Turkish Journal of Agriculture and Forestry

Volume 32 | Number 5 Article 4

1-1-2008

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Turk J Agric For
32 (2008) 381-390

Biological Control of Bacterial Spot Disease of Pepper with *Bacillus* Strains

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Received: 13.09.2007

Abstract: Bacterial spot disease caused by *Xanthomonas axonopodis* pv. *vesicatoria* (*X. axonopodis* pv. *vesicatoria*) is a devastating pepper (*Capsicum annuum*) disease in Turkey. Biological control of *Xanthomonas axanopodis* pv. *vesicatoria* is of great interest, in terms of environmental safety and economic return. In this study, 3 *Bacillus* strains isolated from soil samples of the rhizospheres of peppers grown in greenhouses and fields were used to suppress the size of the population of *X. axonopodis* pv. *vesicatoria*. Results indicated that disease development decreased by 11%-62% and 38%-67% in pepper plants inoculated with the 3 *Bacillus* strains alone and in combination, respectively, in greenhouse and field experiments. In addition, stem diameter, root elongation, root dry weight, shoot dry weight, and yield increased in response to the treatments in the field experiment by 7.0%-20.5%, 7.0%-17.0%, 4.5%-23.5%, 16.5%-38.5%, and by 11.0%-33.0%, respectively. This is the first study to report the successful biological control of bacterial spot disease caused by *X. axonopodis* pv. *vesicatoria* using *Bacillus* species in Turkey.

Key Words: Plant growth-promoting rhizobacteria, biological control, *Xanthomonas axonopodis* pv. *vesicatoria*

Biber Bakteriyel Leke Hastalığına Karşı *Bacillus* İzolatlarıyla ile Biyolojik Mücadele

Özet: *X. axanopodis* pv. *vesicatoria* tarafından neden olunan bakteriyel leke hastalığı Türkiye'de biberin (*Capsicum annuum*) yıkıcı bir hastalığıdır. *X. axanopodis* pv. *vesicatoria*'nın biyolojik mücadelesi, ekonomik dönüşü ve çevre güvenliği nedeniyle büyük önem kazanmıştır. Bu yayında, sera ve tarlada yetiştirilen biberin rizosferinden alınan toprak örneklerinden izole edilen üç *Bacillus* izolatı, *X. axanopodis* pv. *vesicatoria* populasyonunun azaltması için kullanılmıştır. Sonuçlar, üç *Bacillus* izolatının tek başına ve onların kombinasyonlarıyla inokule edilen biber bitkilerinde sırasıyla sera koşulları ve tarla denemelerinde hastalık gelişimini % 11-62 ve % 38-67 oranlarında azalttığını göstermektedir. Ek olarak bu uygulamalarla tarla denemelerinde gövde çapı, kök uzunluğu, kök kuru ağırlığı sürgün kuru ağırlığı ve verim sırasıyla % 7.0-20.0, % 7.0-17.0, % 4.5-23.5, % 16.5-38.5 ve % 11.0-33.0 oranında artmıştır. Bu araştırma bakteriyel yaprak lekesi hastalığına karşı biyolojik mücadelede *Bacillus* türlerinin başarıyla kullanılabileceğini gösteren Türkiye'deki ilk çalışmadır.

Anahtar Sözcükler: Bitki büyüme düzenleyici rhizobakteri, Biyolojik Mücadele, *Xanthomonas axonopodis* pv.*vesicatoria*

Introduction

Pepper (*Capsicum annuum*) is an important greenhouse- and field-grown vegetable in Turkey, with an annual production of 1,750,000 tons in 2002 (DİE, 2002). Bacterial spot disease caused by *X. axonopodis* (syn: *campestris*) pv. *vesicatoria* is a serious pepper disease in the provinces of Adana and Osmaniye, both in the eastern Mediterranean region of Turkey (Aysan and Şahin, 2003). The disease occurs in 52%-100% of the pepper fields in these provinces (Mirik et al., 2005). The disease in pepper plants is characterized by small angular spots with a yellow halo on the leaves. Centers of the spots are desiccated and ripped. Margins of the affected leaves are rimmed with a narrow band of necrotic tissue. Finally, heavily infected leaves drop off the plants while they are still green. Lesions on fruit are blister-like, irregular dark raised spots, frequently surrounded by a water-soaked border. When the fruit lesions enlarge they become brown and rough,

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and have a cracked or warty appearance. Affected fruit may not be marketable. The disease agent survives on pepper seeds (Bashan et al., 1982), volunteer plants, plant debris, and weeds as epiphytes, as well as on pepper leaves (Jones et al., 1986).

