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DEVELOPMENT OF A BIOLOGICAL CONTROL METHOD AGAINST POSTHARVEST DISEASES OF CITRUS FRUITS

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ABSTRACT

Candida oleophila strain O was previously selected for its high and reliable antagonistic activity against *Botrytis cinerea* and *Penicillium expansum*, two important wound pathogens on post-harvest apples. The application of these antagonistic strains on wound pathogens of Citrus was more recently undertaken.

The efficacy of yeast (applied at several concentrations from 10^5 to 10^8 CFU/ml) was assessed against *P. digitatum* and *P. italicum* inoculated after one hours (at a concentration of 10^5, 10^6 and 10^7 spores/ml) on 'Clementine' and 'Valencia late' varieties. The protective levels were positively correlated with high concentration of antagonist and low concentration of pathogen. The antagonistic activity of this strain was also dependent on the incubation time before pathogen inoculation. The protective level increased with time between application of the antagonist and inoculation of fungal spores.

Finally, the efficacy of biomass of *C. oleophila* strain O (produced at an industrial scale), and two different formulations of that biomass was assessed in comparison with fungicidal treatment (Thiabendazole) under semi-practical conditions against *P. digitatum*. This efficacy of strain O (whatever its formulation) was statistically comparable to that for TBZ at commercial dose, indicating that both formulations could be used as an alternative for conventional fungicide in postharvest treatments.

Keywords: Biological control, *Candida oleophila* strain O, Citrus, *Penicillum digitatum*, *Penicillium italicum*.

INTRODUCTION

The most common and serious diseases that affect citrus fruit produced in Mediterranean climates are green mould (*Penicillium digitatum* Sacc.) and blue mould (*P. italicum* Weh.) (Eckert & Eaks, 1989). Both pathogens are responsible of significant economical losses on citrus worldwide (Droby *et al.*, 1989). Application of chemical fungicides is the main method for controlling postharvest decay in citrus fruit including green and blue moulds (Eckert, 1990). However, the consumer demand for pesticide-free food (Norman, 1988) and the proliferation of pathogenic strains that are resistant to currently used synthetic fungicides such as thiabendazole (TBZ) and imazalil (IMZ) (Porat *et al.*, 2002), imposed to develop alternative methods to control postharvest diseases of citrus (Obagwu & Korsten, 2003; Lahlali *et al.*, 2004).

Previous reports have underlined the potential application of biological control agents against postharvest diseases of citrus fruits. Various strains of bacteria and yeasts antagonistic against *P. digitatum* and *P. italicum* were selected such as *Bacillus pumulus* (Huang *et al.*, 1992), *Bacillus subtilis*

(Obagwu & Korsten, 2003), *Candida saitoana* (El-Ghaouth *et al.*, 2000), *Pichia guilliermondii* (Droby *et al.*, 1997), *Pichia anomala* strain K and *Candida oleophila* strain O (Lahlali *et al.*, 2004). Biological control has been considered as a realistic alternative to conventional fungicides to control post-harvest diseases because the application sites are limited to the harvest commodities and the environmental factors are defined and stable in the storage room. Furthermore, the harvested commodities are of higher value, which allow to support potentially higher cost due to biological treatments in comparison with conventional chemical treatments (Fokkema, 1991; Wilson and Wisniewski, 1992, Jijakli *et al.*, 1999). For general acceptance of biological control by farmers, the efficacy must be comparable with the level of control provided by synthetic fungicides (Pusey, 1994; Obagwu & Korsten, 2003). The efficacy of products based only on one antagonistic microorganism is often lower than the efficacy due to products based on mixture of antagonistic bacteria and/or yeasts or products containing an antagonistic agent with other additives (Janisiewicz & Korsten, 2002). In this context, the main objective assigned to this work was to compare the efficacy of different formulations of *C. oleophila* strain O against *P. digitatum* and *P. italicum* with the efficacy of chemical treatment (TBZ).

MATERIAL AND METHODS

Antagonistic and pathogenic microorganisms

C. oleophila strain O was cultured at 25°C for 3 successive generations on Potato Dextrose Agar (PDA, Merck, Darmstadt, Germany) medium with an interval of 24 hours. The final desired concentrations of antagonistic yeast were determined by using Bürker's cell. The biomass of strain O produced at an industrial scale and two formulations based on this biomass were also tested.

