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Evaluation of Root Associated Bacteria for Control of Cotton Root Rot Caused by *Fusarium oxysporum* in Salinated Soils

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ABSTRACT: High salinity of soils in arid and semi arid regions results in desertification and decreased crop yield. In such conditions plants become more vulnerable to diseases caused by pathogenic fungi. The aim of the present work was to select enhanced root colonizing bacteria for their ability to promote plant growth and control root rot of cotton caused by *Fusarium oxysporum* in salinated soil. The best five enhanced cotton root tip colonizer bacteria were selected from the rhizosphere of cotton grown in saline soil and were identified by the 16S rRNA gene sequence as *Pseudomonas spp., Pseudomonas putida, P. chlororaphis, Pseudomonas mendocina and Pantoea agglomerans*. They showed ability to promote plant growth and to control root rot of cotton caused by *F. oxysporum*. Infestation of the soil with *F. oxysporum* resulted in an increase of diseased plants up to 60%. Selected bacterial strains, reduced this proportion to as low as 19 % and also stimulated cotton growth. These results are promising for the application of selected environmentally safe biological control agents in protecting cotton against root rot disease in saline agricultural soils.

Keywords: Biological control, cotton root rot, *Fusarium oxysporum*, plant growth promotion

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Tuzlu Topraklarda *Fusarium Oxysporum*'un Neden Olduğu Pamuk Kök Çürüklüğüne Karşı Kök İlişkili Bakterilerin Etkisinin Değerlendirilmesi

ÖZET: Kurak ve yarı kurak bölgelerde bulunan yüksek oranda tuz içeriğine sahip topraklar çoraklaşmaya yol açmakta ve böylece ürün veriminin azalmasına neden olmaktadır. Bu şartlar altında bitkiler patojenik mantarların neden olduğu hastalıklara karşı daha duyarlıdırlar. Bu araştırmanın amacı, bitkinin gelişme yeteneğini artıran kök koloni bakterisini seçmek ve tuzlu topraklardaki *Fusarium oxysporum*'un sebep olduğu pamuk kök çürüklüğü hastalığını kontrol altına almaktır. Araştırmada, gelişmiş pamuk kök kolonizer bakterisi tuzlu topraklarda büyüyen pamuk bitkisinin rizosferinden seçilmiş ve *Pseudomonas spp., Pseudomonas putida, P. chlororaphis, Pseudomonas mendocina* and *Pantoea agglomerans* gibi 16rRNA geni ile tanımlanmıştır. Araştırmada bitki büyüme süreci iyileştirilmiş ve *F. Oxysporum* 'un sebep olduğu pamuk kök çürüklüğü kontrol altına alınmıştır. *F. Oxysporum*'un bulaşmış olduğu topraklarda hastalık oranı % 60 oranında artmıştır. Seçilen bakteri suşları bu oranı % 19 oranında azaltmış ve aynı zamanda pamuğun gelişmesini artırmıştır. Bu sonuçlar tuzlu tarım topraklarında yetiştirilen pamuk bitkisinde görülen kök çürüklüğü hastalığına karşı biyolojik kontrol etmenlerinin uygulanmasının yararlı olacağını göstermektedir.

Anahtar Kelimeler: Biyolojik kontrol, pamuk kök çürüklüğü, *Fusarium oxysporum*, bitki gelişimi

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INTRODUCTION

Salinity is a major concern for irrigated agriculture in arid and semi-arid regions of the world (Vincent et al., 2006). As a result of soil salinization, plants are under saline or water unbalance stress and become more vulnerable to diseases caused by pathogenic fungi. Uzbekistan is one of the largest cotton producers in the world and pre- or post-emergence cotton seedling root rot is a serious problem which often results in a substantial stand loss (Abd-Elsalam et al., 2007). The pathogenic fungi *Fusarium oxysporum* are the most important soil borne fungi spreading in Uzbekistan and as well as in the world. The pathogens are affecting cotton seedling, causing damping-off and root rot disease of cotton (Chen et al., 1995; Bennett et al., 2008).

Many studies reported that a number of microbial isolates has proven to be an effective biocontrol agent against cotton root diseases caused *by Fusarium, Rhizoctonia, Verticillium and Pythium* (Hagedorn et al., 1993; Chen et al., 1995; Erdogan and Benlioglu, 2010). They are also able to 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 (Lugtenberg and Kamilova, 2004; Mayak et al., 2004). However, most of such beneficial effects of PGPR have been conducted in non-saline agricultural soils and do not address the problems associated with salinity. Present study deals with the selection of salt tolerant enhanced cotton root-tip-colonizing bacteria for their ability to control root rot of cotton seedlings caused by *F. oxysporum* and stimulate growth of cotton in salinated soils.

