Chapter 11
Alleviation of Salt Stress in Legumes by Co-inoculation with *Pseudomonas* and *Rhizobium*

Dilfuza Egamberdieva, Dilfuza Jabborova, and Stephan Wirth

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Abstract Numerous studies have shown that soil salinity decreases nodulation and dramatically reduces N_2 fixation and nitrogenase activity of nodulated legumes. Thus, the development of salt-tolerant symbioses is an absolute necessity to enable cultivation of leguminous crops in salt-affected soils. Dual inoculation of legumes with plant growth-promoting rhizobacteria (PGPR) and rhizobia has been reported to increase the number of nodules compared to those formed by a rhizobial strain alone. The production of IAA by *Pseudomonas* strains represents a beneficial mechanism that promoted enlargement of root system and thereby further enhanced nutrient uptake, nodulation, and shoot growth of leguminous plants. When PGPR are able to alleviate salt stress experienced by the plant, more nodules might develop into nitrogen-fixing ones, thereby enabling the plant to obtain part of its nitrogen from the atmosphere. Co-inoculation techniques could be a new approach to increase

D. Egamberdieva (✉) • D. Jabborova
Faculty of Biology and Soil Sciences, National University of Uzbekistan, Tashkent, Uzbekistan
e-mail: egamberdieva@yahoo.com

S. Wirth
Leibniz-Centre for Agricultural Landscape Research (ZALF), Institute for Landscape Biogeochemistry, Müncheberg, Germany

N.K. Arora (ed.), *Plant Microbe Symbiosis: Fundamentals and Advances*,
DOI 10.1007/978-81-322-1287-4_11, © Springer India 2013

the salt tolerance and yield of legumes used for the food and green manure production in salt-affected soils, providing supply of biologically fixed N at low cost.

Introduction

Salinity is a major concern for irrigated agriculture in arid and semiarid regions of the world (Vincent et al. 2006). In particular, secondary salinity developed from irrigation is widely responsible for reducing soil and water quality, limiting crop growth, and leading to the abandonment of agricultural land (Egamberdiyeva et al. 2007). Salt affects plant growth mainly through toxicity caused by the excessive uptake of salts, especially that of NaCl (FAO 2005). Soil salinity reduces plant growth and photosynthesis due to the complex negative effects of osmotic, ionic, and nutritional interactions (Shannon 1997; Shirokova et al. 2000). Salinity stress increases levels of ethylene that significantly inhibits shoot and root elongation and reduces plant height and overall growth (Ma et al. 1998; Klassen and Bugbee 2002).

Most legumes are rather sensitive to salinity, and only a few agronomical legumes can grow in salt-affected soils (Ashraf and McNeilly 2004). For example, two annual pasture legumes, messina (*Melilotus siculus*) and burr medic (*Medicago polymorpha*), can persist in soils with an electrical conductivity (ECe) up to 36 dS/m (Rogers et al. 2005). Soil salinity particularly disturbs the symbiotic interaction between legumes and rhizobia (Marcar et al. 1991). Numerous studies have shown that soil salinity decreases rhizobial colonization and nodulation and dramatically reduces N_2 fixation and nitrogenase activity of nodulated legumes (Singleton and Bohlool 1984; Zahran and Sprent 1986; Elsheikh and Wood 1995; Zahran 1999). Thus, the development of salt-tolerant symbioses is an absolute necessity to enable cultivation of leguminous crops in salt-affected soils (Velagaleti and Marsh 1989; Mayak et al. 2004). There is now increasing evidence that the use of beneficial microbes can enhance the resistance of plants to adverse environmental stresses, e.g., drought, salts, nutrient deficiency, and heavy metal contaminations (Glick et al. 2007).

In this chapter we describe (1) the effect of salinity on legume-*Rhizobium* symbioses, (2) the *Rhizobium*-*Pseudomonas* interactions, (3) their ameliorative and beneficial effects, and (4) the mechanisms involved in plant growth stimulation and alleviation of salt stress.

