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Full Length Research Paper

Evaluation of bacteria for biological control of early blightdisease of tomato

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Twenty three bacterial isolate out of 190, exhibiting inhibitory affects against *Alternaria solani* in preliminary tests, were screened for their activity towards *A. solani* Ell. and G. Martin) Sor. by a dual-culture *in vitro* assay on nutrient agar (NA) medium and *in vivo* (whole plant) test. *In vitro* studies indicated that all the 23 bacterial isolates inhibited the mycelial growth of *A. solani* by forming inhibition zone ranging from 9.35 to 31.3 mm. The most effective isolate was *Serratia plymuthica* (IK-139) (31.3 mm) based on the *in vitro* test results. Twenty three bacterial isolates were subjected to a whole plant test to investigate their ability to protect the tomato plant against early blight disease. In whole plant tests, 0.5 x 10^8 cfu/ml bacterial suspension was sprayed and one day later, *A. solani* spores suspension were applied on tomato seedlings and plants were incubated in moist chamber at 20°C with 95% relative humidity (RH) and 12 h photoperiods for 21 days. Based on the whole plant tests, *Paenibacillus macerans-GC* subgroup A (1.82), *Serratia plymuthica* (1.78), *Bacillus coagulans (*1.75), *Serratia marcescens-GC* subgroup A (1.50), *Bacillus pumilis –GC* subgroup B (1.50) and *Pantoea agglomerans* (1.32) bacterial isolates reduced the disease severity of early blight significantly when compared with control. These results suggest that the bacterial isolates studied have a good potential to be used as biocontrol agents of *A. solani* in tomato.

Key words: *Alternaria solani,* early blight, biological control, bacterial isolate.

INTRODUCTİON

Early blight of tomato caused by *Alternaria solani* (Ellis and Martin) Jones and Grout, is one of the most common and destructive diseases of tomato in areas of heavy dew, rainfall and relative humidity. The disease becomes wide spread and serious, causing large economic loss to the growers when the season begins with abundant moisture or frequent rains followed by warm and dry weather which are unfavourable for the host and help in rapid disease development (Agrios, 1988). The fungus can cause disease on foliage (leaf blight), stem (collar rot) and fruit, and can result in severe damage during all stages of plant development (Nash and Gardner, 1988).

The leaf blight phase, commonly referred to as early blight, is the most important phase of the disease and can result in complete loss of the crop when incidence is severe (Kallo and Banerjee, 1993). High humidity and the ineffectiveness of fungicides due to frequent heavy rainfall increases the disease intensity. Even under irrigated conditions, susceptible hybrids can be severely damaged by early blight incurring a loss of 50 to 80% (Mathur and Shekhawat, 1986). The control of tomato early blight disease has been almost exclusively based on the application of chemical fungcides. Several effective fungcides have been recommended for use against this pathogen, but they are not considered to be long-term solutions, due to concerns of expense, exposure risks, fungicide residues and other health and environmental hazards. In an attempt to modify this condition, some alternative methods of control have been adopted. Recent efforts have focused on developing environmentally safe, long lasting and effective biocontrol methods for the management of plant diseases. Use of

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Abbreviations: PDA, Potato dextrose agar; **NA,** nutrient agar; **NB,** nutrient broth; **PDA,** potato dextrose agar.

biocontrol agents has been shown to be eco-friendly and effective against many plant pathogens. It has been reported that *Verticillium psalliotae* (Yiğit and Turhan, 1994), *Fusarium sambucinum* Fuckel (*Gibberella pulicaris* (Fr.) Sacc.) (Klikov et al., 1983), *Trichothecium roseum* (Pers. Fr.) Link (Dragoescu, 1986), *Aurebasidium pullulans* (Yiğit, 1993) prevent leaf and stem blight caused by *A. solani* by inhibiting spore germination. The *Streptomyces* species and *Bacillus subtilis* (Sharma and Sharma, 2006; Sid et al., 2005; Mateascu et al., 2002) were also being used to inhibit mycelial growth and spore germination of *A. solani*. Therefore, biocontrol agents may provide a seemingly environmentally friendly alternative to potent and toxic fungicides, which cannot be broken down in the environment.

The aim of present study is to test the *in vitro* and *in vivo* biocontrol abilities of some bacterial agents on *A. solani* causal agent of tomato early blight disease.

MATERALS AND METHODS

Isolation of pathogen and microorganisms

A. solani conidiospore that were taken from infected tissue of diseased tomato fruits were inoculated on water agar medium (2%; 2 g agar/100 ml sterile distillate water). The plates were incubated at 25°C for 3 days. Single spore colony were transferred to the petri dishes containing potato dextrose agar (PDA) to obtain pure culture of the pathogen.

