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Biological control of the potato dry rot caused by *Fusarium* species using PGPR strains

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ABSTRACT

In this study, a total of 17 Plant Growth Promoting Rhizobacteria (PGPR) strains, consisting of eight different species (*Bacillus subtilis*, *Bacillus pumilus*, *Burkholderia cepacia*, *Pseudomonas putida*, *Bacillus amyloliquefaciens*, *Bacillus atrophaeus*, *Bacillus macerans* and *Flavobacter balastinium*), were tested for antifungal activity in *in vitro* (on Petri plate) and *in vivo* (on potato tuber) conditions against *Fusarium sambucinum*, *Fusarium oxysporum* and *Fusarium culmorum* cause of dry rot disease of potato. All PGPR strains had inhibitory effects on the development of at least one or more fungal species on Petri plates. The strongest antagonism was observed in *B. cepacia* strain OSU-7 with inhibition zones ranging from 35.33 to 47.37 mm. All PGPR strains were also tested on tubers of two potato cultivars 'Agria' and 'Granola' under storage conditions. Only *B. cepacia* strain OSU-7 had significant effects on controlling potato dry rot caused by three different fungi species on the two potato cultivars. There were no significant differences in rot diameters among the treatments in comparison to the negative control (with water). This is the first study showing that *B. cepacia* has great potential to be used as effective biocontrol agent of *Fusarium* dry rot of potatoes (*F. oxysporum* and *F. culmorum*) under storage conditions.

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1. Introduction

Potato (*Solanum tuberosum* L.) and its products are known to be the most important source of food for human beings. The world production of potato is 321 million tonnes, of which 4.3 million tonnes are produced in Turkey (Anonymous, 2007). Fungi and bacteria cause diseases on potato tubers under field and storage conditions (Boyd, 1972). Postharvest diseases in potatoes due to fungal pathogens result in significant economic losses in the quality and quantity of the potato yield during storage, transport and marketing process in the World (Eken et al., 2000).

Fusarium dry rot is caused by several species including *Fusarium sambucinum* Fuckel, *Fusarium culmorum* (W.G. Smith) Sacc. and *Fusarium oxysporum* Schlecht, under field and storage conditions (Boyd, 1972; Schisler and Slininger, 1994). Crop losses attributed to the dry rot have been estimated to average 6%, with losses up to 25% reported (Chelkowski, 1989). In addition to destroying tuber tissues, *Fusarium* spp. can produce toxins that have been implicated in mycotoxicoses of humans and animals (Senter et al., 1991; Schisler et al., 1997). *F. sambucinum*, *Fusarium solani* (Mart.) Sacc., *F. culmorum* and *F. oxysporum* are reported as common causes of dry rot of potatoes in Turkey, resulting in partial or almost complete loss of stored commercial potatoes varieties (cvs. Agria, Granola and Morfona) (Eken et al., 2000).

The potato dry rot can be controlled by a combination of storage technologies, physical methods and chemical applications (Eckert and Ogawa, 1988; Ranganna et al., 1997). To prevent *Fusarium* spoilage it is a common practice of different countries to dip harvested potatoes in fungicide solutions prior to storage (Kawchuck et al., 1994). An effective control of Fusarium dry rot has been obtained with the fungicide fenpiclonil and the mixture of thiabendazole and imizalil (Carnegie et al., 1998). The high costs of chemical control, failures in chemical control due to resistance development, and lack of other effective control measures have, therefore, generated considerable interest in biological control that offers an effective and environmentally friendly alternative to the use of synthetic pesticides.

In recent years, intense research efforts have been devoted to the development of antagonistic microorganisms to control postharvest disease. So far, biological controls of dry and soft rots with different biocontrol agents such as fungi, bacteria, and yeasts have been reported as effective under experimental conditions (Bartz and Eckert, 1987; Wilson and Wisniewski, 1992; Schisler, 1994; Schisler et al., 1997; Kotan et al., 1999, 2002; Sadfi et al., 2002). Several microbial antagonists have been identified and shown to reduce *Fusarium* dry rot on potatoes (Schisler et al., 1997; Sadfi et al., 2001, 2002).

