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Antioxidant enzymes stimulation in *Aspergillus parasiticus* by *Lentinula edodes* inhibits aflatoxin production

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Abstract Biosynthesis of aflatoxins, toxic metabolites produced by *Aspergillus parasiticus*, is correlated to the fungal oxidative stress and cell ageing. In this paper, the mechanism underlying the aflatoxin-inhibiting effect of the *Lentinula edodes* culture filtrates was studied by analysing their anti-oxidant activity and β -glucan content. Mushroom β -glucans are pharmacologically active compounds stimulating anti-oxidant responses in animal cells. *L. edodes* lyophilised filtrates stimulate *A. parasiticus* anti-oxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) and aflatoxin inhibition was better correlated with β -glucan content than with anti-oxidant activity of the filtrates. RT-PCR analyses on treated mycelia showed a delay in the activation of *aflR*, and *norA*, genes of aflatoxin cluster and a synchronous activation of *hsf2*-like, a homologue of a yeast transcription factor involved in oxidative stress responses. The first evidence of *hsf2*-like in *A. parasiticus* and its activation during aflatoxin biosynthesis is reported. *L. edodes* filtrates could play a role as external stimulus affecting the anti-oxidant status in the fungal cell that, in turn, leads to aflatoxin inhibition. In the fungal cell, β -glucans present in the filtrates could stimulate the activation of transcription factors related to anti-oxidant response and anti-oxidant enzyme activity with a contemporaneous delay of aflatoxin genes transcription, which led to a marked reduction of aflatoxin production. This research suggests new perspectives to set suitable strategies against aflatoxins and *L. edodes* could be considered a promising tool.

Introduction

Some mushrooms, such as *Lentinula edodes*, *Trametes versicolor*, *Ganoderma lucidum* and *Schizophyllum commune*, can be useful in the treatment of various human pathologies (Yuan et al. 1996; Wasser and Weis 1999; Fisher and Yang 2002). Fungal glucans are pharmacologically classified as biological response modifiers (BRM) and most immunomodulator β -1,3-glucans have been isolated from basidiomycota (Bohn and Bemiller 1995; Yadomae and Ohno 1996). *L. edodes* extracts, in particular polysaccharides such as lentinan, and other β -glucans are involved in the strengthening of the immune system with beneficial effects on human health (Wasser and Weis 1999). Baptista et al. (2004) demonstrated that yeast manno-oligosaccharides are able to attenuate aflatoxicosis and Slamenova et al. (2003) found that fungal β -1,3-glucans can protect DNA from damage due to oxidative stress. Furthermore, the presence of compounds with anti-oxidant activity, such as mannitol and thioproline (Kurashima et al. 1990), was detected in the mycelium and fruit bodies of these mushrooms (Mau et al. 2001; Sun et al. 2004).

Aspergillus parasiticus (Speare) and *A. flavus* (Link) are widespread fungi which contaminate food commodities through aflatoxins. These toxins enter the human food chain and, when consumed, induce acute health problems or chronic toxicity. Amongst their most serious effects on human and animal health are induction of liver cancer and suppression of the immune system (Jackson and Groopman 1999).

Methods to control aflatoxins are mainly based on chemical strategy (pesticides and fungicides). However, excessive use of chemical treatments has many undesirable consequences: (1) marked pollution of the environment, (2) increase in resistant pathogen populations and (3) presence of chemical residues in food commodities. Thus several research groups are now considering "light" or "natural" food grade products with a high efficacy in the inhibition of mycotoxin production but with a low impact on the environment and on human health (Golob 2002).

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In previous studies, we found that aflatoxin production was closely related to the peroxidation of the fungal cell membranes both in vitro (synthetic media) and in vivo (starchy and oily seeds). The induction of unsaturated lipids peroxidation of the fungal cell membranes by oxidising agents (as halogenomethanes) enhanced aflatoxin production in *A. parasiticus* and *A. flavus* (Fanelli et al. 1984; Fanelli and Fabbri 1989). Some anti-oxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) or cysteamine, are able to inhibit aflatoxin stimulation (Fanelli et al. 1985). A few studies also reported the inhibiting effect of an isolate of *L. edodes* on aflatoxin production (Fanelli et al. 2000). More recently, a study (Jayashree and Subramanyam 2000) further underlined the importance of the role played by oxidative stress in the fungal cell in aflatoxin biosynthesis. Moreover, in the seed/fungus interaction model, some authors (Tsitsigiannis et al. 2001) have highlighted the importance of 9- and 13-linoleic acid hydroperoxides in the modulation of aflatoxin production by *Aspergillus* spp., and it has been outlined that lipoperoxides influenced the expression of genes involved in mycotoxin synthesis. However, it has not been definitively demonstrated whether anti-oxidants work by direct interaction with reactive species or by the stimulation of the fungal cell anti-oxidant system. Furthermore, the molecular basis of the relation between cell anti-oxidant defences and aflatoxins formation is not yet fully understood. Knowledge on the intracellular mechanism which leads to aflatoxin synthesis could be useful to achieve control over aflatoxins. Studies on anti-oxidants in animal cells have revealed that many known anti-oxidants act through the stimulation of transcription factors, such as AP-1, that regulate the expression of genes involved in reactive oxygen species (ROS) scavenging such as *sod*, *cat* and *gpx* (Harschman et al. 1988). In yeast, a transcription factor homologue to AP-1, Yap-1, is involved in the same regulatory mechanism. In this situation, other transcription regulatory proteins such as Skn7 and Hsf1-2 are also involved. Hsf-2 plays a role similar to Hsf-1 both in stimulating the transcription of heat shock proteins under heat shock or osmotic stress, but also under oxidative stress conditions (Estruch 2000; Moye-Rowley 2003; He et al. 2003).

