Survival of *Pseudomonas extremorientalis* TSAU20 and *P. chlororaphis* TSAU13 in the rhizosphere of common bean (*Phaseolus vulgaris*) under saline conditions

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ABSTRACT

The abilities of *Pseudomonas extremorientalis* TSAU20 and *P. chlororaphis* TSAU13 to colonise and survive in the rhizosphere of common bean under saline conditions were studied. Four salinity levels (5.0, 7.5, 10.0, and 12.5 dS/m) were maintained in the gnotobiotic system using NaCl salt. Results showed that with increasing salt content root-tip colonization of both bacterial strains was reduced. Both bacterial treatments used in the study increased root and/or shoot length compared to non-treated plants at each NaCl concentration tested, whereas shoot growth was not stimulated at high saline condition (12.5 dS/m). In conclusion, the results of this study indicated that *P. extremorientalis* TSAU20 and *P. chlororaphis* TSAU 13 have the ability to survive in ecologically stressed conditions, such as saline and nitrogen deficient soils, and may positively effect on plant growth of bean. High salinity inhibited their colonisation in the rhizosphere of bean and thus their stimulatory effect on plants was also reduced.

Keywords: Rhizosphere bacteria; root colonisation; *Phaseolus vulgaris*; salinity

Saline soils and saline irrigations constitute a serious production problem for crops as saline conditions are known to suppress plant growth (Shannon and Grieve 1999). It is thought that the repressive effect of salinity on germination and plant growth could be related to a decline in endogenous levels of plant growth hormones or phytohormones (Jackson 1997, Debez et al. 2001). It is also suggested that root-colonising bacteria which produce phytohormones, when bound to the seed coat of a developing seedling, may act as a mechanism for plant growth stimulation and these organisms can prevent the deleterious effects of stresses from the environment (Lindberg et al. 1985, Frankenberger and Arshad 1995). The beneficial attributes of these organisms also include improved nutrient recycling in the rhizosphere microcosm and root colonization are some of the challenges under stress (Mayak et al. 2004, Paul and Nair 2008, Berg 2009, Lugtenberg and Kamilova 2009). *P. extremorientalis* TSAU20 and *P. chlororaphis* TSAU13 showed their capacity to increase the growth of various crops under saline conditions (Egamberdieva and Kucharova 2009, Egamberdieva et al. 2010). Successful soil inoculation of microorganisms requires survival of the introduced strain in soil, which largely depends on the availability of the empty niche, and the capacity of competing with the better adapted native microflora (Van Elsas and Heijnen 1990, Lugtenberg et al. 1999, Rekha et al. 2007).

Understanding of abiotic factors which affect the colonisation of microorganisms in the rhizosphere of plant is of primary importance for the effective use of rhizobacteria as plant growth stimulators (Schroth and Becker 1990). Earlier reports claim that soil salinity has an adverse effect on plant growth promoting bacterial populations by high osmotic strength (low water potential) and toxic effects by salts (Borneman et al. 1996, Sato and Jiang 1996).

The present investigation is designed to investigate the effect of salinity on the colonisation of two selected plant growth-promoting bacteria *P. extremorientalis* TSAU20 and *P. chlororaphis* TSAU13 on the rhizosphere of common bean, using a gnotobiotic sand system and to determine their survival on the plant roots in pot experiments under salinated soil conditions.

MATERIAL AND METHODS

Plant and microorganisms. The common bean (*Phaseolus vulgaris*) was used for the experiments. Strains *P. extremorientalis* TSAU20 and *P. chlororaphis* TSAU13 are from the culture collection of the Department of Microbiology and Biotechnology, National University of Uzbekistan, Tashkent (Egamberdieva and Kucharova 2009). The strains were isolated from the rhizosphere of wheat grown in salinated Uzbek soil after using the enrichment procedure for the isolation of enhanced root tip colonisers described by Kamilova et al. (2005).

Root tip colonization. For the root tip colonisation assay, seedlings were inoculated with bacterial strains and grown under gnotobiotic conditions. The strains were grown overnight in King B (KB) medium; then 1.0 ml of an overnight culture was sedimented by centrifugation (13 000 × g) and the supernatant discarded. The cells were washed with 1 ml phosphate buffered saline and re-suspended in PBS. Cell suspensions were adjusted to OD_{620} = 0.1 that corresponds to cell density of about 10^8 cells/ml.

