Azolla pinnata R.Br.: a fern species that demonstrates satisfactory in vitro antioxidation under herbicidal toxicity

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ABSTRACT

The paper reports the induced antioxidation property of *Azolla pinnata* R.Br. under elevated conditions of the herbicide 2,4-D. With respect to phenolic accumulation, the plant registered varied *in-vitro* antioxidation potentials. Total antioxidation through phosphomolybdenum complex, DPPH (2,2-Diphenyl, 1-picryllydrazyl) and ABTS (2,2'-azino-bis 3-ethylbenzo-thiazoline-6-sulfonic acid) radical scavenging activities were the most significant attributes of this plant species. A gradual fall in phosphomolybdenum complex suggested more involvement in the chelation of 2,4-D with metal ligands. The plant was able to quench the reactive oxygen species (ROS) up to a certain level of 2,4-D but thereafter it failed. Another module with ABTS induced free radical antioxidation, the plant insignificantly responded to any changes of 2,4-D concentrations as compared to the control with reference to BHT (Butylated hydroxytoluene). The most stable phenolic glucoside as flavonoid had a significant and dose-dependent over expression under 2,4-D toxicity. In modules of enzymatic antioxidants, *Azolla* was quite sensitive to peroxidation of H_2O_2 by different isozymic proteins. A significant participation of polyphenol oxidase and catalase were more pronounced whereas peroxidases were least significant in *in vitro* assay. Taking altogether both enzymatic and non-enzymatic antioxidation indices, *Azolla* is an efficient quencher species for herbicide contaminated soils.

Keywords: Antioxidative enzymes; Antioxidants; Herbicide; Aquatic fern

INTRODUCTION

The pharmaceutical and medicinal aspects of certain plants are now given more scientific interest over their nutritive value. The fruits, tubers and even reproductive parts of plants are the major sources of medicinal constituents in the form of special chemical residues. Mostly, the straight chain hydrocarbons and unsaturated polyphenols under the category of secondary metabolites are

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DOI: 10.32945/atr4012.2018

in special chemical reactions (Shahidi & Zhong 2010) such as antioxidation which involves the reduction of reactive oxygen species (ROS) by free electrons donated by organic residues. These antioxidants may either be directly required in quenching of excess energy in ROS or become the part of electron donor for antioxidizing proteins (Das & Roychoudhury 2014). The commonly cultivated crops and most of the wild plant species are the richest sources of antioxidants. Stressors are the important inducers or elicitors for the accumulation of these antioxidants that vary with plant species. It is conventional to use these cultivated species since their physiological and systematic characteristics are more familiar.

Besides higher plants (mostly angiospermic species), few lower taxon members have also been cited as good sources of antioxidants (Agbor et al 2007). Early vascular plants with more diversity of phenolic compounds show a good variation in antioxidation metabolic profiles. *Pteris vittata* L., a member of the Leptosporangiate fern group, possesses improved anti-oxidation pathway which contributes to arsenic tolerance (Kikuchi & Tanaka 2012). A few aquatic ferns are also known for expression of antioxidants. Aquatic ferns have the advantage over other species due to the higher rate of dissolution of the heavy (or toxic) metal ions from tissues in marsh soils (Rai 2009). This also leads to more scientific investigations of other fern species for their tolerance to oxidative stress resulting from metals or xenobiotics.

Herbicides or pesticides are a good source of xenobiotics that lead to increased oxidative stress. In our earlier studies on the duck weed fern *Azolla pinnata* R.Br., we confirmed its hyper accumulation of the systemic herbicide 2,4-dichloro phenoxy-acetic acid (2,4-D) which induced cellular responses (De et al 2016). In the present experiment, *Azolla pinnata* R.Br. was cultured in nutrient media with varying concentrations of 2,4-D. The changes of antioxidative enzymes with the accumulation of antioxidants have confirmed this species as a reliable material for the occurrence antioxidation *in-vitro*. In brief, the present study discusses and explores antioxidant efficiency in addition to the systematic evaluation of different antioxidants as a function of herbicide intensities.

MATERIALS AND METHODS

Plant Growth and Treatments

Azolla pinnata R.Br., a free-floating mosquito fern and a member of Azollaceae was chosen for the present experiment. The plants were collected from the university premises and were grown in cemented tank containing fresh water for 7 days and thereafter, the plants were incubated with ¼th Murashige and Skoog (MS) medium in the laboratory (Murashige & Skoog 1962) for 2 days to acclimatize. This was followed by treatment with varying concentrations (0µM, 100µM, 250µM, 500µM and 1000µM) of 2,4-D (Sigma MB Grade). Plants were kept in such treatment for 7 days under ambient condition of 36±1°C temperature, 75-85% of RH and photoperiod of 14 - 10h light and dark photoperiod.

Preparation of Crude Solvent Extract

Two grams of *Azolla* plants were extracted with 15ml of 80% methanol by shaking at 150 rpm at 25 - 30°C for 24h and filtered the solution through filter paper (Whatmann No.1). The filtrate was stored for further use as methanolic extract.

Determination of Total Phenolic Contents

Estimation of total phenolic content was done according to Velioglu et al (1998). Solution of methanolic extract was mixed with 100µl Folin-ciocalteu reagent followed by addition of 850µl of distilled water and incubated for 5min at 25°C temperature. Thereafter, 500µl of 20 % sodium carbonate was added and incubated for 40min. The absorbance was recorded at 750nm. Total phenol content was determined using a standard curve obtained from the known concentration of gallic acid.