Strains of *P. digitatum* PDRBM1 and *P. italicum* PIRBM1 were isolated from decayed 'Clementine' fruits harvested from Tadla plain in Morocco 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. Spore concentrations were adjusted only with sterile distilled water.

C. oleophila strain O efficacy under controlled conditions

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 4°C during a maximum of 7 days before use. Citrus fruits of both varieties were disinfected by soaking during two minutes in a solution of sodium hypochlorite (10 %) then rinsed twice in 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.
Fruits were treated with 50 µl of *C. oleophila* strain O one hour after yeast application, wounded fruits were inoculated with 50 µl of *P. digitatum* or *P. italicum*. 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'). Two trials were carried out over time in any case.

Comparison between yeast and fungicide efficacy under controlled conditions

Fruits were prepared as described previously. Fifty µl of strain O (dishes production) at 10^7 CFU/ml or strain O (Industrial production) (1 g/3 l) or thiabendazole (0.1%) were applied on wounded fruits. Inoculation of *P. digitatum* or *P. italicum* on treated wounds was realized one hour after previous treatments at a concentration of 10^5 spores/ml. Two trials were carried out over time at 24°C for 7 days with three fruits per treatment.

Population dynamic of *C. oleophila* on wounded 'Valencia-late'

The population dynamic of *C. oleophila* strain O was assessed on wounded citrus fruits 'Valencia-late' variety at 4°C and 25°C. Fourteen fruits of 'Valencia-late' variety were disinfected and wounded as described above. Disinfected fruits of citrus variety 'Valencia-late' were wounded in two sites at their equatorial zone with a depth of 4 mm and 5 mm in diameter. Each wound was inoculated with a suspension of 50 µl of antagonistic yeast applied at 10^7 CFU/ml. Population monitoring of strain O per wound was realized daily at 25°C and every ten days at 4°C. Each wound (two wounds per recovery time) was taken aseptically and placed in 10 ml of KBPT buffer [(KH2PO4 (0.05M), K2HPO4 (0.05M) and 0.05% of Tween 80, pH 6.5)]. After washing during 1 minute and 30 seconds by an ultra-thurrax T25, serial dilutions were carried out and plated in triplicate on Petri dishes containing PDA medium. The plates were incubated at 25°C during 72 hours. The trial was twice repeated.

C. oleophila efficacy under semi-commercial conditions

Fruits of 'Clementine' variety were obtained from Meknès market city in Morocco at the beginning season of yield (September-October) and then wounded (1-2 mm in diameter and 4 mm in the depth) at 4 points with a disinfected cork borer. Wounded fruits were dipped in aqueous suspension of strain O (Industrial production), Formulation 1 of strain O (industrial production + additives), Formulation 2 of strain O (Industrial production + additives) or TBZ at commercial dose (0.3%) for 2 minutes. The control batches were treated with tap water.
The treatments were carried out on four repetitions with 50 fruits per repetition. After 24 hours, fruits were inoculated by pulverization with 10^5

spores/ml of *P. digitatum* whatever the treatment. After drying during one hour, treated fruits were kept in storage room (4°C).
Number of infected fruits or infected wounds were noticed every 10 days. Infected fruits were eliminated during each evaluation. This trial lasted 20 days.

Statistical analysis

The lesion diameter, incidence and severity of decay were analysed by an analysis of variance using SAS software Version 8.12 (SAS Institute, Cary, NC). Statistical significance was judged at $P<0.05$. When analysis revealed statistically significant, Duncan's Multiple Range Test was performed for means separation.
To correct the homogeneity of variance, the data of antagonist populations (CFU/wound) were transformed to logarithm.

