Journal of Food Protection, Vol. 69, No. 11, 2006, Pages 2703–2711
Copyright ©, International Association for Food Protection

Efficacy of *Candida sake* CPA-1 Formulation for Controlling *Penicillium expansum* Decay on Pome Fruit from Different Mediterranean Regions

R. TORRES,[1]* N. TEIXIDÓ,[1] I. VIÑAS,[1] M. MARI,[2] L. CASALINI,[2] M. GIRAUD,[3] AND J. USALL[1]

[1]*Postharvest Unit, Institute of Agro-Food Research and Technology (IRTA), Centre UdL (University of Lleida)-IRTA, Av. Rovira Roure, 191. 25198 Lleida (Catalonia), Spain;* [2]*CRIOF DIPROVAL University of Bologna Via Gandolfi, 19 40057 Cadriano (Bologna), Italy; and* [3]*Ctifl, Centre de Prigonrieux, 24130 Prigonrieux, France*

MS 06-174: Received 10 March 2006/Accepted 29 June 2006

ABSTRACT

The effectiveness of a formulated product of the yeast *Candida sake* CPA-1 for controlling postharvest diseases on pome fruits was demonstrated in laboratory, semicommercial, and commercial trials carried out in the major pome fruit producing region of the European Union. First, one wettable powder and seven liquid formulations were tested in laboratory trials that involved two varieties of apples and two varieties of pears. In all cases, an efficacy similar to that of fresh cells was demonstrated in the control of artificial *Penicillium expansum* infection. After these trials, the formulated product chosen for semicommercial and commercial trials was LF1, a liquid formulation that is particularly suitable for commercial applications. In semicommercial trials, LF1 showed a performance similar to fresh cells in most trials, and the population dynamics of both fresh and formulated cells were quite stable throughout the storage period. This indicates the high viability of *C. sake* CPA-1 in this formulation and the absence of adverse effects during the formulation of the product, which may significantly affect both its ability to grow on fruit and its antagonistic activity. We evaluated the control of natural infection after applying the formulated product in a commercial drencher in different packinghouses. A significant reduction in the incidence of diseases was observed with a recommended dose of around 10^7 CFU/ml when natural infections were greater than 1%. In general, large quantities of yeast were observed on the surface of unwounded fruits of different pome fruit cultivars. Moreover, populations of this biocontrol agent increased rapidly on fruit surfaces and remained quite stable for a long time under commercial storage conditions. Commercial practices used in packinghouses were therefore successfully applied for this formulated product.

The wound-invading fungus *Penicillium expansum* Link is one of the most important postharvest pathogens affecting pome fruits. Control of this postharvest pathogen relies on the use of fungicides applied as drencher or on line-sprayer treatments prior to cold storage *(20).* However, the development of resistance to many fungicides by major postharvest pathogens *(29, 40, 41),* the ban on several pesticides, and the public demand for fungicide-free products have generated an interest in the development of alternatives to synthetic fungicides, though these should be both effective and economically feasible.

Biological control with microbial antagonists has been shown to be one of the most effective alternatives *(8, 16, 42).* Several antagonistic microorganisms have been discovered that reduce postharvest fungal decay on pome fruits *(14, 18, 19, 21, 24, 26–28, 34, 38, 39).* However, before biocontrol agents can be successfully used on a large scale, it is first necessary to optimize several of their key features. For this reason, few yeast- or bacterial-based products have so far been commercialized for the control of postharvest diseases on pome fruits. The first commercial products were registered in the United States under the names BioSave 100 and 110, while in South Africa, the product YieldPlus has also been registered. However, to our knowledge, no biocontrol-based products have at present been registered in Europe. One potential microorganism that could be developed for commercial applications is the naturally occurring yeast *Candida sake* (Saito and Ota) (van Uden and Buckley), strain CPA-1, which was isolated from the surface of apple fruits and exhibited antagonistic activity in vivo against *P. expansum, Botrytis cinerea,* and *Rhizopus stolonifer* in pome fruits *(39).* Commercial amounts of this yeast were obtained with a low-cost culture medium *(2).* The effectiveness of this antagonist, whether with fresh cells *(33, 36, 37)* or formulated cells *(3, 35),* has also been clearly demonstrated, particularly on apples grown in Catalonia (Spain), and no adverse effect on quality parameters has been observed after treatments with this yeast. To our knowledge, no studies have been carried out in other regions of the Mediterranean area.

The main European Union apple-producing countries are Italy (30%), France (27%), Germany (12%), and Spain (10%), while the main pear-producing countries are Italy (39%), Spain (29%), and France (11%) *(4).* On this basis, France, Italy, and Spain could be considered the major pome fruit-producing countries in the European Union.

The goal of this study was to evaluate the efficacy of

* Author for correspondence. Tel: (34) 973003430; Fax: (34) 973238301; E-mail: rosario.torres@irta.es.

C. sake CPA-1 on pome fruits in these three European Mediterranean locations. We were particularly interested in testing different formulations under laboratory conditions and then evaluating the best formulation in semicommercial- and commercial-scale trials, particularly to monitor its effects on *P. expansum* decay.

MATERIALS AND METHODS

Antagonist. We used strain CPA-1 of the yeast *C. sake* (CECT-10817, Colección Española de Cultivos Tipo, University of Valencia, Valencia, Spain). Stock cultures were stored at 5°C on nutrient yeast dextrose agar (nutrient broth, 8 g/liter; yeast extract, 5 g/liter; dextrose, 10 g/liter; and agar, 15 g/liter). For laboratory and semicommercial trials, cells were grown in 5 liters of molasses-urea broth medium in a Biostat A fermentor (MicroDCU 300, B. Braun Biotech International, Melsungen, Germany). The composition of this medium was 40 g of cane molasses, 1.2 g of urea, and 1,000 ml of water. The optimum conditions for obtaining maximum growth in less time were 150 liters/h (0.05 vol of air/vol of medium/min [vol/vol/min]) of aeration at 400 rpm and 25°C for 36 h and an initial population of 10^6 CFU/ml. For commercial trials, production was carried out in a 90-liter fermentor (Ilerfred, Lleida, Catalonia, Spain) with the same medium as described above and under the following growth conditions: 1,500 liters/h (0.5 vol/vol/min) of aeration at 400 rpm at 25°C for 36 h and an initial inoculum of 10^6 CFU/ml. Yeast cells from both fermentors (5 and 90 liters) were harvested by centrifugation at 7,520 $\times$ *g* for 15 min at 10°C in an Avanti J-20 XP centrifuge (Beckman Coulter, Palo Alto, Calif.). Seven liquid formulations (LF1, LF2, LF3, LF4, LF5, LF6, and LF7) and one wettable powder formulation (SF1) of this biocontrol agent were used in laboratory trials. Formulations of this yeast codified as LF1, LF2, and LF3 were prepared in isotonic preservation solution following the methodology described by Abadias et al. *(3).* LF4, LF5, LF6, and LF7 were also prepared in liquid formulations in a sugar preservation solution, as described by Torres et al. *(35).* SF1 was a freeze-dried formulation of *C. sake,* which was protected by lactose and rehydrated with skimmed milk *(1).*

Both the production and formulation processes were carried out at the Postharvest Unit of the Centre UdL-IRTA of Lleida (University of Lleida–Institute of Agro-Food Research and Technology). The fresh cells and the formulations were sent to Bologna and Prigonrieux just before starting the trials.

