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BIOLOGICAL CONTROL OF *PENICILLIUM DIGITATUM* ON CITRUS WITH ANTAGONISTIC BACTERIUM *PSEUDOMONAS FLUORESCENS*

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ABSTRACT

This study was conducted to find out the effect of some antagonistics bacteria isolates on *Penicillium digitatum* the causal green mould of citrus, collected from infected fruits in Rasht, Lahijan, Some – Sara, Fouman, Talesh and Astara in the Guilan province, Iran under *in vitro* conditions. Eighty eight bacterial isolates, from the surface of citrus fruit (cvs. Tamson and Shahsavary) infected by *P. digitatum* and antagonistic ability of 6 isolates of these bacteria (Gram negative) was demonstrated by using the dual culture method. According to biochemical and morphological tests, the six isolates were identified as *Pseudomonas fluorescens*. The effect of volatile metabolites produced by antagonistic *P.fluorescens* was found that all isolates, inhibited growth of *P.digitatum in vitro*. Culture filtrate and antibiotics from these isolates inhibited growth of the pathogen. Under refrigerating room conditions at 4°C, citrus fruits were treated against green mould disease by dipping cell suspensions (1×10^8 cfu/ml) of the bacteria and in Benlate, Rovral TS. fungicides (1000 ppm). Statistical analysis of data indicated that there existed significant differences between all antagonistic isolates and the control ($P< 0.01$). Storage for 25 days at 15°C *in vitro* or after 4 months at 4°C in refrigerating room conditions, mixture of isolate C4 and Benlate fungicide reduced green mould disease by 71.66 and 82.66 percent respectively.

Keywords: Green mould citrus, *Penicillium digitatum*, antagonistics bacteria, refrigerating room conditions

INTRODUCTION

Green mould of citrus, caused by *Penicillium digitatum* (Pers.:Fr.) Sacc., is one of the most economically important post-harvest diseases of citrus worldwide (Smilanick et al., 1997; Caccioni et al., 1998; Holmes and Eckert, 1999; Palou et al., 2002). It is identified by the mass of olivegreen spores produced on infected fruit. The extensive spore production by this pathogen ensures its presence wherever fruit is handled, including the field, packinghouse, equipment, degreening and storage rooms, transit containers and in the marketplace. The disease develops rapidly at warm temperatures, with an optimum of about 27°C (Ismail and Zhang 2004). The disease is primarily controlled by applying synthetic fungicides such as imazalil and thiabendazole (Holmes and Eckert, 1999; Palou et al., 2001). Pathogen resistance to fungicides is a severe problem in the case of green and blue moulds since resistant strains develop readily in large populations of spores. Fungicide resistance in green and blue moulds is also severe when treated fruit is stored repeatedly in a facility for extended durations. *Penicillium* resistance to thiabendazole, imazalil is a problem in California packinghouses (Eckert 1990; Holmes and Eckert, 1999), but has not been reported as a problem with other post-harvest citrus pathogens. Microbial biocontrol agents have shown great potential as an alternative to synthetic fungicides for control of the post-harvest decay of fruits and vegetables (Wilson et al., 1991; Janisiewicz and Marchi 1992; EI-Ghaouth et al., 2000). Two biocontrol products, *Candida oleophila* (Aspire) and *Pseudomonas syringae* (BioSave), have been registered by the US-EPA for commercial post-harvest treatment of citrus fruit for control of green and blue moulds (Brown and Miller 1999). The biocontrol agents show preventive activity against *Penicillium* species with limited or no ability of eradication activity when compared to Imazalil and Thiabendazole and are not potential for the control of fungicide-resistant strains of *Penicillium* species. They are acceptable for use of citrus when customers require non-use of post-harvest chemicals, chemical-free citrus (Ismail and Zhang 2004). Biocontrol of post-harvest decay caused by different epiphytes may need different antagonists (He et al., 2003, Long et al., 2005). Certains fluorescent pseudomonad isolates have antagonistic activity based on producing antibiotic whereas for other isolates such as *Pseudomonas putida* WCS358 are antagonistic because of competition for iron (Vidhysaekaran and Muthamilan, 1999). Strains of the yeast *Cryptococcus laurentii* (Kufferath) Skinner have been studied for the post-harvest biological control of gray and blue mould rots of apples (Roberts, 1990_a) and other fruits such as strawberries, kiwifruits, and table grapes (LIMA et al., 1998), as well as for *Mucor* rot of pears (Roberts, 1990_b). However, alternative methods are needed because of concerns about environmental contamination and human health risks associated with fungicide residues (Wisniewski and Wilson, 1992) and because the widespread use of these chemicals in commercial packing houses has led to the proliferation of resistant strains of the pathogens (Palou et al., 2002). The objectives of the present research was, to isolate of fluorescent pseudomonads from the rhizosphere of citrus trees and surface of oranges infected by the *P.digitatum* and their characterization in term of antagonistic mechanisms used to control the

