Bio indices for 2,4-D sensitivity between two plant species: *Azolla pinnata* R.Br. and *Vernonia cinerea* L. with their cellular responses

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Received: 13 May 2016/Revised: 12 August 2016/Accepted: 16 August 2016/Published online: 30 August 2016
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Abstract In the present experiment a pteridophytic species *Azolla* and an angiospermic species *Vernonia* were evaluated on the basis of cellular reactivity for herbicidal action through ongoing concentrations. Initially, both the species recorded a significant activity of IAA-oxidase as mark of IAA metabolism with herbicidal sensitivity. Still, *Vernonia* species were more affected on 2,4-D mediated auxin catabolism. The loss of auxin concentrations on the tissues by 2,4-D reaction was also reflected on growth parameters including relative growth rate and chlorophyll biosynthesis. In a dose dependent manner *Vernonia* plants were more affected with loss of chlorophyll content and decline in relative growth rate. On the other hand, both those parameters were adjusted significantly with 2,4-D accumulation in *Azolla*. The stability of cellular metabolism was documented by significant down regulation of protein and lipid peroxidation with concomitant moderation to superoxide and hydrogen peroxide accumulation. The later two were more vulnerable to damage in the *Vernonia* plant with profuse accumulation of protein and lipid peroxidation products. Similarly, tissue specific reaction to superoxide and hydrogen peroxide accumulation were distinctly demarcated in two species significantly. As a whole, the cellular responses and metabolite distribution to 2,4-D sensitization are the features to describe bio-indices for

aquatic fern species *Azolla* with comparison to angiospermic species *Vernonia*.

Keywords Herbicide · Oxidative stress · Aquatic fern · Auxin metabolism

Abbreviations

IAA	Indole-3-acetic acid
2,4-D	2,4-dichloro-phenoxy acetic acid
ROS	Reactive oxygen species
O ₂ [−]	Superoxide radical
H ₂ O ₂	Hydrogen peroxide
RH	Relative humidity
TCA	Trichloro acetic acid
NBT	Nitro blue tetrazolium
DAB	Di-amino benzidine
KI	Potassium iodide
MDA	Malondialdehyde
DNPH	2,4 Dinitrophenylhydrazene
RWC	Relative water content

Introduction

Plants being an integral part of terrestrial ecosystem have enough capacity in continue conversion of various xenobiotics of the soil in more degraded forms. Though the xenobiotics with their coherent chemical structure are not completely recycled into environment, still, with the plants chemical modification those are transformed into smaller moieties. Within agricultural fallows restriction of the soil nutrients for the more absorbable forms are the results in indiscriminate use of xenobiotics (Greenberg et al. 2008). Herbicide being of those has significant affects on soil properties, reduction in diversity of habitats as well as

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compromisation of other crop species nutrient use efficiencies. Therefore, use of herbicides is a serious threat to the adjacent or interspersed species within the biodiversity of agricultural field. It has been worked out that herbicides spreading to adjacent or even off-target zones might be effective in sensitive or non-tolerant target plant also (Brazier et al. 2002). Therefore, plant species has been in question to tolerant as well as sequestering residual herbicide moieties or left-over in applied crop plants.

Plant species, mostly those are non-angiospermic are the major focus area in herbicides quenching mechanism. The early vascular land plants, mostly the ferns where metal hyper accumulation, sequestration and quenching its effect have been studied more critically. Studies on *Pteris vitata*, the foremost fern species with adequate arsenic tolerance has thrown insights for metal tolerance in pteridophytes (Wang et al. 2002). In succession the maximisation in probability for fern species to quench metal stress has been established in different ferns mostly in aquatic habitat. *Marsilea*, *Salvinia* and *Azolla* are the cited species for different metal hyper accumulation in more soluble forms in aquatic habitats (Mandal et al. 2013; Das et al. 2013; Oren et al. 2004). With this interaction of metal or organo metallic compounds are also in quest for nullifying their effects by these aquatic fern species. Xenobiotics like herbicides which are either systemic, partly systemic or contact in nature should be interesting in plant herbicide interaction as well as its remediation. Categorically, systemic herbicides with their most established knock-down effects to plants are readily absorbed by roots following its steady transport through vascular tissues in different plant parts. 2,4-D is a major systemic herbicide which alters the auxin metabolism in such a way that almost the affect of later in overall growth processes is compromised. As a whole the growth indices are referred to be down regulated in such a way that very often it is confused with auxin deficiencies in plants.