MATERIAL AND METHODS

Soil sampling and soil characterisation: Soils were sampled from irrigated agricultural site of Syrdarya province, (41000'N, 64000'E, north-east of Uzbekistan), which characterized by weak and strong salinity. According to the WRB-FAO (2006) classification, the soils of selected fields were identified as Calcisol (silt loam seirozem). The soils have been cropped to cotton monoculture for the last 50 to 60 years under flood irrigation without proper drainage facilities using natural flow system. On average, the soil contained 42+9 g sand kg-^{1}, 708+12 g silt kg^{-1}, and 250+13 g clay kg^{-1} (Egamberdieva et al., 2007). The organic matter content of the soil is 0.694 %; total C, 2.506%; total N, 0.091 %; Ca, 63.5 g kg^{-1}; Mg, 20.7 g kg^{-1}; K, 6.2 g kg^{-1}; P, 1.2 g kg^{-1}; Cl, 0.1 g kg^{-1}; Na, 0.7 g kg^{-1}, and the pH is 8.0 (Egamberdiyeva et al. 2007). The climate of the area is continental with a yearly average rainfall of 200+36 mm and more than 90 percent of the total rain falling between October to May. The average minimum monthly air temperature is 0ºC in January, the maximum of 37ºC in July, and the soil temperature ranges between -2 to 35ºC. The average highest relative humidity is slightly more than 80% in January and the minimum is less than 45% in June.

Isolation and enrichment of bacteria: To isolate representative bacterial strains three cotton plants (2 months old) grown in saline soil were sampled. Roots were separated from soil (10 g each) and were shaken for 1.5 h in 100 ml of phosphate buffered saline (PBS) and were plated on TSA/20 with 1.5% of agar supplemented with 1.5 % NaCl. The plates incubated at 28ºC and after 48 hours plates were washed with PBS. Bacterial suspensions were adjusted to an optical density of 0.1 at 620 nm (OD620=0.1) and used for inoculation of sterile cotton seedlings. The inoculated seeds were aseptically planted in the sand column of the gnotobiotic system glass tubes. The seedlings were grown in a climate-controlled chamber (19ºV, 16/8 h day/night cycles, 70% relative humidity) for 7 days. To re-isolate bacteria from the rhizosphere, a length of 1 cm root tip was cut and shaken in 1 ml sterile PBS. The bacterial suspension thus obtained was diluted with PBS and plated on TSA/20 amended with 1.5% NaCl. The whole cycle from seedling inoculation with bacterial suspension to the harvest of root tips was repeated twice for each of three samples. Twenty strains after third cycle of enrichment were chosen and further analyzed. The bacterial strains were stored at 4" C between the experiments. In addition purified strains were frozen in 30% glycerol at -80°C.

Antagonistic activity: The bacterial isolates were tested in vitro to select those with inhibitory effects against *Fusarium oxysporum* using a plate bioassay with PDA agar. Fungal strains grown in agar plate at 28ºC for 5 days and disks of fresh culture of the fungus (5 mm diameter) were cut out and placed in the centre of a 9 cm petri plate. Bacteria (grown in peptone agar plates) were streaked on the test plates perpendicular to the fungi. Plates were incubated at 30ºC for 7 days, until the fungi had grown over control plates without bacteria. Antifungal activity was recorded as the width of the zone of growth inhibition between the fungus and the test bacterium.

Biological control of root rot of cotton: The fungal pathogen *F.oxysporum* was obtained from Tashkent State University of Agriculture. Approximately

one third of a seven day old PDA Petri dish culture of *F. ozysporum* was homogenised and used to inoculate 200 ml of Chapek-Dox medium in a 1 L Erlenmeyer flask. After growth for 3 days at 28ºC under aeration (110 rpm), the fungal material was poured over sterile glass wool to remove the mycelium and the filtrate, containing the spores, was adjusted to a concentration of 5 x 10^6 spores/ml. For soil infestation, spores were mixed thoroughly with salinated soil to 3.0 x 10^7 spores/kg soil. The cotton seeds were sterilized by immersion in 70% ethanol for 5 minutes and subsequently in 0.1% HgC_{l2} for 1 min, washed several times with sterile water, and allowed to germinate for 4 days at room temperature. Subsequently, they were coated with bacteria by soaking them in a suspension of 1x108 CFU/ml bacteria in sterile PBS buffer whereas control seeds were soaked in sterile PBS buffer, both for 15 minutes.