pathogen *in vitro* and under refrigerating room conditions. There is a new opportunity to study biological control of post-harvest diseases of fruits in our country. In this paper the capability of some isolates of *P.fluorescens* to control of green mould of citrus caused by *P. digitatum* was studied.

MATERIALS AND METHODS

Isolation of *P. digitatum*

Infected oranges (*Citrus sinensis* [L.] Osbeck) cultivar Tamson and Shahsavary were collected in 2001 from Rasht, Lahijan, Foman, Talesh and Astara of Guilan Province, Iran. *P. digitatum* was isolated from g rotten citrus tissue, cultured on acidified Potato dextrose agar (PDA), and incubated at room temperature (26 ± 2°C) for a week. The growing colonies of fungi were transfered to new plates for purification and identification.

Isolation and identification of fluorescent pseudomonads

Five g soil samples under a citrus tree and 10 g of skin orange fruit healthy and green mould disease separately were dissolved to 50 ml of sterile water and mixed well. The solution and its diluents were streaked onto KB plates. The plates were incubated at 24°C for a few days. According to the methodology of Schaad et al., (2001), antagonistics isolates of bacteria were identified by biochemical, physiological and biological tests.

Sample preparation for direct PCR from cell culture

Bacterials cells, which were grown on King B medium for 24 h, were resuspened in sterile distilled water. The cell suspensions (approximately 1×10^8 cfu/ml) were boiled for 10 min and were used for PCR assaying (Raaijmakers et al., 1997).

PCR conditions.

Primer were designed by primers PCA1 (5" TTGCCAAGCCTCGCTCCAAC 3") and PCA2 (5"CCGCGTTGTTCCTCGTTGAT 3"). PCR amplifications were carried out in 100 µl reaction volume. A 10 µl volume of boiled bacterial cells was added to 90 µL of PCR mixture contained ,2 mM $MgCl_2$, 20 pmol of each primer, 100 µM (each) dNTP, 0.2 U of *Taq* DNA polymerase (CinnaGene, Inc. Iran), in 10 mM Tris-HCl (pH 9.0), 50 mM KCl and 0.1% Triton X-100. Amplification was performed in a thermal cycler (Mastercycler gradient, Germany) programmed The reaction conditions are: a denaturation step of 94°C for 2 min followed by 37 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min. A final extension step of 72°C for 10 min finishes the reaction. Amplified DNA fragments were examined by horizantal electrophoresis in 1.5% agarose gel in TBE buffer (Sambrook et al., 2001) with 8 µL aliquots of PCR products. Gel were stained with ethidium bromide and were photographed under UV light (312 nm).

In vitro test

Efficacy of *P.fluorescens* isolates to inhibit *P.digitatum in vitro* : Efficacy of the *P.fluorescens* isolates in inhibiting growth of *P.digitatum* were tested by streaking each bacterial isolate on one side of a Petri dish containing potato dextrose agar (PDA) and KB (PDA+ KB) medium (EXPERT 1995). One 5-mm mycelial disc from a 4 days old culture of *P.digitatum* on PDA+ KB was placed at the opposite side of the Petri dish and experiments were independently repeated four times. Growth of fungus was inhibited when it grew toward the bacterial colony and the inhibition zone was measured from the edge of mycelium to the bacterial colony edge. The bacterial isolates that inhibited *P.digitatum* were identified by specific tests for *P.fluorescens* (Stainer et al., 1996).