Total Antioxidant Activity by Phosphomolybdenum Complex Assay

Determination of total antioxidant activity was evaluated according to Prieto et al (1999). 0.1ml of sample solution was taken with 1ml of reagent solution containing 0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate. The Eppendorf tubes were capped and incubated in a water bath at 90°C for 60min. Thereafter, the assay mixture was cooled to room temperature and absorbance was recorded at 820nm against a blank.

Determination of in-vitro Antioxidant Assays of Crude Extract from Azolla pinnata Leaves

1. DPPH radical scavenging activity

Free radical scavenging activity from methanolic extract was done using the 2,2-Diphenyl, 1-picryllydrazyl (DPPH) method as described by Blois et al (1958).100µl of methanolic solution was mixed with freshly prepared 900µl 0.1mM DPPH solution. After 30min of incubation in dark at room temperature, the absorbance was measured at 517nm using a Spectrophotometer. Lower absorbance indicates the higher free radical scavenging activity. The activity of percentage of scavenging was calculated by the following formula-

DPPH radical scavenging percentage (%) = [(O.D control − O.D sample)/O.D control] x 100.

Standard curve was prepared using Butylated hydroxytoluene (BHT), a positive control for the present experiment.

The inhibition of concentration (IC_{50}) was determined from the plots as antioxidant concentration required for providing 50% free radical scavenging activity.

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2. ABTS radical cation decolorization assay

ABTS (2,2'-azino-bis 3-ethylbenzo-thiazoline-6-sulfonic acid), a stable free radical, decolorizes in its non-radical form. The ABTS scavenging activity was determined according to Re et al (1999). ABTS was prepared by recating the 7mM ABTS (dissolved in H_2O) with 3mM potassium persulfate ($K_2S_2O_8$) and storing in the dark for 16h. The freshly prepared ABTS was further diluted for use. The scavenging activity of the sample was determined using the following formula-

ABTS radical scavenging percentage (%) = [(O.D control − O.D sample)/O.D control] x 100.

A standard curve was prepared using BHT, a positive control for the present experiment.

The inhibition of concentration (IC_{50}) was determined similarly as discussed in DPPH radical scavenging method.

Determination of Total Flavonoid Contents

Estimation of total flavonoid content was done according to Zhishen et al (1999) using spectrophotometric method. 250μl of methanolic extract was mixed with distilled water (1.25ml) and 5% $NaNO_2$ solution. The mixture was incubated for 5min and sequentially 10% Aluminium chloride ($AlCl_3$), 1M (Sodium hydroxide) NaOH and distilled water were added to the mixture. After 6min of incubation the absorbance was recorded at 510nm. Quercetein was used as a standard (10mg/ml) solution with methanol to estimate the total flavanoid content.

Determination of Antioxidant Enzymes

Preparation of crude enzyme extract-   500mg of fresh plant samples were crushed in liquid nitrogen and homogenized with 100mM phosphate buffer (pH=7.5) containing 10mM $MgCl_2$ (Magnesium chloride), 1mM PMSF (Phenylmethylsulfonyl fluoride), 100mM EDTA (Ethylene diamine tetraacetic acid), 10mM DTT (Dithiothreitol) and 2% PVP (Polyvinyl pyrrolidone). The homogenate was centrifuged at 15,000 x g for 20min at 4°C. The supernatant was kept for further *in-vitro* assays.

1. Peroxidase activity assay

Guaiacol peroxidase (GPX) activity was determined according to Ammar et al (2008). The freshly prepared supernatant was added in an assay mixture containing 100mM potassium phosphate buffer, 0.5mM o-dianisidine and 0.5% H_2O_2. The absorbance was recorded at 430nm with 15min duration and activity was calculated from the extinction coefficient of o-dianisidine 26.2mM^{-1} cm^{-1}.

2. Catalase activity assay

Catalase (CAT) activity was determined according to Aebi et al (1983). The supernatant was incubated in a assay mixture containing 100mM potassium phosphate buffer (pH=7) and 10mM H_2O_2. The activity was determined by recording the absorbance at 240nm and activity was determined using the extinction coefficient of H_2O_2 0.036mM^{-1} cm^{-1}.

3. Polyphenol oxidase activity assay

Polyphenol oxidase (PPO) activity was determined using catechol as a substrate according to Jiang et al (2002). An aliquot crude enzyme extract (200µl) was added with 800µl of 50mM catechol solution (prepared in 0.1M sodium phosphate buffer (pH=6.5). The absorbance was recorded at 400nm.

Determination of Ferric Reducing Power

Determination of total reducing power of treated plants was done according to Subramanian et al (2015). One milliliter of methanolic samples from all treated plants were mixed with 2.5ml 200mM phosphate buffer containing (pH=6.5) and 2.5ml 1% potassium ferricyanide. The reaction mixture was incubated within water-bath at 50 – 60°C for 25min. Thereafter, 2.5ml of 10% Tri-chloro acetic acid (TCA) was mixed to the reaction mixture and centrifuged at 3000xg for 10min. 2.5ml of upper layer was pipetted out and mixed with 2.5ml of distilled water and 0.5ml of 0.1% $FeCl_3$. The absorbance was recorded at 700nm by using UV-VIS Spectrophotometer. The intensity of reducing power is directly proportionate to the absorbance recorded.

