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MODULATING ROLE FOR ANTIOXIDANT SYSTEM IN DESICCATION TOLERANCE OF *Dicranopteris linearis*

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ABSTRACT

Desiccation stress is a major environmental factor limiting plant growth and productivity. It induces oxidative stress in the cell system, and the plants counteract *via* enzymatic and non-enzymatic antioxidant system. These antioxidant compounds exhibit specific degrees of tolerance against the magnitude of stress. The differential responses of antioxidants may help to understand the functional interplay of them in the defense system. Similarly, it will lead to unravel efficient physiological and biochemical biomarkers of stress tolerance among plants against stresses. In this juncture, the modulating role for antioxidant system of *Dicranopteris linearis* was carried against different duration of desiccation stress (2 – 10 days) induced by polyethylene glycol. Reduced glutathione and ascorbate content exhibits an initial decrease followed by uplift with increasing periods of desiccation duration up to 6 days and further the quantities were maintained till the 10^{th} day of stress when compared to control. Among the antioxidant enzymes, ascorbate peroxidase was up regulated to 3 folds (10^{th} d) while, superoxide dismutase to 2 folds by increasing duration of stress. Similarly, NADPH oxidase (NOX) was increased to 1521.86 (10^{th} d) from 208.5 U/mg protein of control. Catalase, peroxidase and glutathione reductase activities were also increased under desiccation stress. Efficiency of the antioxidant system was also supported by the membrane damage assay through lipid peroxidation. The biochemical parameters were strongly supported by the amounts of H_2O_2 and $O_2^{\cdot-}$ ion contents. Antioxidants of the forked fern are a useful tool for defending against increasing drought stressed environments.

KEYWORDS: Antioxidant System, Reactive Oxygen Species, Ascorbate, Desiccation Stress, Forked Fern.

Plant growth is affected adversely by different environmental abiotic stresses such as cold, salinity, drought and fluctuations in incident light (Berry and Bjorkman, 1982; Andrea et al., 2003). Drought is one of the major environmental factor affecting plant growth development, survival and their distribution. Therefore, it is imperative to understand how the plants respond and adapt to such diverse stress conditions. The mechanism of the plant responding and adapting to desiccation stress include adjusting their cellular metabolism and invoking various defensive mechanism that provide a strategy to protect plants from desiccation stress. Generally, under the drought conditions, oxidative stress was induced resulting from the production and accumulation of reactive oxygen species (ROS) or free radicals (Hussein and Safinaz, 2013;Miller et al.,2010) such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxide radicals ($OH^{\cdot}$), and singlet oxygen ($_1O^2$). The active oxygen species produced during the stress damage cellular components including lipids (peroxidation of unsaturated fatty acids in membranes), proteins (denaturation), carbohydrate and nuclei acid (Blokhina and Virolainen, 2003). Oxidative stress can add to inhibition of the photosynthesis, respiration processes and, thus plant growth become stunded (Jiang and Huang, 2001).

Plant has non-enzymatic and enzymatic antioxidant system to protect cellular membranes and organelles from damaging effects of ROSs. The non-enzymatic compounds mainly are the low molecular mass antioxidants, such as ascorbate, glutathione, β-carotene and α-tocopherol while the enzymatic antioxidant system include the enzymes- monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1), and glutathione reductase (GR, EC 1.6.4.2), and ROS-interacting enzymes, such as superoxide dismutase (SOD, EC1.15.1.1), peroxidase (POD, EC 1.11.1.7) and catalase (CAT, EC 1.11.1.6) (Blokhina and Virolainen , 2003). The non-enzymatic and enzymatic systems in plants together keep the ROS in balanced levels and help the cells not to be injured by accumulation of ROS in normal conditions. There are many reports about flowering species regarding the activities of enzymes and the antioxidant compounds involved in antioxidant metabolism in response to desiccation stress (Sharma et al., 2012) and results suggested that those plants grown under desiccation stress could induce the production of ROS and induce the production of defence systems. However, there is not yet a clear picture about the relationship between the anti-oxidative system and desiccation stress in plants. Little is known about the effect of desiccation stress on the

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ascorbate-glutathione cycle (ASA-GSH cycle) in lower plants, and there is not enough information about the changes of the osmolytes and pigment contents.

Desiccation tolerance is a common property of bryophytes and lichens, but very rare among angiosperms (Alpert and Oliver, 2002). Pteridophytes include almost 70 tolerant species, yet the mechanisms of their desiccation tolerance have been traced only in a limited way (Oliver et al., 2000). The forked fern *Dicranopteris linearis* belongs to Gleicheniaceae and is widespread in tropical and subtropical regions of the world. It is found extensively growing along the road cuttings in shaded or open areas where water availability is scarce. The sporophyte of the fern is up to 3 m tall, with characteristic dichotomously divided leaves and rhizome is several meter long creeping, brown and covered with septate, branched hairs. Their growth pattern and less availability of water in the habitats evoked interest to understand the drought tolerant capacity of this fern. Furthermore, there is a striking lack of data in the literature concerning the desiccation tolerance in the forked fern. For the first time, in the present study the relation between the dehydration-induced changes in evoking the antioxidant mechanism of *Dicranopteris linearis* has been investigated in terms of overall enzymatic and non-enzymatic antioxidant system and their relationship with ROSs.

