

Dissimilitude Response Of Peroxidases Of *Dicranopteris Linearis* (Burm.F.) Underw. Against Desiccation And Rehydration Stress

Kavitha C H¹, Murugan K¹

^{1,2} Plant Biochemistry and Molecular Biology Laboratory,
Department of Botany, University College, Trivandrum, 695 034, Kerala, India

Abstract: Drought is one of the major environmental stress affecting water potential and turgor in the plants leading to physiological imbalance. A wide variety of lower plants are known for their desiccation tolerance during their life cycle. In the present study, desiccation tolerance mechanism of *Dicranopteris linearis* (Burm.f.) Underw commonly called as forking fern was analyzed in terms of ROS production (H_2O_2) and the varied activity of the scavenging enzyme peroxidase. Plants exposed to desiccation-rehydration stress for different periods, i. e., 2 d to 10 d in a controlled growth chambers and the respective controls were maintained in room temperature. Desiccation induced oxidative stress was exhibited as significant rise in the level of hydrogen peroxide (H_2O_2) and it correlated positively with duration. Subsequently, the activity of peroxidases (POX) enzyme (both cytosolic and cell wall bound) was also examined. The activity of cytosolic peroxidase was significantly higher throughout the periods of desiccation compared to wall bounded POX. The role of scavenging potential of this enzyme against ROS generated during stress is established. Histochemical analysis of peroxidase distribution by light microscopy corroborates with the activity of POX in the leaf tissue. Further, comparison of peroxidases activity revealed the insignificant level of cell wall peroxidase suggesting its non involvement in lignification of cell walls during stressed conditions. Here the diversion of peroxidase activity from lignification to its role as ROS scavenger is more ascertained. The present study indicates that the mechanism of desiccation tolerance in the fern is attributed to high activity of antioxidant enzymes involved in reducing the oxidation stress faced by the plant.

Key words: *Dicranopteris linearis*, Desiccation, hydrogen peroxide, peroxidases.

I. Introduction

Water is the central molecule in all physiological processes of plants by being the major solvent for transporting metabolites and nutrients. Drought is a situation that lowers plant water potential and turgor to the extent that plants face difficulties in executing normal physiological functions. Drought, as an abiotic stress, is multidimensional in nature, and it affects plants at various levels of their organization. Drought not only affects plant water relations through the reduction of water content, turgor and total water, it also affects stomatal closure, limits gaseous exchange, reduces transpiration and arrests carbon assimilation (photosynthesis) rates. Tolerance to drought can be defined as the ability of an organism to equilibrate its internal water potential with that of the environment. Plants tolerant to desiccation can survive almost complete protoplasmic dehydration, losing more than 90 percent of their relative water content (RWC), and then resuming normal functions after rehydration. In the plant kingdom, the ability to withstand desiccation of vegetative organs is rather widespread among species belonging to the less complex clades of algae, lichens and bryophytes [1,2] However, a certain number of vascular plants, including pteridophytes and angiosperms, also show this capability [3].

Many ferns and fern allies are desiccation tolerant and are able to recover from complete loss of (80-90%) protoplasmic water. Although it was well documented among angiosperms and *Selaginella* species, no serious attempts were carried among lower vascular and non - vascular plants. Reports are there in *Polypodium polypodioides* about frond curling and folding related with high intensity of light and temperature stress. Similarly, physiological and biochemical mechanisms of desiccation tolerance in *Mohria caffrorum* showed increased levels of oxidative stress and leads to the formation of reactive Oxygen Species (ROS) . Dehydration-related changes of peroxidase and polyphenol oxidase activity in fronds of the resurrection fern *Asplenium ceterach* L. revealed that long term dehydration brought about a remarkable increase in POX and PPO activity which play an important role in the adaptation of the fern to water deficit [4].

