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Full Length Research Paper

Biological control of post harvest disease caused by *Aspergillus flavus* on stored lemon fruits

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Antagonistic activity of 24 selected bacterial strains detected by previous microbiological studies to *Aspergillus flavus* was tested *in vitro* and *in vivo* conditions. Within 24 strains, only ten strains showed remarkable inhibition zone (6-34 mm) against the pathogen in assays carried out in Petri plates. Both cell suspension and culture filtrates of prominent 10 bacterial strains were also tested in order to control *A. flavus* on lemon fruits cvs Meyer and Interdonato under storage conditions. The cell suspension of nine strains and the culture filtrates of three strains led to suppression on disease development on lemon fruits. The highest control was obtained by the cell suspension of *Pantoea agglomerans* RK-153. *Erwinia chrysanthemi* RK-67 and *Bacillus subtilis* RK-6 treatments reduced disease severity on both lemon cultivars. Furthermore, both the cell suspension and culture filtrates of *Burkholderia cepacia* strain RK-277 reduced disease severity on only cvs Interdonato, but not on Meyer. There was no significant difference in decay diameters among those treatments, compared to the negative control at 35 days of inoculation. Even other tested strains also slightly reduced disease severity compared to strains determined as efficient ones; disease severity resulted from other strains were statistically significant, compared to negative control. In conclusion, these strains can be used as new biocontrol agents in controlling postharvest decay of citrus fruit.

Key words: Antibiosis, *Aspergillus flavus* biocontrol, citrus disease, lemon, *Pantoea agglomerans*, postharvest.

INTRODUCTION

Turkey is among the first ten countries in terms of lemon *Citrus limon* L. Burman f.) production with 10.4% of total lemon production in the world (FAO 2006). Postharvest diseases due to fungal infections cause significant economic losses for the citrus industry during storage, transport and marketing. Toker and Bicici (1996) stressed that when mandarin, orange, grapefruit and lemon were stored at ambient temperature for 2 months, and kept at storage conditions for two and four months 16.8, 25.1 and 65.4% crop losses were observed, respectively, due to development of total postharvest diseases.

The most important ones of them are caused by fungi such as *Penicillium* spp, *Aspergillus* spp, *Alternaria* spp, *Botrytis cinerea*, *Monilinia laxa* and *Rhizopus stolonifer* (Ogawa et al., 1995). *Aspergillus flavus* is a wound-invading pathogen that causes decay on stored citrus fruits damaged by insects, animals, early splits, and mechanical harvesting. Furthermore, this pathogen is one of the major fungi species producing aflatoxin, a group of toxic, carcinogenic compounds (Diener et al., 1987; Wilson and Payne 1994; Palumbo et al., 2006).

Traditionally, the postharvest diseases have been controlled by spray of synthetic fungicides such as thiabendazole, imazalil and sodium ortho-phenyl phenate (Poppe et al., 2003). But, alternative control methods are needed because of negative public perceptions about the use of pesticides, development of resistance to fungicides and high development cost new chemicals (Bull et al., 1997). In this respect, microbial biocontrol agents have shown to be great potential as an alternative to synthetic fungicides (Janisiewicz and Korsten, 2002; Zhang et al., 2005), and offers an environmentally friendly alternative to the use of synthetic pesticides.

There are many studies demonstrating postharvest disease control by using biological agents (Huang et al., 1993; Filonow et al., 1996; Bull et al., 1997; Kotan et al.,

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1999; Janisiewicz et al., 2000; El Ghaouth et al., 2001; Zhang and Dou 2002; Aysan et al., 2003; Dahiya et al., 2005; Francés et al., 2006). However, the number of studies concerning biological control of *A. flavus* is too few (Calistru et al., 1997; Bueno et al., 2006; Palumbo et al., 2006), and there is no direct research evidence so far indicating biological control of this pathogen on citrus fruits.

The main purpose of this study is to investigate antagonistic activities of the cell suspensions and culture filtrates of the pre-selected bacterial strains against *A. flavus* under *in vitro* and *in vitro* conditions on lemon fruits.

