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Full Length Research Paper

Screening of rhizobacteria against *Fusarium oxysporum* f. sp. *melongenae*, the causal agent of wilt disease of eggplant

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Rhizosphere isolates of *Bacillus* and *Pseudomonas* species isolated from the roots of eggplants (*Solanum melongena* L.) were collected in eggplant cultivated areas in various geographical districts of Turkey. Isolates were evaluated for siderophore, protease and cyanide production along with the cell wall degrading enzyme production as well as phosphate solubilizing and nitrogen fixing capacities. The isolates showing good performance were tested against *Fusarium oxysporum* f. sp. *melongenae,* a highly destructive fungal agent for eggplants, for the antagonistic activities. The isolates designated as 11-4, 13-1, 56K-3, 65A-3, 67A-2, 76A-1, 85A-2, 10a, 318b, 346a, 379c, 007-1, 007-4, and 048-2 were found promising in inhibition of *F. oxysporum* f. sp. *melongenae in vitro* conditions. The isolates differing in their antagonistic responses to *F. oxysporum* f. sp. *melongenae* would be identified in species level. Also, the best performing isolates for the reduction of disease severity and increased plant growth would be further evaluated via more biochemical parameters such as indole acetic acid production (IAA) and total antioxidant status (TAS).

Key words: *Pseudomonas* (spp.), *Bacillus* (spp.), *Fusarium oxysporum*, rhizobacteria, mycelial inhibition, siderophore production.

INTRODUCTION

Fusarium wilt in eggplants caused by *Fusarium oxysporum Schlecht.* f. sp. *melongenae* has wider geographic distribution than ever before. Fungus initially was described by Matuo and Ishigami (1958) in Japan. Later reports were from Florida, (USA) (Alfieri et al., 1984), Korea (Cho and Shin, 2004), Italy (Richardson, 1990), Greece (Holevas et al., 2000), Spain (Urrutia Herrada et al., 2004); Turkey (Altınok, 2005) and from China (Zhuang, 2005).

The fungal agent enters to the plant from young roots and then blocks the transport of water and nutrients in the vessels via macro and micro conidia. The fungus causes leaf chlorosis and slight vein clearing on outer leaflets, followed by wilting and dropping of leaves, then xylem browning of the stem and finally death of the aboveground parts (Figure 1) (Altınok, 2005).

There is no effective, economical and practical ways to control *Fusarium* wilt disease. Crop rotation, using resistant varieties, soil sterility, fungicides and solarization are recommended against the disease. Efficient soil sterility via chemicals is not effective due to re-colonization of air-borne spores of *Fusarium oxysporum* under favorable conditions (Yücel et al., 2007). Today there are strict regulations on chemical pesticide use because of harmful effects on the environment and their undesirable effects on non-target organisms and possible carcinogenicity of some chemicals (Agrios, 1988; Dikilitas and Kocyigit, 2010). Fungicides with broad spectra also results in imbalances within the microbial community

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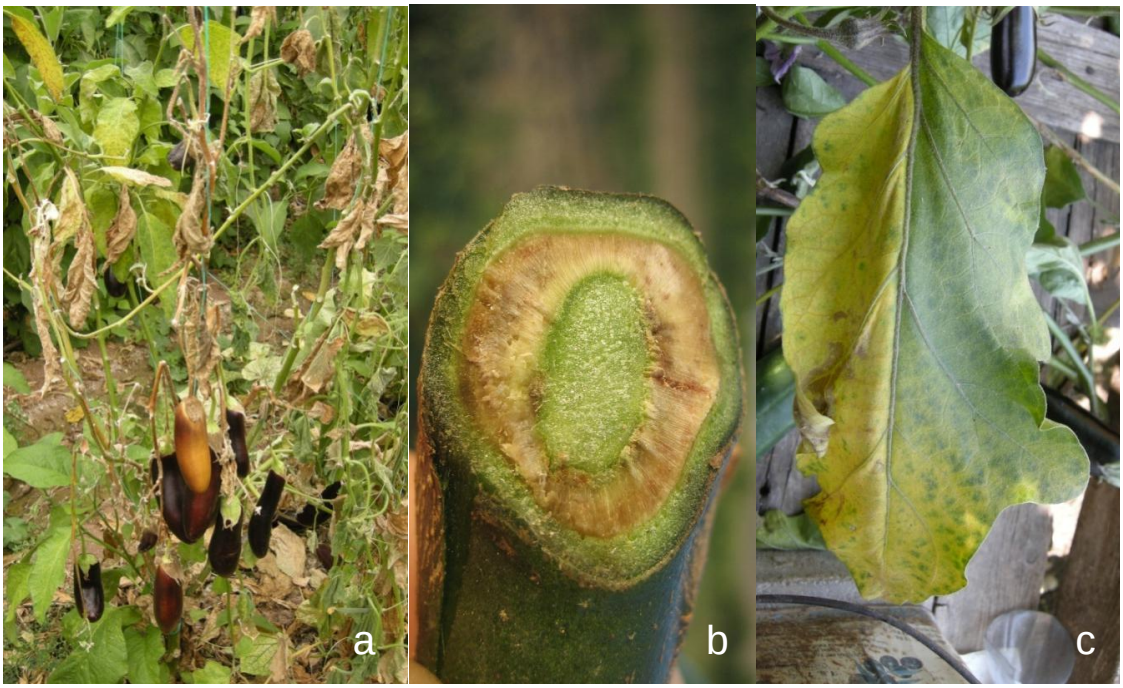