The bean seeds were sterilised by immersion in 70% ethanol for 5 min and subsequently in 0.1% $HgCl_2$ for 1 min, washed several times with sterile water, and allowed to germinate. Subsequently, they were coated with bacteria by soaking them in a bacterial suspension whereas control seeds were soaked in sterile PBS buffer, both for 10 min. Inoculated seedlings were planted in sterile glass tubes with sand as described by Simons et al. (1996). Salinity conditions were established by adding 25, 50, 75, 100, and 125 mmol NaCl into PNS (plant nutrient solution). Electrical conductivity (EC) of these solutions were 0.5 mS/cm for H_2O, 5.0 dS/m for 50 mmol NaCl, 7.5 dS/m for 75 mmol NaCl, 10.0 dS/m for 100 mmol NaCl and 12.5 dS/m for 125 mmol NaCl.

The inoculation treatments were set-up in a randomised design with 10 replications. Plants were grown for 7 days in climate-controlled chambers with 16 h of daylight at 24°C. Then 1 cm of root tip from plantlets was collected. Bacterial cells were removed from the root tip by vortexing in PBS and plated on KB medium.

Survival of bacterial strains in the rhizosphere of plants. Spontaneous and stable rifampicin (200 µg/ml) resistant mutants of the wild type strain were used for the colonisation studies. Mutants of *P. extremorientalis* TSAU20 and *P. chlororaphis* TSAU13 that were marked with antibiotic resistance were obtained by plating the parental strain onto KB agar amended with 200 µg/ml rifampicin. After incubation, isolates were selected based on similarities in colony morphology and growth rate with the parent strain, and were recultured on medium containing rifampicin to ensure stability of the antibiotic resistance marker. The plant seeds were sterilised, allowed to germinate, and coated with rifampicin resistant mutants as described previously. The inoculation treatments were set-up in a randomised design with 10 replications. Plants were grown in pots containing salinated soil in greenhouse conditions which were described above. After four weeks, plants were harvested and the adhering soil was removed from plant roots. One gram of roots were shaken in 9 ml sterile PBS and the resulting suspensions were evaluated for colony-forming units (cfu) according to the dilution-plate method in KB agar with addition of 200 µg/ml rifampicin. After incubation for 2–3 days at 28°C the reisolated, rifampicin-resistant strains were identified for their colony characteristics (Höflich et al. 1995).

Plant growth promotion in gnotobiotic systems and pots. The effects of inoculation with *P. extremorientalis* TSAU20 and *Pseudomonas chlororaphis* TSAU13 on the growth of common bean growing under salt stress was preliminary studied under gnotobiotic conditions. Experiments were carried out in test tubes as described above. Plants were grown in climate-controlled chambers. After 10 days of growth the length of shoots and roots was measured.

Plant tests in the greenhouse were conducted in plastic pots (9 cm diameter; 12 cm deep) containing 350 g of saline soil. The soil was selected from a deep tillage (0–40 cm) irrigated agricultural field affected by salinity from the Sayhunobod district (41°00'N, 64°00'E), Syr-Darya Province, in the North-East of Uzbekistan. The field has an EC value of 659 mS/m soil. The soil surface horizon is calcareous saline, and the deeper levels are mild alkaline in nature. The soil contains 43 ± 9 g sand/kg, 708 ± 12 g sil/kg, and 249 ± 13 g clay/kg. The organic matter content of the soil is 0.694%; total C, 2.506%; total N, 0.091%; Ca, 63.5 g/kg; Mg, 20.7 g/kg; K, 6.2 g/kg; P, 1.2 g/kg; Cl, 0.1 g/kg; Na, 0.7 g/kg, and the pH is 8.0 (Egamberdiyeva et al. 2007).

The plant seeds were sterilised, allowed to germinate, and coated with bacteria as described previously and inoculated seedlings were planted in the plastic pots. The inoculation treatments were set-up in a randomised design with ten replications. The pot experiment had two treatments:

seeds non-inoculated with bacteria, and seeds inoculated with bacteria. Plants were grown at 20–24°C during the day and 12–14°C at night and after four weeks the shoot and root length and dry matter of bean were measured.

Statistical analysis. Data were tested for statistical significance using the analysis of variance package included in Microsoft Excel 98 and comparison was done using a Student's *t*-test. Mean comparisons were conducted using a least significant difference (*LSD*) test ($P = 0.05$). Standard error and *LSD* result were calculated.

RESULTS AND DISCUSSION

The successful inoculation requires bacterial survival in the rhizosphere and it depends on the availability of the empty niche, withstand competition with the often better-adapted native microflora (Bull et al. 1991, Devliegher et al. 1995, Lugtenberg et al. 1999, Rekha et al. 2007) and on the adaptation to physical-chemical conditions of soil (Van Overbeek et al. 1997). Some abiotic factors such as drought and soil salinity could greatly influence the colonization of the plant growth-promoting rhizobacteria.