MATERIALS AND METHODS

Plant Material

Dicranopteris linearis (Burm.f.) Underw. commonly known as forking fern that grows horizontally at ground level with stalked compound fronds. Fresh *D. linearis* was fully hydrated and equilibrated in a controlled environment chamber for 48 h at 20°C and a radiant flux intensity 75 µM /m^2/ s. The samples were desiccated in a desiccator over polyethylene glycol (PEG) in a controlled environment chamber using the same light and temperature regimes as described above. The selected species were subjected to five different desiccation regimes (a) 2 day (b) 4 day (c) 6 day (d) 8 day and (e) 10 day. After the desiccation exposure a set of desiccated samples were subjected to rehydration for 30 min. The samples were divided into two groups: desiccated and desiccated subsequently rehydrated. Control plants were maintained in an optimal water conditions in each case during the whole experimental period.

Quantification of Superoxide Anions ($O_2^{\cdot-}$)

Superoxide anions were quantified, following the method of Doke (1983). The initial absorbance was read at 580 nm and the final absorbance after heating the mixture at 85^oC for 15 min.

Quantification of Hydrogen Peroxide (H_2O_2)

H_2O_2 concentration of the experimental tissues was estimated as per the procedure of Bellincampi et al., (2000) with some modifications. It was based on the peroxidase mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange. The OD was taken at 560 nm. Control was performed by eliminating the H_2O_2 in the reaction mixture without extract.

Quantification of Lipid Peroxidation (LPX)

The level of lipid peroxidation in the cells was measured in terms of malondialdehyde (MDA) content determined by the thiobarbituric acid (TBA) reaction as described by Zhang and Kirkham (1996). The absorbance of the supernatant was read at 532 nm. The value for the nonspecific absorption at 600 nm was subtracted from the 532 nm reading. The concentration of malondialdehyde was calculated using the molar extinction coefficient of 155 mM cm^{-1}.

Assay of NADPH Oxidase (NOX) Enzyme

NADPH oxidase catalyzes the production of superoxides, a type of reactive oxygen species (ROS). Assay was carried out following the procedure of Bestwick et al., (1998). Absorbance was observed at 340 nm on a time scan of 10 sec intervals for 5 min.

Assay of Ascorbate Peroxidase (APX) Enzyme

Ascorbate peroxidase activity was estimated according to the method of Nakano and Asada (1981). It catalyzes the reduction of H_2O_2 using the substrate ascorbate. One mole of H_2O_2 oxidizes one mole of ascorbate to produce one mole of dehydroascorbate. The rate of oxidation of ascorbate was followed by decrease in absorbance at 290 nm. The H_2O_2 dependent oxidation of ascorbate was followed by monitoring the decrease in absorbance at 290 nm.

Assay of Dehydroascorate Reductase (DHAR) Enzyme

Dehydroascorbate reductase activity was measured following the method given by Dalton et al., (1986). Dehydroascorbate reductase catalyzes the reduction of dehydroascorbate involving the oxidation of

reduced glutathione (GSH) to form ascorbate and glutathione disulphide. The increase in absorbance was recorded at 265 nm.

Assay of Monodehydroascorbate Reductase (MDHAR) Enzyme

Monodehydroascorbate reductase activity was determined according to the method of Hossain et al.,(1984).Monodehydroascorbate reductase catalyzes the reduction of monodehydroascorbate involving the oxidation of NADH to form ascorbate. The decrease in absorbance was due to the oxidation of NADH at 340 nm for 1 min at interval of 6 sec.

Assay of Peroxidase (POX) Enzyme

Peroxidase activity was assayed using guaiacol as substrate (Ingham et al., 1998). The increase in absorbance was measured spectrophotometrically at 470 nm for 10 min at 30°C. A set of samples containing reaction mixture without guaiacol was taken as the control. One unit of POX is the amount of enzyme required to oxidise 1 µM guaiacol by H_2O_2 at test condition.

Assay of Superoxide Dismutase (SOD) Enzyme

SOD was assayed according to the method of Kakkar et al., (1984). The assay of SOD is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. The colour formed at the end of the reaction was extracted into butanol and measured at 560nm.The intensity of the chromogen formed gives the activity of the SOD enzyme.

