

BIOCONTROL OF *PHYTOPHTHORA CAPSICI* ON PEPPER PLANTS BY *BACILLUS MEGATERIUM* STRAINS

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SUMMARY

Phytophthora blight or crown blight of peppers, caused by *Phytophthora capsici* Leonian, is one of the most important diseases of pepper in the eastern Mediterranean region of Turkey. The possibility to reduce disease severity using phosphate-solubilizing bacteria was investigated in growth room and field experiments. The pepper plants, inoculated with the pathogen after pre-inoculation with three phosphate-solubilizing strains of *Bacillus megaterium* employed alone or in combination, were monitored for growth parameters and disease severity. Inoculation with the selected strains significantly reduced disease severity in field experiments and two strains increased the yield by 36.2 and 47.7% compared to untreated controls. Bacteria were identified by fatty acid methyl ester (FAME) profiling.

Key words: Phytophthora blight, *P. capsici*, pepper, plant growth-promoting rhizobacteria, biocontrol agents.

INTRODUCTION

Phytophthora blight of peppers is an important disease worldwide. In Turkey it was first detected in the surroundings of Kahramanmaraş city in 1970, from where it rapidly spread to other parts of the country (Cinar and Bicici, 1977). The disease can affect plants at any growth stage, and the damping-off syndrome can kill seedlings within 5 days of infection. The pathogen can also cause crown, leaf and fruit blight, wilting of the whole plant and dark purplish discoloration of the stem. Control methods such as selection of genetically resistant cultivars and planting in well-drained soil are of great importance. Although some fungicides, such as metalaxyl, fosetyl-Al and terrazole propamocarb-hydrochloride can be effective, fungicide applications may not be sufficient for controlling collar blight, if conditions are favourable for disease development. Fungicide use also brings certain environmental hazards (Erwin and Ribeiro, 1996).

Many strategies to control diseases on pepper have been pursued. A promising strategy for the replacement of chemicals has been implementation of induced resistance against plant diseases using certain microorganisms and chemicals. Induced resistance against *P. capsici* blight was obtained with chemicals (Sunwoo *et al.*, 1996; Baysal *et al.*, 2005) and with antagonistic *Trichoderma harzianum* and *Bacillus* spp. (Sid Ahmed *et al.*, 2000, 2003).

However, not enough is known about induced resistance by plant growth promoting rhizobacteria (PGPR) in controlling Phytophthora blight of peppers. PGPR are root-colonizing bacteria with beneficial effects, which include plant growth promotion, biological disease control and induced systemic resistance (Kloepper, 1993; Ramamoorthy *et al.*, 2001; Zehnder *et al.*, 2001). Induced resistance by PGPR has also been studied in recent years using crop plants and different plant pathogens (Zhou *et al.*, 1992; Ramamoorthy *et al.*, 2002).

Phosphorus is an important nutrient for plant development but is generally found in soil in insoluble form; phosphate-solubilizing bacteria can help to make it available. The aim of the study was to assess the possibility of controlling pepper crown blight caused by *P. capsici*, by phosphate-solubilizing bacteria as PGPR under growth room and field conditions.

MATERIALS AND METHODS

Isolation of the pathogen and preparation of inocula.

Plants infected with *P. capsici* were collected from pepper fields in the Adana, Hatay, Kahramanmaraş, Mersin and Osmaniye provinces in 2002. Selected stem sections with active lesions were cut and tissue pieces from the boundary between healthy and discoloured areas were plated on P₁₀ARP CMA medium (Kannwischer and Mitchell, 1978) after surface sterilization with 2% NaOCl for 2 min. The plates were incubated for 3-4 days at 25°C and *Phytophthora* colonies were transferred on potato dextrose agar (PDA). Pathogenicity tests were carried out on pepper plants (*Capsicum annuum* L. local cv. Karaisali salcalik), according to Sunwoo *et al.* (1996).

