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Biological Control of Peach Fungal Pathogens by Commercial Products and Indigenous Yeasts

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ABSTRACT

The potential use of the commercial biocontrol products Serenade (*Bacillus subtilis* QST-713) and Trichodex (*Trichoderma harzianum* Rifai strain T39) to inhibit the postharvest pathogenic molds *Penicillium crustosum* and *Mucor circinelloides* was investigated. Both products exhibited antagonistic activity in vitro against the pathogens, reducing their growth at different levels. In addition, epiphytic yeasts isolated from peaches were identified as *Candida maltosa, Pichia fermentans,* and *Pichia kluyveri* by PCR–restriction fragment length polymorphism of internal transcribed spacer regions and screened for antagonistic activity against the same molds. The efficacy of biocontrol in vitro was dependent on the concentration of the yeast cells. Optimal yeast concentrations were above 10^7 CFU ml^{-1}. However, *C. maltosa* and *P. fermentans* were more effective than *P. kluyveri* in inhibiting molds. The exclusion of antifungal metabolite production and direct competition for nutrients or space with the pathogens was proposed as the mechanism of biocontrol. Application of biocontrol agents directly on artificially wounded peach fruits significantly reduced the incidence of mold rot during storage at 20°C.

Biocontrol in agriculture has been introduced as an alternative to the use of pesticides to minimize losses of products due to insects and microorganisms, with advantages in terms of health and environmental pollution. In the past decade, many studies have been performed in the field of biological control of plant diseases, which often cause significant economic losses.

Product losses are frequently caused by molds, which contaminate fruits in orchards and can grow on wounds of the fruit surface caused by harvesting or postharvest handling. Biocontrol of spoilage microorganisms has mainly been applied on harvested products, because postharvest conditions ensure stable and controllable temperature and humidity. On the other hand, antagonists in field applications must be able to tolerate UV radiation, high temperatures, low nutrient availability, and dry conditions *(17, 19).* Italian and European laws allow only a few chemical principles for postharvest treatments; in particular, no fungicide is permitted on stone fruits. Thus, the fight against fungal rot is commonly performed by means of preharvest chemical treatments. Some studies have tested the antagonistic effects of yeasts, bacteria, and molds toward spoilage agents of different fruits in postharvest storage *(7, 28).* In particular, a few studies have considered the effect of biocontrol on postharvest production of peaches and other stone fruits *(2, 13, 21, 26).* Most of the studies have concluded that some species can have antagonistic effects toward spoilage microorganisms only if colonization is prevented, while the ability to control preexisting infections, as is possible with chemical fungicides, is highly desirable *(5, 8).* The action of antagonistic microorganisms may be due to the secretion of antibiotics, competition for space and nutrients *(3, 4, 25),* production of cell wall lytic enzymes *(9, 32),* and induction of host resistance *(1).*

Italy is one of the main European producers of peaches, for which the major cause of postharvest decay is fungal rot, in particular brown rot caused by *Monilinia laxa,* and soft rot, due to *Rhizopus stolonifer (2, 26).* Organic-farmed, residue-free fruits represent a small percentage of overall production. A particular type of organic cultivation of peaches is performed in Sicily, where a valuable variety, Late Peach of Leonforte, is produced. These peaches are produced under organic farming in parchment paper bags, which protect the fruits from attack by insects (*Ceratitis capitata*) and prevent huge losses without the use of chemical products. The bagging of unripe fruits is performed in July, 3 months before harvesting. After this time, peaches do not receive antiparasitic treatments; hence, the product that is offered to consumers is free from residues.

