The Journal of Animal & Plant Sciences, 21(4): 2011, Page: 758-763
ISSN: 1018-7081

BIOCONTROL OF *BOTRYTIS CINEREA* ON STRAWBERRY FRUIT BY PLANT GROWTH PROMOTING BACTERIA

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ABSTRACT

In the present study, a total of 186 bacterial strains isolated from various soil sources and plant species from Eastern Anatolia region in Turkey were evaluated for their ability to suppress gray mold (*Botrytis cinerea* Pers. ex Fr.) occurred on strawberry cv. Fern. Among 186 bacterial strains, 36 were found effective to inhibit of development *B. cinerea* under in vitro conditions, and thirteen of them which have greater inhibition zone were selected as biocontrol agent. These antagonistic strains were identified as *Bacillus lentimorbus, B. megaterium, B. pumilis, B. subtilis, Enterobacter intermedius, Kurthia sibirica, Paenibacillus polymyxa* and *Pantoea agglomerans*. The inhibition zones among bacteria against *B. cinerea* were found between 0.50 (*Bacillus* C6, *Brevibacterium* MFD-47 and *Pantoea* MFD-232) and 3.75 cm (*Enterobacter* MFD-81) *in vitro*. The strawberry fruits were inoculated with *B. cinerea* in laboratory trials and *Bacillus* MFDÜ-2 (14.41 mm) was found more effective to prevent mycelial development on strawberry fruits in comparison to the control (19.20 mm). In terms of conidia germination on strawberry fruits, the lowest disease incidence was observed in MFD-45 treatment (20.8%), while disease incidence rate for the control was 79.2%. It was shown that antagonistic bacterial strains inhibited *B. cinerea* and that they have a potential use in sustainable strawberry production.

Keywords: Gray mold, *Fragaria x ananassa*, antagonism, postharvest, biocontrol.

INTRODUCTION

The disease can cause important fruit losses on strawberry plants before or after harvest worldwide and it is estimated that they can cause yield losses up to 25% for untreated strawberries (Williamson *et al*. 2007; Zhang *et al*. 2007). Gray mold is also a major cause of postharvest losses of strawberry fruits during storage, transportation or shipment. In strawberry, the fungus can attack flowers, fruits and leaves as well (Sutton and Peng 1993). Infection may occur in the flower, remain quiescent until fruits mature, and then develop abundantly, causing fruit decay accompanied by profuse sporulation of the pathogen (Kovach *et al*., 2000). Therefore *B. cinerea* infections are largely limited postharvest life of strawberry fruits. Control of *B. cinerea* on strawberries can be achieved with the frequent application of fungicide; however, resistance of the pathogen to common fungicides is well known. The fungicide application may also cause to remain toxic residues on the fruits (Rabolle *et al*. 2006; Myresiotis *et al*. 2007). Moreover, commercially available fungicides can reduce pollination and cause misshapen fruits due to time of application (Kovach *et al*., 2000).

Therefore the difficulty in controlling *B. cinerea* has led to researchers to find alternative methods, which include biological control (De Waard *et al*. 1993; Smilanick 1994; Sutton 1995). Biological control is an alternative to reduce *Botrytis* infections and has been shown to be successful in many other crops (Redmond *et al*. 1987; Saligkarias *et al*. 2002). This method was also applied succesfully in strawberries against *Botrytis* (Sutton 1995; Lima *et al*. 1997) and the effectiviness of several biological control agents against gray mold in strawberries are reported (Peng and Sutton 1991; Swadling and Jeffries 1996; Lima *et al*. 1997; Guinebretiere *et al*. 2000; Essghaier *et al*. 2009). In these studies the biological control agents have been used on strawberry flowers and leaves. However there are few studies regarding biological control on strawberry fruits against *B. cinerea*. Therefore, in the present study, selected PGPB antagonists were evaluated for effectiveness in suppressing growth of *B. cinerea* in strawberry fruit under laboratory conditions.