While the experiments were in progress, reisolated

cultures were used for pathogen inoculation. The pathogenic fungal isolates of *P. capsici* was grown on PDA for 7 days at 25°C then the plates were incubated under fluorescent light for 3 days at 25°C to induce sporangial formation. One day before inoculation, the fungal colonies were covered with 20 ml of tap water, and incubated under light overnight. At the day of inoculation, petri dishes were placed for 30 min at 4°C, followed by 60 min at room temperature to enhance zoospore release from sporangia. The flooding water, containing zoospores and mycelium was filtered using two layers of cheesecloth, after which the concentration of the zoospore suspension was adjusted to approximately 10^6 zoospores ml⁻¹ using a haemocytometer. The inoculum was used immediately.

Isolation of candidate antagonistic bacteria. Rhizospheres of healthy pepper plants in the field were sampled from plots not infested and infested with *P. capsici* in Adana, Hatay, Kahramanmaraş, Mersin and Osmaniye provinces in June and July 2002. Bacterial strains were isolated from soil samples by the dilution-preplating method of Aysan *et al.* (2003), using King's medium B (King *et al.*, 1954). Morphologically different bacterial colonies were randomly selected as candidate biocontrol agents after incubation at 25°C for 3 days.

Screening of phosphate-solubilizing bacteria. Several bacterial strains from the rhizosphere of healthy pepper plants were screened by the National Botanical Research Institute for their phosphate-solubilizing ability. Bacterial strains were tested in NBRIP-BPB medium (Nautiyal, 1999; Metha and Nautiyal, 2001), without agar, by tube assays. Four tubes per bacterial strain were inoculated with 100 µl of bacterial suspension (approximately 10^8 cfu ml⁻¹), uninoculated tubes were used as controls. Absorbance values at 400 nm wavelength were measured by a spectrophotometer (Shimadzu UV-120-01, Kyoto, Japan) after 14 days incubation at 25°C. The experiment was done twice and the three strains with the lowest absorbance values were selected for further study.

Identification of bacterial isolates. Bacterial strains were identified using the following tests: gram reaction, including potassium hydroxide wall solubility, fluorescent pigmentation and colony morphology on King's medium B, levan formation on sucrose nutrient agar, oxidase reaction, pectolytic activity on potato slices, and hypersensitive reaction on tobacco leaves. Identification at the species level was confirmed by fatty acid methyl ester (FAME) analysis described by Cattelan *et al.* (1998).

In vitro antagonistic activity of the bacteria. The three phosphate-solubilizing bacterial strains selected (M 1-3, M 3-1, H 8-8) were tested for antagonistic activity against *P. capsici* as described by Radjacommare *et al.*

(2004). Each of the strains was streaked (4 cm line) on one side of a PDA plate. After incubation at 24°C for two days, one mycelial plug (5 mm in diameter) from the edge of a 4 day-old *P. capsici* colony on PDA was placed in each plate at 6 cm from bacterial stripe. Three replicate plates were used for each bacterial strain. All plates were incubated at 25°C for four days. Antagonistic activity was evaluated by measuring the distance between mycelium and streaked bacterial colony after four days. The experiment was repeated twice. Siderephore production of the three phosphate-solubilizing bacteria was tested as described by Kloepper *et al.* (1980).

Effects of phosphate-solubilizing bacteria on disease development.

Preparation of bacterial inocula. Colonies of the selected bacterial strains were grown on King's medium B at 25°C. After 2 days the colonies were washed twice with saline buffer (0.85% NaCl) and the bacterial cells were resuspended in the same buffer. The concentration was adjusted using a spectrophotometer to approximately 10^8 cfu ml⁻¹ and the suspension was used as inoculum.

Growth room experiments. The protective effects of the three selected bacterial strains and their mixture combinations (equal volumes of each) were investigated on the development of crown blight disease on pepper plants cv. Yalova carliston inoculated with *P. capsici*. The pepper seedlings were grown in 15 cm diameter plastic pots, in a mixture of soil: sand: peat (2:1:1 v/v) pre-sterilized by 2 successive autoclave cycles of 1 h each at 121°C.

