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Lentinula edodes enhances the biocontrol activity of *Cryptococcus laurentii* against *Penicillium expansum* contamination and patulin production in apple fruits

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ABSTRACT

Penicillium expansum is a post-harvest pathogen of apples which can produce the hazardous mycotoxin patulin. The yeast *Cryptococcus laurentii* (LS28) is a biocontrol agent able to colonize highly oxidative environments such as wounds in apples. In this study culture filtrates of the basidiomycete *Lentinula edodes* (LF23) were used to enhance the biocontrol activity of LS28. *In vitro L. edodes* culture filtrates improved the growth of *C. laurentii* and the activity of its catalase, superoxide dismutase and glutathione peroxidase, which play a key role in oxidant scavenging. In addition, LF23 also delayed *P. expansum* conidia germination. The biocontrol effect of LS28 used together with LF23 in wounded apples improved the inhibition of *P. expansum* growth and patulin production in comparison with LS28 alone, under both experimental and semi-commercial conditions. The biocontrol effect was confirmed by a semi-quantitative PCR analysis set up for monitoring the growth of *P. expansum*.

1. Introduction

Penicillium expansum is the agent of blue mould, the most common form of post-harvest rot of pome fruits as well as of cherries, nectarines and peaches, which causes considerable economic losses worldwide (Pierson et al., 1971; Prusky et al., 1985; Rosenberger, 1990; Xu and Berrie, 2005). Besides its moulding activity, *P. expansum* is also a producer of patulin, a mycotoxin with toxic immunological (Bourdiol and Escoula, 1990; Escoula et al., 1988; Pacoud et al., 1990), neurological (Deveraj et al., 1982; FAO/WHO, 1995) and gastrointestinal (Broom et al., 1944; Ciegler et al., 1976) effects. The use of fruits contaminated with *P. expansum* greatly increases the risk of patulin contamination of fruit juices (Gonzalez-Osnaya et al., 2007; Moss, 1998; Scott et al., 1977), notably apple juices, which are commonly consumed by infants and children.

The control of fungal diseases during the post-harvest storage of fruits is usually based on chemical treatments (Rojas-Grau et al., 2008; Salomao et al., 2008), cold storage, or modified atmospheres (Rojas-Grau et al., 2007).

However, due to the onset of resistance to fungicides by spoilage fungi, the satisfactory control of patulin in apple fruits and their products has not yet been achieved. Moreover, the currently increasing concern for the environment and the demand for healthy food has stimulated a search for alternatives to fungicides in the control of moulding (Wilson and Wisnieswski, 1992; Sharma et al., 2009; Janisiewicz and Korsten, 2002).

Biological control of fruit decay based on the utilisation of microbial antagonists is considered an effective alternative method. Some components of the microbial community present on the surface of fruits and vegetables, such as bacteria and yeasts, have shown significant antagonistic activity against *P. expansum* (Arras et al., 1996; Droby et al., 2003; Droby, 2006; Chand and Spotts;, 1997). Recent studies have highlighted the possible role played by the yeasts *Cryptococcus laurentii* and *Rhodotorula glutinis* in the control of fungal contamination and patulin production by *P. expansum* on apple fruits (Castoria et al., 1997, 2001, 2002, 2003, 2005). It has been demonstrated that *C. laurentii* LS28 is able to rapidly colonize wounds on apple fruits and thereby to limit *P. expansum* growth. The wound environment is characterised by the presence of oxidant stressors (i.e. hydrogen peroxide) which represent part of the plant defence response to microbial attack. Nevertheless, even in this stressful environment *C. laurentii* LS28 is able to grow rapidly, probably due to its high resistance to the oxidative species present in the wound. This yeast's resistance to oxidative stress is likely to be mainly due to superoxide dismutase (SOD) and catalase (CAT) activity reported in this strain (Castoria et al., 2003). For these reasons, *Cryptococcus laurentii* and *Rhodotorula glutinis* could be used as biocontrol agents of post-harvest pathogens.

However some authors reported that *C. laurentii* cannot always provide satisfactory levels of decay control when used alone. They

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therefore evaluated the effects of compounds such as indole-3-acetic acid (IAA), chitosan or antioxidant compounds on the biocontrol efficacy of the yeast antagonist *C. laurentii* against blue mold rot caused by *P. expansum* in fruits (Yu et al., 2007, 2009; Sharma et al, 2009). In order to further develop this line of research, we evaluated the effect of combining *C. laurentii* with an extract of the basidiomycete *Lentinula edodes* as a new tool for the control of apple decay.