This study was aimed at evaluating the antagonistic effect, in vitro and on artificially wounded fruits, of strain QST-713 of *Bacillus subtilis* (Serenade, AgraQuest Inc., Davis, Calif.), of strain T39 of *Trichoderma harzianum* (Makhteshim Chemical Works Ltd., Beer-Sheva, Israel), and of three yeast strains isolated from peach fruits in controlling Late Peach of Leonforte postharvest pathogens *Penicillium crustosum* and *Mucor circinelloides.*

MATERIALS AND METHODS

Isolation of yeast and mold populations on peaches. Ten grams of moldy, spoiled peaches was aseptically weighed, ho-

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mogenized for 3 min in a Stomacher 400 lab blender (Steward Medical, London, UK) with 90 ml of a sterile physiologic solution (0.9% NaCl), and serially diluted. Yeast and mold isolation was carried out with Sabouraud dextrose agar medium (CM41, Oxoid, Basingstoke, UK) supplemented with chloramphenicol (SR0078, Oxoid) to inhibit bacterial growth; the plates were incubated at 25°C for 4 days. The yeast and mold colonies were randomly picked from the agar plates containing the highest dilutions, selected by different colony and cell morphology, and purified by streaking three times. Identification of mold strains, performed by DSMZ (Braunscheig, Germany), was done, taking into account colony habits and macroscopic and microscopic morphology, under generally accepted standard conditions for the genus (or group) according to the most recent taxonomic monograph available.

Biochemical identification of yeasts. Biochemical identification was carried out by the ID 32C kit (bioMérieux, Marcy l'Etoile, France) following the manufacturer's instructions. Briefly, Analytab Products basal medium ampoules were inoculated with yeast cells picked from individual colonies, and the resulting suspension was standardized to a turbidity equal to a no. 2 McFarland standard solution. Each strip was inoculated and incubated for 24 to 48 h at 30°C. Ampoules that showed turbidity significantly greater than that of the negative control were considered positive. Identification of the isolates was carried out by bioMérieux SA software.

DNA extraction of yeasts. DNA isolation was done following the Hoffman and Winston protocol *(16),* with a few modifications. Pure isolates were grown in YPD (grams per liter of distilled water: yeast extract 10, peptone 10, dextrose 20) at 25°C for 18 h on an orbital shaker. Two milliliters of each cell culture was then centrifuged at 9,000 × *g* for 2 min at room temperature. The cells were resuspended with 400 μl of equilibrated phenol (pH 6) and 400 μl of extraction buffer (10 mM Tris [pH 8], 1 mM EDTA, 100 mM NaCl, 2% Triton, and 1% sodium dodecyl sulfate), and 0.6 g of sterile glass beads (0.45 to 0.50 μm in diameter) was added. The tubes were vortexed for 4 min, centrifuged at 8,000 × *g* for 1 s, and after adding 200 μl of TE buffer (10 mM Tris-HCl and 1 mM EDTA), centrifuged at 14,000 × *g* for 5 min. The supernatant was transferred to sterile tubes, and 500 μl of chloroform–isoamyl alcohol (24:1) was added; the tubes were then centrifuged at 14,000 × *g* for 2 min. The supernatant was transferred to sterile tubes with an equal volume of cold isopropanol and stored at −20°C for 30 min. The tubes were centrifuged at 14,000 × *g* for 10 min at 4°C. The pellets were then washed with 70% ethanol and resuspended in 60 μl of TE buffer. To verify the quality of DNA extraction, electrophoresis in 0.8% (wt/vol) agarose gel containing ethidium bromide with 1× TBE buffer (0.045 M Tris-borate and 0.001 M EDTA [pH 8]) was performed.

PCR conditions. The PCR amplification of the 5.8S-ITS (internal transcribed spacer) region was carried out with the primers ITS1 and ITS4 *(31)* under the following conditions. Each 50-μl reaction mixture contained 5 to 25 ng of template DNA; 10 mM Tris-HCl (pH 9.0); 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM (each) dATP, dCTP, dGTP, and dTTP; 0.5 μM (each) primer; and 1 U of *Taq* DNA polymerase (Invitrogen–Life Technologies, Carlsbad, Calif.). Amplification was performed in a Tpersonal PCR System (Whatman Biometra, Göttingen, Germany) programmed as follows: initial denaturation at 94°C for 5 min; denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min at 72°C, repeated for 35 cycles; and a final extension at 72°C for 5 min. Aliquots of 5 μl of amplified product were separated electrophoretically in 1.4% (wt/vol) agarose gels with ethidium bromide in TBE buffer at 90 V (constant) for 1 h and photographed under transilluminated UV light.