Copper-containing compounds have been routinely applied to control the disease for more than 2 decades in the study region. Chemical control measures have not been effective in many production pepper fields for the last 3 years in the region because sufficient rainfall in spring and summer fosters reproduction, resistance to copper pesticides, and distribution of the bacteria (Mirik et al., 2007); therefore, biological control appears to be the best method for controlling the disease.

Many studies have investigated appropriate approaches to control the disease on pepper plants. Development of varieties resistant to diseases has shown potential as a substitute to chemical use. The use of resistant plant varieties developed via micro-organisms and chemicals is also a potential approach to controlling the disease. According to the results of some studies, biotic and abiotic elements, such as fungi, bacteria, and salicylic acid, can be effectively used to promote plant growth, biological disease control, and systemic resistance (Mauch et al., 1988; Malamy and Klessig, 1992; Kloepper, 1993; Dempsey and Klessig, 1995; van Loon, 1997; Pieterse et al., 2000; Metraux, 2001; Ramamoorthy et al., 2001; Zehnder et al., 2001). Rhizobacteria are identified to be plant growth-promoting rhizobacteria (PGPR) because they stimulate plant growth as a possible disease treatment (Kleopper et al., 1980). The first study about PGPR was conducted in the Soviet Union during the 1940s with 2 bacteria, *Azotobacter chroococcum* and *Bacillus megaterium* var. *phosphaticum*; these were used on cereals over some 10 million hectares (Brown, 1974) for their putative effects via nitrogen fixation and phosphate solubilization, respectively (Bowen and Rovira, 1999). Extensive studies performed by scientists in the People's Republic of China refer to PGPR as yield-increasing bacteria (YIB). In 1990 YIB were used over an area of 3.3 million hectares in 18 provinces, on wheat, rice, corn, sorghum, sweet potato, cotton, oil-seed rape, bean, sugar beet, watermelon, peanut, and vegetables (Bowen and Rovira, 1999). PGPR strains have been used as YIB for about the last 20 years in many countries. In recent years, induced resistance by PGPR has also been intensively studied in crop plants in order to distinguish plant pathogens (Zhou et al., 1992; Ramamoorthy et al., 2002).

Plants require substantial amounts of phosphorus for the basic processes of life. Insoluble forms of phosphorus commonly found in rocks, soil particles, and organic materials are not readily available for plant uptake. Some bacteria known as phosphate-solubilizing bacteria have the ability to solubilize insoluble forms of phosphorus for plant uptake (Dunleavy, 1955; Broadbent et al., 1971; Schippers et al., 1987). Backman et al. (1997) demonstrated the use of 18 PGPR strains of *Bacillus* species in soybean (*Glycine max* L. *Merr*), maize (*Zea mays* L. ssp. *mays*), peanut (*Arachis hypogaea*), and vegetables (Backman et al., 1997). Guo et al. (2004) used *Serratia* sp. (J2), *Bacillus* sp. (BB11), and *Pseudomonas flourescens* to decrease *Ralstonia solanacearum* on tomato and increase yield. Rhizobacteria isolated from the wheat rhizosphere were used to increase stem and ear elongation, the number of roots, and grain weight (Khalid et al., 2003; Khalid et al., 2004). *Bacillus* species induced yield increases in barley (Şahin et al., 2004; Çakmakcı et al., 2007a), sugar beet (Çakmakcı et al., 1999), wheat (de Freitas et al., 1997; Çakmakcı et al., 2007b), and sugarcane (Sundra et al., 2002).

The objective of the present study was to test the ability of phosphate-solubilizing *Bacillus* strains, as PGPR, to control bacterial spot disease caused by *X. axonopodis* pv. *vesicatoria* on peppers growing under greenhouse and field conditions. For this purpose, several bacterial strains were isolated from the rhizospheres of healthy pepper plants in the eastern Mediterranean region of Turkey. These strains were then screened using the National Botanical Research Institute's Phosphate Growth Medium (NPRIP-BPB) broth assays, developed by Nautiyal (1999), to determine whether they were phosphate-solubilizing bacteria.