The induction of mycotoxin production by an oxidative environment has been reported for several post-harvest fungi and, furthermore, it has been widely demonstrated that certain oxidants are able to modulate and trigger the biosynthesis of mycotoxins by such fungi (i.e. *Aspergillus flavus*, *A. parasiticus* and *A. ochraceus*) (Reverberi et al., 2008). As a consequence, natural antioxidants extracted from various plants and fungi have recently been used as novel compounds in the battle against post-harvest development of fungi and production of mycotoxins (i.e. aflatoxins, ochratoxin A) (Reverberi et al., 2005; Ricelli et al., 2002; Zjalic et al., 2006a). Indeed, it has been shown that culture filtrates from basidiomycetes such as *Lentinula edodes* or *Trametes versicolor* can significantly inhibit aflatoxin biosynthesis by *Aspergillus parasiticus* and *A. flavus*, in both *in vitro* and *in vivo* conditions. This control of aflatoxin production by *L. edodes* or *T. versicolor* extracts is linked to their high content of β-glucans and glycoproteins (Reverberi et al., 2005; Zjalic et al., 2006b). In fact, the efficacy of these extracts is due, on the one hand, to the presence of compounds with intrinsic antioxidant activity like β-glucans and glycoproteins, (Slamenova et al., 2003) and. on the other hand, to the stimulation of the antioxidant system of the toxigenic fungi (Reverberi et al., 2005; Zjalic et al., 2006b). It would therefore appear that it is possible to obtain, in a low cost and environmentally friendly way, natural compounds from edible mushrooms which are capable of enhancing the antioxidant properties of treated cells.

The aim of this study was to investigate the influence of *L. edodes* extracts on the control activity of *C. laurentii* against *P. expansum* contamination and patulin biosynthesis in apple fruits in order to improve the biocontrol activity of *C laurentii* (LS28) using a safe, environmental friendly and food grade product. The growth of *P. expansum* was estimated by a semi-quantitative PCR method based on species specific primers which enables the toxigenic fungus to be detected in apples, even when it is in the presence of other microrganims, such as biocontrol agents. Early detection could be just as crucial for ensuring microbiological quality and safety of fruits and juices as is the optimization of preventive strategies, such as good agricultural and industrial practices and the use of biocontrol agents. A preliminary assay under semi-commercial conditions (storage of apple fruits at 4 °C for 40 days) was also carried out to give some indication of the effectiveness and stability of the proposed combination.

2. Material and methods

2.1. Fungal strains

C. laurentii (Kufferath) Skinner (LS28), kindly provided by Department of Animal, Plant and Environmental Science, University of Molise, was originally isolated from apples cv. Annurca collected from local markets in Molise (Italy). This yeast was selected for its protective activity against various post harvest pathogens on different crops (Lima et al., 1998). *C. laurentii* LS28 was maintained at 4 °C on Nutrient Yeast Extract Dextrose Agar (NYDA, DIFCO) before use. Yeast cells were inoculated (10^5 cells/100 µl sterile distilled water) in 50 ml of NYDB, DIFCO and incubated in shaken conditions (120 rpm) at 25 °C in the dark for 48 h.

Lentinula edodes (Berk.) Pegler (LF23), obtained from the collection of the Department of Plant Biology, University "Sapienza", Rome, was kept at 4 °C on Potato Dextrose Agar (PDA, DIFCO) before use. Four discs (1 cm diameter) of LF23 cultured on PDA were inoculated in 500 ml of Potato Dextrose Broth (PDB, DIFCO) and incubated in shaken conditions (100 rpm) at 25 °C for 28 days. The mycelium was separated from culture medium by filtration and the culture filtrate was frozen and lyophilised ($T = -40$ °C; $p = 0.02$–0.03 mbar).

2.2. Isolation of P. expansum from apples

Penicillium expansum Link, patulin producer was isolated from the apple surface (cv. Golden delicious). Apples were superficially washed with sterile distilled water and Triton X100 (0.01% w/v) to collect the surface fungal microflora. Serial dilutions of the mixture were plated on Potato Dextrose Agar (PDA) in Petri dishes (ø 9 cm) in presence of streptomycin (300 ppm) and neomycin (150 ppm) and incubated at 25 °C for 7 days. After the development of fungal colonies, *P. expansum* was isolated in pure culture in PDA medium, incubated at 25 °C for 15 days and identified by both morphological determination following the classical procedure (Pitt and Hocking, 1985) and by molecular identification. Conidia (10^5/100 µl sterile distilled water) from the isolated fungus were inoculated in 50 ml of PDB and incubated at 25 °C for 15 days. The mycelium was recovered, frozen and lyophilised ($T = -32$ °C; $p = 0.02$–0.03 mbar).

2.3. Plant material

Apples cv. Golden Delicious were used in all the experiments. Fruits, obtained from organic agriculture, were kindly provided by Centro di Ricerca per la Frutticoltura (Ciampino-Rome).

