Histochemical Localization of Desiccation Induced Hydrogen Peroxide Production and Its Relation to H$^+$ATPase in *Dicranopteris linearis*

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Abstract

Water scarcity is one of the major environmental stresses that induce a wide array of responses like changes in osmotic potential, malondialdehyde (MDA) level, ion homeostasis, osmolyte accumulation and pigment composition. Research related to plant response to water stress is blooming due to the changing climatic conditions in most parts of the earth. Water stress can vary from a small decrease in water potential to the level of lethality in plants. Currently, it has been proved that drought induced reaction is similar to pathogen-induced responses and that could lead to deleterious tiring by the ROS pathways normally noticed with the hypersensitive responses. The subcellular localization of desiccation induced H$_2$O$_2$ synthesis and its relation to H$^+$ATPase of the desiccated fronds of *Dicranopteris linearis* was studied. Desiccation induced H$_2$O$_2$ accumulation was noticed initially on the plasma membrane, cell wall and subsequently continued into the cytoplasm and cell organelles like chloroplast, mitochondria. Inhibitor analysis reveals that NADPH-dependent superoxide synthase and the cell wall peroxidases were the possible sources for H$_2$O$_2$ production in the cell wall. Symptoms of visible damages like vacuolation, lipid peroxidation were noticed with time span of desiccation. These responses may not be directly connected with defense
against oxidative stress, but may rather indicate changes in oxidative balance within the cells that affect its metabolism and the homeostasis of the whole cell, possibly leading into induction of programmed cell death. Desiccation treatment also caused an inhibition of H^+ATPase activity and that seems to be a correlation between H_2O_2 production and H^+ATPase activity.

Keywords: Dicranopteris linearis, desiccation, hydrogen peroxide, electron microscopy, localization, H^+ATPase.

1. INTRODUCTION

Cellular state represents the total active water level that regulates cells to mitigate their habitats in a narrow manner. Redox state is a part of the cellular response and also a marker for accessing the physiological status of the cell. Intracellular plant antioxidant expressions are closely related to the metabolic state of the cell and are responding against the constantly changing environment. Similarly, the metabolic pathways like photo respiratory, enzymatic and non-enzymatic defense and their responsive-gene regulation and morpho-anatomical features play roles in regulating the above state. Abiotic stress includes drought, salinity, low temperature, UV-B and others. Plant cell metabolism regulate via avoiding photodynamic or reductive activation of molecular oxygen to produce ROS, particularly superoxide, H_2O_2 and singlet oxygen. Generally, in most cases, the formation of ROS is genetically programmed, induced during the course of development and by environmental fluctuations, and has complex downstream effects on both primary and secondary metabolic compounds. Higher plant cells produce ROS, particularly superoxide and H_2O_2 as secondary cascades in many events associated with plant growth and development. Further, higher plants transmit information concerning changes in the environment via the bursts of superoxide at the plasma membrane.

Situations which induce ROS synthesis in an uncontrolled manner leads to oxidative stress, which is a harmful process enhance the oxidation of essential component of the cell. The plant counteract via signals to make appropriate adjustments of gene expression and cell structure in response to environmental and developmental cues. Thus, ROS-antioxidant induction is not a simple signaling cassettes but a complex relationship between metabolism and redox state in the plant system. H_2O_2 is relatively stable, can diffuse freely throughout the cell, and hence may be able to participate in HO formation at sites distant from its synthesis. Bestwick et al., suggested that H_2O_2 accumulation may occur by a reduction in H_2O_2 scavenging and an increase in H_2O_2 production involving coordinated changes in the enzymes like peroxidase, superoxide dismutase, and catalase. H_2O_2 accumulation plays multiple roles like membrane damage, a component of structural defense (lignin synthesis), a signal molecule operating within the challenged cell, and also a defense compound.
However, a little information on the localization of H$_2$O$_2$ production or accumulation during plant-desiccation interactions was known especially among lower plant groups. In this scenario, the present study aims to localize H$_2$O$_2$ in the desiccated fronds of the forked fern and its relation with H$^+$ATPase.

