Salinity: Physiological Impacts on Legume Nitrogen Fixation

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Abstract Salinity is of great concern in arid and semiarid regions, where soil salt content is often high and precipitation is insufficient for leaching. High salinity in soil induces a strong decrease in crop yield and productivity of arable land. According to global change prediction models, salinity is expected to expand in the near future. Legumes represent the world's second major crop of agricultural importance. These plants provide an important source of proteins for human and animal diets and they are widely used as green manure, forage, and others. In comparison with other crops, legumes represent a particular plant group thanks to their capacity to fertilize soils through the atmospheric nitrogen fixation. The symbiotic relationship between *Rhizobiaceae* and legumes leads to the development of root nodules, where bacteria fix the atmospheric nitrogen that the host plant incorporates as organic molecules. In general, rhizobia are more salt tolerant than their respective plant host, but the process of nodule formation is particularly sensitive to salt stress (colonization and infection are highly reduced). Under salt stress, plants face at least three major constraints: water deficit, ion toxicity, and oxidative stress. Some plants develop tolerance mechanisms to overcome it, including changes in gene expression patterns and in metabolic responses related with adaptation/tolerance (ion homeostasis, osmoprotectants, and antioxidant system). In this context, the application of new "omics" technologies in combination with traditional agronomic and physiological studies will improve the salt tolerance of symbiotic processes, and it will optimize the biological nitrogen fixation.

Keywords Legumes crops • Rhizobia • Biological nitrogen fixation • Salt stress • Metabolic responses • Genomic and biotechnology approaches

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1 Introduction to Salinity and N_2 Fixation

1.1 Salinity Types and Causes

Salinity problems are critical worldwide; over 20 % of the cultivated areas and half of the irrigated lands are encountering salinity stress of different magnitudes, reducing greatly yield below the genetic potentials (Flowers 2004). Saline soils are defined by Ponnamperuma (1984) as those that contain sufficient salt in the root zone to impose the growth of plants. However, since salt injury depends on species, variety, growth stage, environmental factors, and nature of the salts, it is difficult to precisely define saline soils. The USDA-ARS (2008) defines saline soils as those ones with an electrical conductivity of the saturation extract (EC_c) equal or higher to 4 dSm^{-1} (≈40 mM NaCl at 25 °C), and soils strongly saline when ECs exceed 15 dSm^{-1}.

Primary salinization occurs naturally when the soil parent material is rich in soluble salts, or in the presence of shallow saline groundwater table. This is caused by two natural processes. The first is the weathering of parent material containing soluble salts of various types, mainly chlorides of sodium, calcium, and magnesium, and, to a lesser extent, sulfate and carbonates. The second is the deposition of microscopic salt particles carried by the wind and rain to oceans (Munns 2009). In arid and semiarid regions, where rainfall is insufficient to wash out soluble salts from the soil, or where drainage is restricted, high concentration of salt is accumulated (USDA-ARS 2008). Secondary salinization results from humane activities that change the hydrologic balance in the soil between precipitation and water used by crops (transpiration). The most common salinity (anthropogenic salinization) occurs when significant amount of poor-quality water is used for irrigation. Therefore salt accumulates in the soil, resulting in saline and thereby unproductive soils (Munns 2009). This process could be avoided with effective irrigation systems that remove salt from the soil profile (Szabolcs and Greenland 1992).

Other causes of salinization are related with the chemical contamination, which often occurs in modern intensive agricultural system as greenhouse and intensive farming systems. This process happens mainly in regions with a poor natural soil cover and high intensive animal husbandry, or by accumulation of airborne/waterborne salts in soils (Shukla et al. 1990; Hastenrath 1991). Szabolcs (1994) has reported that chemicals from industrial emissions may accumulate in the soil, and it can result in salt accumulation in the upper layer of soil.

The long-term consequences of saline irrigation on soil physical fertility are well documented (De Pascale et al. 2003a, b). The saturation level of sodium ion (Na^+) is considered the main cause of clay dispersion with the consequent reduction of impermeability, porosity, and hydraulic conductivity (Amezketa 1999). Climatic factors and water management may accelerate salinization. In arid and semiarid lands evapotranspiration plays a main role in the pedogenesis of saline and sodic soils (Yadav et al. 2011). When an excess of Na^+ content is involved in the salinization process, it is called "sodicity." Sodicity is defined as the exchange sodium percentage (ESP) threshold that causes degradation of soil structure.

The USDA salinity laboratory defines a soil as sodic if it has the ESP greater than 15 (www.ussl.ars.usda.gob). If the concentration of soluble salts is sufficiently low, hydrolysis of the sodic clay will occur, creating a highly alkaline soil. A soil is considered alkaline (type of sodic soils) when it has a high pH due to carbonate salts (pH of 8.5–10 and EPS of 15 or more). The process of sodicity is complex and occurs over a long period of time; however, saline/sodic soils are widespread in arid and semiarid environments (Munns 2009).

Salinity is the most widespread soil degradation process in the earth and it is regarded as a major cause of desertification. Salinization/sodification is a serious form of soil degradation being considered among the major destruction processes endangering the potential use of world soils (Ladeiro 2012). Deforestation is recognized as a major cause of salinization and alkalinization of soils. It affects salt migration in both the upper and lower layers. Deforestation leads to the reduction in rainfall average and increases surface temperature (Shukla et al. 1990). As a consequence, top thin soil rapidly gets eroded in the absence of green cover. Trees can act as a buffer zone between the soil and the rain, and without them, erosion is practically inevitable. Furthermore, soil erosion leads to a greater amount of runoff and increased sedimentation in the rivers and streams (Hastenrath 1991).

The harmful impacts of salinity include low agricultural production, low economic returns (due to high cost of cultivation), and soil erosion. Crop species show a different range of responses to salt stress. Although all species show some growth under salt conditions, eventually, their yield is reduced. Any increase in the agricultural productivity of saline soils, which are often grouped under marginal or waste soils, can be contributed to the socioeconomic improvement of millions of people who sustain their livelihood from them (Ladeiro 2012). Sustainable agriculture involves ecological managements, which implies considering elements, such as biodiversity, nutrient cycling, and energy flux, in order to avoid the loss of nutrients and soils and to prevent the attack of pest and diseases (Spiertz 2010).

1.2 Legumes and Biological Nitrogen Fixation

Legumes (Fabaceae) are the third largest family of flowering plants with over 650 genera and 20,000 species (Lewis et al. 2005). They are second grass in importance to human diets; and among grain crops, legumes rank world's third place behind cereal and oilseed production (Graham and Vance 2003). Moreover, legumes are widely used as green manure since the beginning of agriculture. However, this practice has been diminished since industrially produced fertilizers became available. Green manure adds nitrogen to the soil and improves soil quality by increasing the organic matter content of the soil.