Drought brings about different biochemical responses in plants in order to minimize its deleterious effects. Oxidative stress is a physiological stress response leads to oxidative burst in the cellular system. Oxidative stress leads to the formation of free radicals or ROS (Reactive Oxygen Species). Reactive oxygen species (ROS) or free radicals such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical (OH^{\cdot}), hydrogen peroxide (H_2O_2) and singlet oxygen (1O_2) are major agents causing oxidative stress. In non-stressing conditions, the cellular level of ROS is kept at balanced levels by antioxidant systems including enzymes, such as superoxide dismutase, catalase and peroxidase, and low molecular weight free radical scavengers, such as carotenoids,

tocopherols, ascorbate and glutathione. The uncontrolled production of ROS during stress can significantly affect plant cell growth and metabolism leading to the damage of proteins, membrane lipids, nucleic acids, and chlorophyll directly or through the formation of secondary toxic substances, which, in turn, can lead to destruction of sub cellular structures.

Low cellular hydric potential induces stress in plants. Hydrogen peroxide (H₂O₂) is one of the major reactive oxygen species (ROS) in plant tissues and is a key signaling molecule involved in plant response to both biotic and abiotic stresses, such as pathogen attacks, extreme temperatures, drought, excessive radiation, ozone and wounding .

The present study is focused on the magnitude of stress tolerance in fern *Dicranopteris linearis* due to desiccation followed by rehydration in terms of ROS and activity of peroxidases. Oxidative burst in the plant is evaluated by the amount of hydrogen peroxide formed in the plant tissues during the stress period.

II. Materials And Methods

Plant Material

Dicranopteris linearis (Burm.f.) Underw. was collected from Ponnudi Hills, Thiruvananthapuram, Kerala. The identification was confirmed by referring with the floras and authenticated by comparing with the voucher sample at Department of Botany, University of Calicut. *Dicranopteris linearis* had characteristic forking stem that grows horizontally at ground level with stalked compound fronds. Main rachis was dichotomously branched to forms two secondary rachis, which also subsequently forks. At the end of the upward-growing, forked frond, there are two ultimate leafy branches of equal lengths which are deeply, pinnately lobed and comb-like. The sporangia, produced along the underside of the lobes of the ultimate two branches. The sporangia are non-indusiate with trilete spores. The fern was distributed all over the tropical and subtropical part of the world. 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 /m²/ 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.

Determination Of Cytosolic And Cell Wall Peroxidase

Soluble (cytosolic) and cell wall bound peroxidases were isolated from fern leaves by modifying the method of Ingham *et al* (1998)[5]. Activity of both peroxidases was assayed using guaiacol as substrate. The enzyme activity was measured spectrophotometrically at 470nm.

Localization of POX enzyme

Histochemical localization of POX enzyme was done by DAB method. The sections were incubated for a period up to 10 min in a medium consisting of 0.5 mg/ml DAB and H₂O₂ dissolved in 50 mM phosphate buffer (pH-7.8).A series of control incubations were carried out in the same medium but without H₂O₂.

Determination Of Hydrogen Peroxide (H₂O₂)

H₂O₂ concentration of the experimental tissue was estimated a per the procedure of Bellincampi *et al* (2000)[6] with some modification. It was based on the peroxidase mediated oxidation of Fe²⁺, followed by the reaction of Fe³⁺ with xylenol orange. The absorbance of Fe³⁺ xylenol orange complex was observed spectrophotometrically at 560nm.

III. Result And Discussion

Activity of key enzyme of Phenyl propanoid pathway (PPM) The major enzymes of PPM are phenylalanine ammonia lyase (PAL), cinnamyl alcohol NADPH dehydrogenase (CAD) and peroxidase (POX). Peroxidase, the key enzyme was isolated and assayed, spectrophotometrically in order to ascertain their role in the fern (Table 1). The value was a mean of 15 replicates. The specific activity was expressed in U/mg protein. One unit of POX is the amount of enzyme required to oxidize one micromole H₂O₂ consumed/min.

