Postharvest Biology and Technology 60 (2011) 164–172

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Integrated control of blue mould using new fungicides and biocontrol yeasts lowers levels of fungicide residues and patulin contamination in apples

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ARTICLE INFO

Article history:
Received 3 September 2010
Accepted 20 December 2010

Keywords:
Postharvest diseases
Blue mould
Biocontrol yeast
Fungicides
Integrated control
Patulin

ABSTRACT

We tested the compatibility of the biocontrol yeasts (*Rhodosporidium kratochvilovae* LS11 and *Cryptococcus laurentii* LS28) with the recently developed fungicides boscalid (BOSC), cyprodinil (CYPR) and fenhexamid (FENH) to create an efficient integrated approach to control blue mould on apples. The fungicide thiabendazole (TBZ), which is presently allowed for postharvest treatment of pome fruit in different countries, was also used as the control. Both the biocontrol agents (BCAs) LS11 and LS28 were compatible *in vitro* with BOSC and CYPR, whereas they were strongly inhibited by FENH. TBZ was compatible with LS28, while it strongly inhibited LS11. *In vitro* assays with some isolates of *Penicillium expansum* showed that the majority were resistant to TBZ, whereas they were all markedly inhibited by BOSC and CYPR. Experiments of integrated control were performed on wounded apples kept at 21 °C up to 7 days. After 4 days of storage, the combination of a low BCA concentration (5×10^6 cfu mL^{-1}) with a low dose (25% of the label dose) of commercial formulates of BOSC or CYPR, resulted in an efficient reduction of blue mould incidence (83–100% less infection with respect to the control). Conversely, the combination of BCAs with TBZ was less effective (not more than 60% of rot reduction). When applied alone at low dosage, LS11, LS28, BOSC, CYPR and TBZ reduced Penicillium rot by 35%, 52%, 67%, 72% and 0%, respectively. After 7 days of storage, only the integrated treatment based on BCAs with BOSC or CYPR resulted in a significant rot reduction (as much as 98%). Treatments based on the utilization of the BCA LS28 or low dosage of CYPR alone were much less effective (10% and 28% rot reduction, respectively), whereas both BCAs integrated with TBZ were ineffective. Furthermore, integrated treatments (BCAs + BOSC or CYPR) resulted in lower fungicide residues and patulin (PAT) contamination in apples. Our data show that the integration of biocontrol yeasts with a low rate of the recently commercialized fungicides BOSC or CYPR could be an effective and safer strategy to control *P. expansum* and keep fungicide residues as well as PAT contamination in apples low.

1. Introduction

Apples are grown in many countries and consumed worldwide. Because of their high content in bioactive molecules (e.g., antioxidants and vitamins), such fruit can exert beneficial effects in a balanced diet (Boyer and Liu, 2004; Gallus et al., 2005). The storability and nutritional value of fresh fruit can be heavily affected by postharvest fungal diseases (Snowdon, 1990) and chemical contaminants (Castoria and Logrieco, 2007; WHO, 2008). Blue mould caused by the fungus *Penicilllium expansum* is one of the most important postharvest rots of pome fruit (Rosenberger, 1990). This pathogen is also a major producer of patulin (PAT), a mycotoxin which can reach concentrations of mg/kg in infected apples and pears (Battilani et al., 2008) and is known to have cytotoxic, genotoxic and immunosuppressive activity (Wouters and Speijers, 1996). As a consequence, it is a major health hazard for children, who consume great quantities of fruit juices and/or baby food produced from pome fruit (Beretta et al., 2000). Therefore, many countries have set the highest tolerable levels of PAT in these products (EC Reg., 1881/2006; Moake et al., 2005).

Despite the wide-spread use of modern storage facilities and techniques, synthetic fungicides are still frequently used immediately before or after harvest to control postharvest deterioration of fruit. However, chemical control is being increasingly limited because of environmental and toxicological risks as well as the onset of fungicide-resistant strains of fungal pathogens. Moreover, the legal limits of chemical residues left by pesticides in imported fruit are much lower in some countries, thus discouraging the use of chemical products. In the absence of fully effective postharvest fungicides, alternative or integrative measures are becoming

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doi:10.1016/j.postharvbio.2010.12.010

increasingly important in controlling postharvest fungal disease and in maintaining a high level of quality. Biocontrol by antagonistic microorganisms, including yeasts, yeast-like fungi and bacteria, appears to be a promising tool for preventing postharvest fungal rots and minimizing the use of fungicides (Janisiewicz and Korsten, 2002; Ippolito et al., 2004). However, biocontrol agents (BCAs) are sometimes not sufficient to control fungal decays satisfactorily when applied alone under practical conditions. On the other hand, fungicide-resistant strains of *P. expansum* and other fungal pathogens in packinghouses are constantly increasing as a consequence of prolonged fungicide treatments (Janisiewicz and Korsten, 2002). This is the case with thiabendazole (TBZ), one of the very few synthetic fungicides that is still allowed in many countries for postharvest treatment of pome fruit. Therefore, integrated approaches based on the combination of BCAs and fungicides or alternative means have been suggested to prevent a resistance increase in the pathogen population and limit risks due to intensive use of chemicals (Lima et al., 2005, 2008; Droby et al., 2009).

Several papers have shown the protective activity of selected BCAs from *P. expansum* rot on apples. Surprisingly, very little research has taken into account the influence of BCAs on the accumulation of mycotoxins in fruit. Recently, we found that biocontrol yeasts can lower PAT as well as ochratoxin accumulation in apples and wine grapes, respectively, by preventing attacks by *P. expansum* and *Aspergillus carbonarius*, and these BCAs can detoxify these mycotoxins *in vitro* by transforming them into less toxic compounds (Castoria et al., 2005, 2007; De Felice et al., 2008). On the other hand, to the best of our knowledge, no information is available on the effects of BCAs combined with fungicides on the accumulation of mycotoxins in general, and PAT in particular, and on the persistence of fungicide residues in stored apples. Our previous research showed that the protective activity of the two selected antagonist yeasts, *Rhodosporidium kratochvilovae* LS11 and *Cryptococcus laurentii* LS28, is enhanced by combining them with a low dosage of fungicides and/or natural adjuvants, and that such strategies can control both resistant and sensitive strains of fungal pathogens (Lima et al., 2005, 2008). Therefore, selected biocontrol yeasts are very interesting candidates for their utilisation in integrated control strategies aimed at reducing the use of fungicides and the contamination by chemical residues and mycotoxins (Castoria et al., 2008; Lima et al., 2008).