Materials and Methods

Collection of candidate PGPRs

Soil samples of pepper rhizospheres were collected from production pepper fields in the provinces of Adana, Mersin, Osmaniye, and Kahramanmaraş in the eastern Mediterranean region of Turkey. Soil samples were shaken in sterile distilled water at 200 rpm at room temperature for 1 h. Suspensions were diluted 6 times at a 1:10 ratio and plated on nutrient agar and King's medium B. After incubation at 25 °C for 2-4 days, morphologically different colonies on the medium were selected.

Selection of Phosphate-Solubilizing Bacteria

Colonies were tested for solubilizing of phosphate using NBRIP-BPB broth medium (10 g l^{-1} glucose, 5 g l^{-1} $Ca_3(PO_4)_2$, 5 g l^{-1} $MgCl_2.6H_2O$, 0.25 g l^{-1} $MgSO_4.7H_2O$, 0.2 g l^{-1} KCl, 0.1 g l^{-1} $(NH_4)SO_2$, 0.025 g l^{-1} bromphenol blue) (Nautiyal, 1999) in tube assays at 25 °C. Absorbance values at A_{400} nm were measured with a spectrophotometer (Shimadzu UV-120-01, Kyoto, Japan) 14 days after inoculation of the test tubes (Nautiyal, 1999). The experiment was repeated twice for selection of bacteria based on low absorbance values. Duncan's multiple range test (significance level: P = 0.05) was conducted in analysis of variance (ANOVA) for mean separation. Of the bacteria tested, 3 had minimal absorbance values; therefore, they were chosen for use in the study.

Identification of Phosphate-Solubilizing Bacteria

The 3 selected phosphate-solubilizing bacterial strains were characterized by gram reaction, fluorescent pigmentation, and colony morphology on King's medium B, levan formation on sucrose nutrient agar, oxidase reaction and pectolytic activity on potato slices, and hypersensitive reaction on tobacco leaves. Identification of the strains was confirmed by fatty acid methyl ester (FAME) analysis, as described by Cattelan et al. (1998). FAME studies were carried out in the Biotechnology Application and Research Center of Ataturk University, Erzurum, Turkey.

In Vitro Antagonistic and Siderophore Activity of the Bacteria

The test described by Krishnamurthy and Gnanamarickam (1998) was used to determine the activity of the 3 selected phosphate-solubilizing bacterial strains (M1-3, M3-1, and H8-8) against *X. axonopodis* pv. *vesicatoria*. Each of the strains was streaked on 1 side of King's medium B plates, with 3 replicates, which were incubated at 25 °C for 4 days. Antagonistic activity was evaluated by measuring the distance between the candidate bacteria and streaked bacterial colonies in millimeters. Siderophore production of the 3 phosphate-solubilizing bacteria (M1-3, M3-1, and H8-8) was also tested with King's medium B as described above, but by supplementing the medium with Fe^{+3}, as described by (Kloepper et al. 1980).

Greenhouse Experiment

In the greenhouse experiment, which was conducted in the Plant Protection Department, Adana, in 2003, the effects of the 3 selected bacterial strains alone and in combination (equal volumes of each) on the development of bacterial leaf spot disease caused by *X. axonopodis* pv. *vesicatoria* on pepper plant cultivars were investigated. Bursa Yağlık pepper seedlings (*Capsicum annuum* cv. Bursa Yağlık) were inoculated with the 3 *Bacillus* strains alone, all 3 combined, and in combinations of 2. Root tips of seedlings were trimmed with scissors and dipped into the *Bacillus* species suspension (at 10^9 CFU ml^{-1}) for 10 min. The treated seedlings were planted in pots containing field soil as the growth medium. Seedlings were inoculated with the pathogen 1 week after planting. Inoculations were made using a hypodermic needle to inject 0.2 ml of an inoculum suspension of bacteria (10^8 CFU ml^{-1}) into the epidermis of pepper seedlings. Sterile distillated water and a reference strain (GSPB 224) obtained from Dr. K. Rudolph (Gottingen University, Germany) were used as negative and positive controls, respectively. Relative humidity in the controlled condition was maintained at around 70%, temperature at 25 °C, and illumination at 16:8 h (day:night). A completely randomized design with 6 replicates, each containing 9 plants per treatment, was used. Disease evaluation was made using a 0-5 scale: no symptoms (0); 1-3 necrotic spots (1); coalescence of many spots (2); severe disease symptoms (3); shed and dead leaves (4); dead plant after visible symptoms appeared on positive control plants (5) (Mirik, 2005). The ABBOTT formula was used to calculate percent of bacteria efficiency. Duncan's multiple range test (level of significance: P = 0.05) was conducted in analysis of variance (ANOVA) for mean separation.