Restriction analysis. Aliquots (10 μl) of PCR products were digested with restriction enzymes *Hha*I, *Hae*III, and *Hinf*I (Invitrogen–Life Technologies) in a 20-μl reaction volume, according to the manufacturer's instructions and conditions. Restriction fragment length polymorphism products were visualized by electrophoresis in 2% (wt/vol) agarose gels as previously described. DNA molecular marker, 100-bp DNA ladder-ready to load (Invitrogen–Life Technologies) was used as the standard.

Test of antagonism in vitro with commercial strains. The cultures of the isolated pathogens obtained from the infected peach tissues were tested in vitro for sensitivity to the biofungicides Serenade and Trichodex. *B. subtilis* strain QST-713 produces three groups of lipopeptides that work together to inhibit disease-causing agents. Fungal *T. harzianum* Rifai strain T39 Trichodex contained at 20% in Trichodex was isolated in Israel from the microflora on the surface of cucumbers and was selected for its activity against the gray mold *Botrytis cinerea,* a plant pathogen. T39 is presumed to suppress the penetration of *Botrytis* into plant tissues through competition with the pest for nutrients and space on various plant surfaces.

The commercial strains were cultivated as follows: 0.2 g of each product was suspended in 4 ml of a sterile physiologic solution, and 1 ml of the suspension was included in the appropriate agarized medium. In particular, *B. subtilis* was inoculated in nutrient agar (BO0336, Oxoid) at a final concentration of 2.5×10^8 CFU ml^{-1} and incubated for 3 days at 30°C. *T. harzianum,* inoculated at a final concentration of 5×10^7 CFU ml^{-1}, was grown in malt extract peptone agar with the following composition (in grams per liter of distilled water): malt extract, 30.0; soy peptone, 3.0; and agar, 15.0 (pH 5.6). The plates were incubated at 22°C for 3 days.

Conidia of the spoilage molds were obtained from pure, 5-day-old cultures. Spores were drawn by scraping the surface of the plates with a sterile loop and resuspending the material in 4 ml of a sterile physiologic solution. An aliquot of the suspension (1 ml) was included in both potato dextrose agar (CM0139, Oxoid) and Sabouraud dextrose agar plates.

Before incubation, a 5.0-mm-square plug, taken from the margins of 5-day-old colonies of antagonists with a sterile scalpel, was transferred inverted to the edge of each petri dish containing the spoilage molds. Plates not inoculated with any of the commercial products were used as controls. The plates were examined at 2 and 7 days of incubation at 25°C. The width of the inhibition zone was estimated by measuring the colony diameter of the antagonist and was expressed as follows: +++, ≥20 mm; ++, 11 to 19 mm; +, 2 to 10 mm; ±, ≤1 mm; and −, 0 mm. Each test was performed in triplicate.

Test of antagonism in vitro for indigenous yeasts isolated from peaches. Yeast suspensions were prepared by inoculating a loopful of cells in a sterile physiologic solution from a culture grown on Sabouraud dextrose agar at 25°C for 48 h. The spore suspensions of *P. crustosum* and *M. circinelloides* were prepared by collecting spores from 5-day-old colonies, grown on potato dextrose agar at 25°C, and resuspending them in a sterile physiologic solution. Cell suspensions of yeast cultures and mold spores were enumerated with a Burker chamber.

A top Sabouraud dextrose agar was prepared as reported by Petersson and Schnürer *(24)* by mixing 6 ml of warm agarized

medium and 1 ml of yeast suspension with 10^4, 10^7, and 10^8 cells per ml, in order to determine the lowest effective concentration. The agar-yeast suspension was poured into petri dishes containing 15 to 20 ml of the same agar medium. Once the top agar had been set, 10 μl of a mold suspension with 10^8 spores per ml was inoculated as a spot on each plate; three replicates for each experiment were done. Plates were incubated at 25°C and observed at 3 and 7 days. To evaluate the degree of inhibition by measuring the radial extension of colonies, plates not inoculated with yeast suspensions were prepared and used as negative controls.

