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Influence of antagonist, host fruit and pathogen on the biological control of postharvest fungal diseases by yeasts

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The yeasts *Rhodotorula glutinis* (LS-11), *Cryptococcus laurentii* (LS-28), *Candida famata* (21-D) and *Pichia guilliermondii* (29-A) and the yeast-like fungus *Aureobasidium pullulans* (LS-30), previously selected and characterized for mechanisms of action and antagonistic activity against postharvest pathogens in small and large-scale experiments, were used in this study in order to assess interrelationships among the main factors (antagonist, host fruit and fungal pathogen) involved in biological control of postharvest diseases. The antagonists were evaluated for their inhibitory activity (IA) against six common postharvest fungal pathogens on six different host fruits. Artificially wounded fruits were first inoculated with the antagonist and 2 h later with the pathogen; subsequently they were kept at 20°C for 4–6 days. The IA of each antagonist was evaluated and data were submitted to factorial analysis of variance. The populations of antagonists were also monitored on wounded and unwounded fruits kept at 20°C for 7 days. Each factor examined (antagonist, host fruit and fungal pathogen) as well as their interactions significantly affected the IA. However, among the antagonists, isolates LS-28 and LS-30 were only slightly affected by both host and pathogen, showing a wide range of activity, whereas isolate LS-11 had a variable IA. All the antagonists rapidly colonized the wounds, while their population remained substantially unchanged on unwounded fruits. These results suggest that in order to select yeasts with a broad spectrum of action, more suitable for commercial development, it would be advantageous to perform preliminary assays against several pathogens and in particular on different fruit species.

Keywords: biological control; postharvest diseases; yeasts; fruits; biofungicide

Introduction

Biological control of postharvest disease of fruits and vegetables by antagonistic microorganisms seems increasingly promising to replace or integrate the use of synthetic fungicides which are subjected to some limitations due to development of fungicide-resistant strains of the pathogens, deregistration of some products and risks for consumers and the environment [14,15,36]. The positive role of natural antagonists of the phyllosphere and carposphere in suppressing disease development has been widely demonstrated [1,29]. Several isolates of bacteria and yeasts were selected from the naturally occurring microflora and applied to fruits and vegetables for their high biocontrol activity against postharvest pathogens [3,10,22,23,26,38]. Moreover, in some countries commercial formulations of biocontrol agents, based on the yeast *Candida oleophila* (Aspire™) or on the bacterium *Pseudomonas syringae* (Biosave™), are already available for postharvest applications on pome and citrus fruits [13,19]. Yeasts, including yeast-like fungi, are particularly suitable for postharvest use, because of their high inhibitory capacity, rapid colonization of fruit wounds and modes of action mainly based on competition for nutrients, direct physical interaction with fungal hyphae and production of cell-wall lytic enzymes [4,7,8,12]. However, it is important to point out that most of the selected yeasts have frequently been tested for antagonistic activity on only a few host fruits and against a limited number of postharvest pathogens, while it is essential for the commercial development of a biocontrol agent to have a wide range of activity [38]. Industry is much more likely to invest in and develop a biofungicide that provides both consistent disease control and stable performance [17]. At present, there is a lack of information on the influence of the antagonist, the host fruit and the fungal pathogen on the effectiveness of microorganisms selected and proposed for application as biological control agents against postharvest diseases of fruits and vegetables. Moreover, since antagonists operating as nutrient competitors act prophylactically [1], their survival and colonization on different fruits are also important criteria for selection of an effective biocontrol agent [29,34]. Although considerable research on the microbiology and microecology of antagonists in fruit wounds exists, little information is available for the unwounded surface of fruits, where antagonists are usually applied in commercial conditions.

In this work we examined the combined effect of five yeast isolates, six fruit species, and six fungal pathogens on the biological control of postharvest diseases. We also studied the population dynamics of the yeasts on wounded and unwounded tissues of two different fruit species.

