

Biocontrol of Cotton Damping-off Caused by *Rhizoctonia solani* in Salinated Soil with Rhizosphere Bacteria

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ABSTRACT

Pre- or post-emergence cotton seedling damping-off caused by *Rhizoctonia solani* is a serious problem in many cotton growing countries. Fourteen selected bacterial strains were screened for their ability to control damping-off of cotton seedlings caused by the fungus *R. solani* in slightly saline (E_C 2.3 dS m⁻¹) and saline (E_C 7.1 dS m⁻¹) soils. Based on the results of preliminary screening, four efficient strains, *Pseudomonas alcaligenes* PsA15, *P. chlororaphis* TSAU13, *P. extremorientalis* TSAU20 and *Bacillus amyloliquefaciens* BcA12 were selected among 14 strains. When cotton was grown in both saline soils without addition of *R. solani* 45% and 56% of plants were diseased in slightly saline and saline soils, respectively. In the presence of the fungal pathogen the portion of plants, which had disease symptoms, increased from 67% in slightly saline to 73% in saline soils. All bacterial strains, with the exception of *B. amyloliquefaciens* BcA12, showed statistically significant ($P < 0.05$) disease control (up to 20%) over the *R. solani*-infected plants grown in slightly saline soil. The higher salinity reduced the capacity of bacteria to suppress damping-off of cotton caused by *R. solani*. Only strain *P. extremorientalis* TSAU20 performed well in both saline soils. When cotton seedlings were grown in both saline field soils without addition of the fungal pathogen, all four strains showed a significant ($P < 0.05$) stimulatory effect on cotton dry weight (up to 57%) in comparison to the non-inoculated plants. The mechanisms, by which bacteria may use their plant-beneficial properties are also discussed. Those results showed that *P. extremorientalis* TSAU20 has a great biotechnological potential to stimulate plant growth and protect cotton from damping-off disease under salinated soil condition.

Keywords: biological control, cotton, cotton damping-off, *Rhizoctonia solani*, plant growth promotion, rhizobacteria

INTRODUCTION

Salinity is a major concern for the irrigated agriculture in arid and semi-arid regions of the world (FAO 2002). Uzbekistan, located in Central Asia, is an example of a country, in which the soil salinity is a major concern. In 1990, about 48% of the total irrigated land area was suffering from the soil salinity, and by 2000, the area of salinized soil covered already 64% of the irrigated land area (Shirokova *et al.* 2000; Egamberdiyeva *et al.* 2007). Indiscriminate flood irrigation with poor drainage facilities, deep ploughing of marginal and naturally salinized soils, over-exploitation of the groundwater, recycling of drainage outflows for the irrigation, and monocropping of high water-use crops, such as cotton, are the major factors accelerating secondary soil salinization in Uzbekistan (Egamberdiyeva *et al.* 2007). Salt stress does not only cause a decreased metabolic activity of plant cells but it also increases susceptibility of plants towards various phytopathogens (Kurth *et al.* 1986; Kurth and Finkelstein 1995; Egamberdieva *et al.* 2010).

Cotton (*Gossypium hirsutum*) is the largest agricultural crop in Uzbekistan. Its annual production is about 3.5-4 million tons (2007), making Uzbekistan the world's sixth largest producer and second largest exporter of cotton (UzStat 2007). Damping-off and sore shin of cotton seedlings, killing seeds and seedlings before or after germination is a serious problem in Uzbekistan (Sheraliev *et al.* 2008). In Uzbekistan, estimated annual losses caused by both the soil salinity and fungus-caused root diseases are estimated to be 30%.

The widespread use of fungicides has not managed to control cotton seedlings against damping-off caused by *Rhizoctonia solani* and other fungal pathogens (Garber *et al.*

1979; Bell 1984). According to Pimentel and Levitan (1986), a very small percentage of applied fungicides (0.1%) used for the crop protection reaches the target pathogen. Moreover, the chemicals used to protect plants against fungal pathogens are harmful to the environment. The use of biological control agents, such as plant growth-promoting rhizobacteria (PGPR) to control plant diseases has been considered a viable alternative and environmentally friendly method (Cook and Baker 1983). PGPR are versatile micro-organisms and besides controlling plant diseases, they can increase plant growth, speed up seed germination, improve seedling emergence and protect plants from the deleterious effects of some environmental stresses, including drought and salt (Glick *et al.* 1998; Mayak *et al.* 2004; Lugtenberg and Kamilova 2004; Egamberdieva *et al.* 2008). Many studies have shown that microbial isolates can effectively control *R. solani*-induced damping off of cotton seedlings both in the laboratory and field conditions (Howell and Stipanovic 1979; Howell 1982; Hagedorn *et al.* 1989; Lewis and Papavizas 1991; Hagedorn *et al.* 1993; Zaki *et al.* 1998; Griffin *et al.* 2005; El-Sayed and Embaby 2007; Hassanin *et al.* 2007; Gasoni and Gurfinkel 2009).

However, most studies on biological control of cotton root disease have been conducted in non-saline agricultural soils and have not addressed the problems associated with the salinity. The objective of this study was to screen and select rhizosphere bacteria, which are able both to stimulate plant growth and to control *R. solani*-induced damping-off of cotton seedlings growing in saline soil.