Assessment of antagonistic yeast activity. To assess the possible production of antifungal killer metabolites by yeasts, simple competition plate bioassays were performed with Sabouraud dextrose agar. On half of a petri dish, a 0.5-cm agar plug containing a pathogenic mycelium was placed, while on the other half of the dish, a heavy inoculum of a 48-h yeast culture was streaked *(30).* All plates were incubated at 25°C up to 7 days. Antifungal compound production was considered positive if definite zones of clearing were apparent between the yeast culture and the fungal mycelium, compared to control plates without yeast inocula. Positive results were confirmed by prolonging the incubation time to 14 days.

Test of antagonism on wounded fruits. The commercial strains were prepared as follows: 0.2 g of each product was suspended in 4 ml of a sterile physiologic solution (final concentration, 2.5×10^8 CFU ml^{-1}). Yeast strains were grown on Sabouraud dextrose agar plates at 25°C for 24 h and inoculated into 100 ml of YPD broth in 250-ml flasks. The flasks were incubated on a rotary shaker at 25°C for 16 h, and the cells were then pelleted by centrifugation at 8,000 × *g* and resuspended in sterile distilled water. The concentration was then adjusted to 10^8 CFU ml^{-1}. Spores of the fungal pathogens were obtained from the 5-day-old Sabouraud dextrose agar cultures incubated at 25°C. They were collected by scraping the culture surface with a cotton swab and resuspending the material in a sterile physiologic solution (0.9% NaCl). The concentration of the suspension was adjusted to 10^8 spores ml^{-1}.

Peach fruits used in the experiments were obtained from an orchard located near Enna (Sicily, Italy). The peach cultivar was Late Peach of Leonforte, produced under organic farming. Fruits, free from wounds and rot and homogeneous in maturity and size, were used immediately after harvesting.

Peach fruits were surface disinfected by immersion for 1 min in a diluted solution of sodium hypochlorite (1% active chlorine), washed twice by immersion in distilled water, and left to dry. Then, 50% of fruits were wounded in the equatorial zone (one wound per fruit) with a sterile tip to a uniform depth of 4 mm. In the biological control treatments, the fruits were treated with a suspension of yeast strains (10^8 CFU ml^{-1}) and commercial products following this scheme: PL1, PL3, PL1+PL3, Trichodex, Serenade, and Trichodex plus Serenade. All experiments were carried out on three replicates of eight fruits each. In addition, eight wounded fruits were inoculated in triplicate with each spoilage mold and used as controls. All the treatments were applied by spraying 4 ml of the suspensions on fruits. Two hours after the biocontrol treatments, the fruits were spray inoculated with the 10^8-spore ml^{-1} suspensions. Then, fruits were placed on polystyrene trays and incubated both at room temperature and at 4°C. The incidence of infected wounds (percent) was determined after 7, 15, and 30 days of storage. Environmental humidity (percent) and temperature, monitored by a thermohygrometer HD2301.0 (Delta Ohm, Padova, Italy), gave the following results: room temperature, 18 ± 1°C; room humidity, 68% ± 2%; refrigerated cell temperature, 4 ± 1°C; and refrigerated cell humidity, 75% ± 2%.

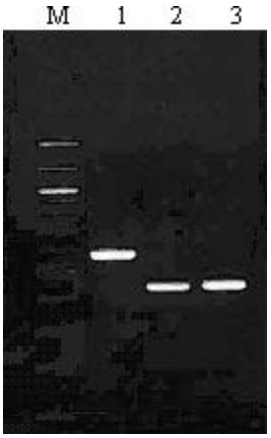

FIGURE 1. *PCR products of the 5.8S-ITS regions. 1 = PL1; 2 = PL2; 3 = PL3; M = 100-bp DNA ladder.*

Symptoms were scored at different days postinoculation as the number of infected fruits in each replica. The mean percent frequency of infected fruits for each treatment was subsequently calculated. The Student's *t* test was applied to test if differences between the treatment means were significant.