Figure 1. Disease symptoms of *Fusarium* wilt of eggplant (a) Symptoms on plant, (b) vascular bundles and (c) leaves.

creating unfavorable conditions for the activity of beneficial organisms (Villajuan-Abgona et al., 1996).

Soil-borne diseases have been controlled more recently by means of certain beneficial bacteria that are indigenous to the rhizosphere of plants (Thomshaw, 1996). The rhizosphere, representing the thin layer of soil surrounding plant roots and the soil occupied by the roots, supports large and metabolically active groups of bacteria known as plant growth promoting rhizobacteria (PGPR) (Kloepper et al., 1980). PGPR rapidly colonize the rhizosphere and suppress the pathogen micro-organisms at the root surface (Rangajaran et al., 2003). These organisms can also be beneficial to the plant by stimulating growth (Bloemberg and Lugtenberg, 2001). Plant growth-promoting ability of these rhizobacteria is possible because of the production of siderophores, antibiotics and phytohormones (Defago and Haas, 1990; Neiland, 1995). Recent studies have demonstrated the importance of strains of non-pathogenic PGPR in enhancing plant resistance (Silva et al., 2004; Maleki et al., 2011; Saharan and Nehra, 2011).

Currently, there is very limited knowledge regarding the biological suppression of *Fusarium* wilt disease in eggplant by the application of PGPR in Turkey. The aim of this study is therefore to isolate the bacteria from the eggplant rhizosphere and screen the organisms for *in vitro* antagonistic activity against *F. oxysporum* f. sp. *melongenae* in Turkey. This study may contribute to the introduction of PGPR systems in biological control of phytopathogenic fungi in eggplant and other crops.

MATERIALS AND METHODS

Soil sample collection and analysis

In 2009, nineteen samples were collected from the rhizosphere of eggplant from Akarsu, Yeniçay and Kelahmet towns of Tarsus in Mersin province. Besides this, seven soil samples from Yamula town from Kayseri province, sixteen samples from Akdeniz, Midik and Havutlu towns of Adana province were collected. Totally, 42 soil samples were collected where eggplant is intensively cultivated.

In 2010, totally 38 soil samples were collected from 17 districts of eggplant cultivated areas belonging to 11 major cities; Serik, Finike, Demre, Manavgat and Kumluca (Antalya), Salihli and Kula (Manisa), Bozyazi (Mersin), Yatağan (Muğla), Bayindir (İzmir), Orhangazi (Bursa), Hatay (İskenderun), Bafra (Samsun), Nazilli (Aydin), Birecik, Akçakale, (Şanliurfa) and Bismil (Diyarbakir) provinces.

Isolation of bacteria

Approximately, 1 kg of soil sample obtained from eggplant root zone was placed in a paper bag and brought to the laboratory. Soil samples were dried and sieved using 0.2 mm pore size soil sieve. Ten g of soil sample was then mixed with 100 ml phosphate buffered saline (PBS) solution and shaken for 2 h at room temperature. This solution was serially diluted and 0.1 ml aliquot of the serially diluted suspension was spread-plated on King's B medium (King et al., 1954) in triplicate. The spread-plate cultures were incubated for 48 h at 25 ± 1°C. Ten to fifteen representative colonies, with different morphological appearances, were selected from the countable plates and re-streaked on a new plate containing the same media to obtain pure colonies. A total of 261 candidate isolates obtained in this manner were maintained on yeast extract dextrose agar slants. Isolates were tested for oxidase

reaction, pectolytic activity and hypersensitive reaction on tobacco plants (Lelliott and Stead, 1987).