Seedlings at the 5-7 true leaf stage were gently uprooted and the roots were washed under running tap water to remove the potting mixture. After draining excessive water, seedling roots were dipped for 15 min in bacterial inoculum.

Each treatment had 12 replicates (1 seedling/replicate). Inoculated plants were re-planted in pots and incubated in a growth chamber at 25±2°C, 70±5% relative humidity, 12/12 h day/night illumination for 7 days. After this period, 25 ml of zoospore inoculum of *P. capsici* were applied per pot by pouring into the crown zone of the treated plants. Seedlings inoculated only with a regional strain of *P. capsici* (P3) were used as positive control.

Seedlings drenched with sterile distilled water instead of zoospore inoculum were used as negative control. Disease severity was evaluated after 15 days using a 0-5 scale (0 = no visible disease symptom; 1 = leaves slightly wilted with brownish lesions beginning to appear on stems; 2 = 30-50% of entire plant diseased; 3 = 50-70% of entire plant diseased; 4=70-90% of entire plant diseased; 5 = dead plant) (Sunwoo *et al.*, 1996). Efficacy of treatments was calculated using Abbott's formula, and statistical analyses were done using Duncan's multiple range test at 5% significance level.

Field trial. The field trial was carried out from mid May to the end of July 2004 in the Mediterranean region of Turkey (University of Cukurova). Pepper seedlings cv. Karaisalı salcalik were purchased from a commercial nursery in Adana. We used a randomized block design with nine treatments, four replicates/treatment and 20 plants/replicate. Bacterial and fungal inocula were applied as previously described for the growth room experiment, except planting, which was in field soil (clay 54%, silt 21%, sand 25%, pH 7.6). Disease severity was evaluated using the 0-5 scale (Sunwoo *et al.*, 1996), 15 days after inoculation. Efficacy of treatments was calculated as above.

Effect of phosphate-solubilizing bacteria on plant growth. Effects of phosphate-solubilizing bacteria on plant growth were studied on pepper plants cv. Karaisalı salcalik under field conditions from mid May to the end of July 2004. The strains M 1-3, M 3-1, H 8-8 alone and their dual and triple combinations were applied to pepper plants by dipping the roots for 15 min in 10^8 cfu ml⁻¹ bacterial inoculum. Twelve replicates were used per treatment. Negative control plants were dipped into sterile distilled water. Stem diameter, root length, root dry weight, shoot dry weight and yield were measured per plant at the end of the vegetation period (end of July 2004). Efficacy of treatments was calculated using Abbott's formula, and statistical analyses were conducted using Duncan's multiple range test at 5% significance level.

RESULTS

Screening and identification of phosphate-solubilizing bacteria. A total of 185 bacterial strains were isolated from rhizosphere samples, collected from Adana, Kahramanmaras, Mersin, and Osmaniye provinces. Absorbance values in NBRIP-BPB broth after 14 days ranged from 0.078 to 0.203.

Strains M 1-3, M 3-1, H 8-8, which had lower absorbance values than the others, (0.078, 0.093 and 0.092, respectively) were selected for further study. The absorbance in control tubes (uninoculated) was 0.212. The selected strains were gram positive, non-fluorescent, oxidase-negative, did not cause soft rot on potato slices and did not produce levan type colonies. Hypersensitive reaction on tobacco leaves was negative.

Identification of bacterial isolates. The strains were identified as *Bacillus* spp. based on the above tests. FAME analysis identified them as *Bacillus megaterium*, with similarity indices ranging from 52 to 55%.

In vitro antagonistic activity. In testing for antagonistic activity against *P. capsici*, all three strains inhibited

mycelial growth of the pathogen on PDA with 12.3, 5.0 and 2.0 mm inhibition zones respectively. However, only H 8-8 produced an inhibition zone on the medium supplemented with Fe⁺³.

