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Desiccation Tolerance in Higher Plants Related to Free Radical Defences

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Summary

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Free radical-induced non-enzymatic peroxidation has the potential to damage membranes, enzymes and nucleic acids and it is likely to be one of the major causes of injury during desiccation of higher plants.

Protective mechanisms to scavenge the peroxidatively-produced free radicals and peroxides have evolved within plants, keeping these deleterious compounds to a minimum.

In this review the early intervention of glutathione, ascorbic acid and tocopherol is considered as a first line of defence to interrupt free radical action in membrane components.

To clarify the link between plant desiccation tolerance and free radical defences we have considered the changes in these major free radical reductants and in membrane status in two resurrection plants, *Boea hygroscopica* and *Sporobolus stapfianus* which offer the advantage that their leaves can exhibit desiccation tolerance depending on the manner in which they are dehydrated.

Introduction

Most plants are able to survive only a mild drought, but prolonged exposure to water deficit conditions usually leads to irreversible damage to cellular structures. The ability to withstand desiccation is a feature more common in lower plants. In higher plants the ability to survive virtual total water loss is limited to embryos of seeds and to a small group of plants (resurrection plants) exhibiting desiccation tolerance throughout their life cycle (GAFF 1989). To evaluate the effect of desiccation on plants it is important to know where and when the actual

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response occurs. Survival may depend on the plant's ability to 1) activate maintenance mechanisms during the desiccation phase; 2) retain integrity in the dry state; 3) develop repair mechanisms upon rehydration.

The production of protective substances, quantitative and qualitative aspects of protein synthesis, full reconstitution of membrane structure and composition, and prevention of oxidation of protein sulphydryl groups, induced during the dehydration and rehydration processes, seems to be requisite for cell survival (GAFF 1989, MARINONE-ALBINI & al. 1994, MICHEL & al. 1994, NAVARI-IZZO & al. 1995, SGHERRI & al. 1994a). Any one of these aspects alone may not be adequate to account for tolerance, but the adaptation shown by many plants could partly be due to changes in membrane composition and phase behaviour, which optimizes the fluidity and membrane function (NAVARI-IZZO & al. 1994, QUARTACCI & al. 1995), so that the ability to protect cellular components and membranes from oxidative stress may be an important aspect of desiccation tolerance. Desiccation injury in higher plants is indeed associated with destructive reactions which are mediated by superoxide radicals ($O_2^{\cdot-}$) and their highly reactive derivatives generated during desiccation and/or rehydration.

As long as the cell has the ability to scavenge and to detoxify the free radicals produced, the membrane components will be protected. Once this capacity is exceeded, radicals will further degrade membrane components such as lipids and proteins causing cell injury far in excess of the initial damage (NAVARI-IZZO & al. 1993, 1995, SENARATNA & al. 1984). For this reason analyses of membrane damage and protection by antioxidants are needed to understand the process.

Free radicals and membrane damage

Several models, among which is the promotion of activated oxygen species, have been considered to explain the nature of the injury to cell membranes during desiccation (LEVITT 1980, MCKERSIE & al. 1990, SENARATNA & al. 1984, 1985).

The active oxygen species superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen are formed in plants as a result of normal aerobic metabolism. When normal pathways and acceptors of electrons are restricted, the possibility of electron leakage to oxygen is enhanced, so increasing the production of superoxide (NAVARI-IZZO & al. 1996b) that initiates the sequential chain reactions that can quickly generate highly reactive free radical species. Superoxide *is a powerful nucleophile and base and also a reducing agent. It can promote* degradative reactions, causing loss of membrane polar lipids by a nucleophilic attack upon the carbonyl groups of the ester bonds linking fatty acids to the polar lipids (NAVARI-IZZO & al. 1991, NIEHAUS 1978). It may even act either as oxidizing agent reacting with protons to give the hydroperoxyl radical ($HOO^{\cdot}$), a species able to initiate peroxidation of lipids, or it can also react as reductant with thylakoid components, such as plastocyanin and cytochrome *f*. The action of a membrane-bound superoxide dismutase (SOD), an enzyme catalysing the removal of the superoxide anion, results in the production of H_2O_2 (ASADA 1994). In the hydrophobic interior of the biological membranes where $O_2^{\cdot-}$ is generated it can