This work was aimed at evaluating the compatibility of the BCAs *R. kratochvilovae* LS11 and *C. laurentii* LS28 with recently developed fungicides in order to create a new, efficient, integrated approach to control *P. expansum* on apples and reduce (i) PAT contamination, (ii) fungicide residue and (iii) risks of the onset of *P. expansum* fungicide-resistant strains.

2. Materials and methods

2.1. Biocontrol agents (BCAs)

Rhodosporidium kratochvilovae strain LS11 (previously reported as *Rhodotorula glutinis* LS11) and *Cryptococcus laurentii* strain LS28, isolated from olives and apples, respectively, were the BCAs used in this study; these antagonists had previously been characterized for antagonistic activity (Lima et al., 1998, 1999) and mechanisms of action (Castoria et al., 1997, 2003). The growth of BCAs and the production of their cell suspensions were carried out as reported elsewhere (Lima et al., 1998, 2006).

2.2. Fungicides

In the experiments *in vitro* and/or *in vivo*, the following fungicides were used: boscalid (Chemical group anilides; trade formulate Cantus®, 50%, w/w a.i., Basf, Milan, Italy); cyprodinil (Chemical group anilinopyrimidines; trade formulate Chorus®, 50% a.i., w/w, Syngenta Crop Protection, Milan, Italy); fenhexamid (Chemical group hydroxyanilides; trade formulate Teldor®, 50% a.i., w/w, Bayer Crop Science, Milan, Italy); thiabendazole (Chemical group benzimidazoles; trade formulate Decco T®, 50%, w/v a.i., Cerexagri, Cesena, Italy).

2.3. Fungal cultures

Isolates of *P. expansum* used in experiments on apples (isolate FS7) and for the assessment of *in vitro* resistance to fungicides (isolates FQ42, FQ44, FQ45, FQ46, FQ47, FQ48) belong to our fungal collection and were obtained from decaying apples. Moreover, isolates P32-R and LB8/99-S, from decaying pears, supplied by CRIOF (*Centro per la Protezione e Conservazione dei Prodotti Ortofrutticoli*), University of Bologna, Italy, were used as reference isolates for their known high resistance or low sensitivity to benzimidazoles, respectively (Baraldi et al., 2003).

In order to obtain conidial suspensions for fruit inoculation the pathogen (isolate FS7) was grown on potato dextrose agar (PDA) under fluorescent light for 5–7 days at 21 °C. Five milliliter of sterile distilled water containing 0.05% Tween 20 were poured into Petri dishes, and conidia were scraped from the agar by using a sterile loop. The suspension obtained was filtered through 4 layers of cheesecloth. The inoculum concentration was adjusted by an haemocytometer to 2×10^4 conidia mL^{-1}.

2.4. Compatibility of BCAs with TBZ and more recent fungicides

The BCAs LS11 and LS28 were tested *in vitro* for their sensitivity to commercial formulates of thiabendazole (TBZ) as well as the more recent developed fungicides boscalid (BOSC), cyprodinil (CYPR) and fenhexamid (FENH). The assays were performed on basal yeast agar (BYA: 10 g bacteriological peptone, 1 g yeast extract, 20 g dextrose, 18 g agar, 1 L1 distilled water). Briefly, each fungicide was suspended in distilled water and mixed with the medium at 45 °C and, according to the full dose suggested by the manufacturers for pre and/or postharvest application on fruit, the following concentrations of fungicide active ingredient (a.i.) were tested: BOSC 375 $\mu g\,mL^{-1}$ (75 $g\,h\,L^{-1}$ of commercial product), 187.5 $\mu g\,mL^{-1}$ (50% of the full suggested dose), 93.8 $\mu g\,mL^{-1}$ (25% of the full suggested dose); CYPR 150 $\mu g\,mL^{-1}$ (30 $g\,h\,L^{-1}$ of commercial product), 75 $\mu g\,mL^{-1}$ (50% of the full suggested dose), 37.5 $\mu g\,mL^{-1}$ (25% of the full suggested dose); FENH 600 $\mu g\,mL^{-1}$ (120 $g\,h\,L^{-1}$ of commercial product), 300 $\mu g\,mL^{-1}$ (50% of the full suggested dose), 150 $\mu g\,mL^{-1}$ (25% of the full suggested dose); TBZ 418 $\mu g\,mL^{-1}$ (100 $g\,h\,L^{-1}$ of commercial product), 209 $\mu g\,mL^{-1}$ (50% of the full suggested dose), 104.5 $\mu g\,mL^{-1}$ (25% of the full suggested dose). Each plate (4 replications per treatment) was poured with 100 μL of yeast suspension containing about 100 cells and incubated for 7 days at 23 °C. In each plate the growing yeast colonies were counted and minimum inhibitory concentration (MIC, i.e. the lowest concentration of fungicide inhibiting the growth of yeast colonies) was assessed.

2.5. Sensitivity of P. expansum isolates to TBZ and more recent fungicides

The resistance of isolates of *P. expansum* to commercial formulates of TBZ, BOSC and CYPR was assessed *in vitro*. The fungicide FENH was not used in this assay because of its incompatibility with (i.e. high inhibiting activity to) both biocontrol yeasts.

The following concentrations of a.i. of each fungicide were tested: BOSC 375 $\mu g\,mL^{-1}$ (75 $g\,h\,L^{-1}$ of commercial product), 187.5 $\mu g\,mL^{-1}$ (50% of the full suggested dose), 93.8 $\mu g\,mL^{-1}$

(25% of the full suggested dose), 37.5 μg mL^{-1} (10% of the full suggested dose); CYPR 150 μg mL^{-1} (30 g h L^{-1} of commercial product), 75 μg mL^{-1} (50% of the full suggested dose), 37.5 μg mL^{-1} (25% of the full suggested dose), and 15 μg mL^{-1} (10% of the full suggested dose); TBZ 418 μg mL^{-1} (100 g h L^{-1} of commercial product), 209 μg mL^{-1} (50% of the full suggested dose), 104.5 μg mL^{-1} (25% of the full suggested dose), 41.8 μg mL^{-1} (10% of the full suggested dose), 20.9 μg mL^{-1} (5% of the full suggested dose), and 4.2 μg mL^{-1} (1% of the full suggested dose).