Production of volatile antibiotic : A 250 µL aliquot of a antagonistic bacterial suspension containing about 1×10^8 colony forming units (cfu/ml) were placed at the Petri dish containing KB, and a 5 mm disk of a four days old pure culture of *P.digitatum* was placed at the center of another Petri dish containing PDA. Both half plates were placed face to face preventing any physical contact between the pathogen and the bacterial suspension, and were sealed to isolate the inside atmosphere and to prevent loss volatiles formed. Plates were incubated at 24 °C for 7 days and the growth of the pathogen was measured and compared to controls developed in the absence of the bioantagonist (mocked inoculation with 6 mm-disk of PDA). Each experiment used a single bacterial isolate that was run in triplicate and repeated at least three times. Results are expressed as % inhibition of the growth of *P.digitatum* in the presence and absents of any bacterial isolate. Percent inhibition was calculated using the following formula (Sivan et al., 1987). % inhibition = [1- (fungal growth / Control growth)] x 100

Secretion of extracellular : These test were performed in 250 ml Erlenmayer flasks containing 100 ml of sterile nutrient broth (NB). 1 ml bacterial suspension isolates (1×10^8 cfu/ml) were inoculated to the flasks containing NB. The flasks were then incubated at 24 °C for 7 days on a rotary shaker at 175 rpm at room temperature (24 ± 2°C). Following incubation, cells were centrifuged at 5000 rpm for 12 minutes and washed twice in order to remove the growth medium. Cell concentration was then adjusted as needed for different experiments (WISNIEWSKI et al., 1995). Culture filtrates were prepared by filtering centrifuged culture of the antagonist through a 0.2 µm polycarbonate membrane filter. Sepateatly, autoclaved culture was prepared by autoclaving a sample containing bacterial cells in culture broth for 20 min at 120°C. The unwashed cells were grown in the 20-h culture filtrate and adjusted to 1×10^8 cfu/ml with additional culture filtrate. The washed cell suspension was prepared as described above (Zhang et al., 2003). Five 15 and 25 % (v/v) of culture filtrate were mixed with PDA and a 5 mm disk of a four days old pure culture of *P.digitatum* was placed at the center of Petri dish. The experiments were independently repeated four times.

Production of diffusible antibiotic : This effect was tested according to Montealegro et al., (2003), PDA plates, covered with a cellophane membrane, were inoculated in the center with 250 µl of a bioantagonistic bacterial suspension (1×10^8 cfu/ml). After incubation for 48 hrs at 24 °C, the membrane with the grown bacterial isolate was removed, and the plate was inoculated in the middle with a 6 mm disk of a pure culture of *P.digitatum* plates were future incubated at 24°C for 5 days and the growth of the pathogen was measured. Control were run with mocked inoculated PDA containing plates on the cellophane membrane (replacing the bacterial suspension by sterile distilled water), and future incubated with *P.digitatum*. Each experiment considering a single bacterial isolate was run in triplicate and was repeated at least three times.

Production of protease : This effect was tested according to Maurhofer *et al*. (1995), efficacy of the *P.fluorescens* isolates in production of protease was tested by streaking each bacterial isolate on Skim milk agar medium (SMA) on the Petri dish. Each experiment considering a single bacterial isolate was run in triplicates and was repeated at least four times. The bacterial isolates that produced protease were identified by a halo zone a rounding of bacterial colony and were measured (Pumarino, 1995).