Forage legumes have been adapted from wild flora and used in managed grazing lands. These include lucerne (*Medicago sativa*), clovers (*Trifolium* spp.), vetch (*Vicia angustifolia*), and birdsfoot trefoil (*Lotus corniculatus*). These kinds of species provide animal husbandry with high-quality forage (Mikić et al. 2011) and they increase soil organic matter when crops are rotated in farming system (O'Hara et al. 2002). Grain legumes, such as pea (*Pisum sativum*), bitter vetch (*Vicia ervilia*), lentil (*Lens culinaris*), chickpea (*Cicer arietinum*), soybeans (*Glycine max*), peanut (*Arachis hypogaea*), faba bean (*Vicia faba*), and grass pea (*Lathyrus sativus*) are commonly recognized as one of the first domesticated plant species and the most ancient crops. They contributed to the "agricultural revolution" in the Fertile Crescent at the end of the last Ice Age (Bellwood 2005). Subsequently and independently, soybean was domesticated in China, cowpea (*Vigna unguiculata*) in Africa, pigeon pea (*Cajanus cajan*) in India, and common bean (*Phaseolus vulgaris*) in Central and South America. The legumes are major source of food, fodder, timber, phytochemicals, phytomedicines, nutriceuticals, and N_2 fertility in agrosystems (Graham and Vance 2003).

Nitrogen is an essential nutrient for plant growth and its availability is a big limiting factor for agricultural systems. For decades, N_2 fertilizers have been continuously used to improve crop yield. However, the use of it accelerates the depletion of large amounts of fossil and nonrenewable energy sources. It substantially contributes to environmental pollution through atmospheric emission and leaching of ammonia, nitrates, and nitrous oxide (Velthof et al. 2009).

In this panorama, it is worthy to pay attention to the biological nitrogen fixation (BNF) which has significantly lower economic and environmental costs than the N_2 chemical fertilizers coming from industrial process. BNF is crucial from the environmental and agricultural points of view; it is the second more important process to maintain a sustainable biosphere. BNF is the assimilation of atmospheric N_2 in the form of organic compounds, and is a sustainable source of N_2 in cropping system. Fixed N_2 can be used directly by plants and it is less susceptible to volatilization, denitrification, and leaching (Garg and Geetanjali 2007). Thus, this process can reduce the use of chemical fertilizers and consequently mitigate global warming and water contamination (Juárez-Santacruz et al. 2013).

The ability to reduce N_2 to ammonia is restricted to some prokaryotes. These species are able to break (with the aid of the key enzyme nitrogenase) the strong triple bond within the N_2 molecule. This process requires high cost of energy and the nitrogenase might be rapidly inactivated by high oxygen (O_2) concentrations (Bruning and Rozema 2013). Many genera of bacteria and archaea are able to fix and transfer the fixed ammonia to plant through the establishment of mutualistic symbiosis (Sprent and Sprent 1990). The most important N_2-fixing agents in agricultural system are the symbiotic associations between legumes and the group of soil bacteria collectively designated as rhizobia. It is estimated that 88 % of legume species examined form N_2-fixing nodules with rhizobia, being responsible for up to 80 % of the BNF in agricultural soils (De Faria et al. 1989).

The first symbiosis took place around 58 Mya when the *Papilionoidea* (a subfamily of the *Fabaceae*) underwent genome duplication (WGD) (Young et al. 2011). This early WGD is important for legume research, as it indicates that many genes in the model and agronomic papilionoid legumes exist as paralogous duplicates (although nowadays they have independently evolved for ≈55 Mya) and perhaps have acquired distinct and new functions (Cannon 2013) such as the communications with rhizobia, thereby enabling the legumes to start this almost unique symbiotic relationship. As such, the WGD seems to have had a crucial role in the success of papilionoid legumes, enhancing their utility to humans (Young et al. 2011).

The rhizobia induce the formation of nodules in the legumes that involves an exchange of molecular signals between the symbiotic partners (host plant and microbe) to communicate with each other. Secondary plant metabolites, mainly flavons or isoflavons, are recognized by compatible rhizobia via chemotaxis. The rhizobia approaches to the root while it induces the expression of several genes involved in the synthesis and secretion of lipochito oligosaccharides NOD factors (NFs). These molecules share a "backbone" structure consisting of two to six β1-4-linked *N*-acetyl glucosamine residues, with a fatty acid amide linked to the nonreducing terminal residue (Lerouge et al. 1990; Spaink et al. 1991). NFs from different *Rhizobium* species differ in the number of *N*-acetyl glucosamine residues, the length and saturation of the acyl chain, and the nature of modifications on the basic backbone (sulfate, acetate, fucose, etc.) (Oren 1999). The NF perception leads to physical attachment of rhizobia to the root hairs. It induces a plasma membrane depolarization, intra- and extracellular alkalinization, ion fluxes, and calcium spiking, triggering several early symbiotic responses in root hair growth ("curling") (Brewin 1991). NFs also provoke infection thread that guides the bacteria to the emerging nodular primordium (Oldroyd and Downie 2008). In addition, NFs induce nodulin gene expression on cortical cells, leading to the nodular organogenesis (Radutoiu et al. 2003; Cooper 2007). In the nodule primordium, bacteria are released inside the infected cortical cells by an endocytosis process. Later, it is encapsulated by a membrane of the host plant, forming a symbiosome (Oldroyd et al. 2011). These symbiosomes work as plant organelles and they are responsible for N_2 fixation and membrane exchange of metabolites (Udvardi and Poole 2013).

In these symbiosomes, the rhizobia can take up gaseous N_2 from the atmosphere and fix it through the nitrogenase to form ammonia or amino acids. Then, it can be transported to the upper parts of the host plant either as amides (mainly asparagine, but also glutamine) or as ureides (Schubert 1986). In return, the plant provides to rhizobia with a carbon (C) source in the form of dicarboxylate (Soussi et al. 2001). The enzyme responsible for the N_2 fixation, nitrogenase, is irreversibly damaged when exposed to O_2. The plant induces three processes to produce a microaerobic environment around N_2-fixing rhizobia in nodules: (1) a barrier to gaseous diffusion in the nodules, outer cell layers limit the rate of O_2 influx to the central infected tissue; (2) bacteroids and plant mitochondria, with their high respiration rates, consume O_2 as fast as it can enter in the nodules; and (3) plant hemoglobins, which have high affinity to bind O_2 in the cytoplasm and rapidly deliver it to mitochondria and bacteroids of nodule cells (L'taief et al. 2007). Leghemoglobins are the most abundant plant proteins in nodules. Steady-state concentrations of free O_2 in the infected zones of legume nodules are typically in the tens of nanomolar, approximately four orders of magnitude lower than equilibrium levels in water (Udvardi and Poole 2013).

2 Impact of Salt Stress on Rhizobial Biology

2.1 Free-Living Bacteria

Rhizobia are a group of diazotrophs, most of which belong to the α-proteobacteria. It includes the genera *Rhizobium*, *Mesorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Bradyrhizobium*, and *Azorhizobium* (Weir 2008). Other non-rhizobial genera showed nodules as *Methylobacterium*, *Devosia*, *Ochrobactrum* (Velázquez et al. 2010; Graham 2008; Rivas et al. 2009), *Phyllobacterium*, and *Shinella* (Trujillo et al. 2005; Valverde et al. 2005). Some nodulating bacteria within the genera *Burkholderia*, *Cupriavidus*, and *Herbaspirillum* (β-proteobacteria) class have also been described (Masson-Boivin et al. 2009).

The sensitivity of different rhizobial species and strain to salt stress varies considerably and some of these bacterial strains can persist and survive in saline soils (Zahran 1999). The growth of many rhizobia was inhibited by 100 mM NaCl, while others (e.g., *S. meliloti*) were able to grow in the range of 300–700 mM NaCl (Talibart et al. 1994). However, the majority of rhizobia are not capable of tolerating the harmful effects of high osmolarity (Talibart et al. 1997). It has been showed that rapidly growing rhizobia (24–48 h) are more tolerant than rhizobia of slow growth (more than 96 h) (Zahran 1999).