In some of our previous studies, a few Plant Growth Promoting Rhizobacteria (PGPR) strains have been shown to be effective

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biocontrol agents of a number of plant pathogenic bacteria and fungi in *in vitro* and *in vivo* conditions, including *Fusarium* spp. (Kotan et al., 1999, 2002). However, there have been no attempts to determine the effect of the PGPR strains on *Fusarium* pathogens causing dry rot under storage conditions. Therefore, the objective of this study was to evaluate the biocontrol effect of 17 previously selected PGPR strains on potato dry rot causing fungi species including *F. sambucinum*, *F. oxysporum* and *F. culmorum* under *in vitro* and *in vivo* (storage) conditions.

2. Materials and methods

2.1. PGPR strains and pathogenic fungal isolates

All PGPR strains (including 1 *Bacillus amyloliquefaciens* (M-47), 1 *Bacillus atrophaeus* (M-35), 1 *Bacillus macerans* (strain M-32), 2 *Bacillus pumilus* (strain M-13 and M-38), 7 *Bacillus subtilis* (strain M-16, M-19, M-34, M-48, M-58, BA-140 and OSU-142), 2 *Burkholderia cepacia* (strain OSU-7 and PR-203), 1 *Flavobacter balastinium* (strain FB-299) and 2 *Pseudomonas putida* (strain OSU-8 and Pp12633)) and fungal isolates (*F. culmorum*, *F. oxysporum* and *F. sambucinum*) were obtained from the culture collection unit in the Department of Plant Protection, Faculty of Agriculture at Atatürk University. The identity of all bacterial strains used in this study was confirmed according to fatty acid methyl esters (FAME) analysis by using Sherlock Microbial Identification System (Microbial ID, Newark, DE, USA) (Sahin, 1997). The bacterial strains were stored at −80 °C in 15% glycerol and Luria Broth (LB), and the pathogenic fungi maintained on potato dextrose agar (PDA) at 4 °C for using further studies.

2.2. Source of potato tubers

The potato cultivars Agria and Granola used in the experiments were obtained from The Department of Crop Science (Atatürk University, Faculty of Agriculture, TR-25240 Erzurum, Turkey). Potato tubers were selected free of wounds and rots and as homogeneous as possible in size, and were stored at 0–1 °C until use. The tubers ranged in size between 80 and 100 mm in diameter.

2.3. Pathogenicity test

The pathogenicity test was performed both for PGPR strains and fungal isolates under storage conditions. PGPR strains were grown on Nutrient Agar (NA) for 27 °C overnight and then bacterial suspension for each one of the PGPR strains was prepared in sterile distilled (sdH_2O) water to a final concentration of 10^8 CFU/ml and then injected into the rind of potato tubers cv. Agria. Absorbance of the bacterial suspension was measured by spectrophotometrically at 600 nm.

For the pathogenicity of fungi isolates, conidia of each isolate grown on Potato Dextrose Agar (PDA) for 7 days were harvested and suspended in sdH_2O at the concentration of 10^6 spores/ml and then injected into the rind of potato tubers. The concentration of spores was determined using a hemacytometer. The inoculated potato tubers (six tubers) for each PGPR strain and fungal isolate were incubated in storage conditions (at 5 °C, in 70% relative humidity, in the dark) for two weeks. After the incubation period, inoculated tubers were evaluated for rot development. In both pathogenicity tests, all treatments consisted of three replicates of six tubers in each replicate, and experiments were repeated two times.

