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Effects of *Pseudomonas fluorescens* and *Candida famata* on blue mould of citrus caused by *Penicillium italicum*

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Abstract

Blue mould caused by *Penicillium italicum* is an important and devastating storage disease of citrus fruits. Control of the pathogen is based on primarily fungicide applications. In present times, biological controlling method of storage diseases is getting more prevalent due to the hazardous effects of fungicide residues on fruits. In this study, 176 *Pseudomonas fluorescens* and 24 yeast isolates were obtained from healthy orange surface. From the total 200 isolates, 40 *P. fluorescens* and 16 yeast isolates were inhibited blue mould development on orange surface. The antagonistic effects of these isolates were tested on PDA. All the *P. fluorescens* isolates that prevented lesion development on orange surface, inhibited the *P. italicum* growth by antibiosis on PDA. On the other hand, the yeast isolates did not produce any inhibition zone, with had not having any antibiosis effect. A *P. fluorescens* isolate (Pf8a) which produced the widest inhibition zone and one isolate from the yeasts were selected for further studies. The yeast isolate was identified as *Candida famata*. The antagonistic isolates were inoculated simultaneously, after 2, 6 and 24 h and before 2, 6 and 24 h the inoculation of *P. italicum*. The research was conducted under the temperature conditions of 10 ^{o}C and 24^{o}C. The results revealed that *P. fluorescens* isolate gradually lost its efficiency and could not inhibit the blue mould development. The yeast, *C. famata* could inhibit the blue mould effectively, but it required a minimum incubation of 6h at 10 ^{o}C and a minimum incubation of 24h at 24^{o}C for sufficient colonization to prevent the infection of *P. italicum*.

Keywords: *Penicillium italicum*, orange, biological control, yeasts, *Candida famata*, *Pseudomonas fluorescens.*
Abbreviations: h_ hour; MEA_ Malt Extract Agar; NA_ Nutrient agar; NYDA_Nutrient Yeast Dextrose Agar; KBA_ King's B Agar; UV_ Ultra violet; cv._cultivar; PDA_Potato dextrose agar; ANOVA_Analysis of Variance .

Introduction

Postharvest losses of fruits and vegetables, including citrus, have been estimated at 25%; much of them caused by fungal and bacterial infections in worldwide (Ghaouth et al., 2002). *Penicillium* decay, caused by green (*Penicillium digitatum* Sacc.) or blue (*Penicillium italicum* Wehmer) mold, is the major cause of postharvest decay of citrus fruits. The extensive spore productions by the pathogens ensure their presence wherever citrus fruits is handled, including the field, packing house, equipments, degreening and storage rooms, transit containers and in the marketplaces. Infections take place only through the wounds where the nutrients are available to stimulate the spore germination. The mycelia of the fungi produce wall-degrading enzymes that cause a break-down of the fruit cell wall. The decay begins at the infected injury sites and within a few days entire fruit can be covered by a mass of green or blue spores. Blue mould develops less rapidly than the green mould in the ambient conditions. However, blue mould is widespread disease in fruit held in cold storage and it can spread in the packed cartons more rapidly than green mould (Ismail and Zhang 2004) and *P. italicum* is more common in the orange especially Valencia cv. (Chalutz and Wilson, 1990). Cultural practices that aimed to reduce inoculums and to prevent wounding the fruits are effective. Pre and post harvest fungicide applications are common and effective practices. The registered pesticides for storage are limited, four fungicides approved for the post harvest diseases of citrus; benomyl, carbendazim, thiabendazole and imazalil. However, the resistance to fungicides is a severe problem in the blue and green moulds, since resistant strains develop readily in large populations of spores. Additionally, the risks to consumers from the use of synthetic chemical fungicides is well known and have let researchers to find alternative control methods, including biological control (Arras, 1993; Droby et al, 1991). Recently, microbial biocontrol agents for the control of postharvest diseases of fruits have gained attention and some commercial products of antagonistic microorganisms have been registered for commercial use against postharvest decay of citrus and pome fruits. Control of moulds caused by *Penicillium* spp. was obtained on citrus fruit with some bacteria like *Pseudomonas* spp. (Bull et al., 1997; Huang et al., 1995; Smilanick and Dennis-Arrue, 1992), *Serratia plymutica* (Meziane et al., 2006) and *Pantoea agglomerans* (Elad et al., 2006; Canamas et al., 2008) and *Bacillus* spp. (Gutter and Littauer, 1953; Singh and Deverall, 1984; Arras and Hallewin, 1994). Antagonistic yeasts; *Saccharomyces cerevisiae* (Cheah and Tran, 1995), *Saccharomycopsis schoenii* (Pimenta et al., 2008), *Candida* spp. (Chalutz and Wilson, 1990; McGuire, 1994; Kinay et al., 2002; El-Neshawy, 2007; Hernandez-Montiel et al., 2010), *Cryptococcus laurentii* (Zhang HongYin et al., 2005), *Kloeckera apiculata* (Long ChaoAn et al., 2005), *Pichia* spp. (Droby et al., 1993, Arras et al., 2002, Lahlai et al., 2004) and *Sporidiobolus parparoseus* (Maharshi et al., 2009) were found effective for the control of blue and green moulds of citrus. The mode of action of bacterial antagonists is associat-