Morphological alterations of bacteria under salt stress have been reported. These include the expansion of cell size, distortion of cell envelope, and change of rhizobial cell structure (Vanderlinde et al. 2010). Besides the surface components, several symbiotic bacteria may suffer additional changes in salty environment: alterations such as decrease in the synthesis of exopolysaccharides (Lloret et al. 1998; Vanderlinde et al. 2010), changes in the length of the side chain of lipopolysaccharide (Soussi et al. 2001), and deletion of periplasmic oligosaccharide involved in bacterial osmotic adaptation (Ghittoni and Bueno 1995).

Salty conditions induced different changes in protein profiles on different rhizobia. Laranjo and Oliveira (2011) detected that tolerant strains *M. huakuii*, *M. plurifarium*, and *M. thiogangeticum* could grow under 1.5 and 3 % NaCl concentrations. These bacterial strains overproduce proteins with molecular weights of 40–85 kDa, which may be related to salt stress tolerance. Interestingly, several previously reported salt stress proteins include high-molecular-weight proteins, such as the alanine-tRNA synthetase (Nogales et al. 2002), some chaperones (Domínguez-Ferreras et al. 2006), and the N_2 regulation proteins (Nogales et al. 2002). Several high-molecular-weight proteins were overproduced under salt stress, mainly in *Mesorhizobium* spp. For instance, this phenomenon has been described in *M. ciceri* (Soussi et al. 2001) and *Rhizobium* sp. (Hastenrath 1991). The appearance of salt stress proteins (SSPs) of low molecular weight after a specific period of time enabled us to speculate that it is quite possible that at preliminary stages of stress, some other mechanisms of salt tolerance are involved (Saxena et al. 1996). The consistent detection of some proteins only after growth under stress may indicate that they were de novo synthesized, suggesting their importance in the survival and growth of *Mesorhizobium* spp. in stress conditions (Guasch-Vidal et al. 2013).

Plasmids are important genetic components for the divergence and adaptation of microbial populations because they contribute to genomic plasticity (Zhang et al. 2001). The correlation between the selection pressure caused by stress and existence of the same plasmids suggests that plasmid plays a major role in the adaptation to environmental stress (Lakzian et al. 2002). Plasmid-mediated salt resistance may be ecologically important since resistance can be rapidly transferred from resistant bacteria to sensitive bacteria; thus extrachromosomal genes can contribute to survival in saline soils (Pereira et al. 2008). Domínguez-Ferreras et al. (2006) showed that plasmid SymB contains a large number of genes upregulated after an osmotic upshift which may have an active role in the osmoadaptation of *S. meliloti*.

Salt stress induces ionic and osmotic stress in rhizobia, through the change in the concentration of solute around cells, producing water deficit and drying the cell (Zahran 1991). Survival and growth in saline environments are the result of adaptive processes, such as ion transport and compartmentation, and osmotic solute synthesis and accumulation. This leads to osmotic adjustment and protein turnover for cellular repair (Munns and Termaat 1986). Under these conditions, rhizobia should maintain turgor positive across the membrane, allowing accumulation of ions. Past experiments have shown an increase in potassium (K^+) levels during early stages of salt stress (Saxena et al. 1996). Under elevated salinity, K^+ ions can also be accumulated in some species of rhizobia (Young et al. 2011).

Other mechanisms for osmotic adaptation when rhizobia is exposed to salt stress are through the production, transport, and compartmentation of compatible solutes or synthesis and intercellular accumulation of other organic compounds of low molecular weight (osmolytes) (Botsford and Lewis 1990). The osmolytes lead to osmotic adjustment (Munns and Termaat 1986) including higher intracellular concentration of carbohydrates, such as sucrose, trehalose, and maltose (Wu et al. 2008), and amino acids, such as free glutamate (Le Rudulier and Bernard 1986; Cordovilla et al. 1995a), proline (Soussi et al. 1999), glycine-betaine (Guo et al. 2014), ectoine (Dong et al. 2013), as well as a dipeptide *N*-acetyl-glutaminyl glutamine amide (Shavrukov 2013) and intracellular accumulation of polyamines (PAs) (López-Gómez et al. 2014a).

It was postulated that trehalose and glycine-betaine are accumulated to prevent starvation rather than as osmotic stabilizers (López-Gómez and Lluch 2012; Oren 1999). Osmoregulation is the main strategy employed by rhizobia to cope with salt stress (Ghittoni and Bueno 1995). Trehalose (α-ᴅ-glucopyranosyl-(1 → 1)-α-d-glucopyranoside) is a nonreducing disaccharide. Trehalose biosynthetic and degradation pathways are widespread throughout bacteria, archaebacteria, fungi, and plant (Avonce et al. 2006). Bacteria are widely distributed among different genera such as *Streptomyces* (Martín et al. 1986), *Mycobacterium* (Elbein and Mitchell 1973), and *Corynebacterium* (Shimakata and Minatogawa 2000). This disaccharide has a structural role as component of the cell wall. It has also been found in other bacteria including *Rhizobium* spp. (Maruta et al. 1996) where trehalose can constitute the sole C source, be used as a compatible osmolyte, or form part of the cell wall structure. *S. meliloti* accumulates a number of osmolytes which help it to deal with osmotic stress, including trehalose, *N*-acetylglutaminylglutamine amide, and glycine-betaine. The glycine-betaine is one of the most powerful osmoprotectants stimulating *S. meliloti* growth in high-salt media (Le Rudulier and Bernard 1986). Glycine-betaine is typically imposed from the environment or synthesized from choline using a pathway encoded by the chromosomal *betCBA* operon. Yurgel et al. (2013) suggest than truncated betB (glycine-betaine aldehyde dehydrogenase BADH) stimulates glycine-betaine catabolism preventing it from being used as an osmoprotectant. *S. meliloti* can use glycine-betaine either as a source of C and N_2 or as a cytoplasmid osmolyte, depending on the osmolarity of its growth medium (Barra et al. 2006).

2.2 Signaling Exchange

A complex series of events coordinated by host and bacterial signaling underlie and lead to the development of symbiotic interaction. Rhizobia respond to flavonoids exuded by the leguminous roots by secreting NFs. These are the molecular key signal to trigger the nodulation program in a compatible host (Bruning and Rozema 2013). This signal exchange can be negatively affected by salinity (Miransari and Smith 2009; Oldroyd and Downie 2004).

Moreover, salt stress also increases the biosynthesis of NFs and alters their structure with a great number of different new biologically active NFs being generated (Estévez et al. 2009). A peculiar bacterium is *Rhizobium tropici* CIAT 899, which can survive in the presence of high concentration of Na^+, by enhanced nod gene expression and NF biosynthesis. This effect is Na^+ specific because high potassium or chloride concentration did not have it. Under salt stress conditions, 14 different new NF structures were identified which were not observed as being produced under neutral or acid conditions (Estévez et al. 2009). Major NF-triggered responses in root hair deformation involved several changes as intra- and extracellular alkalinization, phosphatidic acid and diacylglycerol formation, and accumulation of reactive O_2 species (Mulder et al. 2006; Cooper 2007).