RESULTS

Identification of molds. Strain P1 was identified as *P. crustosum* Thom. The identification procedure gave the following results: (i) colony habit—colony on malt extract agar growing to a diameter of 30 mm at 25°C in 7 days, fine felty green; reverse yellow brown; abundant conidium, forming crustose masses; no growth at 37°C; and (ii) morphology—conidiophores arising from the substrate, 3 μm in diameter, rough walled; rami present, metulae 10 to 15 μm, phialides ampulliform, 9 to 11 μm long; conidia subglobose to ovoidal, up to 4 μm in length, smooth walled, adhering in masses.

Strains P3 and P4 were identified as *M. circinelloides* van Tieghem. The identification procedure gave the following results: (i) colony habit—colony on malt extract agar fast growing, colorless to grayish; reverse yellowish; growth and sporulation at 35°C; no growth at 37°C; sexual reproduction (zygospores) not detected; and (ii) morphology—sporangiophores erect, seldom branched, 15 to 20 μm in diameter, short (1 to 2 mm) or long (up to 12 mm); sporangia at first yellowish and then becoming light brown, up to 80 μm in diameter; walls deliquescing; sporangiospores ellipsoidal, 6 to 11 by 4 to 5 μm; chlamydospores absent.

Identification of yeast strains. Three yeast isolates from spoiled peaches were identified according to the ID 32C strips and ITS polymorphisms. Biochemical tests assigned strains PL1, PL2, and PL3 to *Candida sphaerica, Candida norvegensis,* and *Candida lambica* (synonym of *Pichia fermentans*), respectively. The ITS1 and ITS4 primers amplified the region between 18S and 28S rRNA. The isolates showed different PCR product sizes, ranging from 580 to 440 bp (Fig. 1). The PCR products digested with *Hha*I, *Hae*III, and *Hin*fI enzymes were analyzed for all the

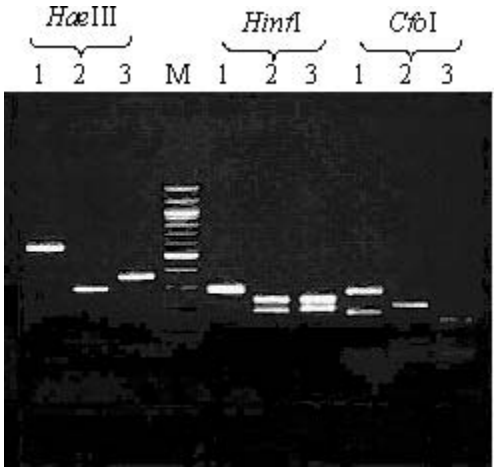

FIGURE 2. *Molecular weight of the restriction fragments of the 5.8S-ITS region. 1 = PL1; 2 = PL2; 3 = PL3; M = 100-bp DNA ladder.*

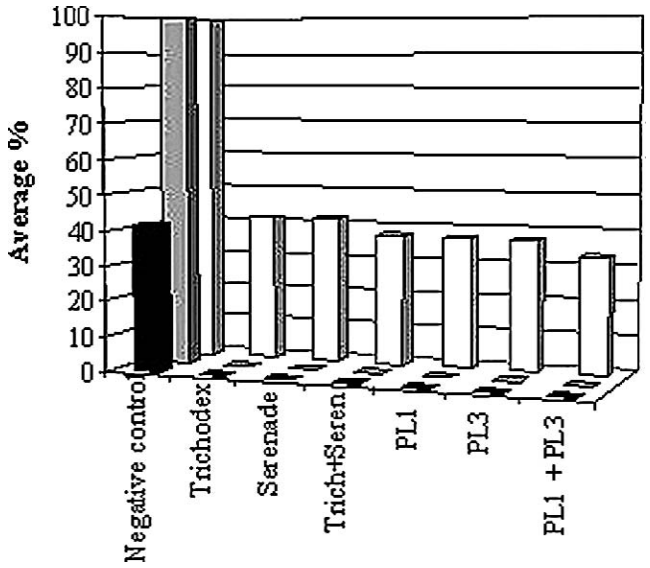

FIGURE 3. *Effect of different biocontrol agents on* P. crustosum *infection of peach fruits at 7 (black bars), 15 (gray bars), and 30 days (white bars) of storage at 20°C.*

isolated strains, whereby three different profiles were obtained (Fig. 2). Yeast strains were identified after comparing the molecular masses of the restriction products with those obtained by Esteve-Zarzoso et al. *(11)* as *Candida maltosa, Pichia kluyveri,* and *P. fermentans,* with identification of the latter being confirmed by the ID 32C strip.