Table. 1. Effect of desiccation stress on peroxidase activity (POX U/mg protein) in *D.linearis* Abbreviations: D-Desiccated; R- Desiccated and subsequently rehydrated *P* < 0.01

	Control	2day D	2 day R	4 day D	4 day R	6 day D	6 day R	8 day D	8 day R	10dayD	10 day R
POXs	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

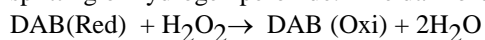
The enzyme POX was found active in the fern from 2nd d of desiccation to 10th d. The higher level of POX activity needs further clarification for establishing its physiological role in the cell system. Apart from the normal function of POX, it is possible to explain the high level of POX activity in the fern as the scavenging enzyme for the removal of H₂O₂, the ROS formed in the cell system as a consequence of desiccation stress for its survival or it may play a role in the general defense mechanism against external barriers. Blokhiana *et al* (2001) [7] had studied the response of anoxic stress towards H₂O₂ formation in plants like rice, wheat and Iris. In their studies they reported the increase in level of H₂O₂ formation in cell system by anoxia. In the present study in *D. linearis*, the high level of POX activity correlating with scavenging potential of H₂O₂ provides further evidence of the desiccation stress. The activities of POX in the rehydrated fern samples followed by different regimes of desiccation also showed an increased profile suggesting the more stress felt by the plant during rehydration. The statistical comparison of the POX activity between the different regimes of desiccation was found significant. The studies on physiological role of enzyme POX in plant system focused to the functional diversity of the enzyme. The physiological role of peroxidase reaction with hydroxycinnamic acids in conservation and protection of cellular constituents of desiccated *Ramonda serbica* leaves was revealed by Veljovic-Jovanovic *et al* (2006)[8]. Suzana zivkovic *et al* (2010)[4] reported an increase in peroxidase activity due to dehydration stress in the rusty back fern *Asplenium ceterach* L. and also suggest its role as an efficient H₂O₂ scavenging system in plant vacuoles in the presence of phenolics and reduced ascorbate. The recalcitrant tea (*Camellia sinensis* L.) seeds showed an increased production of ROS - H₂O₂ combined with decreased activity of POX which resulted in to damage of the embryos which in turn affecting the seed viability. Thus, confirms the role of peroxidase activity in scavenging ROS [9].

Apart from the oxidative polymerization of cinnamyl alcohol to lignin, the enzyme POX was involved in multifunctions (Susumu *et al*; 2001). Hence the occurrence of the enzyme POX and their distributional variation require special emphasis. In order to ascertain the role of POX the ROS H₂O₂ was quantified. It is interested to note that the amount of H₂O₂ was high, which confirms the functional deviation of POX from lignification. The assay data of POX clearly revealed an ambiguous physiological correlation of the key enzyme of the lignin formation.

The study of peroxidase has attracted interest among biochemists because of its capacity to involve in a variety of physiological processes like lignin biosynthesis, pathogen resistance, oxidation of fatty acids and phenols, phytochrome catabolism, fruit ripening, cross linking of cell wall polysaccharides and biosynthesis of extensin and auxin metabolism [10].

Localization Of POX Enzyme

The performance of POX by and large significant, therefore, this major enzyme was histochemically localized in the tissues by DAB method. The frozen section were incubated in the medium having DAB and Hydrogen peroxide, the substrate for POX[11]. During peroxidatic reaction of POX, DAB get oxidized by the splitting of hydrogen peroxide. The dark brown deposits appeared in the cells indicate the oxidized DAB.



All the incubated sections showed positive sign of POX activity. Figure.2,3,4,5,6 shows the POX action in the tissue as brown deposits after specific days of desiccation. The oxidised DAB by the peroxidatic activity of POX show brown deposits on the tissues. For control the tissue sections were incubated in the assay medium without DAB and with H₂O₂ and with DAB and without H₂O₂. Brown deposit was not seen in control sections confirming the peroxidatic activity (Fig. 1). It could be seen from the figures that the enzyme was found active indicating the physiological activeness of the enzyme. The high level of peroxidase activity strongly suggests the role of the enzyme in performing multifunctions in the plant and also in scavenging ROSs. The assay data was supported by histochemical observations.