MATERIALS AND METHODS

The selection of candidate antagonistic bacteria and pathogen fungus

A total of 24 non pathogenic bacterial strains selected of a total of 324 bacterial strains in 76 species of 36 bacterial genera were isolated originally from aerial part of pome fruits from different locations in Eastern Anatolia region of Turkey. It was determined that they had antagonistic activities against some plant pathogenic bacteria *in vitro* and *in vivo* assays in previous study (Kotan, 2002; Kotan et al., 2006). The bacterial cultures were stored at -80˚C in %15 glycerol and Luria Broth (LB).

The pathogen fungus, *A. flavus* was isolated from an infected lemon cultivar Interdonato obtained from a market place located in Erzurum (Turkey). Conidial suspension of the isolated fungus ($1x10^6$ spores/ml) was injected into the rind of lemon fruits, and re-isolated from decayed fruits. The cell suspension of candidate antagonistic bacterial strains and conidial suspension of the re-isolated fungus were adjusted to $1x10^6$ spores/ml and $1x10^8$ CFU/ml in sdH_2O, respectively. Following, they were tested for pathogenicity on lemon fruits belonging to Meyer and Interdonato cultivars. The fungus culture was stored on slants of Potato-Dextrose Agar (PDA) at 4˚C for further studies.

The identification of bacteria and pathogen fungus

The bacterial strains were identified by using both the MIDI (Microbial Identification System (MIS), Inc., Newark, DE, version 5.5) (Paisley 1995) and BIOLOG system by GN2 and GP2 plates (Biolog Inc, Hayward, CA. USA) (Praphailong et al., 1997). The Identity of the pathogen fungus was also conducted by MIS, and confirmed by its morphological and microscopical characteristics (Raper and Fennell, 1965).

Source of fruit material

Lemon fruits cvs Meyer and Interdonato were obtained from market place located in Erzurum (Turkey). The fruits were selected free of wounds and rots and as much as possible homogeneous in maturity and size, and were stored at 5℃ for 3-5 days until use. Following, they were surface-disinfected by immersion for 1 min in a dilute solution of ethanol (70%), washed twice by immersion in distilled and sterilized distilled water (sdH_2O), and left in a dry place to remove excess water on the surface until using for *in vivo* assays.

In vitro assays

Antagonistic activities of the candidate 24 pre-selected bacterial strains were tested against *A. flavus* on PDA medium. They were grown in 50 ml Erlenmeyer flasks containing 20 mL of Nutrient Broth (NB) medium on a rotary shaker at 28˚C for 24 h. Absorbance of the suspensions was measured spectrophotometrically at 600 nm and appropriately diluted to $1x10^8$ CFU/ml in sdH_2O. After this, each bacterial suspensions (20 µl) was separately streaked on the middle of the plates (85 mm diameter) containing PDA medium with a sterile swap as a single line. A plug of agar from the active growing pathogen fungus, cultivated on PDA at 28˚C with a 12 h light/dark cycle seven days before inoculation, was placed on each side of this line (approximately 3 cm from the line). The Plates were incubated at 28˚C with a 12 h light/dark cycles. Inhibition zones (mm), which were determined as the shortest distance between pathogen fungus and antagonist bacterium, were measured after seven days. A plug of only PDA agar was used as a negative control. Only pathogen was used as a positive control. Experiments were repeated three times with three replications for each experiment.

Disease control under standard storage conditions

A total of 10 bacterial strains selected from *in vitro* applications were used for *in vivo* assays as potential biocontrol agents that showed inhibition zone on PDA medium against *A. flavus*. Both cell suspensions and culture filtrates of these strains were tested for antagonistic activity against the pathogen under standard storage conditions, and absorbance of the bacterial cell suspensions was measured as described above. The bacterial culture filtrates were prepared by filtering of their cell suspensions adjusted to $1x10^8$ CFU/ml concentration through 0.45 µm Syringe Driven Filter Unit (MILLEX®-HA).