Test of antagonism with commercial products. *T. harzianum* T39 and *B. subtilis* QST-713 strains inhibited the mycelial growth of *P. crustosum* and *M. circinelloides* in dual culture. The antagonists grew rapidly, preventing growth of the pathogens. Both the commercial antagonists reduced the growth of *P. crustosum,* in terms of growth area in petri dishes, by +++ (21 ± 1 mm). Less activity was shown against *M. circinelloides,* since the antagonistic activity exhibited by the two commercial formulations was, in both cases, ++ (13 ± 2 mm).

Test of antagonism for indigenous yeasts isolated from peaches. The results of biocontrol tests, at different levels of yeast inoculum, are reported in Table 1. Data are expressed as the ratio (percent) between the colony diameter of pathogen in the plates used as negative controls and the colony diameter in the plates inoculated with a biocontrol agent. *C. maltosa* and *P. fermentans* strongly reduced the growth of the molds tested on agar plates (Table 1), and the degree of inhibition was clearly dose-dependent. *P. crustosum* and *M. circinelloides* were very sensitive but only at a concentration of 10^7 yeast cells ml^{-1}. *P. kluyveri* reduced the growth of *M. circinelloides* in a dose-dependent manner but not to the same extent as the other yeast species. In this case, an inoculation of 10^7 or 10^8 cells ml^{-1} of suspension of *P. kluyveri* was required to reduce the growth of *M. circinelloides* by 50%. *P. kluyveri* at the tested concentrations was not able to inhibit *P. crustosum.*

Assessment of antagonistic yeast activity. Competition plate bioassays, performed in petri dishes, showed no zones of clearing between the yeast culture and the fungal mycelium. Pathogens grew in the proximity of the yeast streak for all the tested yeast species but did not overgrow the yeast biomass with complete colonization of the plate. In addition, no morphological changes in molds were observed by prolonging the incubation time to 14 days. From these results, we can exclude any production of antifungal metabolite, killer toxin, or chitinolytic enzymes. It can be hypothesized that biocontrol of these yeast strains against *P. crustosum* and *M. circinelloides* is based, basically, on competition for nutrients or for space.

Bioassays with wounded pathogen-inoculated fruits. In an experimental trial carried out on fruits inoculated with *M. circinelloides* and stored at 20°C (control), the incidence of infections was 41.7% after 7 days and 100% after 15 days. Treatments with PL1 and PL3 yeast strains and with commercial products significantly reduced the incidence of rot in wounded peach fruits (Fig. 3), showing no significant differences among biocontrol agents ($P > 0.05$). In particular, the preventive treatments reduced disease levels to 0%

TABLE 1. *Growth inhibition level of pathogen species in different yeast cell inocula*

	% inhibition level								
	Candida maltosa			*Pichia kluyveri*			*Pichia fermentans*		
Species	10^4 ml^{-1}	10^7 ml^{-1}	10^8 ml^{-1}	10^4 ml^{-1}	10^7 ml^{-1}	10^8 ml^{-1}	10^4 ml^{-1}	10^7 ml^{-1}	10^8 ml^{-1}
Penicillium crustosum	0	100	100	0	0	0	0	100	100
Mucor circinelloides	0	75	80	0	50	55	0	95	100