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Materials and methods

Antagonists

The yeasts used were: *Rhodotorula glutinis* (Fres) Harr (LS-11), isolated from olive; *Cryptococcus laurentii* (Kuff) Skinn (LS-28), isolated from apple; *Aureobasidium pullulans* de Bary (LS-30), isolated from apple; *Candida famata* (Harr) Meyer & Yarr (21-D), isolated from table grapes, and *Pichia guilliermondii* Whicker (29-A), isolated from lemon. The yeasts had previously been isolated [3,23] according to the selective method described by Wilson *et al* [35] and were already used in studies performed to assess their modes of action [2,3,6–8] and their antagonistic activity on some fruits under field and semi-commercial conditions [5,22–24]. Each antagonist was grown on nutrient yeast-extract broth (NYDB) kept on a rotary shaker (150 rpm) for 48 h at 22°C. The cells were collected by centrifugation ($3000 \times g$ for 15 min), washed in sterile distilled water (SDW) and suspended in the same volume of SDW. The yeast concentration was adjusted to 10^8 cells ml^{-1} using a hemocytometer.

Pathogens

The fungal pathogens used were: *Aspergillus niger*, van Thieg; *Botrytis cinerea* Pers; *Rhizopus stolonifer* Ehrenb, isolated from table grapes; *Penicillium expansum* Link, isolated from apple; *P. italicum* Wehm and *P. digitatum* (Pers) Sacc, isolated from orange. In order to obtain the inoculum suspension, each pathogen was grown on potato dextrose agar (PDA) under fluorescent light for 5–7 days at 21°C. Conidia were removed from the agar, suspended in SDW containing 0.05% Tween 20, filtered through four layers of cheesecloth and their concentration was adjusted with a hemocytometer.

Fruits

Fruits, belonging to different species and at maturity suitable for marketing, were harvested from orchards located in Southern Italy and they had not been treated with pesticides. The fruits utilized were: apple (cv Annurca); pear (cv Kaiser); strawberry (cv Pajaro); kiwi fruit (cv Hayward); table grapes (cv Italia); orange (cvs Biondo comune and Vaniglia); mandarin-like (Clementine × Tangelo Orlando, cv Fairchild); and grapefruit (cv Marsh seedless). Before their use, fruits were carefully selected, superficially disinfected by immersion for 1–2 min in a sodium hypochlorite solution (2% active chlorine), rinsed twice with SDW and dried at room temperature for about 2 h.

Inhibitory activity assay

The host-pathogen combinations tested were: apple, pear, strawberry and table grapes inoculated with *B. cinerea* and *P. expansum* (2×10^4 conidia ml^{-1}); kiwi fruit inoculated with *B. cinerea* (1×10^5 conidia ml^{-1}); apple, strawberry and table grapes inoculated with *A. niger* and *R. stolonifer* (1×10^5 conidia ml^{-1}, on apple, and 2×10^4 conidia ml^{-1} on strawberry and table grapes); orange, grapefruit and mandarin inoculated with *P. italicum* and *P. digitatum* (1×10^5 conidia ml^{-1}). The spore concentrations of the pathogen were the lowest ones that in preliminary tests gave 90–100% infection in control fruits after a 4–6 day incubation at 20°C.

Table grape berries were wounded by removing the pedicel, while the other fruits were injured near the equatorial zone with 1–4 wounds (3 mm wide × 3 mm deep) according to fruit size. Thirty microliters of an antagonist suspension (1×10^8 cells ml^{-1}) were placed in each wound, while in the wounds of control fruits was placed a drop of SDW. Two hours later, wounds were inoculated with 15 μl of a pathogen suspension. Fruits were incubated at 20°C and 95–98% RH, and 4–6 days later decay was evaluated, when 90–100% of control wounds were infected. Each treatment included five replications and each replication consisted of ten strawberries or table grape berries or six fruits of the other species tested. The assays were performed at least twice. The infected wounds were assessed daily and the data were transformed into a percentage of inhibitory activity (IA) as follows: $IA = [(T - A)/T] \times 100$, where T was the number of infected wounds in the control, and A was the number of infected wounds inoculated with the antagonist and the pathogen. Values ranged between 0 (no IA, corresponding to 100% infected wounds), and 100 (max IA, corresponding to lack of evident infection). Data assessed on different citrus fruits (orange, mandarin and grapefruit) that were homogeneous in their variability were pooled for each of the two pathogens inoculated in these fruits (*P. digitatum* and *P. italicum*).