Effect of fungicides on *P.digitatum* and antagonistics isolates *in vitro* : This effect was tested according to Horsfall (1956). The development of antagonistic bacteria and *P.digitatum* were tested under Benlate concentrations of 1, 3, 5, 10, 100 and 1000 ppm at room temperature (24 ± 2°C). For *P.digitatum*, 2 ml of each Benlate concentrations which mixed with PDA at 50 Ċ and a 5 mm disk of a four days old pure culture of *P.digitatum* was placed at the center of Petri dish containing PDA. After incubation for 7 days at 24 °C and the growth of the pathogen was measured. Control were run with mocked inoculated PDA on the PDA medium (replacing fungicide concentrations by sterile distilled water). For antagonistics isolates, the 5 mm disk of wathman paper was dipped in the benlate concentrations for 2 minutes and was placed at the Petri dish containing KB. After incubation for 48 hrs at 24 °C the inhibition zone was measured. Four replication per concentration were maintained. In refrigerating room conditions, 2000 ppm concentration of Rovral TS and Benlate 50 WP. were used. Results are expressed as means of % inhibition of growth of *P.digitatum* and zone inhibition of antagonistics bacteria.

***In vivo* test**

Refrigerating room conditions : For evaluate efficacy of *P.fluorescens* isolates to control *P.digitatum* in the refrigerating room works were used orange cultivars Tamson and Shahsavary . The fluorescent pseudomonads isolates that inhibited *P.digitatum in vitro* were tested for their efficacy to control green mould of citrus.

Preparation of bacterial inocula : Cells of antagonistic bacteria for use in Refrigerating room conditions were grown in King's medium B broth (KMB) to late exponential phase at 27°C with shaking at 175 rpm. Cells were harvest by centrifugation (5000 rpm / min, 10 °C, 15 min), washed twice and resuspended in 0.5% sterile NaCl solution. The bacterial suspension was adjusted turbidimetrically to about 1×10^8 cfu/ml for each experiment. The bacterial cells suspensions in KMB medium were used for inoculation of citrus fruit.

Citrus fruits were selected for experiment. They were surface-sterilized with 0.5% NaCl for 5 min and then washed with tap water. After air-drying, citrus were treated with 70% ethanol. The fruits were drenched in the isolates antagonistics suspensions (1×10^8 cfu/ml) and the Benlate and Rovral TS fungicides (1000 ppm) for 2 heurs. The fruits were placed in polyethylene-lined plastic boxes to retain high humidity. Treated fruits were stored at 20°C for 25 days or 4°C for 4 months, then data were recorded as the percentage of decayed fruits. There were

three replicates of 10 fruits per treatment with complete randomization, the experiment was performed three times. (Zhang *et al*., 2004).

Statistical analysis : The incidence and severity of decay were analyzed by analysis of variance (ANOVA) with SAS Software (SAS Institute, version 6.08, Cary, NC). Statistical significance was judged at the level $P < 0.01$. When the analysis was statistically significant, Duncan's Multiple-Range Test (SSR Test) was used to test mean separations among mean values of each treatment.

RESULTS

Isolation of antagonistics bacteria: Eighty eight bacterial isolates, were initially collected from the surface of citrus fruit and rhizoplane and rhizosphere of citrus trees in different areas of the Guilan province-Iran. Among them, 6 isolates were found to inhibit growth of *P.digitatum in vitro*. All of them were identified as *P. fluorescens* according to the methodology of Schaad *et al.*, (2001).

Detection of *Pseudomonas fluorescens* isolates by direct PCR : All isolates of *Pseudomonas fluorescens* were identified by specific primers PCA1 and PCA2. On agarose gel electrophoresis 2%, isolates were produced a band 1110 bp. (expected size). The bands of isolates were similary with isolate standard of 2 – 79 RN (Fig. 1).

Efficacy of antibiosis of *P. fluorescens* isolates to inhibit *P. digitatum* in vitro

Dual culture : No physical contact was observed between any of the antagonistics bacteria tested and *P.digitatum* ; moreever, an inhibitory halo was observed suggesting the presence of fungistatic metabolites secreted by the bacteria. On the other hand, a change in mycelial color was observed close to the colony end of *P.digitatum*, being this one of a darker brown than the one observed at the center of colony. Microscopy observation of this zone, allowed to detect cytoplasmic leakage that could be observed up to the hyphal septum, resulting in deformation and sliming of their apex up to 1/6 of its original size. *P.flourescens* C4 with zone inhibition of 52.3 mm were the most inhibited of *P.digitatum*.

