

REVIEW PAPER

Regulation by arbuscular mycorrhizae of the integrated physiological response to salinity in plants: new challenges in physiological and molecular studies

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Abstract

Excessive salt accumulation in soils is a major ecological and agronomical problem, in particular in arid and semi-arid areas. Excessive soil salinity affects the establishment, development, and growth of plants, resulting in important losses in productivity. Plants have evolved biochemical and molecular mechanisms that may act in a concerted manner and constitute the integrated physiological response to soil salinity. These include the synthesis and accumulation of compatible solutes to avoid cell dehydration and maintain root water uptake, the regulation of ion homeostasis to control ion uptake by roots, compartmentation and transport into shoots, the fine regulation of water uptake and distribution to plant tissues by the action of aquaporins, the reduction of oxidative damage through improved antioxidant capacity and the maintenance of photosynthesis at values adequate for plant growth. Arbuscular mycorrhizal (AM) symbiosis can help the host plants to cope with the detrimental effects of high soil salinity. There is evidence that AM symbiosis affects and regulates several of the above mentioned mechanisms, but the molecular bases of such effects are almost completely unknown. This review summarizes current knowledge about the effects of AM symbiosis on these physiological mechanisms, emphasizing new perspectives and challenges in physiological and molecular studies on salt-stress alleviation by AM symbiosis.

Key words: antioxidant, aquaporin, arbuscular mycorrhizal symbiosis, ionic homeostasis, osmoregulation, photosynthesis, salinity.

Introduction

Excessive salinization of soils is a major ecological and agronomical problem, in particular in arid and semi-arid areas and in Mediterranean ecosystems (Giri *et al.*, 2003; Al-Karaki, 2006; Evelin *et al.*, 2009). Soil salinization can be due to natural causes such as salty rain, waters around the coasts, contamination from parental rocks and oceanic salts, but inadequate cultivation practices have exacerbated the concentration of salts in the rhizosphere (Mahajan and Tuteja, 2005). In this way, salinization of arable land is becoming widespread throughout the world. Estimations indicate that salinization of arable land will result in 30% land loss within the next 25 years, and up to 50% within the next 40 years (Wang *et al.*, 2003; Porcel *et al.*, 2012).

Soil salinity affects the establishment, growth, and development of plants, causing important yield losses (Evelin *et al.*, 2009). Salinity negatively affects three aspects of plant physiology. Firstly the toxic effects of specific ions such as sodium and chloride inhibit protein synthesis, damage cell organelles, disrupt the structure of enzymes, and uncouple photosynthesis and respiration. Secondly, salinity also decreases nutrient uptake and/or transport to the shoot, thus inducing a nutrient imbalance in the plant (Marschner, 1995; Evelin *et al.*, 2009). Finally, accumulation of salts in the soil lowers soil osmotic potentials and hinders the uptake of water by roots, producing a physiological drought in the plant. Moreover, plants must decrease their internal osmotic

potentials to prevent water moving from the roots into the soil.

Under salinity, the availability of atmospheric CO₂ is restricted because stomatal closure is increased and consumption of NADPH by the Calvin cycle is reduced. When ferredoxine is over-reduced during photosynthetic electron transfer, electrons may be transferred from photosystem I (PSI) to oxygen to form superoxide radicals (O₂⁻) by a process known as the Mehler reaction, which initiates chain reactions that produce more harmful oxygen radicals. These include singlet oxygen (¹O₂), hydrogen peroxide (H₂O₂) and hydroxyl radicals (OH[•]). When these cytotoxic reactive oxygen species (ROS) are produced in excess, they can destroy normal metabolism through oxidative damage of lipids, proteins, and nucleic acids (Miller *et al.*, 2010). Thus, salinity also induces an increase in the production of ROS and oxidative damage in the plant (Ding *et al.*, 2010) and there is a constant need for efficient mechanisms to avoid oxidative damage to cells.

As a consequence of all the above mentioned processes, salt stress affects the major plant metabolic processes, such as photosynthesis, growth, energy and lipid metabolisms, and protein synthesis (Ramoliya *et al.*, 2004). However, plants have evolved several biochemical and molecular mechanisms to cope with the negative effects of salinity. Figure 1 summarizes the most important mechanisms involved in homeostasis or damage prevention during salinity. These include the regulation of genes with a role in the transport or compartmentation of Na⁺ and/or K⁺, such as the Na⁺/H⁺ antiporter SOS1, the Na⁺ influx transporter family HKT and the tonoplast Na⁺/H⁺ antiporter family NHX, known for their important role during ionic homeostasis (Munns, 2005). Mechanisms of water/osmotic homeostasis are intended to restore the cellular ion or water content to levels similar to

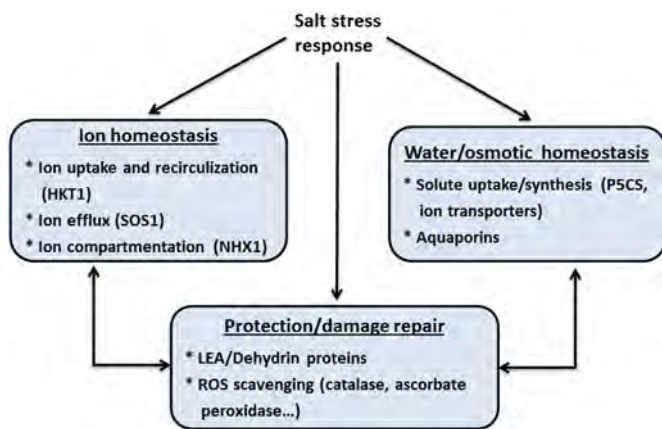


Fig. 1. Homeostasis and protection/damage repair model of the salt stress response. Mechanisms of ion homeostasis and water/osmotic homeostasis are intended to restore the cellular ion or water content to levels similar to those present under unstressed conditions. Protection and damage repair mechanisms attempt to prevent or repair cellular damage caused by altered ion or water content under stress. Adapted from Verslues *et al.* (2006), with kind permission from John Wiley & Sons.