Guasch-Vidal et al. (2013) observed that, in the absence of flavonoid inducers, high concentration of NaCl induced nodulation genes (*nod* genes) and the production of NFs. The higher transcriptional activity of the *nod* operon in the presence of NaCl was revealed by the increasing of the β-galactosidase activity of a *nodP*:*lacZ* fusion. It leads to a higher and detectable production of NFs. There have been a number of previous evidences for preincubation of rhizobia with exogenous oxylipin (NF inducers). It significantly alleviates the stress effects of salinity on *nod* gene expression, enhancing thereby nodulation and N_2 fixation on fenugreek (*Trigonella foenum-graecum*) (Abd-Alla et al. 2014). In common bean (*P. vulgaris*) inoculated with *R. tropici* or *R. etli* under salt stress, it shows a negative effect on the expression of *nod* genes (Dardanelli et al. 2008). Preincubation of *B. japonicum* with the molecular signal genistein (NF inducers) was described as a method to alleviate the stressful effects of high salinity on soybean-*B. japonicum* symbiosis (Miransari and Smith 2009). In addition, the pretreatment of *B. japonicum* with genistein increased nodulation and N_2 fixation of soybean and common bean (Abd-Alla 2011). Preincubation of *R. leguminosarum* with hesperetin and naringenin was found to stimulate nodulation and accumulation of plant dry matter in pea and lentil plants (Begum et al. 2001).

3 The Metabolic Modifications of Nodule N_2 Fixation Under Salt Stress

Legumes can improve saline soil fertility and help to reintroduce sustainable agriculture to these kinds of lands (Crespi and Gálvez 2000). Saline habitats are N poor (Sprent and Sprent 1990) and one of their sources of N input is N_2 fixation by legumes (Zahran 1991). Higher rates of N_2 fixation in saline soils compared to nonsaline and agricultural soils were reported (Wollenweber and Zechmeister-Boltenstern 1989). The low oxygen tension in saline soils may favor the process of N_2 fixation. However, the diffusion of gasses may be impaired at a higher density and water regime in saline soils, and this might reduce N_2 fixation (Rice and Paul 1971).

The effect of salinity on growth and legume nodulation depends strongly on the concentration of NaCl, climatic conditions, soil properties, and growth stage of the plant (Cordovilla et al. 1995a; Flowers 2004). Unfortunately, most crop legumes are on the glycophyte side of the salt tolerance spectrum. Main cereal crops as well as leguminous species are quite sensitive to saline conditions (Cabot et al. 2014). The leguminous plants are classified as sensitive or moderately sensitive (i.e., show 80 % biomass production as compared to nonsaline conditions with 3–6 dSm^{-1} or 30–60 mM NaCl, respectively) (Maas and Hoffman 1977). Some legumes are very sensitive to high salinity, such as *M. truncatula*, *M. sativa*, or *P. sativum*, while other legumes, such as *V. faba*, *G. max*, *P. vulgaris*, or *A. hypogaea*, are more salt tolerant (Läuchli 1984). In general, rhizobia are more salt tolerant than their respective host plant (Manchanda and Garg 2008), but the functional symbiosis is even more sensitive to salinity than either partners is (Mudgal et al. 2010). A review by Zahran (1999) stated that "the best results for symbiotic nitrogen fixation under salt stress are obtained if both symbiotic partners and all the different steps in their interaction (nodule formation, activity, etc.) resist such stress."

The process of nodule formation is particularly sensitive to salt stress, specially the initial phases (Ikeda 1994) (Fig. 1). Under salt stress, the curly hair's root and bacterial colonization are inhibited, infection rate is greatly reduced (Fougère and Le Rudulier 1990), and the weight and number of nodule formation are decreased (Manchanda and Garg 2008). Microscopic observations showed that salinity (1) caused the loss of turgor of the nodule peripheral cells, disintegration of the cytoplasm, and loss of rigidity of the cell wall; (2) changed nodule zonation with a decrease in the volume of intercellular spaces; (3) stimulated infection thread enlargement and expansion; (4) caused disturbances in bacterial release from the infection threads; (5) caused the occurrence of variations that lobulated nuclei and chromatin condensation; and (6) induced synthesis of electron-dense material (EDM) and its deposition in vacuoles (Nielson and Griffith 1978). It was also found that cisternae of rough endoplasmic reticulum were involved in the formation of special cytoplasmic compartments responsible for synthesis of EDM (Borucki and Sujkowska 2008). Autofluorescence studies revealed that salinity also increased accumulation of phenolics in pea nodules (Padilla et al. 2013).