One hundred and ninety bacterial isolate which were provided by Assist. Prof. Dr. Isa Karaman (Department of Biology, Faculty of Science, Gaziosmanpasa University, Tokat, Turkey) from different hosts such as aphid, mosquito's larval, formic, beetle, locust, apple and pear boughs were tested against *A. solani* as an biocontrol agents in *in- vitro* and *vivo* tests.

Effect of bacteria on growth of *A. solani ın vitro* tests

Twenty three bacterial isolate out of 190, exhibiting inhibitory affects against *A. solani* in preliminary tests, were screened for their activity towards *A. solani* by a dual-culture in *in vitro* assay on nutrient agar (NA) medium. Bacterial suspension (0.5×10^8 CFU ml^{-1} culture) was streaked on the center of NA plates. After incubation at 28°C in the dark for 24 h, 5-mm-diameter plugs cut from the leading edge of a 10-day-old culture of *A. solani* on PDA was placed on both side of the plate, 4 cm from the center. NA inoculated with the pathogen alone was used as control. Plates were incubated at 28°C for 5 days, at which time inhibition zones were measured with calipers. Three 9-cm-diameter plates containing 15 ml of NA were used for each treatment. The experiment was repeated twice.

Effect of bacteria on *A. solani*, using whole tomato plants

The bacterial isolates which have inhibitory affects on the pathogen *in vitro* tests were subject to biological test to determine biocontrol efficacy on plant to *A. solani*. Promising bacterial isolates were cultured in line NA medium, the plates were incubated at 28°C for 48 h. Bacterial isolates growing in plates were taken by loop in nutrient broth (NB). Bacterial suspension of 0.5×10^8 cell/ml from each isolates were prepared. *A. solani* (Ellis and Martin) Jones and Grout, previously obtained from naturally infected tomato plants in Tokat, were used in this study. The cultures were grown on potato dextrose agar (PDA) in 9 cm Petri plates and incubated at 21 ± 2°C under a cool-white fuorescent light with a 12 h photoperiod. After 10 to 14 days, conidia were harvested by loading the plates with distilled water (containing 0.01% of surfactant Tween-20) and brushing the agar surface with a paint-brush. The spore concentration in the suspension medium was measured using a haemocytometer, and it was adjusted to 2×10^4 conidia/ml before inoculation.

The seeds of tomato cultivar Yanki F1 were sown in 10 cm diameter pots (1 plants/pot) containing peatmoss. Seedlings with four to five expanded leaves growing in pots in the glasshouse were inoculated with the suspension of bacterial isolate (0.5×10^8 cfu/ml) using a hand sprayer. After one day, *A. solani* spore suspension (2×10^4 conidia/ml) was applied in the same manner to the tomato plants and were kept in a controlled environment cabinet (20°C and 90% relative humidity) for 21 days, for disease development to occur. Positive controls were sprayed with nutrient broth plus *A. solani* spore suspension. The experiment was repeated twice. At 21 days after challenge inoculation, disease severity was recorded using 0 to10 (0- no lesions on leaflets, 10 = 100% lesions on leafs) disease rating scale (Falloon et al., 1995). All data of pot experiments were subjected to one-way analysis of variance (ANOVA) (SAS Institute) and means comparisons using least significant differences (LSD) at $p = 0.05$.

RESULTS AND DISCUSSION

Effect of bacteria on growth of *A. solani ın vitro* tests

A total of 190 bacterial isolates were screened for their ability to suppress *A. solani* in an *in vitro* dual-culture assay. Initially, 23 (about 12%) isolates were active against *A. solani*. Inhibition was clearly discerned by limited growth, or the complete absence of fungal mycelium in the inhibition zone between the fungus and antagonist bacterial colony.

All the twenty three bacterial isolates strongly inhibited the growth of *A. solani* by forming inhibition zones larger than 5 mm on NA. *Serratia plymuthica* (İK-139) isolate had the highest inhibitory effect with inhibition zone of 31.3 mm on *A. solani*. Second most effective bacterium was *S. plymuthica* (İK-150) isolate with inhibition zone of 27.55 mm, followed by *B. subtilis* (İK-92), *Serratia marcescens-GC subgroup A*(IK-174), *Pantoea agglomerans* (IK-147) and *Burkholderia pyrorocinia* (IK-145) with inhibition zone of 26.6, 23.75, 21.05 and 20.25 mm, respectively. On the other hand, *B. subtilis* (İK-159) and *Brevibacillus brevis* (İK-146) produced zone of inhibitions lower than 10 mm (Table 1).

