EFFICACY ASSESSMENT OF *CANDIDA OLEOPHILA* (STRAIN O) AND *PICHIA ANOMALA* (STRAIN K) AGAINST MAJOR POSTHARVEST DISEASES OF CITRUS FRUITS IN MOROCCO

R. LAHLALI*, M.N. SERRHINI & M.H. JIJAKLI***

*Unité de phytopathologie, Faculté Universitaire des Sciences Agronomiques
Passage des Déportés, 2, 5030 Gembloux, Belgium
E-mail: jijakli.h@fsagx.ac.be

**Département de Phytpathologie, Ecole Nationale d'Agriculture de Meknès
BP S/40 50000 Meknès, Maroc,
E-mail: nserhini@enameknes.ac.ma

ABSTRACT

Two yeasts, *Candida oleophila* (strain O) and *Pichia anomala* (strain K), were previously selected for their antagonistic activity against postharvest diseases on apples and pears. The objective of the study was to determine the efficacy of both antagonistic yeast's against wound postharvest pathogens of citrus fruits. The efficacy of both strains (applied at 10^5, 10^6 and 10^8 CFU/ml) was assessed against *Penicillium digitatum* and *P. italicum* inoculated after one hour (at a concentration of 10^5, 10^6 and 10^7 spores/ml) on citrus varieties 'clementine' and 'valencia-late'. Fruits were incubated for one week at 24°C before measurement of lesion diameter. The protective levels were positively correlated with high concentration of antagonist and low concentration of pathogen. Highest protective levels (from 73 to 100%) were detected with the application of strain O or strain K at 10^8 CFU/ml whatever the pathogen (applied at 10^5 spores/ml) and the citrus variety. The antagonistic activity of both strains was also dependent on the incubation period before pathogen inoculation. The protective level increased with time between application of the antagonist and inoculation of fungal spores. Whatever the yeast strain (10^8 CFU/ml), the protective level exceed 70% when wounded oranges were inoculated with *P. digitatum* or *P. italicum* (both at 10^6 spores/ml) 12 hours after yeast treatment. These protective levels reached 100% when the incubation period separating the antagonist application and the pathogenic inoculation was 24 hours. On the other hand, high protective levels (< 80%) were also observed against the sour rot decay on citrus variety 'clementine' caused by *Geotrichum candidum* inoculated at concentration of 10^6 spores/ml when strain O or strain K were applied at 10^8 CFU/ml 24 hours before pathogen. All these results support the potential practical application of both strains against major postharvest pathogens on citrus.

Key words: Biological control, *Candida oleophila* strain O, *Pichia anomala* strain K, citrus, *Penicillium italicum*, *Penicillium digitatum* and *Geotrichum candidum*.

INTRODUCTION

Postharvest green and blue molds and sour decay, caused respectively by *Penicillium digitatum, P. italicum* and *Geotrichum candidum*, are responsible of significant economical losses on citrus worldwide (Droby *et al.*, 1989). Control of postharvest citrus diseases is mainly based on fungicide treatments such as thiabendazole (TBZ) or imazalil (IMZ) sprayed on fruits during packing-house operations. However, fungicide efficacy is frequently decreased by development of resistant strains of postharvest wound pathogens (Viñas *et al.*, 1993). Indeed the growing public concern for human health

safety and environmental hazards associated with high levels of chemical pesticides used in fruit orchards (Wisniewski and Wilson, 1992; Smilanick, 1994), imposed to develop alternative methods to control postharvest diseases of citrus.

Biological control of fruit decay using a microbial antagonist has been considered a desirable alternative to usual synthetic fungicides because the application sites are limited to the fruits, the environmental factors are defined and stable in the storage rooms, and the harvested commodities are of high value which allows to support costs of biological treatments potentially higher than chemical treatments (Fokkema, 1991; Wilson and Wisniewski, 1992, Jijakli *et al.*, 1999). Effective biological control has been reported for postharvest diseases of citrus (Chalutez and Wilson, 1990; El-Ghaout *et al.*, 2000), apples and pears (Janisiewicz, 1987; Jijakli *et al.*, 1993) and other fruits (Lima *et al.*, 1997).

Pichia anomala (strain K) and *Candida oleophila* (strain O) were previously selected for their efficient control of wound postharvest diseases of apples and pears (Jijakli *et al.*, 1993). However, both strains were not yet reported to control postharvest diseases of citrus. In this context, the main objective of this work was to evaluate the antagonistic activity of *C. oleophila* strain O and *Pichia anomala* strain K against major postharvest wound pathogens of citrus (*P. digitatum*, *P. italicum* and *G. candidum*) with respect to (1) relative concentrations at bitrophic level (antagonist and pathogen) and (2) time elapsing between antagonist application and pathogen inoculation.