Field Experiments

The field experiment was performed twice, in 2003 and 2004, whereas the greenhouse experiment was conducted only in 2003. The field experiment was performed in the Research and Implementation Area of the Plant Protection Department, Faculty of Agriculture, University of Çukurova, in the Mediterranean region of Turkey. Pepper seedlings (*Capsicum annuum* cv. Bursa Yağlık) were inoculated with the 3 *Bacillus* strains alone, all 3 combined, and in combinations of 2. Root tips of seedlings were trimmed with scissors and dipped into *Bacillus* species suspension (at 10^9 CFU ml^{-1}) for 10 min. The treated seedlings were then planted in the ground. Seedlings were inoculated with the pathogen 1 week after planting. Inoculations were made using a hypodermic needle to inject 0.2 ml of an inoculum suspension of

bacteria (10^8 CFU ml^{-1}) into the epidermis of pepper seedlings. Sterile distillated water and a reference strain (GSPB 224) were used as negative and positive controls, respectively. The field experiment was arranged in a randomized block design, with 6 replicates consisting of 12 plants per treatment. Application of bacteria and pathogenic bacteria was done in the same way as previously described in the greenhouse experiment. Seedlings were evaluated 12 weeks after planting for stem diameter, root elongation, root dry weight, shoot dry weight, and yield. After all the plants were harvested total weight was measured using an electronic balance accurate to 0.001 g (Vibra). Stem diameter was measured using calipers and root elongation was measured using a ruler. For root and shoot dry weight, as soon as pepper plants were harvested samples were cut and dried in a dry air sterilizer/oven (Nüve FN 500) at 80 °C for 24 h (Nejad and Johnson, 2000). The same scale as described for the greenhouse study was used. The ABBOTT formula was used to calculate percent of bacteria efficiency. Duncan's multiple range test (level of significance: P = 0.05) was conducted in analysis of variance (ANOVA) for mean separation.

Results

Collection of Candidate PGPRs

Analysis of various soil samples taken from the provinces of Adana, Mersin, Osmaniye and Kahramanmaraş revealed that 189 bacterial isolates were collected as candidate antagonists. All the strains did not produce levan-type colonies. The color and number of the 189 strains were cream, yellow, orange, white, and green, and 105, 34, 7, 39, and 4, respectively. Eighty-four of the 189 strains were oxidase-positive and the other 105 strains were oxidase-negative. Only 5 strains caused soft rot on potato slices. Sixteen strains were positive to hypersensitive reaction on tobacco leaves. Twenty-two strains were gram-negative and the others were gram-positive.

Selection of Phosphate-Solubilizing Bacteria

The NBRIP-BPB broth assay was a useful test for screening phosphate-solubilizing bacteria. The absorbance value for the control tube (un-inoculated) was 0.212. The absorbance values for all strains tested were between 0.078 and 0.203. Phosphate was solubilized by 11 strains, namely A3-4, H8-8, 01-2, 02-1, 03-6, 03-20, M1-3, M3-1, M8-4, M10-3, and M11-2 (Table 1). Strains M1-3, M3-1, and H8-8 showed lower absorbance values than the others that were tested. The absorbance values of M1-3, M3-1, and H8-8 were 0.078, 0.092, and 0.093, respectively. These 3 strains (M1-3, M3-1 and H8-8) were chosen for the current study, while the other strains were considered for some future study. The chosen strains caused neither soft rot on potato slices nor levan-type colonies, but were gram-positive, non-fluorescent, and oxidase-negative. Furthermore, hypersensitive reaction on tobacco leaves was negative (Table 1). FAME analysis identified the chosen strains as *Bacillus* species, with similarity indices between 52% and 55%.

In Vitro Antagonistic and Siderophore Activity of the Bacteria

The test performed for antagonistic activity with *X. axonopodis* pv. *vesicatoria* in the field experiment showed that the 3 chosen strains restrained bacterial growth on King's medium B. The inhibition zones were 21.0, 27.0, and 5.0 mm for M1-3, M3-1, and H8-8, respectively; however, for siderophore production, only H8-8 produced an inhibition zone on the medium supplemented with Fe^{+3}. Antagonistic effects were observed with M1-3, M3-1, and H8-8, but siderophore production was only observed with H8-8 against *X. axonopodis* pv. *vesicatoria*.