Table 1. Number of isolates isolated from orange surfaces, number of isolates that inhibit lesion development on orange and number of isolates that produced inhibition zones on agar plates.

Antagonists	Number of isolates	Number of isolates that inhibit lesion development	Number of isolates that produce inhibition zone on agar plate
Pseudomonas fluorescens	176	40	40(13.25-39.75 mm)
Yeasts	24	16	0

Table 2. Mean dimensions (mm) of the lesions on orange fruit treated with antagonists at different inoculation times at 10 ^{0}C

	Antagonist inoculation time	15th day	20th day	25th day	30th day
Control	-	7.02±0.53^{a}	12,89±0,61 ^{a*}	21,89±1,39 ^{ab}	30,33±2,12 ^{ab}
Pf 8a	24 h before pathogen inoculation	0^{d}	8,11±0,95 ^{efg}	15,67±1,51 ^{cdefg}	25,44±2,06 ^{bcd}
	6 h before pathogen inoculation	0^{d}	7,56±0,24 ^{fg}	12,33±2,33 ^{fgh}	22,22±2,01 ^{def}
	2 h before pathogen inoculation	0^{d}	9,56±0,82 ^{cde}	14,11±2,16 ^{efg}	22,89±2,82 ^{cde}
	0 (simultaneous with pathogen)	0^{d}	7,00±0,65 ^{g}	7,00±2,86 ^{hı}	15,67±3,37 ^{fg}
	2 h after pathogen inoculation	5.04±0.25^{bc}	10,44±0,29 ^{bc}	21,33±0,73 ^{ab}	34,44±1,45 ^{a}
	6 h after pathogen inoculation	5.03±0.14^{bc}	10,22±0,78 ^{bcd}	20,11±1,81 ^{abc}	30,22±1,91 ^{ab}
	24 h after pathogen inoculation	4.98±0.29^{c}	13,33±0,65 ^{a}	22,89±1,37 ^{a}	33,44±2,63 ^{a}
C. famata	24 h before pathogen inoculation	0^{d}	0±0 ^{ı}	0±0 ıj	0±0 ^{ı}
	6 h before pathogen inoculation	0^{d}	0±0 ^{ı}	0±0 ıj	0±0 ^{ı}
	2 h before pathogen inoculation	0^{d}	0±0 ^{ı}	5,00±2,59 ^{ij}	15,00±2,74 ^{g}
	0 (simultaneous with pathogen)	4.90±0.39^{c}	8,78±0,60 ^{cdefg}	17,22±2,52 ^{bcdef}	25,33±2,65 ^{bcd}
	2 h after pathogen inoculation	4.95±0.38^{bc}	9,57±0,58 ^{cde}	17,34±2,49 ^{abcdef}	31,56±2,08 ^{ab}
	6 h after pathogen inoculation	4.88±0.44^{c}	9,67±0,73 ^{cde}	14,22±2,91 ^{defg}	24,67±1,80 ^{cd}
	24 h after pathogen inoculation	5.72±0.64^{b}	10,90±0,84 ^{bc}	12,34±2,89 ^{fgh}	29,13±2,42 ^{abc}

*Means followed by with different superscript letters in same columns do not differ significantly at P ≤0.05 (resulted by Oneway-ANOVA, separated by Duncan test).