When bean seedlings were grown in the gnotobiotic sand system under growth cabinet conditions for 7 days with increasing salt content root-tip colonisation of *P. extremorientalis* TSAU20 strain was reduced from 66.5 to 24.0 and *P. chlororaphis* TSAU13 from 79.8 to 16.8 (10^3 CFU/cm of root tip) (Figure 1). There were several studies investigating colonisation of bean root by *Pseudomonas* strains in non saline conditions (Anderson and Guerra 1985, Zdor and Anderson 1992, Miller et al. 2001). Our bacterial strains were able to colonize in the rhizosphere of bean at the highest saline condition (12.5 dS/m). The bacterial strains were also tested for their survival in the root of bean grown in saline soil. The results showed that rifampicin-resistant mutants obtained from bacterial strains *P. extremorientalis* TSAU20 and *P. chlororaphis* TSAU 13 were able to survive in the root of bean. The root colonisation in the rhizosphere of bean was $3.9 \times 10^3 \pm 0.8$ CFU/g of fresh root with *P. extremorientalis* TSAU20 and $1.9 \times 10^3 \pm 0.5$ CFU/g of fresh root with *P. chlororaphis* TSAU 13. Garcia and Hernandez (1996) reported that salinity negatively affects microbiological activity of soil by high osmotic strength and toxic effects of salts. In such harsh condition salt tolerant halophytic bacteria can survive and proliferate in soil and in the rhizosphere (Brown 1976). Our bacterial strains are salt tolerant up to 3% NaCl and temperature resistant (Egamberdieva and Kucharova 2009), thus they were able to survive in the rhizosphere of bean due to their competitiveness and persistence in saline arid soil conditions. Similar results were obtained by Paul and Nair (2008) whereas the root colonization potential of the salt tolerant *Pseudomonas* strain was not hampered with higher salinity in soil. Diby et al. (2005) also observed that the population of the *P. pseudoalcaligenes* MSP-538 did not change considerably with increasing salinity in the soil. According to Lugtenberg et al. (2001) the growth rate of bacterial strains in the rhizosphere will depend on the ability to take up components essential for cell growth and/or maintenance. It is known that pseudomonades are motile by one or several polar flagella and thus allowing bacteria to reach and utilize a large number of carbon

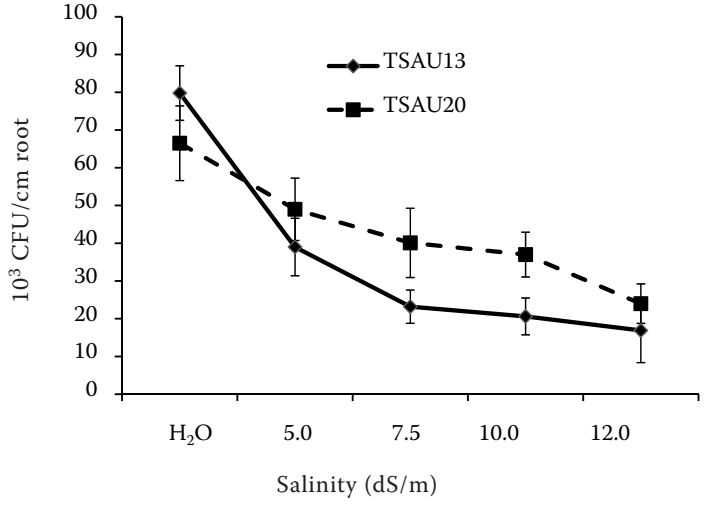

Figure 1. Bean root tip colonisation ability of *P. extremorientalis* TSAU20 and *P. chlororaphis* TSAU13 in a gnotobiotic sand system under increasing salinity (H_2O, 5.0; 7.5; 10.0; 12.0 dS/m)

Table 1. The length of roots and shoots (cm) of common bean when seedlings were inoculated with *P. extremorientalis* TSAU20 and *P. chlororaphis* TSAU13 (plants were grown in a gnotobiotic sand system under increasing salt stress for ten days)

Treatments	H_2O		5.0		7.5		10.0		12.5	
	root	shoot	root	shoot	root	shoot	root	shoot	root	shoot
Control	16.8 ± 0.8	7.4 ± 1.1	15.0 ± 1.6	6.4 ± 0.8	13.4 ± 1.8	5.8 ± 1.6	5.2 ± 0.8	4.8 ± 1.3	2.3 ± 0.3	3.7 ± 1.3
TSAU13	20.5* ± 1.3	10.5* ± 1.2	16.8 ± 1.0	9.6* ± 1.1	16.6* ± 1.1	8.7* ± 0.9	6.7* ± 0.9	5.6 ± 1.5	3.6* ± 1.2	3.8 ± 0.8
TSAU20	18.5 ± 2.3	9.3* ± 0.9	17.6 ± 1.7	9.4* ± 0.8	16.3* ± 0.9	9.0* ± 0.8	7.5* ± 1.3	6.5* ± 0.6	3.7* ± 0.9	4.0 ± 1.1

*significantly different from the negative control at $P < 0.05$

sources and nitrogen compounds as the roots grow (Morales et al. 1996).