also form hydroxyl radicals reacting non enzimatically with hydrogen peroxide in the presence of copper and iron (ELSTNER 1987). The hydroxyl radicals interact with almost all cell components but only at the site where they are generated (ASADA & TAKAHASHI 1987). Activated oxygen species are able to produce chemical modifications and/or damage to proteins, lipids, carbohydrates and nucleotides (BOWLER & al. 1992, LAND & SWALLOW 1968). Proteins in the presence of radicals can suffer fragmentation, cross-linking and amino acid modification (TAKENAKA & al. 1991). Furthermore,protein and non-protein sulphydryl groups are readily oxidized by free radicals leading to profound changes in enzyme activity (GILBERT & al. 1990). In addition lipid-derived radicals may be responsible for protein damage. Free radicals may injure cells through a pathway dependent essentially on membrane damage: 1) by covalent binding to membrane components, enzymes and/or receptors; 2) by impairing transport processes through sulphydryl group oxidation, change in lipid/protein ratios or covalent binding; 3) by de-esterification of polar lipids, and 4) by initiation of lipid peroxidation.

Evidence supporting the above statements derives from the fact that 1) stress conditions increase the production of free radicals (NAVARI-IZZO & al. 1995, QUARTACCI & NAVARI-IZZO 1992); 2) chemical and physical changes observed in membranes isolated from stressed plant tissues can be simulated in vitro by exposure to oxygen free radicals (KENDALL & MCKERSIE 1989, SENARATNA & al. 1987); 3) membranes from tolerant plant tissues are more tolerant of free radical treatment than those from susceptible tissues (KENDALL & MCKERSIE 1989, SENARATNA & al. 1987, 4) membranes from dehydrated plants producing more $O_2^{\cdot -}$ have a greater decrease in fluidity and changes in composition than those producing less $O_2^{\cdot -}$ (QUARTACCI & al. 1995), and 5) the production of $O_2^{\cdot -}$ increases about two orders of magnitude when the integrity of thylakoids is damaged by detergents (TAKAHASHI & ASADA 1982). All the above observations indicate that the integrity of membranes is associated with the acquisition of desiccation tolerance, which makes the membranes more resistant to oxygen free radical attack.

Free radicals and membrane lipids

The generally accepted mechanism of free radical attack involves acyl chain oxidation which should lead to a decrease in unsaturation of membrane lipids. The fact that such a phenomenon has not been observed in water-stressed tissues (KENDALL & MCKERSIE 1989, LILJENBERG 1992, NAVARI-IZZO & al. 1989, QUARTACCI & NAVARI-IZZO 1992, QUARTACCI & al. 1995, SENARATNA & al. 1985), in seeds exposed to free radicals (SENARATNA & al. 1987), and in resurrection plants during desiccation (BIANCHI & al. 1991, NAVARI-IZZO & al. 1994, 1995) suggests that in plants changes in the unsaturation level, which are often considered to be the only result of oxidative stress, are, in fact, a comparatively minor response to the action of free radicals (MCKERSIE & al. 1990, NAVARI-IZZO & al. 1992, QUARTACCI & NAVARI-IZZO 1992). In most cases of oxidative stress lipid peroxidation is probably not the major mechanism by which

increased generation of oxygen-derived species causes primary cellular injury. There are alternative mechanisms of free radical attack on membrane lipids (SLATER 1984, NAVARI-IZZO & al. 1991, 1992) in plant membranes so that the target for oxidative damage need not necessarely be unsaturated lipids. As reported above, the fatty acid ester linkage may be broken as a result of nucleophilic addition of superoxide radical at the ester bond.

The free radical reactions in model membrane systems and plant membranes are quite distinctly different. Liposomes, prepared from soybean asolecithin, exposed to oxygen free radicals resulted in peroxidative reactions leading to degradation of the unsaturated fatty acids. In contrast, in winter wheat treated with free radicals, in spite of the lack of change in fatty acid unsaturation, there was a substantial loss of esterified fatty acids without a subsequent increase in peroxidation, suggesting the presence of terminating antioxidants, preventing the chain reaction from continuing (MCKERSIE & al. 1990). Under conditions of oxidative stress, changes in membrane polar lipids and accumulation of free fatty acids with no changes in unsaturation level was also observed in sunflower, and in barley seedlings (NAVARI-IZZO & al. 1991, QUARTACCI & NAVARI-IZZO 1992).