ed with the production of antibiotics like syringomycin and pyrrolnitrin. But some bacteria are able to compete with the pathogens for nutrients and space in the fruit wound. On the other hand, antagonistic yeasts do not inhibit the pathogens through antibiotic production (Kinay et al., 2002). Yeast isolates have been selected mainly on the basis of their competition ability for nutrients and space. The success of the antagonistic microorganisms is mainly depends on the colonization ability on the wound. They must reach to critical population to avoid the establishment of the pathogen. The protective effects of antagonists often declines with the ripening of the fruit through the storage period (Ghaouth et al., 2002). This study is aimed to isolate antagonistic *P. fluorescens*, besides the yeasts, from the orange surface and compare their effectiveness *in vitro* and *in vivo* against to the *P. italicum*, which is the most common decay fungus on Valencia cv. of orange.

Materials and methods

Pathogen isolate

The causal agent of blue mould, *P. italicum*, was isolated from orange showing blue mould symptoms by taking spores and inoculating into MEA. The isolate maintained as a single spore culture.

Antagonistic isolates

The antagonists were isolated from orange fruits that any postharvest disease control practices had been applied on. Two hundred orange specimens were obtained from different markets from November 2004 to March 2005. The fruit specimens were placed in 500 ml sterile bakers individually with containing of 200 ml sterile water. The bakers were placed on shaker at 100 rpm for 60 minutes. Washing water was then diluted ten-fold and 0.1 ml of dilution was spread on KBA and NYDA. All the agar plates were incubated at 28 ^{o}C. KBA plates were incubated for 24 h and screened out under UV light (264 nm). *P. fluorescens* isolates were selected by means of their fluorescent illumination and streaked on to KBA in triplicate. They were stored on NA after the confirmation of their homogeny. NYDA plates were observed under stereoscopic microscope at 48th h and yeast colonies were selected, homogeny was maintained above.

Preparation of antagonistic suspension

P. fluorescens isolates were streaked on NA while yeast isolates were on NYDA, and incubated in 27 ^{o}C for two days. Bacterial suspensions were prepared in sterile water and adjusted to 1x10^8 cfu spore suspensions according to Mc Farland scale. The conidial suspensions of the yeasts were adjusted to 1x10^8 spores per ml by using a Neubauer hemocytometer.

Preparation of P. italicum spore suspension

Conidia of *P. italicum* were harvested from the two-week old cultures on MEA. The conidiospores were suspended in sterile distilled water containing 0.1% Tween 20 (Merck). The concentration of spore suspension was adjusted to 1x10^7 spores per ml by using Neubauer hemocytometer.

Biocontrol activity assay on orange fruits

The intact orange fruits (Valencia cv.) containing any scar and any wound were surface-disinfected with 0.5 % NaOCl for 5 minutes then washed with tap water. After air-drying, oranges were treated with 70% ethanol. Each fruit was wounded on four points at its equator with a sterile drill tip (3 mm in diameter) in the depth of 3 mm. 20µl of spore suspension of all antagonistic isolates were dropped on the wounds by micropipette. The fruits were placed in plastic

Table 3. Mean dimensions of the lesions on orange fruit treated with antagonists at different inoculation times at +24 ^{0}C