Pathogen. Monoconidial strains of *P. expansum* were isolated from decayed pome fruits from each location. Each laboratory used its own isolates to carry out artificial infection. In all cases, the isolate was maintained on potato dextrose agar for 7 to 14 days at 25°C. Aqueous conidial suspensions of *P. expansum* at 10^4 or 10^3 conidia per ml were prepared, depending on the trial. Spore concentration was determined with a hemocytometer.

Fruit. We studied four cultivars of pears and four cultivars of apples, all grown by standard culture practices, from commercial orchards. Fruits from orchards in Spain (Lleida, Catalonia), Italy (Bologna), and France (Prigonrieux) were used to carry out laboratory, semicommercial, and commercial trials during three consecutive seasons (2001 to 2002, 2002 to 2003, and 2003 to 2004, respectively). In Lleida, the crops studied were Conference and Blanquilla pear cultivars and Golden Delicious and Red-Chief apple cultivars. In Prigonrieux, the crops were Conference and Doyenné du Comice pear cultivars and Golden Delicious and Fuji apple cultivars. In Bologna, the cultivars studied were Kaiser and Conference for pears and Golden Delicious and Red-Chief for apples. After harvest, fruits were either immediately used or stored at 1°C until the time of the assay.

Laboratory trials. Fruits were wounded at the stem (top) and calyx (bottom) with a nail, making injuries that were 2 mm in diameter and 2 mm deep. Fifteen microliters of aqueous suspension of each yeast or fresh cell formulation was then applied to each wound. The yeast concentration was adjusted to a predefined concentration, and after air drying, 15 μl of an aqueous suspension of *P. expansum* at 10^4 conidia per ml was applied. Twenty fruits constituted a single replicate, and each treatment was replicated four times. The number of infected wounds was evaluated after at least 1 month at 1°C for apples and 0°C for pears and at a relative humidity of 85%.

During the first season, laboratory trials were carried out with LF1, LF2, LF3, and SF1 formulations. These were tested at 10^8 CFU/ml in Bologna, at 10^7 or 10^8 CFU/ml in Prigonrieux, and at 10^7 CFU/ml in Lleida. The concentrations tested in Bologna and Lleida were defined by results obtained in previous studies; however, in Prigonrieux, both concentrations were tested during the first season, because this antagonist had never been previously tested. During the second season, LF4, LF5, LF6, and LF7 formulations were tested at 10^8 CFU/ml in Bologna and at 10^7 CFU/ml in Prigonrieux and Lleida.

Semicommercial trials. Two varieties of both pears and apples were selected for each location to test the efficacy of fresh cells and LF1. LF1 was chosen as the best formulation in laboratory and semicommercial trials. Fruits (without artificial wounds) were immersed for 1 min in fresh cell suspension plus a *P. expansum* suspension at 10^3 conidia per ml or in the LF1 formulation plus a *P. expansum* suspension at 10^3 conidia per ml. The antagonist concentration was adjusted to the concentration used at each location: 10^8 CFU/ml in Bologna and 10^7 CFU/ml in Prigonrieux and Lleida. Control fruits were dipped into a conidial suspension of *P. expansum.* After treatment, apples were stored at 1°C and pears at 0°C for at least 1 month. Sixty fruits constituted a single replicate, and each treatment was replicated six times. The number of infected fruits was evaluated at the end of the storage period.

A study was carried out to monitor the population dynamics of both fresh cells and the LF1 formulation. It involved dipping fruits for 1 min in either fresh cells or an LF1 suspension at the same concentration as that used in efficacy trials. No artificial wounds were made to the fruits. After drying, the fruits were stored at 1°C and a relative humidity of 85%. Population dynamics were determined at 0 and 15 days and once a month throughout the storage period. To determine the yeast populations on the fruits, a known surface of peel was removed with a cork borer: for pears, twenty 1.45-cm^2 pieces were taken from four fruits and replicated, and for apples, twenty-five 2.5-cm^2 pieces were taken from four fruits and replicated. The peel surface was shaken in sterile phosphate buffer on a rotatory shaker for 20 min at 150 rpm. It was then sonicated for 10 min in an ultrasound bath. Serial 10-fold dilutions of the washings were carried out with 0.05 M phosphate buffer and plated on nutrient yeast dextrose agar medium supplemented with 0.5 g of streptomycin sulfate per liter. After incubation at 25°C for 2 days, colonies were counted, and their number per square centimeter of fruit surface was calculated.

Commercial trials. The efficacy of the biocontrol agent *C. sake* in controlling the most important postharvest diseases on pome fruits was tested at three commercial packinghouses located in Lleida (Catalonia, Spain), Ferrara (Italy), and Saint-Just (near Montpellier, France). The commercial trials were carried out on

TABLE 1. *Standard controlled atmosphere (CA) conditions for commercial trials*

Locations	Apple	Pear
Lleida	0.5°C, 3% O_2 and 3% CO_2	−0.5°C, 2.5% O_2 and 1.5% CO_2
Ferrara	0.5°C, 2–3% O_2 and 3% CO_2	−0.5°C, 4% O_2 and 2% CO_2
Saint-Just	0.5°C, 2% O_2 and 1.5% CO_2	—