In the absence of changes in fatty acid unsaturation the inherent danger of oxygen reactive molecules lies in their ability to mediate the degradation of polar lipids, with an accumulation of free fatty acids or other uncharged lipids such as triacylglycerols (NAVARI-IZZO & al. 1991, 1993, 1996b, QUARTACCI & NAVARI-IZZO 1992, QUARTACCI & al. 1995). The accumulation of these neutral lipids destabilizes the bilayer leading to the formation of gel phase domains (MCKERSIE & al. 1990). Both nucleophilic attack and peroxidation may result in membrane disorganization due to changes in membrane composition that determine the formation of non-bilayer phases and possible displacement of membrane proteins, the consequence being a loss of functional integrity of membranes.

In thylakoids, lipid damage is an ever present problem due to the high quantity of polar lipids and polyunsaturated fatty acid residues, both particularly susceptible to free radical attack (HALLIWEL & GUTTERIDGE 1984). Therefore, removal of activated oxygen species and hydroxyperoxyl fatty acid radicals is a priority if the functional integrity of thylakoid membranes is to be preserved.

Role of antioxidants in the oxidative chain reaction breaking

Protection against active oxygen is achieved by efficient defence systems, composed of both enzymic and non-enzymic cell constituents. Cellular maintenance would involve the reduction of harmful oxidative molecular species and the synthesis of macromolecules mediating those reductive processes. Repair processes would involve the re-synthesis of damaged macromolecules such as membrane lipids or the re-reduction of soluble -SH-containing enzymes which have been injuried as a consequence of free radicals. The role of antioxidants in protecting cells from degradative and destructive processes has been discussed in many excellent reviews (e.g. ASADA & TAKAHASHI 1987, ALSCHER 1989) and it

should not be necessary to repeat this here in any great depth. It can be pointed out that an important strategy in the ability of antioxidants to protect cell is an early intervention in order to break the sequence of chain reactions determined by the production of free radicals. Thus antioxidant mobility, as well as radical trapping ability, are important in determining antioxidant effectiveness in biological systems. The lipo-soluble antioxidants and membrane-bound antioxidative enzymes of higher plants serve as the first line of defence against activated oxygen species produced within membranes, while the water soluble antioxidants serve to eliminate activated oxygen species in the aqueous phase. Reactive oxygen species, except for hydrogen peroxide, can not diffuse very far from their locus of formation since in biological systems they have a very small average radius of diffusion and a half-life of only few-microseconds (SLATER 1984). For this reason protection measures have to be available in those cellular compartments where the production of reactive oxygen species takes place.

It is reasonable to assume that chloroplasts are a primary site for activated oxygen injury because these organelles both consume and produce oxygen and their functionality depends on redox status of photosynthetic apparatus. The lipophilic antioxidants tocopherol and carotenoids fulfill essential antioxidant action in thylakoid membranes. Furthermore, in the chloroplasts are two separate oxygen-radical scavenging systems: a soluble system comprising glutathione (GSH) and ascorbate (AsA), and a membrane-bound system that comprises SOD and ascorbate peroxidase (ASADA 1994). Ascorbate and glutathione have little influence on the spreading of oxygen radicals along or within the membranes so that it seems reasonable to think that they may intercept only the oxygen radicals spreading outward. The more likely function of GSH is its involvement in the ascorbate-glutathione cycle but a function in the direct removal of oxygen radicals can not be excluded (BARCLAY 1988), so that the reaction between GSH and $O_2^{\cdot-}$ may be potentially of importance in oxidative stress and/or in conditions of lowered SOD activity (WEFERS & SIES 1983). However, both ascorbate and glutathione may serve as mediators between the hydrophilic and lipophilic phase to maintain the antioxidant properties of membrane-localized protective systems. In combination with tocopherol they can result in synergistic inhibition of oxidative damage to cell membranes (FRYER 1992) by trapping the lipid radicals and suppressing lipid peroxidation rather than by scavenging $O_2^{\cdot-}$ or singlet oxygen (NIKI & al. 1982, 1984, PACKER & al. 1979). The chain breaking activity of tocopherol is predominantly maintained by ascorbate, while GSH predominantly acts as a preventitive antioxidant (WEFERS & SIES 1988). Ascorbate has been seen to protect against lipid peroxidation as long as tocopherol is present, but in tocopherol-deficient microsomes ascorbate initiates lipid peroxidation immediately, demonstrating its pro-oxidant effect. On the contrary, GSH is effective at low tocopherol concentrations, thereby providing a protective effect also in tocopherol deficiency. GSH may prevent lipid peroxidation from entering the propagation stage scavenging lipid alkyl or lipoxyl radicals formed during the initiation stage (LIEBLER & al. 1986).