Yeast population dynamics

The population dynamics of the antagonists were evaluated on wounded and unwounded apples and oranges. Fruits were treated as described above, except for unwounded

Table 1 Variance analysis table for inhibitory activity in biological control assays using five antagonistic yeasts, six host fruits and six postharvest fungal pathogens

Factor	Degrees of freedom	Mean square	F value	Significance
Antagonist (A)	4	5321.8	38.7	<0.001
Host fruit (B)	4	1936.4	14.1	<0.001
Pathogen (C)	4	434.3	3.2	0.014
Interaction A × B	16	908.3	6.6	<0.001
Interaction A × C	15	595.5	4.3	<0.001
Interaction B × C	6	2840.2	20.7	<0.001
Interaction A × B × C	24	1005.2	7.3	<0.001
Error	316	137.4		

Table 2 Effect of five antagonistic yeasts, six host fruits and six postharvest fungal pathogens on inhibitory activity (%) in biocontrol assays (three-way interaction)[a]

Host fruit	Pathogen	Antagonist				
		LS-11	LS-28	LS-30	21-D	29-A
Apple	*A. niger*	90.0 A–D	96.3 A–C	98.3 AB	80.0 A–H	80.0 A–J
	P. expansum	41.5 D–L	82.3 A–G	90.1 A–D	76.2 A–F	60.2 B–L
	R. stolonifer	91.3 A–C	87.0 A–D	91.3 A–C	87.0 A–D	78.3 A–H
	B. cinerea	24.5 A–L	91.4 A–D	89.3 A–D	74.6 A–K	75.7 A–G
Pear	*P. expansum*	31.3 G–L	100.0 A	93.6 A–C	25.0 H–L	34.3 E–L
	B. cinerea	78.1 A–J	95.3 A–C	54.7 C–L	82.5 A–F	60.9 B–L
Strawberry	*P. expansum*	66.7 A–L	63.3 B–L	93.7 A–C	70.0 A–L	70.0 A–K
	R. stolonifer	26.7 J–L	63.4 B–L	74.9 A–K	30.0 F–L	63.3 B–L
	B. cinerea	18.6 KL	93.1 A–C	100.0 A	62.1 B–L	82.8 A–F
Kiwi fruit	*B. cinerea*	69.1 A–L	94.2 A–C	92.2 A–D	65.0 A–L	79.2 A–I
Table grapes	*A. niger*	28.2 G–L	86.9 A–E	100.0 A	47.8 C–L	76.1 A–K
	P. expansum	87.0 A–D	78.2 A–H	100.0 A	100.0 A	100.0 A
	R. stolonifer	64.3 A–L	71.4 A–K	100.0 A	77.6 A–J	61.2 B–L
	B. cinerea	16.5 L	95.9 A–C	100.0 A	77.6 A–J	61.2 B–L
Citrus fruits	*P. italicum*	79.3 A–J	78.9 A–J	72.9 A–J	88.9 A–E	93.2 A–D
	P. digitatum	88.9 A–E	83.1 A–G	74.7 A–H	98.7 AB	98.7 AB

[a]Values marked by the same letters are not statistically different at $P = 0.01$, according to Tukey's test.

fruits that were inoculated by spraying the yeast suspension over the whole surface. Control fruits were treated in the same manner with SDW. The yeast population was assessed 0, 12 h, and 1, 2, 4 and 7 days after the treatment on fruits stored at 20°C and high RH.

The peel of unwounded fruits was superficially cut with a cork borer (8 mm, internal diameter) and the sample of skin was removed with a sterile knife; the latter was also used to extract tissue samples containing the whole wound from injured fruits. Three replicate samples were prepared from each treatment. Each sample, consisting of ten portions of tissue taken from two fruits, was placed in a 100-ml Erlenmeyer flask containing 50 ml SDW, and kept on a rotary shaker at 200 rpm for 30 min. In order to assess yeast populations, specimens were processed as described previously [23].

Scanning electron microscopy (SEM)

SEM observations were performed on wounded citrus fruits inoculated with *C. famata*, isolate 21-D, or *P. guilliermondii*, isolate 29-A. Fruits were prepared and treated as described previously for IA assay and incubated at 20°C and 95–98% RH for 5 days; then samples of flavedo and albedo were removed and treated as previously described [6] and observed under a Zeiss DSM 962 scanning electron microscope.