apples and pears. Fruits from two different orchards were used for each apple and pear cultivar, except at Saint-Just, where a large number of orchards had been devastated by a storm, and hence, only one orchard of Granny Smith apples was available. After harvest, fruits were treated in a commercial drencher for application of the following treatments: (i) control, fruits drenched in water; (ii) LF1, fruits drenched in *C. sake* formulation at 10^7 CFU/ml (Lleida and Saint-Just) or 3×10^7 CFU/ml (Ferrara); and (iii) chemical, standard products used in each packinghouse. The chemical treatments used in each packinghouse were as follows. In Lleida, Golden Delicious apples were treated with a solution of etoxiquin (72%, wt/vol; STOP Scald, Sipcam Inagra, Valencia, Spain) plus imazalil (7.5%, wt/vol; Deccozil-S-7.5, Atochem Agri, Plaisir, France) and folpet (7.5%, wt/vol; FOLTENE, Sipcam Inagra) at commercial doses. Conference pears were also treated with etoxiquin plus folpet (20%, wt/vol) and imazalil (7.5%, wt/vol; FRUITGARD F/I, Fomesa Fruitech, Valencia, Spain) at commercial doses. In Saint-Just, Granny Smith apples were treated with thiabendazole (Xedazole 30 crème, 0.2 liters/hl) plus diphenylamine (No-Scald 31, 0.63 liters/hl). In Ferrara, both Golden Delicious apples and Kaiser pears were treated with thiabendazole (0.05%, wt/vol; 'Tecto 20S', Merck Sharp & Dohme, Rome, Italy). After treatment, fruits were stored under local standard controlled atmosphere (CA) conditions until each packinghouse opened its chambers (Table 1). In Ferrara, to increase the incidence of natural infections, a set of fruits was stored under cold conditions (0°C for pears and 1°C for apples). This set of fruits consisted of four boxes chosen at random for replicates and treatments. After storage, the number of fruits infected by each pathogen was evaluated, and the mold types were identified. The sample unit was one bin (around 2,050 apples and 1,250 pears) per four replicates and per treatment.

In commercial trials, the population dynamics of this yeast were examined just after treating the fruits and again at the end of the storage period. The methodology used was the same as that described for semicommercial trials.

Data analysis. Effects of treatments in the incidence of infected wounds and fruits were analyzed by an analysis of variance on data transformed to the arcsine of the square root of the proportion of infected wounds and fruits. This transformation was used to improve the homogeneity of variances. Statistical significance was judged at the level of $P < 0.05$. When the analysis was statistically significant, the least significant difference (LSD) test was used for mean separations.

RESULTS

Laboratory trials. The efficacy of the first four formulations (one solid [SF1] and three liquid [LF1, LF2, and LF3] formulations) of *C. sake* CPA-1 applied at 10^7 or 10^8 CFU/ml to the control *P. expansum* was determined in two varieties of apples and two varieties of pears for each location during the first season (Fig. 1A and 1B). In general, these formulations have shown the same efficacy as fresh cells in regard to controlling diseases due to *P. expansum,* both when artificially infected and when subjected to cold storage conditions for several months. For Blanquilla pears from Lleida, the application of both fresh cells and all the different formulations significantly reduced the number of infected wounds with respect to the control fruits (a reduction of more than 85%) after 3 months under cold storage conditions. Even so, no significant differences were found among *C. sake* treatments (Fig. 1A). In Golden Delicious apples from Prigonrieux, the incidence of disease was low after 4 months of storage; only 29% of decayed fruits were observed in the control fruits. The four formulations and fresh cells showed excellent results, with an efficacy of between 95 and 100% (Fig. 1B). Moreover, trials carried out with the concentration at 10^7 CFU/ml produced results that were the same as those at 10^8 CFU/ml (data not shown). Laboratory trials carried out in Bologna showed a similar tendency, although a concentration of both fresh and formulated cells at 10^8 CFU/ml had to be applied to obtain a control similar to those used at the other locations (data not shown).

During the second season, four new formulations and fresh cells were also evaluated in laboratory trials (Fig. 1C and 1D). In general, LF4, LF5, LF6, and LF7 formulations showed levels of effectiveness similar to fresh cells in controlling blue mold and were always able to significantly reduce the incidence of decay with respect to the control fruits. However, the percent reduction in the number of decayed fruits was always lower with these formulations than with those tested during the previous season. In Conference pears from Prigonrieux, a moderate incidence of disease was observed in the control fruits (around 50%), although a high degree of disease control was observed with fresh cell applications (with a reduction in disease of almost 86%) and with all formulations (a reduction from 52 to 72%). A slight increase in the number of infected fruits was observed in formulated treatments, though no significant differences were observed with respect to fresh cells (Fig. 1C). A higher incidence of blue mold decay was observed in artificially wounded Red-Chief apples from Bologna (Fig. 1D). All treatments—both fresh cells and formulations—produced a significant reduction in the percentage of infected fruits (from 42 to 72%) with respect to the control fruits.

On the basis of the results obtained for the viability and efficacy of all the formulations tested in these trials, and especially the economic aspects related to their practical application, the formulated product chosen for semicommercial and commercial trials was LF1.

Semicommercial trials. In trials carried out in Lleida, a reduction in the number of infected fruits was obtained for both Blanquilla pears and Red-Chief apples with both the fresh cell and LF1 formulation treatments (Fig. 2). Fresh cells in Blanquilla pears showed the greatest reduction in disease (75% reduction), while LF1 was able to reduce the incidence of decay to 41%. In Red-Chief apples,