2.4. Effect of LF23 on the conidia germination of P. expansum

The effect of lyophilised culture filtrate from LF23 (2% w/v) was assayed on conidia germination of *P. expansum*. 1×10^6 conidia of *P. expansum* were inoculated in 5 ml PDB with or without (control) LF23 and incubated at 25 °C for 40 h. Conidia germination was scored by the mean of microscope analysis at different time intervals (8, 16, 20, 24, 28, 32 and 40 h).

2.5. Effect of LF23 on the growth and the antioxidant enzyme activities of LS28

LS28 was inoculated (10^5 cells/100 µl) in 50 ml of NYDB with or without (control) 2% w/v of LF23 lyophilised culture filtrates and the cultures were incubated in shaken conditions (150 rpm) at 25 °C for 48 h. Yeast growth was evaluated by measuring the absorbance value of cultures by spectrophotometer ($\lambda = 600$ nm) after 16, 18, 20, 22, 24, 36, 48 h from inoculum. In order to analyse intracellular enzymatic activity yeast cells were recovered, in the same time intervals as above, by centrifugation at 5000 rpm for 15 min at 4 °C (Spellman et al., 1998). The collected cells were then suspended in 1 ml of lysis buffer (PBS), vortexed for 1 minute in the presence of glass beads ($Ø = 106$ µm) in order to break the cell walls and centrifuged at 4000 rpm for 15 min at 4 °C. The activities of some antioxidant enzymes, such as SOD, CAT and glutathione peroxidase (GPX) were analysed as previously described (Reverberi et al., 2005). The same extraction and analytical procedures were used for evaluating the activities of SOD, CAT and GPX into *P. expansum* and LF23 mycelia.

2.6. Apple inoculation

Four wounds (ø 3 mm × 3 mm) were made on the surface of apple fruits (for each treatment 5 apples cv. Golden Delicious, 20 wounds, were used), previously surface-disinfected with 2% v/v sodium hypochlorite, rinsed 3 times with sterile distilled water and dried with sterile paper. Wounds were treated with 30 µl of water suspension containing 10^6 cells/ml of LS28, or with 30 µl of 2% w/v water suspension of lyophilised culture filtrates of LF23, or with 30 µl

of 2% w/v water suspension of LF23 containing 10^6 cells/ml of LS28. After 2 h the same wounds were also inoculated with 15 µl of water suspension containing 10^4 conidia of *P. expansum*. Untreated wounds represented the internal control. Apples were incubated in the dark for 6, 12, 24, 48, 72, 96, 144 h at 25 °C and 90% of relative humidity.

In order to evaluate the antagonistic activity of LS28 and LF23 on *in vivo* mould extension and patulin production in semi-commercial conditions 5 apples, inoculated as previously described, were incubated in dark conditions at 4 °C and 90% RH for 40 days. The apples were stored in a commercially available plastic box. After 40 days the apples were incubated at 25 °C for 3 and 6 days and then analysed.

2.7. Assay of biocontrol activity of LS28 and LF23

In order to evaluate the antagonistic activity of LS28 and LF23 *in vivo*, the growth of *P. expansum* and its patulin production on apples were quantified up to 6 days after inoculation.

Mould extension was evaluated by measuring rot diameter (mm), the inhibitory activity (I.A.) was calculated by the equation reported by Lima et al. (1999):

$$\text{Inhibitory Activity} = \frac{\text{fungal growth in the control–fungal growth in the treatment}}{\text{fungal growth in the control}} \times 100$$

For patulin assay, cylinders (15×10 mm) of apple tissue were recovered from each wound by a sterile borer, homogenized into a mortar and centrifuged at 13,000 rpm for 30 min at room temperature. The supernatant was recovered, filtered through a 0.45 µm filter and 20 µl of the sample were injected into HPLC 1100 (Agilent) equipped with a Synergy Hydro C18 column (4.6×250 mm) with a pre-column of the same material, as previously described (Ricelli et al., 2007).

2.8. DNA extraction

Genomic DNA of fungi in pure culture was extracted from 50 mg of lyophilized mycelium with TRIS-SDS lysis buffer with slight modifications (Marek et al., 2003). Apple wounds (15×10 mm) were recovered with a sterile borer, lyophilized and DNA was extracted from 100 mg of tissue with the same method described below. The samples were incubated with extraction buffer for 60 min at 65 °C overnight. After incubation, samples were put in ice for 10 min and centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was collected in a 2 ml tube and 3/10 volume of sodium acetate 4 M was added. This solution was placed on ice for 30 min and centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatant was transferred, extracted with phenol-chloroform-isoamylic alcohol (25:24:1) and precipitated by adding 0.5 volume of cold 2-propanol.

2.9. DNA amplification

Species-specific primers (P*epg1_for* 5′-GGT AAA AAC TCC CTC CAA ACC-3′, P*epg1*_rev 5′-GAA ACG GGA AAA CTT AGT CAT TA-3′) were designed on the basis of the consensus conserved sequence of the P*epg1* gene of *P. expansum* (NCBI GeneBank accession number AF047713), which encodes for a polygalacturonase enzyme responsible for fruit tissue rot. Primers P*epg1* used in PCR amplified a 747 bp DNA fragment.