So, target oriented investigation in focusing the physiological traits for herbicide sensitivity or tolerance should be prospect full. With reference to aquatic fern species the solvation of herbicides in soil into more portable forms for plants would raise the possibility for quenching of such xenobiotics directly from crop fallows. Therefore, the status of one common terrestrial fern *Pteris vitata* would no less be supportive in gathering information for herbicide tolerance with pteridophytic flora under simulated condition of herbicides. In addition, extent of variability on such reaction in this fern species as compared to the higher angiospermic plant would also be a comparative account in literature how the former one would be advanced than another. In more hope the fern species will be assigned for bioremediation of xenobiotics where application is common in fallows for crops. The herbicide used as 2,4-D

typically applied for broad leaf plants and thus *Vernonia cinerea* L., a member of compositae was selected against *Azolla pinnata* R.Br. on this comparative account.

Materials and methods

Plant material

Azolla pinnata R.Br., a free-floating aquatic fern, member of Azollaceae and *Vernonia cinerea* L., a broad leaf weed, member of Compositae was chosen as the plant material for the present experiment.

Plant growth and treatment

Azolla pinnata R.Br. the plant material was collected from the Regional Fodder Station, Kanchrapara, Kalyani, W.B, India and the *Vernonia cinerea* L. was collected from the University campus. Plants were grown in fresh water, then collected and washed with de-ionized water before using those for experimental purpose. Plant samples were then transferred into ¼ th strength of Murashige and Skoog's media (Murashige and Skoog 1962) for 7 days to acclimatize. Thereafter, those plants were treated with varying concentrations (0, 100, 250, 500 and 1000 µM) of herbicide 2,4-Dichlorophenoxyacetic acid (2,4-D). The experimental sets were kept in a greenhouse condition under 37 ± 1 °C of temperature, 65–85 % of relative humidity (RH) and photoperiod of 14–10 h light and dark for 7 days. Thereafter, the plants were harvested and frozen in liquid nitrogen and stored at –80 °C for further biochemical assays.

Determination of relative growth rate

For the determination of RGR, the plants were recovered from 2,4-D treatments and washed thoroughly in de-ionized water for recording the fresh plant weights. Thereafter, the plant samples were completely dried in hot air oven under 80 °C for 5 days. On completion, the dried plant weights were recorded and determined the relative growth rate according to Jampeetong and Brix (2009).

Determination of chlorophyll content

Chlorophyll content was determined according to Porra et al. (1989). For the estimation of chlorophyll content 0.5 g of each plant samples were taken into test tube containing 10 ml of methanol and kept in dark for 24 h. After this the supernatant from each test tube was taken for measurement of absorbance at 652 and 665 nm by the help of a UV–VIS Spectrophotometer (Cecil, Model No. CE7200).

Calculation ($\mu\text{g/ml}$) =

$\text{Chl } a = 16.29 A_{665} - 8.54 A_{652}$

$\text{Chl } b = 30.66 A_{652} - 13.58 A_{665}$

$\text{Chl } (a + b) = 22.12 A_{652} + 2.71 A_{665}$

Determination of superoxide (O_2^-) content

For determination of the extracellular generation of O_2^- content from the control and treated plants, 1 g of plant tissue was homogenized in liquid nitrogen by adding 100 mM Tris-Cl buffer (pH 6.5). The homogenate was centrifuged at $7000 \times g$ for 20 min at 4 °C. Thereafter, the supernatant was collected and added within 3 ml of assay mixture containing 50 mM Tris-Cl (pH 6.5), 0.2 mM NBT, 0.2 mM NADH and 250 mM sucrose for 12 h in dark. The absorbance was read at 530 nm. The concentration of O_2^- was derived by using the extinction coefficient of $12.8 \text{ mM}^{-1} \text{ cm}^{-1}$ (Achary et al. 2007).