Effects of Salinity on Legume-*Rhizobium* Symbioses

Many studies reported the negative effects of soil salinity on crop yield and total nitrogen fixation of leguminous plants such as bean, chickpea, lentil, and soybean (van Hoorn et al. 2001). Similar results were observed by Mensah and Ihenyen (2009) on mung bean (*Vigna mungo* L. Hepper), where they observed decreases in percentage germination and seedling emergence with increases in salinity. The existence of inter- and intraspecific variability in the sensitivity of N_2 fixation to salinity has also been reported in legumes (Serraj et al. 2001). Subbarao et al. (1990) observed that nodule initiation by *Rhizobium* was the most salt-susceptible aspect of pigeon pea than growth. Rhizobial species *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Mesorhizobium* lead to symbiotic interactions with legumes and result in root nodule formation. However, root nodulation in legumes is dependent on numerous soil and environmental factors, and very often the introduced *Rhizobium* has to overcome intense competition from native microorganisms that colonize the rhizosphere (Mishra et al. 2009). Salinity leads to a failure in the establishment of rhizobia in the rhizosphere, by reducing survival and proliferation of rhizobia in the soil and rhizosphere, or by inhibiting very early symbiotic events, such as root hair colonization (Singleton and Bohlool 1984; Hashem et al. 1998). Cordovilla et al. (1999) reported that *R. leguminosarum* formed an infective symbiosis with faba bean under saline conditions, and that N_2 fixation was more sensitive to salinity than plant growth. The reduction of N_2-fixing activity is usually attributed to a reduction in respiration of the nodules and leghemoglobin production (Delgado et al. 1994; Walsh 1995). An explanation for the reduction in symbiotic legume growth might be that the salt stress causes a failure of the infection and nodulation process. For example, according to Bouhmouch et al. (2005), salt inhibits the absorption of Ca, which reduces the growth of roots, root tips, and root hairs, thereby decreasing sites for potential rhizobial infection and further nodule development. Cordovilla et al. (1995) observed that the depressive effect of salt stress on N_2 fixation by legumes is directly related to the salt-induced decline in dry weight, N content in the shoot, and the salt-induced distortions in nodule structure (Zahran and Abu-Gharbia 1995).

According to Rekha et al. (2007), colonization of the inoculated bacteria in the rhizosphere largely depends on the availability of the empty niche and the capacity of competing with other microflora. The colonization of leguminous root hairs by rhizobial cells is fundamental for the establishment of the legume-*Rhizobium* symbiosis (Gulash et al. 1984). The very early symbiotic events, colonization and infection of root hairs by rhizobial cells, are especially sensitive to environmental stresses (Räsänen et al. 2003). A decrease in the number of rhizobial cells was demonstrated to occur in the root of soybean, common bean, and chickpea (*Cicer arietinum*) grown under salt stress (Zahran and Sprent 1986; Bouhmouch et al. 2005). Since the symbiotic performance of legumes depends upon the population size and survival of introduced rhizobia in the root, the improvement of their colonization in saline conditions is important to develop salt-tolerant symbioses (Velagaleti and Marsh 1989).

Plant Growth-Promoting Rhizobacteria

Beneficial rhizosphere bacteria are of two general types: those forming a symbiotic relationship with the plant and those that are free living in soil and root (Barriuso et al. 2005; Lugtenberg and Kamilova 2009). The use of plant growth-promoting rhizobacteria (PGPR) in improvement of crop yield started long time ago, and there are many reports where beneficial microbes can enhance plant growth, development, nutrient uptake, and yield (Lugtenberg et al. 2001; Arora et al. 2008; Egamberdieva et al. 2010). Treatments with PGPR like *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Enterobacter*, *Pseudomonas*, *Burkholderia*, *Bacillus*, and *Serratia* increase germination percentage, emergence, root and shoot growth, total biomass of the plants, seed weight, grains, and yields (Mantelin and Touraine 2004; Joseph et al. 2007; Yasmin et al. 2007). Further studies also confirmed enhanced growth, nodulation, and yield of chickpea by *Rhizobium* (Carter et al. 1994; Elsheikh and Elzidany 1997; Akhtar and Siddiqui 2009; Khosravi et al. 2010).

The plant growth promotion activity of rhizobacteria is primarily related to its impact on root growth and morphology (Dobbelaere et al. 2001). Creus et al. (2004) reported that bacterial inoculation caused the production of lengthy root hairs, stimulated the production of lateral roots, and improved the root diameter and surface respectively. The ability of other PGPR species to improve growth, nodulation, and nitrogen fixation is documented for many legume species (Burdman et al. 2000; Tanimoto 2005; Egamberdieva et al. 2010).