those present under unstressed conditions. This depends of the action of genes involved in solute biosynthesis and water channels (aquaporins). However, the precise role of aquaporins in the response to osmotic stress is not completely understood. Protection and damage repair mechanisms attempt to prevent or repair cellular damage caused by altered ion, water content, or ROS under stress.

As well as the intrinsic adaptation mechanisms developed by plants, under natural conditions they grow in association with a number of soil microorganisms. Some of these microorganisms, especially bacteria and fungi, can improve plant performance when environmental conditions are adverse (Barea *et al.*, 2005; Aroca and Ruíz-Lozano, 2009). Arbuscular mycorrhizal (AM) fungi establish a symbiotic association with the roots of 80% of terrestrial plants (Van der Heijden *et al.*, 1998; Smith and Read, 2008). This AM symbiosis can be defined as a specialized system for nutrient uptake and transfer that is more efficient than roots alone. However, the uptake and transfer of nutrients to the host plant is not the only physiological role of AM symbiosis. Indeed, in most cases studied, the association between an AM fungus and a plant makes the host plant more tolerant to abiotic stresses (Dodd and Ruíz-Lozano, 2012). In addition, AM fungi can be found under extreme saline conditions, and they can be adapted to these conditions (Wilde *et al.*, 2009).

The literature about the effects of salinity on AM fungi and their capacity to colonize plants is somewhat controversial. Some studies state that salt inhibits germination of spores or other fungal propagules, colonization of the plant roots, and sporulation of AM fungi (Juniper and Abbott, 2006; Giri *et al.*, 2007; Jahromi *et al.*, 2008; Sheng *et al.*, 2008). However, several publications report that AM fungi in saline soils can increase plant salt tolerance, decreasing plant yield losses (Ruíz-Lozano *et al.*, 1996; Al-Karaki *et al.*, 2001; Cantrell and Linderman, 2001; Hajiboland *et al.*, 2010). These studies have suggested some mechanisms to explain the enhanced salt tolerance of AM plants (e.g. better ability for nutrient and water uptake due to an extended explored soil surface by fungal hyphae, greater root hydraulic conductivity and osmotic adjustment, maintenance of enhanced K⁺/Na⁺ ratios, and lower accumulation of sodium in the shoots of the host plants). However, the intimate mechanisms that allow AM plants a higher tolerance to salinity are far from being understood. In addition, studies in the field are faced with the problem that such protective traits are generally encoded by gene families. Thus, any investigation on the impact of AM colonization on the expression of genes with products involved in salt tolerance is faced with the multiplicity and complexity of the traits. Hence, in gene expression studies, efforts should concentrate on the few that are seemingly of paramount relevance in salt tolerance. In this review, we discuss our current knowledge of the regulation by AM symbiosis of plant responses to salt stress and propose new perspectives for physiological and molecular studies, which should shed further light on the intimate tolerance mechanisms induced by AM symbiosis.

Regulation by AM symbiosis of plant responses to salt stress and perspectives for future studies

Plant salt tolerance itself is a complex trait to which many different factors may contribute. Plants have evolved biochemical and molecular mechanisms, which may act in a concerted manner and constitute the integrated physiological response to soil salinity. The most important plant strategies are: (i) synthesis and accumulation of compatible solutes; (ii) control of ion uptake by roots, compartmentation, and transport into plant tissues, which constitutes the ion homeostasis strategy; (iii) fine regulation of water uptake and distribution to plant tissues by the action of aquaporins; and (iv) reduction of oxidative damage through improved antioxidant capacity. Additional plant responses can include selective build-up or exclusion of salt ions, maintenance of photosynthesis at values adequate for plant growth, changes in membrane structure, and synthesis of phytohormones (Türkan and Demiral, 2009).

Osmotic adjustment

As salts accumulate in soil, the soil water potential becomes more negative. Thus, plants must respond by decreasing their water potential in order to maintain a favourable gradient for water flow from the soil into the roots and to avoid cell dehydration. The most important mechanism used to achieve such an effect is decreasing the plant osmotic potential by active accumulation of organic ions or solutes, known as osmotic adjustment or osmoregulation (Morgan, 1984; Hoekstra *et al.*, 2001). Osmotic adjustment allows cells to maintain turgor and related processes, such as cellular expansion and growth, stomatal opening, and photosynthesis. It also serves to keep a favourable gradient of water potential, which permits water entrance into the plant. The inorganic ions that participate in osmotic adjustment are mainly K^+ and Cl^- . Among the organic solutes, the most important are uncharged organic compounds such as proline, glycine betaine (*N,N,N*-trimethylglycine betaine), soluble sugars, pinitol, and mannitol (Flowers and Colmer, 2008).