MATERIALS AND METHODS

Source and maintenance of antagonistic bacteria and pathogenic fungus: A total of 186 Plant growth promoting bacteria (PGPB) strains including genera *Acinetobacter, Aeromonas, Agrobacterium, Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Brevibacillus, Brevibacterium, Brevundomonas, Enterobacter, Hydrogenophaga, Kurthia, Paenibacillus, Pseudomonas, Erwinia, Pantoea, Rhodococcus, Variovorax, Microbacterium* and

Flavobacterium found at Department of Plant Protection of Ataturk University were used as antagonistic bacteria. These bacteria were isolated from soil and various plant species (walnut, bean, grape and strawberry) from Eastern Anatolia Region in Turkey. Bacteria were grown on Nutrient Agar (NA, Difco) for routine use, and maintained in Nutrient Broth (NB, Difco) with 15% glycerol at –80ºC (Nuaire, USA) for long-term storage. *B. cinerea* isolate has been supplied by Department of Plant Protection, University of Cukurova, Adana, Turkey. This isolate was cultivated on Potato Dextrose Agar (PDA, Difco) medium.

Detection of antagonistic activity in vitro assay: The 186 PGPB strains were used for pre-evaluation against an isolate of *B. cinerea in- vitro* plate assay. PDA plates were inoculated by a streak of the antagonistic bacterial strains. A disc (7 mm in diameter) of the fungi was punched out with a sterilized corkborer from advancing zones of the fresh culture, and placed on either side of bacteria inoculated plates. The petri plates were incubated at 25±2°C for 5 days. The diameters of hyaline inhibition zones were measured and experiment was repeated three times. After pre-evaluation, 36 strains formed various levels of inhibition zone against *B. cinerea* (Table 1).

Detection of inhibition of mycelia growth on strawberry fruit: The study was conducted at the Ataturk University, Department of Horticulture and Plant Protection in Turkey in 2007 and 2008. Day-neutral strawberry cv. 'Fern' was used as material. Among pre-selected 36 PGPB strains, 13 strains which formed higher inhibition zone against *B. cinerea* were used as antagonists in this experiment (Table 1). Green strawberry fruits were sampled from cv. Fern in the field conditions. A disc (7 mm in diameter) of fungi was punched out with a sterilized corkborer from advancing zone of the fresh culture, was placed on strawberry fruits. A disc without fungi was used as a negative control. Negative and positive control fruits were sprayed with sterile distillated water. After inoculation of pathogenic fungi, antagonistic bacteria were sprayed on inoculated fruits. These bacterial strains were grown on nutrient agar. A single colony was transferred to 500 ml flasks containing NB, and grown aerobically in flasks on a rotating shaker (150 rpm, Gerhard, Germany) for 48 h at 27ºC. The bacterial suspension was then diluted in sterile distilled water to a final concentration of $1x10^9$ CFU/ml, and the resulting suspensions were used to treat strawberry fruit. Inoculated fruits were incubated in damp chambers in a climatic cabinet (25°C and dark). Four days after inoculation of fungi and bacteria, the diameter of mycelial growth on fruit were measured and mycelial growth area was calculated. The experiment was conducted as completely randomized design with three replicates per treatment, 6 fruits in each replicate (14 treatments x 3 replicates x 6 fruits = 252 fruits in total) and experiment was repeated three times.

Detection of inhibition of conidial germination on strawberry fruit: The thirteen cultures (Table 1) that inhibited mycelial growth in vitro were screened for inhibition of conidial germination on freshly harvested strawberry fruit that had been artificially inoculated with *B. cinerea*. Strawberry fruits were harvested from field, sorted and transported to the laboratory. Fruits were dipped in a suspension of 10^4 *B. cinerea* conidia mL^{-1}, allowed to dry for 1 h and inoculated with 1 h bacterial suspensions ($1x10^9$ CFU/ml). Control fruits dipped in conidia, dried and dipped in nutrient broth diluted 1:1 with sterile distilled water, were placed in damp chambers in a climatic cabinet. Fruits were incubated for 4 days at 25°C, before being scored for percent of fungal infection, by examining each fruit for visual signs of infection. The experiment was conducted as completely randomized design with three replicates per treatment, 8 fruits in each replicate (14 treatments x 3 replicates x 8 fruits = 336 fruits in total) and experiment was repeated three times.

Data analysis: Data were evaluated by analysis of variance, and means were separated by Duncan's multiple range tests (Duzgunes *et al.* 1993).