RESULTS

Determination of Total Phenol Content and its Variation

The *Azolla* plants recorded a well sensitivity to 2,4-D in terms of non-enzymatic antioxidant through a significant variation in total phenol content (Figure 1). The methanolic extract of the tissues when compared with gallic acid as equivalent showed a dose-dependent increase in phenol content. Thus, the variations were in the order of 0.2 fold, 0.3 fold, 1.0 fold and 1.3 fold as compared to control condition against 2,4-D treatments. The values were all significant at p≤0.05. Therefore, the toxicity level of herbicides induced the antioxidation reaction in the tissues. With gallic acid as standard, total phenolics was correlated (R^2= 0.02 & 0.83) with mean values of phenol content and free radical scavenging efficiencies against increasing order of herbicides (Table 1).
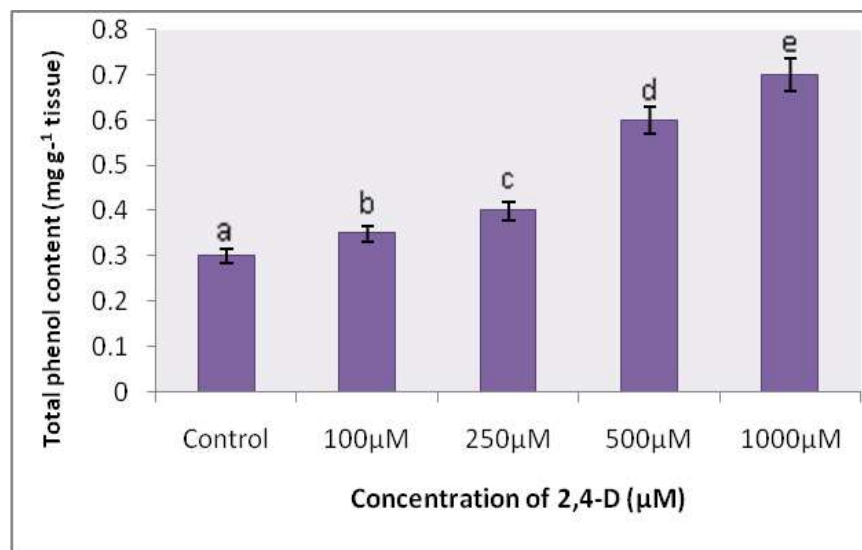
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Figure 1. Total phenol content assay in *Azolla* plants under different concentrations (0, 100, 250, 500, 1000μM) of 2,4-D treatments. The data were plotted from means of three replications (n=3) ±SE. Bars showing different letters indicate significant differences between treatments according to Duncan's test at p≤0.05.

Table 1. Table representing the correlation between different 2,4-D treated *Azolla* plants for different parameters.

Co-relation between different Parameters at $p \leq 0.05$ and 0.01	
Flavonoids & DPPH	0.34
Flavonoids & ABTS	0.312
Phosphomolybdenum & Catalase	-1.25
Phenol & Phosphomolybdenum	-3.9
Phenol & ABTS	0.02
Phenol & DPPH	0.83
Phenol & Flavonoids	1.08
Phenol & Reducing Power	2.1

Total Antioxidation Activity by Phosphomolybdic Acid Method

Total antioxidation activity was determined following the reduction of molybdenum [Mo(VI) − Mo(V)] by the methanolic extract with a green colored phosphomolybdate complex produced. Figure 2 presents the considerable antioxidation activity against standard cinnamic acid. At the concentration of 50µl/ml, the changes of optical density (O.D.) of methanolic extract for phosphomolybdate complex were in the order of 23.8%, 40.9%, 71.6% and 87.05% when compared to the control. The highest absorbance value thus directly indicates a higher reducing power for tissue extract in antioxidation. It is interesting to note that though plants had been affected significantly ($p \leq$ 0.05 & 0.01) through initial concentrations, but at maximum concentration, plants hardly had any changes and thus the correlation values for total phenolic content through phosphomolybdenum complex was inconsistent.
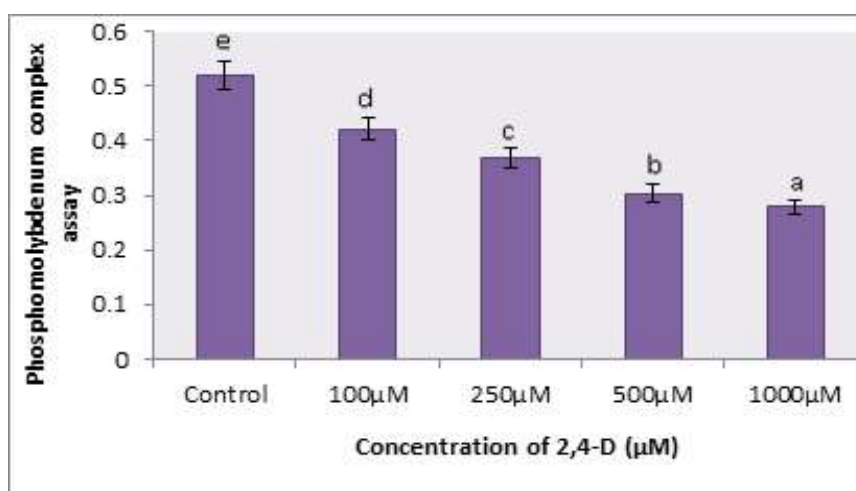

Figure 2. Phosphomolybdenum complex assay in *Azolla* plants under different concentrations (0, 100, 250, 500, 1000µM) of 2,4-D treatments. The data were plotted from means of three replications (n=3) ±SE. Bars showing different letters indicate significant differences between treatments according to Duncan's test at $p \leq 0.05$.