	Antagonist inoculation time	5th day	10th day	15th day	20th day
Control	-	9.25±0.16^{a}	15,67±0,29 ^{a*}	33,11±1,01 ^{a}	42,56±2,89 ^{a}
Pf 8a	24 h before pathogen inoculation	0^{f}	4,56±0,18 ^{f}	8,22±1,39 ^{f}	11,11±2,63 ^{hı}
	6 h before pathogen inoculation	0^{f}	5,00±0,29 ^{f}	10,89±0,59 ^{ef}	23,89±3,99 ^{cde}
	2 h before pathogen inoculation	0^{f}	5,01±,024 ^{ef}	10,11±0,54 ^{f}	13,11±1,11 ^{ghı}
	0 (simultaneous with pathogen)	0^{f}	6,56±0,44 ^{e}	14,22±1,04 ^{e}	19,22±2,12 ^{efgh}
	2 h after pathogen inoculation	4.85±0.36^{e}	10,67±0,37 ^{cd}	23,00±1,03 ^{b}	32,00±4,07 ^{bc}
	6 h after pathogen inoculation	5.81±0.38^{cd}	10,78±0,52 ^{cd}	21,78±1,47 ^{bc}	34,00±3,68 ^{b}
	24 h after pathogen inoculation	6.22±0.52^{bc}	9,44±0,63 ^{e}	18,44±1,27 ^{d}	28,78±3,65 ^{bcd}
C. famata	24 h before pathogen inoculation	0^{f}	0±0 ^{g}	0±0 ^{h}	0±0 ^{j}
	6 h before pathogen inoculation	0^{f}	0±0 ^{g}	3,33±1,37 ^{g}	6,11±2,73 ^{ij}
	2 h before pathogen inoculation	0^{f}	4,44±0,18 ^{f}	10,00±0,75 ^{f}	12,00±2,82 ^{ghı}
	0(simultaneous with pathogen)	0^{f}	4,57±0,18 ^{f}	9,44±0,50 ^{f}	19,57±2,24 ^{efg}
	2 h after pathogen inoculation	0^{f}	4,33±0,17 ^{f}	8,78±0,52 ^{f}	12,11±1,16 ^{ghı}
	6 h after pathogen inoculation	5.18±0.43^{de}	9,22±0,68 ^{e}	19,33±1,25 ^{cd}	23,22±3,26 ^{def}
	24 h after pathogen inoculation	5.68±0.63^{cd}	11,56±0,63 ^{c}	22,33±1,27 ^{bc}	29,00±3,51 ^{bcd}

*Means followed by with different superscript letters in same columns do not differ significantly at P ≤0.05 (resulted by Oneway-ANOVA, separated by Duncan test).

trays which contain moistened filter paper at the bottom; all the plastic trays were covered with polyethylene stretch film to maintain humidity. After the 24 h incubation at 24 ^{o}C, the fruits were inoculated with *P. italicum*. The fruits, inoculated only antagonists and only *P. italicum* were used as two different – and + controls of the experiment. Each treatment was done with 4 replications and each replication consisted of 3 orange with 12 inoculated scars. The fruits were incubated for 5 days at 24 ^{o}C. At the end of this incubation period, all the fruits were examined for the decay. Lesion diameters, caused by *P. italicum*, were measured in two different directions and then recorded.

Determination of antagonism on agar plates

The spore suspension antagonistic isolates were dropped 20µl to each of four point of per PDA plates (9 cm). After the 24 h incubation, conidial suspension of *P. italicum* was sprayed to the plates and incubated at 27 ^{o}C in the dark. The plates were checked daily. At the 4th day of incubation, the plates were evaluated and the inhibition zones of the antagonists were measured and then recorded. The experiment was done triplicate for every antagonistic isolates.

Comparisons of antagonists at different conditions

One *P. fluorescens* isolate (Pf 8a) which exhibit high antagonistic activity on orange and agar plates, and one yeast isolate (C 59a) which inhibited lesion development on orange were used for the bioassays. The selected yeast isolate was identified as *C. famata* by using API test kits (Biomerioux) and its morphological characters in University of Hacettepe, Faculty of Medicinary, Microbiology and Clinical Microscopy Laboratory (Ankara, Turkey). The fruits were inoculated at different times to determine the effects of inoculation times and temperature of the antagonists on *P. italicum* infection. The inoculation times were described below and illustrated in Fig 1.

1. 24 h before (Antagonists were inoculated 24 h before the pathogen inoculation)
2. 6 h before (Antagonists were inoculated 6 h before the pathogen inoculation)
3. 2 h before (Antagonists were inoculated 2 h before the pathogen inoculation)
4. 0 (simultaneous) (Antagonists and *P. italicum* were inoculated at the same time)
5. 2 h after (Antagonists were inoculated 2 h after the pathogen inoculation)
6. 6 h after (Antagonists were inoculated 6 h after the pathogen inoculation)
7. 24 h after (Antagonists were inoculated 24 h after the pathogen inoculation)

This experiment was performed in both 10 ^{o}C and 24 ^{o}C. Lesion developments were observed and lesion diameters were measured for every five days interval untill the up to control fruits totally decayed. The data were subjected to ANOVA at the *P=0.05* level and comparisons were done by using Duncan's Multiple Range Test.