Determination of phosphate-solving capacity of bacteria

Phosphate-solubilization test was conducted by inoculating the bacteria on National Botanical Research Institute's phosphate growth medium (NBRIP) (Nautiyal, 1999) with sterile toothpick in three replicates. Plates were incubated at 25 ± 1°C for 4 days. The presence of clearing zone around the bacterial colonies was determined as phosphate-solving isolates.

Determination of nitrogen fixation capabilities

Jensen growth medium (Ahmad et al., 2005) was used to determine nitrogen fixing capacity for bacterial isolates obtained from the soil. Plates containing this medium were inoculated with six different bacteria with three replicates and incubated at 27 ± 1°C for seven days. Growing isolates on Jensen growth medium were recorded as positive for nitrogen fixation.

***In vitro* antagonistic activity**

The *in vitro* inhibition of mycelial growth of *F. oxysporum* by the bacterial isolates was tested using the dual culture technique as described by Landa et al. (1997). Four 50 µl drops from the 10^8 cfu/ml suspension of the bacterial colonies were equal distantly placed on the margins of potato dextrose agar (PDA) (BioLab) plates and incubated at 28°C for 24 h. One agar disc (0.5 cm) from fresh PDA cultures of *F. oxysporum* was placed at the centre of the PDA plate for each bacterial isolate and incubated at 27 ± 1°C for seven days. Inhibition zones of the fungal colony towards and away from the bacterial colony were measured. The percentage growth inhibition was calculated using the following formula (% inhibition = [(R - r)/R*100]): where, *r* is the radius of the fungal colony opposite the bacterial colony and, *R* is the maximum radius of the fungal colony away from the bacterial colony.

Production of secondary metabolites by the bacterial isolates *in vitro*

Siderophore production

Isolates were assayed for siderophore production on succinate medium containing indicator chemicals (Chaiharn et al., 2008). Plates were spot inoculated with 48 h old bacterial cultures of the test organism and incubated at 30°C for 5 days. Development of yellow-orange halo around the colony was determined as positive for siderophore production.

Cell wall degrading enzyme production

Bacterial isolates were tested for production of protease activity by growing them on skimmed milk agar medium (Cattelan et al., 1999). An ability to clear the skimmed milk suspension in the agar plates was taken as evidence for the secretion of protease. Non inoculated plates were used as control.

For chitin degradation, plates containing chitin agar were used. Cellulase activity was tested on CMC agar plates. Plates were spot inoculated with bacteria and incubated at 30°C for 5 days. Development of halo zone around the colony was considered as positive for cell wall degrading enzyme production.

Production of hydrogen cyanide

To determine hydrogen cyanide production, test organisms (100 µl) were streaked on tryptic soy agar medium. Sterilized filter papers in 1.5 cm diameter were saturated with Picric acid (5.0%, w/v) and placed in the upper lid of the inoculated Petri dishes. Plates were covered with Parafilm and incubated at 30°C for 4 days. Hydrogen cyanide (HCN) production was determined by evolution of reddish brown color change of yellow filter papers. Reactions were evaluated as weak (yellow-light red), medium (brown) and strong (reddish brown). Non inoculated plates with bacteria were used as control.

RESULTS

Isolation and screening

Totally 261 candidate PGPR isolates were obtained from the analysis of soil samples obtained from various locations (Mersin, Adana, Antalya, Kayseri, Manisa, Muğla, Izmir, Hatay, Bursa, Şanliurfa and Diyarbakir) of eggplant plantation areas in Turkey. Strains were negative in producing levan type colonies, oxidase reaction and hypersensitive reaction on tobacco leaves. Colony colors were cream in 147 out of 261 isolates, white in 45, yellow in 61, orange in 3, pink in 2, green in 2 and light brown in 1 isolate.

Phosphate-solving capacity

Two rhizobacteria designated as 007-1 and 007-2 were capable of solubilizing phosphate isolated from Hatay (İskenderun) region (Figure 2). Their phosphate solubilizing index were 1.50 and 1.79 respectively (Table 2).

Nitrogen fixing capacity

Another important trail of PGPR is nitrogen binding capability. Twenty-four isolates were able to grow on Jensen's medium (Table 2).

Antagonistic activity

Antagonistic activities of 261 isolates were tested against *F. oxysporum* f. sp. *melongenae* in dual culture in laboratory. Thirty isolates were found to inhibit the growth of the pathogen ranging from 60-87 % (Table 1). The isolates designated as 11-4, 13-1, 56K-3, 65A-3, 67A-2, 76A-1, 85A-2, 10a, 318b, 346a, 379c, 007-1, 007-4, and 048-2 were found promising in inhibition of *F. oxysporum* f. sp. *melongenae in vitro* conditions.