Assay of Catalase (CAT) Enzyme

Catalase activity was assayed by measuring the initial rate of H_2O_2 disappearance using the method of Aebi (1984).The decomposition of H_2O_2 to give H_2O and O_2 is catalysed by CAT. The decomposition of H_2O_2 can be followed directly by the decrease in extinction per unit time at 240 nm. The difference in extinction per unit time is a measure of CAT activity.

Assay of Glutathione Reductase (GR) Enzyme

Glutathione reductase activity was determined by using the method of Carlberg and Mannervik (1975). GR catalyzes the reduction of oxidized glutathione (GSSG) involving the oxidation of NADPH. Though this reaction is reversible, but formation of GSH is strongly favoured. Catalytic activity of GR can be measured by following the decrease in absorbance at 340 nm due to the oxidation of NADPH.

Quantification of Ascorbate (Asc) and Dehydroascorbate (DHA)

Asc and DHA were measured according to Kampfenkel et al., (1995).The assay is based on the reduction of Fe^{3+} to Fe^{2+} and the spectrophotometric detection of Fe^{2+} complexed with 2,2-dipyridyl. Dehydroascorbate is reduced to ascorbate by pre incubation of the sample with dithiothreitol (DTT). Subsequently the excess DTT is removed with N-etylmaleimide (NEM) and total ascorbate is determined by the 2,2- dipyridyl method. The concentration of dehydroascorbate is calculated from the difference of total ascorbate and ascorbate (without pretreatment with DTT).

Estimation of Reduced Glutathione (GSH)

Estimation of total reduced glutathione was carried out according to the method of Moron et al., (1979). A homogenate of the leaf tissue was prepared in 5% tri chloro acetic acid (TCA) and the precipitated protein was used for the estimation of GSH. 0.1ml of the precipitated protein was made up to 1.0ml using 0.2M phosphate buffer (pH-8).To this 2.0 ml of Ellman's reagent(5,5'-dithiobis(2-nitrobenzoic acid)DTNB) was added. The intensity of yellow colour developed was read at 412 nm within 2 min against the reagent blank. Standard GSH corresponding to concentrations ranging between 2 and 10 µm were also prepared.

Statistical Analysis

All data from desiccated, rehydrated and control plants were subjected to analysis of variance using SPSS 17.0 software. The values were expressed as the mean of three replicates ± standard error (SE). Student t-test ($p < 0.05$) was used to check statistical significance.

RESULTS AND DISCUSSION

Desiccation, due to its osmotic effect in natural and agricultural ecosystems can induce diverse responses such as retardation of growth and synthesis of non-toxic metabolites to enhance the osmotic potential of the cell and their by permitting the metabolic processes to continue to enhance the antioxidant enzyme activities.

Hydrogen Peroxide (H_2O_2) and Superoxide Anion ($O_2^{\cdot-}$) Concentration

The H_2O_2 concentration of fern fronds was significantly increased with increasing desiccation period, i.e.; the highest H_2O_2 concentration was noticed during the highest duration of desiccation. The 4, 6 and 8th day of desiccation treatments caused enormous increases in H_2O_2 concentration in the fern (Fig. 1). $O_2^{\cdot-}$ level is also showed a similar trend with duration of treatment (Fig. 2). Kachout et al., (2010) compared the effects of heavy metals on ROSs and antioxidant activities among *Atriplex hortensis* and *A. rosea*, i.e.; ROSs increased in proportion to heavy metal concentrations.

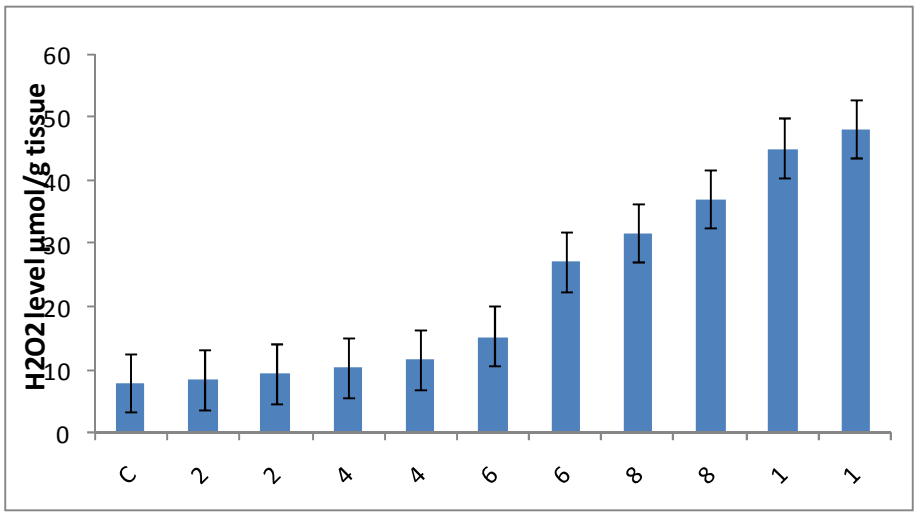

Figure 1: Changes in H_2O_2 level of *D.linearis* from 2nd day to 10th day of desiccation.