Proline, glycine betaine, pinitol, and mannitol are important osmoprotectant osmolytes that are synthesized by many plants in response to dehydration stresses, including salinity. These compounds help in maintaining the osmotic status of the cell and protect against detrimental effects of salinity. It has also been suggested that proline plays roles in stabilizing subcellular structures, in buffering cellular redox potential under stresses, and in scavenging free radicals (Chen and Dickman, 2005). The biosynthesis of proline in higher plants starts from glutamic acid and is catalysed by two enzymes; pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) (Delauney and Verma, 1993). Overexpression of the P5CS gene in tobacco plants increases both the production of proline and the tolerance to salinity or drought (Kishor *et al.*, 1995). In the case of glycine betaine, this compound has been shown to protect plants against salinity by acting both as an osmotic regulator and as a protecting agent for

PSII (Jagendorf and Takabe, 2001). The biosynthesis of glycine betaine in plants starts from choline through two oxidative reactions catalysed by choline monoxygenase (CMO) and betaine aldehyde dehydrogenase (BADH), respectively (Chen and Murata, 2011). Pinitol is synthesized from *myo*-inositol through a two-step reaction. The methyl group from *S*-adenosyl-L-methionine is first transferred to *myo*-inositol by inositol methyl transferase, resulting in the production of ononitol. Ononitol is then converted to pinitol by an epimerase that has yet to be isolated and characterized (Chiera *et al.*, 2006). Mannitol is synthesized from mannose-6-phosphate by the action of mannose-6-phosphate reductase (Zhifang and Loescher, 2003).

So far, investigations carried out on osmoregulation in AM symbiosis are scarce and somewhat contradictory. Several studies have reported a higher proline concentration in AM plants than in non-AM plants at different salinity levels (Sharifi *et al.*, 2007; Talaat and Shawky, 2011), while, in contrast, other studies have reported that non-AM plants accumulate more proline than AM plants under salt stress (Wang *et al.*, 2004; Rabie and Almadini, 2005; Jahromi *et al.*, 2008; Sheng *et al.*, 2011). These results suggest that proline accumulation in plants may be due to salinity and not necessarily the result of mycorrhizal colonization, or that proline accumulation may be a symptom of stress in less-salt-tolerant species. In any case, these studies involve different plant species such as soybean, wheat, bean, lettuce, and maize, and also different AM fungi such as *Glomus intraradices*, *Glomus mosseae*, or a mixture of *Glomus* spp., and even different treatments such as salt pre-treated mycorrhizal fungi (Sharifi *et al.*, 2007), which may explain the contrasting results obtained. In fact, different symbiotic efficiencies may be found in different plant–fungus combinations (Ruíz-Lozano *et al.*, 1995). However, accumulation of betaines under salt stress was found to increase when the plant was colonized by AM fungi (Al-Garni, 2006). Betaines can stabilize protein complexes and the structures and activities of enzymes, as well as maintaining the integrity of membranes against the damaging effects of salt stress (Evelin *et al.*, 2009).

The accumulation of sugars in plants colonized by AM fungi has been proposed as a defence mechanism against drought stress (Porcel and Ruíz-Lozano, 2004) and against salinity (Sheng *et al.*, 2011; Talaat and Shawky, 2011). However, Sharifi *et al.* (2007) observed no role of soluble sugars in the responses to salinity of *Glomus etunicatum*-colonized soybean plants. However, it is not clear whether or not the accumulation of sugars in the roots of AM plants is due to the sink effect of the AM fungus demanding sugars from shoots (Augé, 2000). The increased sugar accumulation observed in some studies may also be due to hydrolysis of starch to sugars in mycorrhizal seedlings.

To illustrate further the complex response of AM plants in terms of solute accumulation, it was noted that in a recent study by Ruíz-Lozano *et al.* (2011) with lettuce plants that, under drought stress, non-AM plants accumulated more proline in their shoots than AM plants. In contrast, in the roots, AM plants accumulated more proline than non-AM

plants. This suggests that, in root tissues, AM plants accumulate more proline in order to cope with the low water potential of drying soil and to keep a water potential gradient favourable to water entrance into the roots (Porcel and Ruiz-Lozano, 2004), and the same may occur under salt-stress conditions. Moreover, Sheng *et al.* (2011) found enhanced reducing sugar accumulation in the leaves of AM maize plants subjected to salt stress, while the content of proline was lower than in non-AM plants. The authors proposed that the high levels of sugars in AM plants might be the result of an increase in the photosynthetic capacity of the plants and that these sugars contributed to the osmotic adjustment of the plants (Sheng *et al.*, 2011).

The studies carried out so far on osmoregulation in AM plants subjected to salt stress have not determined whether changes in the accumulation of osmoregulatory compounds translate into a better water status in plant tissues in terms of water potential or relative water content. Thus, we can conclude that the role of AM symbiosis on host-plant osmotic adjustment, as well as on the regulation of key genes for the biosynthesis of these compounds, is not clear, and this aspect should be investigated further. In this regard, we propose that the regulation of genes encoding major plant osmoprotectants, such as $\Delta 1$ -pyrroline-5-carboxylate synthetase (proline biosynthesis), choline monoxygenase (glycine betaine biosynthesis), mannose-6-phosphate reductase (mannitol biosynthesis) and inositol methyl transferase (pinitol biosynthesis), should be examined in the roots and shoots of AM and non-AM plants subjected to increasing salinity levels. The expression patterns of these genes should be correlated with the amounts of proline, glycine betaine, mannitol and pinitol that accumulate in these plant tissues and with their water status under the different treatments.