Greenhouse Experiment

Disease development in the greenhouse was suppressed by about 62% when all 3 bacteria (M1-3 + M3-1 + H8-8) were used in combination. The combination of the 3 selected strains (M1-3 + M3-1 + H8-8) significantly suppressed disease by 62%. Dual combinations of M3-1 + H8-8, M1-3 + M3-1, and M1-3 + H8-8 reduced disease by 51%, 46%, and 33%, respectively, when compared to the positive control. Using only 1 *Bacillus* strain, disease development was reduced 43%, 32%, and 11% for M1-3, M3-1, and H8-8, respectively. The results are given in Table 2.

The plant parameters measured and positively affected by the *Bacillus* strains are presented in Table 3. Increases in stem diameter due to the *Bacillus* strains ranged from 19% to 74%. The minimum increase (19%) in stem diameter was observed for both M1-3 + H8-8 strains, while the M1-3 + M3-1 isolates exhibited the maximum increase (74%). Stem diameters increased 30%, 41%, 30%, 30%, and 67% for the remaining M1-3, M3-1, H8-8, M3-1 + H8-8, and M1-3 + M3-1 + H8-8, respectively. Root elongation also improved using the strains. The

Table 1. Phenotypic properties of the phosphate-solubilizing bacteria.

Strains	Gram Reactions	F/NF*	Colony Morphology on King's B	Color	L	O	P	T	Phosphate Reduction	Absorbance Values	Inhibition Zone (mm)
A 3-4	+	NF	Flat	Cream	–	–	–	–	+	0.163^{b**}	0.0
H 8-8	+	NF	Flat	Cream	–	–	–	–	+	0.092^{c}	5.0
M 1-3	+	NF	Flat	Cream	–	–	–	–	+	0.078^{c}	21.0
M 3-1	+	NF	Flat	White	–	–	–	–	+	0.093^{c}	27.0
M 8-4	+	NF	Mucoid	Cream	–	+	–	+	+	0.200^{ab}	18.0
M 10-3	+	NF	Flat	Yellow	–	–	–	–	+	0.176^{b}	121.0
M 11-2	+	NF	Mucoid	Cream	–	+	–	–	+	0.198^{ab}	12.0
O 1-2	+	NF	Flat	Cream	–	+	–	–	+	0.203^{ab}	9.0
O 2-1	+	NF	Flat	White	–	+	–	–	+	0.203^{ab}	0.0
O 3-6	–	F	Mucoid	Cream	–	+	–	–	+	0.159^{b}	11.0
O 3-20	+	NF	Flat	Cream	–	–	–	–	+	0.176^{b}	87.0
Water										0.212^{ab}	

The symbol '+' denotes a positive result and the symbol '–' denotes a negative result.

L: levan formation; O: oxidase reaction; P: pectolytic activity on potato slices; T: hypersensitive reaction on tobacco leaves.

*Fluorescent pigment production on King's B medium.

**Means with the same letter are not significantly different according to Duncan's multiple range test at $P < 0.05$.

Table 2. Effects of the *Bacillus* strains on scale value and disease severity (%) in greenhouse and field experiments.

Treatment	Greenhouse experiment		Field experiments			
			2003		2004	
	Scale	%	Scale	%	Scale	%
Positive Control	3.32	0^{a}	2.11	0^{a}	2.04	0^{a}
M1-3	1.89	43^{bc}	0.69	67^{c}	0.77	62^{b}
M3-1	2.25	32^{bc}	1.11	47^{b}	0.75	63^{b}
H8-8	2.96	11^{a}	1.31	38^{b}	0.86	58^{b}
M1-3 + M3-1	1.79	46^{bcd}	1.09	48^{b}	0.88	57^{b}
M1-3 + H8-8	2.21	33^{bc}	1.29	39^{b}	0.79	61^{b}
M3-1 + H8-8	1.61	51^{cd}	1.22	42^{b}	0.85	58^{b}
M1-3 + M3-1 + H8-8	1.25	62^{d}	1.04	51^{b}	0.86	58^{b}

*Means with the same letter are not significantly different according to Duncan's multiple range test at $P < 0.05$.