Effect of bacteria on *A. solani*, using whole potato plants

The 23 bacterial isolates used were initially selected from a population of 190 by *in vitro* screening for antagonism to *A. solani* on NA media. Foliar application of *Paenibacillus macerans-GC subgroup A* (İK-36), *S. plymuthica* (İK-139), *Bacillus coagulans* (İK-22), *S. marcescens-GC subgroup A* (İK-174), *Bacillus pumilis* –

Table 1. Effect of bacterial isolates on growth inhibition of *A. solani*.

Code	Bacterial isolates	Inhibition zone (mm)
İK–159	*Bacillus subtilis*	9.35
İK-146	*Brevibacillus brevis*	9.65
İK–88	*Cellulomonas turbata*	10.35
İK–164	*Bacillus lentimorbus*	12.35
İK–22	*Bacillus coagulans*	12.4
İK–89	*Bacillus coagulans*	13
İK–34	*Bacillus cereus-GC* subgroup A	13.35
İK–104	*Bacillus amyloliquefaciens*	13.45
İK–178	*Brevibacillus agri*	13.5
İK–16	*Burkholderia cepacia*	14.4
İK–132	*Bacillus lentimorbus*	14.6
İK–36	*Paenibacillus macerans-GC* subgroup A	14.95
İK–81	*Micrococcus luteus-GC* subgroup C	16.05
İK–57	*Brevibacillus laterosporus*	16.45
İK–55	*Paenibacillus apiarius*	16.65
İK–83	*Bacillus subtilis*	17.8
İK–91	*Bacillus pumilis –GC* subgroup B	18
İK–145	*Burkholderia pyrorocinia*	20.25
İK–147	*Pantoea agglomerans*	21.05
İK–174	*Serratia marcescens-GC* subgroup A	23.75
İK–92	*Bacillus subtilis*	26.6
İK–150	*Serratia plymuthica*	27.55
İK-139	*Serratia plymuthica*	31.3

GC subgroup B (İK-91) and *P. agglomerans* (İK-147) significantly (P < 0.05) reduced the disease severity of early blight with 1.82, 1.78, 1.75, 1.50, 1.50 and 1.32, respectively, when compared with that of control treatment (Table 2). On the other hand, foliar application of *B. subtilis* (İK-83 and İK-159), *Bacillus cereus* (İK-34), *Bacillus amyloliquefaciens* (İK-104) and *Paenibacillus apiarius* (İK-55) were not different from the control (Table 2).

Twenty three bacterial isolates were initially selected from all isolates tested; and these isolates showed antagonistic properties against the isolate of *A. solani* in the dual culture experiments. No physical contact was observed between any of the bacteria tested and *A. solani*; moreover, an inhibitory halo was observed suggesting the presence of fungistatic metabolites secreted by the bacteria. In the present study, the highest level of inhibition zone (31.3 mm) was obtained between *S. plymuthica* (IK-139) and *A. solani*. The second most largest inhibition zone was produced by *S. plymutica* (IK-150). Similarly, Gould et al. (2008) reported that *S. plymuthica* 5-6 suppressed growth of *F. sambucinum* by 60 and 67% at 15 and 25°C, respectively, when compared to *F. sambucinum* alone. Stanley et al. (1994) reported that *S. plymuthica* CL43 strain reduced rotting of *Botrytis cinerea* and *Alternaria brassicicola* on cabbage under storage conditions. Production of antifungal metabolites, competition for nutrients and space may account for supression of *A. solani in vitro* by the *S. plymutica* isolates. It has been shown that *S. plymutica* A 153 isolate from the roots of winter wheat, produces antifungal metabolites including chlorinated macrolides and haterumalide (NA, B, NE and X), which suppressed apothecial formation in *Sclerotinia sclerotiorum* and slowed spore germination of several filamentous fungi. *S. plymuthica* also produces pyrrolnitrin and 1-acetyl-7-chloro-1-H-7-chloro-1-H-indole which suppresses fungal spore germination (Kamensky et al., 2003). Similarly, *B. subtilis* (IK-92) and (IK-83) also inhibited *A. solani* with inhibition zone of 26.6 and 17.8 mm, respectively. Production of zones of inhibition at the boundary with the pathogen agrees with the report of Basım (1990) that *in vitro A. solani* interactions of *B. subtilis* AB-27 and AB-2 strains resulted in production of the highest zone of inhibition. The zones of inhibition produced might be due to the production of antifungal metabolites by the *B. subtilis* isolates. It was reported that *B. subtilis* can secrete several antifungal metabolites such as subtiline, bacitracin, bacillin and bacillomycin, which belong to the iturine family (Alippi and Mónaco, 1994).