Table 1 Soil chemical properties of slightly saline and saline field soils used in this study. under cotton monoculture. The soil samples were taken from a depth of 0-30 cm (Egamberdieva *et al.* 2010)

Soil salinity	K ⁺	Ca ⁺²	Mg ⁺²	CO ₃ ²⁻	N	P	C _T	Na ⁺	Cl ⁻
E _c (dS m ⁻¹)	(g kg ⁻¹)							(mg kg ⁻¹)	
Slightly saline (E _c 2.3 dS m ⁻¹)	5.92	53.4	23.7	16.1	1.06	1.30	24.8	600.2	52.0
Saline (E _c 7.1 dS m ⁻¹)	6.58	67.4	24.6	17.6	0.95	1.23	24.8	813.1	94.2

Table 2 List of microorganisms used in this study.

Bacterial strain	Species	Origin	Reference or source
TSAU22	<i>Pseudomonas aureantiaca</i>	Wheat	Egamberdieva and Kucharova 2009
TSAU13	<i>P. chlororaphis</i>	Wheat	Egamberdieva and Kucharova 2009
TSAU6	<i>P. extremorientalis</i>	Wheat	Egamberdieva and Kucharova 2009
TSAU20	<i>P. extremorientalis</i>	Wheat	Egamberdieva and Kucharova 2009
TSAU1	<i>P. putida</i>	Wheat	Egamberdieva and Kucharova 2009
ArG1	<i>Arthrobacter globiformis</i>	Melon	Egamberdieva and Höflich 2004
ArS50	<i>A. simplex</i>	Alfalfa	Egamberdieva and Höflich 2004
ArT16	<i>A. tumescens</i>	Alfalfa	Egamberdieva and Höflich 2004
BcA12	<i>Bacillus amyloliquefaciens</i>	Cotton	Egamberdieva and Höflich 2004
BcP26	<i>B. polymyxa</i>	Wheat	Egamberdieva and Höflich 2004
MbP18	<i>Mycobacterium phlei</i>	Wheat	Egamberdieva and Höflich 2004
PsA15	<i>P. alcaligenes</i>	Melon	Egamberdieva and Höflich 2004
PsD6	<i>P. denitrificans</i>	Alfalfa	Egamberdieva and Höflich 2004
PsM13	<i>P. mendocina</i>	Tomato	Egamberdieva and Höflich 2004
Fungi			
NUUF1	<i>Rhizoktonia solani</i> causes damping-off of cotton	Cotton	Culture Collection of National University of Uzbekistan (CCNUU)
NUUF10	<i>Fusarium oxysporum</i> causes root rot of cotton	Cotton	CCNUU, Uzbekistan
TSAUF1	<i>F. solani</i> causes tomato foot and root rot	Tomato	CCNUU, Uzbekistan

MATERIALS AND METHODS

Study site, soil sampling and characterisation of soil

Two soils, saline (EC) and slightly saline (EC) soils were sampled from an irrigated agricultural site located in Syrdarya Province (41° 00' N, 64° 00' E.) in north-eastern Uzbekistan. Both slightly saline soils and strong salinity are found at this site. According to the WRB-FAO (2006) classification, the soils of selected fields were identified as Calcisol (silt loam seirozem). The surface soil horizon was calcareous saline whereas the deeper soil horizons were only mildly alkaline (Egamberdieva *et al.* 2007). In these soils, cotton has been grown for the last 50 to 60 years under a continuous monoculture production system and under flood irrigation without proper drainage facilities but using a natural flow system. In general, high concentration of Ca²⁺, K⁺, and Na⁺ are associated with CO₃²⁻ and Cl⁻ ions, reflecting the dominance of carbonates and chlorides in saline soil. On average, the two kinds of soils was taken contained 42 ± 9 g of sand kg⁻¹, 708 ± 12 g of silt kg⁻¹, and 250 ± 13 g clay kg⁻¹ (Egamberdieva and Kucharova 2009). Chemical properties of the two soils are shown in **Table 1**. The climate of the sampling site is continental, with a annual average rainfall of 200 ± 36 mm, more than 90% of the total rain falling between October and May. The average monthly minimum air temperature is 0°C in January, and the maximum one is 37°C in July. During the year, the soil temperature ranges between -2 and +35°C. The average maximum relative humidity is slightly more than 80% in January and the minimum one is less than 45% in June. Under a dry continental climate, the combination of high temperature and low rainfall during the growth season makes irrigation essential for crop production.

Plant and microorganisms used

Seeds of the salt-tolerant cotton cultivar 'Namangan' were obtained from the Tashkent State University of Agriculture, Faculty of Plant Production. Bacterial strains used are listed in **Table 2**. All bacterial isolates were obtained from the Culture Collection of the National University of Uzbekistan (CCNUU). The strains *Pseudomonas aureantiaca* TSAU22, *P. chlororaphis* TSAU13, *P. extremorientalis* TSAU6, *P. extremorientalis* TSAU20, *P. putida* TSAU1 were previously isolated from the rhizosphere of wheat grown in salinated Uzbek soil (Egamberdieva 2009; Egamberdieva and Kucharova 2009) after using the enrichment procedure for