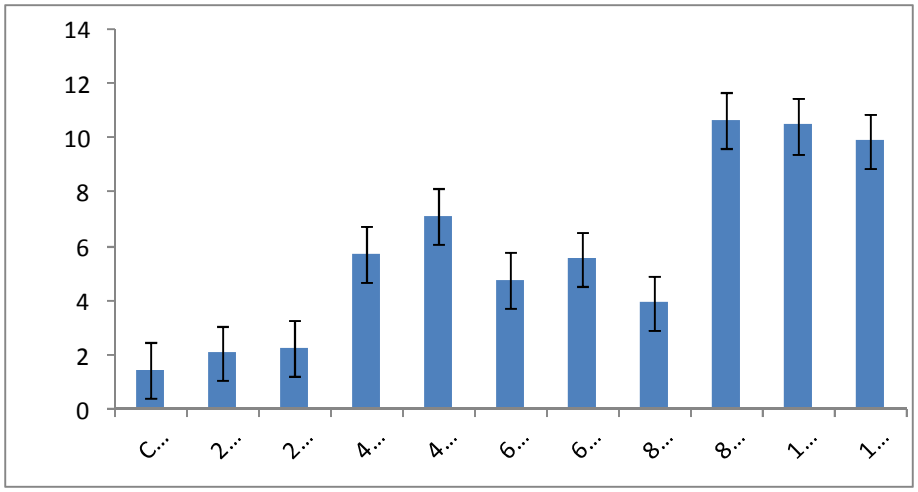

Figure 2: Change in $O_2^{\cdot-}$concentration of *D.linearis* from 2nd day to 10th day of desiccation.

Lipid Peroxidation

Efficiency of antioxidant defense may be correlated with the degree of membrane damage due to peroxidation of unsaturated fatty acids in the lipids structure of cell membranes. Oxidative stress factors induce enormous amounts of ROSs and alkyl peroxides, if these molecules are not effectively detoxified by specific antioxidant enzymatic and non-enzymatic compounds, oxidative membrane damage results. This leads to the formation of lipid peroxide derivatives, the most common and toxic of these being the malondialdehyde (MDA). The present results reflect that desiccation stress exerted on forked fern was significantly mitigated by antioxidants so that the membrane damage by lipid peroxidation was

marginal. Interestingly the enhanced membrane lipid peroxidation, manifested in increased generation of malondialdehyde and other related, thiobarbituric acid-reactive substances, occurs upon exposure to 2nd day of desiccation stress condition compared to 4,6,8 and 10 days (Fig.3) ($P < 0.05$). This data is corroborating with the results indicated by the APX, SOD, CAT, POX and GR enzyme activities at this levels. Ascorbate and glutathione concentrations also support the antioxidant enzyme activity. Contrarily, in crops like wheat, rice and lettuce with heavy metal toxicity it was reported that the antioxidant defense system could not prevent peroxidation of membrane lipids which leads to severe oxidative stress (Eraslan et al., 2007; Panda et al., 2003; Shah et al., 2001).

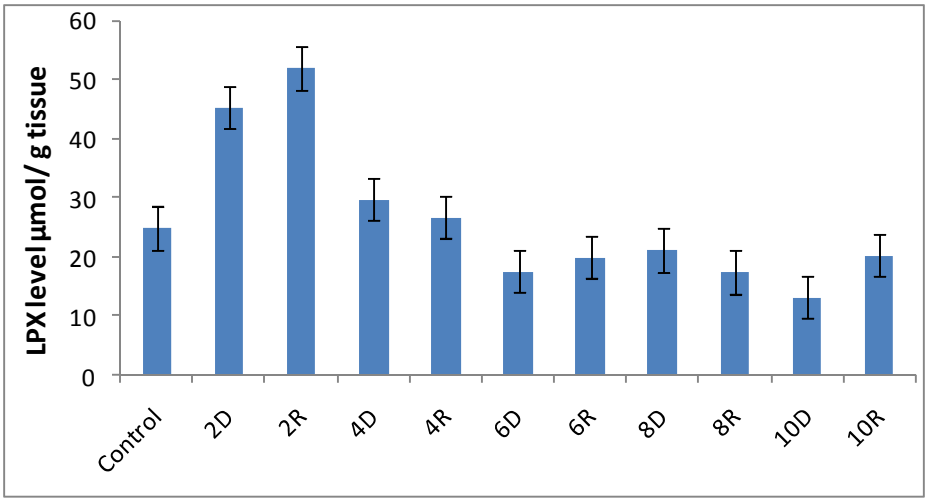

Figure 3: Level of lipid peroxidation(LPX) of *D.linearis* from 2nd day to 10th day of desiccation.