Tocopherol is an amphipatic molecule with its hydrophobic phytyl tail located in the membrane, associated with the acyl chains of fatty acids while its polar "head" group (chromanol) is oriented towards the membrane surface, with the phenolic hydroxyl group located near the polar group of the lipid matrix (PERLY & al. 1985). Tocopherol is an non-specific trap for activated oxygen species, but it reacts rapidly (2.4×10^6 $M^{-1}s^{-1}$) with alkyl peroxyl radicals formed in the lipid phase (BURTON & al. 1983), becoming irreversibly oxidized (NEELY & al. 1988). The tocopheroxyl radical must be reduced to re-function as chain breaking antioxidant, but no enzyme systems for such a function have been described. Fast regeneration of tocopheroxyl radical by ascorbate and GSH has been detected by electron spin resonance spectroscopy (NIKI & al. 1982, SLATER 1984). Direct interactions at the membrane surface with either ascorbate (LIEBLER & al. 1986) or reduced glutathione (WEFERS & SIES 1988) allows tocopherol many chain breaking events before its degradation, producing the monodehydroascorbate and glutathyl radicals. However, ascorbate and GSH will act as radical scavengers only when there is an efficient removal of their radical forms. The radicals formed to regenerate tocopherol are in turn recycled by available reducing equivalents such as NAD(P)H (ASADA & TAKAHASHI 1987), in association with their specific reductase enzymes. This mechanism creates a link between the free radical reactions initiated in the lipid phase and the scavenging activity in the aqueous phase. The regeneration of tocopherol by reduced ascorbate and glutathione is important also because tocopherol seems to influence significantly the fluidity of membranes in a manner similar to cholesterol (FRYER 1992), and may even form complexes with fatty acids (ERIN & al. 1987). Thus, in addition, the action of tocopherol can be regarded as a molecular mechanism responsible for the stabilization of biomembranes exposed to the damaging action of free fatty acids.

Protective mechanisms in the desiccation tolerance of resurrection plants

During dehydration and rehydration of the resurrection plants *Boea hygroscopica* and *Sporobolus stapfianus* peroxidative damage to polyunsaturated fatty acids does not occur. However, in these plants desiccation determined increased deesterification of polar lipids and accumulation of free fatty acids and triacylglycerols with concomitant changes in the packing, fluidity, and/or physical arrangement of the membranes (NAVARI-IZZO & al. 1994, 1995, QUARTACCI & al. 1995, 1996). In addition, an increased or a maintained unsaturation level was