The PCR was carried out in 25 µl reaction mixture by using 100 ng of DNA extracted from fungus or 250 ng of DNA extracted from apple. All reagents were provided by Sigma-Aldrich, USA. The amplification was carried out in an Eppendorf Mastercycler. Optimal PCR conditions: 94 °C for 3 min, 94 °C for 45 s, 65 °C for 45 s, 72 °C for 1 min (steps 2 to 4 repeated for 32 cycles), 72 °C for 8 min. In order to obtain a semi-quantitative value of the amount of DNA amplified by PCR, the software UVI doc was used to correlate fluorescence intensity of fragment's signals to known DNA amount.

A test of the method sensitivity with serial dilutions (range 0.02 pg–2 µg) of fungal DNA with P*epg1* primers was carried out. The relative luminescence intensity of the different quantity of fungal genomic DNA was quantified by using the software UVI-Doc Mw Version 10.01 and these data were used to generate a relative luminescence intensity standard curve (semi-quantitative analysis). The amplification of *P. expansum* DNA with P*epg1* primers in a 0.02 pg–2 µg range was carried out. The results show that the sensitivity was 5 pg/µl when P*epg1* primers were used on fungal DNA derived from *in vitro* culture and it was 25 pg/µl if DNA was extracted from apples contaminated with *P. expansum* (treated or untreated with the biocontrol agents). The regression curves generated with the different relative luminescence intensity values showed a positive and good correlation ($R^2 = 0.99$) between intensity and DNA amount and this was expressed by the function {Intensity = 0,133 * ln(DNA) + 0.28}. This curve was then used as a reference standard for extrapolating quantitative information for DNA targets of unknown concentrations. PCR amplification reactions were carried out in triplicate from 3 independent experiments.

3. Statistical analysis

All the data presented are the mean value (±SE) of three determinations from three separate experiments. In all experiments, mean values were compared using Student's *t* test.

4. Results

4.1. Effect of LF23 on growth and antioxidant enzyme activities of C. laurentii

The effect of LF23 (2% w/v) on growth and antioxidant enzyme activities of LS28 inoculated in synthetic liquid medium, (NYDB), was assayed in order to evaluate the possible use of these filtrates to increase yeast antagonistic activity in wounded apples. The use of LF23 led to a stimulating effect on the growth of yeast cells for a period up to 25 h of incubation (LS28: 0.33 ± 0.02 OD_{600} vs. LS28 ± LF23: 0.46 ± 0.05 OD_{600}), then at the end of the incubation period (48 h) yeast cell number became similar in treated and untreated samples (data not shown).

The antioxidant enzyme activities (SOD at pH 7.8 and 10.0, CAT and GPX) were significantly higher ($p<0.01$) in the yeast cells treated with LF23 up to 20 h. From 22 to 48 h only the activity of SODs was higher in the sample treated with LF23 compared with the untreated ones (Fig. 1).

4.2. Effect of LF23 on the germination of P. expansum conidia

The effect of LF23 on the germination of *P. expansum* conidia was assayed by adding these extracts to the fungal cultures at the same concentration used in all the experiments (2% w/v). LF23 completely inhibited fungal conidia germination up to 16 h of incubation (control: 46% vs. LF23:0%), then the germination process was significantly delayed in comparison with untreated samples until 32 h of incubation (control: 97% vs. LF23: 75%).

4.3. Effect of LF23 on antioxidant enzymes activities of P. expansum

The activity of SOD at pH 7.8 and 10.0, CAT and GPX was assayed after different incubation periods in *P. expansum* mycelia grown in PDB at 25 °C up to 7 days (Fig. 2). The activity of CAT and GPX was significantly higher during all the experiments in the mycelia treated with LF23, whereas the activity of SOD in the treated mycelia was not stimulated during the experiment (data not shown). In the samples