RESULTS

In vitro experiments: In vitro experiments showed that 36 of 186 PGPB strains, including species *Acinetobacter, Bacillus, Brevibacterium, Brevibacillus, Brevundomonas, Enterobacter, Erwinia, Kurthia, Pantoea, Paenibacillus* and *Pseudomonas* inhibited *B. cinerea* growth in various levels (Table 1), producing inhibition zones on PDA plates. Of these, 7 bacterial strains produced an inhibition zone greater than 3.00 cm in diameter, 11 produced inhibition zones between 2.00 and 3.00 cm in diameter and 18 formed an inhibition zone less than 1.00 cm in diameter. 5 days later from incubation, the biggest hyaline inhibition zone was found in *Enterobacter* MFD-81 (3.75 cm), followed by *Bacillus* T33 (3.30 cm). The other 155 PGPB strains did not inhibit the growth of *B.cinerea* on PDA medium.

Effects of PGPB on mycelial growth on strawberry fruits: The data of mycelial growth on strawberry fruits are summarized in Table 2. The bacterial treatments significantly reduced mycelial growth compared to positive control ($P<0.001$). The results also showed that the highest (20.02±1.47 mm) and lowest (14.41±0.83 mm) diameter of mycelial on fruit was observed in CD-8 and MFDÜ-2, respectively. Similarly to mycelia diameter, mycelium area on fruits of CD-8 (317.1±18.44 mm^2) and MFDÜ-2 (166.6±16.54 mm^2) was the highest and the lowest. In the case of bacterial strains, MFDÜ-2,

CD-9, T33, T26, MFD-4, MFD-1, MFD-45, MFD-113 and MFD-18 significantly decreased mycelial growth on fruits compared with the control. The other bacterial strains were found to be ineffective.

Effects of PGPB on conidia germination on strawberry fruits: Bacterial isolates, which inhibited *B. cinerea* growth *in-vitro*, were also tested for their ability to reduce grey mold on strawberry fruits inoculated with *B. cinerea* conidia (Figure 1). Significant reduction of gray mold rot on fruits was observed with the CD-9, MFD-1, MFD-20, MFD-45, MFD-81, MFD-113, MFDÜ-2, T26 and T33 compared to control (P<0.001). However, there were no significant differences between CD-8, MFD-4, MFD-18, MFDÜ-1 and control (Figure 1). The results showed that the highest percentage of gray mold infection (79.2%) was observed in the control and the lowest (20.8%) was in MFD-45, followed by MFD-81 (25.0%) and T26 (37.5%), with no statistically differences between MFD-45 and MFD-81. MFD-45 and MFD-81 reduced the amount of gray mold rot on fruits by 73.7 and 68.4% compared with the control, respectively. Percent of gray mold rot MFDÜ-2, MFD-113, CD-9, T33, MFD-20 and MFD-1 treatments were 41.5, 41.7, 50.0, 50.0, 54.2 and 58.3%, respectively.