2. MATERIALS AND METHODS

2.1. Plant material

*Dicranopteris linearis* (Burm.f.) Underw. commonly known as forking fern belongs to Gleicheniaceae and is widespread along the tropical and subtropical regions of the earth. It grows horizontally at ground level with stalked compound fronds. It is found dominating along the road cuttings in shaded or open areas where water availability is scarce. The sporophyte is characteristic with dichotomously divided leaves and creeping rhizome covered with seaptate, branched hairs.

2.2. Desiccation treatment protocol

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 species was subjected to five different desiccation regimes (a) 2 day (b) 4 day (c) 6 day (d) 8 day and (e) 10 day. Control plants were maintained in an optimal water conditions in each case during the whole experimental period.

2.3. Histochemical localization of H$_2$O$_2$ by transmission electron microscopy (TEM) using CeCl$_3$ staining

Leaf sections were fixed with 2.5% glutaraldehyde in 50 mM pH 7.0 sodium cacodylate buffer, first under vacuum and then at 4°C overnight for TEM analysis. Subsequently, the samples were washed with buffer and stored at 4°C. The localization of H$_2$O$_2$ accumulation by CeCl$_3$ staining and subsequent pre-fixation was carried by the protocol of Bestwick *et al*.$^5$ Cerous ions (Ce$^{3+}$) react with H$_2$O$_2$ results in to electron dense cerium perhydroxide deposits.$^6$

\[
\text{H}_2\text{O}_2 + \text{CeCl}_3 \rightarrow \text{Ce(OH}_2\text{)OOH}
\]

The CeCl$_3$-treated and control sections were post-fixed in 1% osmium tetroxide, dehydrated in ascending ethanol series and embedded in Epon LX 112 and polymerized. Ultrathin sections were cut with a diamond knife on a Reichert Ultracut microtome and mounted on copper slot grids (2 X 1 mm). Sections were observed...
with transmission electron microscope at an accelerating voltage 60 kV. Intensity of electron-dense was quantified using an image analyzer.

2.4. Inhibitor treatments

Twelve 2-month-old fronds were exposed to different regimes of desiccation 2-10 days using polyethylene glycol (PEG). Leaves were collected at 0, 2 to 10 days and sectioned. Sections were first vacuum infiltrated with buffer alone (50 mM Mops pH 7.2) or with buffer containing NADPH oxidase and peroxidase inhibitors, as described by Bestwick et al.\(^5\), for 30 min at room temperature. \(\text{H}_2\text{O}_2\) accumulation was analyzed from the leaf tissue.

2.5. Determination of H+ATPase activity

Plasma membrane H+ATPase was isolated from the leaf samples of *Dicranopteris linearis* under desiccation rehydration stress according to the protocol of Gallaghard and Leonard\(^7\). The released inorganic phosphate (Pi) was determined according to the method of Fiske and Subbarow\(^8\). After termination the reactive solution was mixed with 0.1 ml of 1-amino1-naphthol-sulphonic acid (0.125% in 15% NaHSO3 1% Na\(_2\)SO\(_3\)). This mixture was shaken at 25°C for 30 min. The absorbance was recorded at 750 nm. The protein content of the enzyme was estimated by Bradford method\(^9\).

2.6. Statistical analysis

Results were presented as mean ± SD. All data were subjected to analysis of variance performed on the window based Genestat package (6th ed., NAG Ltd., England).

3. RESULTS AND DISCUSSION

The control cells appeared highly vacuolated with maturity. Chloroplast was intact showing grana with packed thylakoids and well defined starch grains were also noticed. Mitochondria were normal (Fig.1). No \(\text{H}_2\text{O}_2\) deposits were observed.
During the initial period of desiccation i.e.; 2 days, the cells showed many vesicles, vacuolation and H$_2$O$_2$ deposition along the cell wall (Fig.2a&b). Endoplasmic reticulum (ER) and mitochondria were intact.