Each fungicide, was mixed with PDA at 43 °C, and the mixture was immediately poured into Petri dishes (100 mm diameter). The plates were left to cool and mycelial plugs (6 mm diameter) of each fungal strain were withdrawn from actively growing colonies (4–5 days PDA dark-grown cultures at 23 °C), and placed in the centre of the Petri dishes containing the fungicide. Four replicates for each fungicide concentration were prepared. Cultures were incubated in the dark at 21 °C and colony diameters were measured on a daily basis until they reached their highest value in fungicide-free control dishes (after 7–10 days, depending on the fungal strain). The percentage of inhibition of radial mycelial growth on amended PDA was calculated and data obtained were subjected to Probit Analysis to determine the EC_{50} value (i.e. concentration of each fungicide causing 50% reduction in mycelial growth).

2.6. Integration of BCA and fungicide for the control of P. expansum on apples

Apples cv Golden delicious, from an orchard conducted by the integrated crop management method, were purchased from a local supplier and kept at 3 °C, and 95–98% RH prior to use. The fruit was then removed from cold storage 24 h before performing each experiment. During storage (from 5 to 20 days) average values of fruit firmness and soluble solid concentration were 4.5 kg and 11.5° Brix, respectively, with a low variability throughout the time of the experiments.

Apples were superficially disinfected by dipping them in a sodium hypochlorite solution (2% active chlorine) for 1 min; the fruit were then rinsed twice with sterile distilled water and dried at room temperature. Four wounds (5 mm wide by 3 mm deep) on each fruit were made around the blossom end. For biocontrol treatments, 30 μL of yeast cell suspension in distilled water at 5×10^6 cells mL^{-1} were placed in each wound. The combined treatments (BCA + fungicide) were performed by applying 30 μL of yeast at 5×10^6 cells mL^{-1} suspended in low dosage (25% of the full suggested dose) of each fungicide (TBZ, BOSC or CYPR) in each wound.

In each experiment, controls were represented by fruit in which wounds were treated with 30 μL of sterile distilled water alone, 30 μL of fungicide alone at low dosage or 30 μL of fungicide applied alone at full dose. After 2 h at room temperature, each wound was inoculated with 15 μL of a conidial suspension of *P. expansum* at 2×10^4 conidia mL^{-1}. The apples were incubated in the dark at 21 °C for 7 days at 95–98% RH. Each treatment included 3 replicates and each replicate consisted of 8 fruit. The number of wounds showing rot symptoms as well as the diameter of rotting lesions in each wound were assessed on a daily basis.

2.7. Analysis of fungicide residues and patulin contamination in apples

2.7.1. Preparation of samples

After 7 days at 21 °C, apples were sampled to determine residues of fungicides (CYPR and BOSC) and PAT accumulation. Each fruit was cut in half at the equatorial line, and infected or uninfected wounds from the wounded half were withdrawn with a cork borer (15 mm diameter). For each replicate (8 apples), withdrawn wounds were pooled, and each sample (each consisting of 32 wounds) was stored at −20 °C. Extraction and analysis of fungicide residues and PAT were performed as described below.

For the analysis of fungicides 10 g of each sample were homogenized using a Ultra-Turrax T25 (IKA-WERKE, Germany) and homogeneously mixed with 10 g of fine diatomaceous earth at pH 10. The mixture was then placed onto a SPE polypropylene tube and the sample eluted with 100 mL of a dichloromethane/ethyl acetate (80:20, v/v) solution. The extract was completely dried and then dissolved in 10 mL of a methanol/water (70:30, v/v) solution, centrifuged at 10,000 rpm for 3 min and used for LC/MS/MS analysis of fungicides. The standard working solutions of fungicides were prepared by appropriate dilutions with the same methanol/water (70:30, v/v) solution.

For the analysis of PAT, 10 g of sample were transferred into a falcon tube containing 15 g of Na_2SO_4, 2 g of $NaHCO_3$ and 10 mL of extraction solution (ethyl acetate/hexane 60:40, v/v). The tube was shaken for 3.5 min on a mechanical shaker and then centrifuged at 2000 rpm for 1 min to force separation of layers. Since PAT is not stable at alkaline pH, this step was carried out as quickly as possible. The centrifuged extract (2.5 mL) was immediately placed onto an unconditioned Strata C18-E solid phase extraction column (Phenomenex, USA) (parameter: Surface Area 500 m^2 g^{-1}; pore size 70 Å; pore volume 0.88 mL g^{-1}; average particle size 58 μm) which was washed with 3 mL of extraction solution. The eluate was collected in a test-tube containing 50 μL of acetic acid (Merck, Darmstadt-Germany), and the flow was adjusted to 1 drop per second by using slight air pressure. The solvent was evaporated at max 40 °C for about 35 min in a vacuum centrifuge Thermo Savant (SVPT Srl-JOUAN) and 1 mL of acidulated water (pH 4) was added to the dried sample. This solution was vortexed in order to fully dissolve PAT and then transferred into a vial before injection.

2.7.2. LC/MS/MS analysis of fungicides

Chromatographic separation was performed using an HPLC apparatus equipped with two micropumps Series 200 (Perkin Elmer, Canada, USA) and a Gemini 5 μm C18, 110 Å column (150 mm × 2 mm) (Phenomenex, CA, USA). The eluents were: (A) water 0.1% formic acid; (B) acetonitrile. The gradient program was as follows: 35–50% B (5 min), 50–95% B (7 min), 95% B (2 min), 95–35% B (2 min), at a constant flow of 0.2 mL min^{-1}. Injection volume was 20 μL.

MS/MS analyses of BOSC and CYPR were performed on an API 2000 triple quadrupole mass spectrometer (Applied Biosystems, Canada) equipped with a Turbolonspray source. Analyses were performed in the positive ion mode in MRM (multiple reaction monitoring).

The calibration curve displayed good linearity in the range 5–500 ng mL^{-1}. All chromatographic points of the calibration curve were run in triplicate, and the standard deviation was lower than 0.05.

The limits of detection (LOD, with a signal to noise ratio of 3) for CYPR and BOSC were 0.5 and 2.5 ng g^{-1} respectively, limits of quantification (LOQ, with a signal to noise ratio of 6) were 2 ng g^{-1} for CYPR and 5 ng g^{-1} for BOSC.

2.7.3. HPLC analysis of patulin

Patulin (4-Hydroxy-4H-furo[3,2-c]pyran-2(6H)-one) contamination in apples was assessed by HPLC according to Arranz et al. (2005) and Katerere et al. (2007) with slight modifications as described below. Analyses were carried out by using an HPLC apparatus (Shimadzu-Japan) equipped with an autosampler SIL-20A, two pumps LC-20AD and a UV/vis detector SPD-20A set at 276 nm wavelength. The column was a Gemini 5 μ C18, 110 Å (150 mm × 2 mm) (Phenomenex, CA, USA). The mobile phase was isocratic: (A) (95%) was water containing 1% acetic acid and

(B) (5%) was methanol. The flow rate was set at 1 mL min^{-1}. In these chromatographic conditions, the retention time for PAT was 14.0 ± 0.1 min. The quantification was carried out by including signal area values into a linear calibration curve, in the range of 5–1000 μg kg^{-1}, and by correcting errors imputable to variable sensitivity of instrument.