2.4. In vitro assays on Petri plates

The potential PGPR strains were grown on NA at 25 °C for 24 h to obtain fresh culture for Petri plate assay. The fungal pathogenic isolates were also cultivated on PDA at 25 °C for 7–10 days until sporulation. PGPR strains were streaked as single line onto PDA medium in Petri dish (9-cm diameter) and the two adjacent sides of the Petri plates were inoculated with a 5-mm disc of the pathogen fungi simultaneously. These plates were incubated at 25 °C for one week. After an incubation of 7 days, antagonistic activities were evaluated by measuring (in mm) the zones between pathogens and tested bacteria. All treatments consisted of three Petri plates, and experiments were repeated three times. Petri plates with disk of fungi in the absence of PGPR strains were used as negative control. The inhibition zone between fungal colonies and PGPR strains were evaluated when mycelium colonies merged on negative control plates, which was after seven days. The data were collected as inhibition zone values as mm in each replication, and evaluated by one-way analysis of variance (ANOVA) by using SPSS 10.0 for Windows.

2.5. In vivo assays in storage condition

Based on the antifungal activity on Petri plates, selected PGPR were selected as potential biocontrol agents. Cell suspensions of PGPR were tested for activity against pathogenic fungi on potato tubers under storage conditions. Bacterial strains were grown on NA for 24–48 h at 27 °C and suspended in sdH_2O. Concentration of bacterial cell suspensions was adjusted to 10^8 CFU/ml. Conidia of the pathogenic fungal isolates were obtained from the pure cultures. Conidia were collected from 7-days-old PDA agar cultures incubated at 25 °C under a photoperiod of 12-h light and 12-h dark. Inoculum was prepared by scraping spore material from the surfaces of the colonies with a wet cotton swab and resuspending the material in distilled water containing 0.5% Tween 80 (Merck, Germany). The conidia of each isolates were suspended in sdH_2O at the concentration of 10^6 spores/ml.

The storage assays were performed at 5 °C and at 70% humidity. The selected potato tubers of Agria and Granola were washed in running tap water, dipped in ethanol (70%) for 2 min, rinsed twice with double distilled sterile water (10 min each) and air-dried. Cell suspensions of bacteria individually were assayed against the pathogens by co-inoculating (25 μl) into wounds (6 × 6 mm) in two cultivars potatoes. Sterile water was used as negative control and fungal suspensions were used as positive control. After 6 weeks incubation at 5 °C under storage condition, diameters of decayed area on potato tubers were measured. All treatments consisted of three replicates of six tubers in each replicate, and experiments were repeated three times. The data were obtained after 42 days and collected as average sizes of lesions in each replication. The data were evaluated by one-way analysis of variance (ANOVA) by using SPSS 10.0 for Windows.

2.6. Statistical analyses

An analysis of variance (ANOVA) and Duncan's multiple range test (at $p = 0.05$) were performed to analyze statistical differences and to discriminate between means.

3. Results

3.1. Pathogenicity tests

In the pathogenicity tests, all PGPR strains were negative pathogenicity on potato tubers, whereas all fungal isolates were able to cause decay (rot) with 10–15 mm diameter on potato tubers after 7 to 10 days (Table 1).

3.2. In vitro assays

The effects of PGPR strains on fungi isolates tested in *in vitro* and *in vivo* were given in Table 2. In the Petri plate assays results PGPR

Table 1
Pathogenicity test results for PGPR strains and fungal isolates on potato tubers of Agria.

PGPR bacteria	Strains	Pat.	Pathogen fungi	Isolates	Pat.
Bacillus amyloliquefaciens	M-47	–	*Fusarium sambucinum*	PF-97	+
Bacillus atrophaeus	M-35	–	*Fusarium oxysporum*	Foxy 1	+
Bacillus macerans	M-32	–	*Fusarium culmorum*	Fcul 1	+
Bacillus pumilus	M-13	–			
Bacillus pumilus	M-38	–			
Bacillus subtilis	BA-140	–			
Bacillus subtilis	OSU-142	–			
Bacillus subtilis	M-16	–			
Bacillus subtilis	M-19	–			
Bacillus subtilis	M-34	–			
Bacillus subtilis	M-48	–			
Bacillus subtilis	M-58	–			
Burkholderia cepacia	OSU-7	–			
Burkholderia cepacia	PR-203	–			
Flavobacter balastinium	FB-299	–			
Pseudomonas putida	OSU-8	–			
Pseudomonas putida	Pp12633	–			