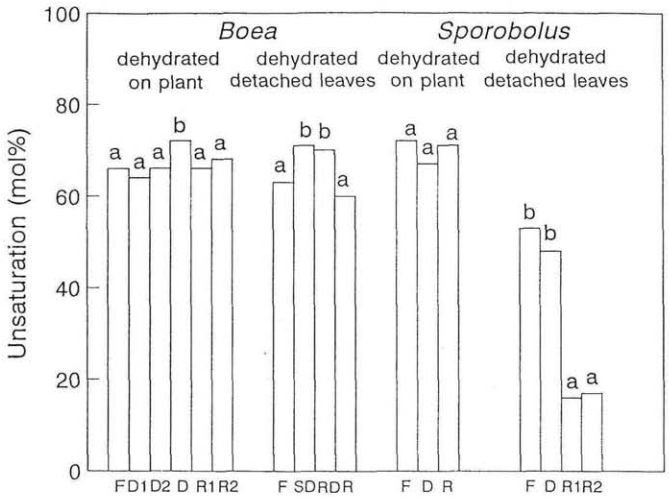

Fig. 1. Polar lipid unsaturation levels of *Boea hygroscopica* and *Sporobolus stapfianus* leaves. For comparisons among means analysis of variance was used. Histograms accompanied dy different letters are significantly different at $P \leq 0.01$ level. F, fresh leaves; D1, dehydrated at 78.7% RWC; D2, dehydrated at 48.0% RWC; D, completely dehydrated; R1, rehydrated to 37.5% RWC; R, completely rehydrated; RD, rapidly dehydrated; SD, slowly dehydrated.

observed upon desiccation in these (Fig. 1) as well as in other resurrection plants. Following rehydration the unsaturation level returned again to that of undesiccated leaves (BIANCHI & al. 1991, NAVARI-IZZO & al. 1994). The changes in unsaturation upon desiccation may be the result of the decrease of both enzymic oxidation (BIANCHI & al. 1992) and oxidation due to activated oxygen forms, and upon rehydration, of the increase in superoxide radicals (NAVARI-IZZO & al. 1994). Furthermore, in dried *Craterostigma plantagineum* and in *Sporobolus stapfianus* the inhibitors of lipoxygenase, colneleic and colnelenic acids, have been found (BIANCHI & al. 1992, MARINONE-ALBINI & al. 1994). This further supports the ability of resurrection plants to minimize membrane damage. According to these results the target for oxidative damage need not necessarily be unsaturated lipids and the free radical-induced polar lipid deesterification hypothesis has to be considered (NAVARI-IZZO & al. 1991, 1992).

The enhanced formation of reduced glutathione has been seen to play an important role in limiting lipid peroxidation (ALBRECHT & WIEDENROTH 1994, NAVARI-IZZO & al. 1994, SGHERRI & al. 1994a). Evidence that desiccation injury is related to the levels of GSH comes from studies on *Tortula ruralis* and *Selaginella lepidophylla* where the $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase activity was severely reduced following the oxidation of GSH pools and was restored by adding GSH (DHINDSA 1987a,b, LEBKUECKER & EICKMEIER 1992, STEWART & LEE 1972). Notably, in *Boea hygroscopica* the activity of the enzyme

remained constant in spite of the high dehydration level (NAVARI-IZZO & al. 1996a).

Detached leaves of *Boea hygroscopica* have the ability to increase the amount of constitutive glutathione up to 50 times both upon slowly and rapidly drying and to utilize reduced glutathione when rehydrated (SGHERRI & al. 1994a). Dehydration of *Boea hygroscopica* plants to 80% relative water content (RWC) brought about a dramatic decrease in GSH, which upon further dehydration began to accumulate till complete dehydration (NAVARI-IZZO & al. 1996a). The release of feedback inhibition of the synthesis of GSH through an initial decrease in GSH might have been involved (SMITH & al. 1985). The observation that in *Boea hygroscopica* dehydrated to 80% RWC an induction in ABA was also observed is interesting (BOCHICCHIO & al. unpublished results), with ABA presumably involved in the induction of gene expression during turgor loss (MICHEL & al. 1994). By accumulating GSH its redox status (GSH^2/GSSG) increased up to 20 times during drying (NAVARI-IZZO & al. 1996a).