2.8. Data analysis

The percentages of apple wounds infected with *P. expansum* were converted to Bliss angular values (arcsine square root) before analysis of variance. Each experiment was performed at least twice. Homogeneity of variance for repetitions of each experiment was evaluated and data from separate experiments with homogeneous variances were pooled.

Data were subjected to one-way analysis of variance (ANOVA univariate, SPSS release 15.0 for windows; SPSS Inc., Chicago, IL) and the means were compared by using Tukey's multiple range test.

In sensitivity fungicide assays, the EC_{50} values were determined by subjecting to Regression Probit Analysis the results of fungal growth inhibition by using the software SPSS release 11.0 for windows.

In order to compare the integrated treatments with those applied separately, the type of effect (additive, synergistic or antagonistic) was evaluated. For this purpose, percentage values of infected wounds were transformed into percentages of protection efficacy (PE) as follows: $PE = [(C - T)/C] \times 100$, where C is the number of infected wounds in the control (water + pathogen) and T is the number of infected wounds in the examined treatment (BCA alone or BCA + fungicide). Values ranged from 0 (no PE), to 100 (maximum PE). The synergy factor (SF) was calculated according to Abbott's formula (Levy et al., 1986), as follows: SF = E(obs.)/E(exp.), where E(obs.) and E(exp.) are observed and expected PE of the mixture (BCA + fungicide), respectively. E(exp.) was calculated as follows: $a + b - a \times b/100$, where a = PE of the factor a (BCA) applied alone; b = PE of the factor b (fungicide) applied alone. If SF = 1, the interaction between BCA and fungicide is additive; if SF < 1, the interaction between BCA and fungicide is antagonistic; if SF > 1, the interaction between BCA and fungicide is synergistic.

3. Results

3.1. Compatibility of BCAs with TBZ and more recent fungicides

In vitro, both the tested biocontrol yeasts LS11 (*R. kratochvilovae*) and LS28 (*C. laurentii*) were highly resistant to the fungicides BOSC (MIC > 375 μg mL^{-1}) and CYPR (MIC > 150 μg mL^{-1}), whereas they were strongly inhibited by FENH (no growth observed at lower concentration tested; MIC < 150 μg mL^{-1}); isolate LS28 also showed a good compatibility with TBZ (MIC > 418 μg mL^{-1}), while LS11 was very sensitive to this fungicide (MIC < 104.5 μg mL^{-1}) (Table 1).

Table 1
Minimum inhibitory concentration (MIC: μg mL^{-1} of active ingredient) of the fungicides boscalid, cyprodinil, fenhexamid and thiabendazole toward the *in vitro* growth of the biocontrol yeasts *R. kratochvilovae* LS11 and *C. laurentii* LS28.

Fungicide[a]	Yeast isolate	
	LS11	LS28
Boscalid (Cantus®, 50)[b]	>375	>375
Cyprodinil (Chorus®, 50)	>150	>150
Fenhexamid (Teldor®, 50)	<150	<150
Thiabendazole (Decco T®, 50)	<104.5	>418

[a] For each fungicide, concentrations of 25%, 50% and 100% of the full label dose for the respective commercial product were tested.
[b] In brackets: name of the commercial product and percentage (w/v) of active ingredients.

On the basis of these results BOSC and CYPR were chosen for the following *in vitro* and *in vivo* assays.

3.2. Sensitivity of P. expansum isolates to TBZ and more recent fungicides

The effects of the fungicides TBZ, BOSC and CYPR on mycelia growth of isolates of *P. expansum* from pome fruit were tested on PDA medium amended with different concentrations of fungicide. The results of these experiments are reported in Table 2.

Toward TBZ, isolates FQ44, FQ45, FQ48, and LB8/99-S, reporting EC_{50} values below 4.2 μg mL^{-1} of a.i.) were sensitive, whereas isolates FQ42, FS7, FQ46, FQ47 and P32-R with EC_{50} values ranging from 160 μg mL^{-1} to higher than 418 μg mL^{-1} of a.i. were resistant.

As regards the more recently developed fungicides, all the tested isolates of *P. expansum* were strongly inhibited by CYPR (EC_{50} < 18.8 μg mL^{-1} of a.i.) and BOSC (EC_{50} < 46.9 μg mL^{-1} of a.i.).

3.3. Integration of BCAs and fungicides for the control of P. expansum on apples

The results of the experiments carried out on wounded apples with the biocontrol yeasts *R. kratochvilovae* LS11 and *C. laurentii* LS28, applied alone or in combination with low rates of TBZ, BOSC or CYPR for the control *P. expansum* are reported in Fig. 1.

After 4 days of storage, 100% of the wounds were infected with the pathogen in control apples (water + *P. expansum*). TBZ, either at

Table 2
Sensitivity of some isolates of *P. expansum* toward the fungicides thiabendazole, boscalid and cyprodinil, expressed as EC_{50} (μg mL^{-1} of active ingredient reducing by 50% the growth *in vitro* of the fungal mycelium).

Isolate of *P. expansum*[a]	Source	Fungicide			
		Thiabendazole (Decco T®, 50)[b]		Boscalid (Cantus®, 50)	Cyprodinil (Chorus®, 50)
		EC_{50}		EC_{50}	EC_{50}
FQ42	Apple	160.0	(94–263)[c]	<46.9	<18.8
FS7	Apple	>418		<46.9	<18.8
FQ44	Apple	<4.2		<46.9	<18.8
FQ45	Apple	<4.2		<46.9	<18.8
FQ46	Apple	>418		<46.9	<18.8
FQ47	Apple	250.0	(123–455)	<46.9	<18.8
FQ48	Apple	<4.2		<46.9	<18.8
P32-R	Pear	194.5	(105–298)	<46.9	<18.8
LB8/99-S	Pear	<4.2		<46.9	<18.8

[a] **FS7**: isolate of the pathogen used for artificial inoculations in experiments on apples; **P32-R** and **LB8/99-S**: reference isolates known for high resistance or high sensitivity to benzimidazoles, respectively.
[b] In brackets: name of the commercial product and percentage (w/v) of active ingredients.
[c] In the presence of exact values of EC_{50}, numbers in parenthesis indicate 95% confidence limits as determined by probit analysis.