MATERIAL AND METHODS

Fruit preparation

Citrus fruits 'clementine' (*Citrus reticulata* Blanco) and 'valencia-late' (*Citrus sinensis* [L] Osbeck) were harvested from commercial orchards in Tadla plain, Morocco. These fruits were stored, washed and only healthy fruits were selected for the experiments. Fruits were stored at temperature of 4°C during a maximum of 7 days before a later use. Citrus fruits of both varieties were disinfected by soaking during two minutes in a solution of sodium hypochlorite (10%) then rinsed twice in the sterile distilled water. After drying for one hour, citrus fruits 'valencia-late' were wounded in two equidistant points at the equatorial site. Each wound was 5 mm in diameter and 4 mm in deep. On the other hand, citrus fruits 'clementine' received a single wound with 5 mm in diameter and a depth ranged from 1 to 2 mm.

Antagonistic and pathogenic microorganisms

P. anomala strain K and *C. oleophila* strain O were cultured at 25°C for 3 successive generations on Potato Dextrose Agar (PDA) medium with an interval of 24 hours. The final concentrations of both yeast's were determined by Bürker's cell.

Strains of *P. digitatum* PDRBM1, *P. italicum* PIRBM1 and *G. candidum* GCRBM1 were isolated from decayed 'clementine' fruits harvested from Tadla plain in Morocco by the laboratory of plant pathology (ENA-Meknes) and stored onto PDA medium at 4°C in darkroom. The conidial suspension was

prepared from 9±2 day-old cultures of pathogen cultivated on PDA medium by scraping the surface of the colonies recovered with Tween 20 (0.05%). Spores were counted with a Bürker's cell and these concentrations were adjusted only with sterile distilled water.

Determination of biocontrol efficacy in relation with relative concentrations of pathogens and yeast's strains

Fruits previously prepared were treated by 50 µl of *C. oleophila* strain O or *Pichia anomala* strain K at concentrations of 10^5, 10^6 or 10^8 CFU/ml. One hour after yeast application, wounded fruits were inoculated by 50 µl of *P. digitatum* or *P. italicum* (10^5, 10^6 or 10^7 spores/ml). Fifty µl of sterile distilled water were applied on the control before pathogen inoculation. Fruits were stored in plastic boxes during 7 days at 24°C under 16 hours of photoperiod and high relative humidity. Three fruits were used per treatment (6 wounds for citrus variety 'valencia-late' and 3 wounds for citrus variety 'clementine') and two trials were carried out over time.

Determination of biocontrol efficacy in relation with time separating yeast application and pathogen inoculation

Prepared fruits were treated by one of the yeast's strains applied at a concentration of 10^8 CFU/ml. This application was made on citrus varieties 12 hours after pathogen inoculation, at the same time or 12 or 24 hours before pathogen inoculation. Two trials were carried out over time and each treatment contained 3 replicates per trial.

Determination of biocontrol efficacy of both yeast's strains against sour rot decay

Regarding *G. candidum*, two concentrations of both yeast's strains (10^5 and 10^8 CFU/ml) have been tested against this pathogen (10^6 spores/ml) on citrus variety 'clementine'. The yeast's strains were applied on citrus variety 'clementine' at the same time or 24 hours before the pathogen inoculation. The inoculated fruits were kept during 7 days at 24°C.

Evaluated parameters and statistical analysis

The lesion diameter due to pathogen infection was measured. All statistical analyses were performed using SAS software Version 8.12 (SAS Institute, Cary, NC). The data were analyzed separately for each combination (antagonist-pathogen concentration and variety) by GLM (General Linear Model). Mean separations were performed following the Ducan's multiple range test (P=0.05). The protective levels (Y%) were calculated with respect to the following formula:

$$D_T - D_x / D_T \times 100 = Y\%$$

Where D_T = diameter lesion of control and
D_X = diameter lesion of treatment.