greatest increase in root elongation (21%) was observed with the M1-3 + M3-1 combination, while the M1-3 + H8-8 combination decreased root elongation by about 10%. The other M1-3, H8-8, M3-1, M3-1 + H8-8, and M1-3 + M3-1 + H8-8 treatments increased root elongation 16%, 10%, 8%, 8%, and 19%, respectively. Increases in root dry weight due to the *Bacillus* strains ranged from 7% to 33%. The maximum increase (33%) in root dry weight was recorded for the M3-1 + H8-8 combination, while the M1-3 + M3-1 + H8-8 combination decreased root dry weight by 13%. The remaining M1-3, M1-3 + M3-1 and M1-3 + H8-8 treatments increased root dry weight by 7%, 7%, and 27% respectively. M3-1 and H8-8 strains did not affect root dry weight.

Table 3. Effects on plant growth of the *Bacillus* strains in the greenhouse experiment.

Treatment	Stem diameter		Root elongated		Root dry weight		Shoot dry weight	
	mm	%	mm	%	g	%	g	%
Positive Control	2.7	–	103.3	–	1.5	–	1.2	–
M1-3	3.5	30^{bc*}	120.0	16^{ab}	1.6	7^{b}	1.7	42^{cd}
M3-1	3.8	41^{ab}	111.7	8^{ab}	1.5	0^{b}	3.0	250^{a}
H8-8	3.5	30^{bc}	113.3	10^{ab}	1.5	0^{b}	2.0	67^{b}
M1-3 + M3-1	4.7	74^{a}	125.0	21^{a}	1.6	7^{b}	1.6	33^{d}
M1-3 + H8-8	3.2	19^{c}	93.3	-10^{b}	1.9	27^{a}	1.8	50^{cd}
M3-1 + H8-8	3.5	30^{bc}	111.7	8^{ab}	2.0	33^{a}	1.7	42^{cd}
M1-3 + M3-1 + H8-8	4.5	67^{a}	123.3	19^{a}	1.3	-13^{b}	1.8	50^{cd}

*Means with the same letter are not significantly different according to Duncan's multiple range test at $P < 0.05$.

The greatest increase (250%) in shoot dry weight was observed for M3-1, while H8-8 increased shoot dry weight by 67%. Shoot dry weight increased 42%, 33%, 50%, 42%, and 50% using the remaining M1-3, M1-3 + M3-1, M1-3 + H8-8, M3-1 + H8-8 and M1-3 + M3-1 + H8-8 combinations, respectively. Treatment with each *Bacillus* strain alone, all 3 strains combined, and dual combinations of the strains (M3-1 + H8-8, M1-3 + M3-1, and M1-3 + H8-8) resulted not only in the suppression of disease symptoms, but also increases in plant growth parameters, such as stem diameter, root elongation, root dry weight and shoot dry weight, as compared to the control plants.

Field Experiments

Disease severity was decreased by the *Bacillus* strains in the field experiments of 2003 and 2004 (Table 2). About a 38%-67% reduction in disease development was observed in the field experiment in 2003. Using only strain M1-3, disease severity decreased by about 67%, while the other treatments, M3-1, H8-8, M1-3 + M3-1, M1-3 + H8-8, M3-1 + H8-8, and M1-3 + M3-1 + H8-8, reduced disease development by 47%, 38%, 48%, 39%, 42%, and 51% respectively. As the statistics indicate, these strains were less effective for reducing the symptoms of bacterial spot disease when compared to M1-3 in the field experiment. No symptoms appeared on the negative control plants. In 2004, the reductions in disease development ranged from 57% to 63%. The M1-3 and M3-1 isolates reduced disease development almost equally, 63% and 62%, respectively. Symptoms of bacterial spot disease decreased by 58%, 57%, 61%, 58%, and 58% using the H8-8, M1-3 + M3-1, M1-3 + H8-8, M3-1 + H8-8, and M1-3 + M3-1+H8-8 treatments, respectively. The negative control plant exhibited no symptoms.