One seed was sown per plastic pot (9 cm diameter; 15 cm deep), each containing 300 g of saline soil, at a depth of approximately 1.5 cm. Each treatment contained four groups of twelve plants. The plants were grown under open natural conditions at 21-24ºC and were watered when necessary. The number of diseased plants was determined when 50 to 70% of the plants in the control without bacteria were diseased, usually four weeks after sowing. Plants were removed from the soil, washed, and the plant roots were examined for root rot symptoms as indicated by browning and lesions. Roots without any disease symptoms were classified as healthy.

Plant growth promotion: The effect of the bacterial strains on seedling and plant growth was measured in petri plates and also plastic pots containing 300 g of the salinated soil mentioned above. The inoculation treatments were set-up in a randomized design with 10 replications. The cotton plants were grown under open natural conditions and after four weeks of growth the dry weight of the whole plants was determined. The best five bacterial strains were identified.

Identification, DNA isolation, PCR amplification: Bacterial strains were grown at 28ºC under vigorous aeration on Luria-Bertani medium amended with 10mM MgSO4. Total DNA from bacterial strains was isolated using the technique of Souza et al., (2003). An approximately 1,440 bp DNA fragment encoding part of the 16S rDNA sequence was amplified with primers 27fm (5'-AGAGTTTGATCMTGGCTCAG-3') and r1522 (5'-AAGGAGGTGATCCAGCCGCA-3') using a PCR (polymerase chain reaction). Total DNA of the strains was used as a source of template DNA. The nucleotide sequence of the PCR fragments was determined by ServiceXS (Leiden, The Netherlands). Sequences were assembled with DNAMAN Software. Homology searches with 16S rDNA sequences in GeneBank were performed with the BLASTN program (version 2.2.1) (Altschul et al., 1997).

Statistical analysis: 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 AND DISCUSSION

Successful soil inoculation of microorganisms requires survival and/or growth of the introduced strain in a highly competitive environment (Van Elsas and Heijnen, 1990; Lugtenber et al., 1999). After the third cycle of enrichment, twenty best cotton root tip colonisers were randomly chosen. They were evaluated for their ability to promote plant growth and control root rot of cotton caused by *F. oxysporum* in salinated soil. Twelve strains were antagonistic towards the phytopathogenic fungi *F.oxysporum*. Plant growth promoting properties of strains were tested in pot experiments using saline soil. Ten strains were able to stimulate root, shoot length and dry weigh of cotton in a statistically significant way in comparison with the untreated control and they were taken for further study (Table 1). The best performer was strain RC9, which increased the dry weight by 65%.

It is suggested that root colonizing 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 (Frankenberger and Arshad, 1995). Ten selected bacterial strains were evaluated for their ability to control cotton root rot caused by *F. oxysporum* in salinated soil. Thirty eight percent of the cotton plants which had grown in soil to which no Fusarium spores had been added were diseased, whereas in the presence of the pathogenic fungus 60% of the plants had disease symptoms (Figure). Five out of ten strains namely RC2, RC4, RC5, RC8 and RC13 showed disease reduction in comparison to the Fusarium-infected control plants (Figure 1). Among them only three bacterial strains RC4, RC8 and RC13 were antagonistic against *F.oxysporum*. Since efficient root colonization is the delivery system for antibiotics around the root in case strains control a disease through antibiosis, these strains are likely to

Table 1. Overview of antagonistic activity and plant growth promotion properties of the twenty newly isolated enhanced cotton root tip colonizers

Bacteria	Shoot length, cm	Root length, cm	Dry weight, g/plant	*F. oxysporum*
Control	9.5	7.0	0.60	-
RC1	10.6	11.2*	0.77	-
RC2	12.2*	15.7*	0.86*	-
RC3	9.7	7.7	0.61	+
RC4	9.0	14.0*	0.88*	+
RC5	10.7	13.0*	0.95*	-
RC6	9.4	11.0*	0.85*	+
RC7	7.5	12.5*	0.96*	+
RC8	10.7	12.7*	0.89*	+
RC9	11.5*	11.0*	0.99*	-
RC10	10.5	6.7	0.76	-
RC11	9.6	6.1	0.66	-
RC12	9.8	9.5	0.75	+
RC13	11.5*	9.7	0.96*	+
RC14	9.6	6.7	0.76	+
RC15	10.7	6.5	0.70	+
RC16	10.0	7.5	0.67	+
RC17	10.2	6.7	0.74	-
RC18	12.4*	8.6	0.90*	-
RC19	10.5	9.7	0.79	+
RC20	8.2	10.8*	0.85*	+