Conidia of *A. flavus* were collected from 7-days-old PDA agar cultures incubated at 28˚C with a 12 h light/dark cycle. The conidial suspension was prepared by scraping spores from the surfaces of the colonies with a wet cotton swab and re-suspending the spores in distilled water containing 0.5% Tween 80. The spore concentration was determined by using a hemocytometer, adjusted to $1x10^6$ spores/ml by appropriate dilution and used fresh as a stock suspension. Lemon fruits were washed in running water, dipped in ethanol (70%) for 2 min, rinsed twice with double distilled sterile water (10 min each) and air-dried. The surface-sterilized fruits were wounded (5 mm in diameter and 5 mm in depth from surface) by puncturing each fruit once with a cork borer. Each fruit was wounded one times halfway between the calyx and the stem end. Fruits wounds were inoculated with both 25 µl of conidial suspension of pathogen fungus and 25 µl of the cell suspensions or culture filtrates of antagonistic bacterial strains individually paired with the pathogen by co-inoculating. The fruits were sealed in polyethylene-lined plastic boxes, and they were stored at 10˚C, in 80% humidity under a photoperiod of 12-h light and 12-h dark. Treatments were arranged in a randomized block design. Each treatment was applied to three replicates of 3 fruit each within each experiment. Diameters of decay on fruits surface were measured 15 and 35 days later. NB medium alone was used as negative control, and the fungal suspension alone was used as positive control.

Statistical analyses

An analysis of variance (ANOVA) and Duncan's multiple range test (at $P<0.05$) were performed to analyze statistical differences and to discriminate between means (Stat Soft Inc, 1998).

RESULTS AND DISCUSSION

Totally 24 pre-selected candidates antagonistic bacterial

Table 1. MIS and BIOLOG identification results of bacterial strains tested for antagonistic activity, their similarity index (SIM) and minimum-maximum inhibition zones as millimeter (MMZ) against *A. flavus* (*In vitro*).

Strain no.	MIS results	SIM	BIOLOG results	SIM	MMZ (mm)
RK-137	*Alcaligenes piechaudii*	0.786	*Enterobacter aerogenes*	0.54	10-12
RK-157	*Alcaligenes piechaudii*	0.393	*Pantoea agglomerans*	0.66	10-12
RK-143	*Alcaligenes piechaudii*	0.251	*Pantoea agglomerans*	0.47	0
RK-156	*Alcaligenes piechaudii*	0.461	*Pantoea agglomerans*	0.26	0
RK-136	*Alcaligenes piechaudii*	0.409	*Pantoea agglomerans*	0.42	0
RK-105	*Alcaligenes piechaudii*	0.420	*Pantoea agglomerans*	0.34	0
RK-86	*Pantoea agglomerans*	0.698	*Pantoea agglomerans*	0.68	8-10
RK-160	*Pantoea agglomerans*	0.517	*Pantoea agglomerans*	0.35	12-15
RK-80	*Pantoea agglomerans*	0.763	*Pantoea agglomerans*	0.56	8-10
RK-169	*Pantoea agglomerans*	0.782	*Pantoea agglomerans*	0.35	0
RK-84	*Pantoea agglomerans*	0.718	*Pantoea agglomerans*	0.56	0
RK-85	*Pantoea agglomerans*	0.857	*Pantoea agglomerans*	0.71	0
RK-92	*Pantoea agglomerans*	0.889	*Pantoea agglomerans*	0.58	0
RK-123	*Pantoea agglomerans*	0.489	*Raoultella terrigena*	0.45	0
RK-153	*Pantoea agglomerans*	0.418	*Enterobacter aerogenes*	0.18	6-10
RK-91	*Enterobacter intermedius*	0.605	*Pantoea agglomerans*	0.12	0
RK-67	*Erwinia chrysanthemi*	0.610	*Pantoea agglomerans*	0.61	12-16
RK-103	*Bacillus pumilus*	0.626	*Bacillus subtilis*	0.27	0
RK-6	*Bacillus subtilis*	0.660	*Bacillus subtilis*	0.57	24-25
RK-240	*Bacillus subtilis*	0.735	*Bacillus subtilis*	0.65	0
RK-277	*Burkholderia cepacia*	0.409	*Burkholderia cepacia*	0.64	21-34
RK-135	*Erwinia rhapontici*	0.867	*Raoultella terrigena*	0.15	16-18
RK-142	*Pseudomonas putida*	0.277	*Brevundimonas vesicularis*	0.37	0
RK-102	*Serratia liquefaciens*	0.572	*Acinetobacter calcoaceticus*	0.14	0