RESULTS

Effect of relative concentration of both microorganisms on biocontrol effictiveness

The statistical analysis of lesion diameters revealed a significant effect of yeast's strains concentrations on lesion diameter development provoked by *Penicillium* pathogens inoculated at various concentrations whatever the pathogen and the citrus variety (Table 1 and 2). The protection offered by each strain of yeast's was higher with increasing concentrations of antagonist and decreasing concentrations of pathogen *P. italicum* or *P. digitatum*. Whatever yeast strain applied on wounded sites, the lesion diameters due to both pathogens were significantly reduced in comparison to the lesion diameter of control treatment. The lowest concentration of yeast (10^5 CFU/ml) only offered a weak protection against both wound citrus pathogens. This protection did not exceed 35% whatever the concentration of pathogen. The highest concentration of both strains (10^8 CFU/ml) allowed a higher efficacy on citrus variety 'valencia-late' against both wound pathogens inoculated at 10^5 spores/ml: 100% of protection was observed when strain O was applied whereas the use of strain K allows to reach 73 to 85% depending on the pathogen. In opposite, strain K allowed a slightly higher protective level (100% against both pathogens) on citrus variety 'clementine' than strain O (93 and 95% respectively against *P. digitatum* and *P. italicum*).

Effect of time elapsing between antagonist application and pathogen inoculation on biocontrol effectiveness

A significant difference was observed between lesion diameter means (evaluated on citrus variety 'valencia-late') corresponding to different periods separating the antagonist application and the pathogen inoculation (Table 3). The application of the antagonistic strain 12 hours after the pathogen reduced already significantly the lesion diameter with regard to the control (just inoculated with the pathogen) whatever the antagonist-pathogen combination. When antagonist and pathogen were simultaneously applied to the wound, the protective level observed for strain O was ranged between 70 and 78 % whatever the pathogen while this protective level did not exceed 50% for strain K. Compared with the results obtained during the first assay, where both antagonistic yeasts were applied one hour before wound pathogens inoculation (10^6 spores/ml) at a concentration of 10^8 CFU/ml, a difference in the protective levels was noticed and estimated around 10 to 20%. Both antagonistic yeasts offered a protective level up to 70% or 95% on citrus variety 'valencia-late' against both pathogens when they were applied respectively 12 or 24 hours before pathogen inoculation.

The influence of growing time between antagonist application and pathogen inoculation was also performed on citrus variety 'clementine' (Table 4). All treatments were significantly different from the control except when strain K was applied 12 hours after *P. digitatum* on 'clementine'. The protective level varied from 35% to 57% when strain K or strain O and one of the pathogen were applied simultaneously. These protective levels were lower than those detected during the first experiment where the pathogen was inoculated one

hour after the antagonist. The protective levels were superior to 80% when the antagonists were applied 12 hours before the pathogens and reached 100% when the time separating antagonist application and pathogen inoculation was 24 hours.

Efficacy of strain K and strain O against *G. candidum*

The highest concentration (10^8 CFU/ml) of both yeast strains K and O allowed a higher efficacy against sour rot decay than the lowest concentration (10^5 CFU/ml) (Table 5). This efficacy was equal or superior to 80% if the pathogen was inoculated 24 hours after yeast application (at 10^8 CFU/ml). Whatever the yeast strains applied at 10^5 CFU/ml, the protective level was ranged between 14 and 23% when pathogen and antagonist were applied at the same time and between 40 and 60% when yeast was applied 24 hours before the pathogen inoculation. In all cases a significant difference was observed between yeast treatments and the untreated treatment (P=0.05). Strain K allowed a higher control against *G. candidum* than strain O.

Table 1. Lesion diameter development (mm) on wounded citrus variety 'valencia-late' inoculated with various spores concentrations of *P. digitatum* or *P. italicum* one hour after treatment by different concentrations of *C. oleophila* (strain O) or *P. anomala* (strain K).

Yeast concentration (CFU/ml)	*P. digitatum* spores concentration (spores/ml)			*P. italicum* spores concentration (spores/ml)		
	10^5	10^6	10^7	10^5	10^6	10^7
C. oleophila						
10^5	38.3^{xb}	48.8^{b}	60.4^{b}	37.3^{b}	42.6^{b}	54.6^{b}
10^6	22.4^{c}	34.1^{c}	44.2^{c}	31.6^{c}	37.6^{ab}	51.4^{b}
10^8	0.0^{d}	7.4^{d}	28.9^{d}	00.0^{d}	12.4^{c}	34.7^{c}
Control^{y}	54.0^{a}	67.5^{a}	75.3^{a}	44.0^{a}	50.5^{a}	60.9^{a}
P. anomala						
10^5	42.0^{b}	54.0^{b}	62.3^{b}	29.9^{b}	35.4^{b}	39.5^{b}
10^6	36.7^{b}	47.5^{b}	47.8^{c}	25.2^{b}	34.1^{b}	37.6^{b}
10^8	15.6^{c}	24.9^{c}	39.3^{d}	05.6^{c}	19.1^{c}	31.0^{c}
control	55.6^{a}	65.8^{a}	71.6^{a}	40.0^{a}	42.5^{a}	49.75^{a}