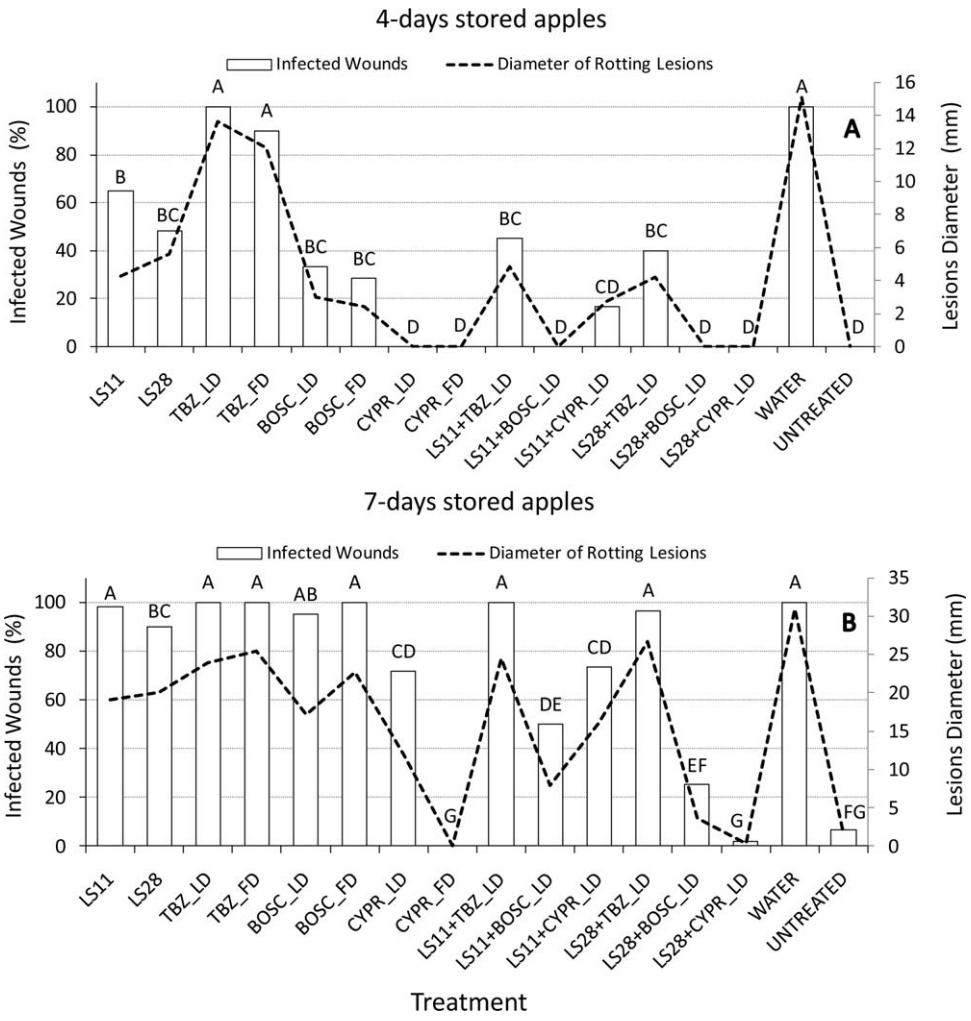

Fig. 1. Activity of the biocontrol yeasts *R. glutinis* LS11 and *C. laurentii* LS28 applied in combination with a low dosage (LD) of the fungicides thiabendazole (TBZ), boscalid (BOSC) or cyprodinil (CYPR) against *P. expansum* on apples kept at 21 °C for 4 days (A) and 7 days (B). Values marked by the same letter are not statistically different at $P = 0.01$ (Tukey's test). FD = full dosage of fungicide, as suggested by the manufacturer. For each fungicide, the LD corresponded to 25% of the FD. The BCAs LS11 and LS28 were applied at a low concentration of cells (5×10^6 cfu mL^{-1}). Except for the untreated control, all the apples were artificially inoculated with *P. expansum* isolate FS7 (2×10^4 conidia mL^{-1}). Two separate experiments were performed and in each assay three replicates per treatment were used.

full or at low dosage, was ineffective against the fungus (90% and 100% of infected wounds, respectively). Conversely, BOSC at full and low dosage (72% and 67% reduction of infections as compared to the control, respectively) and, particularly, CYPR at full and low dosage (100% less infections at any dose), were highly effective against the pathogen. The BCAs LS11 and LS28 alone also appreciably reduced the level of infection with respect to the control (by 35% and 52%, respectively), although they were applied at a relatively low concentration of cells (5×10^6 cfu mL^{-1}). When combined with TBZ, BOSC or CYPR at low dose, both the BCAs yielded significant reductions of infected wounds. In particular, LS11 plus BOSC and LS28 plus BOSC or CYPR were the most effective treatments against the pathogen (100% reduction of infected wounds).

After 7 days of storage, diameters of rotting lesions consistently increased on control fruit, which had already reached 100% of infected wounds at 4 days (see above). The protection of the BCAs and the fungicides BOSC and TBZ applied alone decreased consistently or was completely lost. Only CYPR applied alone at full dosage was still effective (0% infected wounds). As regards the integrated treatments, the combination of the BCAs with TBZ proved to be ineffective. Conversely, the treatments based on the combination of the BCAs with a low dosage of BOSC or CYPR were very effective against the pathogen. In particular, the best results were obtained with LS11 plus BOSC (50% reduction of infected wounds) and with LS28 plus BOSC or CYPR (75% and 98% of reduction, respectively).

In both assessments (4 and 7 days), the diameters of rotting lesions on apples seem in substantial agreement with the respective percentages of infected wounds (Fig. 1A and B).

At the end of the experiments (after 7 days of storage), the synergy factor (SF) was calculated in order to ascertain whether the different BCA-fungicide combinations were synergistic, additive or even antagonistic (Table 3). The integration of both BCAs with low rate of TBZ evidenced a clear antagonistic effect, with SF values < 1 (0.0 and 0.2 for LS11 and LS28, respectively). The integrated treatments LS11 + BOSC, LS28 + BOSC and LS28 + CYPR were clearly synergistic, with SF average values markedly > 1 (6.7, 3.2 and 2.4, respectively). Lastly, the combination of LS11 and CYPR, which resulted in a SF value around 1, can be considered as additive.

3.4. Fungicide residues and patulin contamination in apples

After 7 days, both fungicide residues and PAT contamination were determined in apple subjected to different treatments. The results of these analyses are reported in Fig. 2.