Different levels of H$_2$O$_2$ deposits was noticed as electron-dense granules in various parts of the cell wall. The morphology of ribosome, nuclei and endoplasmic reticulum, does not appear to have been adversely affected by desiccation treatments up to 6 days. Meanwhile, damaged membrane of organelles and plasmolysis with separations of the plasma membrane from the cell wall was noticed in the cells. The chloroplasts were flattened with scattered H$_2$O$_2$ (Fig.3).
Subsequent days of desiccation (8th and 10th days) displayed prominent deposits of cerium perhydroxides along the membrane (Fig.4a and 4b).

In addition, 8 day desiccated frond cells displayed massive irregular sized vacuoles. Chloroplasts become oblong and more stretched. \(H_2O_2\) could be seen prominently along the intercellular region of the cells (Fig. 5). At higher desiccation periods, the cells appeared highly vacuolated. Relatively large \(H_2O_2\) deposits were observed in the surface between cytoplasm and cell walls (Fig.6). Plastids were deformed with irregular shape. Mitochondria also displayed irregularities (Fig.7).
Histochemical localization of hydrogen peroxide (H$_2$O$_2$) using the cerium chloride technique provided ample evidence of an enhanced cerium (IV) perhydroxide staining along the cell wall and plasma membrane and subsequently into the cytoplasm. Higher duration of exposure (8 to 10 d) showed noticeable increase in the accumulation of H$_2$O$_2$. In the control, the staining of cell wall and plasma membrane due to cerium (IV) perhydroxide deposits was negligible and fairly uniform (Fig.1). The staining of these cell structures was noticeably more pronounced in the cells that had been exposed to higher desiccation periods (Fig.8a and 8b).

Figure 8a and 8b reveal the desiccation impact at higher magnitude, where dense granular particles can be seen along the plasma membrane and also within adjacent cell wall regions in the desiccated cells, but not in the control cell (Fig.1). Figures 2a and 2b show the presence of cerium per hydroxide particles marginally in the cytoplasm, which is filled with membranous vesicles, and showed signs of degeneration.
Desiccation in the apoplast region of the cell can lead to the formation of diverse short-lived ROS that accumulates along the cell wall and the plasma membrane components. Visibility of CeCl₃ precipitation in the membranes observed via the TEM suggests the active H₂O₂ production by the cells as a consequence of desiccation resulted oxidative stress in the cell wall.

To confirm that CeCl₃ had penetrated into the cells and particularly the chloroplasts, and that chloroplastic H₂O₂ production can be visualized with the CeCl₃ staining, leaf sections were infiltrated with methyl viologen that sets off ROS production in the chloroplasts. In these leaves, H₂O₂ accumulation was visible in the chloroplasts stroma of the cells. These fronds also displayed severe ultra-structural damages. The appearance of methyl viologen-induced H₂O₂ in the cytoplasm, mitochondria, and chloroplasts shows that CeCl₃ obviously penetrates biological membranes and can be used to detect intracellular H₂O₂ production in plant cells in a similar way as in mammalian cells, where cerium-derived deposits have been detected inside subcellular structures, including mitochondria.

To elucidate the possible sources of the H₂O₂ accumulation in desiccated leaves, the fronds were exposed to desiccation for 10 d and infiltrated with inhibitors of possible sources for radical production in the cell wall and subsequently with CeCl₃, as described in Bestwick et al. Inhibitor effects were analyzed from the cells. Results from these inhibitor experiments presented in Table 1 show that precipitation of CeCl₃ is H₂O₂-specific and that both cell wall peroxidases and the plasma membrane NADPH oxidase are possible sources for the H₂O₂. Catalase removed the staining almost completely, confirming that the precipitate detected is derived from H₂O₂. DPI, an inhibitor of flavin containing oxidases, such as the plasma membrane NADPH oxidase, reduced the percentage of H₂O₂ producing cells and abolished the strong staining completely. KCN and NaN₃ were used to inhibit peroxidases. KCN was roughly as effective in inhibiting the desiccation induced H₂O₂ production as DPI, whereas NaN₃ abolished H₂O₂ accumulation detectable with the CeCl₃ staining almost completely.