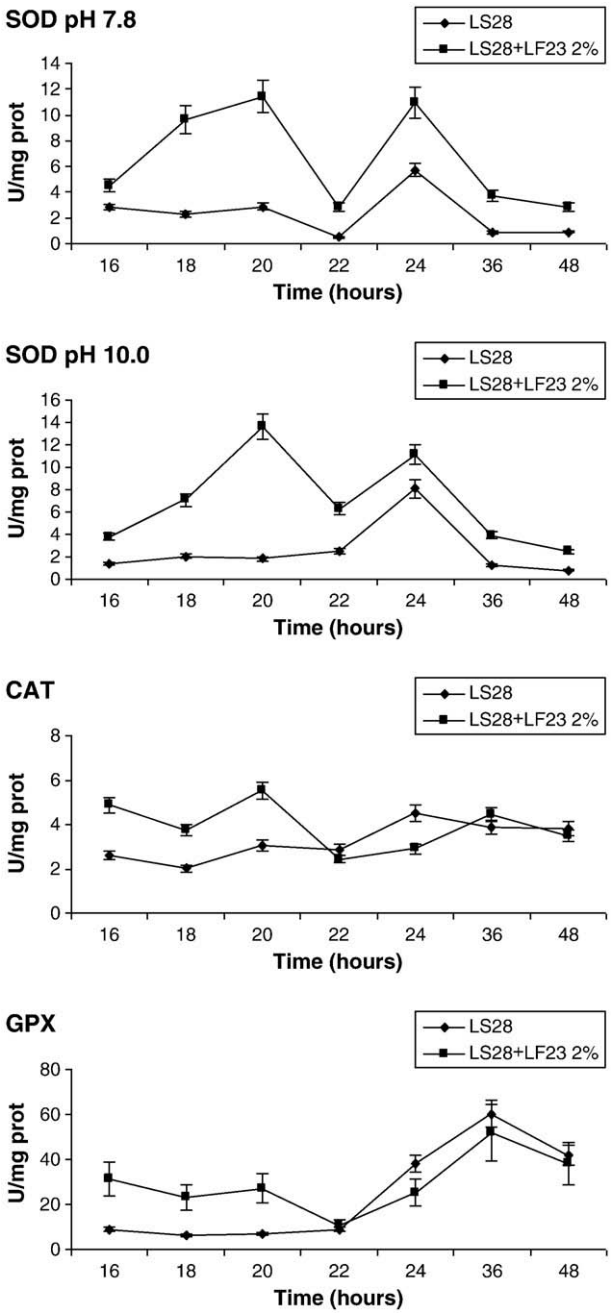

Fig. 1. Influence of LF23 (2% w/v) on antioxidant enzyme activities Superoxide dismutase (SOD) pH 7.8 and 10.0; Catalase (CAT) and Glutathione peroxidase (GPX) of *C. laurentii* (LS28) grown in liquid synthetic medium (PDB) for different periods at 25 °C. Data represent the mean of 3 independent replicates ± SE.

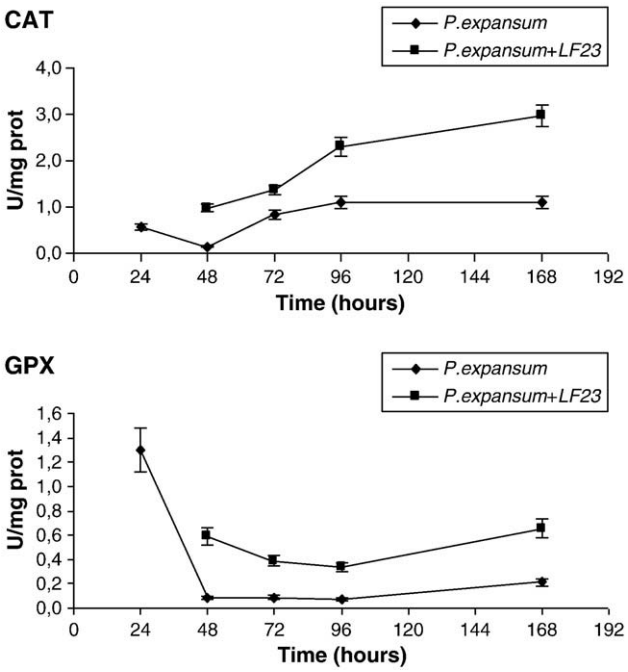

Fig. 2. Influence of LF23 (2% w/v) on antioxidant enzyme activities of *P. expansum* grown in liquid synthetic medium (PDB) for different periods at 25 °C. Catalase (CAT) activity and Glutathione Peroxidase (GPX) activity. Data represent the mean of 3 independent replicates ± SE.

treated with LF23 the first assay was performed after 48 h instead of 24 h, since the inhibiting effect on conidia germination and thus on mycelial growth occurred during the first incubation period, as already reported (data not shown).

4.4. Biocontrol activity of LS28 in the presence and in the absence of LF23 in wounded apples

Rot severity was measured 6 days after inoculation of wounded apples with *P. expansum* and the results indicated different effectiveness of the treatments (Fig. 3). Treatment with biocontrol agent LS28 led to inhibition of 85% of rot extension, while treatment with LF23 alone showed an inhibiting effect of 25% (Fig. 3). When wounded apples were treated with both LS28 and LF23, rot inhibition was significantly ($P<0.05$) increased, achieving 100% inhibition in controlling blue mould. These results suggest that *P. expansum* growth could be completely inhibited by this treatment of apple wounds under these experimental conditions (Table 1).