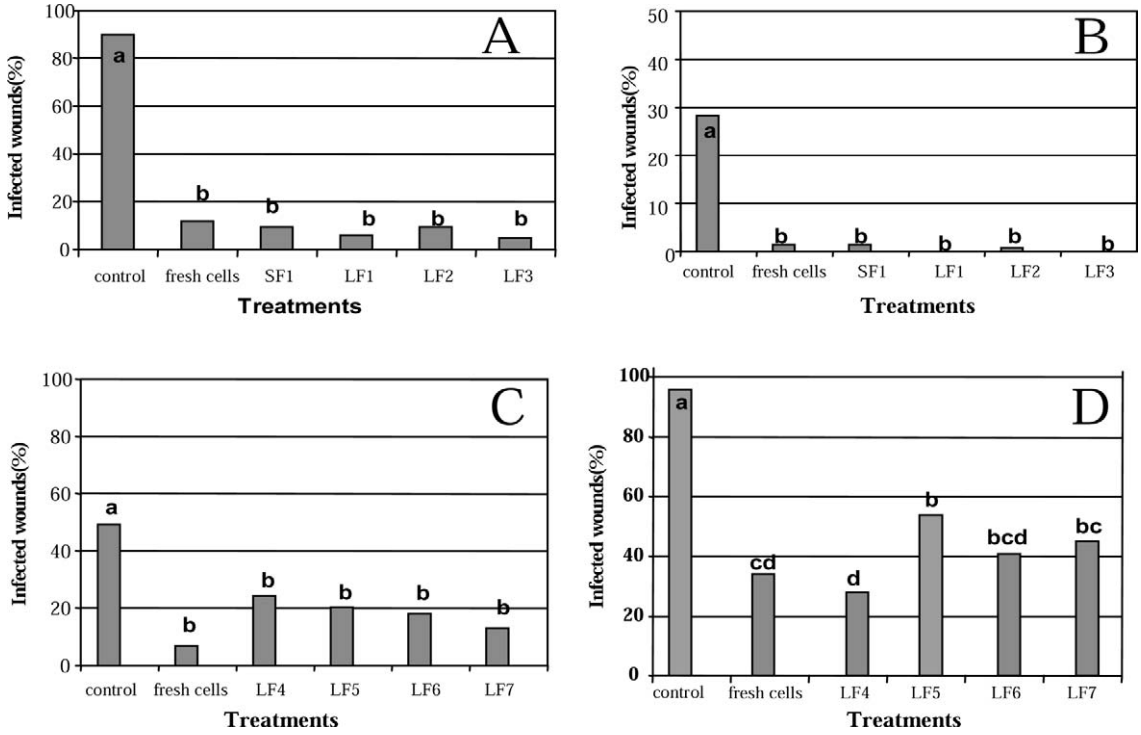

FIGURE 1. *Effect of the biocontrol agent* Candida sake *against* Penicillium expansum *decay on Blanquilla pears from Lleida (A), Golden Delicious apples from Prigonrieux (B), Conference pears from Prigonrieux (C), and Red-Chief apples from Bologna (D). Treatments with fresh cells and different formulations (SF1, LF1, LF2, and LF3 in A and B; LF4, LF5, LF6, and LF7 in C and D) were applied, and then* P. expansum *was suspended at* 10^4 *conidia per ml. The concentration of the biocontrol agent was* 10^7 *CFU/ml in A and C and* 10^8 *CFU/ml in B and D. Twenty fruits constituted a single replicate, and each treatment was replicated four times. The number of infected fruits was determined after cold storage at 0°C for 3 months and 45 days for Blanquilla and Conference pears, respectively, and at 1°C for 4 months for both varieties of apples. Columns with the same letters indicate no significant differences (*$P < 0.05$*) according to the LSD test.*

no significant differences were found between fresh cells (65% reduction) and the LF1 formulation (48% reduction) with respect to the control fruits. Similar behavior was observed in Conference pears, in which the reductions associated with fresh cells and LF1 were 47 and 29%, respectively (data not shown). No reduction was observed in Golden Delicious apples when the fruits were treated with both fresh cells and LF1 because of the low incidence of decay (less than 6%).

Trials carried out in Bologna and Prigonrieux showed a very low incidence of disease (lower than 5% in the control batches); hence, a poor effect was obtained after the biocontrol agent treatment. Fresh cells significantly controlled only blue mold on Kaiser pears from Bologna.

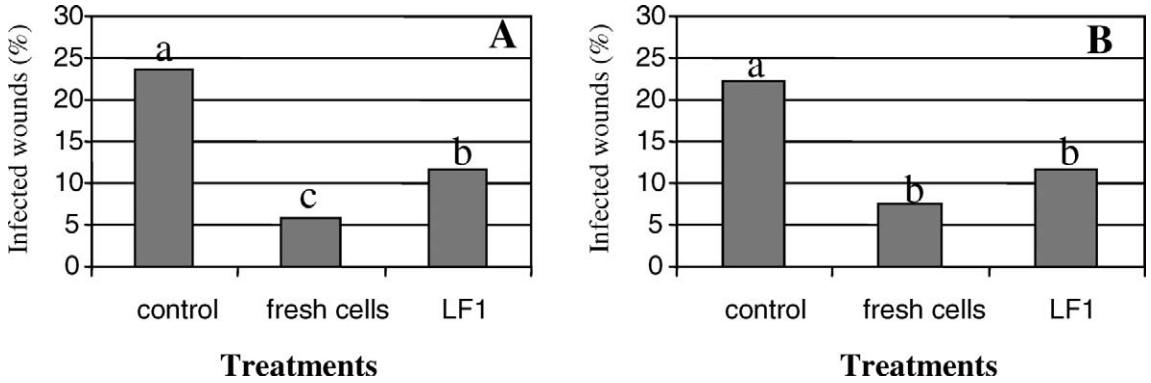

FIGURE 2. *Effect of the biocontrol agent* Candida sake *against* Penicillium expansum *decay in semicommercial trials on Blanquilla pears (A) and Red-Chief apples (B) from Lleida. Fruits were immersed for 1 min in a suspension containing a mixture of fresh cells and conidia of* P. expansum *or in an LF1 formulation and conidia of* P. expansum. *The pathogen concentration was* 10^3 *conidia per ml. Sixty fruits constituted a single replicate, and each treatment was replicated six times. The number of infected fruits was evaluated after cold storage at 0°C for 3 months for pears and at 1°C for 8 months for apples. Columns with the same letters are not significantly different (*$P < 0.05$*) according to the LSD test.*

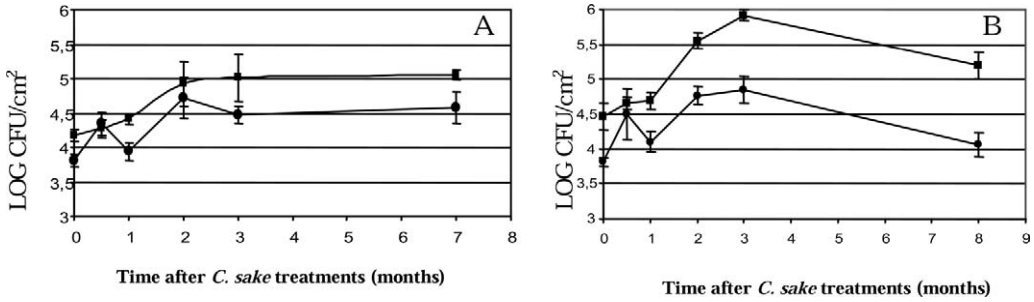

FIGURE 3. *Population dynamics of* Candida sake *CPA-1 on unwounded Conference pears (A) and Golden Delicious apples (B) treated with fresh cells (●) or the LF1 formulation of* C. sake *(■). Fruits were kept under cold storage conditions for several months, and a population evaluation was carried out each month. Four fruits constituted a single replicate, and each treatment was replicated four times. Vertical bars indicate mean standard errors.*

These showed levels of around 10% of infected fruits in the control. There was a reduction in disease of 90% (1% of infected fruits) in treated fruits (data not shown).