Production of secondary metabolites of PGPR *in vitro*

Two isolates produced siderophore (007-1, 007-4). Eight

Figure 2. Zones by rhizobacteria on NIBRIP medium.

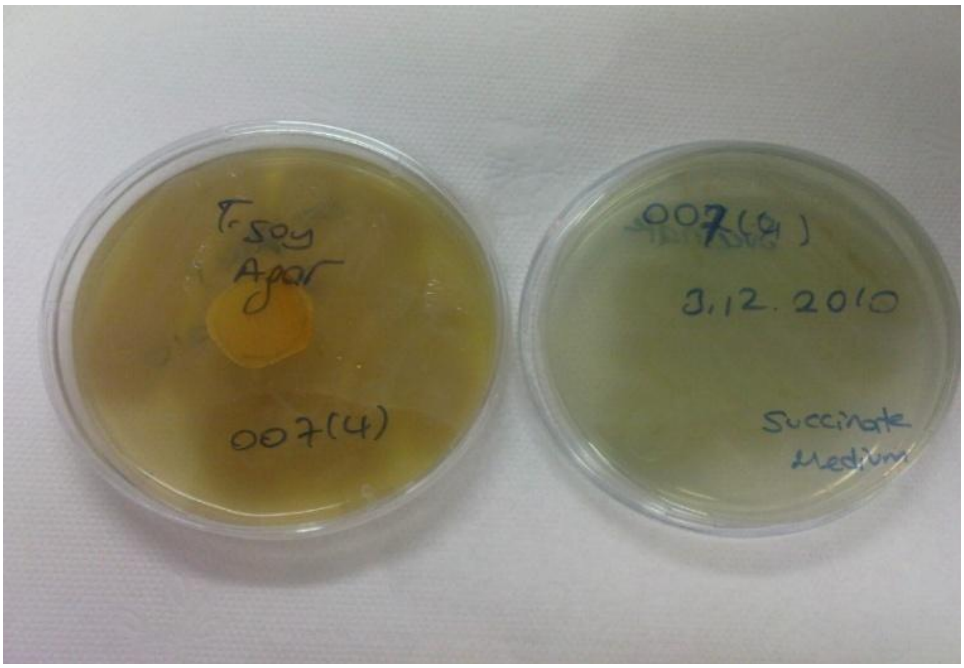

Figure 3. Production of HCN on tryptic soy agar medium.

isolates showed protease activity (85A-2, 318b, 320b, 346a, 379c, 007-1, 007-4, 048-2. All isolates failed to produce chitinase and cellulase activities. Three of them produced hydrogen cyanide (76A-1 007-1, 007-4) (Figure 3 and Table 2).

DISCUSSION

Due to the nature of soil-borne diseases, it is difficult to control the disease progress via the use of pesticides. Although using resistant cultivar is the best option, however, the possibility of emerging of new pathogen races and breaking of resistance of the host defence, led us to use of PGPR, which has recently received more attention because of their activity in plant growth promotion and disease control (Kloepper and Schroth, 1981; Mirik et al., 2008; Akhtar et al., 2010). They are colonizers of plant roots and their flexible metabolism directed toward the dominating substances released by young parts of the roots. Colonization with PGPR results in enhanced plant growth through accumulation of hormones, mineral and water supply (Whipps, 2001). For example, Egamberdieva (2008) reported that the positive effect of PGPR increased the growth of wheat and pea. Similarly, Akhtar et al. (2010) demonstrated that the use

Table 1. Antagonistic activities of potential PGPR isolates on *F. oxysporum* f. sp. *melongenae in vitro.*

Bacterial isolate	Locations	Mycelial inhibition (%)
11-4	Mersin/Tarsus/Alifakı	72.85^{cdef}
13-1	Mersin/Tarsus/Kelahmet	78.37^{bc}
16-2	Mersin/Tarsus/Akarsu	66.09^{efghi}
21-1	Mersin/Tarsus/Akarsu	64.52^{fghi}
56K-3	Kayseri/Yamula	72.56^{cde}
62K-2	Kayseri/Yamula	68.32^{defgh}
62K-3	Kayseri/Yamula	60.54^{hi}
65A-3	Adana/Akdeniz	72.07^{cdef}
67A-2	Adana/Akdeniz	67.08^{efghi}
73A-1	Adana/Havutlu	64.15^{fghi}
73A-2	Adana/Havutlu	62.23^{ghi}
75A-4	Adana/Havutlu	61.02^{hi}
76A-1	Adana/Havutlu	67.87^{defghi}
81A- 4	Adana/Mıdık	60.14^{i}
85A-2	Adana/Mıdık	73.06^{cde}
2c	Antalya/Serik	62.39^{ghi}
3b	Antalya/Serik	72.94^{cdef}
8b	Antalya/Demre	60.27^{hi}
9b	Antalya/Manavgat	61.89^{hi}
10a	Antalya/Manavgat	86.21^{a}
12b	Antalya/Kumluca	68.6^{defgh}
318a	Manisa/Kula	70.62^{cdefg}
318b	Manisa/Kula	74.78^{cde}
320d	Mersin/Bozyazı	70.45^{cdefg}
320b	Mersin/Bozyazı	62.16^{ghi}
346a	Manisa/Kula	73.49^{cde}
379c	Muğla/Merkez	78.31^{bc}
007-1	Hatay/İskenderun	85.05^{ab}
007-4	Hatay/İskenderun	87.47^{a}
048-2	Bursa/Orhangazi	75.09^{cd}
Control		0.00^{j}

