence, while H2DCFDA and MCB showed green and blue fluorescence respectively.

**Fourier transform infrared spectroscopy (FTIR) analysis**

FT-IR analysis was performed to confirm the changes in the potential plant components present in the cellular membrane.
due to fluoride stress (Figure 4). FTIR spectra of shoot biomass without fluoride (control) showed a number of absorption peaks which implies the complexity of biomass. FTIR spectra of control and treated plant samples are compared and displayed the following shifts. The analysis showed that untreated sample (control) exhibited characteristic IR absorption bands at 3338 cm$^{-1}$ for OH group (broad signal stretching vibration) which is shifted to 3390 cm$^{-1}$, indicating complexation of $-\text{OH}$ groups. The band for $-\text{C}−\text{H}$ group at 2918 and 2851 cm$^{-1}$ (symmetric stretch), indicating the presence of carbohydrates and lipids also gets significantly shifted in the treated sample. $-\text{SH}$ group at 2359 cm$^{-1}$ (symmetric stretch) shifted to 2357.5 which may be due to complexation of fluoride ions with $-\text{SH}$ group. The next absorption peak exhibited $-\text{C} = \text{O}$ group (oxygen attached to a benzene ring, vibrational bands) at 1651 cm$^{-1}$ shifted to 1634 cm$^{-1}$ and $-\text{C} = \text{C}$ group at 1319.7 cm$^{-1}$ shifted to 1321 cm$^{-1}$ (asymmetric stretch) attributed to lipids. Another shift was observed from 1254 cm$^{-1}$–1243 cm$^{-1}$ implying complexation of nitrogen from the amino group with fluoride ion. Also, the other weak absorption peaks shifted from 782.7 cm$^{-1}$–780.9, 669.1 cm$^{-1}$–667.2 cm$^{-1}$ indicating the interaction of thiol or sulhydryl group with fluoride ion. Hence, the above changes revealed the complexation of amide, hydroxyl, amino and thiol groups with that of fluoride ion, accumulated by treated plants (Castaldi et al. 2010; Giri et al. 2012).

**Principal component analysis**

In PCA analysis, three factors were extracted which explained 90.12% variation in the data set. PC1 showed explained 53.35% of the variance with strong positive loading SOD, APOX, CAT, POD, and GR. PC2 exhibited 22.64% of the variance with strong positive loading of phenols, proline and moderate loading of electrolyte leakage. PC3 showed strong positive loading on anthocyanin pigment explaining 14.19% of the total variance in the data set (Table 3). Positive loading implies that all the variables under study are positively correlated with each other which also supported the fact that all these parameters play a pivotal role in maintaining plant homeostasis during stress.

**Pearson’s correlation coefficient analysis**

Correlation between different parameters studied in our investigation Pearson’s correlation coefficient. In Pearson’s correlation coefficient analysis, a significant positive correlation was found between SOD-APOX (0.922), SOD-CAT (0.903), SOD-POD (0.901), SOD-GR (0.903), APOX-CAT (0.837), APOX-POD (0.930), APOX-POD (0.950), CAT-POD (0.864), CAT-GR (0.869), Phenols-proline (0.780) and Phenols-electrolyte leakage (0.526) at 0.05 level of significance whereas a significant negative correlation was found between APOX-electrolyte leakage (−0.394) at 0.05 level of significance.

**Discussion**

The entire investigation revealed that the fluoride accumulation by *S. polyrhiza* triggered a couple of cellular changes, consequently increasing fluoride tolerance ability of the plant. The experimental plant *S. polyrhiza*, exhibited significant potentiality with respect to fluoride accumulation in concentration and duration-dependent manner. The results obtained are in agreement with the earlier findings on aquatic plants that have shown significant fluoride accumulation and its removal ability with increasing concentrations (Sinha et al. 2000; Zhou et al. 2012; Sharma and Kaur 2017). Bioaccumulation of toxic metals from polluted water by members of the duckweed family (*L. minor* and *L. trisulca*) is well documented (Samardakiewicz and Woźniy 2000). Since accumulated toxic metals are generally retained by the roots of aquatic plants, subsequently led to less transport to aerial portions. As *S. polyrhiza* possess thread-like a root system, it is obvious that the plants absorb most of the fluoride content from the nutrient medium. Stevens et al. (1998) reported that the concentration of fluoride in plants significantly increased after achieving the threshold of F ion activity in the nutrient medium. Further, fluoride
uptake from culture medium is dependent on its ionic strength and plant species. Fluoride uptake can be explained as an increased apoplastic transport of fluoride through passive diffusion from water, in a unidirectional flow, eventually led to the accumulation of fluoride in plants (Pant et al. 2008).