Pat: Results of pathogenicity tests on one potato cultivar 'Agria' following injections of rinds of six tubers with spores of each Fusarium species (106 spores/ml) and 10^8 cells/ml of each bacterium and incubation at 5 °C for two weeks. The experiment was conducted two times.

strains had inhibitory effects on the development of at least one fungal species. Ten of the 17 PGPR strains (*B. pumilus* M-38; *B. atrophaeus* M-35; *B. subtilis* M-19, M-34, M-48, M-58, BA-140 and OSU-142; *B. cepacia* OSU-7 and *P. putida* OSU-8) evaluated in Petri plate assay were antagonistic against all three fungi species (*F. culmorum*, *F. oxysporum* and *F. sambucinum*). *B. cepacia* strain OSU-7 was the stronger antagonist with inhibition zone values ranging from 35 to 43 mm (Table 2). The remaining seven PGPR strains (*B. amyloliquefaciens* M-47; *B. atrophaeus* M-35; *B. macerans* M-32; *B. pumilus* M-13, *B. subtilis* M-16; *B. cepacia* PR-203; *F. balastinium* FB-299 and *P. putida* Pp12633) did not show significant inhibitory effect against pathogenic fungi. For this reason, they were not tested for *in vivo* assays.

3.3. In vivo assays

Effects of antagonistic PGPR strains determined by Petri plate assay were tested on tubers of two potato cultivars in storage conditions were given in Table 2. Only *B. cepacia* strain OSU-7 had significant effect on controlling potato dry rot caused by three different fungi species on two potato cultivars. There was a significant difference in rot diameters of this treatment to the positive control treated with all three *Fusarium* isolates, but not negative control treated with water. *P. putida* OSU-8 and *B. subtilis* M-48 were effective in controlling only *F. culmorum* on potato cultivar Granola, but not Agria (Table 2). Some strains such as *B. subtilis* M-16, BA-140 and OSU-142 also reduced rot diameters on potato tubers, but there was a significant difference in rot diameters among those treatments in comparison to the negative control. No consistent of between *in vitro* an *in vivo* test results was observed for the remaining 15 PGPR strains. Some reduced disease severity of the treatments was not important in comparison to the negative control. Positive control applications inoculated with *F. oxysporum*, *F. culmorum* and *F. sambucinum* isolates showed decay with 56, 47 and 25 mm diameter on potato cultivar Agria, 36, 49 and 28 mm diameter on potato cultivar Granola, respectively (Table 2).

4. Discussion

B. cepacia strain OSU-7 among 17 PGPR strains tested had significant effects on controlling of all three fungi species (*F. sambucinum*, *F. oxysporum* and *F. culmorum*) causing potato dry rot in

Table 2
Results of *in vitro* (on Petri plates) and *in vivo* (on potato tubers) tests of biological control of three Fusarium species by 17 selected bacterial strains from eight different species.