Statistical analysis: Inhibitory activity data were factorially analyzed by a three-way ANOVA using the software SPSS 7.5 for Windows (SPSS Inc, Chicago, IL, USA) with antagonist, host fruit and pathogen as main factors. Mean values of single effects and interactions were compared using Tukey's test. The percentages of IA were converted into Bliss angular values (Arcsin ✓ %) before analysis.

Results

Inhibitory activity

The factorial analysis of variance of IA data revealed that the single factors analyzed (antagonist, host fruit and pathogen) as well as their two-way and three-way interactions had a significant influence on the biological control experiments (Table 1). In particular, among the factors tested, the antagonist and host fruit showed a higher level of significance ($P < 0.001$) than the pathogen ($P = 0.014$).

IA average values of all possible combinations of the single factors (three-way interaction) are reported in Table 2. Values ranged from 16.5% (isolate LS-11 on table grapes inoculated with *B. cinerea*) to 100% (different combinations of treatments) and showed significant differences among several values included in this range. Considering the antagonists, notice the high variability of isolate LS-11, with IA values ranging from 16.5% to 91.3%, and the more stable activity of isolates LS-28 and LS-30 whose IA values ranged from 63.3% to 100% and from 54.7% to 100%, respectively. IA values of isolates 21-D and 29-A ranged from 25.0% to 100% and from 34.3% to 100%, respectively.

The effect of the single factors on IA is shown in Figure 1. The average IA value of each antagonist (Figure 1a) ranged from 56.4% of isolate LS-11 to 90.1% of isolate LS-30. Isolates LS-28, 21-D and 29-A showed values of 85.0%, 71.4% and 73.4%, respectively. As regards the host fruit factor (Figure 1b), the IA values were statistically similar for apple (79.3%), kiwi fruit (79.9%), table grapes (76.5%) and citrus fruits (86.9%), whereas significantly lower values were observed on pear (65.6%) and strawberry (65.2%). The IA values related to the fungal pathogen (Figure 1c) were not statistically different (ranging from 71.2% for *R. stolonifer* to 82.6% for *P. italicum*) except