Antioxidant Enzymes

NADPH oxidase (NOX) enzyme belongs to transmembrane proteins able to transport electrons across a membrane usually the plasma membrane from a cytosolic electron donor to oxygen the extracellular acceptor thus catalyzing the generation of superoxide anion ($O_2^{\cdot-}$) (Jimenez-Quesada, 2016). Life span of $O_2^{\cdot-}$ molecules was short, which are rapidly dismutated to H_2O_2 either spontaneously or through the action of superoxide dismutases (SOD). Duration dependent increase in the activity of NOX was noticed i.e., in the control the activity level was 208.5 U/mg protein, while at 10th day of desiccation it increased to 1523.86 U/mg protein (Table.1). The level of NOX activity correlates positively with $O_2^{\cdot-}$ content in the ferns. Similarly, the level of H_2O_2 is indirectly linked to the plasma membrane-bound NADPH oxidase activity (Petrov and Van Breusegem, 2012). Further, it is also hypothesized that NOX activity is required for proline accumulation a response to abiotic stress (Marino et al., 2012; Hu et al., 2006; Thiery et al., 2004).

The most versatile AOX enzyme in Asada-Halliwell enzyme cycle is ascorbate peroxidase (APX) and is effectively involved in mitigating oxidative stress in plants. This enzyme is distributed in chloroplasts, mitochondria, peroxisomes, cytosol and cell wall. The present results revealed that APX activity increased steadily from 2 day of desiccation to 10th day i.e., 240.5 to 509.6 U/ mg protein (Table.1), and therefore this enzyme is a suitable marker of stress tolerance against desiccation stresses. The values were statistically significant at 5% level. This up regulation is most probably related to the increased amount of reduced ascorbate availability under high desiccation conditions and thus, revealing that the APX activity is dependent on the concentration of reduced ascorbate. Its increased activity may also be correlated to poor accumulation of hydrogen peroxide, taking into account that APX scavenges hydrogen peroxide effectively (Shigeoka et al., 2012).

APX scavenges ROSs by converting ascorbate (Asc) to dehydroascorbate (Sharma et al., 2012). In the present results, desiccation stress increased APX activity remarkably. Higher activity of APX suggests the more effective H_2O_2 removal in this fern. Similar results were also reported in Okapi canola cultivar compared to other cultivars (Omidi, 2010) and other plant species such as

wheat (Abdullah and Ghamdi, 2009), olive tree (Sofo et al., 2008), and Kentuky blugrass (Bian and Jiang, 2009).

Ascorbate (Asc) the pivotal molecule involved in the removal of H_2O_2 *via* Asc-GSH cycle (Pinto et al., 2003). Oxidation of Asc occurs sequentially *via* monodehydroascorbate (MDHA) and subsequently dehydroascorbate (DHA). In the Asc-GSH cycle, 2 molecules of Asc are consumed by APX to reduce H_2O_2 to H_2O with concomitant generation of MDHA. MDHA, a short life time radical and can spontaneously dismutate into DHA and Asc or is reduced to Asc by NADP(H) dependent enzyme MDHAR (Miyake and Asada, 1994). DHA is also highly unstable at pH above 6.0 and is decomposed to tartaric acid and oxalic acid. To inhibit this, DHA is drastically reduced to Asc by the enzyme DHAR using reducing equivalents from GSH (Asada, 1996). The balance between the GSH and glutathione disulfide (GSSG) is pivotal in maintaining the redox status of the cell. GSH can also reduce DHA by a non-enzymic mechanism at pH above 7 and at GSH optimal concentrations (> 1 mM). This pathway in crucial in chloroplasts, where in the presence of light, pH is almost 8 and around 5 mM GSH concentration (Foyer and Halliwell, 1976). Generation and maintenance of reduced GSH pool, either by *de novo* synthesis or *via* GR recycling, using NADPH as a cofactor and electron donor, is significant for the cell. The role of GSH in the antioxidative defense system provides a rationale for its use as marker of stress. In the present study the changes in the cellular concentration of these non-enzymatic anti-oxidants ascorbate and glutathione ascertain their role in maintaining the Asada-Halliwell enzyme cycle. The ascorbate content was at par with control in all the treated samples except 6D and 6R which shows a slight increase. The results indicate the constant supply of H^+ donor to the enzyme APX which is a potent H_2O_2 scavenger. The AsA/DHA profile is also supported by the APX activity. Increase in accumulation of DHA shows the active utilisation of AsA to scavenge H2O2.Reduction of DHA during 10^{th} day of desiccation is due to the regeneration of ascorbate by the enzymes DHAR and MDHAR. Reduced glutathione is a low molecular weight thiol antioxidant which helps in regeneration of ascorbate mediated by enzymes like MDHAR, DHAR and GR. Its assay showed an increase in two fold by during 6D of desiccation followed by a decrease. Active utilisation of reduced glutathione to regenerate ascorbate is the reason for its decrease during prolonged desiccation up to 10 days (Table-1).