[a]28 days old plants. Significantly different $\alpha = 0.05$

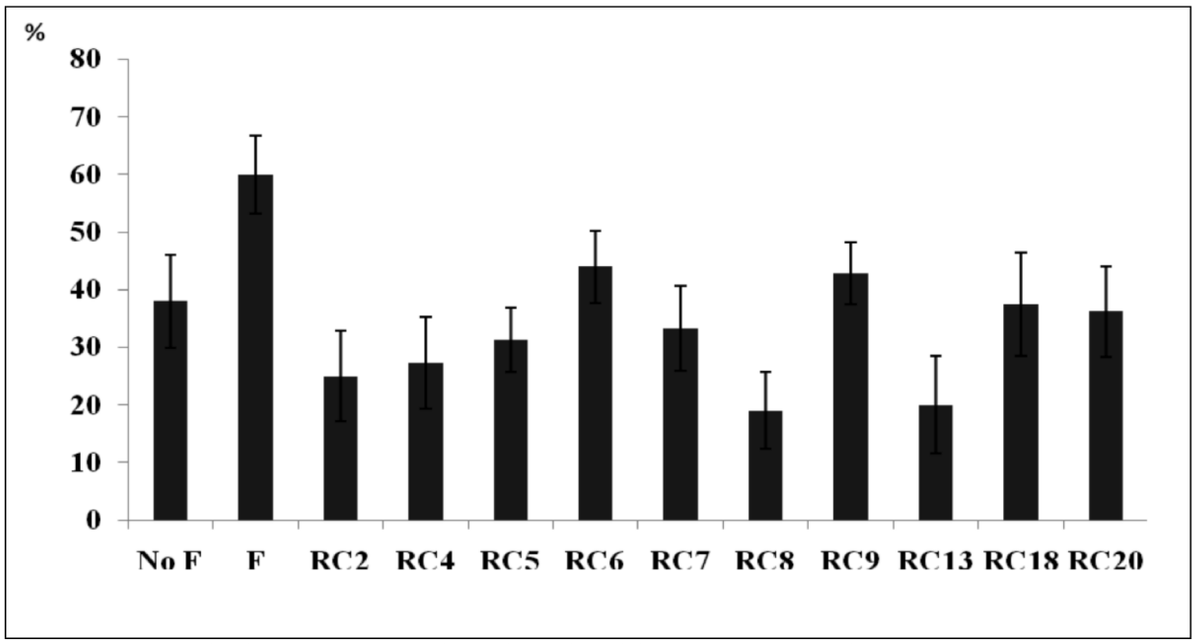

Figure 1. Control of cotton root rot in salinated soil by selected bacterial isolates (Plants were grown under open natural conditions in pots containing salinated soil infested with *F. oxysporum* spores (3.0×10^7 spores per kg), except for the positive control in which no spores were added to the soil).

Table 2. Identification of putative plant-growth stimulating bacterial strains isolated from the rhizosphere of cotton

Isolate #	Identified as	Similarity (%)	Accession #	Organism, strain
RC2	*Pseudomonas sp.*	99	AY040872	*Pseudomonas* sp. WBC-3
R4	*P. putida.*	99	DQ133506	*P. putida* GM6
R5	*P. chlororaphis*	99	AM158279	*P. chlororaphis* CDAE5
R8	*P. mendocina*	99	DQ178223	*P. mendocina* LGM1223T
E13	*Pantoae agglomerans*	99	AY616179	*P. agglomerans* TWC95.VI.3

be also good root colonizers (Lugtenberg and Kamilova, 2004). So, in addition to a probable role for antibiosis, competition for nutrients and niches is likely to also play a role in the beneficial effects of these bacterial strains.

Molecular characterisation based on 16S rDNA homology of a partial sequence (1,440 bp) with the sequences in GeneBank Nucleotide sequencing of amplified 16S rDNA fragments obtained after colony PCR, and comparative analysis using DNA databases, revealed that the isolated strains belong to the genera Pseudomonas. The 16S rDNA sequences of the isolates show very high homology with the strains listed in Table 2.

We conclude that screening and application of the enhanced potential root colonizing bacterial strains *Pseudomonas sp., P. putida, P. chlororaphis, P. mendocina* is essential for developing sound strategies to manage the root rot of cotton and stimulate plant growth in salinated soils.

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