**Table 1.** Effect of inhibitors on the cell wall and plasma membrane H₂O₂ localized in 8d desiccated fronds of *D. linearis.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intensity of CeCl₃ (% of scored cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strong</td>
</tr>
<tr>
<td>8d desiccated+ Buffer</td>
<td>10</td>
</tr>
<tr>
<td>8d desiccated+ CAT</td>
<td>0</td>
</tr>
<tr>
<td>8d desiccated+ DPI</td>
<td>0</td>
</tr>
<tr>
<td>8d desiccated+ KCN</td>
<td>0</td>
</tr>
<tr>
<td>8d desiccated+ NaN₃</td>
<td>0</td>
</tr>
</tbody>
</table>
H^+ATPase activity

The present study shows that desiccation causes a significant reduction in the activity of plasma membrane associated H^+ATPase in the cells (Table.2).

Table.2. Effect of desiccation stress on H^+ATPase activity (U/mg protein) in D.linearis

<table>
<thead>
<tr>
<th></th>
<th>control D</th>
<th>2 day D</th>
<th>2 day R</th>
<th>4 day D</th>
<th>4 day R</th>
<th>6 day D</th>
<th>6 day R</th>
<th>8 day D</th>
<th>8 day R</th>
<th>10 day D</th>
<th>10 day R</th>
</tr>
</thead>
<tbody>
<tr>
<td>H^+ATPase</td>
<td>0.131±</td>
<td>0.128±</td>
<td>0.346±</td>
<td>0.25±</td>
<td>0.228±</td>
<td>0.104±</td>
<td>0.174±</td>
<td>0.098±</td>
<td>0.155±</td>
<td>0.057±</td>
<td>0.124±</td>
</tr>
<tr>
<td></td>
<td>0.09</td>
<td>0.12</td>
<td>0.12</td>
<td>0.07</td>
<td>0.02</td>
<td>0.11</td>
<td>0.13</td>
<td>0.04</td>
<td>0.09</td>
<td>0.08</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Abbreviations: D-Desiccated; R- Desiccated and subsequently rehydrated. P < 0.01.