In apples treated with BOSC, an average of 1103 and 521 μg kg^{-1} of residual fungicide were recorded for full dosage (FD) or low

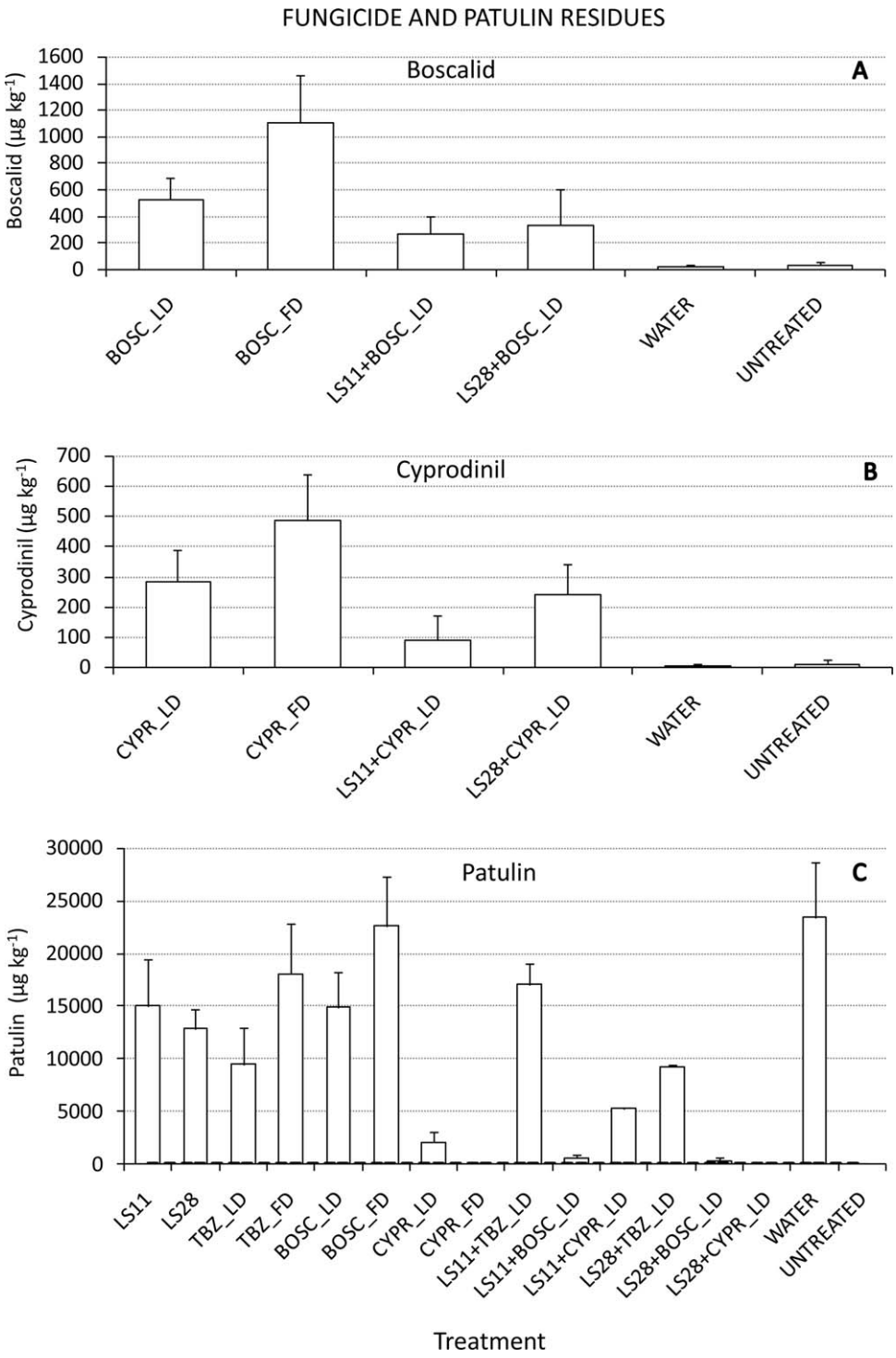

Fig. 2. Residues of the fungicides boscalid (A) and cyprodinil (B) and contamination with the mycotoxin patulin (C) in apples kept for 7 days at 21 °C. Fruit were subjected to different treatment as in Fig. 1. Bars on hystograms represent standard deviation from the mean. Two separate experiments were performed and in each assay three replicates per treatment were used.

dosage (LD) treatments, respectively. When a low dosage of BOSC was used in the integrated treatments with LS11 or LS28, fungicide residues were 269 and 338 $\mu g\,kg^{-1}$, respectively (Fig. 2A).

In apples treated with full and low dosages of CYPR, fungicide residues were 488 and 282 $\mu g\,kg^{-1}$, respectively. In the integrated treatments with the two doses of CYPR plus LS11 or LS28, the residual concentrations of fungicide on apples were 91 and 240 $\mu g\,kg^{-1}$, respectively (Fig. 2B).

As regards contamination of apples with the mycotoxin PAT, significant differences were found in the various treatments (Fig. 2C). In particular, the highest level of PAT (24437 $\mu g\,kg^{-1}$) was recorded in the control apples (water + *P. expansum*), followed by BOSC at full dose (22579 $\mu g\,kg^{-1}$) and TBZ at full dose (18,078 $\mu g\,kg^{-1}$). A significantly lower level of PAT was detected in the following treatments: CYPR at low dose (2080 $\mu g\,kg^{-1}$), CYPR at full dose (45 $\mu g\,kg^{-1}$), LS11 plus BOSC at low dose (556 $\mu g\,kg^{-1}$), LS28 plus BOSC at low

Table 3
Values of the synergy factor (SF)[a] for the activity of the biocontrol yeasts *R. kratochvilovae* LS11 and *C. laurentii* LS28 combined with a low dose of commercial formulates (25% of the full dose suggested by the manufacturers) of the fungicides thiabendazole, boscalid or cyprodinil against *P. expansum* on apples kept for 7 days at 21 °C. Average values ± standard deviation are reported.