When the apples stored for 40 days at 4 °C were incubated at 25 °C after inoculum with *P. expansum* and treatment with LS23 and LS28, rotting appeared earlier (after 3 days), but after 6 days the rot severity measured was similar (data not shown) to the results obtained without the cold storage step. The outcome of this experiment suggests that low temperature storage did not significantly influence the growth either of the pathogen or of the biocontrol yeast.

4.5. Monitoring of P. expansum grown on inoculated apples by PCR

To give a rough indication of *P. expansum* growth on apples in the presence and in the absence of the different biocontrol agents, specific primers (P*epg1*), designed on the polygalacturonase (PG) encoding gene, were used for a semi-quantitative PCR amplification. First, the growth of *P.* expansum on untreated apples was analysed by PCR in the time interval 6 h up to 6 days. In Fig. 4a the PCR amplification results *per* time and the respective visual analysis of rot

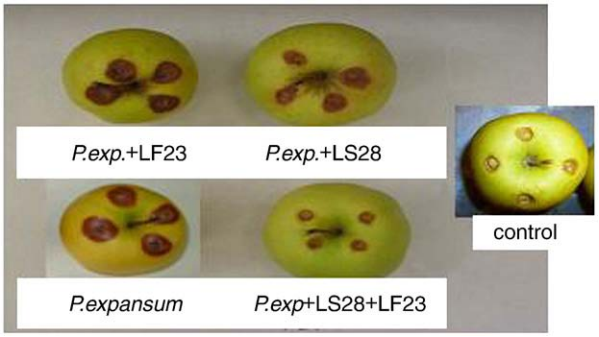

Fig. 3. Different inhibitory activities of LS28 and LF23 (2% w/v) on rotting due to *P. expansum* inoculated on wounded apples after 6 days of incubation at 25 °C.

Table 1
P. expansum rotting (lesion diameter, mm) on apples and inhibitory activity of rotting by LS28 and LF23, alone or in combination. Data represent the mean of lesion diameters ± SE.

	Lesion diameter (mm)	Inhibitory Activity (%)
P. expansum	12.0 ± 0.85	0
P. expansum + LF23	9.0 ± 0.61	25
P. expansum + LS28	1.8 ± 0.72	85
P. expansum + LF23 + LS28	0.1 ± 0.01	100
Untreated wounded apple	0.2 ± 0.01	0

development are shown. Second, the amplification was performed using DNA extracted from apples artificially inoculated with the pathogen, yeast and LF23 and incubated for 6 days at 25 °C (Fig. 4b). The results, obtained through UVIdoc software quantification, confirmed the differences in rot extension on apple fruits previously observed in Fig. 4a–b. In fact, the amplification signal of the sample *P. expansum* + LS28 was less evident than both control (*P. expansum* alone in the wounds) and *P. expansum* + LF23 sample, whereas no visible amplification was produced by *P. expansum* + LS28 + LF23 sample. The match of the fluorescence values registered through the UVIdoc system with the standard curve obtained by DNA extracted from the contaminated matrix lead to a rough quantification of the *P. expansum* DNA present in the contaminated apples in the presence or in the absence of the different biocontrol agents. The quantity of DNA of *P. expansum* after 6 days of incubation was 0.54 ± 0.005, 0.045 ± 0.012 and 0.036 ± 0.015 ng/mg apple respectively, in the contaminated apples in the absence of biocontrol agents, in the presence of LF23, and in the presence of LS28. Using this same approach on the *P. expansum* + LF23 + LS28 sample, no pathogen was detected.

4.6. Patulin assay

The data concerning patulin accumulation in wounded apples contaminated with *P. expansum* and treated or untreated with biocontrol agent LS28 and with LF23 are showed in Fig. 5. It was evident that, after 6 days of incubation at 25 °C, LS28 significantly inhibited patulin production by 80% (0.08 ± 0.01 ng/mg) in comparison with the control inoculated with *P. expansum* (0.41 ± 0.14 ng/mg). On the other hand, LF23 did not significantly control patulin production, even if the treatment with LF23 reduced patulin accumulation by 54% (0.224 ± 0.09 ng/mg). When apple wounds were treated with LS28 + LF23 the inhibiting effect on patulin production was significantly enhanced (about 99%, 0.004 ng/mg ± 0.001).

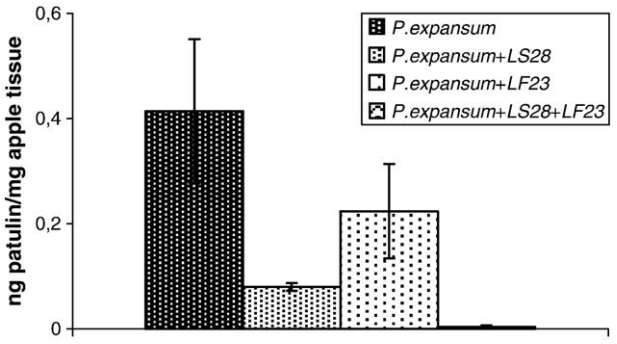

Fig. 5. Patulin accumulation, after 6 days of incubation at 25 °C, in wounded apples inoculated with *P. expansum* conidia, treated with biocontrol agent LS28 and LF23 (2% w/v), alone and in combination. Data represent the mean of 3 replicates ± SE.