Ionic homeostasis

When the concentration of sodium salts in the soil rises, plants take up more Na^+ and less K^+ . Na^+ ions directly compete with K^+ for binding sites that are essential for various metabolic functions (Evelin *et al.*, 2009). In addition, Na^+ is toxic to cell metabolism and has a deleterious effect on some enzymes. High Na^+ levels also reduce photosynthesis and lead to ROS accumulation (Mahajan and Tuteja, 2005). Hence, Na^+ uptake and distribution within the plant are major determinants for the salt sensitivity of a plant. Prevention of Na^+ entry into the root, sequestration into the vacuole, and transport to and allocation within the leaf are strategies by which plants cope with a high-salt environment. In contrast, potassium is one of the essential elements and plays a key role in plant metabolism. K^+ has a role in opening and closing of stomata, is required for maintaining the osmotic balance, is a co-factor for many enzymes, and is involved in the biosynthesis of proteins, as K^+ participates in the binding of tRNA to the ribosomes (Blaha *et al.*, 2000). These functions cannot be replaced by Na^+ ions (Giri *et al.*, 2007) and, consequently, a lower $\text{K}^+:\text{Na}^+$ ratio generated as a result of salinity disrupts various metabolic pathways and

the cytoplasmic ionic balance (Giri *et al.*, 2007; Evelin *et al.*, 2009). Thus, in recent years, the significance of maintaining a high cytosolic $\text{K}^+:\text{Na}^+$ ratio in salinity tolerance is becoming widely accepted. To this end, plants have evolved several strategies that allow them to limit the entrance of Na^+ into the root, its sequestration into the vacuole, and its transport and distribution to the leaves. The most important transporters contributing to Na^+ and K^+ homeostasis in *Arabidopsis* are represented in Fig. 2 and comprise:

- Transporter type HKT, which allows the transport of Na^+ and K^+ and is involved in the transport of Na^+ from the root to the shoot, as well as in the maintenance of the $\text{K}^+:\text{Na}^+$ ratio in the root (Munns, 2005).
- Transporter type AKT, which is a channel for K^+ with higher selectivity for K^+ than for Na^+ (Munns, 2005).
- Transporter type NHX, which is a Na^+/H^+ antiporter localized in the vacuole. It is expressed in roots and leaves and sequesters Na^+ into the vacuole (Munns, 2005).
- Transporter type SOS1, which is a Na^+/H^+ antiporter involved in Na^+ expulsion from the cell. It may also participate in Na^+ extrusion from the root to the external medium (Munns, 2005).

AM fungi can be found under severe saline conditions in nature, both in saline inlands (Aliasgharzadeh *et al.*, 2001; Nasr, 2003) and in salt marshes (Wolfe *et al.*, 2007; Wilde *et al.*, 2009). Several greenhouse experiments have shown that AM fungi can promote plant growth under salinity stress (Zuccarini and Okurowska, 2008; Wu *et al.*, 2010a) by providing nutrients (Ruiz-Lozano and Azcón, 2000) or by alleviating the additional water stress (Sheng *et al.*, 2008). Mycorrhizal colonization of host plants has been shown to prevent Na^+ translocation to shoot tissues, while enhancing K^+ absorption under saline conditions (Alguacil *et al.*, 2003; Giri *et al.*, 2007; Sharifi *et al.*, 2007; Zuccarini and Okurowska, 2008; Talaat and Shawky, 2011). Thus, AM plants maintain a higher $\text{K}^+:\text{Na}^+$ ratio, preventing the disruption of cellular enzymatic processes and inhibition of protein synthesis. However, there are also contrasting

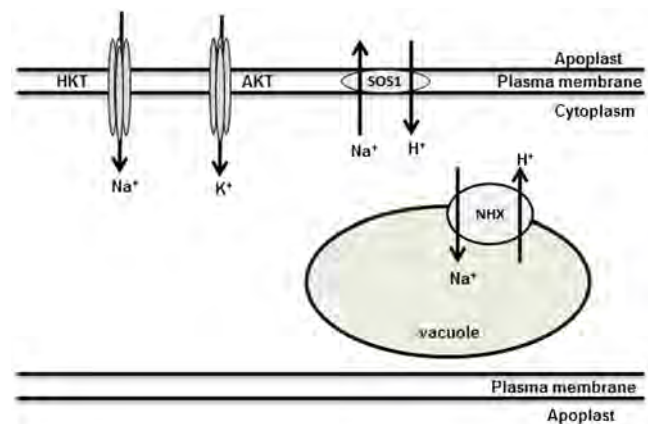


Fig. 2. Schematic representation of some important transporters involved in Na^+ and K^+ homeostasis in plants subjected to salinity. Adapted from Türkan and Demiral (2009).

reports that AM fungi sometimes enhance Na^+ uptake (Allen and Cunningham, 1983). Recent results by Hammer *et al.* (2011) indicated that AM fungi can selectively take up elements such as K^+ and Ca^{2+} , which act as osmotic equivalents while they avoid the uptake of toxic Na^+ . The concentration of Na^+ appeared to increase in AM plants with increasing salinity levels up to a certain level, decreasing subsequently when the salt concentration was higher. These results suggest a possible buffering effect of AM fungi on the uptake of Na^+ when the content of Na^+ is within acceptable limits (Evelin *et al.*, 2009; Hammer *et al.*, 2011). This could make them important in the alleviation of salinity stress in the host plants. Indeed, a recent article has shown that the colonization of fenugreek plants by *G. intraradices* prevented excess uptake of Na^+ at increasing salinity in the soil (Evelin *et al.*, 2012). Mycorrhization also contributed to overcome the Na^+ -induced Ca^{2+} and K^+ deficiencies. Thus, AM plants maintained higher $\text{K}^+:\text{Na}^+$, $\text{Ca}^{2+}:\text{Na}^+$, and $\text{Ca}^{2+}:\text{Mg}^{2+}$ ratios in their tissues than their non-AM counterparts. Moreover, AM plants had also higher concentrations of other nutrients such as Cu, Fe, Mn^{2+} , and Zn^{2+} compared with non-AM plants.