In general, a high presence of yeast was observed on the surface of unwounded fruits, and this remained stable throughout storage at all locations (10^4 to 10^6 CFU/cm^2). The population dynamics of *C. sake* during the cold storage period, for both fresh and formulated cells in pome fruits from Lleida, are presented in Figure 3. Excellent growth of *C. sake* under cold storage conditions was observed in both pears and apples, especially with the LF1 formulation, and on Golden Delicious apples (Fig. 3B). On pears, the level of the yeast on the fruit surface was lower (around 10^5 CFU/cm^2) but remained quite stable throughout the storage period (Fig. 3A).

Commercial trials. We evaluated the control of natural infection after applying the biocontrol agent in different packinghouses. In Conference pears from Lleida, the incidence of decay was slightly higher than 3% in orchard 1 and 2% in orchard 2, in the control fruits, after a commercial storage period of 10 months under CA conditions (Fig. 4A). In orchard 1, no significant differences were found between the control and LF1; chemical treatment was the only treatment that was able to reduce decay (62%). In orchard 2, the LF1 treatment showed a reduction in decay to 56%; however, this reduction was not significant with respect to the control or chemical treatment. The main molds found on this cultivar were *P. expansum, B. cinerea,* and *Alternaria* spp. Treatment efficacy on Golden Delicious apples from Lleida is shown in Figure 4B. For orchard 1, LF1 application produced an important reduction in incidence with respect to the control fruits (57%). No significant differences were found between chemical and biological applications. For orchard 2, no significant differences were found between treatments in relation to the control fruits, probably because of the low incidence of disease in these fruits (0.6%). The most important pathogens found in this cultivar were *P. expansum, B. cinerea, R. stolonifer,* and *Alternaria* spp.

As far as population dynamics were concerned, the population of this biocontrol agent remained at around 10^3 CFU/cm^2 from the beginning ($t = 0$) to the end of the assay (after the shelf life) in apples and increased from 6×10^3 to 6×10^4 CFU/cm^2 in pears. In both cultivars, a slight decrease in population was observed by the end of the CA period (Table 2).

Commercial trials carried out in a packinghouse at Ferrara (Italy) showed that after 3 months of storage under CA conditions, the percentage of infected fruits was less than 0.4% for apples (control, 0.4%; LF1, 0.2%; and chemical,

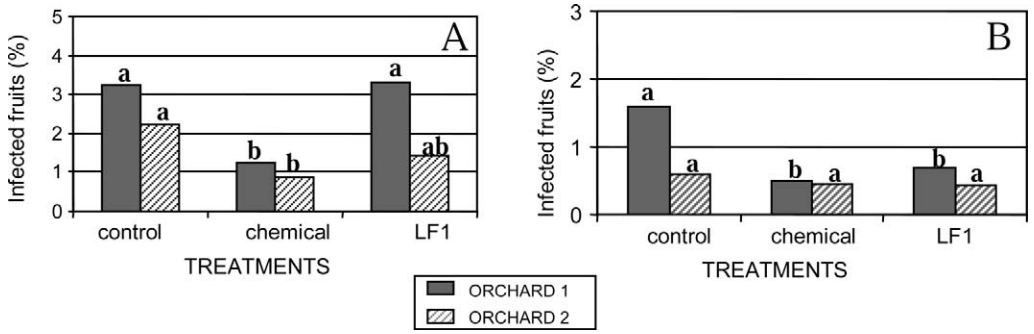

FIGURE 4. *Incidence of natural decay on Conference pears (A) and Golden Delicious apples (B) in a commercial trial carried out in a packinghouse at Lleida. After harvest, fruits were treated in a packinghouse with one of the following treatments: (i) water (control); (ii)* C. sake *formulation at 10^7 CFU/ml (LF1); or (iii) chemical treatments usually used in the packinghouse (chemical). The application system was a commercial drencher. Two orchards were monitored for each variety of pome fruit used (■, orchard 1, and ▨, orchard 2). The sample unit was one bin per four replicates and per treatment. After the treatments, fruits were stored under CA conditions, and after the storage period, the percentage of decayed fruits was recorded. For each orchard, columns with the same letters are not significantly different ($P < 0.05$) according to the LSD test.*

TABLE 2. *Population of* C. sake *formulation (LF1) on unwounded pome fruits from different packinghouses*[a]

Location	Pome fruit variety	Viable cells of *C. sake* formulation (LF1) after different storage periods (CFU/cm^2)			
		0	CA	Cold storage (°C)	Shelf life
Lleida	Golden Delicious apples	1.6×10^3 A	9.2×10^2 A	ND[b]	1.5×10^3 A
	Conference pears	6.9×10^3 B	1.3×10^3 C	ND	6.2×10^4 A
Ferrara	Golden Delicious apples	6.0×10^3 B	1.6×10^3 C	2.4×10^4 A	2.0×10^3 C
	Kaiser pears	1.1×10^4 B	2.1×10^4 C	8.4×10^4 A	2.4×10^4 B
Saint-Just	Granny Smith apples	1.9×10^3 A	1.4×10 B	ND	ND

[a] The study was carried out just after treatment (0), after a commercial storage period in a controlled atmosphere (CA), after cold storage, and after 7 days at 20°C (shelf life). LF1 fruits were drenched in *C. sake* formulation at 10^7 CFU/ml (Lleida and Saint-Just) or 3×10^7 CFU/ml (Ferrara). The sample unit was four fruits per four replicates. For each orchard and cultivar, files with the same letters are not significantly different ($P < 0.05$) according to the LSD test.

[b] ND, not detectable.

0.1%) and 4% for pears (control, 3.2%; LF1, 2.3%; and chemical, 1.2%). In Ferrara, after 4 months plus the shelf life, the incidence of disease was evaluated again. In all trials, the predominant pathogen was *P. expansum* (Fig. 5). In Kaiser pears, *P. expansum* spread quickly at the end of the shelf life (Fig. 5A). In orchard 1, the LF1 treatment significantly reduced disease when compared to the control (65%) and the chemical treatment. No differences were found between the control and the chemical treatment. In orchard 2, the reduction in the number of decayed fruits with LF1 was significantly greater than with the chemical treatment. The presence of blue mold was considerably lower in Golden Delicious apples than in pears (Fig. 5B). The LF1 treatment significantly reduced the incidence of disease in both orchards, but especially in orchard 2. In this orchard, no control was observed with the chemical treatment.

As for the population dynamics of apples from orchard 1, the population of *C. sake* decreased during the period of CA storage (from 6.1×10^3 to 1.6×10^3 CFU/cm^2), whereas the population among fruits from orchard 2 tended to remain stable (2.8×10^3 CFU/cm^2). After storage under cold conditions, the population size in fruits from the two orchards increased by about 1 log. In pear fruits from different sources (orchards 1 and 2), the population increased, with only slight differences during CA storage, and increased by almost 1 log under cold conditions, though it subsequently decreased during the shelf life (Table 2).