strains were tested for antagonistic activity against pathogen *A. flavus* on Petri plate assay. All tested biocontrol bacteria were identified by using both MIS and BIOLOG systems. The identification of tested biocontrol bacteria and their antifungal activity results on PDA medium under *in vitro* conditions were given in Table 1. A total of 24 bacterial strains were consisted of 10 different species and 8 genera according to MIS, and 7 different species and 7 genera according to BIOLOG. Ten strains gave the same identification results, but the others did not give similar. These results show that MIS and BIOLOG identification systems alone are not yet accurate enough to serve as a primary method for identifying some of the bacterial species. On the other hand, the pathogen fungus was identified as *A. flavus* according to MIS (Similarity index: 0.635), BIOLOG (Similarity index: 0.875) and its morphological or microscopical characteristics.

Pathogenicity test results of re-isolated fungus were positive on both cultivars lemon fruits. A total of ten bacterial strains (RK-6, RK-67, RK-80, RK-86, RK-135, RK-137, RK-153, RK-157, RK-160 and RK-277) tested led to inhibition zone (6-34 mm) against the pathogen on Petri plates. Both cell suspension and culture filtrates of selected 10 bacterial strains were also tested for management against *A. flavus* on lemon fruits cvs Meyer and Interdonato under storage conditions. These results were given in Table 2. The cell suspension of nine strains and the culture filtrates of three strains reduced disease severity on lemon fruits. The most successful results were obtained from the cell suspension of *Pantoea agglomerans* RK-153 (Figure 1), *Erwinia chrysanthemi* RK-67 and *Bacillus subtilis* RK-6 strains reduced disease severity on both lemon cultivars. Furthermore, both the cell suspension and culture filtrates of *Burkholderia cepacia* strain RK-277 reduced disease severity on only cvs Interdonato, but not Meyer. There was no significant difference in decay diameters among those treatments in comparison to the negative control on the 35 days later. Other the tested strains also slightly reduced disease severity, but there was a significant difference in decay diameters in comparison to the negative control.

A total of ten strains of tested 24 bacterial strains showed antifungal activity on Petri plate assays against *A. flavus*. All of them reduced disease severity on lemon fruits cvs Meyer or Interdonato under storage conditions. In other words, there was a correlation between *in vitro* and *in vivo* results of tested bacterial strains. A comparison of *in vitro* and *in vivo* data indicates that the *in vitro* assay was useful for identifying antifungal activity of tested bacteria against *A. flavus.*

The majority of the effective strains according to MIS results belonged to *P. agglomerans* (4 strains). Similar

Table 2. Decay diameter measured 15th and 35th days of storage on lemon fruits cvs. Meyer and Interdonato treated with the cell suspensions or the culture filtrates of tested bacterial strains against *A. flavus*.

Treatments	Treated with cell suspensions of bioagents				Treated with culture filtrates of bioagents			
	Interdonato		Meyer		Interdonato		Meyer	
	15th day	35th day	15th day	35th day	15th day	35th day	15th day	35th day
Negative control (only NB)	06.0	06.0	06.0	06.0	06.0	06.0	06.0	06.0
Alcaligenes piechaudii RK-157	06.0*	06.0*	16.0**	28.0**	14.0**	21.3**	19.3**	27.0**
Pantoea agglomerans RK-153	06.6*	06.6*	07.3*	07.3*	32.6**	37.0**	24.3**	28.3**
Bacillus subtilis RK-6	06.6*	06.6*	08.0*	09.3*	29.0**	37.0**	21.0**	28.3**
Burkholderia cepacia RK-277	08.0*	08.0*	17.6**	30.3**	06.0*	07.3*	15.6**	30.6**
Erwinia chrysanthemi RK-67	06.0*	09.0*	06.0*	08.0*	12.0**	13.3**	06.0*	18.6**
Pantoea agglomerans RK-86	08.0*	10.0*	18.6**	27.6**	17.6**	29.6**	20.6**	33.3**
Erwinia rhapontici RK-135	08.0*	10.0*	22.6**	34.3**	33.0**	38.6**	12.6**	21.3**
Pantoea agglomerans RK-80	10.0*	11.6*	20.3**	27.6**	29.3**	35.3**	14.0**	20.3**
Pantoea agglomerans RK-160	08.3*	12.0*	16.3**	25.6**	27.3**	37.0**	14.6**	16.0**
Alcaligenes piechaudii RK-137	11.3**	16.0**	10.6**	22.3**	27.6**	31.6**	06.0*	06.0*
Positive control (only pathogen)	30.3	52.3	29.0	44.3	30.3	38.3	29.0	37.0