DPPH Radical Scavenging Activity

Figure 3 demonstrates the free radical scavenging activity through DPPH reaction with methanolic extract of *Azolla* as well as standard BHT. Among the extract from different 2,4-D treatments, a discriminating trend of DPPH scavenging activity under various 2,4-D treatments was observed. Thus, IC_{50} value of extract against DPPH had maximum at 500 µM (1.04 fold) against the control (Figure 5 & Table 2). On comparative basis it is well understood that the plant extract had the defined scavenging activity when it recorded 4.73%, 1.174%, 2.121%, 1.947% and
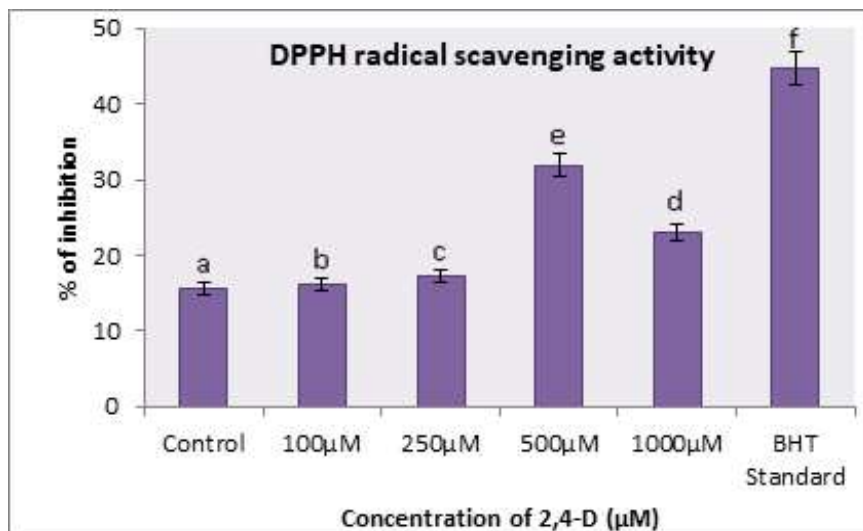
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Figure 3. Percentage of inhibition by DPPH radical scavenging assay in *Azolla* plants under different concentrations (0, 100, 250, 500, 1000μM) of 2,4-D treatments. The data were plotted from means of three replications (n=3) ±SE. Bars showing different letters indicate significant differences between treatments according to Duncan's test at p≤0.05.

Table 2. Table representing the antioxidant effect of phenolic concentrations of different 2,4-D treated *Azolla* plants on reduction of DPPH radical scavenging.

DPPH radical scavenging	
Different concentrations of 2,4-D treated *Azolla* Plants	IC 50 value: in μg phenolic contents
Control	0.96
100 μM	0. 92
250 μM	0.87
500 μM	0.47
1000 μM	0.65

ABTS Cation Decolourization Efficiency

In more modified way the scavenging of free radicals was deduced by ABTS[+] through the percentage of inhibition for its de-colourization (Figure 4). The activity of ABTS de-colourization from 100mg/μl methanolic extract from 2,4-D contamination was in a more discriminating trend. Though activity with BHT as standard through percentage of inhibition was dose-dependent, still, for the experiment the maximum inhibition was recorded as the initial concentration (100μM). As compared to the control it was significantly (p≤0.05) over-expressed

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by 1.63 fold and thereafter it was almost plateau towards higher concentration of 2,4-D. The values of percentage inhibition of ABTS scavenging were in the order of 0.83 fold, 0.94 fold and 1.12 fold against the control and was significant ($p \leq 0.05$). Thus, the pattern of IC_{50} value was equivalent to percent inhibition of ABTS decolourisation that was deduced in Table 3. The analysis of plant anti-oxidation for ABTS was more inconsistent, particularly, at the highest concentration of 2,4-D. In another way when total flavonoid was quantified which may be responsible for antioxidation, it recorded a non-significant correlation ($R^2 = 0.312$) at both $p \leq 0.05$ and $p \leq 0.01$ level of significance.
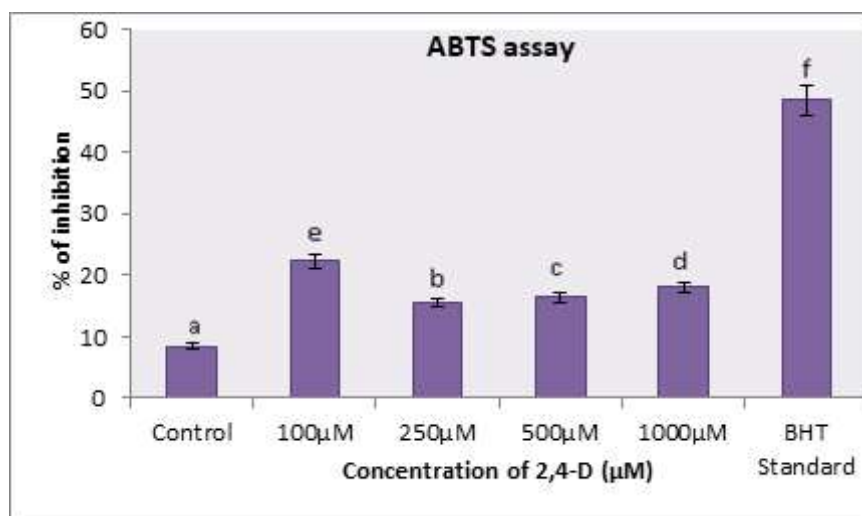

Figure 4. Percentage of inhibition by ABTS radical scavenging assay in *Azolla* plants under different concentrations (0, 100, 250, 500, 1000µM) of 2,4-D treatments. The data were plotted from means of three replications (n=3) ±SE. Bars showing different letters indicate significant differences between treatments according to Duncan's test at $p \leq 0.05$.