Table 1: Influence of desiccation rehydration stress on Ascorbate(AsA), Dehydroascorbate(DHA)(µmol g-1) and Reduced Glutathione(GSH))(µmol g-1) contents of *D.linearis*

	Control	2D	2R	4D	4R	6 D	6R	8D	8 R	10D	10R
Ascobate(AsA)	662.6 ±0.11	659.2 ±0.24	657.6 ±0.17	672.14 ±0.15	666.3 ±0.22	898.64 ±0.11	887.56 ±0.34	648.6 ±0.09	649.77 ±0.21	653.8 ±0.15	650.21 ±0.07
DehydroAscorbate (DHA)	12.23 ±0.08	15.61 ±0.02	15.68 ±0.07	39.69 ±0.17	40.82 ±0.07	84.01 ±0.11	82.13 ±0.11	66.89 ±0.06	69.76 ±0.12	35.4 ±0.01	37.9 ±0.07
Reduced glutathione(GSH)	14.72 ±0.22	18.15 ±0.34	20.24 ±0.07	21.51 ±0.19	20.03 ±0.17	32.45 ±0.09	19.87 ±0.16	23.07 ±0.06	16.07 ±0.03	22.49 ±0.017	15.44 ±0.22

MDHA radical produced in APX catalyzed reaction has a short lifetime, and if not rapidly reduced, it disproportionate to AsA and DHA (Ushimaru et al., 1997). Monodehydroascorbate reductase is a FAD enzyme that catalyzes the regeneration of Asc from the MDHA radical using NAD(P)H as the electron donor (Hossain and Asada, 1985). It catalyzes the reduction of DHA to Asc using GSH as the reducing substrate (Ushimaru et al., 1997) and, thus, plays unique role in maintaining Asc in its reduced form. Despite the possibility of enzymic and non-enzymic resurgence of Asc directly from MDHA, some DHA is always produced when Asc is oxidized. DHA is oxidized in a reaction catalyzed by DHAR. Glutathione reductase (GR), another NAD(P)H dependent enzyme catalyzes the reduction of GSSG to GSH and, thus, maintains high cellular GSH/GSSG ratio.

Interestingly, monodehydro ascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) showed 5 and 4 folds of activities respectively (10^{th} day of desiccation) suggesting the active recouping of reduced ascorbate content for APX enzyme to scavenge the ROS H_2O_2 molecules effectively (Table.2). The values are statistically significant at 5% level.

The activities of POX were much higher in the desiccated fronds of the ferns examined. Generally, POX activity was higher in oxidative stressed plants than in the control. The activity of POX ranged from 13.45 to 45 U/mg protein (Table.2). Wang et al., (2010) reported that heavy metals induce POX activity more efficiently than CAT in eliminating H_2O_2.

Peroxidases are stress enzymes in plants and were used as a potential biomarker for sub lethal toxicity in spruce seedlings (Radotic et al., 2000). Wang et al., (2010) found that lead treated *Vicia faba* showed more enhancement of POX and ascorbate peroxidase activity as an intrinsic and major defense tool responsible for H_2O_2 degradation under higher concentrations of lead in contrast to CAT, whose activity was reduced. Nadgorska-Socha et al., (2015) also correlated the accumulation of heavy metals and antioxidant responses in *Vicia faba* plants grown on monometallic contaminated soil.

Superoxide dismutase (SOD) is a multiple form enzyme and present in different plant cellular compartments. In contrast to APX, SOD is a stable enzyme and exhibits a high catalytic turn over number (Alscher et al., 2012). In this context, it is explainable that under the present experimental conditions the SOD activity exhibit modification upon low stress exerted by 2 day desiccation, i.e.; it increased moderately under exposure to 6^{th} day and 8^{th} day and at high desiccation level of 10 days it registered a significant increase in the catalytic activity (2 fold) (Table.2). Bartha et al., (2010); Mahmoudi et al., (2010); Wang *et al*., (2009) reported insignificant levels of SOD activities in many terrestrial plants under moderate desiccation stress conditions. Therefore, the present increase in the SOD activity in the forked ferns as a consequence of desiccation stress in commendable.

Table 2: Activity of enzymes (Umg^{-1} protein) - NADPH oxidase (NOX), Ascorbate peroxidase (APX), Monodehydroascorbatereductase (MDHAR), Dehydroascorbatereductase (DHAR), Peroxidase (POX) and Superoxide dismutase (SOD) in *D.linearis*during 2 to 10 day of desiccation (D) and rehydration (R) treatments.