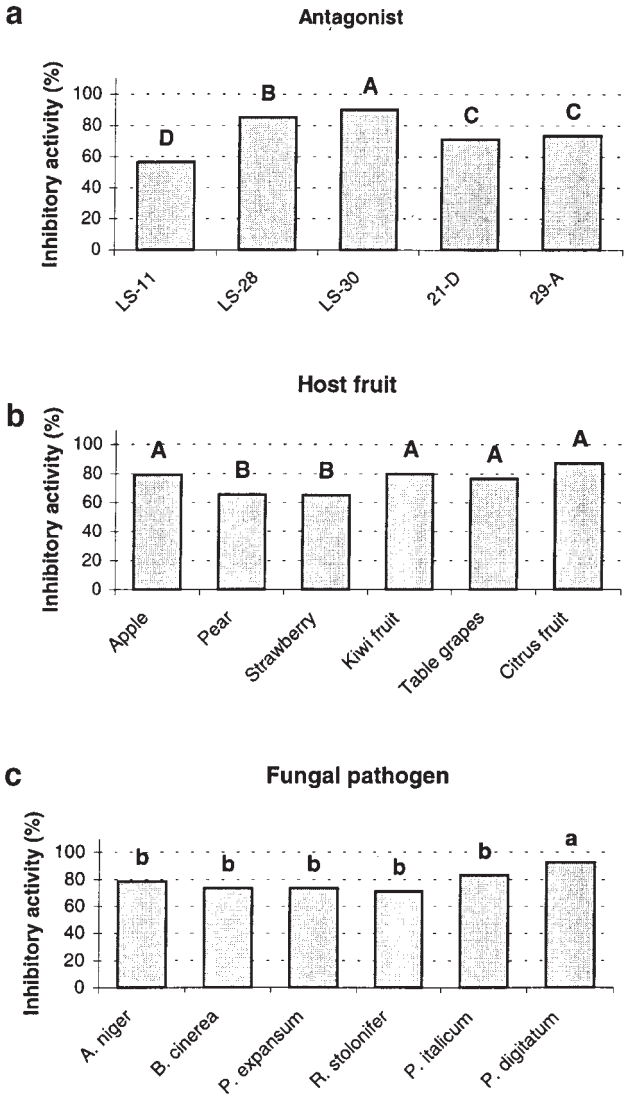

Figure 1 Bar charts showing the effect of the main factors: antagonistic yeast (a); host fruit (b) fungal pathogen (c) on the inhibitory activity (%) in biocontrol assays. In each graph values marked by the same letters are not statistically different at $P = 0.05$, small letters, and at $P = 0.01$, capital letters (Tukey's test).

for *P. digitatum* which showed a significantly higher value (92.3%).

Yeast population dynamics

Population dynamics of the antagonists, assessed on wounded and unwounded apple and orange fruits, are shown in Figure 2. On control fruits (not reported) no yeast cell was recovered.

On wounded orange population sizes ranged from 1.6×10^5 CFU wound^{-1} (LS-30 after 12 h) to 5.5×10^6 CFU wound^{-1} (29-A at day 7) (Figure 2a). Population sizes of isolates LS-11, LS-28, 21-D and 29-A increased rapidly within 24 h after inoculation, while they remained substantially stable or decreased (LS-11) from 24 h to day 7. Moreover, isolate 29-A increased its population at a higher rate with respect to the other isolates, showing in particular, significantly higher sizes at days 2 and 4. The population size of LS-30 decreased slightly within the first 12 h, then it increased rapidly till day 2 followed by a decrease again up to day 7.

On wounded apples yeast populations ranged from 2.3×10^5 CFU wound^{-1} (LS-30 at time 0) to 3.5×10^7 CFU wound^{-1} (LS-11 at day 4) (Figure 2b). Population sizes of isolates LS-11, LS-28, 21-D and 29-A increased rapidly till day 2 after the treatment, while they remained substantially steady up to day 7. Isolate LS-30 showed significantly lower population sizes until day 4 after its application; however, at day 7 it reached the same population size as the other antagonists.

As regards unwounded fruits, the yeasts on orange behaved differently from those on apple. On orange, populations of all antagonists remained prevalently steady at 10^5 CFU cm^{-2} fruit skin (Figure 2c); significantly lower values were observed only for isolates LS-30 and 21-D at days 4 and 7 after application of the treatments. On apple, populations ranged from 1.2×10^3 CFU cm^{-2} to 1.0×10^7 CFU cm^{-2} (Figure 2d). The yeast populations decreased consistently within the first 24 h, while they increased constantly up to day 7; isolate LS-11 showed significantly higher values at days 2, 4 and 7.

Scanning electron microscopy (SEM)

SEM observations, performed on wounded orange tissues treated with cells of *C. famata*, strain 21-D, or *P. guilliermondii*, isolate 29-A, showed that both yeasts consistently colonized fruit wounds (Figure 3).

Discussion

The antagonists used in this study are well known since they are representative of the yeasts occurring naturally on fruit and vegetable surfaces and have been reported for their high antagonistic activity against postharvest diseases [6,9,20,21,23,37]. The fruits and pathogens examined are not only some of the most widespread but also some of the most important.

Results indicate that the factors investigated as well as their interactions significantly influenced the IA in biological control assays. However, among the antagonists, isolates LS-28 of *C. laurentii* and LS-30 of *A. pullulans* were only slightly affected by both host and pathogen, showing a wide range of activity, whereas isolate LS-11 of *R. glutinis* had a variable IA. Isolates 21-D of *C. famata* and 29-A of *P. guilliermondii* showed intermediate values. The presence, on the same or different fruits and vegetables, of antagonistic yeasts with different degrees of activity has been reported in several studies [6,11,22,25,30].

In addition to antagonist diversity, the variability of IA could be related to differences of fruit susceptibility and/or pathogen virulence in agreement with Spott and Sanderson [28], who found that fruit crop and pathogen diversity are some of the most important factors affecting performance of antagonistic microorganisms in the postharvest environment.