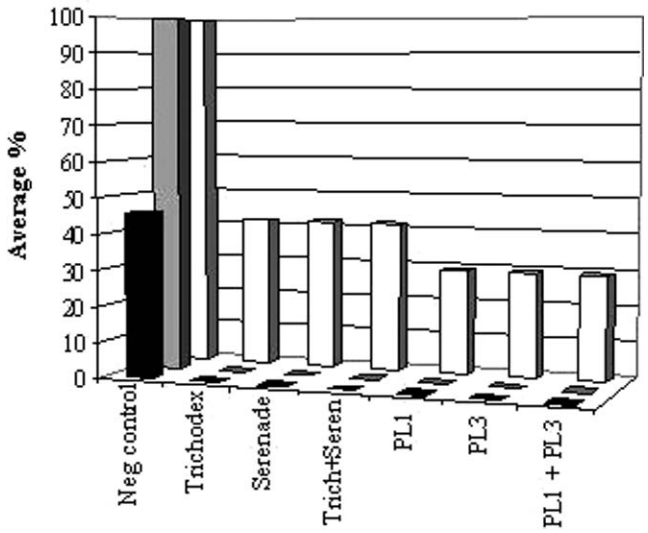

FIGURE 4. *Effect of different biocontrol agents on* M. circinelloides *infection of peach fruits at 7 (black bars), 15 (gray bars), and 30 days (white bars) of storage at 20°C.*

(efficacy of 100%) after 15 days of storage and from 33.3 to 41.7% (efficacy of 66.7 to 58.3%) after 30 days of storage. In an experimental trial carried out on fruits inoculated only with *P. crustosum* at 20°C (control), the incidence of infections was 45.8% after 7 days and 100% after 15 days. Treatments with all biological control agents reduced disease levels to 0% (efficacy of 100%) after 15 days of storage and from 29.2 to 41.7% (efficacy of 70.8 to 58.3%) after 30 days of storage (Fig. 4). No significant differences ($P > 0.05$) in the control levels of *Mucor* or *Penicillium* rot were observed.

Fruit samples stored at 4°C did not show any mold infection in treated or control samples for up to 30 days of storage. In none of the experimental trials did the application of yeast biocontrol agents produce any negative effect on the quality of fruits.

DISCUSSION

The use of biocontrol agents to manage the postharvest decay of fruits was explored as an alternative to the use of synthetic fungicides. The present study aimed at evaluating the antagonistic effect in petri dishes of two commercial products, Trichodex and Serenade, basically to widen the list of target-spoilage species, since the financial and time costs involved in the registration of a new product are very high. From the results of this investigation, it is evident that strain T39 of *T. harzianum,* which is commercialized as Trichodex, and strain QST-713 of *B. subtilis,* commercialized as Serenade, show antagonistic activity against the postharvest spoilage agents *P. crustosum* and *M. circinelloides.*

T. harzianum T39 was found to be effective against *Phytophthora erythroseptica* on tomato and potato *(12)*; against *B. cinerea* on tomato *(22),* cucumbers *(6),* strawberries *(14, 15),* and table grape *(18)*; against *Spaerotheca fusca* on cucumbers *(6)*; against *Phytophthora infestans* on potato leaves *(29)*; against *Mucor piriformis* on strawberries *(15)*; and against *Colletotrichum acutatum* on strawberries. *B. subtilis* QST-713 showed biocontrol activity against infection of blueberry flowers by *Monilinia vaccinii-corymbosi (23).* The effectiveness of *T. harzianum* T39 and *B. subtilis* QST-713 against *P. crustosum* and *M. circinelloides* has not, to our knowledge, been reported in the spectrum of activity of the commercial formulations or in any scientific report to date.

Moreover, in the present research, we identified three yeast strains, isolated from the same peaches, that exhibited biocontrol efficacy against the two mold species. The species *C. maltosa, P. kluyveri,* and *P. fermentans* showed remarkable biocontrol activity, which was clearly dose-dependent, against the two mold species. Nutrient or space competition appeared to be the principal mode of action, as the production of lytic enzymes or antimicrobial metabolites was not detected. The biocontrol ability of *C. maltosa, P. kluyveri,* and *P. fermentans* has been observed previously against *B. cinerea (27), Cephalosporium maydis (10),* and *Aspergillius ochraceus (20),* but it has not, to our knowledge, been demonstrated against the spoilage molds of the peaches isolated in the present study.

Further identification of new antagonists is always desirable, because antagonists identified in specific geographic areas, and especially from the same source, may be more effective against the pathogen strains present in that location. In addition, access to several biocontrol yeast species offers the possibility of employing combinations to take advantage of their different biological properties or just to avoid resistance phenomena, thus making biological control more effective.

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