To our knowledge, only Ouziad *et al.* (2006) have analysed the expression of genes involved in ion homeostasis in mycorrhizal plants. They studied the effect of AM symbiosis on the expression of two Na^+/H^+ antiporters in tomato plants subjected to salinity stress. The results showed that, under the conditions assayed, AM symbiosis did not alter the expression of *LeNHX1* and *LeNHX2*. Recently, cyclic nucleotide-gated ion channels (CNGCs) have been proposed as candidate genes for studies of salt-stress amelioration in AM plants (Porcel *et al.*, 2012). The CNGC family is composed of non-selective cation channels that enable the uptake of Na^+ , K^+ , and Ca^{2+} (Kaplan *et al.*, 2007). It has been suggested that CNGCs contribute to sodium reallocation within the plant tissues, assisting the plant in coping with salinity stress (Porcel *et al.*, 2012).

In summary, there are several studies showing that AM plants have better $\text{K}^+:\text{Na}^+$ ratios than non-AM plants. However, the molecular mechanisms involved in such an effect are almost completely unknown. Thus, studying the possible regulation of genes encoding known ion transporters such as HKT, AKT, SOS, and NHX, and probably also CNGCs, during the response of AM symbiosis to salinity is a promising field. These studies should be accomplished in combination with measurements of sodium and potassium content and their ratios in the different plant tissues. Together, this should allow a better understanding of whether AM symbiosis affects Na^+ and K^+ uptake, distribution, and compartmentation within the plant cell, and should shed further light on new mechanisms involved in the enhanced tolerance of AM plants to salt stress.

Regulation of root water uptake and redistribution along plant tissues by aquaporins

The lowering of water potential in saline soils obliges the plants to face the problem of acquiring enough water from

the soil (Ouziad *et al.*, 2006). Exposure to salinity challenges the plant water status and triggers specific strategies for control of water uptake and loss (Boursiac *et al.*, 2005). One of the primary responses of plants to salt is inhibition of their root water uptake capacity (i.e. root hydraulic conductivity). Although some exceptions have been reported in barley and tobacco, this response can be observed in a large variety of plant species (Martínez-Ballesta *et al.*, 2003). The exact mechanism by which salinity reduces the hydraulic conductance in cells and roots is still unknown. However, it has been suggested that it could be due to changes either in the aquaporin function or in the amount of this protein present in the membrane (Carvajal *et al.*, 2000; Martínez-Ballesta *et al.*, 2000; Sade *et al.*, 2010).

Aquaporins are a group of water-channel proteins that facilitate and regulate the passive movement of water molecules following a water potential gradient (Kruse *et al.*, 2006). However, water is not the only molecule transported by aquaporins. In fact, low-molecular-weight compounds such as glycerol, urea, ammonium, and CO_2 can also cross these proteins (Flexas *et al.*, 2006; Katsuhara *et al.*, 2008; Maurel *et al.*, 2009). These proteins belong to the large major intrinsic protein (MIP) family of transmembrane proteins and are represented in all kingdoms (Maurel *et al.*, 2008). In plants, aquaporins are subdivided into five evolutionarily distinct subfamilies: the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the small basic intrinsic proteins (SIPs), the nodulin26-like intrinsic proteins (NIPs) (Chaumont *et al.*, 2001; Johanson *et al.*, 2001), and the uncharacterized X intrinsic proteins (XIPs) (Gupta and Sankararamkrishnan, 2009), which have been shown recently to transport a variety of uncharged substrates (Bienert *et al.*, 2011). The most abundant information relates to PIPs and TIPs and demonstrates their important role in the regulation of root hydraulic conductivity and in osmoregulation of the cell cytoplasm (Luu and Maurel, 2005).

The notion that aquaporin water channels serve as a major path for the uptake of water by roots was raised from early experiments showing that root water transport can be inhibited by the general aquaporin blocker mercury ions (Maggio and Joly, 1995). Recent physiological and molecular studies have confirmed conclusions based on mercury inhibition and provide compelling evidence for a role of aquaporins in the regulation of water transport under stress (Maurel *et al.*, 2008; Aroca *et al.*, 2012). Hence, in the case of roots under salt stress, the role of aquaporins in the plant response to salinity must be considered.

As AM fungi have been shown to transfer water to the root of host plants (Marulanda *et al.*, 2003; Ruth *et al.*, 2011), it could be expected that the host plant increases its permeability for water, and aquaporin genes could be upregulated in order to allow a higher rate of transcellular water flow (Javot and Maurel, 2002). In fact, in recent years, it has been demonstrated that AM symbiosis regulates root hydraulic properties, including root hydraulic conductivity, and these effects have been linked to the regulation of plant aquaporins (Ruíz-Lozano and Aroca, 2010).