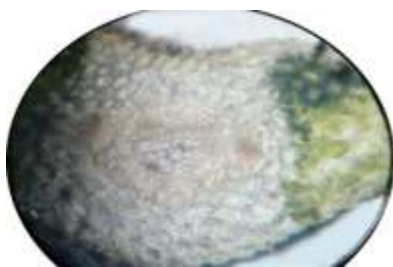


Fig. 1. Leaf tissue of the control plant without brown deposits

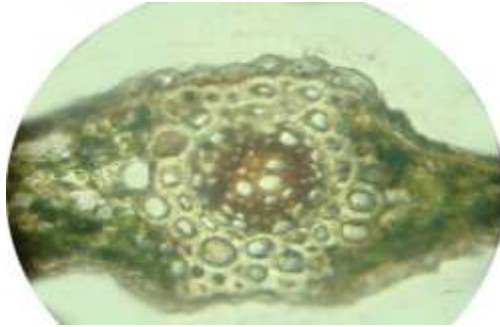


Fig 2: Leaf tissue of Desiccated for 2 Days

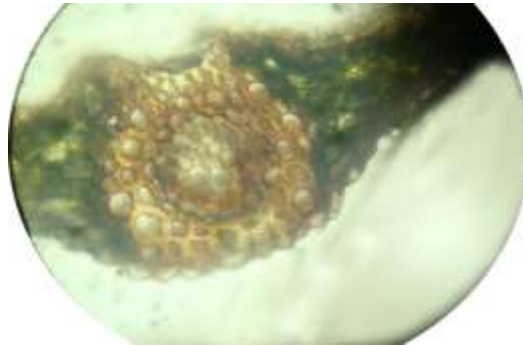


Fig 3: Leaf tissue of Desiccated for 4 Days

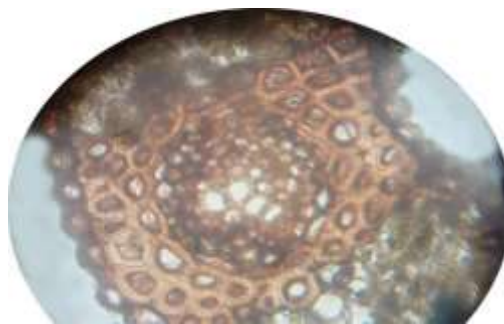


Fig 4: Leaf tissue of Desiccated for 6 Days



Fig. 5. Leaf tissue desiccated for 8 days

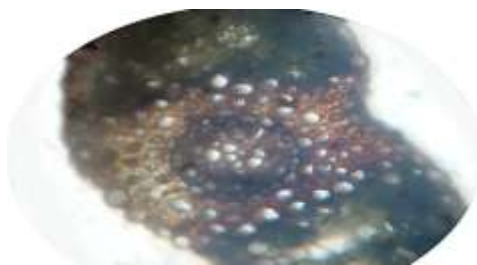


Fig. 6. Leaf tissue desiccated for 10 days

Role Of Cytosolic And Cell Wall Bound Peroxidases

Since the fern shows high POX activity, it is necessary to establish the specific function of POX. The POX was fractionated and was isolated separately in soluble and cell wall bound forms and both were subjected to assay studies. The phenolic interference during extraction of POX fraction from tissue was suppressed by adding PVPP in the extraction buffer. Activity of cytosolic and cell wall bound POX was shown in table 2 and 3 respectively. Cytosolic POX, expressed remarkable activity than cell wall bound POX. The data makes clear that cytosolic POX has a pertaining role other than lignification; while the minimal assay data of cell wall bound POX suggests the possible low lignified nature of the cells.