	Control	2D	2R	4D	4R	6 D	6R	8D	8 R	10D	10R
NOX	208.5 ±1.6	450.2 ±0.09	312.2 ±0.12	860.275 ±0.42	828.91 ±0.34	1277.57 ±0.07	883.57 ±0.04	1258.7 ±0.03	986.64 ±0.09	1523.86 ±0.12	1333.42 ±0.07
APX	150.72 ±0.02	240.5 ±0.3	175.19 ±0.04	278.08 ±0.05	260.62 ±0.13	251.55 ±0.12	241.23 ±0.46	477.22 ±0.15	465.89 ±0.15	509.55 ±0.32	281.31 ±0.43
MDHAR	12.3 ±0.56	63.07 ±0.17	31.83 ±0.21	55.02 ±0.26	22.05 ±0.16	65.28 ±0.17	70.6 ±0.31	83.64 ±0.24	51.2 ±0.53	78.92 ±0.31	52.57 ±0.33
DHAR	50.625 ±0.09	56.85 ±0.06	33.3 ±0.11	230.31 ±0.13	309 ±0.14	218.875 ±0.10	243 ±0.22	414.45 ±0.07	376.96 ±0.17	266.57 ±0.24	225.97 ±0.22
POX	9.85 ±0.54	13.45 ±0.43	14.9 ±0.78	19.8 ±0.65	19.21 ±0.21	30 ±0.17	26.7 ±0.87	40 ±0.38	31.7 ±0.94	45 ±0.13	36.4 ±0.43
SOD	6.19 ±0.08	7.71 ±0.05	6.7 ±0.02	7.4 ±0.16	6.84 ±0.13	13.14 ±0.09	13.05 ±0.21	14.77 ±0.23	11.16 ±0.20	13.55 ±0.06	12.66 ±0.04

Like APX, catalase (CAT) also regulates hydrogen peroxide and scavenges it only when it accumulates in micromolar concentrations in micro-bodies like peroxisomes, glyoxysomes and uricosomes. CAT is easily photo-inactivated, but possesses high turnover rate vlaue (Mittler, 2002). Accumulation of H_2O_2 under desiccation stress in the forked fern exposed for a period 2- 10 days induce significant changes in CAT activity as compared to control conditions i.e., 3.25 U/mg protein of control to 21.04 U/mg protein at 10th day of desiccation stress (an obvious increase of this enzyme's catalytic activity, with severe desiccation stress – 7 folds (Fig.4). The present results are in disagreement with the concept of sensitivity of this enzyme only to higher concentrations of hydrogen peroxide accumulation as reported by Fodorpataki et al., (2015) in duckweed (generated by more severe desiccation stress).

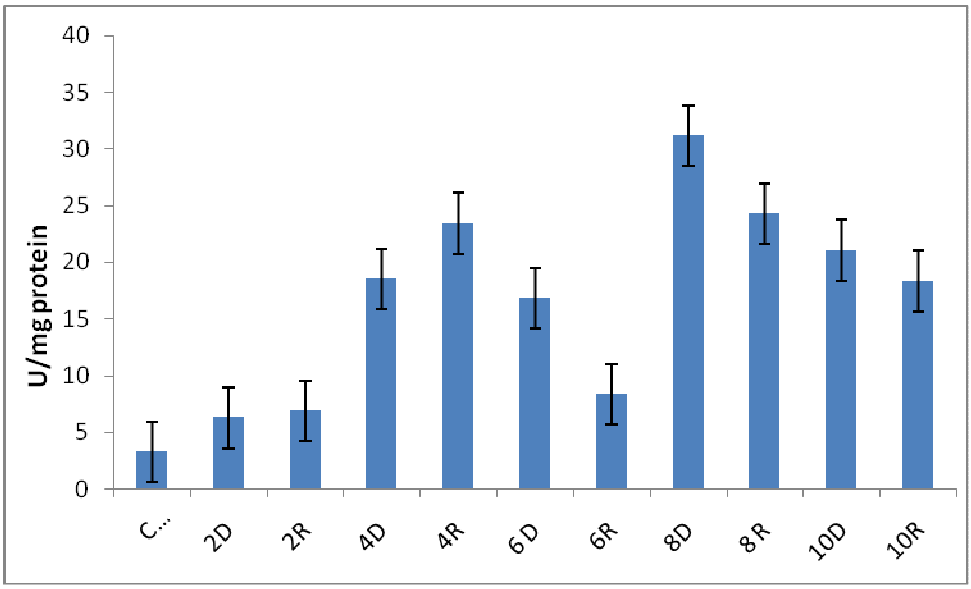

Figure 4: Activity of catalase(CAT) enzyme in the forked fern subjected to desiccation stress.