RESULTS

Assessment of strain O efficacy in relation with pathogen and strain O concentrations

The statistical analysis of lesion diameters revealed a significant effect of concentrations of *C. oleophila* strain O on lesion diameter development provoked by *Penicillium* pathogens inoculated at various concentrations whatever the pathogen and the citrus variety (Table 1). The efficacy was higher with increasing concentrations of strain O and decreasing concentrations of *P. italicum* or *P. digitatum*. The lesion diameters due to both pathogens were significantly reduced by applying strain O 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. Whatever the citrus variety, the highest concentration (10^8 CFU/ml) allowed a higher protection against both wound pathogens inoculated at 10^5 spores/ml.

Assessment of strain O efficacy in relation with time separating its application and pathogen inoculation.

The influence of growing time between application of *C. oleophila* strain O and pathogen inoculation was performed on citrus varieties 'Valencia-late' and 'Clementine' (Table 2). All treatments were significantly different from the control. When antagonist and pathogen were simultaneously applied to the 'Valencia-late' wounds, the protective level observed ranged between 70 and 78% whatever the pathogen. However, for 'Clementine' wounds, this level reached 57%. These protective levels were lower than those detected during the first experiment where the pathogen was inoculated one hour after applied strain O. On the other hand, the protective levels were superior to 80% when the strain O was applied 12 hours before the pathogens and reached 100% when the time separating antagonist application and pathogen inoculation was 24 hours.

Table 1. Lesion diameter development (mm) on wounded citrus varieties inoculated with various spores concentrations of *P. digitatum* or *P. italicum* one hour after treatment with *C. oleophila* strain O applied at several concentrations.

	P. digitatum spores concentration (spores/ml)			*P. italicum* spores concentration (spores/ml)		
Yeast concentration (CFU/ml)	10^5	10^6	10^7	10^5	10^6	10^7
'Valencia-late'						
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}
'Clementine'						
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}

x: Data are the mean of lesion diameters (mm) measured 7 days after pathogen inoculation.
y: Untreated citrus variety inoculated with the pathogen.
For each citrus variety-pathogen concentration combination, values associated with the same letter are not significantly different according to Duncan'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 diameter development (mm) on wounded citrus varieties treated with *C. oleophila* strain O, applied 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)
'Valencia-late'		
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}
'Clementine'		
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}

x: Data are the mean of lesion diameters (mm) measured 7 days after pathogen inoculation.
y: Untreated citrus variety inoculated with the pathogen.

For each citrus variety-pathogen combination, values associated with the same letter are not significantly different according to Duncan'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.

Assessment of strain O and fungicide efficacy under controlled conditions

Whatever the pathogen and citrus variety, the protective level offered by strain O (from dishes production or industrial production) remained lower as compared with that reported for thiabendazole (0.1%) (Figure 1, 2). In case of 'Valencia-late', treatments based on strain O were significantly lower than TBZ application except for treatment with industrial production of strain O against *P. digitatum*. On 'Clementine' variety and against *P. italicum*, the efficacy of strain O (industrial production) was slightly higher than that ob-

served for fresh cells. This efficacy was equal to 74 % and 83 % respectively against blue and green mould.