the isolation of enhanced root tip colonizers developed by Validov *et al.* (2006). Briefly, wheat roots were separated from soil (10 g each) and were shaken for 1.5 h in 100 ml of phosphate buffered saline (PBS; 20 mM sodium phosphate, 150 mM NaCl, pH 7.4) and were plated on TSA/20 (1/20 of Tryptic Soya Broth with 1.5% of agar (Difco Laboratories, Detroit, MI, USA) supplemented with 1.5% NaCl. The plates were incubated at 28°C. After 48 h plates were washed with PBS. Bacterial suspensions were adjusted to an optical density of 0.1 at 620 nm (OD₆₂₀ = 0.1) and were used for the inoculation of sterilized and germinated wheat seedlings. Germinated seeds were placed into the bacterial suspension with sterile forceps and shaken gently for approximately 10 min. Inoculated seeds were aseptically planted into the sand column of glass tubes of the gnotobiotic system, 5 mm below the sand surface. Six seedlings were inoculated with the bacterial suspension obtained from each sample. Subsequently, a high quality, sterilized sand (quartz sand, with particle size 0.1-0.3 mm (Wessem BV, The Netherlands) was treated with 10% Plant Nutrient Solution (PNS) (Kuiper *et al.* 2001). The seedlings were grown in a climate-controlled chamber (19°V, 16/8 h day/night cycles, 70% relative humidity) for 7 days, or until the root tips penetrated the gauze. To re-isolate bacteria from the rhizosphere, the complete sand column was carefully removed from the tube. Most of the still adhering rhizosphere sand was removed from the roots and a length of 1 cm root tip was cut off with caution, in order to prevent cross-contamination from upper root parts. Root tips were shaken in 1 ml of sterile PBS and the bacterial suspension hereby obtained was diluted with PBS and plated on TSA/20 amended with 1.5% NaCl. After 48 h of incubation at 28°C, bacteria were washed from the plates with PBS and bacterial suspensions originating from the same sample were pooled together. For the inoculation of seedlings, bacterial suspensions were adjusted as it was mentioned previously. The whole cycle, starting from the inoculation of seedlings with bacterial suspension and ending to the harvest of root tips was repeated three times. After the third enrichment cycle, root colonising *Pseudomonas* strains were chosen for further experiments (Egamberdieva 2009; Egamberdieva and Kucharova 2009).

Other strains used in this study, *Arthrobacter globiformis* ArG1, *A. simplex* ArS50; *A. tumescens* ArT16, *P. alcaligenes* PsA15, *P. denitrificans* PsD6, *P. mendocina* PsM13, *Bacillus amyloliquefaciens* BcA12, *B. polymyxa* BcP26, *Mycobacterium phlei* MbP18, were previously isolated from the rhizosphere of various plants grown in salinated soil using a conventional method (Egamberdieva and Höflich 2004). For isolation of bacteria from

the rhizosphere, 1 g of washed roots were macerated and shaken with 9 ml of sterile distilled water. The resulting suspensions were spread over the surface of a glycerol-peptone agar plate: peptone – 10 g, glycerol – 10 ml, NaCl – 5 g, KH_2PO_4 – 0.1 g, agar – 15 g/l sterile water. After incubation for 4 days at 28°C, the bacterial strains were isolated from the plate and identified. The fungal pathogens of cotton, *R. solani*, and *Fusarium oxysporum* and tomato root pathogen *F. solani* used in this study were also obtained from the CCNUU.

Pseudomonas strains were cultured for 2 days for on King's medium B (KB) (King *et al.* 1954) and *Arthrobacter*, *Bacillus*, and *Mycobacterium* strains were grown for 2 days on LC medium (containing per liter of distilled water: tryptone (Difco Laboratories) 10 g; Bacto-yeast extract (BD Biosciences) 5 g; NaCl (Sigma Chemical Co.) 10 g and agar (Difco Laboratories) 18 g) at 28°C under vigorous shaking for 2 days. The solid growth medium contained 1.8% agar (Difco Laboratories). The fungal cultures were maintained on potato dextrose agar (PDA) (Difco Laboratories) with regular sub-culturing at 1-month intervals.

Preparation of fungal inoculants and infecting soils with fungi

Six strains of the fungal pathogen *R. solani* Kuhn were used for the inoculation of soil by the method of Zheng and Sinclair (2000). Briefly, *R. solani* strains were cultured in potato-dextrose broth (PDB) (Difco Laboratories) in a shaker at 100 rpm. After growth for 5 days at 28°C under aeration (110 rpm), the mycelial mats were harvested by filtering culture suspensions through a single sheet of sterile filter paper (Whatman No. 1). Filter paper was dried, and the mycelial mat was weighed and homogenized in sterile distilled water. The soil was sterilized at 100°C for 24 h and mixed with the mycelial suspension using a soil mixer, to give an inoculum density of 100 mg of mycelia per kg of soil. To ensure growth of fungal strains, fungus-infected soils were kept moist for 1 week before cotton seeds were sown. To re-isolate fungal strains, a piece of root of a sick plant was removed after 5-6 weeks and plated on PDA medium in Petri dishes and incubated at 28°C under a 12-h photoperiod for 5 days.

This procedure was repeated twice with each fungal strain. All six fungal strains were morphologically indistinguishable and caused similar symptoms, typical of damping off. The procedure for infecting soil with *R. solani* was similar to that described above, except that soils used for biological control experiment were not sterilised.

Preparation of bacterial inoculants

Pseudomonas strains were grown overnight in KB broth King's B medium (KB), whereas other *Bacillus*, *Arthrobacter*, and *Mycobacterium* strains were grown in Luria-Bertani broth (LB) (Difco Laboratories). 1.0 ml of an overnight culture was pelleted (13,000 × g) and the supernatant was discarded. Cells were washed with 1 ml of PBS and re-suspended (Leeman *et al.* 1995). Cell suspensions were adjusted to $\text{OD}_{620} = 0.1$ for *Pseudomonas* and $\text{OD}_{620} = 0.3$ for other bacterial strains. Both OD_{620} values corresponded to a cell density of $10^7 - 10^8$ cells/ml and were used for inoculation of sterile cotton seedlings.