When the expression of aquaporin genes was analysed in salt-stressed AM and non-AM plants, the results obtained were not conclusive. Aroca *et al.* (2007) analysed the expression pattern of four aquaporin genes in AM and non-AM *Phaseolus vulgaris* plants subjected to drought, cold, or salinity. The results showed that three of these PIP genes were differentially regulated by AM symbiosis depending on the specific conditions of the osmotic stress applied. However, in mycorrhizal bean plants subjected to salinity, enhanced water flux through their roots was observed compared with non-AM plants, and this effect also correlated with the enhanced expression of three out of four aquaporin genes analysed in these plants and with the amount of PIP2 protein and its phosphorylation state (Aroca *et al.*, 2007). In another study, Jahromi *et al.* (2008) studied two PIP genes in *Lactuca sativa* plants subjected to increasing salinity levels. The expression of *LsPIP1* and *LsPIP2* genes was downregulated by mycorrhization in the absence of salinity. In contrast, under saline conditions, in mycorrhizal plants the expression level of the *LsPIP2* gene was almost unaffected, while the expression of the *LsPIP1* gene was upregulated, mainly at 100 mM NaCl. Ouziad *et al.* (2006) also described a differential effect of AM symbiosis on tomato aquaporin isoforms under salinity conditions. Thus, the *LeTIP* and *LePIP1* genes were upregulated in the leaves and downregulated in the roots of tomato plants subjected to salt stress, although no data about root hydraulic values were shown. Hence, these results by Ouziad *et al.* (2006), Aroca *et al.* (2007), and Jahromi *et al.* (2008) suggest that each aquaporin gene responds differently to AM colonization depending on the nature of the stress imposed. These differences may be a consequence of the variety of plant and fungal species tested, the mode of salt-stress application, and the complexity of expression patterns of different members of the large family of aquaporins (Sarda *et al.*, 1999). This highlights that fact that regulation of aquaporin genes by AM symbiosis in response to different abiotic stresses with an osmotic component is rather complex (Ruíz-Lozano and Aroca, 2010) and demonstrates the need for a comprehensive study that specifically analyses the role of AM symbiosis in the regulation of plants aquaporins under salt-stress conditions. As a first approach, analyses should focus on those aquaporins with a proven capacity for water transport such as PIPs (mainly the PIP2 subgroup) and TIPs. These analyses should be correlated with measures of root and leaf hydraulic conductivities and water status in order to determine the final influence of the regulated aquaporins on the tissue water permeability and content.

Antioxidant defence

Adverse environmental conditions such as salinity may uncouple different pathways in plant metabolism. Thus, electrons are transferred to molecular oxygen to form ROS (Ding *et al.*, 2010). ROS are also produced at a low level under optimal growth conditions. However, because of the accumulation of NADPH and ATP that have not been

consumed, their rate of production is dramatically elevated during stress. Under these conditions, ROS accumulation depends greatly on the balance between ROS production and ROS scavenging (Miller *et al.*, 2010). For this reason, plants have evolved efficient systems for ROS removal, which include non-enzymatic molecules that act as ROS scavengers such as ascorbate, glutathione, α -tocopherol, flavonoids, anthocyanines, and carotenoids, as well as specific ROS-scavenging antioxidative enzymes acting in synchrony (Fig. 3). For recent reviews of antioxidant systems in plants, see Miller *et al.* (2010) and Scheibe and Beck (2011).

On the other hand, ascorbate is an important antioxidant that serves as an electron donor to many important metabolic reactions. Ascorbate was shown to play an important role in the protection of photosynthesis during salt stress (Noctor and Foyer, 1998; Shao *et al.*, 2008). Glutathione is a tripeptide found abundantly in all cell compartments in its reduced form (Foyer and Noctor, 2005). The ratio of glutathione (GSH) to its oxidized form, GSSG, plays an important role in maintaining redox equilibrium in the cell during H_2O_2 degradation and other processes (Shao *et al.*, 2008). Additionally, glutathione plays a key role in the regeneration of reduced ascorbate in the ascorbate–glutathione cycle (Halliwell and Foyer, 1976).

There is a correlation between antioxidant capacity and salinity tolerance, which has been reported in several plant species (Türkan and Demiral, 2009). In the field of AM symbiosis, several studies have suggested that AM symbiosis helps plants to alleviate salt- or water-deficit stresses by enhancing the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and peroxidase (POX), or the accumulation of antioxidant compounds such as ascorbate and glutathione (Alguacil *et al.*, 2003; Porcel *et al.*, 2003; Porcel and Ruíz-Lozano, 2004; Zhong *et al.*, 2007; Garg and Manchanda, 2009; Ruíz-Sánchez *et al.*, 2010; Wu *et al.*, 2010b; Talaat and Shawky, 2011). Thus, mycorrhizal plants possess enhanced activity of several antioxidant enzymes,

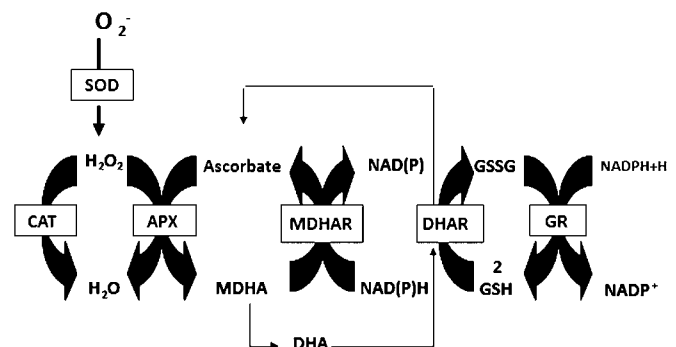


Fig. 3. Methods of ROS removal in plants. APX, ascorbate peroxidase; CAT, catalase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; SOD, superoxide dismutase.