Determination of hydrogen peroxide (H_2O_2) content

Determination of H_2O_2 content of control and treated plants were done according to Ghosh et al. (2011). 500 mg of plant tissue was homogenized in liquid nitrogen by adding 2 ml of 1 % (w/v) trichloro acetic acid (TCA). The homogenate was centrifuged at $10,000 \times g$ for 15 min at 4 °C. Thereafter, the supernatant was collected and added within assay mixture containing 10 mM potassium phosphate buffer (pH 7) and 1.0 M potassium iodide (KI), 1 mM 2,4-dichlorophenol indophenol, 50 mM manganese chloride and 0.2 mM NADH followed by incubation in dark for 20 min. The absorbance was read at 390 nm. The H_2O_2 content was derived using a standard solution.

Determination of IAA-oxidase activity

IAA oxidase enzyme activity was determined according to Ebrahimzadeh and Abrishamchi (2001). 0.5 g of fresh roots were washed with de-ionised water, blotted to dry and homogenized with 0.1 M phosphate buffer (pH 7) in prechilled mortar and pestle. Homogenate was filtered through 4 layered muslin cloths and centrifuged at $15,000 \times g$ for 20 min at 4 °C. The supernatant was used as an enzyme source. Thereafter, the supernatant was collected and added within assay mixture containing 3 ml 0.1 M phosphate buffer (pH 6.5), 2 ml para-coumaric acid (0.5 mg ml^{-1}), 0.5 ml 0.002 M MnCl_2 and 4 ml IAA (0.05 mg ml^{-1}) was added to start the reaction. Incubate the reaction mixture in dark with shaking at 30 °C. During the enzymatic reaction 2 ml of assay mixture was pipetted out after 0 and 50 min of incubation and 5 ml mixture of sulphuric acid and FeCl_3 was added to it and mixed well

and kept in dark for 30 min. After incubation absorbance of each reaction mixture was then recorded at 535 nm.

For in-gel analysis of IAA-oxidase the enzyme extract was loaded in a 10 % native PAGE at 10 V/lane under cold condition. The detection of specific bands of polypeptide were resolved in 250 mM phosphate buffer (pH 7) and stained by o-dianisidine solution (20 mg o-dianisidine stain added in 1 ml of glacial acetic acid) followed by addition of 500 μl of 0.5 % H_2O_2 with gentle agitation.

Determination of lipid peroxidation (MDA content)

For determination of malondialdehyde content 1.0 g of fresh tissue from control and treated plants were homogenized in liquid nitrogen by adding 20 % (w/v) trichloroacetic acid (TCA) and 0.5 % thiobarbituric acid (TBA). The homogenate was incubated at 90 °C for 30 min, thereafter it was placed on ice for another 30 min followed by centrifugation at $12,000 \times g$ for 15 min. The absorbance of the supernatant was read at 532 and 600 nm as suggested by Dhindsa et al. (1981).

Determination of protein oxidation (carbonyl content)

Determination of carbonyl content was conducted by dinitrophenyl hydrazene (DNPH) reagent according to Verbeke et al. (2000). 1.0 g of fresh tissue from control and treated plants were homogenized in liquid nitrogen by adding 10 % (w/v) sodium dodecyl sulphate (SDS) followed by incubation at room temperature for 30 min. To this homogenate 10 mM DNPH and 1.5 mM TCA was added followed by gentle agitation. The pellet was suspended with 0.2 M sodium phosphate buffer (pH 7.0) and the absorbance was read at 360 nm. Finally, the carbonylated derivative of protein was calculated using the molar extinction coefficient ($530 \text{ M}^{-1} \text{ cm}^{-1}$) of DNPH. The protein content of the sample was determined with Bradford reagent (Bradford 1976).