Interestingly a positive relationship between was seen H^+ATPase and the elevated production of H_2O_2 as indicated by cerium perhydroxide deposits. Penetration of cerium chloride across tissues as a result of the reaction between CeCl_3 and H_2O_2, is likely to take more time and, therefore, this protocol may not be used to evaluate the dynamic events related to H_2O_2 synthesis and their gradient across the plasma membrane. However, it has been established as an ideal tool in assessing the concentration differences in H_2O_2 over time. H_2O_2 deposits along the membranes correlated with adverse effects of desiccation on H^+ATPase. The occurrence of increased concentrations of H_2O_2, initially in the cell walls and along the cell membrane, suggests that both the cell wall and the plasma membrane may be the source of H_2O_2. Even though a direct link has yet to be proved, it seems to be that elevated level of H_2O_2 leads alterations in membrane permeability, and also its integral protein complexes. Kititorova et al., 14 reported H_2O_2-mediated decrease in hydraulic conductivity in roots of wheat in relation to salinity stress, and the results provided in the present analysis, showing a close relationship between H_2O_2 level and H^+ATPase activity in isolated the plasma membrane, substantiate this view. Prasad et al., 15 showed a correlation between H_2O_2 concentration and chilling stress in corn plants. Similarly, Frahry and Schopfer 16 also revealed that H_2O_2 content in the soybean root could be induced significantly by exogenous NADH or NADPH. Thus, the present results tempt to suggest that in addition to decrease of the H^+ATPases, the water-channel proteins, aquaporins, may be altered. Decrease of H^+ATPase activity may reduce cytoplasmic pH, and this in turn lead to a decrease in root hydraulic conductivity. Javot and Maurel17, revealed that the sensitivity of water-channel activity to environmental parameters may affect the water uptake and the water balance among plants. Zhang and Tyerman18, compared the activity of aquaporins against chilling with the cell pressure probe in algae and also in the cortical cell of the roots. Wan and Zwiazek19 reported that, mercurial compounds inhibits water transport.
by reacting with sulphhydryl groups of the proteins and thereby block the channels. This further leads to decrease of hydraulic conductivity and thereby water transport. Elevated levels of H$_2$O$_2$ may interfere with the activity of H$^+$ATPase and aquaporins. Meanwhile, a direct correlation of the aquaporins activity (open/closed state) to H$_2$O$_2$ remains to be experimentally elucidated. The increased desiccation period may be involved in the closing of aquaporins. Further, the decrease in the hydraulic conductivity due to the increase in the water viscosity with desiccation, which may inhibit water uptake. Cochard et al. have proved that chilling remarkably increased the resistance to water flow of *Populus tremuloides* seedlings, and this feature may not be explained by the corresponding enhancement in the water viscosity. As noticed here, H$_2$O$_2$ caused a decrease in H$^+$-ATPases activity in the plasma membranes. Meanwhile, the relationship of this to water channel activity is not yet clear, although the extrusion of protons from cells should be connected to the uptake of nutrient ions. Zhang and Tyerman have proved that the hydraulic pressure conductivity was not altered by K$^+$ channel blocker tetraethylammonium at doses that usually block K$^+$ channels. The ways in which desiccation inhibits H$^+$ATPase are not yet clear. H$_2$O$_2$ may influence ATP hydrolysis and/or disulphide exchange of oxidized glutathione with the reactive cysteine in V-ATPase. Studies in jack pine seedlings revealed that the inhibition of the plasma membrane H$^+$ATPase activity by direct freeze and thaw was resulted by the thiol oxidation of plasma membrane proteins. Reduced glutathione level prevented lipid peroxidation through glutathione-mediated ROS scavenging system. Similarly, plasmodesmata can also facilitate movement of water between adjacent cells, desiccation induced closure of plasmodesmata may be another possibility for the observed reduction in H$^+$ATPase activity. Rapid accumulation of H$_2$O$_2$ initially at the plasma membrane may also be relevant in this regard.

H$_2$O$_2$ formation in the present study appears to be an alternative to the desiccation-induced O$_2^-$ based oxidative burst. Minibayeva and Beckett reported similar trend in many lichens, thalloid liverworts and hornworts species. Runeckles and Vaartnou proved that the ozone derived O$_2^-$ signal disappeared in few minutes after removing the plants from ozone source using Electron paramagnetic resonance studies.

Schraudner et al. have shown that O$_3$ induces early bursts of active H$_2$O$_2$ production in the cell walls of the O$_3$-sensitive tobacco cultivar Bel W3. The number and distribution of these oxidative bursts correlated with the discrete sites of local cell death and visible symptoms developed later. Similarly, Kettunen et al. and Rao and Davis reported O$_2^-$ formation similarly in ozone exposed *Arabidopsis thaliana*. The oxidative burst is one of the earliest sign in plant-pathogen interactions. It has been shown with H$_2$O$_2$-specific CeCl$_3$ staining that the oxidative burst takes place only in the immediate vicinity of the invading pathogen. The subcellular location of
the desiccation induced H$_2$O$_2$ and O$_2$– synthesis, however, has not been documented among ferns. In the present analysis, desiccation induced active H$_2$O$_2$ production in the fronds of the fern in different subcellular locations was visualized. Initially, the H$_2$O$_2$ accumulation was seen in the cell wall and plasma membrane and subsequently, in the cytoplasm, chloroplast and mitochondria (Fig.2-8.) i.e., apoplastic phase followed by symplasic regions. Continuing H$_2$O$_2$ accumulation in the fronds that can be visualized with DAB (3,3-diaminobenzidine)- staining takes place only at the locations that later develop macroscopically visible necrosis.