but the response of the individual enzymes has been shown to vary with respect to the fungal species and the host plant. This variation may also depend on the micronutrients available to some of the enzymes. For instance, CAT, ascorbate peroxidase (APX), and SOD are metalloenzymes whose activities can be determined by the availability of the metals they utilize. This also suggests that the effect of AM symbiosis inducing activities of several antioxidant enzymes may be the indirect result of the mycorrhizal effects on host plant growth and acquisition of phosphorus and nitrogen (Alguacil *et al.*, 2003; Evelin *et al.*, 2009). Hence, elucidating the mechanisms that control the enhanced antioxidant capacity in AM plants during salt stress could provide a powerful strategy to improve the tolerance of crops to these environmental stress conditions. However, so far, the influence of AM symbiosis on the accumulation of non-enzymatic antioxidants such as ascorbic acid, glutathione, carotenoids or tocopherols in the host plant has been much less studied. Therefore, this aspect requires an in-depth investigation. Moreover, the existence of genes encoding different proteins that may be involved in cellular defence against oxidative stress has been described in AM fungi. This includes genes encoding SOD (Lanfranco *et al.*, 2005; González-Guerrero *et al.*, 2010), glutaredoxins (GRXs), which are small proteins with glutathione-dependent disulfide oxidoreductase activity involved in cellular defence against oxidative stress (Benabdellah *et al.*, 2009b), pyridoxine 5'-phosphate synthase (PDX), which is involved in the biosynthesis of vitamin B6, an essential metabolite for defence against cellular oxidative stress (Benabdellah *et al.*, 2009a), and even a metallothionein (MT)-encoding gene, which was suggested to play a role in regulation of the redox status of the extraradical mycelium of *G. intraradices* (González-Guerrero *et al.*, 2007). Some of these genes have been shown to be induced by oxidative stress, but their responses to salinity have never been studied.

We propose to investigate in AM and non-AM plants subjected to increasing salinity levels the activities of SOD, CAT, APX, GR and glutathione peroxidase (GPX) enzymes, as well as the accumulation of reduced ascorbate and glutathione in plant tissues. These data should be correlated with the levels of H₂O₂ and oxidative damage to lipids and proteins in the same tissues. From a molecular point of view, as a first approach, the expression pattern of genes encoding these antioxidant enzymes should be analysed in the different treatments. The expression of key genes involved in the biosynthesis of ascorbate and glutathione should be also analysed. To this end, the genes GDP-D-mannose 3',5'-epimerase (GME), which is key in the biosynthetic pathway of ascorbate (Watanabe *et al.*, 2006), and the gene cysteine synthase (CS), involved in the biosynthesis of cysteine and glutathione (Harada *et al.*, 2001; Choi *et al.*, 2007), are proposed. From the fungal side, the above mentioned genes encoding SOD, GRXs, PDX or MTs should be also included. Additional fungal genes may arise from analysis of transcriptomic data provided recently by Tisserant *et al.* (2012) regarding the AM fungus *G. intraradices*.

Maintenance of photosynthetic capacity

Salt stress inhibits photosynthetic ability, which leads to a decrease in crop production (Pitman and Läuchli, 2004). In fact, increasing salinity causes a reduction in chlorophyll content due to suppression of specific enzymes that are responsible for the synthesis of photosynthetic pigments. A reduction in the uptake of minerals (e.g. magnesium) needed for chlorophyll biosynthesis also reduces the chlorophyll concentration in the leaf and contributes to reduction of photosynthesis (Giri and Mukerji, 2004; Murkute *et al.*, 2006; Sheng *et al.*, 2008). The lowering of the photosynthetic rate induced by salt stress can lead to an over-reduction of the reaction centres in PSII. If the plant is unable to dissipate the excess energy, this may damage the photosynthetic machinery. This damage, as well as the ability of the plant to dissipate or not the excess energy, can be quantified by measuring the fluorescence of chlorophyll *a* (Baker, 2008).

Few studies so far have investigated the influence of AM fungi on photosynthesis and particularly on leaf photochemical properties under salt stress. However, improvements in physiological processes such as photosynthetic activity or water-use efficiency have been reported in mycorrhizal plants growing under salt-stress conditions (Sheng *et al.*, 2008; Zuccarini and Okurowska, 2008; Hajiboland *et al.*, 2010). Sheng *et al.* (2008) studied the effects of AM symbiosis on maize tolerance to salinity. They found that AM symbiosis improved the photosynthetic capacity of maize leaves, mainly through elevating the capacity of gas exchange and the efficiency of photochemistry and non-photochemistry of PSII and regulating the energy bifurcation between photochemical and non-photochemical events. Similar results were found by Zuccarini and Okurowska (2008) in experiments with sweet basil plants. Under salt stress, mycorrhizal maize plants also had a higher dry weight of shoot and root, higher relative chlorophyll content, and better water status compared with non-mycorrhizal maize plants. However, Sheng *et al.* (2008) suggested that the influence of AM symbiosis on the photosynthetic capacity of maize plants was due to a mycorrhizae-mediated enhancement of water status, and not to a mycorrhizae-mediated enhancement of chlorophyll concentration.