Histochemical detection of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) accumulation

In vivo detection of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in control and treated plants were done according to Mostofa et al. (2015). The roots were collected from two species and thoroughly washed. The tissues were incubated in a solution of 0.2 % NBT solution (0.1 g NBT in 50 mM phosphate buffer, pH 7.5) for overnight. The excess stain was drained out and washed with double distilled water followed by fixation and mounting with 60 % glycerol. For in vivo detection of H_2O_2 , the tissues were complete immersed 1 mg/ml DAB

aqueous solution diluted prior used (50 mg DAB in 50 ml of distilled water) and kept overnight. The demarcation of brown coloration in affected zones were marked through microscopic observation and recorded.

Determination of proline content

1 g of fresh plant tissue was crushed in 3 % aqueous sulphosalicylic acid. Thereafter, the homogenates were centrifuged at $10,000\times g$ for 10 min, the supernatant was collected and reaction was started by adding acid-ninhydrin solution (1.25 g ninhydrin in 30 ml glacial acetic acid). Reaction mixtures were heated at 95 °C for 1 h after getting cooled; they were extracted with 4 ml toluene by vortexing for 1 min. The upper toluene layer was then pipetted out and absorbance was read at 520 nm, using toluene as a blank and expressed as μM proline/g of fresh tissue (Bates et al. 1973).

Determination of relative water content (RWC)

Relative water content was measured according to Weatherley (1965). 1 g of control and treated plants were taken after 7 days of treatment and fresh weight (FW) was recorded. Then the plant samples were dried at 80 °C for 3 days and dry weight (DW) was recorded. Those dried samples were rehydrated freshly for 3–4 h, until the weight remains constant and thereafter, the turgid weight (TW) was recorded.

Calculation:

$$\text{RWC} = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW})$$

Statistical analysis

All the data were recorded with three replications ($n = 3$) and data were expressed as mean $\pm$ SE. The statistical analysis was performed by one-way (ANOVA) followed by least significance difference (LSD) test taking $p \leq 0.05$ levels of significance (Gomez and Gomez 1984). Windows Microsoft Excel 2007 software was employed for computation and data analysis.

Results

The present investigation compares the physiological responses of herbicides in *Azolla* and *Vernonia* species. The two species have reacted with varying degrees of sensitivity through some physiological expressions. Initially, *Azolla* recorded normal sustainability over 2,4-D regardless of concentrations, whereas, *Vernonia* had developed few syndromes via growth and chlorophyll

biosynthesis. Thus, at highest 2,4-D concentration the changes in RGR recorded 74.60 % decline in *Vernonia*. In case of *Azolla* the RGR was more stable initially followed by a decline maximally by 24.37 % as compared to the control (Fig. 1). The fractions of chlorophyll-a, b and its total content vary significantly between two species at all the 2,4-D concentrations. For *Vernonia*, total chlorophyll was subdued by 57.63 % in accumulation over control, whereas the same was significantly ($p \leq 0.05$) up regulated with 2.78 fold over control in *Azolla* (Fig. 2). The development of oxidative stress under herbicide interaction in sensitive species is quite common in plant sustenance over xenobiotic contamination. In accompany with tissue specific O_2^- accumulation, the maximum values with 4.26 fold and 3.38 fold were recorded for *Vernonia* and *Azolla* respectively over control (Fig. 3). Similarly, another ROS, the H_2O_2 was found more accumulated in *Azolla* (6.57 fold) than *Vernonia* (10.31 fold) as compare to control (Fig. 4). A linear and significant ($p \leq 0.05$) relationship exists between concentration of 2,4-D with O_2^- and H_2O_2 accumulation for both species. Thus, for tissue specific O_2^- a clear indication of O_2^- accumulation more with *Vernonia* than *Azolla* was evident from in vivo staining. The cortical tissues were demarked with formazon accumulation by NBT reaction for *Azolla*, whereas for *Vernonia* it was the central conductory tissues was more intense with same reaction (Fig. 5A, B). In distribution of H_2O_2 with tissue specificity was found with varying coloration with DAB on the hypodermal region to the outer cortical tissues for *Azolla*. However, the *Vernonia* assumed to be more sensitized with H_2O_2 only within the cortical tissues and more diffused althrough the xylary strands (Fig. 6A, B). In consequence of ROS accumulation by 2,4-D treatment, the oxidative degradation of bio molecules mostly in lipid and proteins were analyzed in the present study. A dose