Under semi-commercial conditions no patulin was detected after the 40 days-storage at 4 °C. Nevertheless, when the *P. expansum*-contaminated apples were brought to 25 °C, patulin was already detected 3 days after the start of incubation (0.35 ng/mg apple tissue) and after 6 days the quantity of the toxin was similar (0.47 ng/mg apple tissue) to that produced in the apples not stored at 4 °C. All samples treated with the biocontrol agents showed a large inhibiting effect on patulin biosynthesis (45%, 77% and 99% in LF23, LS28 and LS28 + LF23-treated apples respectively); including those which were cold-stored and then incubated at 25 °C for 6 days.

5. Discussion

Previous studies have provided evidences that *L. edodes* culture filtrates display a significant effect in the control of some mycotoxins whose biosynthesis is related to oxidative stress (Reverberi et al., 2005). The biocontrol effect of the culture filtrates of this basidiomycete is exerted by the antioxidant activity of some of the compounds, mainly polysaccharides such as β-glucans, present in the filtrates. These compounds demonstrated both an antioxidant activity *per se* and an ability to stimulate the activity of SOD, CAT and GPX of aflatoxin producer fungi like *Aspergillus parasiticus*. In particular, SOD was assayed

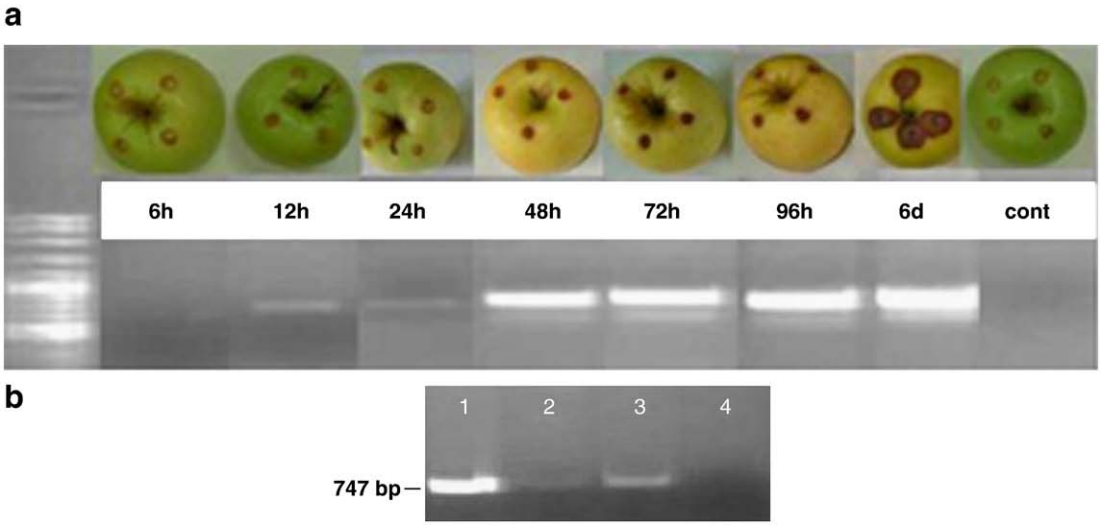

Fig. 4. a) Agarose gel electrophoresis of PCR products from *P. expansum* extracted from apples at different times of incubation; b) Agarose gel electrophoresis of PCR products from *P. expansum* extracted from apples (lane 1) inoculated with biocontrol agent *Cryptococcus laurentii* (lane 2), LF23 (lane 3) or both (lane 4) at 6 days after infection. The images are representative of 5 repetitions for each experiment.

at pH 7.8 and 10.0 to investigate its activity in both the cytoplasm and peroxisome. To strengthen the capacity of scavenging the reactive species inside the fungal cell leads, in turn, to the inhibition of some oxidative-stress-related mycotoxins such as aflatoxins (Reverberi et al., 2008). A direct correlation between oxidative stress and patulin production by *P. expansum* has not yet been demonstrated and only one study reports the inhibiting effect exerted by phytoalexins like quercetin and, to a lesser extent, resveratrol, against patulin biosynthesis but without considering the antioxidant properties of the phytoalexins assayed (Sanzani, 2007). Another study (Mossini et al., 2004) reports the inhibiting effect of *Azadiracta indica* leaf extracts on the growth and patulin production of a *P. expansum* strain without making any mention of a correlation between antioxidants and patulin inhibition. The results reported in our study suggest a correlation between oxidative stress and patulin production. In fact a mean inhibition of 50% in patulin biosynthesis by *P. expansum* treated with LF23 was obtained in apple fruits. This result seems to be in accordance with the CAT and GPX stimulation carried out by LF23 on *P. expansum* mycelium *in vitro*.