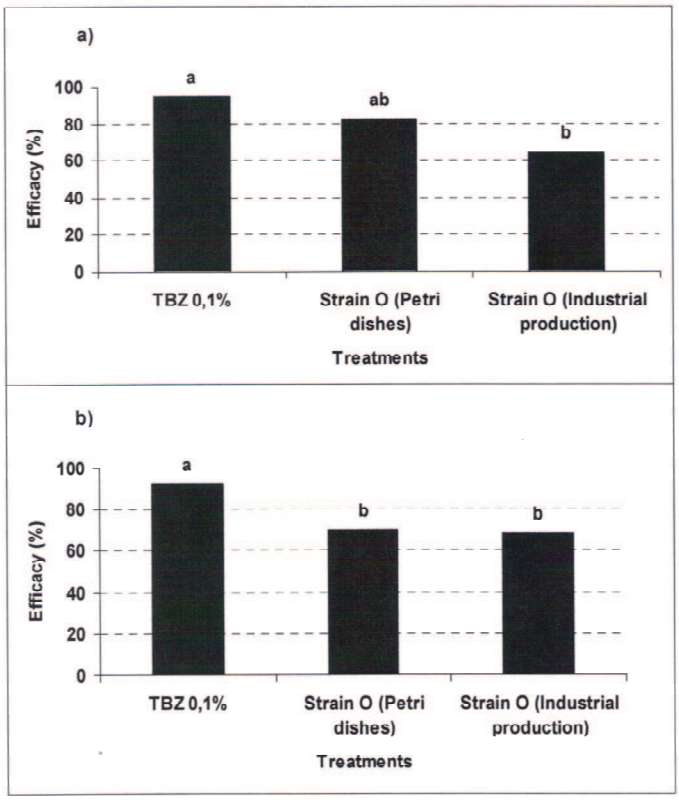

Figure 1. Percentage of protection observed on 'Valencia-late' (after 7 days of incubation at 24°C) treated with *C. oleophila* strain O produced in Petri dishes (10^7 CFU/ml) or at an industrial scale (1 g/3 l) or TBZ (0.1 %) then inoculated one hours later with *P. digitatum* (a) or *P. italicum* (b) (10^5 spores/ml). The average protective level was calculated from two trials carried out over time with three replicates per treatment. Histograms sharing the same letter belong to the same homogenous group according to Duncan's Multiple Range Test ($P<0.05$).

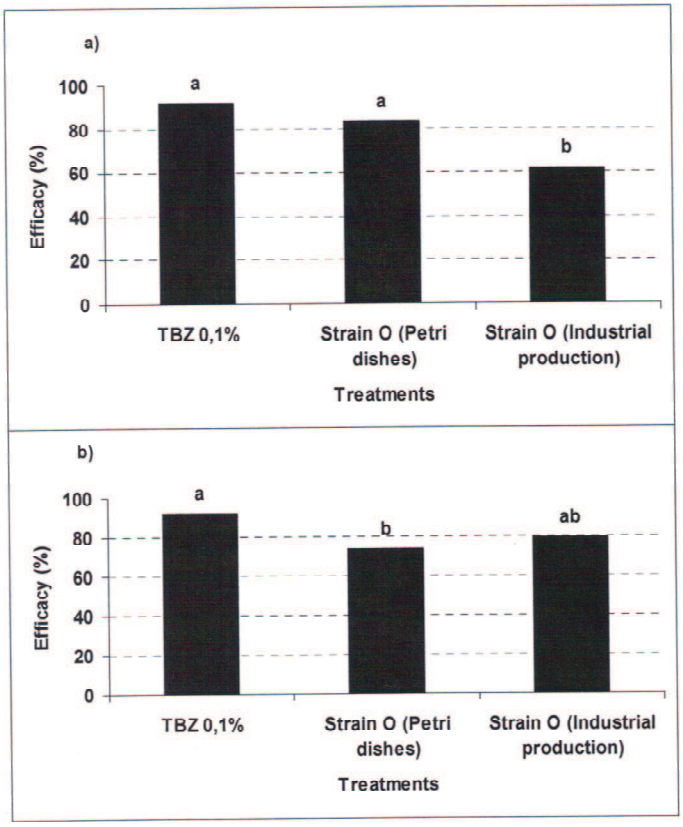

Figure 2. Percentage of protection observed on 'Clementine' variety (after 7 days of incubation at 24°C) treated with *C. oleophila* strain O produced in Petri dishes (10^7 CFU/ml) or at an industrial scale (1 g/3 l) or TBZ (0.1%) then inoculated one hours later with *P. digitatum* (a) or *P. italicum* (b) (10^5 spores/ml). The average protective level was calculated from two trials carried out over time with three replicates per treatment. Histograms sharing the same letter belong to the same homogenous group according to Duncan's Multiple Range Test ($P<0.05$).

Assessment of strain O density on wounded fruit 'Valencia-late'

The ability of cells of *C. oleophila* strain O to multiply and survive on wounds of citrus fruits was studied at 25°C and 4°C (Figure 3). The density of strain O remained stable at 25°C during the first 24 hours and increased to reach a maximum of 2.5×10^7 CFU/ wound after 4 days of incubation. At 4°C, the density of antagonistic yeast decreased after two first days then increased to reach a density of 3.7×10^6 CFU/wound after 10 days of incubation. The population in wounded sites remained stable until thirteen days ($\pm10^7$ CFU/wound).