In our study, many of the bacteria that showed direct antagonism toward *A. solani* on Petri dishes were also effective in whole plant tests by reducing disease severity. Based on the results of whole plant tests, particularly

Table 2. Effect of bacterial isolates on early blight severity of tomato.

Code	Bacterial species	Average disease severity[1]
	Control	5.27 ab[2]
İK-83	*Bacillus subtilis*	5.60 a
İK-34	*Bacillus cereus-GC* subgroup A	5.55 a
İK-92	*Bacillus subtilis*	5.47 a
İK-159	*Bacillus subtilis*	5.42 a
İK-104	*Bacillus amyloliquefaciens*	4.73 abc
İK-55	*Paenibacillus apiarius*	4.23 bc
İK-57	*Brevibacillus laterosporus*	4.00 c
İK-89	*Bacillus coagulans*	3.75 cd
İK-164	*Bacillus lentimorbus*	2.85 ed
İK-132	*Bacillus lentimorbus*	2.58 ef
İK-88	*Cellulomonas turbata*	2.50 efg
İK-16	*Burkholderia cepacia*	2.48 efg
İK-150	*Serratia plymuthica*	2.45 efg
İK-178	*Brevibacillus agri*	2.28 efgh
İK-81	*Micrococcus luteus-GC* subgroup C	2.22 efgh
İK-145	*Burkholderia pyrorocinia*	2.10 efgh
İK-146	*Brevibacillus brevis*	2.10 efgh
İK-36	*Paenibacillus macerans-GC* subgroup A	1.82 efgh
İK-139	*Serratia plymuthica*	1.78 efgh
İK-22	*Bacillus coagulans*	1.75 efgh
İK-174	*Serratia marcescens-GC* subgroup A	1.50 gh
İK-91	*Bacillus pumilis –GC* subgroup B	1.50 gh
İK-147	*Pantoea agglomerans*	1.32 h

[1]Disease severity on a 0 to 10 rating scale was measured 21 days after inoculation where (0 = no lesions on leaflets, 10 = 100% lesions on leafs); [2]values followed by the same letter within each column do not differ significantly according to LSD test at $P < 0.05$.

P. macerans-GCsubgroup A (İK-36), *S. plymuthica* (İK-139), *B. coagulans* (İK-22), *S. marcescens-GC subgroup A* (İK-174), *B. pumilis –GC subgroup B* (İK-91) and *P. agglomerans* (İK-147) strains reduced disease severity of *A. solani* significantly when compared with control. It was reported that *S. marcescens* isolate reduced disease severity of *Sclerotiorum rolfsii* up to 75% in bean, peanut and cicer grown in *S. rolfsii* infested soil (Ordenlich et al., 2002). Several isolates of *S. plymuthica* have been reported as being highly effective biocontrol agents for a variety of plant diseases including *Verticillium dahliae* on strawberry (Kurze et al., 2001; Scherwinski et al., 2007) and *Phytophthora capsici* blight on pepper (Shen et al., 2007). Kamensky et al. (2003) demonstrated that spray-ing of *S. plymuthica* strain IC14 to cucumber seedling leaves efficiently reduced disease incidence caused by *B. cinerea* and *S. sclerotiorum* (about 80% disease reduc-tion). Similar to the findings of the present study, Kiewnick et al. (2000) reported that *P. agglomerans* reduced seed blight severity of barley caused by *Pseudomonas syringae pv. syringae* up to 74% in field. Even though several bacteria such as *B. subtilis* (IK-83, IK-92 and IK-159), *B. cereus-GC* subgroup *A* (IK-34) and *B. amyloliquefaciens* (IK-104) were very effective against *A. solani in vitro* tests, they provided no protection on whole plant when challen-ged with *Phytophthora infestans*. This suggests that these bacteria are perhaps weak competitors when cha-llenged with *A. solani*.

Even though a relatively small number of bacteria isolates were tested, promising results were obtained regarding the selection of tomato early blight biocontrol agents. Further field studies must be conducted to analyze the real potential of these bacteria agents in field conditions. Studies are also needed to determine the modes of action of those bacteria, the population density of the applied bacteri, and the best form of introduction into the host.

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