Experiments for controlling damping off of cotton seedlings by bacteria in field soil

Cotton seeds were surface-sterilised by immersion in 70% ethanol for 5 min and subsequently in 0.1% HgCl_2 for 1 min, rinsed several times with sterile water, and allowed to germinate for 3 days at room temperature. The sterility of seeds was tested on KB agar by incubation plates for 3 days at 28°C.

Seedlings were inoculated with bacteria by soaking surface-sterilised and germinated seeds in a bacterial suspension whereas uninoculated control seeds were soaked in sterile PBS buffer, both for 15 min.

One seedling was planted to a plastic pot (capacity 500 ml, diameter 9 cm), containing 300 g of field soil, at a depth of approximately 1.5 cm. The treatments were arranged in a randomized complete block design with 12 replications. The plants

were grown in pots under field conditions at 24-26°C during the day and between 12 and 14°C at night, and were watered when necessary. The number of diseased plants was determined when 40 to 60% of the fungus-infected plants but without controlling bacteria were diseased, usually 6 weeks after sowing. At harvest, plants were removed from the soil, roots were separated from shoots, were washed and examined for the symptoms of damping-off e.g. indicated by browning and lesions of root. Roots without any disease symptoms were classified as healthy.

Plant growth promotion by bacteria

The effect of the bacterial strains on the growth of cotton was measured in plastic pots containing 300 g of the weak saline and saline soil mentioned above. The inoculation treatments were set-up in a randomised design with 10 replications. After six weeks of growth the root and shoot length and dry weight of the whole plants was determined.

Colonization of cotton roots by bacterial strains

Spontaneous and stable rifampicin (rif) (200 µg/ml) (Sigma Aldrich, St Louis, MO, USA) resistant mutants of the wild type *Pseudomonas* strains were used for the colonization studies. Antibiotic-resistant mutants of *P. alcaligenes* PsA15, *P. extremorientalis* TSAU20 and *B. amyloliquefaciens* BcA12 were selected by adding rif (200 µg/ml) at the start of exponential growth in KB agar at 28°C and by repeating the process with successive subcultures until a spontaneous mutant was selected which was resistant to rif. Prior to the root colonisation experiment, the growth rate of the mutants on KB agar (with or without rif) was compared with the growth rate of the parental strains.

The cotton seeds were surface-sterilised, germinated and inoculated with rif-resistant mutant bacteria as described above. The inoculation treatments were set-up in a randomised design with 10 replications. Plants were grown in plastic pots (9 cm diameter; 12 cm deep) containing 300 g of both saline field soils under open, natural conditions in which the temperature ranged between 24 and 26°C during the day and between 10 and 12°C at night. After 6 weeks, plants were harvested and the adhering soil was removed from roots; 1 g of roots was shaken in 9 ml of sterile PBS. The resulting suspensions were evaluated for colony-forming units (cfu) according to the dilution-plate method on KB agar to which 200 µg/ml rif was added. After incubation for 2-3 days at 28°C, the bacterial colonies were enumerated and rif-resistant strains were identified for their colony characteristics (Höflich *et al.* 1995).

Antagonistic activity

The antagonistic activity of bacterial strains was tested *in vitro* against the plant pathogenic fungi, *F. oxysporum*, *F. solani* and *R. solani* using a plate bioassay supplemented with 1.5% NaCl. Fungal strains were grown on PDA agar plates at 28°C for 5 days. Disks containing a fresh culture of the fungus (approx. 5 mm in diameter) were cut out of the edge of the fungal growth and placed in the centre of a 9 cm diameter Petri dish. Bacteria grown on solid LC medium, a modification of Luria broth base Miller (Difco) (containing per liter of distilled water: tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g and agar-agar, 18 g) supplemented with NaCl to a final concentration of 1.5%, were streaked on the test plates perpendicular to the fungus at 2.5 cm from the disc. Plates were incubated at 28°C until the fungi had covered the control plates without bacteria (7 days). Antifungal activity was recorded as the width of the zone of growth inhibition between the fungus and the test bacterium.

1-Aminocyclopropane-1-carboxylic acid (ACC) deaminase activity

1-Amino cyclopropane-1-carboxylic acid (ACC) deaminase is an enzyme that degrades the precursor of the plant hormone ethylene, which is produced by the plant during environmental stress. Some PGPR bacteria are able to promote plant growth by lowering the endogenous ethylene synthesis in the roots through their ACC

deaminase activity (Glick *et al.* 1998). The method of analysing bacterial strains for their ability to use ACC as the sole nitrogen source is a trait that is a consequence of the presence of the activity of the enzyme, ACC deaminase. Synthetic Basal Medium (BM) (Lugtenberg *et al.* 1999) was used to check whether bacterial strains had ACC deaminase activity. BM medium was supplemented with 3.0 mM ACC (Sigma Chemical Co., St. Louis, MI, USA) (EC 4.1.99.4) and 3.0 mM (NH₄)₂SO₄ as the sole N source (positive control) or without an added N-source (negative control).

