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

Giuseppe Lima[a,*], Raffaello Castoria[a], Filippo De Curtis[a], Assunta Raiola[b], Alberto Ritieni[b], Vincenzo De Cicco[a]

[a] Dipartimento di Scienze Animali, Vegetali e dell'Ambiente, Università del Molise, Via F. De Sanctis, 86100 Campobasso, Italy
[b] Dipartimento di Scienza degli Alimenti, Università di Napoli 'Federico II', Parco Gussone, 80055 Portici, Napoli, Italy

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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

* Corresponding author. Tel.: +39 0874 404659; fax: +39 0874 404855.
E-mail address: lima@unimol.it (G. Lima).

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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 $\mu g\,mL^{-1}$