Commercial trials carried out in Prigonrieux (France) showed a very low incidence of decay in the only orchard tested (less than 0.1% for *Penicillium* spp. and 0.2% for the other pathogens); therefore, no differences were observed between the control and LF1 treatment (0% for the chemical treatment). Furthermore, about 50% of the fruits from the control and LF1 treatments were affected by superficial scald. A low population of yeast was observed on Granny Smith apples (Table 2).

DISCUSSION

Detailed pilot-scale trials were carried out with the biocontrol agent *C. sake* CPA-1 to define its most appropriate future applications in packinghouses. Other authors have reported the efficacy of fresh cells of this biocontrol agent under different pre- and postharvest conditions *(33, 36, 37, 39).* However, the scope of this study was also expanded to evaluate the antagonistic activity of formulated yeast-

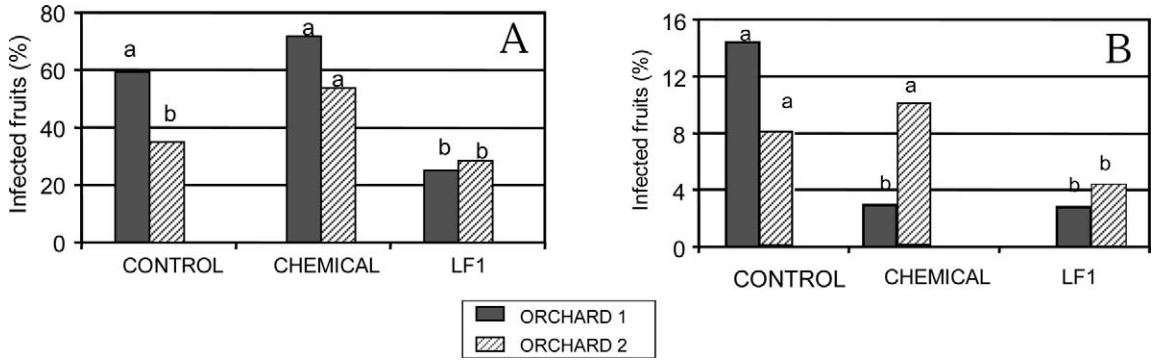

FIGURE 5. *Incidence of natural decay due to blue mold on Kaiser pears (A) and Golden Delicious apples (B) in a commercial trial carried out in Ferrara. After harvest, fruits were treated in a packinghouse with one of the following treatments: (i) water (control); (ii)* C. sake *formulation at 3 × 10^7 CFU/ml (LF1); or (iii) chemical treatments usually used in the packinghouse (chemical). The application system was a commercial drencher. Two orchards were monitored for each variety of pome fruit used (■, orchard 1, and ▨, orchard 2). The sample unit was one bin per replicate and treatment. After the treatments, fruits were stored for 3 months under CA, for 4 months under cold conditions, and for 7 days at 20°C. After the storage period, the decayed fruits were recorded. For each orchard, columns with the same letters are not significantly different (*$P < 0.05$*) according to the LSD test.*

based products at different locations within the Mediterranean area. This biocontrol agent therefore had to work over a wide range of conditions and combat different pathogen strains, in different environmental conditions, at points of origin and in packinghouses where it has never been tested before. Furthermore, the cultivars tested in this study included some of the most important pome fruit varieties produced in the European Union, such as Golden Delicious apples and Conference pears.

This study showed the effectiveness of a formulated product developed for commercial applications and used to control postharvest disease, particularly blue mold, on pome fruits. The development of this biocontrol product involved several different steps, some of which have been described in detail by other authors. An economical mass production method that involves the use of industrial waste materials has already been developed for *C. sake (2).* The next step was to develop a shelf-stable formulated product that retains a level of biocontrol activity similar to fresh cells of the microorganism *(17).* All of the formulations of *C. sake* tested in this study showed a level of efficacy similar to fresh cells in controlling disease due to *P. expansum,* even for different varieties and locations. To be commercially acceptable, the production and formulation of the product must be cheap, and its application must be compatible with packinghouse operations *(25).* Not all of the formulations tested in this study were viable for commercial trials from a practical and economic point of view. Only one liquid formulation, LF1, was selected for semicommercial and commercial trials because it was cheap to produce, easily suspended in water, and as effective as fresh cells in semicommercial applications. Furthermore, it could be applied as successfully as chemical treatments in drencher applications. This formulation had a final concentration of 5×10^9 to 7×10^9 CFU/ml after 2 to 3 months of storage at 4°C. Thus, a 1,000-liter drencher (for a treatment dose of 10^7 CFU/ml) requires 1.5 to 2.5 liters of product, which is usual for postharvest applications of chemicals. The storage and transport of formulated products at 4°C appear not to be an obstacle to their commercialization, as other products, such as BioSave 100 and 110, are sold in that way. Other studies will be required to examine the feasibility of extending the shelf life of the product.

In several commercial trials, LF1 reduced the incidence of natural infection to less than 50%. Moreover, the applied dose of this formulation exhibited good pathogen control in a variety of pome fruit cultivars. This is an important factor to take into account for future commercial applications, because not all biocontrol agents have this property. For example, the biocontrol agent *Pseudomonas syringae* had to be applied at a higher dose in pears than in apples to obtain the same level of control against *P. expansum (15).* Other yeasts used as biofungicides in semicommercial and commercial trials were applied at a concentration of 10^8 CFU/ml to achieve effective biocontrol activity *(9–11).*

The recommended dose of *C. sake* CPA-1 for commercial trials was 10^7 CFU/ml in both Lleida and France and was a little higher in Italy (3×10^7 CFU/ml). In Italy, the greater virulence of *P. expansum* strains meant that it was necessary to apply a higher dose of *C. sake* to control blue mold *(22).* Doses of around 10^8 CFU/ml of a strain of *Metschnikowia pulcherrima* were also required to control *P. expansum* on apples in trials carried out in Italy *(30).* Over 80% of *P. expansum* isolates from pears in Italian packinghouses are resistant to thiabendazole and, moreover, show a higher level of pathogenic fitness on fruits and produce larger lesions than more sensitive isolates *(5).* This also explains the poor performance of thiabendazole, which is frequently associated with its synergic effect on resistant strains. Results obtained from pears treated with thiabendazole confirmed the inefficiency of the thiabendazole treatment when resistant strains are present *(23).* In the presence of resistant biotypes of *P. expansum,* thiabendazole also proved ineffective against blue mold on d'Anjou pears *(9)* and on Golden Delicious, Red Delicious, and Empire apples *(11).*