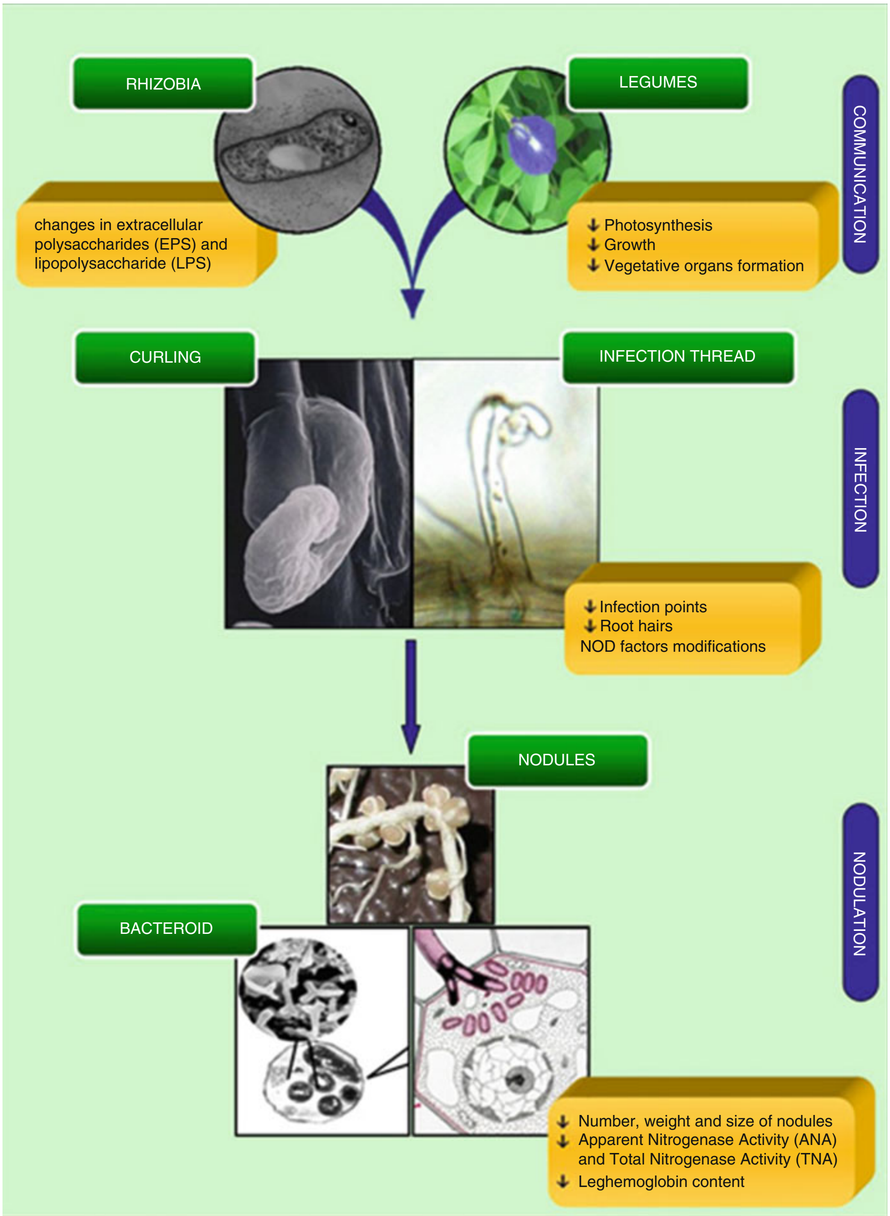

Fig. 1 Effects of salt stress in different stages of symbiotic process

High salinity affects plant growth, especially shoot growth, and thereby symbiotic relations in legumes (Tejera et al. 2004; López et al. 2008). The negative effect of salinity on *Rhizobium*-legume symbiosis is reflected in inhibition of specific nitrogenase activity. This activity is conditioned by the reduction of the protein leghemoglobin, by oxidative stress and by carbohydrate contents of both the cytosol and the bacteroids (Delgado et al. 1993). Salt stress can also be attributed to reducing C supply to bacteroids, mainly in the form of malate limitation, likely as a result of the salt-induced inhibition of sucrose synthase activity and phosphoenolpyruvate carboxylase. This suggests that under low salinity, host growth is slightly reduced growth of the host (Delgado et al. 1994; Soussi et al. 1999). The supply of O_2 to the nodules is a limiting factor. Salt tolerance was associated with higher stability of O_2 nodule conductance of the tolerant rhizobia when increasing soil salinity (L'taief et al. 2007).

3.1 Mechanisms of Salt Stress Tolerance

Salinity imposes at least three primary stresses on plants. The first is a hyperosmotic stress caused by the reduction of water potential of the external soil solution. This solution can exceed their osmotic potential of the plant cell, reducing the water availability for the plant. The second consist in a hyperosmotic stress related to the toxic effects of the accumulated ions, specially Na^+ and Cl^- that produce a nutritional imbalance (Munns and Tester 2008). The third effect stems from the generation of reactive oxygen species (ROS). The combination of all of these factors causes adverse effects on plant growth and development at physiological, biochemical, and molecular levels (Coba de la Peña et al. 2010).

3.1.1 Synthesis of Compatible Solutes

The presence of salt in the growth media often results in the accumulation of low-molecular-mass compounds, termed as compatible solutes or osmoprotectants. These osmoprotectants do not interfere with normal biochemical reactions (Zhifang and Loescher 2003). These compatible solutes are nontoxic and they have low-molecular-weight substances which include mainly simple sugars, disaccharides, sugar alcohols or polyols, amino acids, and sulfonium compounds (Ben Salah et al. 2009). The accumulation of compatible osmolytes works as an osmotic adjustment (raise osmotic pressure) to counteract the high concentration of inorganic salts in the vacuole and the root medium (Zhu 2001). Another function of these accumulated compounds under salinity stress is to act as an energy sink or reducing power, such as C and N_2 source, or scavenging ROS (Sairam et al. 2005). Additionally, these compounds also protect some structures against denaturation (Trinchant et al. 2004).

Sucrose is supplied to the nodule via phloem, and its metabolism leads to the synthesis of dicarboxylic acids. These are the main products of sucrose degradation supplied to bacteroids to support N_2 fixation in legume nodules (Ghittoni and Bueno 1995). However, sucrose metabolism is inhibited under salt stress resulting in a lower supply of dicarboxylic acids (as respiratory substrates) by bacteroids (Soussi et al. 2001). Bacteroids isolated from nodules of pea plants have the respiratory capacity diminished when they are exposed to salt stress. It is associated with a lower content of malate in the nodule cytosol, and it can be reversed by the addition to the incubation medium of exogenous respiratory substrates, such as malate, succinate, proline glutamate, or lactate (Delgado et al. 1994; Ferri et al. 2000). The increase in total soluble sugars has been frequently related to osmoregulation process (Zhu 2002).

Trehalose was described in 1980 as the first major carbohydrate in soybean root nodules (Streeter 1985). In later studies with senescent nodules, trehalose becomes the most abundant carbohydrate. The sucrose content (84 %) is reduced during the nodule senescence, while the amount of trehalose remains constant (Müller et al. 2001). The role of trehalose in legume root nodules under salt stress conditions has been studied by the addition of the trehalase inhibitor validamycin A. Validamycin A caused an increase in the amount of trehalase that improved the response to salinity by increasing the biomass production of species as *M. truncatula* (López et al. 2009) and *L. japonicus* (López et al. 2006). In addition, salt stress increased (especially in roots, bacteroids, and nodular trehalose concentration) about 40–100 % in the legumes *L. japonicus* and *M. truncatula*, respectively. These data support a role for trehalose acting as osmoprotectant under stress conditions in the rhizobia-legume symbiosis (López-Gómez et al. 2013; Domínguez-Ferreras et al. 2009). Barraza et al. (2013) propose that breeding for the genetic modification or trehalose degradation in nodules could be a valuable strategy for improving symbiotic N_2 fixation.

Transgenic plants that accumulate high levels of *proline* are reported to display increased tolerance to salt and osmotic stress (Kishor et al. 2005). *M. sativa* is one of a few plants, which accumulates simultaneously large amounts of both proline-betaine and proline as osmoprotectant solutes. Proline-betaine within the nodules can significantly alleviate (directly or indirectly) the negative effect of salt on nitrogen fixation (Pocard et al. 1984). It has been accepted that symbiosome behaves as osmometer and the peribacteroid membrane serves to maintain and accommodate changes in osmotic pressure (Ouyang and Day 1992). Bacteroids isolated from *P. vulgaris* root nodules inoculated with *R. tropici* strain CIAT 899 could use proline or lactate for bacteroidal respiration after exposure of plant to salinity (Ferri et al. 2000). Under saline stress, proline levels increase in soybean nodules resulting in high ratios of NADP/NADPH and thereby the activation of the pentose phosphate pathway and the eventual production of purine (Miransari et al. 2013). The purine derivates can act as transporters of fixed N_2. Proline can be a transporter of redox reaction product from plant cytoplasm to the bacteroids. It has been verified in the bacteroids of root nodules by the high activity of proline dehydrogenase (ProDH) (Kohl et al. 1990). Within the amino acids, proline shows the largest increase in the nodular tissue of salt-stressed *M. truncatula* and *L. japonicus* plants (López-Gómez et al. 2012).

Salinity induces expression of osmoprotectant-related genes, one of which has been shown to be involved in *pinitol* synthesis (Szabolcs 1994). Pinitol proved to be another carbohydrate that accumulates in nodules of *L. japonicus* plants subjected to salt stress (López-Gómez et al. 2012). Previously, this polyol was described as a compatible solute in plants (Obendorf et al. 2008) and a major carbohydrate (up to 50–60 % of soluble sugar) in soybeans under water stress conditions (Streeter et al. 2001). Palma et al. (2013) suggested that higher increase of pinitol synthesis in nodules of *M. sativa* could be one of the adaptive features used by the plants under salt stress.