Rhizobium-Pseudomonas **Interactions**

In the rhizosphere, a synergism between various bacterial genera such as *Bacillus*, *Pseudomonas*, *Arthrobacter*, and *Rhizobium* has been shown to promote plant growth of various plants such as peanut, corn, soybean, and maize (Dey et al. 2004; Ratti et al. 2001). Available reports indicate improved yield of legumes health, and nodulation when co-inoculated with PGPB, compared to inoculation with *Rhizobium* alone (Valverde et al. 2005; Egamberdieva et al. 2010; Yadegari and Rahmani 2010). In other studies the co-inoculation with *Pseudomonas* spp. and *Rhizobium* spp. enhanced nodulation and nitrogen fixation, plant biomass, and grain yield in various leguminous species including alfalfa (Bolton et al. 1990), soybean (Dashti et al. 1998), chickpea (Goel et al. 2002), and pea (Tilak et al. 2006).

There are several reports on the positive effects of co-inoculation of legumes with *Pseudomonas* and *Rhizobium* spp. A significant increase in N content of root and shoot of *Galega orientalis* was also observed after co-inoculation of *Pseudomonas trivialis* strain 3Re27 with *Rhizobium galegae* HAMBI 540 which significantly increased the N content of the roots by 20 % and of the shoots by 52 % compared to *R. galegae* HAMBI 540 alone. Shoot and root growth was also increased by co-inoculation of both strains (Egamberdieva et al. 2010). Improved mineral nutrition would explain the promotion of root and shoot growth (Burdman et al. 1997; Cakmakci et al. 2005). Similar results were observed by Khurana and Sharma (2000) and Siddiqui et al. (2001) where combined inoculation of *Rhizobium* and *Pseudomonas* increased nodulation, nitrogenase activity, growth, and yield of chickpea under greenhouse conditions. In other studies a greater number of nodules and dry weight was recorded in soybean and alfalfa when the co-inoculation with *B. japonicum* and *Pseudomonas* was observed by Rosas et al. (2006).

Alleviation of Salt Stress in Plants

The ameliorative effects of PGPR on plant growth under saline conditions have been shown for various plant species, such as tomato, pepper, canola, bean, and lettuce (Barassi et al. 2009; Kang et al. 2009; Egamberdieva 2009). Salt-stressed soybean plants had significantly decreased plant growth, photosynthesis, and mineral uptake with increasing salinity, and inoculation of salt-stressed plants with PGPR strains could alleviate salinity stress (Han and Lee 2005). These PGPR (e.g., *Rhizobium*, *Azospirillum*, *Pseudomonas*, *Flavobacterium*, *Arthrobacter*, and *Bacillus*) utilize osmoregulation, oligotrophic, endogenous metabolism, resistance to starvation, and efficient metabolic processes to adapt under dry and saline environments (Lugtenberg et al. 2001; Egamberdiyeva and Islam 2008). These bacteria, with a physiological adaptation and genetic potential for increased tolerance to drought, increased salt concentration, and high temperatures, could improve plant production in degraded sites. The inoculation of bean with bacterial strains *P. extremorientalis* TSAU20 and *P. chlororaphis* TSAU13 increased shoot length of bean significantly at 5.0, 7.5, and 10.0 dS/m up to 50 % (Egamberdieva 2011). The *Pseudomonas* strains *P. trivialis* 3Re27 and *P. extremorientalis* TSAU20 have an excellent root-colonizing capability and plant growth-promoting activity. They are also salt tolerant, capable of growing in 4 % NaCl, and able to alleviate salt stress in pea and soybean plants (Egamberdiyeva and Hoflich 2002; Egamberdiyeva et al. 2004; Egamberdieva et al. 2010). Both a gnotobiotic sand system test and the greenhouse experiment with low-fertilized potting soil demonstrated that the salt tolerance of *Galega officinalis* clearly improved when the plant was inoculated besides its own specific symbiont *R. galegae* sv. *officinalis*, with either of the two PGPR strains, *P. extremorientalis* TSAU20 or *P. trivialis* 3Re27 (Fig. 11.1) (Egamberdieva et al. 2013). In earlier studies Hasnain and Sabri (1996) showed that inoculation of wheat with *Pseudomonas* sp. stimulated plant growth by reducing plant uptake of toxic ions and increasing the auxin content. Heidari et al. (2011) also reported that plant growth, auxin and protein contents of *Ociumum basilicm* inoculated by

Fig. 11.1 The effect of *R. galegae* R1141 combined with *Pseudomonas* strain TSAU20 on nodulation of *Galega officinalis* (pot experiments, 0 and 50 mM NaCl)