Results

Isolates and their antagonistic effects to P. italicum on orange and agar plate

From the 200 orange specimens, 176 *P. fluorescens* and 24 yeast isolates obtained. Of the *P. fluorescens* isolates, 40 isolates showed antagonistic activation and produce inhibition zones and isolate Pf 8a exibited the widest zone of inhibition (39.75 mm). Yeast isolates did not produce any inhibition zone (Table 1).

Effects of antagonists on lesion development of P. italicum at different inoculation times and at 10 ^{o}C

The first lesion was developed at the 15th day on the fruits that *P. fluorescens* and *C. famata* inoculated following pathogen inoculation (Table 2). Generally, preinoculations of antagonists caused less lesion development, but only 24 and 6 h preinoculations of *C. famata* wholly inhibited the lesion. 2 h preinoculation of the antagonist inhibited lesion development at 20th day, but at 25th day *P. italicum* caused decay symptoms on the orange and lesion spread to a 15 mm of dimension. Subsequent inoculations of *C. famata* were unsuccessful (Fig 2). The bacterial antagonist, Pf 8a, which was very effective *in vitro* studies, had a limited inhibitory effect when they inoculated 15 day before the pathogen inoculation, but the inhibition of the antagonists failed at the 20th day of the incubation (Fig 3).

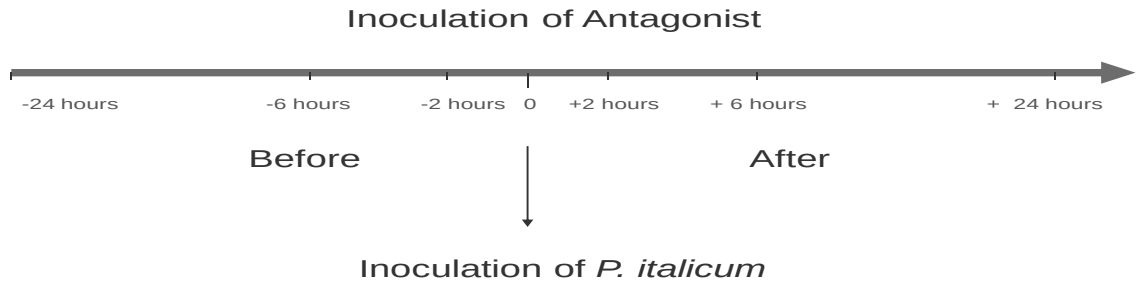

Fig 1. Application procedure to determine the effects of antagonistic isolates at different inoculation times

Effects of antagonists on lesion development of P. italicum at different inoculation times and at 24 ^{0}C

Effects of antagonists on the lesion development of *P. italicum* at 24 ^{o}C can be seen on Table 3. The first lesion developed in the antagonist inoculated oranges at 5th day of the inoculation. Pf 8a inhibited lesion development at 5th day when inoculated at the same time and previous inoculations, but from 10th day of incubation, it failed to inhibit the lesion development for all inoculation times (Fig 4). The lesion dimensions were significantly low at preinoculations of Pf 8a. *C. famata* could not prevent the lesion development at 5th day of incubation when it inoculated 6 and 24 h following pathogen inoculation. Six h preinoculation of *C. famata* prevented *P. italicum* infection at 10th day, but at the 15th day, the lesion development was observed. The 24 h preinoculation of *C. famata* totally inhibited infection of *P. italicum* up to 20th day of incubation (Fig 5), at that time the lesion dimension reached up 42.56 mm on control oranges.