Fig. 1 Relative Growth rate in *Azolla* (A) and *Vernonia* (B) grown under varying concentration 0 μM (Control), 100, 250, 500 and 1000 μM of 2,4-D. The experimental data are plotted from means of three replications ($n = 3$) $\pm$ SE. Bars showing different letters indicate significant differences according to Duncan's test at $p < 0.05$

Fig. 2 Chlorophyll content in *Azolla* (A) and *Vernonia* (B) grown under varying concentration 0 µM (Control), 100, 250, 500 and 1000 µM of 2,4-D. The experimental data are plotted from means of three replications ($n = 3$) $\pm$ SE. Bars showing different letters indicate significant differences according to Duncan's test at $p < 0.05$

Fig. 3 Generation of superoxide in *Azolla* (A) and *Vernonia* (B) grown under varying concentration 0 µM (Control), 100, 250, 500 and 1000 µM of 2,4-D. The experimental data are plotted from means of three replications ($n = 3$) $\pm$ SE. Bars showing different letters indicate significant differences according to Duncan's test at $p < 0.05$

dependent relationship for both lipid peroxidation and protein oxidation is evident for both *Vernonia* and *Azolla*, however, variably. Thus, the extent of MDA content ranged from 1.46 to 3.46 fold and 1.28 to 3.01 fold for *Vernonia* and *Azolla* (Fig. 7) respectively although the 2,4-D concentrations. The product of protein oxidation (carbonyl content) was more moderated in *Azolla* (2.78 fold) than *Vernonia* (3.41 fold) at highest concentration of 2,4-D as compare to control (Fig. 8). Physiologically the variation in compatible solutes in status of plant water relation was evident from *Azolla* and *Vernonia*. The dehydration stress on 2,4-D accumulation was recorded in RWC by a steeper rise with 1.73 and 2.44 fold maximally in *Azolla* and *Vernonia* respectively (Fig. 9). 2,4-D could also impart the effects of plant water relation as evident from compatible solute like proline for both in *Azolla* and *Vernonia*

Fig. 4 Accumulation of hydrogen peroxide in *Azolla* (A) and *Vernonia* (B) grown under varying concentration 0 µM (Control), 100, 250, 500 and 1000 µM of 2,4-D. The experimental data are plotted from means of three replications ($n = 3$) $\pm$ SE. Bars showing different letters indicate significant differences according to Duncan's test at $p < 0.05$

(Fig. 10). The accumulation for the proline recorded an significant changes ($p \leq 0.05$) with maximum value 2.33 and 1.09 fold for *Azolla* and *Vernonia* respectively. The extraction and partial purification of the total protein for analysis of IAA-oxidase activity both in vitro and in-gel staining (for polymorphic variation of protein) were done in these species. Activity of IAA-oxidase in between two species was most discriminating in support of role of auxin in growth as interrupted by 2,4-D. So, *Vernonia* appeared most sensitive to induce 4.61 fold over expression activity than control. This was quite inconsistent for *Azolla* through an initial up regulation followed by a decline, however, 3.37 fold increased at highest concentration of 2,4-D (Fig. 11). In case of *Azolla*, at the protein level though a single band representing the polymorphism for IAA-oxidase, still, its variation through treatments proved its inductive nature. On the contrary, *Vernonia* was more responded in expression of polymorphism with two alternative bands varying in concentrations through 2,4-D concentrations (Fig. 12A, B). In close observation, the first polymorphic band was distinctly expressed in highest concentration of 2,4-D, whereas, the next one in succession was weak in expression at the same concentration over control. This arises the possibility for the first band having more involvement for increased activity attributing the IAA metabolism, if any, for both the species under 2,4-D toxicity.