Antagonist mechanisms of action could also contribute to the variability of IA in biocontrol assays. In fact, some mechanisms of the yeasts, ie, induction of resistance and direct interaction with the pathogen, might be mediated by

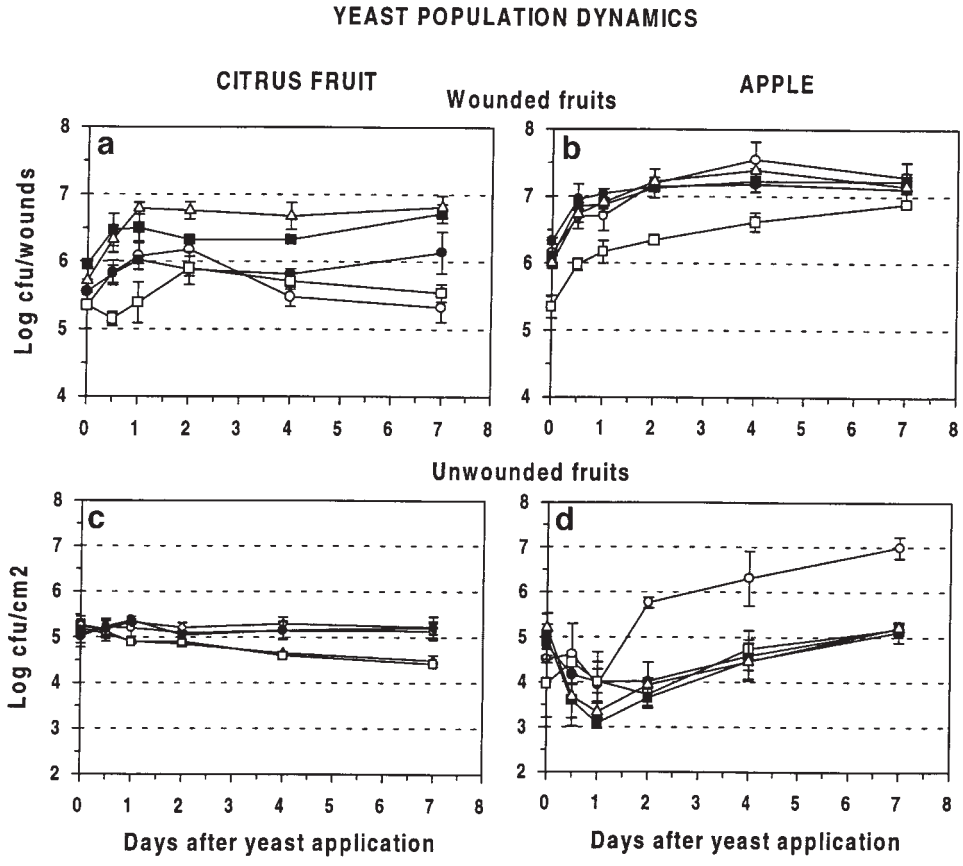

Figure 2 Population dynamics of the yeasts *Rhodotorula glutinis* (LS-11, –○–), *Cryptococcus laurentii* (LS-28, –●–), *Aureobasidium pullulans* (LS-30, –□–), *Candida famata* (21-D, –■–) and *Pichia guilliermondii* (29-A, –△–) on wounded (a, b) and unwounded (c, d) apple and orange fruits kept at 20°C for 7 days. Bars represent ± standard deviation of the mean using three replicates for each treatment.

the host and/or the pathogen [6,7,12,32,37] thus subjecting the performance of these antagonists to modifications, changing the host and/or the pathogen. Some compounds responsible for resistance can be differently elicited by the antagonists and some yeasts attach themselves to the pathogen hyphae, while others do not [2,7,8,12,16].

Monitoring populations of the five yeasts on wounded and unwounded orange and apple fruits showed that all isolates colonized wounded tissues rapidly and survived at an appreciable level on unwounded surfaces. On wounded fruits, isolate LS-30 of *A. pullulans* at almost all sampling times showed lower CFU than other isolates. Since this isolate behaved as one of the most effective and stable antagonists, the lower wound colonization could be balanced by more efficient mechanisms of action. In particular, as regards the comparison of this isolate with the less effective isolate LS-11, some differences were found in the production of cell-wall lytic enzymes; in fact, LS-30 produced a significantly higher level of β-1,3-glucanase and also produced chitinases [7,8]. Isolates of *C. famata* and of *P. guilliermondii*, elicited phytoalexins (scoparone and scopoletin) in citrus fruit wounds [2,6,27]. In particular, in agreement with Rodov *et al* [27] scoparone reached fungitoxic concentrations 48 h after yeast inoculation. Moreover, the mode of action of isolate 29-A in orange fruit wounds probably involves rapid colonization of the tissues during the first 24 h after inoculation (Figure 2).