Fungicide	Biocontrol agent	
	LS11	LS28
Thiabendazole	0.0 ± 0.0	0.2 ± 0.4
Boscalid	6.7 ± 2.0	3.2 ± 0.4
Cyprodinil	1.1 ± 0.8	2.4 ± 0.5

[a] According to Abbott's formula (Levy et al., 1986), when SF = 1, the interaction is additive; when SF < 1, the interaction is antagonistic; when SF > 1, the interaction is synergistic.

dose (338 μg kg^{-1}) and LS28 plus CYPR at low dose (13 μg kg^{-1}). The other treatments (i.e. BCAs alone, TBZ at low dose, BOSC at low dose, LS11 plus TBZ at low dose, LS28 plus TBZ at low dose and LS11 plus CYPR at low dose, resulted in intermediate levels of PAT, and were not statistically different from the control. In general, apples with higher amount of PAT accumulation (Fig. 2C) also had larger diameters of rotting lesions (Fig. 1A and B).

4. Discussion

An integrated strategy based on the combination of BCAs with natural compounds or reduced dosage of fungicides appears to be one of the most reliable options for large-scale utilisation of microbial antagonists in the control of postharvest fungal rots of fruit and vegetables (Lima et al., 2008; Droby et al., 2009). Therefore, the optimisation of biocontrol efficacy also depends on survival and colonisation of BCAs in wounded and unwounded fruit surfaces in the presence of low quantities of fungicides applied separately or in combination with these microbial antagonists. Moreover, the widespread diffusion of fungal pathogen isolates that have become resistant to fungicides used for a long time in the field and/or in packinghouses (e.g. benzimidazoles), has led to the need of assessing the compatibility and efficacy of BCAs with new and recently developed fungicides.

In the present study, the two biocontrol yeasts *R. kratochvilovae* isolate LS11, and *C. laurentii* isolate LS28, known for their wide spectrum of activity against major postharvest fungal pathogens on various crops (Lima et al., 1998, 1999) were tested in combination with low rates of the recently developed fungicides BOSC and CYPR in comparison with TBZ, which has been on the market for many years.

CYPR is a broad-spectrum fungicide belonging to the chemical class of anilinopyrimidines. In the fungal cell, this compound inhibits the biosynthesis of methionine and other aminoacids and inhibits the secretion of hydrolytic enzymes associated with pathogenesis, such as pectinases, cellulases and proteases (Leroux, 1996). Recently, CYPR has also been shown to be effective in the control of both benzimidazole-resistant and -sensitive isolates of *P. expansum* (Errampalli and Brubacher, 2006).

BOSC, a chemical compound belonging to the anilide class, is a fungicide showing a good effectiveness against several plant fungal pathogens. Its mechanism of action differs from that of previous similar fungicides (e.g. strobilurines) and is based on the inhibition of the enzyme succinate ubiquinone reductase (complex II) in the mitochondrial electron transport chain (Stammler et al., 2007).

Both fungicides, CYPR and BOSC, are good candidates to replace TBZ and/or other old fungicides in the control of pre and/or postharvest fungal rots of fruit, because they from toxicological and environmental points of view are also considered as low-risk fungicides (Adaskaveg et al., 2006).

The biocontrol yeast isolates LS11 and LS28 exhibited good compatibility *in vitro* with BOSC and CYPR. These fungicides also showed clear and strong inhibitory activity on different isolates of *P. expansum*, whereas TBZ was less effective or ineffective. For these reasons, BOSC and CYPR were subsequently used in experiments of integrated control of blue mould on apples. To our knowledge, this is the first study showing the compatibility of selected biocontrol yeasts with the fungicides BOSC or CYPR. Only two previous studies showed a good level of compatibility of CYPR with biocontrol bacteria (*Pseudomonas syringae*, isolate MA-4 or ESC-10) that provided a more efficient control of *P. expansum* in integrated application on apples (Zhou et al., 2002; Errampalli and Brubacher, 2006).

The fungicide TBZ was highly toxic toward the BCA *R. kratochvilovae* LS11, whereas it appeared to be compatible with *C. laurentii* LS28. This result is in full agreement with our previous research in which isolates LS11 and LS28 were tested for their compatibility with benzimidazoles (Lima et al., 2006).

Penicillium expansum FS7, the challenging isolate of the pathogen used in this study, as compared with *P. expansum* reference strains (P32-R, resistant, and LB8/99-S, sensitive) and also with other isolates collected from decaying fruit, was highly resistant (EC50 > 418 μg mL^{-1} of a.i.) to TBZ *in vitro*. In other studies (Viñas et al., 1991; Errampalli et al., 2006) it was found that a majority of *P. expansum* isolates collected from packinghouses and tested *in vitro* toward fungicides was resistant to TBZ because of their ability to grow on PDA amended with 5 or 40 μg mL^{-1} of a.i. Accordingly, the majority of isolates we tested in this work can be considered highly resistant to TBZ since their EC_{50} values resulted higher than 160 μg mL^{-1} of a.i.

Conversely, our *in vitro* assays showed high antifungal activity of the recent developed fungicides BOSC and CYPR against all tested isolates of *P. expansum*, yielding EC_{50} values below 46.9 and 18.8 μg mL^{-1} of a.i., respectively, which correspond to 10% of the full dose suggested by the manufacturers for these fungicides. Our aim was to assess the antifungal activity of these fungicides at a concentration corresponding to 25% of the full dosage (to be used in BCA + fungicide integrated combinations); therefore, we did not test concentrations of BOSC and CYPR lower than 10% of the full dosage. However, other research has shown that the EC_{50} toward fungal pathogens is below 5 μg mL^{-1} for BOSC (Avenot et al., 2008) and 1 μg mL^{-1} for CYPR (Sholberg et al., 2005).

In the assays on apples, the application of the BCAs LS11 and LS28 in combination with small doses of BOSC or CYPR consistently enhanced the efficacy and persistence of the control of *P. expansum* rot with respect to the treatments (biological and chemical) applied separately; the BCAs combined with BOSC or CYPR were highly effective against *P. expansum* after 4 days and even at the end of the experiments (7 days of apple storage) when most of the treatments (BCAs or fungicides) applied individually at the same doses yielded unsatisfactory control. After 7 days, however, only reduced and full doses of CYPR still showed a significant reduction of infected wounds among the individual treatments, thus confirming the efficacy of this fungicide against *P. expansum* on apples as previously reported by Errampalli and Brubacher (2006).

Interestingly, the combination of LS11 with BOSC, and LS28 with either BOSC or CYPR, also yielded a synergistic effect on the protective activity. This result is in agreement with our previous integrated control experiments, in which combined applications of BCAs with fungicides (Lima et al., 2006) or other compounds (Lima et al., 2005) displayed a comparable synergistic improvement of protective activity.