Bacterial application*	*Fusarium oxysporum*			*Fusarium culmorum*			*Fusarium sambucinum*		
	Zone** (mm)	Decay diameter (mm)***		Zone** (mm)	Decay diameter (mm)***		Zone** (mm)	Decay diameter (mm)***	
		Agria	Granola		Agria	Granola		Agria	Granola
Negative control		6.00a	6.00a		6.00a	6.00a		6.00a	6.00a
OSU-7	45.37	6.25a	6.00a	41.62	6.00a	6.00a	35.33	6.50a	12.25a
BA-140	37.12	19.75b	21.37b	35.87	50.50d	40.62b–e	29.00	28.62b–f	31.00b
M-58	36.50	35.75c–f	Nd	34.50	36.50b–d	38.12bc	32.00	33.50ef	32.00b
M-47	36.25	34.37c–e	Nd	35.62	49.37d	49.87f	0.00	Nt	Nt
OSU-142	34.75	29.62bc	21.75b	31.75	49.75d	46.87ef	27.66	32.25d–f	26.25b
M-16	21.00	28.62bc	Nd	29.62	21.37b	45.12d–f	0.00	Nt	Nt
M-34	19.62	40.37c–g	Nd	29.00	45.37cd	39.50b–d	27.33	35.25f	28.62b
M-38	19.25	33.87cd	Nd	19.62	29.87bc	42.25c–e	19.66	24.62bc	27.25b
Pp12633	13.87	29.50bc	27.87c	0.00	Nt	Nt	0.00	Nt	Nt
M-19	15.37	49.12f–h	Nd	19.00	46.50cd	34.00b	26.33	33.50ef	32.37b
M-13	15.62	46.37d–h	Nd	18.75	32.37b–d	39.00b–d	0.00	Nt	Nt
M-35	19.62	47.50e–h	Nd	23.12	35.37b–d	45.12d–f	23.00	22.12b	32.75b
OSU-8	23.75	48.87f–h	Nd	11.25	48.12d	6.25a	23.00	27.12b–e	28.62b
M-48	24.50	53.37gh	36.37d	19.25	35.87b–d	9.12a	24.33	25.75b–d	27.25b
M-32	0.00	Nt	Nt	15.00	26.62b	35.00b	19.50	31.50c–f	28.62b
PR-203	0.00	Nt	Nt	38.00	48.00d	44.50c–f	0.00	Nt	Nt
FB-299	3.12	Nt	Nt	6.00	44.87cd	39.12b–d	3.33	Nt	Nt
Positive control		56.12h	36.37d		47.50cd	49.87f		25.25b–d	28.75b

Nt: The bacterial strains were not tested, since they had weak inhibition zone on Petri plates; Nd: Decay diameter was not determined, since the potato tubers decayed completely.

* Sterile water was used as negative control and only fungal suspensions were used as positive control. M-47: *B. amyloliquefaciens*; M-35: *B. atrophaeus*; M-32: *B. macerans*; M-13 and M-38: *B. pumilus*; M-16, M-19, M-34, M-48, M-58, BA-140, BA-142: *B. subtilis*; OSU-7 and PR-203: *B. cepacia*; FB-299: *F. balastinium*; OSU-8 and Pp12633: *P. putida*.

** The mean zone of inhibition measured as mm between pathogens and tested bacteria onto PDA medium after an incubation of seven days at 25 °C. The data are presented as the means of three experiments.

*** The diameters of decayed area on potato tubers (Agria and Granola) were measured after six weeks incubation at 5 °C under storage condition. Data are presented as the means of three experiments in which rinds were inoculated with spores of each fungus and each strain. All Numbers within a column followed by the same letters are not significantly different according to one-way analysis of variance (ANOVA) by using SPSS at $p = 0.05$). All treatments consisted of three replicates of six tubers in each replicate, and experiments were repeated three times.

storage. This strain had its inhibitory effects against some plant pathogenic fungi and bacteria in previous studies (Miller et al., 1997; Kotan, 1998; Kotan et al., 1999, 2002; Sahin et al., 2004). There are a lot of studies in the literature indicating that strains of PGPR species (*Pseudomonas* sp., *Pantoea* sp., *Enterobacter* sp., *Bacillus* sp., *Trichoderma* sp.) may be used as potential biocontrol agents against potato dry rot (Schisler, 1994; Schisler et al., 1997; Sadfi et al., 2001, 2002; van den Boogert and Luttikholt, 2004). Organisms of the *B. cepacia* complex have also been the focus of considerable research as effective biocontrol agents against soilborne (McLoughlin et al., 1992; Milus and Rothrock, 1997; Heydari and Misaghi, 1998; Quan et al., 2006), foliar (Joy and Parke, 1994), and post harvest diseases (Janisiewicz et al., 1991; Smilanick et al., 1993). *B. cepacia* has not been previously reported as an antagonist dry rot of potato except *F. sambucinum* (Kotan et al., 1999). This is the first study showing that *B. cepacia* has great potential to be used as effective biocontrol agents of *Fusarium* dry rot of potatoes in the storage conditions. *B. subtilis* BA-140 and OSU-142 strains also showed strong antifungal activities on Petri plate assays, but had no reduction of disease development on *in vivo* assay.