Data are means of at least three replicates. Means followed by different letters in columns are significantly different at $P<0.05$ as determined by Duncan's multiple range test.

of *Bacillus pumilus, Pseudomonas alcaligenes*, and *Rhizobium* sp. improved the growth of *F. oxysporum*-inoculated plants by competing with the pathogen and the production of essential nutrients and antibiotics. General concept of the success of PGPR was attributed to the inhibitory effects of antagonistic organisms. For example, *Bacillus* and *Pseudomonas* spp. significantly inhibited the propagation and mycelial colonization of the pathogens by rapidly colonizing in the vascular stele of roots of crop plants in advance of the pathogens and resulting in induced against the fungal pathogens such as *F. oxysporum* and *Macrophomina phaseolina* (Akhtar and Siddiqui 2007). The rhizobacteria also increase the content of nitrogen, which leads to increase of plant growth and therefore, they indirectly result in reduced disease severity (Akhtar et al., 2010). Secondary metabolites such as siderophores and hydrogen cyanide production by the rhizobacteria are suspected to be responsible for the suppression of several soil-borne pathogenic fungi (Kloepper et al., 1980; Scher and Baker, 1982). In our study, we screened and selected rhizobacteria against *F. oxysporum* f. sp. *melongenae* from rhizosphere of eggplant out of 261 isolates of bacteria for the first time. Thirty of these isolates caused considerable reduction in growth of the fungi ranging from 60 to 87%. Two of these isolates produced siderophore in culture. Two of them solved phosphate, which was found established in the rhizosphere. However, they were low in numbers to compete with other rhizospheric bacteria. Beside this, 20 of them were found in fixing nitrogen. Eight of them showed protease activity (casein degradation) and three of them produced hydrogen cyanide. No isolates were able to

Table 2. The production of secondary metabolites produced by bacterial isolates.

Bacterial isolates	Production of secondary metabolites				
	Nitrogen binding	Phosphate solubilization	Siderophore production	HCN production	Protease activity
11-4	+	-	-	-	-
13-1	+	-	-	-	-
16-2	+	-	-	-	-
21-1	-	-	-	-	-
56K-3	+	-	-	-	-
62K-2	-	-	-	-	-
62K-3	+	-	-	-	-
65A-3	-	-	-	-	-
67A-2	+	-	-	-	-
73A-1	-	-	-	-	-
73A-2	+	-	-	-	-
75A-4	-	-	-	-	-
76A-1	+	-	-	WR	-
81A- 4	+	-	-	-	-
85A-2	+	-	-	-	+
2c	+	-	-	-	-
3b	+	-	-	-	-
8b	+	-	-	-	-
9b	-	-	-	-	-
10a	+	-	-	-	-
12b	+	-	-	-	-
318a	+	-	-	-	-
318b	+	-	-	-	+
320d	+	-	-	-	-
320b	+	-	-	-	+
346a	+	-	-	-	+
379c	+	-	-	-	+
007-1	+	+	+	+	+
007-4	+	+	+	+	+
048-2	+	-	-	-	+
Control					

WR = weak reaction.

produce chitinase and cellulase. In the light of our preliminary work, we hypothesize that the application of PGPR could help in biocontrol of *F. oxysporum* f. sp. *melongenae* in eggplant cultivated areas. We would also seek the possibility and the ratio of mixture of rhizobacteria with the combination of resistant eggplant cultivars to ensure the highest plant defense response against *Fusarium* wilt. Genetical studies aiming to improve the colonization capacity and enhancing the production of secondary metabolites would also enable the plants to defend themselves more efficiently. We would also seek the possibility and the ratio of mixture of most successful rhizobacteria including 007-1 and 007-4 with the combination of eggplant cultivars to ensure the highest plant defense response against *Fusarium* wilt.

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