C. sake demonstrated a high level of adaptation for growing in pome fruit tissue and for achieving similar control on a number of different pome fruit cultivars, including Conference, Blanquilla, Golden Delicious, and Red-Chief. Population studies of this antagonist showed that it grows rapidly on the surface of unwounded pome fruits and is adapted to develop under commercial conditions, at low temperatures, and under CA conditions. In semicommercial trials, there was a greater population of LF1 than of fresh cells; this indicated the vitality of this strain in the formulated product and the lack of adverse effects during the formulation process that might otherwise have limited the adaptation of this microorganism to the wound environment. Our results confirm those obtained by Janisiewicz and Jeffers *(17)* for the biofungicide BioSave 11, based on a strain of *P. syringae.*

Our study showed an important degree of variability in postharvest decay due to this biocontrol agent, which was applied under a wide range of conditions. In some locations, the main obstacle to observing differences between this formulation and the standard fungicides used in packinghouses was the low incidence of decay in commercial trials. Because *P. expansum* requires a wound to initiate infection, resistance of the epidermis to breakage could be a key factor in the resistance of pome fruits to decay. Spotts et al. *(32)* reported that the incidence of wounding in d'Anjou and Bosc pears could be due to a lack of care when harvesting the fruits. In addition to their sensitivity during harvest and postharvest treatments, the skin of fruits could play an important role in resistance to pathogens. A study about the level of resistance to decay among apple cultivars, which evaluated epidermal resistance to breakage and sinus opening, showed that Golden Delicious apples have a tender epidermal layer and that their cortical tissue is moderately susceptible to *P. expansum (31).* More in-depth research should be conducted to investigate the high degree of variability in the results obtained from semicommercial and commercial trials.

An understanding of the modes of action of biological control agents is important for the commercial development of biopesticides and especially for fulfilling certain requirements of the registration procedure for commercial use.

Various mechanisms have been described, including antibiosis, the production of lytic enzymes, induced resistance, competition for nutrients and space, and even resistance to oxidative stress *(6).* Competition for nutrients could play the main role in the biocontrol of *C. sake* CPA-1 on pome fruits *(13).* This mode of action has also been recently described for several yeasts *(7, 12, 30, 38).* Neither in vitro nor in vivo inhibition of *P. expansum* was detected in culture filtrates of *C. sake* CPA-1 grown in different media *(13).* This observed lack of antagonism for *C. sake* suggests that the experimental conditions did not exert any antibiotic activity against *P. expansum.* The putative mode of action of this yeast could be an advantage for expediting its registration as a biofungicide product.

The clear need to develop new and alternative methods for controlling postharvest disease has prompted the development of three different biological control products: Aspire (*Candida oleophila* I-182, Ecogen, Langhorne, Pa.) for citrus fruits and BioSave 110 (*P. syringae,* EcoScience, Worcester, Mass.; formerly BioSave 11) and YieldPlus for apples and pears, although none of these products has been developed in Europe. The development of a practical biological product for Europe would be an important way of enabling growers to reduce their dependence on chemical treatments. One of these fungicides could be a *C. sake*–based product.

ACKNOWLEDGMENTS

The authors are grateful to the European Community (QLK5-1999-01065) for its financial support and to the Nufri (Lleida, Catalonia, Spain), Apofruit (Ferrara, Italy), and Fruivial/Cofruid'oc (Saint-Just, France) packinghouses for their technical assistance in commercial trials.