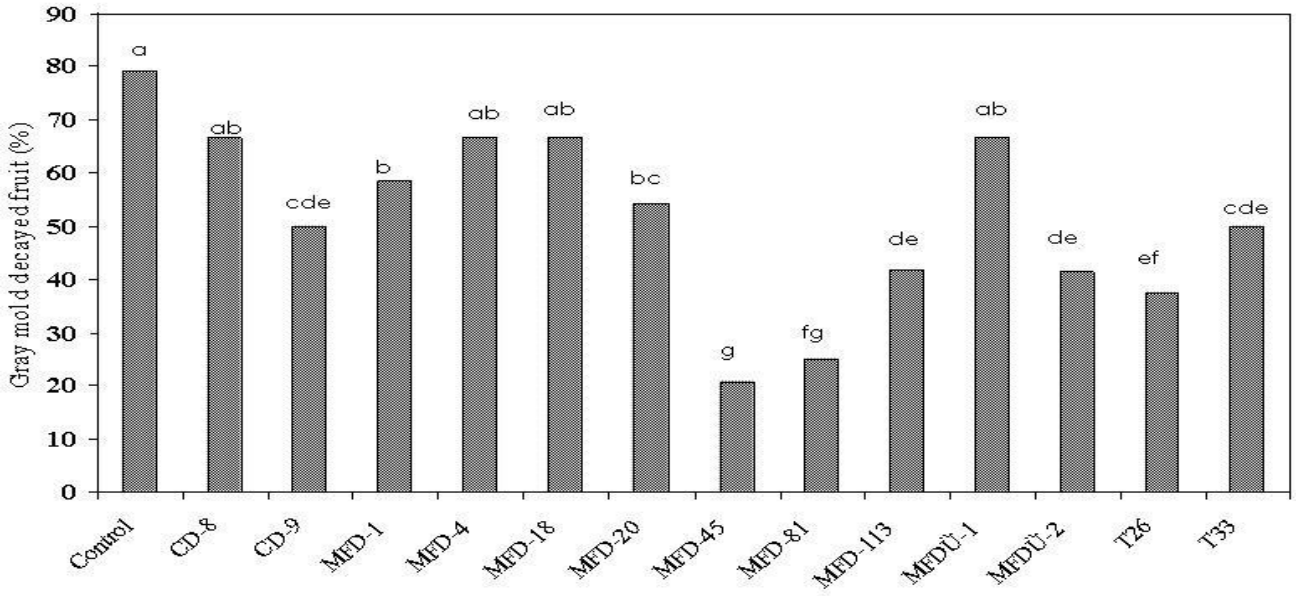

Figure 1. Effect of some bacterial strains on conidia germination on strawberry fruit (P<0.001).

Table 1. Effect of PGPB on *Botrytis cinerea* mycelia development in *in vitro* conditions.

PGPB	Inhibition zone(cm)	PGPB	Inhibition zone(cm)
Bacillus subtilis C6	0.50	***Bacillus subtilis* MFD-4**	**2.65**
Brevundomonas vesicularis C18	2.10	*Bacillus cereus Gc subgroup A* MFD-9	0.95
Bacillus cereus Gc subgroup A C22	2.10	***Kurthia sibirica* MFD-18**	**2.25**
Bacillus megaterium GC subgroup A CD-3	1.00	*Bacillus megaterium GC subgroup A* MFD-19	2.10
***Bacillus megaterium GC subgroup B* CD-8**	**2.40**	***Bacillus subtilis* MFD-20**	**3.25**
***Paenibacillus polymyxa* CD-9**	**2.50**	*Bacillus atrophaeus* MFD-22	2.10
Bacillus thurungiensis kurstaki E1	0.95	***Enterobacter intermedius* MFD-45**	**2.15**
Bacillus subtilis E2	1.00	*Brevibacterium luteum* MFD-47	0.50
Bacillus cereus Gc subgroup B E3	1.00	***Enterobacter intermedius* MFD-81**	**3.75**
Bacillus cereus Gc subgroup B E4	0,90	***Pantoea agglomerans* MFD-113**	**3.15**
Bacillus atrophaeus E6	1.95	*Pantoea agglomerans* MFD-232	0.50
Acinetobacter calcoaceticus E15	0.90	*Brevibacterium casei* MFD-408	0.75
Bacillus thurungiensis kurstaki E17	0.75	*Brevibacterium casei* MFD-419	1.00
Enterobacter agglomerans GC subgroup III F3-88	1.05	*Brevibacterium casei* MFD-455	0.70
Brevibacillus centrosporus FD-2	0.75	***Bacillus subtilis* MFD-Ü1**	**3.20**
Erwinia crysanthemi biotype III FF4	1.00	***Bacillus subtilis* MFD-Ü2**	**3.00**
***Bacillus lentimorbus* MFD-1**	**3.20**	***P. fluorescens biotype G* T26**	**2.95**
Bacillus megatorium GC subgroup A MFD-2	2.00	***Bacillus pumilis* T33**	**3.30**

Table 2. Effect of some bacterial strains on mycelia development on strawberry fruit.

Treatments	Mycelia diameter (mm)	Mycelia area (mm²)
Negative Control	0	0
Positive Control	19.20±1.53 ab	292,9±31.65 abc
Bacillus CD-8	20.02±1.47 a	317.1±18.44 a
***Paenibacillus* CD-9**	**14.68±0.71 fg**	**173.1±18.34 g**
***Bacillus* MFD-1**	**15.89±0.95 defg**	**201.7±9.36 efg**
***Bacillus* MFD-4**	**15.63±0.58 defg**	**203.5±36.94 efg**
***Kurthia* MFD-18**	**17.01±0.72 cde**	**228.8±21.67 de**
Bacillus MFD-20	18.62±1.20 abc	273.0±35.12 bc
***Enterobacter* MFD-45**	**16.42±1.02 defg**	**219.9±26.57 def**
Enterobacter MFD-81	17.75±1.98 bcd	260.9±27.87 cd
***Pantoea* MFD-113**	**16.68±1.10 cdef**	**221.0±20.72 def**
Bacillus MFDÜ-1	19.15±2.28 ab	291.3±17.99 abc
***Bacillus* MFDÜ-2**	**14.41±0.83 g**	**166.6±16.54 g**
***Pseudomonas* T26**	**15.11±1.18 efg**	**182.2±11.02 fg**
***Bacillus* T33**	**14.87±1.12 efg*****	**174.4±13.88 g*****