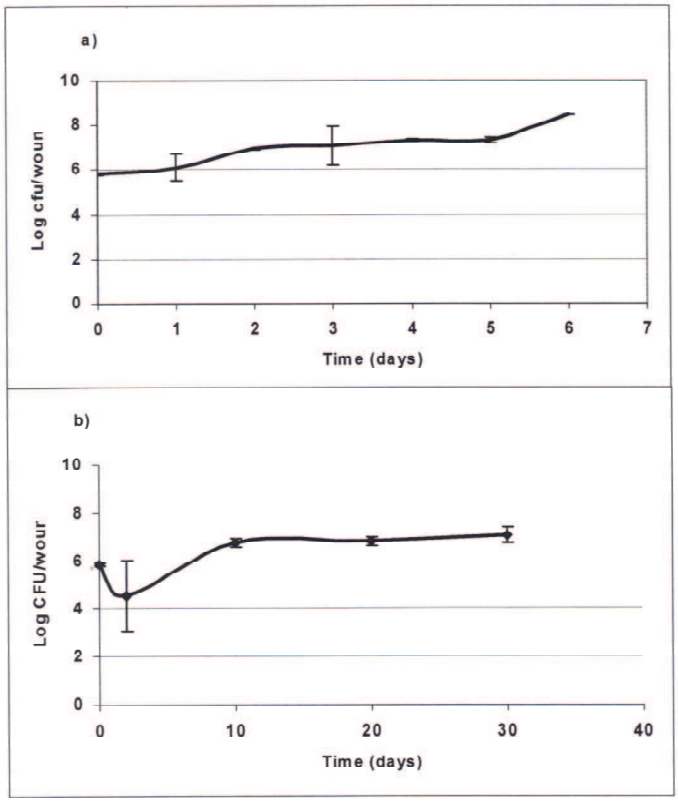

Figure 3. Population density of *C. oleophila* strain O, applied at 10^7 CFU/ml, on wounded sites of 'Valencia-late' at 25°C (a) and 4°C (b). Vertical bars represent the standard errors between two trials carried out over time and relating to their respective averages.

Assessment of strain O efficacy under semi-commercial conditions

The evaluation of the efficacy of strain O produced at an industrial scale with or without formulations additives was determined against *P. digitatum* on 'Clementine' variety (Figure 4). All applied treatments were significantly different from the control treatment and no significant difference was observed between TBZ treatment and strain O with or without additives. The percentage of infected fruits was ranged between 7 and 19% and, between 3 and 8% for infected wounds.

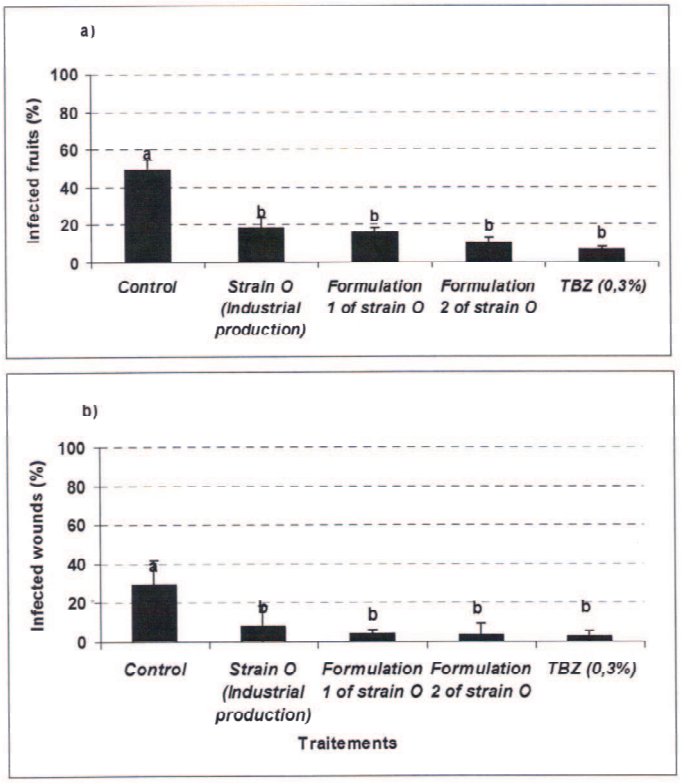

Figure 4. Percentage of infected fruits (a) and infected wounds (b) on 'Clementine' variety by green mould at various treatments. All treatments were pulverised with *P. digitatum* at 10^5 spores/ml. Four repetitions were carried out by treatment (50 fruits per repetition). Histograms sharing the same letter belong to the same homogenous group according to Duncan's Multiple Range Test ($P<0.05$).