Table 3. Table representing the antioxidant effect of phenolic concentrations of different 2,4-D treated *Azolla* plants on reduction of ABTS radical scavenging.

ABTS radical scavenging	
Different concentrations of 2,4-D treated *Azolla* Plants	IC 50 value: in µg phenolic contents
Control	0.29
100 µM	0.11
250 µM	0.16
500 µM	0.15
1000 µM	0.13

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Determination of Flavonoid Content

The most commonly occurring antioxidant, the flavonoid, was more consistent in its accumulation with increasing concentration of 2,4-D (Figure 5). It indicates that the plants responded to oxidative stress in a more inductive manner that was recorded maximum (1000µM) 0.52 fold against the control. However, the intermediate concentrations may not be significant (p≤0.05) and thus, plants insensitivity to minimum dosses of 2,4-D for oxidative stress (Figure 9). For the contribution of non-enzymatic quenching of ROS the flavonoid content may be tallied total anti-oxidation capacity in a linear manner (R^2=0.34 & 0.312 for DPPH and ABTS respectively). However, the intra-herbicidal concentrations were not readily significant (p≤0.05) as deduced from Table 1.
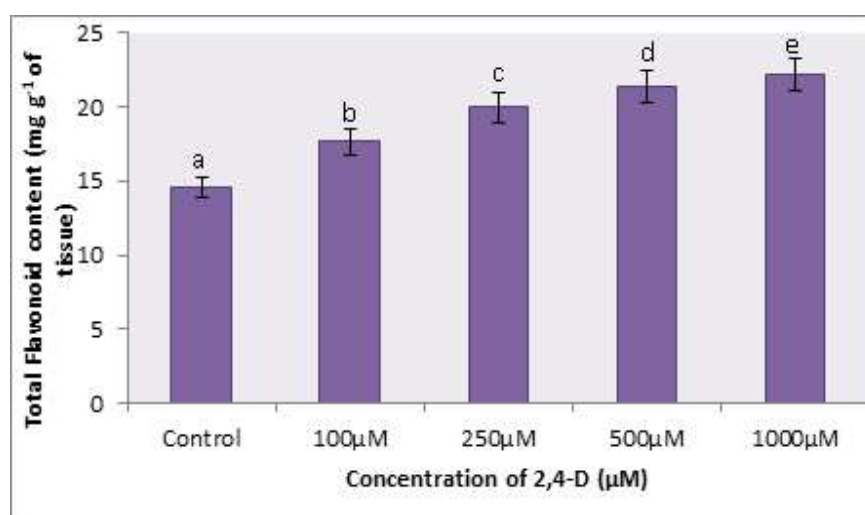

Figure 5. Total flavonoid content assay in *Azolla* plants under different concentrations (0, 100, 250, 500, 1000µM) of 2,4-D treatments. The data were plotted from means of three replications (n=3) ±SE. Bars showing different letters indicate significant differences between treatments according to Duncan's test at p≤0.05.

Effect of 2,4-D on In-vitro Enzymatic Anti-oxidation

In another module the activity of plant extract recovered from different 2,4-D concentrations recorded also some variations in their effectivity against ROS. Initially, when peroxidase activity was concerned (Figure 6), taking guaiacol reduction the range of variations were 0.02 fold to 0.14 fold. Statistically it was insignificant irrespective of 2,4-D concentrations at both levels (p≤0.05 and p≤0.01). Therefore, the 2,4-D mediated oxidative stress may not be directly influencing the antioxidation however, enzymatic through total antioxidation pathway. Table 3 presents the contribution of enzymatic path with other modules of antioxidation profiles. Catalase, the enzyme not requiring any electron donors was found to be more sensitive regardless of 2,4-D concentrations. The maximum and minimum activity of catalase was recorded at 100µM and 1000µM as

to control by 0.151 fold and 0.365 fold respectively (Figure 7). Still, catalase from its highest concentration of 2,4-D are found to be more contributory to phosphomolybdenum assay which otherwise reflects the total antioxidation, however, in negative manner (R^2=-1.25) (Table 1). The intermediate concentrations of 2,4-D might have no effective potential on reduction of activity and thus remains not significant ($p \le 0.05$ and $p \le 0.01$).