*There was no significant difference in decay diameters among those treatments in comparison to the negative control (P=0.05)
**There was significant difference in decay diameters among those treatments in comparison to the negative control (P=0.05.

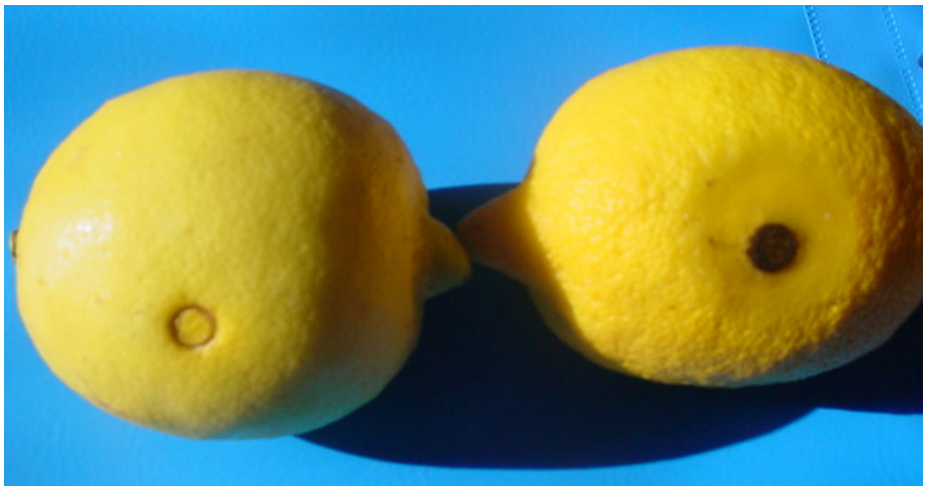

Figure 1. Lemon fruits cvs Interdonato treated with together the cell suspension of antagonistic bacterium *P. agglomerans* strain RK-153 and pathogen fungus *A. flavus* (on the left), and treated with only pathogen fungus *A. flavus* (on the right) under storage conditions (35th days).

information about *P. agglomerans* strain CPA-2 as potential biocontrol agents of postharvest pathogens *Penicillium digitatum* and *Penicillium italicum* has also been reported by Poppe et al. (2003). The cell suspension of *P. agglomerans* strains reduced completely disease severity on especially cvs. Interdonato lemon fruits in comparison to positive control under storage condition. Furthermore, one of these strains (RK-153) showed a weak antifungal activity on Petri plate, but they were effective extremely under storage conditions. In this study, while the *in vitro* assays carried out at 28˚C, the *in vivo* assays carried out at more low temperature (10˚C) and high moisture (80%). It is known that enzymes are the more stabile at low temperatures and high moisture ratio (Wang et al., 2002; Brenchley, 2005).

The cell suspensions of tested bacteria, exception of *A. piechaudii* RK-137, were generally more effective especially on Interdonato lemon cultivars than their culture filtrates against pathogen fungus (Table 2). In addition, antifungal activity of the both cell suspension and culture filtrates of *B. cepacia* RK-277 was important on cvs Meyer, but not on cvs Interdonato. These differences in biocontrol activity can be due to the susceptibility to pathogen of lemon cultivars. According to means of decay diameters applied only pathogen on fruits, cvs Meyer was the more resistant cultivar than cvs

Interdonato against *A. flavus*. It has been known that lemon posses antimicrobial fatty acids such as palmitic acid and linoleic acid (Lee et al., 2002; Dale et al., 2004). These essential oils may play an important role in inhibition of pathogens. Besides, importantly, this study demonstrated that the specific interactions between the biocontrol agents and pathogen or host may be an important factor in biocontrol.