The plant parameters measured and positively affected by the *Bacillus* strains are presented in Table 4. Increases in stem diameter due to the *Bacillus* strains ranged from 7.0% to 20.5%. The minimum increase in stem diameter (7.0%) was observed for both M1-3 and H8-8 strains, while the M3-1 isolate exhibited the maximum increase (20.5%). Stem diameters increased 14.5%, 8.0%, 12.5%, and 14.5% in response to M1-3 + M3-1, M1-3 + H8-8, M3-1 + H8-8, and M1-3 + M3-1 + H8-8 combinations, respectively. Root elongation also improved using the strains. The greatest increase in root elongation (17.0%) was observed for the M3-1 strain, whereas the M1-3 isolate caused the lowest increase (7.0%). The H8-8, M1-3 + M3-1, M1-3 + H8-8, M3-1 + H8-8, and M1-3 + M3-1 + H8-8 treatments increased root elongation by 9.0%, 12.5%, 11.5%, 12.0%, and 13.0%, respectively. Increases in root dry weight due to the *Bacillus* species varied from 4.5% for the H8-8 strain to 23.5% for the M3-1 strain. The M1-3, M1-3 + M3-1, M1-3 + H8-8, M3-1 + H8-8, and M1-3 + M3-1 + H8-8 treatments increased root dry weight by about 7.0%, 13.5%, 11.5%, 13.5%, and 12.0%, respectively.

The greatest increase in shoot dry weight (38.5%) was observed for both M3-1 and M1-3 + M3-1, while H8-8 decreased shoot dry weight by 8.5%. Shoot dry weight increased 16.5%, 27.0%, 15.5%, and 36.0% using the M1-3, M1-3 + H8-8, M3-1 + H8-8, and M1-3 + M3-1 + H8-8 treatments, respectively. The maximum increase in yield (33.0%) was caused by M1-3 + M3-1, followed by

Table 4. Effects of the *Bacillus* strains on plant growth in field experiments (these are mean values of 2 experiments conducted in 2003 and 2004).

Treatment	Stem diameter		Root elongated		Root dry weight		Shoot dry weight		Yield	
	mm	%	mm	%	g	%	g	%	g	%
Positive Control	10.7		122.8		63.4		288.9		1771.6	
M1-3	11.5	7.0^{c*}	131.4	7.0^{b}	67.8	7.0^{b}	336.6	16.5^{ab}	1966.5	11.0^{ab}
M3-1	12.9	20.5^{a}	143.7	17.0^{a}	78.3	23.5^{a}	400.0	38.5^{a}	2303.1	30.0^{a}
H8-8	11.5	7.0^{c}	133.9	9.0^{b}	66.3	4.5^{b}	264.3	-8.5^{b}	1993.1	12.5^{ab}
M1-3 + M3-1	12.2	14.5^{ab}	138.2	12.5^{ab}	72.0	13.5^{ab}	400.0	38.5^{a}	2356.2	33.0^{a}
M1-3 + H8-8	11.6	8.0^{c}	125.8	11.5^{ab}	70.7	11.5^{ab}	366.9	27.0^{ab}	1966.5	11.0^{ab}
M3-1 + H8-8	12.0	12.5^{bc}	137.5	12.0^{ab}	72.0	13.5^{ab}	333.7	15.5^{ab}	2090.5	18.0^{ab}
M1-3 + M3-1 + H8-8	12.2	14.5^{b}	138.8	13.0^{ab}	71.0	12.0^{ab}	292.9	36.0^{a}	2010.8	13.5^{ab}

*Means with the same letter are not significantly different according to Duncan's multiple range test at $P < 0.05$.

M3-1 (30.0%), M3-1 + H8-8 (18.0%), M1-3 + M3-1 + H8-8 (13.5%), H8-8 (12.5%), and M1-3 and M1-3 + H8-8 (11.0%). The bacteria M3-1 increased plant growth in the field experiments. According to these field experiments the M3-1 strain can be considered as an effective PGPR. The use of *Bacillus* strains in field experiments resulted not only in the suppression of disease symptoms, but also increased plant growth parameters when compared to the control plants.