3.1.2 Ionic Homeostasis

The legumes usually respond to salinity by excluding Na^+ and Cl^- ions. In fact, it is thought that salt tolerance in legumes is related to their ability to avoid salt ion accumulation (Läuchli 1984). The relationship between salt tolerance and macronutrient accumulation in legumes was reported in 1995 by Cordovilla et al. (1995b). Later, NaCl tolerance associated with retention of Na^+ and maintenance of K^+ selectivity seemed to be a strategy used against the salt stress in common symbiotic bean plants (Tejera et al. 2005). Tejera et al. (2006) suggested that maintenance of high tissue K^+/Na^+ ratio is a criteria for salt tolerance. The capacity to accumulate Na^+ in the root might be related to a protection of the photosynthetic apparatus from Na^+ damage (Soussi et al. 1998). The salt effect in nodule functioning can lead to toxic accumulation of ions such as Cl^- and, in particularly, Na^+ ions in the cytosol (Fernández-Pascual et al. 1996). Differential distribution of these ions across nodule cell layers (infected and uninfected cells) of two lines of faba bean has been described (Abd-Alla et al. 2001). Unless ions are stored in vacuoles, Na^+ and Cl^- are concentrated in compartments such as the cytoplasm. Here ion homeostasis needs to be controlled in order to avoid damage of cellular components, inhibitory effects on cell metabolism, and disturbance of enzymatic activity (Cabot et al. 2014). The disproportionate presence of Na^+ (in both cellular and extracellular compartments) negatively impacts on the acquisition and homeostasis of essential nutrients such as K^+ and Ca^{2+}. Indeed, high levels of Na^+ inhibit the Ca^{2+} and K^+ absorption which results in an antagonium cation. Na^+ and K^+ are known to be of the osmotic potential (Asch et al. 1999). K^+ is a vital contributor to effective FBN (Høgh-Jensen 2003) and Ca^{2+} signaling is critical for the establishment of a successful plant-microbe symbiosis (Garg and Geetanjali 2007).

3.1.3 ROS Scavenging

Salinity, but not osmotic stress, affects apoplastic and intercellular production of ROS, inhibiting root curling and inducing root hair death (Muñoz et al. 2012). Moreover, salt stress affects negatively physiological process in legume-nodulating plants as stomatal conductance, the maximum quantum yield of photosystem II, and total chlorophyll content (Latrach et al. 2014). Some of these effects may involve the oxidative damage of cellular components although they could be prevented by antioxidants. Nodules have a high potential of ROS production due to the elevated rates of bacteroid respiration, the highly reducing conditions required for N_2 fixation, as well as the presence of many electron transfer components, including ferredoxin, uricase, and hydrogenase (Dalton et al. 1998). However, ROS production has been shown to play a key role in the rhizobia-legume interaction and maintenance of the symbiosis between the plant and the bacteria (D'Haeze et al. 2003).

Oufdou et al. (2014) suggest that the salinity effect on the ascorbate-glutathione cycle enzymes depended strongly on the rhizobia strain used for inoculation of the faba bean plants. This specific rhizobia strain involved in nodulation appears to play a role in the changes of enzyme activities against the NaCl stress. Ascorbate and glutathione are molecular antioxidants that play important functions on plant growth and stress responses. Both antioxidants can be found in nodules at concentrations ranging from 0.5 to 2.0 mM, which is consistent with its multiple and essential functions (Dalton et al. 1998). They are potent water-soluble antioxidants, acting as direct ROS scavengers, and as ascorbate-glutathione pathway metabolites from hydrogen peroxide detoxification. Although nodule host cells synthesize their own glutathione, some amounts of this critical antioxidant needs to be produced by the bacterial partner to achieve optimal N_2 fixation. This is evident because when glutathione (GSH; γ-glutamyl-cysteinyl-glycine) is deficient in rhizobia, it forms nodules with early senescence and diminished symbiotic performance (Muglia et al. 2008).

Under salt stress, N_2 fixation and antioxidant enzyme activities in nodules were affected: in *P. vulgaris* catalase (CAT) and ascorbate peroxidase (APX) were inhibited by salt stress (Tejera et al. 2004), whereas superoxide dismutase (SOD) and peroxidase were activated (Jebara et al. 2010). Another study in *P. vulgaris* and *M. sativa* nodules indicated an inhibition of the nodule CAT activity when plants were exposed to NaCl (Tejera García et al. 2007). This inhibition was proportional to the N_2 fixation, confirming the correlation between CAT activity and the functioning of symbiosis (Mhadhbi et al. 2004). The protective role of antioxidants in salinity conditions was investigated in *L. japonicum*. This species has several markers of salt stress and it got more tolerant with the expression of antioxidant genes. The result indicates that *L. japonicum* is more tolerant to salt stress than the other legumes which can be attributed to the capacity of the plant to prevent Na^+ reacting the shoot and to activate antioxidant defense (Rubio et al. 2009).

3.1.4 Other Molecules Involved

The study of the salt stress effects on the growth of nodulated plants and thus, on their ability to fix N_2, has been of considerable interest to analyze the interaction and the alterations of signaling molecules. Molecules, such as indole-3-acetic acid (IAA), PAs, salicylic acid (SA), and abscisic acid (ABA), have been researched in symbiotic plant responses. IAA is expressed in both free-living bacteria and bacteroids, and the IAA overproducing rhizobia strain can accumulate a higher level of trehalose (Bianco and Defez 2009). Bianco and Defez (2009) suggest a different system to enhance salt tolerance. They found evidences about overexpression of IAA in *S. meliloti* 1021. This strain can accumulate a higher level of trehalose and play a positive role in the adaptation to osmotic stress.

PAs are polycationic compounds widespread in many organisms and particularly in plants. They have been implicated in the regulation of many physiological processes and stress responses. Spermidine (Spd), spermine (Spm), and putrescine (Put), for example, have been accumulated under abiotic stress conditions (Bachrach 2010). Hernández-Lucero et al. (2008) studied the effect of salt stress on the expression of many genes involved in PA biosynthesis, including *S*-adenosylmethionine decarboxylase (SAMDC) and in common bean cultivars, and suggested that Spm accumulation might be part of the mechanism conferring salt tolerance. Similar results were described by Echeverria et al. (2013). These authors found that Spd and Spm contributed to salt-induced root PA increment in roots of nodulated plants. This suggests that these PAs might mediate an adaptative role to salinity of *M. tianshanense* symbiosis with *L. tenuis* plants. Recently, López-Gómez et al. (2014a) have found homospermidine (Homspd) as the most abundant PA in nodules of *P. vulgaris*-*R. tropici* symbiosis. The presence of 4-aminobutylcadaverine (4-ABcad) was only described before in nodules of *V. angularis*. Both PAs have been detected in bacteroids which indicate the production of these compounds by the bacteria in symbioses. These results indicated that bacterial metabolism alteration towards the production of uncommon PAs (such as 4-ABcad) is one of the mechanisms to tolerate salt stress in the rhizobia-legume symbiosis. However, in *M. sativa*–*S. meliloti* symbiosis, the PAs did not accumulate in nodules during the initial response to salt treatment while proline accumulation has prevalence over PAs at the earliest response to salinity. This is due to N_2 limitation under salt stress conditions and the existence of a common nodular precursor for both compounds (López-Gómez et al. 2014b).

Some endogenous low-molecular-weight molecules, called hormones, can regulate protective responses of salt stress in symbiosis. ABA is one of the key hormones regulating plant responses to abiotic stress (Addicott et al. 1968). Under salinity stress, the ABA content increases in *P. vulgaris* and *M. ciliaris* nodules (Ben Salah et al. 2011). Some reports have demonstrated the relation between ABA and proline nodular accumulation (Khadri et al. 2007) and PA metabolism (Ben Hassine et al. 2009) under salt stress. They suggest a role for ABA as a signal involved in the maintenance of the cellular PAs. ABA pretreatment improved the N_2 fixation capacity under salt stress conditions by the induction of the nodular antioxidant defenses which may be mediated by the common PAs Spd and Spm that seem to be involved in the antistress responses induced by ABA (Palma et al. 2014).