Hajiboland *et al.* (2010) showed that mycorrhization improved the net assimilation rates both by elevating stomatal conductance and by protecting photochemical processes of PSII against salinity. However, the enhancement of the PSII photochemistry by AM fungi did not occur in plants without salt treatment. Thus, the authors suggested that AM colonization acted only in maintenance of photochemical capacity in stressed leaves and did not increase its potential for energy trapping.

We propose that comprehensive studies aimed at determining the influence of AM symbiosis on net photosynthesis activity, stomatal conductance, transpiration, water-use efficiency, and intercellular CO₂ concentration should be carried out. These data should be complemented with measurements of chlorophyll fluorescence to calculate different parameters related to quantum yield both in the dark-adapted state and

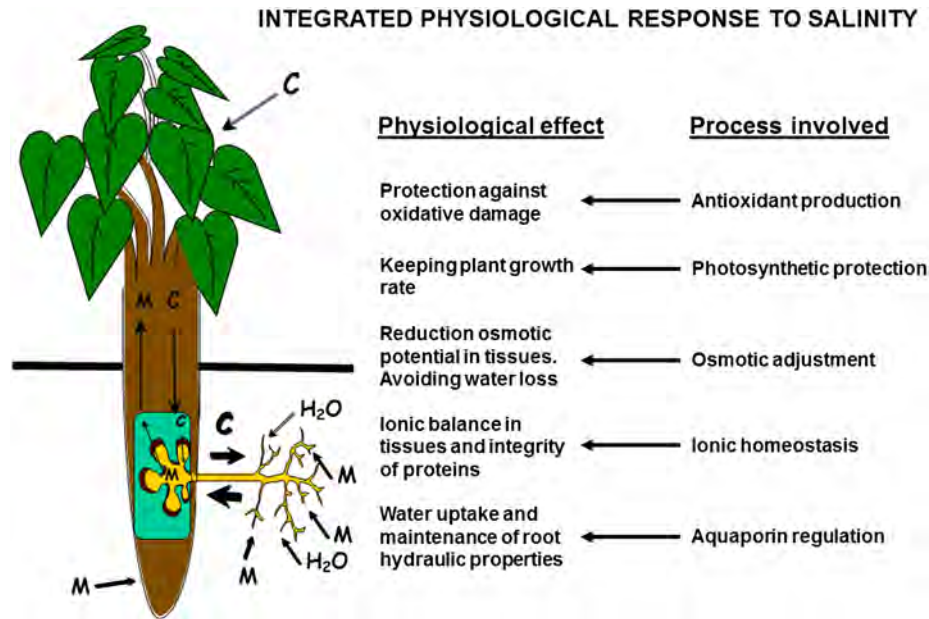


Fig. 4. Summary of the main processes by which AM symbiosis can regulate the integrated physiological plant response in order to improve tolerance to salinity. The exchange flux of water, minerals (M) and carbon compounds (C) between the plant and the fungus is also represented. Minerals include nutrients and salt ions presents in the soil solution.

in the light-adapted state [non-photochemical quenching (NPQ), F_v/F_m , F_v'/F_m' , optimum quantum yield of PSII (Φ_{PSII}) and photochemical quenching (qP)] according to Maxwell and Johnson (2000) and Sheng *et al.* (2008). Moreover, the accumulation of photosynthetic pigments should be also recorded by measuring the levels of chlorophyll *a* and *b*, total chlorophyll, and carotenoids in the different treatments.

Photosynthetic efficiency also depends on the activity of enzymes involved in carbon assimilation such as ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Masumoto *et al.*, 2005). Valentine *et al.* (2006) found that grapevine plants inoculated with an AM fungus and subjected to drought had higher water-use efficiency and Rubisco activity than non-AM plants. In contrast, no data are available about Rubisco activity in AM plants subjected to salinity. Thus, this aspect deserves to be examined in future studies. The activity of this enzyme should be also measured in AM and non-AM plants under different salt levels, in combination with molecular studies aimed at evaluating the expression pattern of genes encoding the small (*rbcS*) and large (*rbcL*) subunits of Rubisco (Tsutsumi *et al.*, 2008).

Conclusion

The integrated physiological response of plants to salinity involves several mechanisms that may act in a concerted manner. These include synthesis and accumulation of compatible solutes, control of ion homeostasis, regulation of water uptake and distribution to plant tissues, reduction of oxidative damage, and maintenance of photosynthesis (Fig. 4). There is evidence that AM symbiosis affects and regulates several of these mechanisms, but many physiological

aspects and the molecular bases of such regulation are almost completely unknown. This is especially evident in the case of genes involved in ionic homeostasis, where only one study has been conducted in relation to AM symbiosis (Ouziad *et al.*, 2006), with no conclusive results. Indeed, prevention of Na^+ entry into the root, sequestration into the vacuole, and transport to and allocation within the leaf are major determinants for the salt sensitivity of a plant. Thus, a challenge in future studies on salt-stress alleviation by AM symbiosis should be to determine the way in which the symbiosis modifies at the molecular level these physiological processes key for plant survival and development under saline conditions. Current transcriptomic analysis of some AM fungi (Tisserant *et al.*, 2012) is a promising tool that could provide new data regarding fungal genes that may also participate in the response of AM symbiosis to salinity stress. These candidate genes should be included in future studies aimed at identifying the intimate mechanisms determining AM improvement of plant salt tolerance.

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