The addition of additives to strain O biomass allowed obtaining a higher protection (around 4% of infected wounds) in comparison with strain O biomass applied alone (8%). However, the percentage of infected wounds observed in formulation based on strain O was slightly higher than that for fungicide treatment (3%).

DISCUSSION

Results of the present work showed that *C. oleophila* strain O reduced significantly the diameter lesion of both green and blue moulds on laboratory scale trials on 'Valencia-late' and 'Clementine' orange varieties. However the efficacy of strain O remains dependent on the incubation time between its application on citrus wound and the pathogen inoculation and the ratio of concentrations between pathogen and antagonist. An antagonistic yeast concentration of 10^8 CFU/ml gave higher protection than a concentration of 10^5 CFU/ml whatever the pathogen and citrus variety. 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-Ghaouth *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. Nunes *et al.* (2001) tested the biocontrol effectiveness with respect to relative concentration of antagonistic bacteria *Pantoea agglomerans* CPA-2 and *P. expansum* and *R. stolonifer*, two postharvest diseases of pears and apples and they concluded a total protection with higher concentration of antagonist (10^8CFU/ml) and low concentration of both pathogens (10^3 spores/ml).

The potential of *C. oleophila* strain O as biological control agents has been previously reported against *P. italicum* and *P. digitatum* (Lahlali *et al.*, 2004; Jijakli *et al.*, 2004). However, its efficacy was not evaluated in semi-practical trials or compared with that of a conventional fungicide such as imazalil or thiabendazole. In some cases, strain O produced in dishes or at industrial scale was not as effective as the fungicide treatment against green or blue moulds in citrus. Obagwu & Korsten (2003) also underlined that applying isolates of *Bacillus spp.* alone offered a lower protection against postharvest citrus diseases as compared to the use of conventional fungicides. To improve their efficacy, they suggested to use them in combination with sodium bicarbonate or hot water. Previous reports highlight the beneficial role of GRAS substances such as sodium carbonate and sodium bicarbonate to reduced conidial germination of *P. digitatum* on citrus fruits (Smilanick *et al.*, 1997; Smilanick *et al.*, 1995). Smilanick *et al.* (1999) reported that combining 0.3% of sodium carbonate and *P. syringae* ESC-10 gave higher protection than the use of each treatment alone against green mould citrus. The use of calcium chloride (2 % w/v) in mixture with *P. anomala* (strain K) or *C. oleophila* (strain O) increased the protection against *B. cinerea* and *Penicillium sp.* (Jijakli *et al.*, 1999). In our study, the efficacy of two different formulations of strain O (industrial production) containing some additives was tested against green mould in semi-practical trials on 'Clementine' variety. No significant difference was observed between biological (strain O produced at an industrial level with or without additives) and chemical treatments in terms

of rates of infected fruits and infected wounds. The use of additives in formulated product based on strain O improved slightly its efficiency. The maximal period of 'Clementine' fruits storage, estimated between 3 to 4 weeks (Serrhini, unpublished data), is compatible with the efficacy of formulated and unformulated strain O observed after 20 days.

The study of survival ability of *C. oleophila* strain O in wounded citrus fruits indicates a good adaptation of this strain to cold storage temperatures. Furthermore, it was observed that strain O is able to grow at the wounded sites which are the main entry of *P. italicum* (green mould) and *P. digitatum* (blue mould).

In conclusion, the effectiveness of strain O produced at an industrial scale and formulated was comparable to that of thiabendazole at commercial dose, indicating that the formulations based of this biocontrol agent could be used as a substitute for conventional fungicides to control green and blue decays of citrus fruits.

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