Volatile antibiotics: All antagonistics isolates showed there are significant differencebetween them and the control ($P<0.01$). *P.fluorescens* C4 were the antagonistic bacteria isolate that showed the best inhibitory effect on the growth of *P.digitatum.* The % inhibition of *P.flourescens* C4 at 72 h culture of antagonistic isolate was 61.6, while although all bacteria showed inhibitory effect on *P.digitatum* grow (Table 1).

Diffusible antibiotics: Results similar to those obtained when the effect of volatile antibiotics, were obtained when the effect of diffusible antibiotic was tested (Table 1). Isolate C4 and C5 with % inhibition of 74.6 and 66.3 respectively were the most inhibited of *P.digitatum,* while isolate C1 with % inhibition 56.6 was the less inhibited by diffusible antibiotics on the growth of *P.digitatum*.

Secretion of extr cellular: All antagonistics isolates were seen there are significant different between isolates and concentration of juices ($P< 0.01$). Autoclave and non-autoclave of *P.fluorescens* C4 % inhibition 75.7 and 45.6 respectively (15% v/v) were the most inhibited of *P.digitatum* (Table 1).

Production of protease: All six antagonistics isolates were able to produce protease on SMA medium. Among isolates, *P.fluorescens* C4 was found most effective which had 23 mm of halo zone arounding bacterial colony. All of the six isolates were able to secrete the enzymes involved in biocontrol, and that all had the ability to control *P.digitatum* through secretion of diffusible and volatile metabolites, it may be concluded that they use these two latter mechanisms of biocontrol as opposite to some fungal biocontrol microorganisms that also use fungal cell wall hydrolyzing enzymes within their biocontrol mechanisms (Perez *et al.,* 2002)

Table 1. Effect of antibiosis of *Pseudomonas fluorescens* isolates in inhibition of growth of *P.digitatum in vitro*

	***Pseudomonas fluorescens* isolates**					
Antibiosis (Inhibition) (%)	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**
Daul culture	39.5 c	40.4 c	47.3 b	52.3 a	46.3b	39.2 c
Volatile antibiotics simultaneously	41.3 c	41.3 c	49.6 b	57.6 a	48.5 b	41.5 c
Volatile antibiotics 72 h	46.6 c	45.6 c	54.6 b	61.6 a	55.6 b	46.3 c
Antibiotics	56.6 c	57.3 c	65.3 b	74.6 a	66.3 b	56.2 c
Secretion of extracellular (non autoclave 15% v/v)	54.5 c	55.6 c	65.6 b	75.7 a	66.7 b	54.1 c
Secretion of extracellular (autoclave 15% v/v)	35.66 b	35.66 b	36.33 b	45.66 a	37.33 b	35.3 c

Means followed by a common letter in a row are not significantly different according to Duncan's multiple range test (*P=0.01*).

Table 2. Effect of *Pseudomonas fluorescens* isolates and fungicides in control of green mould decay of citrus *in vitro* and refrigerating room conditions