x: Data are the mean of lesion diameters (mm) measured 7 days after pathogen inoculation.
y: Untreated citrus variety inoculated with the pathogen only.
For each yeast-pathogen concentration combination, values associated with the same letter are not significantly different according to Ducan's multiple range test (P <0.05).
The data result from two separates trials and no significant difference were observed between both trials in any cases.

Table 2. Lesion development (mm) on wounded citrus variety 'clementine' inoculated with various spores concentrations of *P. digitatum* or *P. italicum* one hour after treatment by different concentrations of *C. oleophila* (strain O) or *P. anomala* (strain K).

Yeast concentration (CFU/ml)	*P. digitatum* spores concentration (spores/ml)			*P. italicum* spores concentration (spores/ml)		
	10^5	10^6	10^7	10^5	10^6	10^7
C. oleophila						
10^5	31.9[xb]	48.6[a]	60.3[ab]	29.5[b]	35.4[b]	43.4[b]
10^6	12.8[c]	23.3[b]	54.4[b]	21.9[b]	31.3[b]	35.6[b]
10^8	3.3[c]	17.8[b]	24.4[c]	2.0[c]	8.0[c]	18.3[c]
Control[y]	61.1[a]	69.3[a]	74.4[a]	42.9[a]	49.6[a]	55.1[a]
P. anomala						
10^5	37.6[a]	45.1[ab]	56.8[ab]	29.6[b]	38.5[b]	43.4[b]
10^6	18.4[b]	25.3[bc]	42.9[bc]	18.9[c]	30.8[b]	38.5[b]
10^8	0.00[c]	14.1[c]	23.6[c]	0.00[d]	14.1[c]	19.9[c]
Control	52.3[a]	64.6[a]	72.5[a]	45.8[a]	52.9[a]	59.1[a]

x: Data are the mean of lesion diameters (mm) measured 7 days after pathogen inoculation.
y: Untreated citrus variety inoculated with the pathogen only.
For each yeast-pathogen concentration combination, values associated with the same letter are not significantly different according to Ducan's multiple range test ($P < 0.05$). The data result from two separates trials and no significant difference were observed between both trials in any cases.

Table 3. Lesion diameter development (mm) on wounded citrus variety 'valencia-late' treated with antagonistic yeast suspension (at 10^8 CFU/ml), and inoculated with pathogen suspension (10^6 spores/ml) in relation with time separating both operations.

Incubation time	*P. digitatum* (10^6 spores/ml)	*P. italicum* (10^6 spores/ml)
C. oleophila strain O applied: (10^8 CFU/ml)		
12 h after the pathogen	59.2[xb]	36.0[b]
0 h after the pathogen	19.4[c]	22.3[c]
12 h before the pathogen	10.8[c]	15.4[d]
24 h before the pathogen	0.0[d]	1.2[e]
Control	77.4[a]	50.2[a]
P. anomala strain K applied: (10^8 CFU/ml)		
12 h after the pathogen	57.9[b]	34.3[b]
0 h after the pathogen	37.5[c]	27.4[b]
12 h before the pathogen	13.0[d]	8.9[c]
24 h before the pathogen	1.6[e]	4.1[c]
Control[y]	66.4[a]	54.4[a]

x: Data are the mean of lesion diameters (mm) measured 7 days after pathogen inoculation.
y: Untreated citrus variety inoculated with the pathogen only.
For each yeast-pathogen combination, values associated with the same letter are not significantly different according to Ducan's multiple range test ($P < 0.05$). The data result from two separates trials and no significant difference were observed between both trials in any cases.

Table 4. Lesion diameter development (mm) on wounded citrus variety 'clementine' treated with antagonistic yeast suspension (at 10^8 CFU/ml), and then inoculated with pathogen suspension (10^6 spores/ml) in relation with time separating both operations.