Effect of phosphate-solubilizing bacteria on disease development.

Growth room experiment. Strains M 1-3, M 3-1, H 8-8, alone and in combination (Table 1) reduced disease severity compared to the positive control (pathogen inoculated but non-treated) plants (Table 1). While disease severity in these plants was 43.3%, it ranged from 11.6% to 33.3% in PGPR-treated plants. The combination of all three bacterial strains was the most effective treatment, decreasing disease severity by 73.3%. The mixture of M 1-3 and H 8-8 had a similar effect to that of the triple combination, and fell within the same significance group. All treatments, except strain H 8-8 alone, were significantly effective on disease develop-

Table 1. Effects of phosphate-solubilizing bacteria on *Phytophthora capsici* on pepper grown under climatized room conditions.

Treatments	Disease severity (%)	Efficacy (%)
<i>Phytophthora capsici</i> (positive control)	^(a) 43.3 ± 4.41 c	-
M 1-3	23.3 ± 6.01 ab	46.2
M 3-1	18.3 ± 4.41 ab	57.7
H 8-8	33.3 ± 4.41 bc	23.1
M 1-3 + M 3-1	16.6 ± 4.41 ab	61.7
M 1-3 + H 8-8	15.0 ± 2.89 a	65.4
M 3-1 + H 8-8	21.6 ± 9.28 ab	50.1
M 1-3 + M 3-1 + H 8-8	11.6 ± 1.67 a	73.2
Negative control	0	

^(a)Means with the same letter are not significantly different by Duncan multiple range test at $p < 0.05$.

Table 2. Effects of phosphate-solubilizing bacteria to *Phytophthora capsici* on pepper in the field.

Treatments	Disease severity (%)	Efficacy (%)
<i>Phytophthora capsici</i> (positive control)	^(a) 47.2 ± 2.06 b	-
M 1-3	23.4 ± 1.50 a	50.4
M 3-1	26.3 ± 1.05 a	44.3
H 8-8	26.2 ± 4.74 a	44.5
M 1-3 + M 3-1	27.8 ± 1.59 a	41.1
M 1-3 + H 8-8	29.4 ± 1.14 a	37.7
M 3-1 + H 8-8	26.7 ± 1.64 a	43.4
M 1-3 + M 3-1 + H 8-8	28.2 ± 1.04 a	40.3
Negative control	0	

^(a)Means with the same letter are not significantly different by Duncan multiple range test at $p < 0.05$.

Table 3. Effects of phosphate-solubilizing bacteria on stem diameter, root elongation, root dry weight, shoot dry weight and yield.

Treatments	Stem diameter (mm)	Efficacy (%)	Root elongation (mm)	Efficacy (%)	Root dry weight (g)	Efficacy (%)	Shoot dry weight (g)	Efficacy (%)	Yield (g)	Efficacy (%)
Not treated	^{a)} 10±1.1 d	0	88.6±14.6 c	0	59.2±11.1 ab	0	235.4±140.7 ab	0	1142.6±361.5 c	0
M1-3	10.7±0.8 cd	7.0	97.2±5.3 b	9.7	62.3±20.5 ab	5.2	255.9±109.7 ab	8.7	1324.3±473.6 abc	15.9
M3-1	12.8±0.4 a	28.0	109.0±6.7 a	23.0	87.9±20.1 a	48.4	359.2±178.18 a	52.6	1556.3±495.7 ab	36.2
H8-8	10.3±1.0 cd	3.0	99.8±6.7 b	12.6	56.3±7.2 b	-4.9	178.0±55.6 b	-24.3	1286.2±400.6 bc	12.6
M1-3+M3-1	11.8±0.7 b	18.0	103.3±7.7 ab	16.6	64.4±14.6 a	8.8	358.1±195.3 a	52.1	1687.7±581.4 a	47.7
M1-3+H8-8	10.5±0.8 cd	5.0	100.8±5.7 ab	13.8	63.3±198.9 ab	6.9	316.1±194.8 ab	34.3	1332.1±44.5 abc	16.6
M3-1+H8-8	11.3±0.5 bc	13.0	100.5±3.6 ab	13.4	62.7±5.8 ab	5.9	258.8±83.86 ab	9.9	1533.8±479.7 abc	34.2
M1-3+M3-1+H8-8	11.0b±0.6 cd	10.0	102.8±2.9 ab	16.0	63.6±5.6 a	7.4	326.4±133.7 a	38.7	1267.1±456.6 bc	10.9