Production of indole-3-acetic acid

Indole-3-acetic acid (IAA) is the most abundant endogenous auxin produced by plants, and which regulates aspects such as stem elongation and root growth. The production of IAA by bacteria is as an important factor in direct plant-growth-promoting abilities of rhizosphere bacteria (Frankenberger and Arshad 1995; Woodward and Bartel 2005). The production of IAA was determined according to the method of Bano and Musarrat (2003). *Pseudomonas* strains were grown in KB medium and *Bacillus* strain were grown in Luria-Bertani (LB) medium. medium supplemented with 1-4% NaCl with and without tryptophan (500 µl/ml) and incubated at 28°C. After three days of cultivation, 2-ml aliquots of bacterial cultures were centrifuged at 13,000 × *g* for 10 min. One ml of supernatant was transferred to a fresh tube to which 100 µl of 10 mM orthophosphoric acid and 2 ml of reagent (1 ml of 0.5 M FeCl₃ in 50 ml of 35% HClO₄) were added. After 25 min, the absorbance of the developed pink color was measured at 530 nm using a Perkin-Elmer Lambda 3A spectrophotometer (Perkin-Elmer Corp., Norwalk, CT, USA). The concentration of IAA in culture was calculated from a calibration curve of pure IAA standard.

Production of cell wall degrading enzymes

The production of lytic enzymes such as lipase, cellulase, protease and glucanase by rhizosphere microorganisms can result in the direct suppression of plant pathogenic fungi (Lugtenberg *et al.* 2001). Lipase (EC 3.1.1.3) activity of bacterial strains was determined by the Tween lipase indicator assay according to Howe and Ward (1976). *Pseudomonas* strains were grown on KB agar and the *Bacillus* strain was grown on LB agar supplemented with 1.5% NaCl and containing 2% Tween-80 at 28°C After 5 days, the degradation of Tween by lipase was detected as a clear halo around the bacterial inoculum. Protease secretion was revealed by growing strains on agar plates described above but amended with skimmed milk to a final concentration of 5%. After 1-2 days, the halo appearing around bacterial colonies indicated the presence of extracellular protease (Brown and Foster 1970). Cellulase (EC 3.2.1.4) activity was detected using the substrate carboxymethylcellulose (Sigma Aldrich) in top-agar plates (Hankin and Anagnostakis 1977).

Statistical procedures

Data were tested for statistical significance using the analysis of variance package included in Microsoft Excel 98. Comparisons were done using Student's *t*-test. Mean comparisons were conducted using a least significant difference (LSD) test (*P* = 0.05).

RESULTS

The fourteen bacterial strains were evaluated for their ability to control damping-off of cotton seedlings caused by the fungus *R. solani* and to promote plant growth in slightly saline (E_C 2.3 dS m⁻¹) and saline (E_C 7.1 dS m⁻¹) soils (Table 1). The bacterial candidates were previously isolated from the rhizosphere of various plants.

Based on the results of a preliminary screening experiment, four efficient PGPR strains namely, *P. alcaligenes* PsA15, *P. chlororaphis* TSAU13, *P. extremorientalis* TSAU20, and *B. amyloliquefaciens* BcA12 showed statistically significant (*P* < 0.05) capacity to control damping-off in comparison to the *R. solani*-infected control plants without added bacteria. These four strains were selected for

Table 3 Controlling of damping-off of cotton by three *Pseudomonas* spp. and *Bacillus amyloliquefaciens* in slightly saline and saline soil.

Treatments	Slightly saline soil	Saline soil
	Diseased plants (% ± SD)	Diseased plants (% ± SD)
Positive control	45 ± 3.1	56 ± 7.2
Negative control	67 ± 8.8	73 ± 10.7
<i>P. alcaligenes</i> PsA15	30 ± 6.0*	56 ± 5.1
<i>P. chlororaphis</i> TSAU13	22 ± 6.3*	46 ± 6.0
<i>P. extremorientalis</i> TSAU 20	20 ± 3.6*	31 ± 10.8*
<i>B. amyloliquefaciens</i> BcA12	41 ± 9.4	55 ± 8.1

^aSurface-sterilized and Pre-germinated cotton seeds were inoculated with bacteria and plants were grown under open natural conditions in pots containing slightly saline (E_C value 2.3 dS m⁻¹) and saline field soil (E_C value 7.1 dS m⁻¹) infected with *R. solani*, positive control - without *R. solani*, negative control - *R. solani* added to the soil, * Significantly different from the negative control at *P* < 0.05

more detailed investigations.

The four strains were tested for their ability to suppress damping-off of cotton seedlings grown in slightly saline (E_C 2.3 dS m⁻¹) and saline field soils (E_C 7.1 dS m⁻¹) in order to determine how salinity affected the ability of bacterial strains to biologically control damping-off of cotton seedlings. When no *R. solani* was added to saline soils, the portion of diseased plants was 45% in slightly saline soil and 56% in saline soil. In the presence of the added pathogen, the portion of plants which showed disease symptoms increased to 67% in slightly saline soil and to 73% in saline soil (Table 1).

In slightly saline soil, all selected bacterial isolates, with the exception of *B. amyloliquefaciens* BcA12, showed the capacity to control damping-off in comparison to the *R. solani*-infected control plants without bacteria (Table 3). In saline soil, the performance by *R. solani* to suppress damping-off of cotton plants was reduced. However, *P. extremorientalis* strain TSAU20 performed well both in slightly saline and saline soils, reducing diseased plants by as much as 20%.