Fluoride provoked oxidative stress accelerated the generation of reactive oxygen species (ROS), stimulated the activation of the antioxidant defence system of plants to counteract enhanced oxidative stress. Stimulation of increased SOD activity at all the exposure periods in our present investigation confirms its crucial role in detoxifying ROS levels generated during fluoride induced stress. SOD in chloroplast degrades $O_2^-$ to $H_2O_2$, thus counteract the deleterious effect (Upadhyay et al. 2014). Our results are in coherence with the observation made by Allen et al. (1997). Authors observed that an increase in SOD activity may be due to the de-novo synthesis of enzymatic proteins. Chakrabarti and Patra (2015) also reported a similar increasing trend in SOD activity in paddy (Oryza sativa) under fluoride stress at elevated concentrations. The probable reason behind increased SOD activity may be due to the increased pace of SOD biosynthesis in the plant under fluoride exposure for a longer duration (Alscher et al. 2002). Meanwhile, activities of CAT, APOX, POD, and GR was also found to be increased concomitantly during fluoride stress conditions indicating that S. polyrhiza could tolerate stress by activating antioxidant defence mechanisms. Since peroxidases are considered as stress markers during toxic metal stress conditions and help in effective scavenging of reactive oxygen species derivatives (Radotić et al. 2000). Both POD and APOX are potent $H_2O_2$ scavengers, regulate its level in the plant cells, as the excessive presence of $H_2O_2$ in cytosol led to hydroxyl

Table 3. Factor loading values, eigen values, variance% and cumulative % for the first three factors extracted from PCA.

| Parameters          | Components | PC1  | PC2  | PC3  |
|---------------------|------------|------|------|------|
| SOD                 |            | 0.953| 0.220| -0.055|
| APOX                |            | 0.973| -0.030| -0.149|
| CAT                 |            | 0.931| 0.037| 0.192 |
| POD                 |            | 0.968| -0.026| -0.044|
| GR                  |            | 0.951| 0.021| -0.152|
| PHENOL              |            | 0.085| 0.931| -0.215|
| ANTHOCYANIN         |            | -0.204| -0.048| 0.932 |
| PROLINE             |            | 0.121| 0.914| 0.107 |
| Electrolyte leakage |            | -0.417| 0.530| -0.507|
| Eigen values        |            | 4.802| 2.038| 1.271 |
| %Variance           |            | 53.35| 22.64| 14.11 |
| Cumulative %        |            | 53.35| 76   | 90.12 |
formation via Haber–Weiss cycle and impair various cellular functions (Singh et al. 2006). Our results are in accordance with increased POD activity recorded in other aquatic plants viz; L. gibba, L. minor and S. polyrhiza under Cu and Cd stress (Banu Doganlar 2013). Increased APOX activity helps in the adaptation of plant under fluoride stress by activation of antioxidative enzymatic system triggered by increased ROS levels. Saini et al. (2013) also noticed increased APOX expression in Prosopis juliflora which when cultured in 20–50 ppm concentration of fluoride containing medium for the duration of seven days. Enhanced POD activity implies that the synthesis of POD increased during stress and genes encoding peroxidases show their increased expression. Moreover, dramatically increased activity of APOX and POD also confirmed that both these enzymes are responding well by concurrently removing H$_2$O$_2$ generated in fluoride-treated $S$. polyrhiza (Singh et al. 2006). Furthermore, the stimulating effect of fluoride on the CAT activity revealed that plants encountered elevated oxidative damage. Our results corresponded well with the previous reports showing that oxidative stress either cause elevation or inhibition of CAT activity which depends on duration, type, and intensity of stress (Sharma et al. 2012). Karmakar et al. (2016) also observed increased catalase activity in three aquatic macrophytes viz; Pistia stratiotes, Eichhornia crassipes, S. polyrhiza under 20 ppm fluoride concentration exposed for 10 days. Increased CAT activity implies effective dismutation of H$_2$O$_2$ to H$_2$O and O$_2$ and confirmed enhanced plant tolerance capability in stressed situations (Gill and Tuteja 2010). Moreover, our present results reported an increase in GR activity upon F$^−$ exposure which can be understood by the mechanism of ACH-GSH (ascorbate-glutathione) cycle, which at its high pace, detoxify ROS formation during fluoride exposure. Thus, GR plays a defensive role by generating GSH and regulating GSH pool to counteract the oxidative damage (Reddy and Raghaven德拉 2006). Present study results are inconsistent with the enhanced GR activity reported in Vicia faba and O. sativa under Pb and Cd stress (Verma and Dubey 2003; Cordova et al. 2003).