The wound environment is characterised by a marked presence of Reactive Oxygen Species (ROS), in fact during wounding the plant activates several oxidising enzymes such as peroxidases and lipoxygenases. The ROS formed during the wounding process are necessary both for reinforcement of the cell wall and for preventing pathogen infections, however an excess of ROS during this period can promote fungal infection and the biosynthesis of patulin. Thus the use of biocontrol agents in the apple wound for controlling soft rot agents such as *P. expansum* needs to take into account the agent's ability to grow in such a hostile environment. As a matter of fact the competitiveness of *C. laurentii* as a biocontrol agent in apple wounds is correlated to its levels of SOD and CAT production, as it is which enable the yeast to resist oxidative stress (Castoria et al., 2003, 2005). The metabolic requirement of resistance to a heavily oxidised environment can represent a limiting factor for a potential biocontrol agent. *Rhodotorula glutinis*, for example, despite its ability to metabolize patulin, cannot be proposed as an effective biocontrol agent on fruits due to its poor ability to grow in a highly oxidized environment (Castoria et al., 2005).

In this paper we have described the role of *L. edodes* culture filtrates in reinforcing the competitiveness of the biocontrol agent *C. laurentii* through the enhancement of its antioxidative potential. Various authors are currently studying a strategy to improve the biocontrol ability of *C. laurentii.* However the reported studies make recourse to the use of chemical compounds such as silicates or indole acetic acid which promote plant defence response to stress (Yu and Dong Zheng, 2007). Here we propose a novel strategy of biocontrol using agents capable of resisting or inhibiting the oxidants present in the wound This strategy involves boosting the biocontrol activity of *C. laurentii* by complementing it with LF23 extract. The role of LF23 extract consists of enhancing the antioxidant enzyme activity of the yeast colonising the apple wounds and controlling patulin biosynthesis by promoting the antioxidant activity of *P. expansum*. In fact, it has been reported that one of the main reasons for the still limited use of biocontrol strategies in post harvest prevention is that most of the potential biocontrol agents are not able to exert sufficient control of post-harvest diseases when used alone (Janisiewicz and Korsten, 2002; Droby et al., 2003). Our study has demonstrated that in wounded apples treated with *C. laurentii* and *L. edodes* culture filtrates, an almost complete control of rotting can be achieved during 6 days of incubation at 25 °C. Moreover, in samples treated with the biocontrol yeast and *L. edodes* the presence of patulin was significantly inhibited in comparison with the samples treated with *C. laurentii* alone. This control of rotting was due to the inhibition of *P. expansum* development and this is confirmed by the results obtained with *PG1* semi-quantitative PCR for monitoring fungal growth. The biocontrol agents also showed promising results when tested on apples after 40 days of cold storage inhibiting apple rot and patulin biosynthesis by *P. expansum*.

The results obtained might appear to suggest that *L. edodes* lyophilised filtrates could also be considered a biocontrol agent, since they promote a significant delay in the conidia germination of the pathogen *P. expansum* and an inhibition of patulin production. Nevertheless, the performance of LF23 in the control of patulin in our experiments was not sufficient to ensure a significant effect when used alone but it did prove itself very useful as an "enhancer". The use of LF23 together with *C. laurentii* improved the efficiency of the biocontrol activity of *the yeast* leading to an almost total control of *P. expansum* growth and patulin production and promoting a significant increase of the growth rate of the biocontrol yeast. These observations are in agreement with the study of Droby et al. (2003), who found that there was a direct relationship between the concentration of the antagonist and the induction of biocontrol ability. LF23 could be considered a beneficial additive, able to enhance the biocontrol activity of other microrganisms which are not as well structured as *C. laurentii* in their antioxidant asset. In particular this agent could represent a reinforcement of the enzymatic antioxidant potential of yeasts like *Rhodotorula glutinis* (Castoria et al., 2005) which are able to prevent patulin biosynthesis or to degrade it but are less competitive in highly oxidative environments.

This study demonstrates that the use of culture filtrates from the edible mushroom *L. edodes,* can greatly improve the biocontrol activity exerted by *C. laurentii* and can also contribute directly to the control of patulin contamination. Moreover, LF23 extracts are not only non toxic for human health but better still, they can have a positive healthy effect as reported by several authors (Wasser and Weis, 1999; Xu, 2001; Zjalic et al., 2008). The edible mushroom *L. edodes* could have a useful role in the formulation of a commercial product for rot disease and patulin control in apple fruits.

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