Treatments	**Rot reduction (%)**			
	Tamson		**Shahsavary**	
	25 days	**4 months**	**25 days**	**4 months**
C1 + *Penicillium*	41.33 g	31.55 g	32.66 g	40.55 g
C2 + *Penicillium*	41.33 g	32.66 g	31.55 g	41.66 g
C3+ *Penicillium*	52.66 f	42.75 f	42.66 f	51.55 f
C4+ *Penicillium*	59.66 ef	50.33 ef	49.66 ef	58.65 ef
C5 + *Penicillium*	43.55 g	34.66 g	33.55 g	42.45 g
C6 + *Penicillium*	41.22 g	31.25 g	32.35 g	40.45 g
C4 + C5 + *Penicillium*	64.75 e	55.66 e	54.55 e	63.66e
Rovral TS + *Penicillium*	76.33 c	67.55 c	65.66 c	75.66 c
Benlate + *Penicillium*	73.66 d	63.55 d	62.66 d	72.75 d
Thiabendazol + *Penicillium*	65.75 e	56.55 e	56.66 e	65.66 e
C1 + Rovral TS + *Penicillium*	81.65 b	72.66 b	70.55 b	80.66 b
C2 + Rovral TS + *Penicillium*	83.33 b	74.66 b	72.66 b	82.66 b
C3 + Rovral TS + *Penicillium*	83.66 b	74.75 b	73.66 b	83.75 b
C4 + Rovral TS + *Penicillium*	88.75 a	78.66 a	77.55 a	88.66 a
C5 + Rovral TS + *Penicillium*	81.75 b	71.66 b	70.55 b	82.66 b
C6 + Rovral TS + *Penicillium*	81.35 b	72.33 b	70.35 b	80.33 b
C1 + Benlate TS + *Penicillium*	78 66 c	69.55 c	68.55 c	77.66 c
C2 + Benlate TS + *Penicillium*	79.66 bc	68.66 bc	68.66 bc	79.33 bc
C3 + Benlate TS + *Penicillium*	79.75 bc	68.66 bc	68.55 bc	79.33 bc
C4 + Benlate TS + *Penicillium*	82.66 b	71.66 b	70.55 b	81.75 b
C5 + Benlate TS + *Penicillium*	79.75 bc	69.55 bc	68.66 bc	79.66 bc
C6 + Benlate TS + *Penicillium*	78.25 c	69.2 c	68.25 b	77.55 c
C1 + Thiabendazol + *Penicillium*	71.33 d	60.55 d	59.66 d	70.75 d
C2 + Thiabendazol + *Penicillium*	72.75 d	61.66 d	61.55 d	72.66 d
C3 + Thiabendazol + *Penicillium*	72.75 d	60.66 d	59.66 d	72.75 d
C4 + Thiabendazol + *Penicillium*	78.33 c	66.55 c	65.55 c	77.66 c
C5 + Thiabendazol + *Penicillium*	72.66 d	61.55 d	60.66 d	72.33 d
C6 + Thiabendazol + *Penicillium*	71.25 d	60.25 d	59.33 d	70.25 d
Control	0 h	0 h	0 h	0 h

Means followed by a common letter in a columne are not significantly different according to Duncan's multiple range test (*P=0.01*).

Refrigerating room conditions

In refrigerating room conditions, statistical analysis of data showed there existed significant differences between antagonistics isolates and fungicides. All of the isolates in refrigerating room conditions at 20 days are more effective. Among these six isolates, *P.flourescens* C4 was the most effective for control green mould of citrus on Tamson cultivars. The rot reduction in C4 isolate at 25 and 4 months were 43.66 and 34.66 % respectively (Table 2). The results of used the mixture of isolates (C4 and C5) on Tamsons and Shahsavary cultivars showed that there existed significant differences between application isolates antagonistics with were used these isolates alone. During the 25 days and 4 month in the refrigerating room conditions, there are no significant difference were seen between Tamsons and Shahsavary cultivars on the rot reduction for all treatments, also there are no significant difference between application of C1, C2, C5 and C6 isolates in all treatments. Maximum control was obtained Tamsons and Shahsavary cultivars with rot reduction 88.75 and 77.55 % respectively when C4 was combined Rovral TS after 25 days inoculation (Table 2).