Table 11.1 The length of roots and shoots, biomass of whole plants, and the number of nodules of soybean when seedlings were inoculated with *Bradyrhizobium japonicum* strains USDA110 alone and together with *Pseudomonas putida* TSAU1

Bacterial strains	Root[a] length (cm)	Shoot[a] length (cm)	Biomass (g)[b] weight	Nodule numbers
0 mM NaCl				
USDA110	11.7	20.6	0.086	6.3
USDA110+TSAU1	13.4*	23.4	0.100	8.0
50 mM NaCl				
USDA110	10.2	10.6	0.067	4.2
USDA110+TSAU1	12.4*	16.0*	0.088*	4.6
75 mM NaCl				
USDA110	9.0	8.2	0.053	3.0
USDA110+TSAU1	10.2	12.2*	0.084*	4.0

Plants were grown in the gnotobiotic sand system under salt stress for 3 weeks. Values represent means for six plants ($N=6$)

[a]cm

[b]g/plant

*Significantly different from plants inoculated with *B. japonicum* alone at $P<0.05$

Pseudomonas sp. under drought stress conditions increased compared to the control. The combined inoculation of *Azotobacter*, *Azospirillum*, *Pseudomonas*, and *Mesorhizobium* resulted in promotion of grain yield and biomass in chickpea (Rokhzadi et al. 2008). Parmar and Dadarwall (1999) also observed that co-inoculation of *Pseudomonas* and *Bacillus* sp. with *Rhizobium* strains enhanced the nodule weight, root length, shoot biomass, and total plant nitrogen in chickpea, when grown in sterilized jars or under pot culture conditions. We have observed that the co-inoculation of salt-stressed soybean with *B. japonicum* USDA110 and *P. putida* TSAU1 improved root and shoot length, dry weight, and nodulation compared to those plants inoculated with *B. japonicum* alone (Table 11.1).

Increasing the salt content decreased the ability of *B. japonicum* cells to colonize soybean roots, colony-forming units (CFU) counts decreased from $\log_{10}$ 3.9 CFU to $\log_{10}$ 3.5 CFU (Table 11.2). However, the co-inoculation of *B. japonicum* USDA110 with *P. putida* TSAU1 increased the number of rhizobial cells colonizing soybean roots. Competitive root tip colonization test showed that the *Pseudomonas* strain was a better colonizer than *B. japonicum* (Table 11.2). In other study we demonstrated that the colonization of *G. officinalis* root tips by *Rhizobium* cells increased almost twofold under saline conditions when the plants were inoculated besides *Rhizobium* with *Pseudomonas* strains (Egamberdieva et al. 2013). Such combined inoculation could also enhance formation of nodules on legumes grown in salinated potting soil. In addition, we observed that though salt stress decreased the proportion of big nitrogen-fixing nodules, enhanced nodulation achieved by dual inoculation compensated this decrease and the number of big nodules was duplicated compared to the plants inoculated with *Rhizobium* alone (Egamberdieva et al. 2013).

Table 11.2 The competitive root tip colonization of *B. japonicum* strain USDA110 and *Pseudomonas putida* TSAU1 in the rhizosphere of soybean

	Root colonization Log CFU/1 cm root ± SD	
Bacterial strains	USDA110	TSAU1
0 mM NaCl		
USDA110	3.9 ± 0.06	
USDA110 + TSAU1	4.1 ± 0.08	4.2 ± 0.10
50 mM NaCl		
USDA110	3.7 ± 0.15	
USDA110 + TSAU1	4.0 ± 0.05	4.1 ± 0.10
75 mM NaCl		
USDA110	3.5 ± 0.19	
USDA110 + TSAU1	3.8 ± 0.20	3.9 ± 0.10

Plants were grown in the gnotobiotic sand system under salt stress for 3 weeks

Biomechanisms to Enhance Plant Growth

Mechanisms by which bacteria are able to promote plant growth and prevent damage caused by salinity include production of phytohormones like indoleacetic acid (IAA), gibberellic acid, cytokinins, and ethylene (Spaepen et al. 2009; Mishra et al. 2010), production of ACC-deaminase to reduce the level of ethylene in the roots of developing plants (Dey et al. 2004), asymbiotic nitrogen fixation (Ardakani et al. 2010), and production of exopolysaccharides (EPS) (Upadhyay et al. 2011).