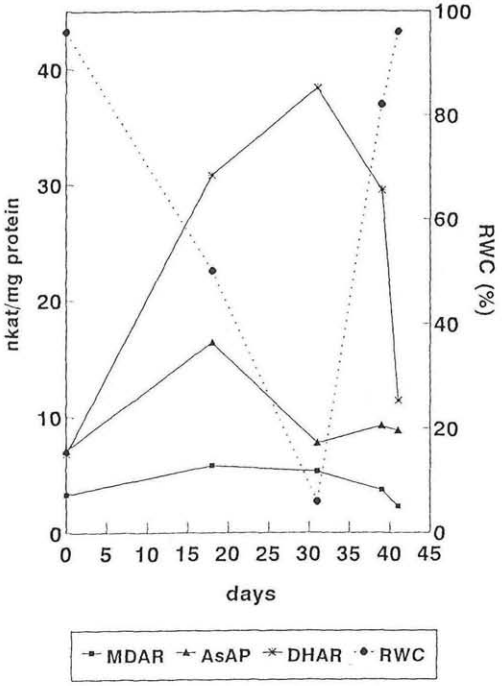

Fig. 2. Activities of the enzymes related to ascorbate metabolism in *Sporobolus stapfianus* leaves dehydrated and rehydrated on the plant. Data are expressed as means ± SE. AsAP, ascorbate peroxidase; DHAR, dehydroascorbate reductase; MDAR, monodehydroascorbate reductase; RWC, relative water content.

A more reduced status of the cell during desiccation has also been observed by an induction of the synthesis of ascorbate (SGHERRI & al. 1994a). The ability to maintain a low dehydroascorbate/ascorbate ratio through regeneration of AsA is probably more important than the absolute amount of total ascorbate present during drying. The activation of monodehydroascorbate reductase, dehydroascorbatereductase and ascorbate peroxidase has been indeed observed during dehydration of *Sporobolus stapfianus* plants (Fig. 2). The accumulation of reduced ascorbate and glutathione during drying might constitute a reserve which allows resurrection plants to tolerate oxidative damage during rehydration, when the injury caused by desiccation must be repaired. Indeed detached leaves of *Sporobolus stapfianus* which during dehydration did not accumulate reduced ascorbate and glutathione (SGHERRI & al. 1994b) are not able to revive upon rehydration (GAFF 1989). Both GSH (NAVARI-IZZO & al. 1996a, SGHERRI & al. 1994a) and AsA (Fig. 3) may have helped to break the chain of

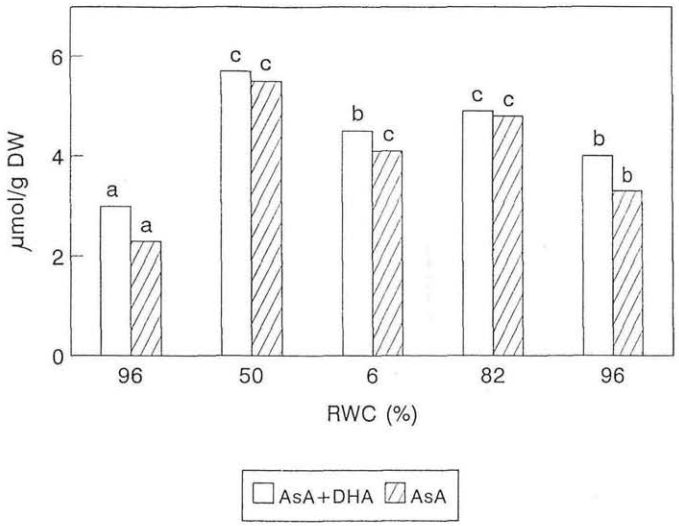

Fig. 3. Ascorbate content of *Sporobolus stapfianus* leaves dehydrated and rehydrated on the plant. For statistical analysis see Fig. 1. AsA, ascorbate; DHA, dehydroascorbate; RWC, relative water content.

peroxidative reactions by maintaining tocopherol in a reduced form thus preventing lipid peroxidation and/or oxidation of protein sulphydryl groups in desiccated resurrection plants (NAVARI-IZZO & al. 1996a). This could have important implications with regard to the preservation of the integrity of thylakoid membranes, which otherwise may be susceptible to peroxidative damage and will, consequently, affect the ability of resurrection plants to recover photosynthetic activity upon rehydration.

The hypothesis based on membrane damage resulted from hydration-dependent changes in polar lipid transition temperature must also be remembered (CROWE & CROWE 1992). In this case high concentrations of sugars, mainly sucrose, found in dried resurrection plants (BIANCHI & al. 1991, 1992) may give protection to lipid membranes either by stabilizing their polar lipids (CROWE & CROWE 1992) and/or by acting as hydroxyl radical scavengers (SMIRNOFF 1992). Particularly, arbutin as a source of hydroquinone might reduce the rate of peroxidation of unsaturated lipids (SUAU & al. 1991).

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