The apoplastic ROS induction may be via the plasma membrane NADPH oxidase associated with an extracellular SOD and extracellular POXs, was addressed here with inhibitor experiments (Table 1), which suggest that both are involved in the H$_2$O$_2$ production in the cell walls of desiccation exposed fronds. The plasma membrane located NADPH oxidase is activated by biotic or elicitor challenge in various plant species 34. The O$_2$– synthesized via NADPH oxidase has to dismutate rapidly to H$_2$O$_2$ either spontaneously or by the SOD activity 35.

Extracellular CuZn SOD is connected with the plasma membrane NADPH oxidase and that may induce H$_2$O$_2$ production 36. Previously Kavitha and Murugan 39 proved an increase in the total cellular AOX activity in desiccation exposed fern fronds at different duration of desiccation stress. Furthermore, the increase in AOX activity was parallel with periods of desiccation. It may be proposed that the enzyme activity profile was related to the cellular damages.

Other possibility of apoplastic H$_2$O$_2$ synthesis is by the extracellular POXs. They are involved in lignin biosynthesis and catalyze a reaction where H$_2$O$_2$ is produced at the expense of NAD(P)H 37,38. In the fronds of the forked fern, the guaiacol peroxidase activity was increased by desiccation 39. The mode of increase was similar to that of other enzyme activity and the increase was more pronounced in the 10th day after desiccation. Further, DPI has a peroxidase inhibiting activity, especially at high concentrations 40. In the French bean and rose cells, the increased concentration of DPI reduced NADPH oxidase dependent H$_2$O$_2$ production compared to control 40. Thus, the ability of DPI to partly inhibit peroxidases does not exclude the possibility for involvement of NADPH oxidase as a source of H$_2$O$_2$ in the desiccated ferns. This is also substantiated by two different locations for the apoplastic H$_2$O$_2$ accumulation detectable with the CeCl$_3$ staining; in Fig.3-8, CeCl$_3$ precipitation is clearly visible on the cell wall and also on the surface of the plasma membrane. Furthermore, the CeCl$_3$ precipitates on the plasma membrane have a distinct spatial pattern which suggests the presence of a single origin, presumably the oxidase protein complex, for each precipitate. It must be proposed that the inhibitors used are not specific and that they may have H$_2$O$_2$ scavenging properties as reported by Barcelo 41. KCN, NaN$_3$ 41 and DPI 42 have an ability to scavenge H$_2$O$_2$ in addition to their inhibitor activity. However, it has been shown in other systems, by combining localization, enzyme
activity and inhibitor studies that the two most likely candidates for stress-induced H$_2$O$_2$ synthesis are plasma membrane NADPH oxidase and cell wall POXs. Although the results from the inhibitor studies (Table 1) must be interpreted in correlation with the histochemical localization of the H$_2$O$_2$ synthesized and with enzyme assay. Tuomainen et al., suggested that these parameters provide an ambient system in dissecting the subcellular sites for ROS synthesis, thus leading the way to more specific studies. Furthermore, the inhibitory effect of DPI on nitric oxide (NO) synthesis and the interaction of NO and ROS production substantiates the present results.

In the present study, desiccation induced H$_2$O$_2$ production in the chloroplasts was comparatively less may be probably due to the efficient H$_2$O$_2$ scavenging mechanisms in the chloroplasts of these exposed plants. Chronic desiccation stress, results in overexpression of SOD in the chloroplast reduced the leaf damage in tobacco. In tobacco, chloroplastic SOD activity may be the rate-limiting enzyme for ROS scavenging and that was sufficient for detoxification of the H$_2$O$_2$ produced via SOD. Methyl viologen-induced O$_2^-$ synthesis in the chloroplasts, on the other hand, resulted in H$_2$O$_2$ accumulation that seemed to exceed the scavenging capacity in chloroplasts, since increased CeCl$_3$ precipitation was visible in the chloroplast stroma.