Discussion

It is quite obvious that *Azolla* plants have developed some in-built strategies or induced in expression of tolerance against xenobiotics as studied with reference to 2,4-D in the present experiment. The later refers that herbicide

Fig. 5 A Detection of superoxide anion accumulation in roots (marked in circle) of *Azolla* plants under varying concentrations 0 μM (Control), 100, 250, 500 and 1000 μM of 2,4-D for 7 days.

B Detection of superoxide anion accumulation in roots (marked in arrow) of *Vernonia* plants under varying concentrations 0 μM (Control), 100, 250, 500 and 1000 μM of 2,4-D for 7 days

which is an auxin derivative in nature. Therefore, the summation of auxin mediated physiological responses might have changed under 2,4-D treatments. The RGR imparts a direct relationship to the dry matter accumulation, allocation and partitioning of biomass in plants (Weraduwaage et al. 2015). In *Azolla*, a concomitant change of RGR, however, maintained a stable rate initially and thereafter it declined. This differs in *Vernonia* where the RGR is compromised throughout the incubation period that denotes more sensitivity in growth concerned. Therefore, *Vernonia*, a broad leaf 2,4-D sensitive species are trailed by *Azolla* in accommodation of photosynthetic dry matter accumulation. In connection with growth parameters of plants, these are demarked by photosynthetic activity as preliminarily indicated by chlorophyll accumulation. This is also reflected in biosynthesis of chlorophyll through its different fractions but with a discriminating trend in *Azolla* and *Vernonia* as found in the present study. Initially, the down regulation of chlorophyll might be attributed in two ways: retarded biosynthesis and its concomitant breakdown or turnover. On the other hand, the sustenance of chlorophyll accumulation on prolong treatment of 2,4-D may also be traced as genotypic potential of *Azolla* species. Regulation in ethylene biosynthesis even under metal pollution

has also been marked in some aquatic ferns to nullify the chlorophyll breakdown (Grossmann et al. 2001). Auxin herbicide causes accumulation of ethylene following ABA up regulation and has also been a bio indicator in varied manner with other cellular responses to tolerate xenobiotics (Hansen and Grossmann 2000). This behaviour might also fit well with the *Azolla* species herein for its accommodation of 2,4-D via cellular mode of resistance. 2,4-D with its impairment on auxin metabolism would also have some involvement in oxidative stress. It is somehow related to the imbalance of auxin and ethylene inter-relationship which results an elevated redox in the tissues (Csiszar et al. 2004). In earlier literature it has also been referred that auxin herbicide like 2,4-D indirectly modulates the ethylene biosynthesis that changes in signalling for ROS. In the present experiment, both superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are the studied ROS to be fairly accumulated in *Azolla* and *Vernonia*. Expectedly, the sensitivity of 2,4-D was more relevant with the significant accumulation of O_2^- and H_2O_2 in *Vernonia*. The tissue specific distribution so concerned is mostly on root cap region for the *Azolla*, which is more in cortical tissues in *Vernonia*. H_2O_2 appears to be beneficial in some indirect connectivity for other forms of stress responses. Besides inducing

Fig. 6 A Detection of hydrogen peroxide accumulation in roots (marked in arrow) of *Azolla* plants under varying concentrations 0 μM (Control), 100, 250, 500 and 1000 μM of 2,4-D for 7 days.