Discussion

In the isolation studies, 176 *P. fluorescens* (71.43%) and 26 yeast like fungi (28.57%) were obtained. Janisiewicz (1988) stated that the antagonistic bacteria were more prevalent than yeast like organisms. In this research, similarly, *P. fluorescens* was more frequent on intact orange surface than yeasts. The results of the studies aimed to determine and compare the effectiveness of *Pseudomonas fluorescens* and yeasts on the orange fruits revealed that *C. famata* has longer lasting inhibitory effect on lesion development than the antagonistic *P. fluorescens*, despite yeasts did not produce antibiosis on agar plate (Table 1). Janisiewicz (1988) reported that a *Pseudomonas* sp. isolate reduced spore germination of *Penicillium expansum* and inhibited the lesion development when inoculated 70 h before the *P. expansum* inoculation. The lesion evaluations were done in the 7th day of incubation. In our study Pf 8a inhibited lesion development when applied simultaneously and before the *P. italicum* inoculation. Inhibitory effect continued up to 20th day in 10 ^{o}C and up to 10th day in 24 ^{o}C and after then lesion development started. *Pseudomonas* species produce antibiotics like syringomycin (Bull et al., 1998) and pyrrolnitrin (Smilanick and Dennis-Arrue, 1992). But *P. digitatum* isolates with very high levels of pyrrolnitrin resistance were found (Smilanick and Dennis-Arrue, 1992). Pf 8a produced antibiotics on PDA and wide inhibition zone against *P. italicum.* The antibiotics that produced by Pf 8a could not be sufficient to prevent the lesion development for a long incubation period. Furthermore, the antibiotic residues are not desired in the food because of their adverse side effects. (Droby et al., 1989). As a contrast to the bacterial antagonists, yeasts don't produce any antibiotics. Competition for nutrients and space is the major component of mode of action of the antagonistic yeasts. Application of *Candida famata*, 24 and 6 h before the pathogen inoculation inhibited lesion development in 10^{o}C, but, 6 h preinoculation was not sufficient for long incubation period in 24^{o}C. The yeast, *C. famata*, controlled the decay, when it colonizes properly on the wounds. *C. famata* could colonize, enough to control decay development, in 24 h at 24^{o}C, and in 6 h at 10^{o}C. Arras (1996) indicated that *C. famata* can compete with *P. digitatum*, green mould of orange, for space and nutrients, the *Penicillium* hypha were rapidly colonized by yeast cells, hyphal tissues were altered by possibly lytic enzymes like *β*-1,3 glucanase and chitinase (El-Neshawy, 2007). Two phytoalexin, scopoletin and scoparone, were elicitated in the orange tissue after 24 h by the yeast inoculation. These phytoalexins were inhibitory to both spore germination and germ tube elongation of *P. digitatum* (Kim et al., 1991; Rodov at al., 1994). According to Kim et al. (1991); inhibitory concentration of the scoparone was 29 µg/g for germ tube elongation and 46 µg/g for spore germination. Arras (1996) also stated that maximum elicitation of scoparone (188.7 µg/g fresh weight) observed on the orange fruits treated with *C. famata* and 24 h later inoculated with *P. digitatum*. The biosynthesis of the phytoalexins was significantly lower when the pathogen inoculated at the same time with yeast inoculation, or 24 h before. In our research, inhibition of the lesion development by 24h preinoculation of *C. famata* may be explained by both strengthening the defence mechanisms by elicitation of phytoalexins and inhibiting the pathogen directly by production of lytic enzymes and competition for space and nutrients. The performance of the yeasts is partially linked to a rapid consumption of available sugars in the medium (Hernendez-Montiel et al., 2010). *C. famata* presented a good protection when applied 24 h before the pathogen inoculation in this research. There are some other reports on that the yeasts need at least 24 h for enough colonization to inhibit the lesion development. Jijakli et al., (2004) stated that protection level of the *Candida oleophila* against to blue and green mould of citrus increased with the time between the application of the antagonist and the inoculation of the spores of pathogens. Hernández-Montiel et al., (2010) stated that a *C. famata* strain significantly reduced blue mould on Mexican lemon more than fifteen days of storage at 13°C. The authors declared the isolate was an alternative for biological control of blue mold decay of Mexican lemon. Karimi et al. (2006) reported that *C. famata* isolates reduced decay percentage of blue mould from 90% (control) to 6% in Valencia orange. In our research *C. famata* prevented the blue mould development totally more than 30 days when it was applied 6h before the pathogen inoculation at 10 °C.