SA is a water-soluble phenolic compound which can regulate plant growth and has an important role in biotic and abiotic stresses (Palma et al. 2014). SA treatment negatively affected the responses of pea plant to NaCl and this is correlated with an imbalance in antioxidant metabolism (Barba-Espín et al. 2011). Exogenous SA application inhibited the growth of rhizobia and the production of NFs, and reduced the number of total nodules (Mabood and Smith 2007). When the *nahG* gene was expressed in *L. japonicum*, a correlation between reduction of SA level and number of infection was observed. *nahG* gene encodes a SA-hydrolase that degrades SA to catechol (Stacey et al. 2006). It has been reported that SA mitigates the adverse effect of salinity (Nazar et al. 2011). Other reports have demonstrated that the application of SA may promote the ROS formation during salt and osmotic stresses (Nazar et al. 2011). The mechanisms by which SA enhances H_2O_2 content are not clear. It could involve inactivation of removing enzymes by this compound as described in bean by Palma et al. (2009) and/or activation of SOD enzyme as reported by Khan et al. (2010). Manipulation of hormone level and/or its signaling could be a useful strategy for selecting/improving legume plant growth and FBN under salt stress condition (Ben Salah et al. 2011) (Fig. 2).

Fig. 2 Damage and responses induced by salt stress in rhizobia-legume symbiosis

4 Application of Biotechnology for Improved N_2 Fixation Under Salinity

Identification of mechanisms regulating salt tolerance in plant species has become a priority of research in several countries in order to efficiently develop salt-tolerant crops. It is important to use ecophysiological approaches (Mekhaldi et al. 2008) that can provide an alternative to the attenuation of the soil salinity effect on crop plants. This will enable us to identify salt-tolerant species or varieties to expand our knowledge on salinity adaptation mechanisms. The adaptability of plants may be related to constitutive expression of genes encoding protein synthesis and osmoprotective molecules (Cushman et al. 1990). Changes in the concentration of proteins under salt stress may suggest new lines of research into the molecular mechanisms of salt stress adaptation.

Knowledge of the molecular and physiological basis of plant-microbe interactions and their responses to abiotic stress is of vital importance. It can lead to better and more efficient N_2-fixing culture. Recent advances in nodule proteomics, metabolomics, transcriptomics, and fluxomics have provided novel information concerning nodule functioning within a salinity environmental context (Aranjuelo et al. 2014).

4.1 Genomics Approach

Over the last 20 years, many genes that confer salt tolerance were introduced into various crops and test plants (Sun et al. 2013). Traditionally, improved tolerant legumes have been chosen using crosses for higher adaptation to stressed environments and novel methodologies, as omics technology. It has enabled significant progress on C and N exchange understanding between plants and nodules (Aranjuelo et al. 2013). Actually, biochemical tools and cellular and molecular biology are used to modify rhizobia legumes or to make them more resistant to adverse conditions. To make use of genetic engineering, molecular and physiological processes must be studied in stressful situations: circumstances where the plant requires toleration or damage mitigation machinery (Bhatnagar-Mathur et al. 2008). The genome mapping techniques are accelerating the accurate gene position and function identification, genes controlling agronomic traits such as salinity tolerance. The range and accuracy of current breeding programs are enhanced through the use of linked markers with the desirable alleles, such as targeted quantitative trait loci (QTLs) and marker-assisted selection (MAS).

QTLs are stretches of DNA containing or linked to the genes that underlie a quantitative trait. By integrating physiological and genetic strategies, one can get a better understanding of the molecular basis of adaptation of crops to improve abiotic stress tolerance in plants. QTL mapping is revealing genetic components of salt tolerance for genetic improvement of existing varieties (Karan and Subudhi 2012). QTL is associated with salt tolerance at various stages of plant development. It is needed to obtain a more stable plant performance in areas affected by high salinity (Turan et al. 2012). Numerous molecular marker-related techniques have been used in legumes in relation to abiotic stresses. Random amplified polymorphism (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and derivatives have been reported to be used for abiotic stress studies (Lee et al. 2004; Kassem et al. 2004).

MAS is the use of molecular markers linked to useful traits to select individuals with desirable genetic makeup during the variety development process (Karan and Subudhi 2012). The use of genetic and genomic analysis can facilitate breeding strategies for crop improvement. These analyses help to identify regions of DNA tightly linked to agronomic traits of crops, which are called molecular markers (Varshney et al. 2005). The MAS is the result of combining traditional breeding techniques and molecular biology and allows choosing directly the individuals carrying the genes of interest (Collard and Mackill 2008). Combined with traditional selection techniques, MAS is a valuable tool for selection of traits of interest. Besides backcross introgression of characters (controlled by one or a few genes), MAS is used to accumulate QTLs and make genetic improvement in characteristics encoded by several loci (Karan and Subudhi 2012).

As a result, genetic maps for many species with potential stress resistance-related and/or tolerance-related QTLs have been established (Collins et al. 2008). This improved knowledge of the genetic resistance/tolerance control in many vegetables provides information on the number, chromosomal location, and individual or interactive effects of QTLs involved (Dita et al. 2006). Also, Lee et al. (2004) reported that soybean genotypes that are tolerant to salinity have the same QTL allele (*Sat*091). In addition, it has been suggested that the SSR markers Satt237, Satt339, and Satt255 could be used for MAS in soybean breeding program (Hamwieh and Xu 2008). The use of MAS can be useful for crop improvement, such as selecting the common bean drought tolerant (Schneider et al. 1997). However, in legume breeding, its practical application for resistance or stress tolerance has been usually limited by the genetic complexity of traits associated with stress (Dita et al. 2006).

MicroRNAs (miRNAs) are small (20–24 nucleotides) noncoding RNAs which bind to the coding DNA sequences (CDS) or untranslated regions (UTR) of target genes. As a result, they reduce protein expression by accelerating the decomposition of mRNA or repressing the translation of the target mRNAs (Brodersen et al. 2008). Being sessile organisms, plants have to cope with a constantly changing environment to guarantee their survival and reproductive success. Currently, there are several evidences that make us strongly suggest that miRNAs are involved in abiotic stress. The spatial and temporal patterns of miRNA expression during various biological processes to stress responses demonstrate critical regulatory roles for miRNAs (Jones-Rhoades et al. 2006). Recent reports have revealed important regulatory roles of small RNAs in controlling nodulation and BNF in *M. truncatula* and soybean. MTR-miR169 was found to regulate nodule development through regulation of expression of the transcription factor in *M. truncatula* MtHAP2-1 (Combier et al. 2006).

Therefore, miRNAs are involved in different steps during the establishment of symbiosis and BNF. However, the expression dynamics of *miRNA*s are not known, nor it is clear whether expression of them is specifically regulated in N_2-fixing nodules, controlling their BNF efficiency.

4.2 Transgenic Approach

Abiotic stresses generally involve the disturbance of diverse cellular functions, activation of complex metabolic pathways, and polygenic traits conferred by Popelka et al. (2004). This complexity, coupled with the lack of good sources of natural tolerance, makes this an area that is not easily amenable to conventional breeding strategies. The successful use of genetic transformation requires better physiological and molecular understanding of these tensions. Recent advances in understanding the physiological and molecular events and pathways involved in abiotic stress tolerance in legumes, along with advances in the development of gene transfer protocols, have helped to generate transgenic legumes with greater abiotic stress tolerance (Coba de la Peña and Pueyo 2012).