Many studies revealed that under high desiccation period, the cytoplasmic free radical scavenging by antioxidant system was in safe guarding plants. It is well known that peroxisomal catalase is a sink also for cytoplasmic H$_2$O$_2$, in addition to the H$_2$O$_2$ produced via photorespiration in peroxisomes. This scavenging mechanism was crucial for defense against oxidative burst in the cell system. The H$_2$O$_2$ accumulation in the cytoplasm, chloroplast and mitochondria suggest that cytoplasmic organelles could be a sink for the desiccation induced H$_2$O$_2$ produced, but not for the apoplastic H$_2$O$_2$. Intensity of H$_2$O$_2$ staining in chloroplast was marginal than in the surrounding cytoplasm, which would be seen if catalase scavenges cytoplasmically synthesized H$_2$O$_2$.

The mitochondrial H$_2$O$_2$ accumulation coincided with the symptoms of visible damage formation. Naton et al., reported similar changes with pathogen infected parsley cells, where the mitochondria were swollen and their tubuli were damaged. Subsequently, they reported the loss of mitochondrial membrane integrity, ROS accumulation and the death of the infected cells. Jabs, also suggested that in mammals the mitochondria are the major source of ROS metabolism leading to programmed cell death (PCD). The imbalance in mitochondria was through a reduction in transmembrane potential followed by uncoupling of electron transport from ATP synthesis. The latter causes O$_2^-$ production and subsequent bursting of mitochondrial integrity.
The role of mitochondria in oxidative stress has not been extensively analyzed in plants. Oxidative stress induced accumulation of heat shock proteins; especially the mitochondrial HSP22 in tomato cell cultures was also reported. Similarly, *Betula pendula* the transcript levels of the mitochondrial phosphate translocator (*Mptl*) were significantly increased by ozone impact was reported. These reactions may not be directly linked with defense against oxidative stress, but rather indicate changes in the oxidative balance of the cell that affect mitochondrial metabolism and the homeostasis of the cell. The relationship of ROSs: AOX machinery in plants to cell organelle permeability transition, subsequent hypergeneration of O$_2^-$ radicals and following PCD, as in animal cells, remains to be elucidated. Michaeli and Galili reported the degradation of organelles or specific organelle components via selective autophagy in plant cells induced by ROSs. Pellinen et al., reported ozone-induced hydrogen peroxide production and its localization at subcellular level in *Betula pendula* leaf cells. Lee et al., noticed rapid accumulation of H$_2$O$_2$ in cucumber roots due to exposure to low temperature appears to mediate decreases in water transport. Induction of an oxidative burst is a general feature of sensitive plants exposed to the air pollutant ozone. Slesak et al., stated the role of hydrogen peroxide in regulation of plant metabolism and cellular signaling in response to environmental stresses. Dat et al., noticed changes in H$_2$O$_2$ homeostasis trigger an active cell death process in tobacco cells. D’Haeez et al., correlated reactive oxygen species and ethylene and also their positive role in lateral root base nodulation of a semiaquatic legume. Schutzendubel et al., visualized cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiation in scots pine roots. Earlier reports of histochemical demonstration and localization of H$_2$O$_2$ in organs of higher plants by tissue printing on nitrocellulose paper was carried by Schopfer. Shao et al., compared primary antioxidant free radical scavenging and redox signaling pathways in higher plant cells. Dunand et al., connected distribution of superoxide and hydrogen peroxide in *Arabidopsis* root and their influence on root development: possible interaction with peroxidases. Rubio et al., localized superoxide dismutases (SOD) and H$_2$O$_2$ in legume root nodules. All these data substantiate the role of H$_2$O$_2$ accumulation as a signaling cascade to induce antioxidant machinery and there by counter balance oxidative burst of cells. In the present study, the forked fern produced enormous amount of H$_2$O$_2$ during increased periods of desiccation and decreased H*ATPase activity. This further reduce water transport between the cells.

4. CONCLUSION

The localized accumulation of H$_2$O$_2$ is consistent with the existence of a physically close link between the putative receptor controlling recognition of the challenging abiotic stress namely desiccation and activation of ROS production. Confirmation of
the molecular organization of the signaling cascade leading to ROS production will require the identification of both the desiccation induced ligand and the plant’s receptor, which are effective in generating drought resistant species.

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