*** P<0.001, mean±standard deviation.

DISCUSSION

The present study showed that gray mold rot can be effectively controlled by PGPB due to its antagonistic capability to inhibit spore germination and penetration of the fungus, *B. cinerea,* on strawberry fruits. Previous studies also confirmed that these bacteria had a broad spectrum of antimicrobial activity against several plant pathogenic fungi and bacteria species in vitro and in vivo (Esitken *et al*. 2002; Altindag *et al*. 2006). Kloepper *et al*. (2004) studied several strains of *Bacillus* spp and they found elicit induced resistance in 11 different host plants and caused reductions in a spectrum of diseases (foliar, stem and soil-borne fungal diseases). Nevertheless, it seems that there were no direct correlation between inhibition zone diameter determined in vitro and biocontrol effects on fruit. For example, MFD 45 had the smaller inhibition zone among selected bacteria however it was found one of the most effective bacteria on mycelium development and conidia germination.

For controlling fungal plant pathogens, a variety of mechanism has been considered (Kim and Kim 1994). Cherif *et al*. (1992) suggested that cell-wall-degrading enzymes such as b - 1, 3 - glucanases, cellulases, proteases and chitinases are involved in the antagonistic activity of some biological control agents against phytopathogenic fungi. Essghaier *et al*. (2009) indicated that suppression of *B. cinerea* growth *in vitro* by the selected moderately halophilic isolates and formation of inhibition zones were presumably due to the metabolites being released from bacteria into the culture medium. Antagonists that compete with saprophytic growth of *Botrytis* spp. may reduce pathogen growth and/or sporulation in crop debris (Köhl *et al*. 1995; Morandi *et al*. 2003), resulting in the reduction of disease progress rate. Using these antagonists is advantageous because of the continuity of the interaction between pathogen and antagonist in the crop debris (Fokkema 1993). Suppressing either colonization or sporulation of *B. cinerea* is a valid strategy to biologically control the pathogen in strawberry and other hosts (Sutton and Peng 1993; Köhl and Fokkema 1998; Morandi *et al*. 2003). In strawberry production, young leaves can be infected by *B. cinerea*, therefore, the high levels of suppression of pathogen sporulation in leaves will effectively reduce inoculum produced in crop debris and consequently contribute to reduce disease incidence on both flowers and fruits (Mertely *et al*. 2002; Legard *et al*. 2005).

Among PGPB agents, *P. polymyxa* was found to be the most effective in suppressing germ tube growth of *B. cinerea* in a strawberry fruit pulp suspension culture (Pichard *et al*. 1995; Helbig 2001). They found that the bacteria have antibiotic and enzyme production which is vital for biocontroling of disease. The involvement of substances, enzymes or antibiotics, seems to play a role in the active principle of the present isolate as symptoms of lysis were observed at germ tubes of conidia of *B. cinerea*.

But, we cannot say that the antagonistic behavior of bacteria was a result of such activity because we did not make antibiotic and enzyme production tests of bacteria. However, our isolation and assay techniques selected based on a bacterium's ability to grow on fungal mycelium wall material and the fact that these bacteria are able to grow on fungal cell walls infers that antibiotic activity was probably not solely responsible for the antagonism we observed.

On the other hand, there is a new strategy being investigated is increasing competition for nutrients on leaf surfaces by enhancing saprophytic fungal, bacterial and/or yeast populations. This approach shows promise

for controlling grey mould, *Botrytis cinerea*, on grapes, tomato and potted plants (Farber et al. 2006), but is limited to pathogens that require nutrients to grow and infect the plant.

Acknowledgements: The authors wish to thank to TUBITAK for their financial support for this study through project, TOVAG 106O049.

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