B Detection of hydrogen peroxide accumulation in roots (marked in arrow) of *Vernonia* plants under varying concentrations 0 μM (Control), 100, 250, 500 and 1000 μM of 2,4-D for 7 days

Fig. 7 Malondialdehyde (MDA) content in *Azolla* (A) and *Vernonia* (B) grown under varying concentration 0 μM (Control), 100, 250, 500 and 1000 μM of 2,4-D. The experimental data are plotted from means of three replications ($n = 3$) $\pm$ SE. Bars showing different letters indicate significant differences according to Duncan's test at $p < 0.05$

Fig. 8 Protein carbonyl content in *Azolla* (A) and *Vernonia* (B) grown under varying concentration 0 μM (Control), 100, 250, 500 and 1000 μM of 2,4-D. The experimental data are plotted from means of three replications ($n = 3$) $\pm$ SE. Bars showing different letters indicate significant differences according to Duncan's test at $p < 0.05$

lignifications moieties on cell wall, H_2O_2 also becomes an indicative for water stress (Dawood 2016). The activity gradient of these two ROS are supposedly indicative of the fact for 2,4-D mediated other degenerative responses in plants. The tissue specific distribution so concerned is mostly on root cap region for the *Azolla*, which is more in cortical tissues in case of *Vernonia*. This may be suggestive for possibility for restriction of ROS invasion more towards meristems in fern species (Schroder and Atkinson 2006). It

is interesting to note that the path for ROS generation is significantly varied in *Azolla* and *Vernonia* plants. The oxidative stress at cellular level in *Azolla* was more validated when it recorded a substantial amount of malondialdehyde and carbonyl content. Herbicidal affects on cellular level and its indications have been quite a good trait for bioremediation efficacy especially in higher plants (Das et al. 2013). Still, in lower vascular species, like ferns

Fig. 9 Relative water content in *Azolla* (A) and *Vernonia* (B) grown under varying concentration 0 μM (Control), 100, 250, 500 and 1000 μM of 2,4-D. The experimental data are plotted from means of three replications (n = 3) ± SE. Bars showing different letters indicate significant differences according to Duncan's test at $p < 0.05$

Fig. 11 IAA-Oxidase enzyme activity in *Azolla* (A) and *Vernonia* (B) grown under varying concentration 0 μM (Control), 100, 250, 500 and 1000 μM of 2,4-D. The experimental data are plotted from means of three replications (n = 3) ± SE. Bars showing different letters indicate significant differences according to Duncan's test at $p < 0.05$

Fig. 10 Proline content in *Azolla* (A) and *Vernonia* (B) grown under varying concentration 0 μM (Control), 100, 250, 500 and 1000 μM of 2,4-D. The experimental data are plotted from means of three replications (n = 3) ± SE. Bars showing different letters indicate significant differences according to Duncan's test at $p < 0.05$

(*Azolla* in the present case) the lipid and protein oxidation with concomitant variation in ROS would be a new approach for bio indication. Still, aquatic fern species have already displayed well the cellular traits under metal and metalloids in soil which might pioneer these species as bio indicators (Mandal et al. 2013). For another ROS like H₂O₂ the distribution was found throughout the root, still, maximised in central vascular cylinder in *Azolla*. Undoubtedly, H₂O₂ is proven as a safer ROS within a threshold concentration. The inductive role of H₂O₂ against xenobiotics through modifications of cellular functionals is well established in higher plants (Raghavan et al. 2005). For *Vernonia*, the activity of H₂O₂ is more in cortical and central vascular strands. ROS accumulation in the tissues under any form of abiotic stress is also corroborated with status of osmotic stress (Hossain et al. 2015). In the present experiment, the RWC physiologically reflects the tissue dehydration status in a most simplified way and has been

another feature for herbicide sensitivity (Obidiegwu et al. 2015). *Azolla* being an aquatic fern retains more water holding capacity and less sensitive to changes of RWC. Still, from the water potential and related tissue hydration of *Vernonia* it showed some osmotic adjustment although 2,4-D application with an linear increase in RWC. Very contextually the stabilization of osmotic activities is concerned with generation of compatible solute like proline. The 2,4-D concentrations had realized the similar trend in proline accumulation both in *Azolla* and *Vernonia* in a compatible way. Thus, at physiological level of *Vernonia* shows a bio indication in their stability by retaining the hydration as well as osmotic adjustment. The later refers to the compatible solutes like proline, glycine-betaine etc. as also documented in fern species under metal toxicity (Mandal et al. 2014). In the present study, *Azolla* and *Vernonia* recorded physiological compatibilities in retention of hydration and that circumvented through osmotic adjustment by proline.