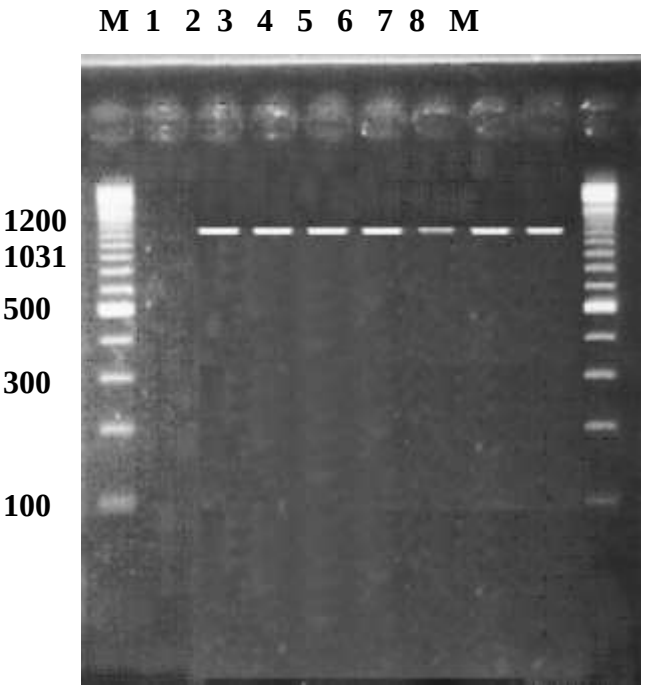

Fig.1. Agarose gel electrophoresis of products from polymerase chain reaction (PCR) performed on DNA 16S of *Pseudomonas fluorescens* isolates, M, 100 bp DNA marker; lane 1, control negative (distilled water), lane 2 is posetive control (*P. fluorescens* 2 – 79 RN) showing the amplification the approximately 1110 bp; lanes 3 to 8, antagonistic strains of *P. fluorescens* isolates.

DISCUSSION

Citrus is one of the world's most important fruit crops. Like many other fresh fruits and vegetables, citrus fruits are susceptible to a number of decay causing organisms (Ismail and Zhang, 2004). Green and blue moulds are the most prevalent post-harvest decays affecting citrus. Implementation of an integrated decay control program that include post-harvest measures as discussed in this article, should greatly minimize post-harvest losses, enhance consumer acceptance and increase economic returns to the grower (Ismail and Zhang, 2004). In general, decay control is not equivalent to that achieved with fungicides, and combination treatments appear more promising (Mitcham, 2000). Although isolates of *P.fluorescens* could be obtained from different orange cultivars, antagonistic potential of these isolates appears to vary a great deal (Hagedron *et al.,* 1989; Wilson *et al.,* 1992). The ability of *P.fluorescens* isolates to serve as biocontrol agent of green mould is described here**.** The results of dual culture studies showed that *P.fluorescens* isolates were inhibited the growth of *P.digitatum* on plates. Members of the genus *Pseudomonas* spp. are well known antagonistic bacteria (Vidhysaekaran and Muthamilan, 1999). They are known to produced volatile compounds such as hydrogen cyanide (Castric and Castric, 1983). Culture filtrate and autoclaved cell culture co-cultured with pathogen spores affect spore germination or germ tube elongation. In associated studies, autoclaved cell culture of *P.fluorescens* isolates and its culture filtrate had antagonist activity against artificially inoculated pathogens growth of *P.digitatum* on plates. This suggests that *P.fluorescens* isolates does

produce antibiotics as *B. subtilis* (Chalutz and Wilson, 1990). Immersion of citrus fruit in isolates suspension of antagonistics controls green and blue moulds (Smilanick *et al.,* 2003).

The results of in refrigerating room trial in the current study indicate the potential usefulness of with *P.fluorescens* isolates suspension when added fungicides in controlling green mould of citrus. *P.fluorescens* effectively controlled green mould of citrus when it was applied in refrigerating room conditions. *P.fluorescens* isolates effectively controlled green mould decay when it was applied to fruit immersion with mixed by isolates C4 and Rovral TS. Most biocontrol trials have dealt with use of *P.fluorescens* C4 against green mould. *In vivo,* under refrigerating room conditions, combination of C4 and C5 was comparable to Rovral TS application alone. A combination of *P.fluorescens* isolates and fungicides could be a reliable solution to control green mould rot on oranges.An antagonist is very promising if it can be combined with routine post-harvest treatments. Cold storage treatment is a routine practice in orchards to prolong the storage period of fruits. The demonstrated biocontrol effect of *P.fluorescens* at low temperature (4°C) indicates that *P.fluorescens* isolates can be combined with cold storage to enhance control efficacy.

In short, combination of fungicides with the biological control agent overcomes significant limitations of either of these treatments alone. Overall, this study offers a viable strategy by which combinations of different alternative control methods, whose modes of action complement one another, can effectively control post-harvest decay of citrus fruit. The low disease intensity of green mould of citrus with C4 isolate suggestes that the antibiosis metabolites are conductive for rapid inhibition agent, *P.digitatum*. The multiple activity may be useful under natural conditions in which the same fruit suffer from disease other than green mould.

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