Incubation time	*P. digitatum* (10^6 spores/ml)	*P. italicum* (10^6 spores/ml)
C. oleophila strain O applied: (10^8 CFU/ml)		
12 h after the pathogen	54.0^{xb}	41.1^{b}
0 h after the pathogen	21.6^{c}	29.3^{c}
12 h before the pathogen	18.3^{c}	5.3^{d}
24 h before the pathogen	0.0^{d}	0.0^{d}
Control	70.0^{a}	53.1^{a}
P. anomala strain K applied: (10^8 CFU/ml)		
12 h after the pathogen	67.^{a}	40.3^{b}
0 h after the pathogen	48.6^{b}	32.0^{c}
12 h before the pathogen	2.5^{c}	10.3^{d}
24 h before the pathogen	0.0^{c}	0.0^{e}
Control^{y}	75.3^{a}	51.6^{a}

x: Data are the mean of lesion diameters (mm) measured 7 days after pathogen inoculation.
y: Untreated citrus variety inoculated with the pathogen only.
For each yeast-pathogen combination, values associated with the same letter are not significantly different according to Ducan's multiple range test ($P < 0.05$). The data result from two separates trials and no significant difference were observed between both trials in any cases.

Table 5. Lesion diameter development (mm) on wounded citrus variety 'clementine' inoculated with *G. candidum* (10^6 spores/ml) at the same time or 24 hours after treatment by both yeast's strains *C. oleophila* (strain O) and *P. anomala* (strain K).

Treatments	*C. oleophila* strain O	*P. anomala* strain K
10^5 CFU/ml:		
0 h after pathogen	33,3^{xa}	31,9^{b}
24 h before the pathogen	23,15^{b}	17,3^{c}
10^8 CFU/ml:		
0h after the pathogen	22,1^{b}	17,1^{c}
24 h before the pathogen	7,8^{c}	6,8^{d}
Control^{y}	38,5^{a}	41,1^{a}

x: Data are the mean of lesion diameters (mm) measured 7 days after pathogen inoculation.
y: Untreated citrus variety inoculated with the pathogen only.
For each yeast strain, values associated with the same letter are not significantly different according to Ducan's multiple range test ($P < 0.05$). The data result from two separates trials and no significant difference were observed between both trials in any cases.

DISCUSSION

In our experiments, *C. oleophila* strain O and *P. anomala* strain K isolated from apples surface (Jijakli *et al.*, 1993) effectively reduced infection of three major postharvest diseases (*P. digitatum*, *P. italicum* and *G. candidum*) on both citrus varieties at laboratory scale. However, the efficacy obtained by both antagonistic yeast's against sour rot decay remained less important than this observed for *Penicillium* decayed. Chalutz and Wilson (1990)

showed also that *Debaryomyces hansenii* inhibited incidence of green and blue mold and sour rot on several citrus cultivars.

Our results demonstrated that antagonistic activity of both yeast strains depends on the incubation time between their application on citrus wound and the pathogen inoculation and the ratio of concentrations between pathogen and antagonist. These observations suggest that colonisation of the wound sites is a prerequisite to effective protection. An antagonistic yeast concentration of 10^8 CFU/ml gave higher protective level than lower concentrations whatever the pathogen and the citrus variety. In this respect, Droby *et al.* (1989) reported that an increase of *D. hansenii* concentration resulted in more effective biocontrol against *P. digitatum* on citrus. Our results are also in accordance with those of El-Ghaout *et al.* (2000) who observed a more effective control of post-harvest decay with *Candida saitoana* applied at 10^8 CFU/ml and often no control of decay when this biocontrol agent was applied at 10^5 CFU/ml. As, protective level obtained by both antagonistic yeast's increased with time separating their application and pathogen inoculation, this parameter must be considered for the development of an effective biocontrol method against postharvest diseases of citrus. Chalutz and Wilson (1990) reported also that the efficacy of *D. hansenii* was maintained when applied simultaneously or prior to inoculation with *Penicillium digitatum*. However, this efficacy was reduced when *D. hansenii* was applied after pathogen inoculation. Jijakli *et al.* (1993) showed already that the incubation time was one of the major parameters influencing the efficacy of both strain K and strain O against postharvest diseases of apples.

Our results do not clarify the mode of action of *C. oleophila* strain O and *P. anomala* strain K in the inhibition of citrus postharvest decay nor do they fully assess their efficacy under commercial practices. These areas need further studies. If required, other techniques will be considered to increase the protective level of biocontrol method such as preharvest application, manipulation of the environment and physiological and genetic manipulation (Janisiewicz and Korsten, 2002). The design of the yeast formulation is also important and in some cases allows a higher and more stable efficacy of the antagonist (Jijakli *et al.*, 2002).

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