All four strains (*P. alcaligenes* PsA15, *P. chlororaphis* TSAU13, *P. extremorientalis* TSAU20, and *B. amyloliquefaciens* BcA12) significantly (*P* < 0.05) increased the length and dry weight of cotton roots and shoots in both saline soils relative to uninoculated control plants (Table 4). Nevertheless, the stimulation of growth of cotton plants by bacterial strains was higher in saline soil than in slightly saline soil. Again, the best performer was strain *P. extremorientalis* TSAU20, which increased the root and shoot length by 67% and their dry weight by 47% (Table 4).

P. alcaligenes PsA15, *P. chlororaphis* TSAU13, *P. extremorientalis* TSAU20 and *B. amyloliquefaciens* BcA12 were tested for their ability to colonize cotton roots when grown in slightly saline and in saline soil. This experiment was performed by using rif-resistant mutants of the four parental strains. All four bacterial strains were able to survive in the rhizosphere of two 2-month-old cotton plants (Table 5). The population of the rif-resistant mutants was log 4.01 CFU/g cotton roots in slightly saline soil and log 3.42 CFU/g cotton roots of cotton grown in saline soil. Among the strains tested, *P. extremorientalis* TSAU20 showed the highest ability to colonise the rhizosphere of cotton grown in slightly saline and saline soil.

The production of auxin (IAA) of the strains *P. alcaligenes* PsA15, *P. chlororaphis* TSAU13, *P. extremorientalis* TSAU20 and *B. amyloliquefaciens* BcA12 was tested by growing in KB medium under saline condition (1-4% NaCl). All strains tested appeared to produce IAA in media containing up to 3% NaCl (Table 5). The presence of tryptophan stimulated auxin production in *P. alcaligenes* PsA15 (up to 71%), *P. chlororaphis* TSAU13 (80%), *P. extremorientalis* TSAU20 (97%) and *B. amyloliquefaciens* BcA12 (56%).

When four strains were tested for their antagonistic activity towards *F. oxysporum*, *F. solani* and *R. solani*, only *P. chlororaphis* TSAU13 was antagonist against all tested pathogenic fungi (Table 5). This strain was also able to

Table 4 Promotion of plant growth of cotton by three *Pseudomonas* spp. and *Bacillus amyloliquefaciens* in slightly saline and saline field soil.

Treatments ^a	Slightly saline soil			Saline soil		
	Root length, cm	Shoot length, cm	Dry matter g/plant	Root length, cm	Shoot length, cm	Dry matter g/plant
Control	100 (7.4) ^b	100 (7.3) ^b	100 (0.760) ^c	100 (6.4) ^b	100 (5.9) ^b	100 (0.57) ^c
<i>P. alcaligenes</i> PsA15	136*	125*	119	155*	159*	157*
<i>P. chlororaphis</i> TSAU13	136*	135*	144*	124	149*	146*
<i>P. extremorientalis</i> TSAU20	153*	143*	144*	142*	167*	147*
<i>B. amyloliquefaciens</i> BcA12	146*	145*	128*	145*	161*	136*

^a Pre-germinated cotton seeds were coated with bacteria, and plants were grown under open natural conditions in pots containing slightly saline (EC value 2.3 dS m⁻¹) and saline field soil (EC value 7.1 dS m⁻¹), * Significantly different from the control at $P < 0.05$

Table 5 Traits possibly involved in biocontrol and/or plant growth-promoting activity of bacterial strains

Strains	HCN	ACC deaminase	Antagonistic activity against ^a			Production of exoenzymes ^a			Production of IAA ^b			Bacterial colonization (Log (CFU)/g root ⁻¹) ^c	
			<i>R. solani</i>	<i>R. solani</i>	<i>F. oxysporum</i>	Lipase	Protease	Cellulase	1 % NaCl	2 % NaCl	3 % NaCl	Slightly saline	Saline
<i>P. alcaligenes</i> PsA15	-	-	-	-	-	-	-	-	2.4	10.0	8.5	3.82 ± 0.12	3.71 ± 0.22
<i>P. chlororaphis</i> TSAU13	+	-	+	+	+	+	+	+	8.5	7.8	7.1	3.80 ± 0.13	3.61 ± 0.18
<i>P. extremorientalis</i> TSAU20	-	-	-	-	-	-	+	-	7.4	6.0	4.7	4.01 ± 0.18	3.76 ± 0.10
<i>B. amyloliquefaciens</i> BcA12	-	-	-	-	-	-	-	-	6.7	6.3	6.0	3.68 ± 0.10	3.42 ± 0.14

a) All tests conducted with addition of 1.5%NaCl

b) Auxin (IAA) level in µg/ml of after 5 days of incubation at 28°C. *Pseudomonas* strains were grown in KB medium and *Bacillus* strain were grown in Luria–Bertani (LB) medium supplemented with 1-4% NaCl.

c) The number of bacteria in the rhizosphere of cotton grown in slightly and saline field soil for 2 months

produce fungal cell wall-degrading enzymes, namely lipase, protease, cellulase and HCN. The remaining strains, i.e., *P. alcaligenes* PsA15, *P. extremorientalis* TSAU20 and *B. amyloliquefaciens* BcA12, did not show antagonistic activity. They were also negative for HCN production and lacked fungal cell wall-degrading enzyme activities except for *P. extremorientalis* strain TSAU20, which was able to produce cellulase (**Table 5**).