PPO though not recognized as antioxidative enzymes, its contribution to oxidised polyphenol is effective on total antioxidation pathway. Thus, when the reduction in concentration of polyphenol was indexed through PPO activity it had a correlation of 1.08 with both phenol and flavonoid concentration in the *Azolla* tissues (Table 3). The activity kinetics of PPO recorded to be limited with a threshold concentration of 2,4-D and the highest activity was 1.57 fold over expressed as compared to the control (Figure 8). Moreover, the limitation of activity for phenol oxidation was insensitive at maximum 2,4-D concentration which may otherwise reflects either the disintegration of antioxidising moieties or its *de-novo* biosynthesis.
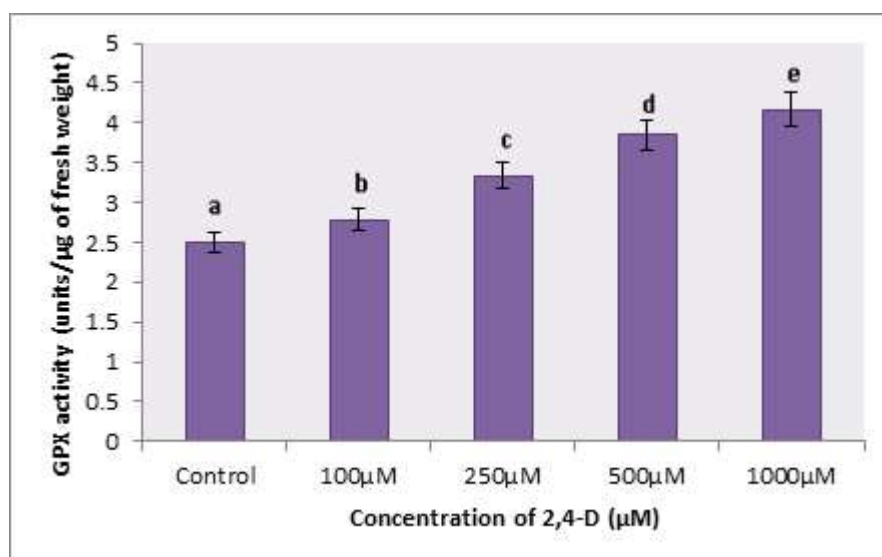

Figure 6. Peroxidase activity in *Azolla* plants under different concentrations (0, 100, 250, 500, 1000µM) of 2,4-D treatments. The data were plotted from means of three replications (n=3) ±SE. Bars showing different letters indicate significant differences between treatments according to Duncan's test at $p \le 0.05$.
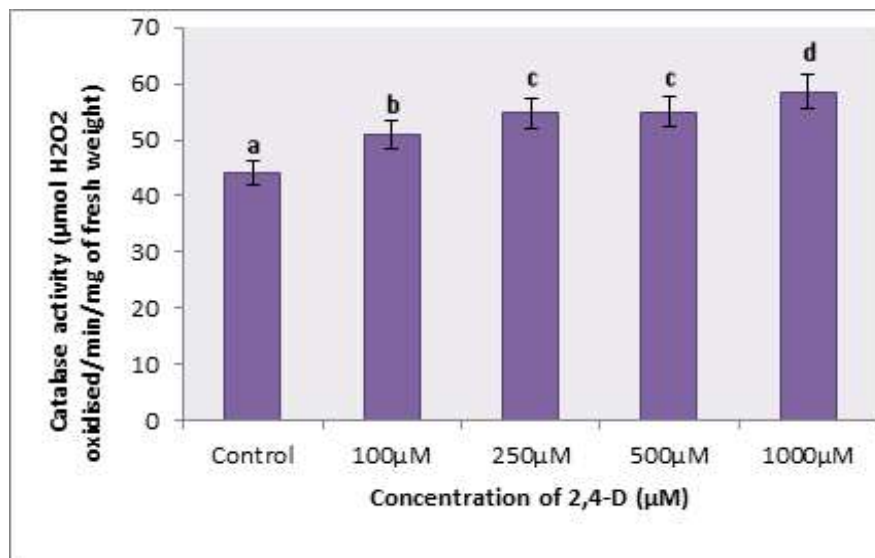
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Figure 7. Catalase activity in *Azolla* plants under different concentrations (0, 100, 250, 500, 1000µM) of 2,4-D treatments. The data were plotted from means of three replications (n=3) ±SE. Bars showing different letters indicate significant differences between treatments according to Duncan's test at p<0.05.
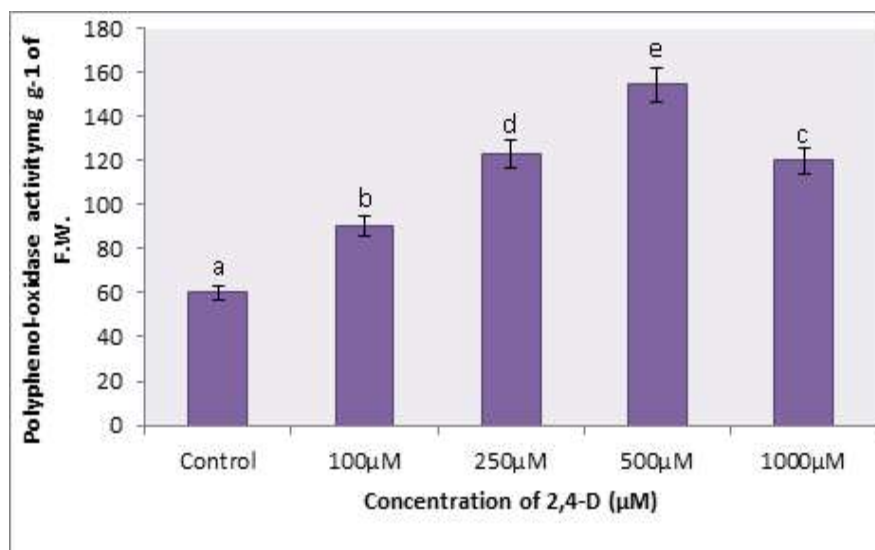

Figure 8. Polyphenol-oxidase activity in *Azolla* plants under different concentrations (0, 100, 250, 500, 1000µM) of 2,4-D treatments. The data were plotted from means of three replications (n=3) ±SE. Bars showing different letters indicate significant differences between treatments according to Duncan's test at p≤0.05.