It is consistent that plant's responses to auxin would have been secured by a homeostasis in its biosynthesis and its degradation in a reversible way (Normanly et al. 2010). 2,4-D so far reported in effects to plants is based on enzymatic lysis of the indigenous auxin pool. Incidentally, this has also got supported in the present study for IAA-oxidase activity from *Vernonia* and *Azolla*, however, in a discriminating manner. In earlier studies, the increasing concentration of 2,4-D has induced the IAA-oxidase both at transcriptional and translational levels (Parvaiz 2014). With this the *Azolla* plants are more inconsistent in IAA-oxidase activity and thus not bearing any association in auxin metabolism with effects of 2,4-D. On the contrary, a steeper up regulation of enzyme activity in *Vernonia* undoubtedly refers to the auxin hydrolysis with effects of herbicides. As an analog of auxin 2,4-D is assumed to be

Fig. 12 **A** Effect of varying concentrations 0 μM (Control), 100, 250, 500 and 1000 μM of 2,4-D on separation of different isozymes of IAA-oxidase [IAA-O1] in *Azolla*. **B** Effect of varying concentrations 0 μM (Control), 100, 250, 500 and 1000 μM of 2,4-D on separation of different isozymes of IAA-oxidase [IAA-O1 and IAA-O2] in *Vernonia*

metabolised by few enzymes as in higher plants and thus might score the compatibility with IAA-oxidation even in the fern species (Mierziak et al. 2014). More so, the activity of IAA-oxidase is displayed as a pool of varying molecular forms of enzyme and their differential expression in tissues. The IAA-oxidase, one class of peroxidase oxidises the indole ring of auxin in accompanying with H_2O_2 (Savitsky et al. 1999). The activity of those forms is also required to subsidise other cellular reactions where lysis of H_2O_2 into free anionic forms are most important. The later are more involved in some appoplastic and cell wall lignification processes and thus correlates the IAA-oxidase activity in varying amounts (Ebrahimzadeh and Abrishamchi 2001). In the present experiment, the inconsistent nature of activity as well as single band polymorphism in *Azolla* species would have also circumvented the probability of lesser IAA-oxidation. This is quite opposed in case of *Vernonia* where the over expression of IAA-oxidase coupled with variations in polymorphic bands were the features. This also holds the more likelihood for auxin degradation as a factor of 2,4-D sensitivity in target species. Contextually, this is more relevant with illustration of *Vernonia* as observed in the present experiment. IAA-oxidase is more abundant in apoplastic spaces, however, few in cytosolic with multiple polymorphic forms do also exist in aquatic species (Cheng and Cheng 2015). This possibly may attribute in any way for growth and development as well as any sort of cellular strains in environment. Taking

together the elucidation of IAA-oxidase polymorphism and its nature might be purposeful for bio indication in tracing of herbicide tolerance.

Conclusion

The present study has got some relevances with its objective to show *Azolla* plants as a tolerant aquatic fern species against herbicidal toxicity. This study was conducted against a susceptible species (*Vernonia*) as positive control under increasing concentration of 2,4-D to analyze some physiological attributes. Successfully, *Azolla* was evident as more withstanding species against the herbicidal concentrations through mostly cellular responses for oxidative stress management. Besides the changes in water relations and IAA metabolism, both the species observed a significant modulation in their redox potential that might support their varied tolerance. Moreover, polymorphism in IAA metabolic enzymes was more discriminately characterised the varied sensitivity of these species. Therefore, 2,4-D is evident as an potent xenobiotic that might be bio indicated by this aquatic fern *Azolla* with selected physiological and cellular activities.

Acknowledgments This work is financially supported by DST-PURSE programme, DST, Govt. of INDIA, New Delhi, for University of Kalyani.

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