Only strain *P. alcaligenes* PsA15 could utilize ACC as the sole N source, indicating the presence of ACC deaminase, which plays a role in reducing the levels of the stress compound ethylene in plants. Other strains were negative the ACC deaminase activity (**Table 5**).

DISCUSSION

Biological control of damping-off of cotton by rhizosphere bacteria

This is the first report of the use of bacterial strains to control damping-off of cotton caused by *R. solani* in salinated soil. Four of the selected bacterial strains tested in this study significantly ($P < 0.05$) reduced the damping-off of cotton caused by *R. solani* in saline soil. The bacterial strains *P. alcaligenes* PsA15 and *B. amyloliquefaciens* BcA12, known to reduce *Verticillium* wilt of cotton (Egamberdiyeva 2003), and two other strains, *P. chlororaphis* TSAU13 and *P. extremorientalis* TSAU20, were isolated from the rhizosphere of wheat grown in salinated Uzbek soil after using an enrichment procedure for the isolation of enhanced root tip colonizers.

Biological control of damping-off of cotton caused by *R. solani* using PGPR was observed by other authors (Wather and Gindrat 1988; Alagesaboopathi 1994; Hassanin *et al.* 2007). The beneficial effects on cotton stand were also obtained with seed treatments of *Trichoderma viride*, *T. harzianum* (Sivan and Chet 1986; De Vay *et al.* 1987), *Gliocladium roseus* (Howell 1982), *Coniothyrium minitans* (El-Sayed and Embaby 2007) and *Burkholderia cepacia* (Heydari and Misagli 1998). It has been also reported that a number of microbial isolates have proven to be an effective biocontrol agent against damping-off of various crops. Mavrodi *et al.* (2012) studied the suppression of *Rhizoctonia* disease in wheat by *Pseudomonas* strains. Other authors observed that PGPR suppress symptoms of dam-

ping-off cucumber (*Cucumis sativus*) seedlings (Jung *et al.* 2003), potato (*Solanum tuberosum*) (Brewer and Larkin 2005) and tomato (*Lycopersicon esculentum*) (Yangui *et al.* 2008) caused by *R. solani*. Patil *et al.* (2011) found that antagonistic actinomycetes could biologically control *R. solani* in tomato under greenhouse conditions.

However, these strains were used as biological control agents under favourable soil conditions. The negative effect of abiotic factors on the biological control ability of bacterial strains was reviewed by Burpee (1990) and Kalifa *et al.* (2012). These factors include temperature, pH, moisture, soil type, inorganic and organic constituents, but the problem associated with salinity was not addressed. Rangarajan *et al.* (2003) studied antagonistic *Pseudomonas* strains that were screened for *in vitro* antibiosis against *Xanthomonas oryzae* pv. *oryzae* and *R. solani* – the bacterial leaf blight (BB) and sheath blight (ShB) pathogens of rice (*Oryza sativa*), respectively. They suppressed disease by 46 to 82% under saline soil condition. In our study we observed that higher saline soil may inhibit the ability of bacterial strains to control damping-off of cotton. Salt stress does not only cause a decline in the metabolic activity of plant cells but it also results in an increased susceptibility of plants towards phytopathogens (Kurth *et al.* 1986; Werner and Finkelstein 1995). This result is consistent with our finding that a higher percentage of diseased plants (56%) were observed in saline soil compared to plants grown in slightly saline soil (45%) and that were not infested with *R. solani*. All four selected strains, with the exception of *B. amyloliquefaciens* BcA12, showed significant ($P < 0.05$) repression of damping-off of cotton caused by *R. solani* in slightly saline soil relative to the *Fusarium*-infected control plants. In saline soil they were not effective, whereas only one strain, *P. extremorientalis* TSAU20, was able to control damping-off of cotton in saline soil.

Plant growth promotion by rhizosphere bacteria

In the absence of a pathogen, all four strains significantly ($P < 0.05$) increased root and shoot growth and dry weight of cotton in both slightly saline and saline soil. Plant growth was higher in saline soil than in uninoculated control plants. In previous studies, Rashid *et al.* (2000), Hafeez *et al.* (2002) and Anjum *et al.* (2007) observed that bacterial inoculum significantly increased seed cotton yield, and

plant height over their respective controls under normal soil conditions. Similar results were reported by Yue *et al.* (2007) for *Klebsiella oxytoca*, which was able to relieve salt stress and promote the growth of cotton seedlings in salinated soil. After treatment with bacterial strains, plant height and dry weight of cotton increased by 14.9 and 26.9%, respectively, compared to the control. In K-deficient soil, *Bacillus edaphicus* also stimulated growth of cotton and the N and P content of above-ground plant components (Sheng 2005).

According to Paula *et al.* (1992), the magnitude of a plant's response to any microbial inoculation can be greatly affected by the soil condition. The greatest benefits occurred when crops encountered stressful conditions (Lazarovits and Nowak 1997); for example, high pH makes nutrients less available to plants. Bacterial inoculation stimulated taproot growth and increased the number of lateral roots which may result in better absorption of water and nutrients from the soil (Hoflich and Kuhn 1996). Similar results we observed in our previous work (Egamberdiyeva 2007) in which in nutrient-poor arid soil, bacterial strains significantly ($P < 0.05$) enhanced early plant growth of maize, and the inoculation could compensate for nutrient deficiency and improve plants' development.