At the end of the experiments, the combined application of the BCA LS11 with TBZ proved to be ineffective against *P. expansum*. As observed in *in vitro* assays, this result is most likely due to the high resistance of *P. expansum* isolate FS7 to TBZ as well as the strong sensitivity of LS11 to TBZ. Actually, TBZ is so toxic to

LS11 that our previous assays (Lima et al., 2006) show that *in vitro* the growth of this BCA was inhibited even at 1.2 $\mu g\,mL^{-1}$ of a.i. Although isolate LS28 was compatible with TBZ *in vitro*, this BCA-fungicide combination also failed to control *P. expansum* on apples. We have previously reported that this BCA-fungicide integration, with respect to treatments applied separately, provided a more efficient control of *B. cinerea* on apples (Lima et al., 2006). The worse performance of isolate LS28 combined with TBZ recorded in this study can be explained by both the higher virulence of *P. expansum* on apples with respect to *B. cinerea* (Jones and Aldwinckle, 1990) and by the high degree of resistance to TBZ of isolate FS7 of *P. expansum*, the challenging pathogen used in our experiments.

Chemical analyses of apples at the end of the experiments showed that the most effective integrated treatments (i.e. BCAs plus BOSC or CYPR) achieved in fruit a level of fungicide residues consistently lower (about 3–4 times lower) with respect to residues recovered in fruit treated with CYPR or BOSC at full dosage (Fig. 2A and B).

With the exception of BOSC applied at full dosage, fungicide residues were abundantly below the maximum residue limit (MRL) established for CYPR and BOSC (MRL = 1000 $\mu g\,kg^{-1}$ for both fungicides). Interestingly, the integrated treatment based on the BCA LS11 resulted in lower levels of fungicide residues, as compared with residues found in control apples treated with the fungicides alone at low dosage. These results are worthy of further studies aimed at assessing the possible capability of LS11 to degrade the fungicides used in this study. In our previous research we found that isolate LS11 can resist in the presence of a toxic compound such as patulin, and this resistance could rely on the yeast ability to degrade this mycotoxin (Castoria et al., 2005, 2007).

Integrated treatments with BOSC and CYPR also achieved significant lower levels of PAT contamination as compared to the untreated control (Fig. 2C). For apple-derived products, the European Community (EC) has set the highest tolerable levels of PAT in the range from 10 to 50 $\mu g\,kg^{-1}$, depending on foodstuffs (solid apple products, fruit juices, fermented drinks, etc.) and population group (adults, babies or infants) (EC Reg., 1881/2006). In our research, values of PAT in untreated control apples (water + *P. expansum*) and other ineffective treatments (i.e. the fungicides TBZ and BOSC alone) were consistently higher (around from 18000 to 24000 $\mu g\,kg^{-1}$) and considerably above the maximum tolerable level. These very high levels of PAT are due to the drastic experimental conditions used in this work, i.e. artificial pathogen inoculation, wide size of fruit wounds and fruit keeping at room temperature, all of which are very conducive to high levels of infection and PAT contamination (Morales et al., 2010). The high level of PAT in apples treated with TBZ and BOSC individually applied is in agreement with the lower activity against blue mould displayed by these fungicides with respect to the most effective fungicide CYPR. These results partially disagree with those reported by Morales et al. (2007). Although a significant reduction of blue mould rot was recorded, they found that the application of some fungicides on PAT accumulation in apples had no effect. On the other hand, our results showed that CYPR not only was highly effective against Penicillium rot, but also kept PAT accumulation low in fruit.

Nevertheless, even under the drastic conditions used in our experiments, the best integrated treatments (i.e. LS11 with BOSC, and LS28 with either BOSC or CYPR) were highly effective against *P. expansum*, since they led to a dramatic reduction of both rot incidence and PAT contamination; in particular, in apples subjected to integrated treatments with CYPR and BOSC, PAT contamination was from 78% to 100% lower than in those of the untreated control.

In previous reports (Castoria et al., 2005) we showed that the biocontrol yeast LS11 determined a significant reduction of PAT accumulation in apples pretreated with this BCA and inoculated with *P. expansum*. Conversely, in this work, the utilization of isolate LS11 as a stand-alone treatment did not achieve a significant reduction of PAT contamination on apples. This may be due to the lower number of cells of the biocontrol yeasts used in our experiments (5×10^6 cfu mL^{-1} vs 10^8 cfu mL^{-1}) and/or to the different apple cultivars that were used in the two studies.

Recently, reduced sensitivity to CYPR and BOSC in the population of some fungal pathogens on crops treated with these fungicides has been reported (Babij et al., 2000; Avenot et al., 2008). Our work shows that the integrated strategy based on BCAs combined with BOSC or CYPR can control isolates of *P. expansum* that are sensitive or resistant to TBZ more efficiently with respect to the chemical control applied alone. At the same time, this type of integrated strategy may also reduce the risk of the onset of pathogen strains toward more recent fungicides such as BOSC or CYPR, since control also relies on the different and multifaceted modes of action that are involved in the protective activity of biocontrol yeasts such as LS11 and LS28: competition for space and nutrients and wound competence based on resistance to oxidative stress caused by reactive oxygen species generated in fruit wounds (Castoria et al., 2003) as well as production of lytic enzymes (Castoria et al., 1997). These mechanisms are complex and make it unlikely for a fungal pathogen to develop resistance.

In conclusion, the integration of the selected biocontrol yeasts isolates *R. kratochvilovae* LS11 and *C. laurentii* LS28 with low dosages of the recently developed fungicides BOSC and CYPR appears to be a very promising method to control blue mould and keep low both fungicide residues and patulin accumulation in apples. Further investigations are needed to test this integrated strategy under large-scale conditions.

Acknowledgements

This work was funded by MIUR (Italian Ministry of Education, University and Scientific Research) – Project PRIN 2006072204_001, 2007–2009: Innovative systems aimed at reducing patulin and fungicide contamination in pome fruit and pome fruit-based products.

We are sincerely grateful to both Dr Alex Idnurm at the University of Missouri-Kansas City, USA for sequencing the ITS region of strain LS11, and Dr José Paulo Sampaio at the University of Lisbon, Portugal for his taxonomic studies on LS11, since these investigations allowed us to re-classify *Rhodotorula glutinis* LS11 as the new species *Rhodosporidium kratochvilovae*. We also thank Prof. Paolo Bertolini at the CRIOF (*Centro per la Protezione e Conservazione dei Prodotti Ortofrutticoli*), University of Bologna, Italy for providing us with isolates P32-R and LB8/99-S of *P. expansum*, and Janice Beccaria, from the Faculty of Foreign Languages and Literature at the University of Salento, Lecce, Italy for her valuable assistance in the linguistic revision of the manuscript.

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