Determination of Ferric Reducing Power

The ability of the plants to overproduce non-thiol proteins and conjugates is required to reduce macromolecules. *Azolla* exhibited some striking behaviour in a discriminate manner to reduce the Fe^{3+} ions. It is interesting that at initial concentrations, the plant was showed its potential to elevate its reducing power by 0.553 fold, 0.245 fold and 0.0943 fold respectively for 100, 250, 500μM at p≤0.05 as compared to the control (Figure 9). Thereafter, the plant was down regulated by 18.65 % of activities, but not significant against the control. At 1000μM of 2,4-D, the plant again geared up the reducing activities by against the penultimate concentration. This rising in activities may not be any significant change over the control, yet it still indicates the plant's recovery from oxidative exposure to 2,4-D.
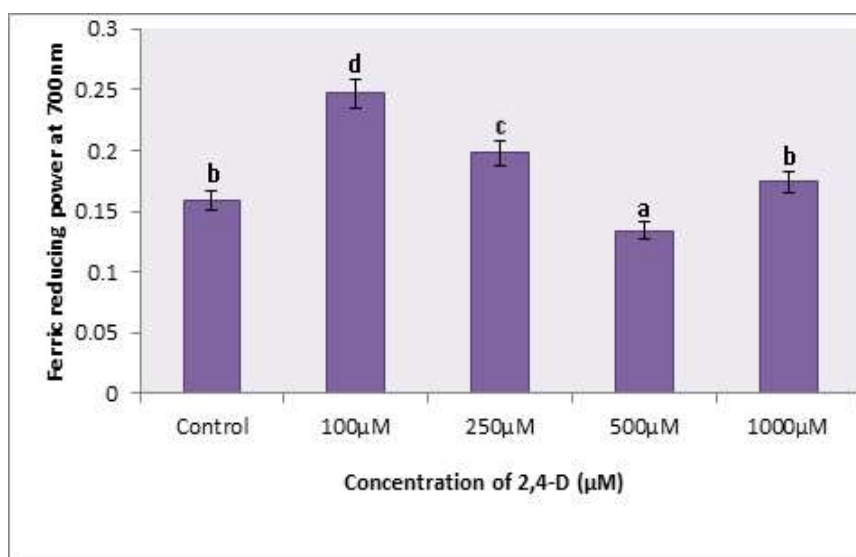

Figure 9. Ferric reducing power in *Azolla* plants under different concentrations (0, 100, 250, 500, 1000μM) of 2,4-D treatments. The data were plotted from means of three replications (n=3) ±SE. Bars showing different letters indicate significant differences between treatments according to Duncan's test at p≤0.05.

DISCUSSION

2,4-D is absorbed by the roots of *A. pinnata* quickly and is partitioned at varying concentrations in the tissues (Bhupinder Dhir 2013). From earlier studies, *Azolla* showed differential response to 2,4-D phytotoxicity in terms of physiological activities like chlorophyll loss, nutrient imbalance, impairment of nitrogen metabolism and restriction of growth (De et al 2017). However, the plant exhibited a suite of detoxification paths particularly to minimize oxidative stress in response to a number of xenobiotics and pollutants. In our earlier experiment under varying concentrations of 2,4-D, *Azolla* plants showed a gradient of oxidative stress responses (De et al 2016). This indicates the probability of antioxidation activity in the tissue extract as assessed different *in-vitro* methods.

be appropriate to reach any conclusion, particularly under the influence of any abiotic stress factors.

In the present experiment, *Azolla* may have produced a sufficient amount of ROS when induced with 2,4-D. Initially, we have measured the different particles of ROS in different parts of tissues and the activity of 2,4-D appeared to be tolerated by this species (De et al 2016). The phenolic content of this plant species, irrespective of the concentration of 2,4-D, revealed a dose-dependent total antioxidant activity. This was attributed both to DPPH and ABTS radical scavenging system *in-vitro*. It is interesting to note that the total antioxidant is dependent on the efficiency of the plant species of its hydrogen donation reactions. Their effectiveness either by enzymatic or through reducing agents (antioxidants) in those reactions are also important (Amarowicz et al 2004). In both cases of DPPH and ABTS with BHT as pure antioxidants, the plant extract under 2,4-D concentrations had a significant lesser antioxidation effectivity. Still, *Azolla* showed its inductive potential for antioxidation in a gradient manner of 2,4-D. Mostly the phenolic constituents are the predominant residues in the plant extract that contribute to the antioxidant effect. The study also showed that the correlation coefficient between phenolic concentration and DPPH/ABTS system was not consistent. This suggests that other pathways are involved in 2,4-D mediated oxidative stress remediation.