Discussion

Several researchers have successfully employed *Bacillus* species to control disease development (Capper and Campbell, 1986; Sung and Chung, 1997; Guo et al., 2004). In our study, the M1-3, M3-1, and H8-8 isolates were effective against bacterial spot disease in pepper plants. Researchers have argued that the combination of a few strains was more efficient in reducing disease development when compared to an individual strain (Kloepper and Schroth, 1981; Ramamoorthy et al., 2001; Romero et al., 2003; Anith et al., 2004; Silva et al., 2004). Application of combinations of the 3 PGPR strains resulted in much more intensive plant growth promotion and disease reduction when compared to the strains tested singly. This might have been due to a different mechanism of action for each PGPR strain (Raupach and Kloepper, 1998). This situation may explain why combinations of strains provide more consistency in disease suppression. These results are in agreement with studies by Pierson and Weller (1994), and Duffy and Weller (1995), both of which demonstrated that certain mixtures of fluorescent pseudomonads suppressed take-all disease to a significantly greater degree than either treatment used alone. Our study demonstrated that a combination of all 3 selected *Bacillus* strains (M1-3 + M3-1 + H8-8) significantly decreased the severity of bacterial spot disease. Several control mechanisms, such as phytohormones, antibiotic, siderophore, nitrogenase, etc. were activated and disease development was controlled using combined biological control agents (Brown, 1974; Weller, 1988; Shishido and Chanaway, 1997). Sung and Chung (1997) found that a mixture of *Streptomyces* species and *Bacillus cereus* isolates, which synthesize chitinase, and *P. flourescens* and *Burkholdere cepecia* isolates, which synthesize antibiotics, had a synergistic effect in controlling *Pyricularia oryzae*.

The fluorescent pseudomonads produce a range of secondary metabolites, many of which exhibit antibiotic or phytotoxic activity. Antagonistic effect and siderophore production of the tested *Bacillus* strains were determined against *X. axonopodis* pv. *vesicatoria*.

The production of siderophore by PGPR in the rhizosphere could efficiently complex iron, inhibiting the growth of certain components of the native microplane. The addition of the siderophore and pseudobactin to soil caused increases in plant growth and reductions in target colonization of the rhizosphere, similar to that achieved by inoculation of seeds with PGPR (Schroth and Hancock, 1982). All 3 *Bacillus* species were controlled using the solubilizing phosphate, and synthesized antibiotics and siderophores. Antagonists (Byrne et al., 2005) and PGPR (Romero et al., 2003, 2005) were effective against bacterial spot disease on pepper. Our study showed that the *Bacillus* species were important agents for decreasing disease development through increasing biological activity.

An antagonistic effect was observed in 2 of 3 tested isolates, while siderophore production was observed in the other strain.

Rhizobacteria isolated from different plant rhizospheres were used to increase stem elongation, the number of roots, and yield (Broadbent et al., 1977; de Freitas et al., 1997; Çakmakcı et al., 1999; Sundra et al., 2002; Khalid et al., 2003; Guo et al., 2004; Khalid et al., 2004; Şahin et al., 2004; Çakmakcı et al., 2007a, 2007b.). Differences in the level of protection against *Xanthomonas campestris* pv. *vesicatoria* were observed among the antagonists tested. It is possible that each rhizobacterium activates different defense mechanisms within the induced resistance pathway, resulting in differential reduction in symptoms. Possible defense mechanisms include physical (lignification) and chemical (quinones) barriers arising from increased enzyme activity associated with induced systemic resistance (Agrios, 1997; Hammerschmidt et al., 1996). Plant growth varies in response to inoculation with phosphate-solubilizing bacteria in laboratory and field studies (Schippers, 1988). It is apparent that enhanced crop yields can be explained by one or several mechanisms (Kloepper et al., 1988). For example, Lahevrte and Berthelin (1988) inoculated maize seeds with isolates of the phosphate-solubilizing *Enterobacter agglomerans* and assessed root exudates for their ability to solubilize phosphate. They observed that there wasn't a relationship between the leakage of these root exudates and solubilization of phosphate from rock phosphate. They suggested that the phosphate-solubilizing *Enterobacter agglomerans* metabolized root exudates to plant growth substances, which enhanced plant growth (de Freitas et al., 1997). Our study showed that the *Bacillus* species, M3-1, M1-3, and H8-8, increased all plant parameters, such as stem diameter, root elongation, root dry weight, and shoot dry weight, due to their ability to solubilize phosphate. To the best of our knowledge this is the first work conducted on the biological control of bacterial spot disease caused by *X. axonopodis* pv. *vesicatoria* in Turkey. Suitable formulations of the *Bacillus* strains tested in the present study should be further investigated to identify those with the potential for commercial use in the pepper production areas of Turkey.

Acknowledgements

This study (TOGTAG-2498) was supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK) and a grant from Çukurova University, Turkey (project number ZF 2003 D6). We are grateful to Prof. Dr. F. Sahin (Yeditepe University, Faculty of Engineering and Architecture, Department of Genetic and Bioengineering) for identification of the bacterial strains by FAME analysis.

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