In biological control, various mechanisms have been described, including antibiosis, production of hydrolytic enzymes, parasitism, induced resistance and competition for nutrients and space. Biocontrol agents in their native habitat may compete with other microorganisms for space and food, produce toxic substances, parasitize and/or kill other plant pathogens. These results showed that nutrient competition can be one of the modes of these in question strains. The mode of action in biocontrol provided by this antagonist is possibly due to the production of an antibiotic and to spatial separation of the fungal spores from the damaged fruit tissue. Furthermore, it is reported that *P. agglomerans* possess strong chitinolytic activity, and suppressed postharvest disease (Chernin et al., 1995). Chitinolytic enzymes have been considered important in the biological control of the postharvest pathogens because of their ability to degrade fungal cell walls, of which as major component is chitin (Chet, 1987). In the present study we also assume involving of some enzymes produced by *P. agglomerans* playing an important role in the inhibition of *A. flavus.*

In addition, several strains of *P. agglomerans* produce some antibiotics such as pantocin A and B (Wright et al., 2001). It has been reported that these strains strongly inhibits the growth of various plant pathogenic bacteria as *Erwinia* species, but they do not inhibit the growth of *P. digitatum* (El-Goorani et al., 1992). Another antibiotic such as pyrrolnitrin and herbicolin produced by *P. agglomerans* strains have a fungicidal effect against fungi such as *B. cinerea* (Chernin et al., 1996), *Penicillium expansum*, *Monilinia fructicola* and *Rhizopus stolonifer* (Ritte et al., 2002), *Botrytis cinerea* (Winkelmann et al., 1980), *Fusarium culmorum* (Kempf et al., 1993).

In this study, one of the effective strains was *B. subtilis* strain RK-6 which cell suspension reduced disease severity on both cultivars. *Bacillus* genus bacteria have great potential as biological control agents because they keep their viability when stored for long time. Considering the cases mentioned above, use of Gram-positive bacteria having a natural formulation (production of endo-spores tolerant to heat and desiccation) in this disease suppression would have great advantage and contribution in order to overcome the drawbacks incurred by the use of other biocontrol agents.

One of the most important alternative control methods of plant disease is the use of biological control agents. The biological control offers a powerful and environmentally friendly alternative to the use of synthetic pesticides. So far, a lot of studies have successfully employed to determine antagonistic bacteria to control postharvest disease. These studies have been generally focused on *P. agglomerans* (Teixidó et al., 2001; Zhang and Dou, 2002; Poppe et al., 2003; Plaza et al., 2004), *B. subtilis* (Rodile and Prakash, 1996; Sailaja et al., 1997; Zhang and Dou, 2002), *B. cepacia* (Huang et al., 1993; Quan et al., 2006; Palumbo et al., 2006) and *Pseudomonas aeruginosa* (Kishore et al., 2006). In this study, the majority of effective bioagents also belonged to *P. agglomerans*. But, this manuscript provides the first evidence that *A. piechaudii*, *E. chrysanthemi* and *E. rhapontici* are antagonists of *A. flavus*. Furthermore, our results showed that bacterial strains isolated from rhizosphere of pome fruits can be used for as a potential biocontrol agent against *A. flavus* on lemon fruits under storage condition.

In conclusion, this study showed that the cell suspensions and/or culture filtrates of 4 strains of *P. agglomerans* (RK-80, RK-86, RK-153 and RK-160), 2 *A. piechaudii* (RK-137 and RK-157), 1 *B. subtilis* (RK-6), 1 *B. cepacia* (RK-277), 1 *E. rhapontici* (RK-135) and 1 *E. chrysanthemi* (RK-67) may be useful as potential biocontrol agent against *A. flavus*. These strains may be developed as new biocontrol agents for postharvest decay control of citrus fruit. But, further study is necessary to understand the mode of action mechanism of these bioagents.

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