Root colonisation and survival of bacterial strains

Inoculation of plants with PGPR will not result in significant effects unless the environment supports growth and survival of the introduced microorganisms in a highly competitive environment (Wessendorf and Lingens 1989; Van Elsas and Heijnen 1990; Devliegher *et al.* 1995). Lugtenberg *et al.* (1999) and Thomashow and Weller (1995) reported that competitive root colonisation by rhizosphere bacteria is considered to be one of the mechanisms of biological control of root disease by PGPR. The successful colonization of the rhizosphere by introduced beneficial bacteria usually requires that the bacteria not only be well-adapted to the rhizosphere, but that it also have some selective advantage over numerous indigenous bacteria with the potential to colonize that rhizosphere (Kawaguchi *et al.* 2003). The survival and growth of the inoculated bacteria in soil largely depends on the availability of empty niches, and the capacity of competing with the better adapted native microflora (Rekha *et al.* 2007). In our study the rif-resistant mutants of four effective strains were able to colonize the rhizosphere of cotton due to their persistence in saline soil. The strain *P. extremorientalis* TSAU20, which was isolated as an enhanced wheat root colonizer (Egamberdiyeva and Kucharova 2009) showed higher colonisation rate in the rhizosphere of cotton, whereas *B. amyloliquefaciens* BcA12 was demonstrated to have lower colonization ability. This is consistent with data on root colonization by PGPR in the root systems of corn, tomato, broad bean, barley, and canola (Shaw *et al.* 1992; Ramos *et al.* 2000). *Pseudomonas fluorescens* CS85, which was previously isolated from the rhizosphere of cotton seedlings, acts as both a PGPR and as a biocontrol agent against cotton pathogens, including *R. solani*, *Fusarium oxysporum* f sp. *vasinfectum*, and *Verticillium dahlia* which colonize all surfaces of the young plant root zones, such as roots hairs and lateral roots during the period of plant growth (Wang *et al.* 2004).

Factors that can influence the survival of microorganisms in soil include soil-type, condition, pH, temperature, water potential as well as the presence of other soil organisms (Benizri *et al.* 2001). According to Diby *et al.* (2005), root colonisation potential of a strain was not hampered by higher salinity of soil. The strains used in this study were able to colonize the rhizosphere of cotton due to their competitiveness and persistence in saline soil.

Plant growth promotion and biological control traits

In the present work we tried to evaluate on which mecha-

nisms the observed biological control of damping-off of cotton and plant growth stimulation in saline soil can be based. Some mechanisms such as antagonism (Kuiper *et al.* 2001; Bloemberg and Lugtenberg 2004; Lugtenberg and Kamilova 2009), competition for nutrients and niches (Kamilova *et al.* 2005) and production of phytohormones (Frankenberger and Arshad 1995; Spaepen *et al.* 2009) were demonstrated.

Only strain *P. chlororaphis* TSAU13 was antagonistic towards pathogenic fungi *F. oxysporum*, *F. solani* and *R. solani* under laboratory conditions. Safiyazov *et al.* (1995) reported antagonistic activity of bacterial strains suppressing the development of cotton seedling disease. The potential root colonising bacteria *P. extremorientalis* strain TSAU20 did not show antagonistic activity against *F. oxysporum*, *F. solani* and *R. solani* even though this strain was able to control damping-off of cotton in both saline soils. In our previous work, this strain was also able to control cucumber foot and root rot through competition for nutrients and niches (Egamberdiyeva *et al.* 2010). Thus, our result suggests that competition for nutrients and niches might be an important factor in controlling plant diseases in saline soils. Wang *et al.* (2004) also observed that *P. fluorescens* CS85, which did not inhibit the growth of several fungal pathogens in the laboratory, was an effective biocontrol agent against cotton seedling diseases through competition for nutrient and niches.

All four bacterial strains tested were able to produce IAA under saline condition (Egamberdiyeva 2009). It is known that unfavourable environmental factors such as salinity and drought cause sharp changes in the balance of phytohormones associated with a decline in the level of growth-activating hormones such as IAA (Zholkevich and Pustovoytova 1993; Jackson 1997; Sakhabutdinova *et al.* 2003). In such a condition, the IAA-producing bacteria may supply additional phytohormones to the plant, thus may help stimulate root growth and reverse the growth-inhibiting effect of salt stress to a certain extent in both shoot and root growth (Kabar 1987; Afzal *et al.* 2005).

Strain *P. alcaligenes* PsA15 was able to utilize ACC as the sole nitrogen source. This suggests that strain synthesizes ACC deaminase, which can cleave the plant ethylene precursor ACC, and thereby lower the level of ethylene in a developing or stressed plant (Glick *et al.* 1998; Penrose *et al.* 2001; Glick 2005). Yue *et al.* (2007) demonstrated that *Klebsiella oxytoca* strain Rs-5, which had ACC deaminase activity, could relieve salt stress and promote cotton seedling growth more in saline than in non-saline soil.

The differential effects of salinity to bacterial control of cotton disease, as shown in this study, may explain importance of the selection of isolates whose biocontrol activity is not adversely affected by an increase in salinity. It is possible to recommend selected enhanced root-colonizing *P. extremorientalis* strain TSAU20 bacteria to control damping-off of cotton caused by *R. solani* and to stimulate plant growth under conditions of soil salinity.

ACKNOWLEDGEMENTS

This study was supported by the Academy of Sciences for the Developing World (TWAS 07-271RG/BIO/AS). The authors thank Leena Rasanen and Dr. Jaime A. Teixeira da Silva for significant improvements to grammar.

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