REFERENCES

1. Abadias, M., N. Teixidó, J. Usall, A. Benabarre, and I. Viñas. 2001. Viability, efficacy and storage stability of freeze-dried biocontrol agent *Candida sake* using different protective and rehydration media. *J. Food Prot.* 64:856–861.
2. Abadias, M., N. Teixidó, J. Usall, and I. Viñas. 2003. Optimization of growth conditions of the postharvest biocontrol agent *Candida sake* CPA-1 in a lab-scale fermenter. *J. Appl. Microbiol.* 95:301–309.
3. Abadias, M., J. Usall, N. Teixidó, and I. Viñas. 2003. Liquid formulation of the postharvest biocontrol agent *Candida sake* CPA-1 in isotonic solutions. *Phytopathology* 93:436–442.
4. Agricultural University of Lublin (Poland). 2004. Available at: http://www.prognosfruit2004.ar.lublin.pl. Accessed 23 February 2006.
5. Baraldi, E., M. Mari, E. Chierici, M. Pondrelli, and G. C. Pratella. 2003. Studies on thiabendazole resistance of *Penicillium expansum* of pears: pathogenic fitness and genetic characterization. *Plant Pathol.* 52:362–370.
6. Castoria, R., L. Caputo, F. De Curtis, and V. De Cicco. 2003. Resistance of postharvest biocontrol yeasts to oxidative stress: a possible new mechanism of action. *Phytopathology* 93:564–572.
7. Castoria, R., F. De Curtis, G. Lima, L. Caputo, S. Pacifico, and V. De Cicco. 2001. *Aureobasidium pullulans* (LS-30) an antagonist of postharvest pathogens of fruits: study on its modes of action. *Postharvest Biol. Technol.* 22:7–17.
8. Chalutz, E., and S. Droby. 1997. Biological control of postharvest diseases, p. 157–170. *In* G. J. Boland and L. D. Kuykendall (ed.), Plant-microbe interactions and biological control. Marcel Dekker, Inc., New York.
9. Chand-Goyal, T., and R. A. Spotts. 1997. Biological control of postharvest diseases of apple and pear under semi-commercial and commercial conditions using three saprophytic yeasts. *Biol. Control* 10: 199–206.
10. Droby, S., L. Cohen, A. Daus, B. Weiss, B. Horev, E. Chalutz, H. Katz, M. Keren-Tzur, and A. Shachnai. 1998. Commercial testing of Aspire: a yeast preparation for the biological control of postharvest decay of citrus. *Biol. Control* 12:97–101.
11. El-Gaouth, A., J. L. Smilanick, G. E. Brown, A. Ippolito, M. Wisniewski, and C. L. Wilson. 1999. Application of *Candida saitoana* and glycolchitosan for the control of postharvest diseases of apple and citrus fruit under semi-commercial conditions. *Plant Dis.* 84: 243–248.
12. Fan, Q., and S. Tian. 2001. Postharvest biological control of grey mold and blue mold on apple by *Cryptococcus albidus* (Saito) Skinner. *Postharvest Biol. Technol.* 21:341–350.
13. Höfte, M., L. Poppe, U. Druvefors, J. Schnürer, A. De Cal, P. Melgarejo, V. Stépien, and M. H. Jijakli. 2004. Modes of action of *Candida sake* CPA-1, *Pantoea agglomerans* CPA-2, *Epicoccum nigrum* 282 and *Pichia anomala* J121, effective antagonists of postharvest pathogens, p. 27. *In* International Workshop of Development of Biocontrol Agents of Diseases for Commercial Applications in Food Production Systems, Sevilla, Spain.
14. Ippolito, A., A. El-Ghaouth, C. L. Wilson, and M. Wisniewski. 2000. Control of postharvest decay of apple fruit by *Aureobasidium pullulans* and induction of defense responses. *Postharvest Biol. Technol.* 19:265–272.
15. Janisiewicz, W. J. Personal communication.
16. Janisiewicz, W. J. 1997. Biological control of postharvest diseases of temperate fruits: challenges and opportunities, p. 171–198. *In* G. J. Boland and L. D. Kuykendall (ed.), Plant-microbe interactions and biological control. Marcel Dekker, Inc., New York.
17. Janisiewicz, W. J., and S. N. Jeffers. 1997. Efficacy of commercial formulation of two biofungicides for control of blue mold and gray mold of apples in cold storage. *Crop Prot.* 16:629–633.
18. Janisiewicz, W. J., and A. Marchi. 1992. Control of storage rots of various pear cultivars with a saprophytic strain of *Pseudomonas syringae. Plant Dis.* 76:555–560.
19. Janisiewicz, W. J., T. J. Tworkoski, and C. P. Kurtzman. 2001. Biocontrol potential of *Metschnikowia pulcherrima* strains against blue mold of apple. *Phytopathology* 91:1098–1108.
20. Koffmann, W., and L. J. Penrose. 1987. Fungicides for the control of blue mold (*Penicillium* spp.) in pome fruits. *Sci. Hortic. (Canterbury)* 31:225–232.
21. Mao, G. H., and R. A. Cappellina. 1989. Postharvest biocontrol of gray mold of pear by *Pseudomonas gladioli. Plant Pathol.* 79:1153.
22. Mari, M. Personal communication.
23. Mari, M., L. Casalini, and G. C. Pratella. 2001. Relative fitness of thiabendazole-sensitive and -resistant strains of *Penicillium expansum* on pear fruit. Proceedings of the VIII National Congress S.I.Pa.V. Potenza, Italy, 3 to 5 October 2001.
24. Mari, M., M. Guizzardi, and G. C. Pratella. 1996. Biological control of gray mold in pears by antagonistic bacteria. *Biol. Control* 7:30–37.
25. McGuire, R. G. 1994. Application of *Candida guilliermondii* in commercial citrus coatings for biocontrol of *Penicillium digitatum* on grapefruits. *Biol. Control* 4:1–7.
26. Mercier, J., and C. L. Wilson. 1994. Colonization of apple wounds by naturally occurring microflora and introduced *Candida oleophila* and their effect on infection by *Botrytis cinerea* during storage. *Biol. Control* 4:138–144.
27. Nunes, C., J. Usall, N. Teixidó, and I. Viñas. 2001. Biological control of postharvest pear diseases using a bacterium *Pantoea agglomerans* CPA-2. *Int. J. Food Microbiol.* 70:53–61.
28. Roberts, R. G. 1990. Postharvest biological control of gray mold of apple by *Cryptococcus laurentii. Phytopathology* 80:526–530.
29. Rosenberg, D. A., and F. W. Meyer. 1981. Postharvest fungicides for apples: development of resistance to benomyl, vinclozolin and iprodione. *Plant Dis.* 65:1010–1013.
30. Spadaro, D., R. Vola, S. Piano, and M. L. Gullino. 2002. Mechanisms of action and efficacy of four isolates of the yeast *Metschni-*

kowia pulcherrima active against postharvest pathogens on apples. *Postharvest Biol. Technol.* 24:123–134.
31. Spotts, R. A., L. A. Cervantes, and E. A. Mielke. 1999. Variability in postharvest decay among apple cultivars. *Plant Dis.* 83:1051–1054.
32. Spotts, R. A., P. G. Sanderson, C. L. Lennox, D. Sugar, and L. A. Cervantes. 1998. Wounding, wound healing and staining of mature pear fruit. *Postharvest Biol. Technol.* 13:27–36.
33. Teixidó, N., J. Usall, and I. Viñas. 1999. Efficacy of preharvest and postharvest *Candida sake* biocontrol treatments to prevent blue mould on apples during cold storage. *Int. J. Food Microbiol.* 50: 203–210.
34. Torres, R., N. Teixidó, J. Usall, M. Abadias, and I. Viñas. 2005. Postharvest control of *Penicillium expansum* on pome fruits by the bacterium *Pantoea ananatis* CPA-3. *J. Hortic. Sci. Biotechnol.* 80: 75–81.
35. Torres, R., J. Usall, N. Teixidó, M. Abadias, and I. Viñas. 2003. Liquid formulation of the biocontrol agent *Candida sake* by modifying water activity or adding protectants. *J. Appl. Microbiol.* 94: 330–339.
36. Usall, J., N. Teixidó, E. Fons, and I. Viñas. 2000. Biological control of blue mould on apple by a strain of *Candida sake* under several controlled atmosphere conditions. *Int. J. Food Microbiol.* 58:83–92.
37. Usall, J., N. Teixidó, R. Torres, X. Ochoa de Eribe, and I. Viñas. 2001. Pilot test of *Candida sake* (CPA-1) applications to control postharvest blue mould on apple fruits. *Postharvest Biol. Technol.* 21:147–156.
38. Vero, S., P. Mondina, J. Burgueño, M. Soubes, and M. Wisniewski. 2002. Characterization of biocontrol activity of two yeast strains from Uruguay against blue mold of apple. *Postharvest Biol. Technol.* 26:91–98.
39. Viñas, I., J. Usall, N. Teixidó, and V. Sanchis. 1998. Biological control of major postharvest pathogens on apples with *Candida sake. Int. J. Food Microbiol.* 40:9–16.
40. Viñas, I., N. Vallverdú, S. Monllao, J. Usall, and V. Sanchis. 1993. Imazalil resistant *Penicillium* isolated from Spanish apple packinghouses. *Mycopathologia* 123:27–33.
41. Wicks, T. 1977. Tolerance to benzimidazole fungicides in blue mold (*Penicillium expansum*) on pears. *Plant Dis. Rep.* 61:447–449.
42. Wisniewski, M. E., and C. L. Wilson. 1992. Biological control of postharvest diseases of fruits and vegetables: recent advances. *Hortscience* 27:94–98.