Production of the auxin phytohormone indole-3-acetic acid (IAA) by bacterial inoculants might be responsible for the enlarged root system and number of infection sites prior to nodulation (Tanimoto 2005; Tilak et al. 2006). Rhizobacteria synthesize and release auxin as secondary metabolites because of the rich supplies of substrates exuded from the roots (Lugtenberg et al. 2001; Shahab et al. 2009; Egamberdieva and Kucharova 2009). Bacterial strains which belong to genera such as *Pseudomonas*, *Bacillus*, *Rhizobium*, and *Microbacterium* are among the most active IAA producers (Wang et al. 1982; Costacurta and Vanderleyden 1995; Mehnaz and Lazarovits 2006; Tsavkelova et al. 2007). The IAA that is secreted by bacteria, together with endogenous plant IAA, is taken by plant cells which can stimulate plant cell proliferation (Glick et al. 2007). The exogenous application of auxins to alfalfa (Gruodien and Zvironaite 1971) and groundnut (Srinivasan and Gopal 1977) promoted plant growth and nodulation. Earlier reports showed that *Rhizobium meliloti* associated with alfalfa produced 20 mg/ml of IAA (Williams and Singer 1990), whereas *Rhizobium leguminosarum* produced 2.0 mg/ml of IAA (Beltra et al. 1980). IAA produced by nodule bacteria is transported to other parts of the plant and might be involved in several stages of the symbiotic relationship (Wheeler et al. 1979; Hunter 1989).

In early studies, the depressive effect of salinity on plant growth was explained by decline in endogenous levels of hormones in the rhizosphere (Zholkevich and Pustovoytova 1993; Jackson 1997), whereas phytohormones released by rhizobacteria effect positively to seedling development (Frankenberger and Arshad 1995;

Afzal et al. 2005). Low concentration of pure IAA or low titer of IAA-producing bacteria enhanced root growth and nodulation (Remans et al. 2008), whereas high concentration of pure IAA or high titer of IAA-producing bacteria inhibited root growth and nodulation (Plazinski and Rolfe 1985). Bacterial IAA can also act as signal molecule in bacteria-bacteria communication (Spaepen et al. 2009). Another explanation for enhancement of nodule formation by the rhizobia in legumes might be the production of hydrolytic enzymes such as cellulases by root-colonizing *Pseudomonas* strains, which could make penetration of rhizobia into root hairs or intercellular spaces of root cells easier, leading to increased numbers of nodules (Sindhu and Dadarwal 2001).

Plant stress can be reduced by 1-aminocyclopropane-carboxylate (ACC) deaminase-producing bacteria (Glick et al. 1997). The ACC-deaminase enzyme can cleave the ethylene precursor ACC to α-ketobutyrate and ammonium and thereby lower the level of ethylene in developing or stressed plants (Glick 1995; Glick et al. 1998; Hontzeas et al. 2005). PGPR releasing the enzyme ACC-deaminase may decrease the ethylene level and enhance the survival of seedlings (Glick et al. 1998). It has been reported that PGPR strain *P. trivialis* 3Re27 was able to utilize ACC as N source indicating the presence of ACC-deaminase and increased salt tolerance of goats' rue, stimulating shoot and root growth under salinated soil conditions (Egamberdieva et al. 2013). Similar results were observed by Shaharoona et al. (2006) where co-inoculation of *Bradyrhizobium* with PGPR isolates strains possessing ACC-deaminase activity enhanced the nodulation in mung bean compared with inoculation with *Bradyrhizobium* alone. Arshad et al. (2008) observed that inoculation with PGPR containing ACC-deaminase was highly effective in removing the effects of water stress on growth, yield, and ripening of peas.

Conclusion

As discussed in this review, salinity decreases nodulation, reduces N_2 fixation and nitrogenase activity of legumes, and leads to a failure in the establishment of rhizobia in the rhizosphere by inhibiting very early symbiotic events. The co-inoculation of legumes with *Rhizobium* and PGPR *Pseudomonas* strains was able to alleviate salt stress of plants grown in salt-affected soils. The phytohormone auxin produced by root-colonizing bacteria plays an important role in alleviating salt stress in plants. Co-inoculation techniques could be a new approach to increase the salt tolerance and the yield of leguminous plants used for food and green manure production in salt-affected soils, providing supply of biologically fixed N at low cost. The future direction in research needs to include (1) the mechanisms involved in alleviation of salt stress in plants, (2) the potential competition between PGPR strains and indigenous soil microflora in the rhizosphere of plants grown under stressed environments, and (3) more research on the interaction between PGPR and rhizobia, as the latter are known to confer resistance to salt stress and drought while promoting growth of the host plant.

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