Some higher values of populations reached by antagonists in the wounded tissues of apples, with respect to those found in citrus fruit wounds, are probably due to the higher content of nutrients, ie, readily available sugars [31]. On unwounded tissues the antagonists survived at a substantially stable level of population on orange fruits, whereas their population decreased within 1–2 days after inoculation and increased up to day 7 on apple; isolate LS-11 of *R. glutinis* showed significantly higher values at days 2, 4 and 7 on apple. These differences on antagonist populations could also be due to nutritional characteristics of the two host fruit surfaces. Isolate LS-11, according to our previous investigation [23], has a particular ability to survive and colonize unwounded fruits.

The results of this study, in agreement with those of other research [3,22,25], found no relationship between the origin where the antagonist was isolated and its effectiveness against postharvest diseases on different fruits.

In conclusion, this investigation indicates that antagonist isolate, host fruit and fungal pathogen can affect significantly the IA in the biological control of postharvest diseases. Thus, in order to select yeasts with a broad spectrum of action it would be advantageous to perform preliminary assays against several pathogens and, in particular, on different fruit species, since the host fruit showed a higher influence on biocontrol compared to that of the fungal pathogen. Consequently, experiments on a wider scale

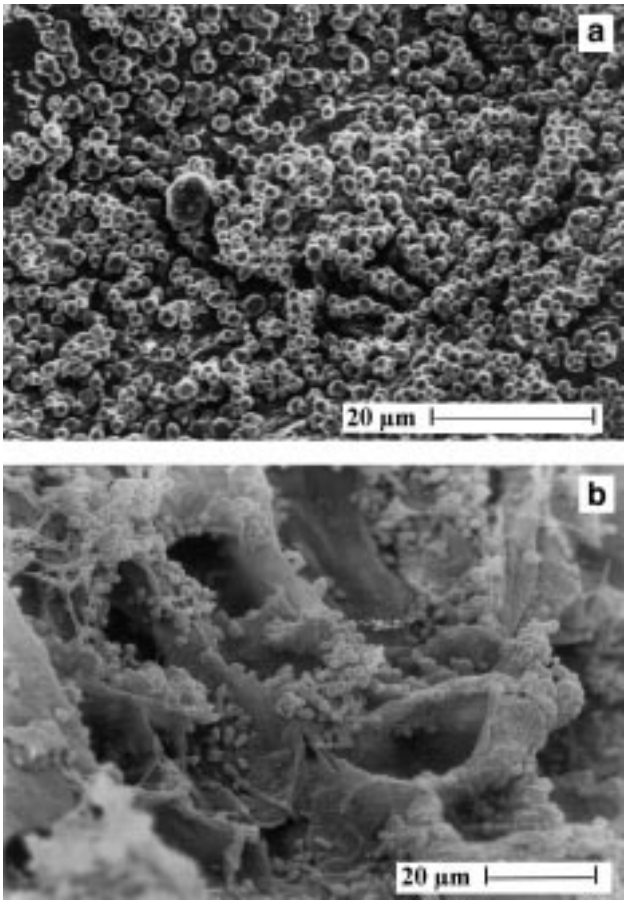

Figure 3 Scanning electron micrographs showing the colonization of orange fruit wounds by the yeasts *Candida famata*, isolate 21-D (a), and *Pichia guilliermondii*, isolate 29-A (b).

should be performed choosing the selected broad-spectrum antagonists which should be more suitable for commercial development. If these isolates are not available the variability in the IA could be stabilized and/or enhanced using a mixture of different antagonists [18,33] or using integrated control strategies [15]. However, it is known that other characteristics, ie, survival and host colonization, modes of action, compatibility with fungicides and safety for consumers could also be decisive factors [38].

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