The use of biotechnological methods is among the most effective techniques to improve plant and bacterial symbiotic performance under stress (Miransari et al. 2013). Improvement of BNF by biotechnology can be achieved in different ways. In this part of the chapter, we focus on (1) improvement of the plant and (2) rhizobia tolerance to salt stress with emphases on the mechanisms mentioned above.

4.2.1 Improvement of Legumes Under Salt Stress

Genes responsible for osmolyte synthesis would be one of the key gene groups important for genetic engineering due to the increasing of BNF under saline conditions. In transgenic *M. truncatula* plants overexpressing the *Δ1-pyrroline-5-carboxylate synthetase* (*P5CS*) gene, which encodes an enzyme involved in proline biosynthesis, more proline is accumulated in leaves, roots, and nodules, resulting in enhanced tolerance to salt and osmotic stresses, improving BNF (Verdoy et al. 2006). The modification of trehalose content in *Rhizobium* is another approach. This modification is due to the overexpression on *Rhizobium* of the *OtsA* gene, which codes for trehalose phosphate synthase (TPS). As a result, expression of several genes involved in N assimilation, C assimilation, O_2 transport, and H_2O_2 or ROS detoxification was affected in common bean nodules during the symbiotic interaction. It gave rise to improvements in grain yield, N_2 fixation, and stress tolerance (Suárez et al. 2008). Suárez et al. (2008) demonstrated that the expression of a bifunctional yeast TPS1-TPS2 in transgenic alfalfa plants increased salinity resistance and the nitrogenase activity having a higher number of nodules and infected cells. DREB is a well-characterized transcription factor known to play an important role in regulating gene expression in plant responses to abiotic stresses. Overexpression of the soybean *DREB1* gene in alfalfa produced significantly higher amount of free proline and total soluble sugars, which might contribute to their higher salt tolerance (Jin et al. 2010).

The expression of a cyanobacterial flavodoxin gene in *S. meliloti* resulted in oxidative stress tolerance in alfalfa pellets, which was attributed to the ROS-facilitating detoxification and protection against oxidative damage. Flavodoxin overproduction in transgenic *M. truncatula* plants also confers salt tolerance (Coba de la Peña et al. 2010).

Another consequence of salinity in plants is the ion damage, caused by the increase of ions, mainly Na^+. Genetic manipulation of genes encoding ion channels, contributing to maintenance of K^+/Na^+ ratio, has been very successful to increase salt tolerance in plants (Conde et al. 2011). Overexpression of the *Arabidopsis vacuolar H^+-pyrophosphatase* (*AVP1*) gene in peanut plants was able to maintain higher photosynthetic rates and transpiration rates under salt stress as compared with control plants (Qin et al. 2011).

4.2.2 Genes That Enhance BNF

The development of bacterial strains tolerant to salt stress is one of the targets for genetic engineering to enhance rhizobial symbiosis with leguminous plants to improve yield and BNF (Zahran 2001). Genes involved in stress responses are highly conserved among bacteria and they codify a large set of proteins (Gottesman et al. 1997). The gene of the molecular chaperonin Hsp60 of *E. coli* (*groEL*) is probably one of the best studied genes. This gene is found in a large number of bacteria, and is required for the proper folding of many proteins (Lin and Rye 2006).

BNF is an energy-consuming process that takes place under microaerobic conditions, since nitrogenase activity is inhibited by O_2. Overexpression of the *B. japonicum* terminal oxidase *cbb3* (essential for N_2-fixing endosymbiosis) in a *R. etli ntr*C mutant increased N_2 fixation (Soberón et al. 1999). Castillo et al. (1999) found that increasing the number of *nodD1* regulatory gene copies in *S. meliloti* strains could significantly improve the activity of nitrogenase, leading to enhanced N_2 content in plants and plant growth. Insertion of additional copies of *nifA* and *dctABD* genes in a *S. meliloti* recombinant strain also increased N_2 fixation, and thus the yield of alfalfa (Bosworth et al. 1994).

As mentioned above, one of the organism mechanisms used for stress adaptation is the enhancement of the synthesis of osmoprotectants. Boscari et al. (2006) observed that an *S. meliloti* strain overexpressing *betaine transporter* (*betS*), achieving an increase of proline and glycine betaine in bacteroids, resulted in increased N_2 fixation of nodulated alfalfa plants.

5 Future Perspectives

The BNF is one of the possible solutions of N-source for farmers who do not want to use artificial fertilizer, and plays a key role in the sustainable production of legumes (Miransari et al. 2013). The salt stress causes extensive crop losses in many parts of the world due to lack of salt tolerance in major field crops. Improving salinity tolerance in crops is a major goal of plant breeders to ensure the food supply for a growing world population (Flowers 2004). The combination of the current phenotypic selection and the recent addition of new molecular breeding markers could accelerate the improvement of legumes. Therefore, it is important to take into account individually (1) the genetic variability in the resistance/tolerance to abiotic stresses and (2) genetic variability in the interaction between plant and bacteria (Coba de la Peña and Pueyo 2012). Recently, techniques have been developed for use in MAS breeding programs in legumes. The application of “omics” methodologies, either alone or in combination with more traditional agronomic and physiological studies, may contribute to the elucidation of the mechanisms responsible for plant responses to climate changes. The combination of physiology, transcriptomics, metabolomics, and proteomics will provide key information regarding the plant mechanisms that determine the best or worst performance in a wide range of climate conditions (Ahuja et al. 2010). An interesting perspective on the genetic manipulation is the accumulation of osmoprotectants to improve the salt tolerance. Several candidates have been identified in legumes, such as proline, pinitol, glycine betaine, and trehalose (Shavrukov 2013). The manipulation of genes involved in the metabolism of these compounds has received special attention to increase the accumulation of these osmoprotectants in plant.

Conventional breeding technologies, together with molecular genetic analysis including QTL mapping studies, are revealing important genetic components to improve salt tolerance in field crops (Karan and Subudhi 2012). QTL mapping and MAS have been increasingly used in the effort of the systematic dissection and the use of natural variation in the germplasm available to improve crop yield in saline environments (Collins et al. 2008). In particular, wild crop and land races have enormous potential to remove the upper alleles for improving crop adaptation to salinity (Feuillet et al. 2008). It is expected that genomics technologies will contribute significantly towards discovery of candidate genes for various useful trait components. It can be used to improve elite cultivars using transgenic pyramiding (Takeda and Matsuoka 2008). The utility of transgenic technology can be further improved through the discovery and exploitation of stress-inducible promoters. These promoters could improve salt tolerance with minimal undesirable pleiotropic effects on plant growth, getting a productivity as under normal conditions. However, the collaboration of geneticists, molecular breeders, physiologists, and genomicists is required to implement an integrated approach. It would allow discovering, testing, and integrating superior alleles for improving salt tolerance in major food crops.

In conclusion, salinity tolerance is too complex to be easily amenable for improvement just through selection as a trait itself. However, hypothesized traits to contribute salinity tolerance are genetically more tractable and genes underlying these processes can be discovered using molecular genetics tools and genomics (Roy et al. 2014). This new research would improve the BNF.

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