^{a)}Means with the same letter are not significantly different by Duncan multiple range test at $p < 0.05$.

ment. No symptoms were observed in the negative (non-treated and non-inoculated) control plants.

Field experiment. The three phosphate-solubilizing bacterial strains and their combinations were effective in reducing disease severity in field conditions, albeit the efficacy was lower in the field conditions than in growth room conditions (Table 2). Treatment with strain M 1-3 reduced crown blight severity to half, having an efficacy of 50.4%, higher than all other treatments, although not significantly so. The difference between the treatments and the positive control was significant.

Effect of phosphate-solubilizing bacteria on plant growth. The bacterial strains and their combinations affected plant growth and yield of pepper cv. Karaisali salcalik under field conditions (Table 3). Bacterial applications increased stem diameter by 3.0-28.0%, but only the dual combinations M 1-3 + M 3-1 and M 3-1 + H 8-8 and the strain M 3-1 significantly increased stem diameter compared to the untreated control. All three strains and their combinations significantly affected root elongation but not root dry weight. M 3-1 and the combination M 1-3 + M 3-1 significantly increased yield compared to untreated controls. There were no significant differences in root dry weight and in shoot dry weight due to the application of bacteria.

DISCUSSION

Root colonizing and plant growth promoting bacteria referred to as PGPR affect plant growth by increasing nutrient cycling, suppressing pathogens and producing biologically active compounds (Khalid *et al.*, 2004). The beneficial effects of PGPR and PGPR-mediated disease resistance have been demonstrated under field conditions in recent years (Sid Ahmed *et al.*, 2000; Ramamoorthy *et al.*, 2001). Several biocontrol agents alone or in combination (Sid Ahmed *et al.*, 1999, 2003; Ezziyyani *et al.*, 2004, 2007) have been employed to control pepper blight caused by *Phytophthora capsici* and the root-rot disease complex of chickpea caused by *Meloidogyne incognita* and *Macrophomina phaseolina* (Siddiqui and Akhtar, 2007). This is the first study on biological control of pepper blight in Turkey.

In the study, two methods were used for screening PGPR strains: *in vitro* studies with NBRIP-BPB broth assay and *in vivo* studies under growth room and field conditions. In accordance with Siddiqui and Akhtar (2007), our results indicate that inoculation with phosphate-solubilizing bacteria is useful in controlling phytophthora crown blight under field conditions and in increasing yield. It is known that application of phosphate-solubilizing bacteria to soil or seed helps to improve solubilization of fixed soil phosphorus and to induce plant growth (Broadbent *et al.*, 1977). Treatments

with the strains M 1-3, M 3-1 and their combination significantly reduced disease severity in the two experiments and promoted plant growth and yield. H8-8 was effective in field conditions but not in growth room conditions. It is generally believed that growth room/field experiments are the most reliable methods for selection of beneficial microorganisms, but their results are not always correlated.

Our results showed that all the bacterial strains used and their combinations are effective in controlling pepper blight caused by *P. capsici* in the field. They may be used as promising antagonists for successful management of Phytophthora blight in our region. However, additional studies should be carried out for successful adaptation to commercial pepper production and to explain the mechanisms of action of PGPR bacteria. A root dipping inoculation technique, using phosphate-solubilizing bacterial strains, can be used in high-value transplant crops and seed treatments with these strains may also be useful, effective and easy for many crops (Wei *et al.*, 1991).

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