Glutathione reductase (GR) has an indirect role in the antioxidant defense i.e., it does not scavenge any of the ROSs, but it regenerates the reduced form of glutathione, which is required for sustained scavenging of excess amounts of H_2O_2 in the Asc-GSH redox chain, along with ascorbate (Chattopadhyay, 2014). Activity profile of GR is in accordance with the results concerning variations of glutathione content of the forked fern exposed to different days of desiccation stress, GSH level exhibits significant reduction at lower desiccation level (2^{nd} day), and it increases from 4^{th} day of desiccation onwards to ensure an efficient regeneration of the elevated amount of glutathione in the effort of plants to pace with oxidative stress associated with higher desiccation stress. At higher desiccation (10^{th} day), glutathione content continues to increase at par with GR activity. Like catalase, this enzyme is also suitable for the early detection of desiccation stress, but its activity varies in plants with intensity of stress (Fig.5). Zhang et al., (2013) have also reported that GR activity increases progressively with the concentrations of organic xenobiotic substance - 1-octyl-3-methylimidazolium bromide of water pollution.

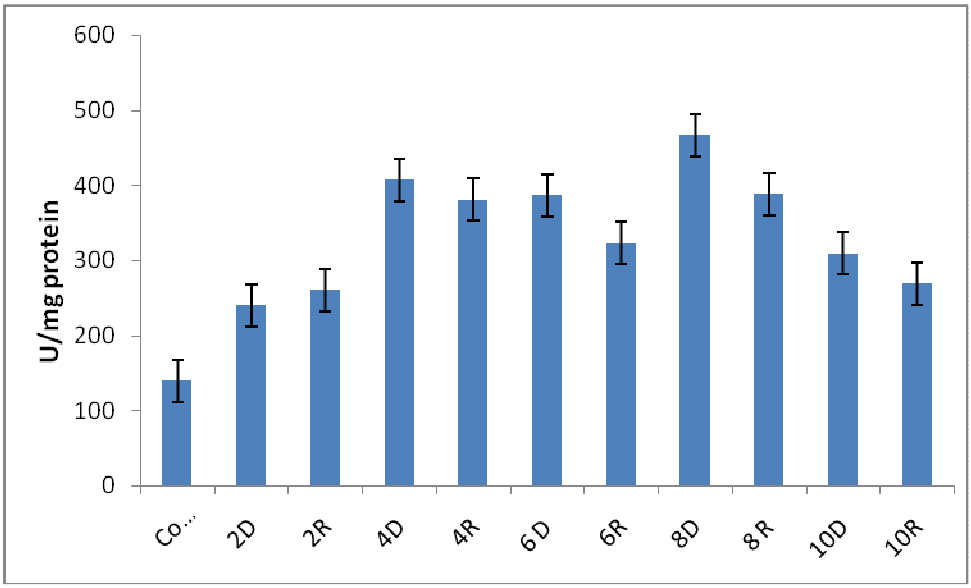

Figure 5: Activity of Glutathione Reductase (GR) enzyme in *D.linearis* from 2^{nd} day to 10^{th} day of desiccation

Aldoobie and Beltagi (2013) analyzed the physiological, biochemical and molecular responses of common bean plants to heavy metals stress. Gao et al., (2008) reported the effects of salt stress on growth, antioxidant enzyme and phenylalanine ammonia-lyase activities in *Jatropha curcas* seedlings. Kranner and Birtic (2005) reviewed the modulating role for antioxidants in desiccation tolerance. Mirzaee et al., (2013) reported the effects of drought stress on the lipid peroxidation and antioxidant enzyme activities in two canola cultivars. Recently, Sarkar et al., (2016), analyzed antioxidative changes in *Citrus reticulata* induced by drought stress and its effect on root colonization by arbuscular mycorrhizal. Kavya Naik and Devaraj (2016) evaluated the effect of salinity stress on antioxidant defense system of *Guizotia abyssinica*. Abu-Muriefah (2015) also reported the effects of silicon on membrane characteristics, photosynthetic pigments, antioxidative ability, and mineral element contents of faba bean. All these data substantiate the application of antioxidant as modulators of stress tolerance among plants.

CONCLUSION

Selected antioxidants of forked fern may be useful biochemical markers of oxidative stress induced by increased desiccation stress. The amount of ascorbate and glutathione were maintained progressively with enhanced desiccation stress caused by PEG. Enzymatic activities of APX, SOD, CAT, GR, POX were stimulated by desiccation stress and showed significant levels. Oxidative membrane damaged, evaluated by the degree of lipid peroxidation of unsaturated fatty acids, intensifies only at low desiccation periods, due to the effective protection ensured by different antioxidants at higher desiccation periods. The ratio between reduced and oxidized form of ascorbic acid is a sensitive stress marker than variation of the total amount of ascorbate. The results also demonstrate that the compounds of the antioxidative defense system behave differently under different degrees of oxidative damage caused by increased PEG. Therefore, the measurement of changes in the dynamics of antioxidants gives an insight in the whole defense system against various degrees and forms of oxidative stress, and integrated stress tolerance of plants. Future studies are designed to evaluate the expression of isozymes of these antioxidant enzymes.

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