Table. 2. Effect of desiccation stress on cytosolic peroxidase activity (CY-POX U/mg protein) in *D.linearis*
Abbreviations: D-Desiccated; R- Desiccated and subsequently rehydrated. $P < 0.01$.

	Control	2 dayD	2 dayR	4 dayD	4 dayR	6dayD	6 dayR	8 dayD	8 dayR	10 dayD	10 dayR
CY-POX	6.2 ± 0.88	9.25±0.32	14.6± 0.48	12.9± 0.09	15.61± 0.26	19.8± 0.39	21.5± 0.99	25.7± 0.35	26.3± 0.12	29.4± 0.08	30.2± 0.45

Table. 3. Effect of desiccation stress on cell wall peroxidase activity (CW-POX U/mg protein) in *D.linearis*
Abbreviations: D-Desiccated; R- Desiccated and subsequently rehydrated $P < 0.01$.

	Control	2 dayD	2 dayR	4 dayD	4 dayR	6dayD	6 dayR	8 dayD	8 dayR	10 dayD	10 dayR
CW-POX	3.65 ± 0.02	4.2± 0.04	4.3± 0.65	6.9± 0.92	5.6± 0.3	10.2± 0.18	5.2± 0.43	14.3± 0.056	5.4± 0.38	15.6± 0.72	6.2± 0.01

Determination Of Hydrogen Peroxide (H₂O₂)

H₂O₂ acts as signaling cascade and regulator of the expression of genes encoding antioxidants, cell rescue/defense proteins, signaling proteins of kinase, phosphatase and also transcription factors [12]. H₂O₂ content in *D. linearis* showed a low pace of increase up to 4 d of desiccation but subsequently increased drastically from 6 d to 10 d desiccation when compared with control. H₂O₂ peak reached maximum at 10 d (Table 3). This increase in H₂O₂ might be due to enhanced activities of NADPH oxidase and superoxide dismutase enzyme systems in cells against desiccation stress [13]. Desiccation followed by rehydration of all the samples also showed an increase in H₂O₂ content and interestingly the content was greater than respective period of desiccation (Table 4). The enhanced level reflects the severe stress felt by the plant when rehydrated after desiccation. Lubaina *et al.*, (2013)[14] reported an induction of H₂O₂ along with antioxidants as signal response to dehydration stress in the moss *Octoblepharum albidum* and thereby suggesting its protective role as stress tolerance. Jin-Fen Wen *et al.*, (2012)[1] also reported H₂O₂ accumulation in cultured tobacco cells subjected to heavy metal cadmium stress.

Table. 4. Effect of desiccation stress on amount of H₂O₂ (µmol g⁻¹ FW) in *D.linearis*
Abbreviations: D-Desiccated; R- Desiccated and subsequently rehydrated; H₂O₂ – hydrogen peroxide.
 $P < 0.01$.

	Control	2 dayD	2 dayR	4 dayD	4 dayR	6dayD	6 dayR	8 dayD	8 dayR	10 dayD	10 dayR
H ₂ O ₂	7.9± 0.02	8.46± 0.18	9.41± 0.52	10.42± 0.27	11.69± 0.09	15.39± 0.04	27.2± 0.81	31.86± 0.55	37.18± 0.34	45.22± 0.76	48.22± 0.07

IV. Conclusion

The present findings indicate that *D.linearis* responded to water deficit stress by enhancing their anti-oxidative capacity. POX activity and differential accumulation of H₂O₂ in the tested samples under different regimes of desiccation suggests that POX play an important role in the overall oxidative stress tolerance potential in the fern. Comparatively higher specific activity of cytosolic POX under drought stress further substantiate the role of it to be of critical importance for the detoxification of stress induced H₂O₂. It is reported that the extent and magnitude of growth recovery after re-watering, depends on the intensity and duration of drought, as prolonged dry spells may cause irreversibly injury to tissues. Further studies are warranted to analyze the entire spectra of free radicals, lipid peroxidation and other antioxidant enzyme system to mitigate oxidative burst in the cells.

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