Formerly known as *Pseudomonas cepacia*, the bacterium now known as *B. cepacia* Palleroni and Holmes, was first described in 1950 as the cause of sour skin of onions (Burkholder, 1950). Subsequently, *P. cepacia* was renamed *B. cepacia* (Yabuuchi et al., 1992) and transferred to the beta subdivision of the proteobacteria (Olsen et al., 1994). Many strains of the *B. cepacia* complex produce one or more antibiotics active against a broad range of plant pathogenic fungi (Roitman et al., 1990; Rosales et al., 1995). Organisms of the *B. cepacia* complex produce inhibitory metabolites such as pyrrolnitrin (Hwang et al., 2002), siderophores (Stephan et al., 1993), cepaciamide A(B), cepacidine A(B), cepacin A(B), and lipopeptides (Parker et al., 1984; Kang et al., 1998). These antibiotics appear, in many cases, to be important for disease suppression. Compounds such as cepacin A and cepacin B exhibit only antibacterial activity, whereas pyrrolnitrin is effective against fungi, yeasts, and gram positive bacteria. Lipopeptide strongly inhibit the growth of some soil borne fungi, especially *Pythium ultimum* Trow and *Collectotrichium* sp., but it has little effect on *Candida albicans* Robin, several other yeasts, and some pathogenic and nonpathogenic filamentous fungi. Cepacidine is a glycopeptide with a potent antifungal activity, but no activity against bacteria. Cepaciamide A and B are fungitoxic compounds that exhibit strong activity against *Botrytis cinerea* Pers. Ex Fr. and *Penicillium expansum* Link (Quan et al., 2006). The data in this study has confirmed the evidence of the previous studies suggesting *B. cepacia* OSU-7 has capability of suppressing a wide range of plant pathogens on several important crop plants (Kotan, 1998; Kotan et al., 1999, 2002; Sahin et al., 2004; Altindağ et al., 2006). Therefore, *B. cepacia* OSU 7 may be producing a complex or some novel antimicrobial compounds in order to have a broad spectrum of antifungal and antibacterial activities.

The use of bacterial and fungal biocontrol agents to control potato dry rot disease has received a great deal of attention, because postharvest conditions provide an ideal niche for biocontrol agents. In this present study, we observed that the biocontrol agents can show different activity against the same pathogen on different cultivars. The different efficacy of the biocontrol agents could be due to the influence of several factors. These factors include the efficiency of the type or strain of biocontrol agent, the type or aggressiveness of pathogens, the susceptibility of the host to the pathogen(s) of host and environment (Francés et al., 2006).

In conclusion, our results showed that *B. cepacia* OSU-7 has great potential to be used as biocontrol agents for management of *Fusarium* species causing dry rot on potato as well as many other fungi and bacterial pathogens previously (Miller et al., 1997; Kotan et al., 1999, 2002; Sahin et al., 2004). The *in vitro* and *in vivo* data are supporting that antagonism is the main mechanism of *B. cepacia* OSU-7 for biological control of disease, but the antimicrobial compounds have not been isolated and identified yet. Even though some strains of *B. cepacia* have been reported as plant or opportunistic humans pathogens such as cystic fibrosis (Parke and Gurian-Sherman, 2001), *B. cepacia* OSU 7 is an environmental organism and identified as non-pathogen for plant (Kotan, 1998; Kotan et al., 1999, 2002; Sahin et al., 2004; Altindağ et al., 2006) as well as animals or/and humans (unpublished data) since it does not grow at 36 °C. Thus, further study is necessary for determining the mechanisms of antagonistic action of OSU-7.

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