In an earlier paper, we reported that lower vascular plants, particularly, pteridophytes are good sensitive species to induced antioxidation, mostly by metal or metalloid toxicity (De et al 2016). In fact, pteridophytic are least known in terms of their antioxidation pathways under xenobiotics stress except for *Pteris vittata* (Kaur et al 2017). *Azolla pinnata*, an aquatic N_2 fixing fern, has also enriched its genetic plasticity to accommodate more xenobiotics (like 2,4-D) establishing it as a good plant for hyper accumulation. Besides its sequestering efficiencies for xenobiotics within cellular or non-cellular spaces the quenching ability for 2,4-D is also noticeable. Thus, the higher values of IC_{50} at higher concentrations of 2,4-D for DPPH and ABTS may be a reliable bio-indication to the herbicide. In pteridophytic species, development and accumulation of phenolics mostly in the form of flavonoids and iso-flavonoids are indexed against a few abiotic stressors like water, salinity and even factors inducing oxidative stress (Kang 2013). In the present experiment, the correlation of total flavanoids and phosphomolybdenum complex are the result of complex reaction with phenolic concentration. Molybdenum from its oxidized state Mo(VI) is reduced to Mo(V) state. The formation of phosphomolybdenum complex may suggest flavonoid accumulation and its chelation to 2,4-D. Thus, the optimum concentration for 2,4-D inducing the maximum ROS generation may be regulated and plants are stabilised in ROS homeostasis.

For the crop species, the terpenoids (eg, carotenoids) are required to contribute to the structural defence against ROS in the cell organelles (like chloroplast) (Ahuja et al 2012). On the other hand, antioxidants are the major chemical moieties for direct and indirect lysis of ROS. The flavonoids with their unsaturated nucleus absorb excess energy of ROS. This could be attributed to single electron reduction through hydrogen (H^+ + e^-) donation. Aquatic environment is the major source of xenobiotics due to more dissolution and rapid absorption processes. Similarly, *Azolla* might have equal chance to absorb the residual herbicides like 2,4-D from the soil and may thereby induce flavonoid biosynthesis for non-enzymatic antioxidation. Related studies on aquatic ferns

antioxidation pathway. Thus, we also evaluated the *Azolla* leaf extract and its purified fraction for *in-vitro* enzymatic antioxidation. Three enzymatic proteins in partial purified forms were analyzed with reference to lysis of H_2O_2 and oxidation of polyphenolics. Peroxidase with phenolic residue (guaiacol as electron donor) was important which has been considered against herbicidal treatments. It is interesting to note that *Azolla* when exposed to higher concentration of H_2O_2 as ROS resulted in changes in the activity of GPX although the changes were not significant. The insignificant changes of GPX activity may suggest that the accumulated H_2O_2 is tolerable to the *Azolla* at a certain tissue concentration. H_2O_2 when it does not cross the threshold value of toxicity is an inducer for different cellular processes (Bhaskaran & Kannappan 2017). The H_2O_2 may also serve as a modulator for other antioxidants and enzymes in downstream order of metal toxicity (Mandal et al 2013). Therefore, the stability of peroxidase activity over extended doses of 2,4-D may also be bio-indexed for *Azolla* species.

A similar trend of activities was also recorded in the case of catalase where plants required no phenolic residue as electron donor for lysis of H_2O_2 (Molina & Kahmann 2007). Catalase intolerance to xenobiotics is taken as good biomarkers in many plants, particularly, for its number of iso-forms varying in cellular compartments. In earlier studies, catalase has been more sustainable among the antioxidative proteins when ROS mediated damages are confirmed by xenobiotics (De et al 2016). The reducing capacity of *Azolla* under 2,4-D stress is interesting to note for its overall maintenance of cellular homeostasis towards optimum redox. It enables the plant's genetic plasticity to maintain a steady ratio of reduced to oxidised non-protein thiol. This imparts the sustenance of bio molecules more in their reduced state for thermodynamic stability (Martínez & Araya 2010). The results confirm the findings of an increased conversion of Fe^{3+} to Fe^{2+} (R^2= 2.1) (Table 3). The reducing capacity is a reflection of the number of reductants in a compound promoting antioxidation by donation of electron ($H^+ + e^-$). Therefore, the *Azolla* species despite of xenobiotics mediated oxidative stress is likely to curtail the radical chain reactions of unsaturated moieties (Farkas 2008). With this view, the accumulation of reductants in *Azolla* might cause the reduction of iron-ferricyanide complex to its ferrous form in significant amounts of polyphenolics. This could add another biomarker with polyphenolics in *Azolla*, already been evident as a xenobiotics quencher (Prasad et al 2015).

CONCLUSION

The study revealed that *Azolla pinnata* R.Br. possesses major antioxidant property. Importantly, this plant species showed dual effectivity through hyper accumulation of the herbicide 2,4-D and its concomitant induction of antioxidation. The fern species has equivalent bioaccumulation of flavanoids, anthocyanin and other polyphenolics which might be linked to its tolerance to ROS. In modules of enzymatic antioxidants, *Azolla* was quite sensitive to peroxidation of H_2O_2 by different isozymic proteins. A significant participation of polyphenol oxidase and catalase were more pronounced whereas peroxidases were least significant in *in vitro* assay. Taking altogether both enzymatic and non-enzymatic antioxidation indices, *Azolla* is an efficient quencher species for herbicide contaminated soils. It has potential for use in environmental cleanup particularly in soils contaminated with herbicides and other xenobiotics.

Azolla pinnata R.Br.

ACKNOWLEDGMENT

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