Salt stress also reduced the plant growth, where the shoot length decreased up to 50% and root length up to 7% at 12.5 dS/m condition presumably due to the reduced availability of nutrients required for the growth (Table 1). Inhibition of plant growth by salinity was also explained as a result of toxic effects of the NaCl, and an ability of the root system to control entry of ions to the shoot and to slowing down water uptake of plants (Hajibagheri 1989, Lambers 2003). Both bacterial treatments used in the study increased shoot and/or root length compared to non-treated plants at each NaCl concentration tested (Table 1). 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. 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% (Table 1). High salinity (12.5 dS/m) reduced the plant growth promoting activity. Siddqui et al. (2003) also reported that high level of salinity may cause a dramatic fall in bacterial populations in the rhizosphere and roots and consequently may reduce the growth-promoting potential on plants. The root length was stimulated significantly by bacteria strains at 7.5, 10.0, and 12.5 dS/m up to 59% compared to control plants.

Plant growth promoting properties of strains were also tested in pot experiments using saline soil. *P. extremorientalis* TSAU20 and *P. chlororaphis* TSAU 13 stimulated root length (47%) and dry weight (50%) significantly in saline soil (Figure 2). The shoot growth of plants was not affected by bacterial inoculation, whereas only *P. chlororaphis* TSAU 13 significantly stimulated shoot dry weight (33%) of bean. It is thought that the depressive effect of salinity on plant growth could be related to a decline in endogenous levels of hormones (Zholkevich and Pustovoytova 1993, Jackson 1997, Debez et al. 2001, Sakhabutdinova et al. 2003). In

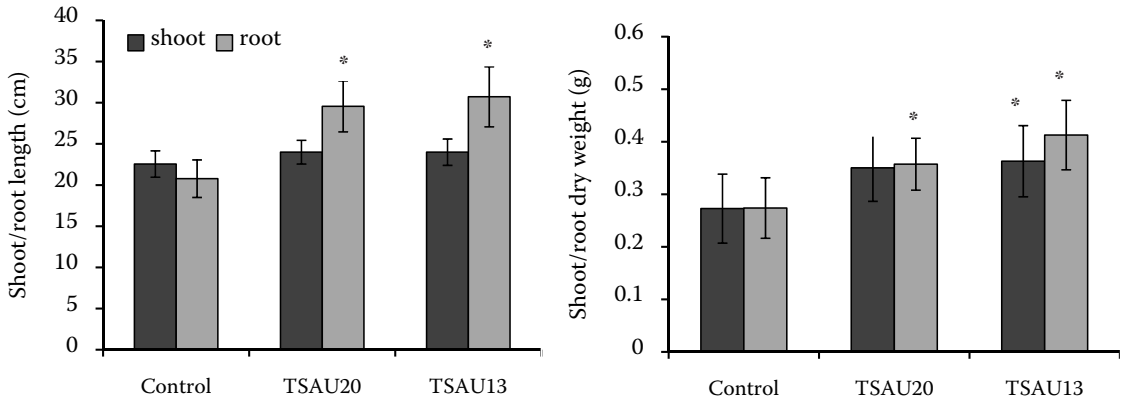

Figure 2. The effect of *P. extremorientalis* TSAU20 and *P. chlororaphis* TSAU13 strains on shoot, root length and dry matter of common bean in salinated soil (plants were grown in pots for four weeks). *significantly different from the negative control at $P < 0.05$

such conditions, application of additional natural phytohormones such as bacterial auxins in the root will affect positively seedling development in salinated conditions (Frankenberger and Arshad 1995, Afzal et al. 2005). Both tested *Pseudomonas* strains in this work are able to produce IAA in saline conditions (Egamberdieva and Kucharova 2009).

In conclusion, the results of this study indicated that *P. extremorientalis* TSAU20 and *P. chlororaphis* TSAU 13 have the ability to survive in ecologically stressed conditions, such as saline soils, and may positively affect on plant growth of bean. High salinity inhibited their colonisation in the rhizosphere of bean and thus their stimulatory effect on plants may also reduce.

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