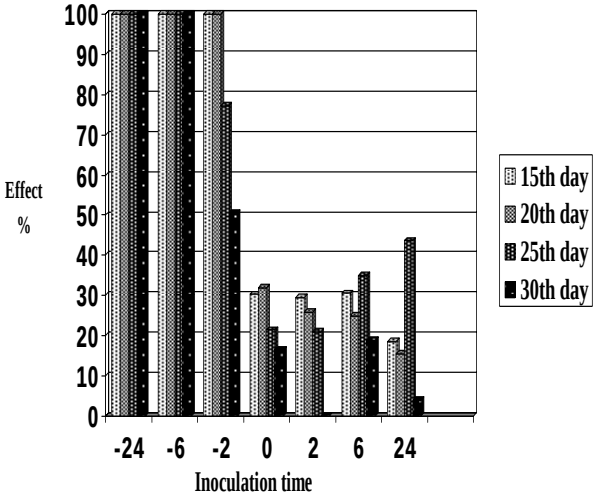

Fig 2. Percent effects of different inoculation times of *Candida famata* on the lesion development of blue mould on orange at 10°C.

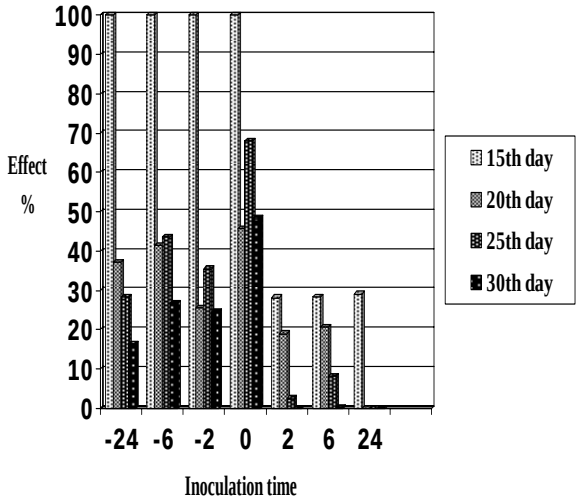

Fig 3. Percent effects of different inoculation times of *Pseudomonas fluorescens* on the lesion development of blue mould on orange at 10°C.

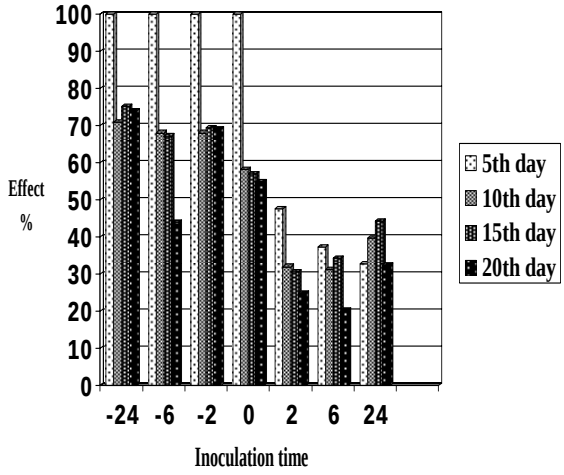

Fig 4. Percent effects of different inoculation times of *Pseudomonas fluorescens* on the lesion development of blue mould on orange at 24°C.

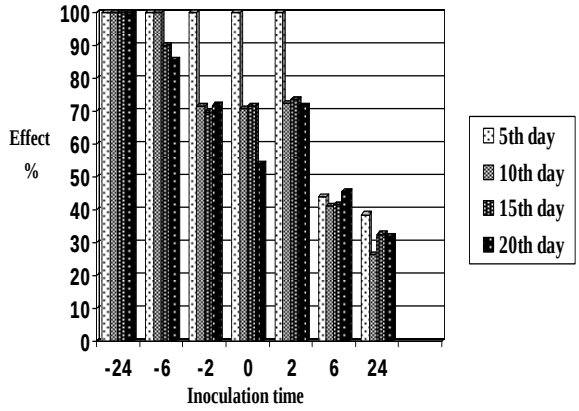

Fig 5. Percent effects of different inoculation times of *Candida famata* on the lesion development of blue mould on orange at 24°C.

Conclusion

The yeast; *C. famata* provided an effective control for the citrus postharvest decay caused by *P. italicum*. It can easily colonize on the wounds and inhibits the decay development. The antagonistic bacterium, *P. fluorescens* which have high antibiotic production capacity *in vitro* studies, controlled the decay development for 5-10 days, but it failed to continue its efficiency. This paper indicated that, the antagonistic yeast, *C. famata* can be used for the control of the important postharvest decay, blue mould of citrus caused by *P. italicum*.

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