A considerable increase in anthocyanin pigment is also an indicator of stress. Our findings indicated that increased anthocyanin accumulation in S. polyrhiza during fluoride mediated stress might help in protecting the plants from detrimental effects of superoxide radicals generated during fluorideinduced stress without limiting its photosynthesis ability. Mobin and Khan (2007) also reported a similar increasing trend in two cultivars of Brassica juncea under Cd-induced stress. Likewise other parameters, phenolic content also increased in fluoride stressed plants of S. polyrhiza in our present study. Phenolics do have antioxidant properties and are present in abundant quantity in plant tissues (Grace and Logan 2000). Accumulation of phenols might be due to increased activity of phenylalanine lyase (PAL) enzyme, during stress conditions (Schützendübel et al. 2001). Increased phenolic content was also observed in cadmium stressed B. juncea (Kapoor 2016). Moreover, our data also revealed an increased accumulation of proline and leakage of electrolytes during stress. Being an osmolyte proline can be regarded as nonenzymatic antioxidants to combat the ROS generated effects (Yilmaz and Parlak 2011). Increased proline level under fluoride stress means that plants maintain their osmotic potential by removing –OH via accumulation of proline and protect the plant from being damaged (Smirnoff and Cumbes 1989). Also, there is a dramatic accumulation of proline during stressful conditions due to its increased formation in the plant cells to maintain homeostasis (Mansour and Ali 2017). Similar changes were reported in the tea plant (Cai et al. 2016) and barley (Gautam and Bhardwaj 2010) under fluoride stress. Permeability of the plasma membrane is reflected by electrolyte leakage and it was increased with excessive lipid peroxidation. Moreover, fluoride stress in S. polyrhiza led to increased lipid peroxidation as reported by Sharma and Kaur (2017). Cai et al. (2016) observed similar increased electrolyte leakage percentage in tea plants due to fluoride toxicity. Enhanced electrolyte leakage due to increased exposure to fluoride was also reported in Populus deltoides (Singh et al. 2013).

In order to gain insights into toxicological implications induced by fluoride at the cellular level, scanning electron microscopy and confocal microscopy was performed. In addition electron, dispersive X-ray analysis was done to determine fluoride accumulation by plants (Figure 2). Scanning electron micrographs of stressed frond showed much variations as compared to unstrained frond. Electron micrographs of ventral surface of fluoride-treated frond showed mostly closed stomata, while they remained open in unstrained one. The reason behind is reduced or no ATP supply increased abscisic acid accumulation during stressful conditions which eventually led to their closure. Literature data also showed the effect of fluoride on tea leaves, caused changes in stomatal aperture as imaged by SEM by Cai et al. (2016). Yang et al. (2004) reported that heavy metals have significantly affected stomatal movements which involved its stomatal opening and closing. It is plausible that S. polyrhiza attained fluoride stress tolerance attributes by showing alterations in stomatal movements. Confocal micrographs depicted no or less fluorescence in control roots of the plant while, high fluorescence was observed in treated roots which indicated that S. polyrhiza responded to the stress and fluorescence spectroscopy helps in determining F$^−$ localization (Figure 3).

Furthermore, FTIR analysis was used as a tool to observe structural changes induced by fluoride. Our findings indicated that the aforementioned peaks in the treated plant sample were not merged and showed less intensity that revealed reduced protein and carbohydrate composition (D’Souza et al. 2008). However, initiation of lipid peroxidation was also observed in the treated sample that corresponded to initial broad signal with no peak tracing in the wavenumber. The result of FTIR confirmed chemically reduced carbohydrate and protein content along with increased lipid peroxidation which justified the potential effect of fluoride on S. polyrhiza. Results are in agreement with the IR band spectra observed in Padina tetrastromatica (Hauck) under cadmium stress using FTIR (D’Souza et al. 2008). Since heavy metals perturbed the IR band spectra and led to alterations of chemical composition in plants. This technique proved to be useful in determining probable fluoride binding sites in S. polyrhiza.

An integrated schematic representation of present study demonstrating fluoride-induced changes in S. polyrhiza is presented in Figure 5.

Conclusions and future prospects
The present investigation on S. polyrhiza summarized that fluoride stress influenced the plant, well evidenced by the
activation of antioxidant enzymatic defence system (SOD, CAT, APOX, POD, and GR) and other parameters which include increased proline, anthocyanin, phenolic content and electrolyte leakage. Our data is also supported by microscopic observations and FTIR analysis. Moreover, *S. polyrhiza* showed significant accumulation of fluoride in dose-duration dependent manner. It can be concluded that this plant has sufficient ability to tolerate fluoride stress conditions. Furthermore, our findings suggested that plant adopts fluoride stress tolerance attributes to adapt well. Hence, defence strategies adopted by *S. polyrhiza* prove to be imperative in combating the detrimental effects generated during fluoride mediated oxidative stress. Also, it would be interesting to study more adaptive mechanisms and mechanisms of fluoride uptake by the plant at molecular and genetic levels to determine its exact potential for phytoremediation.

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**Ethical approval**

This article does not include any studies related to human subjects or animal models performed by any of the authors.

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