In this study the correlation amongst *L. edodes* filtrates, their anti-oxidant activity and ability to stimulate anti-oxidation processes in *A. parasiticus* and to inhibit aflatoxin formation is investigated by a physiological and molecular approach.

Materials and methods

Fungal strains

L. edodes (Berk.) Pegler, strains CF21, 22, 23, 24, 42 and 43, were obtained from the collection of the Laboratory of Plant Pathology, Department of Plant Biology, Università "La Sapienza"—Roma. The strains were cultured on potato dextrose agar (PDA; Difco) in Petri dishes, and incubated at

25°C for 10 days. Potato dextrose broth (PDB; Difco) cultures of different isolates were prepared from Petri dishes and used as inocula after 10 days of incubation at 25°C.

A. parasiticus (Speare) (NRRL 2999), producer of B₁, B₂, G₁, G₂ aflatoxins, was cultured on PDA and incubated at 30°C for 7 days. A suspension of 1×10⁶ conidia in 0.2 ml of sterilised distilled water was used as inoculum.

Assays of different mushroom culture filtrates on aflatoxin production

PDB (500 ml) was inoculated with 10 ml of homogenised mycelia from 10-day liquid cultures of *L. edodes* isolates and incubated at 25°C under rotary shaken conditions (100 rpm) for 21 days. The mycelium was separated from the substrate by filtration through Millipore filters (0.2 µm); then the filtrate was lyophilised (LF, lyophilised filtrate) or dialysed (membrane cut off 10,000 Da) and, for the strain CF42, separated in different fractions. LF were added (final concentration, 2% w/v) to 25 ml of PDB. The flasks were inoculated with *A. parasiticus* conidia, as previously reported, and incubated at 30°C. The fungal growth was assayed after 3, 6 and 9 days of incubation and aflatoxins were monitored in the same time intervals from the mycelia and culture filtrates.

Determination of *A. parasiticus* growth

The *A. parasiticus* growth was measured by collecting the mycelium from the PDB cultures supplemented with lyophilised filtrates (LF), extracellular material (EM) and esopolysaccharides trypsinated (EPSt), after filtration (Millipore filters, 0.45 µm); it was washed three times via a physiologic saline solution (NaCl 0.9% w/v), once in distilled water and by weighting the mycelium after drying at 80°C for 48 h.

Extraction of esopolysaccharides from *L. edodes* culture filtrates

To obtain the esopolysaccharides present in the culture filtrates from the *L. edodes* CF42, the fungal strain which showed the best inhibiting effect on aflatoxin production, the protocol of Krcmar et al. (1999) was followed. The filtrates were reduced in volume by heating at 45°C for 24 h and then precipitated with cold absolute ethanol (1:1 v/v) for 12 h at 4°C, and centrifuged at 11,000 rpm for 30 min. The pellets were dissolved in distilled water at 45°C and then precipitated again with ethanol (1:1 v/v) for 12 h and centrifuged. According to Krcmar et al. (1999), the pellets are mainly composed of esopolysaccharides containing about 20% proteins. They were collected, re-suspended and dialysed (membrane cut off 10,000 Da) against distilled water and lyophilised; this fraction represents the extracellular material (EM). An aliquot of EM (150 mg) was

dissolved in 40 ml of distilled water, and trypsin (2 mg) was added. This solution was stirred overnight at 40°C, dialysed (membrane cut off 10,000 Da) against distilled water and lyophilised to obtain esopolysaccharides trypsinated (EPSt). The EM and EPSt fractions were assayed on aflatoxin production by *A. parasiticus*.

Quantification of β -glucans present in *L. edodes* lyophilised filtrates, extracellular material and esopolysaccharides trypsinated

The amount of β -glucans present in LF (from six isolates) and EM, EPSt (from CF42) was performed as described by Vis and Lorenz (1997). The samples were incubated at 40°C for 1 h with 1 ml of sodium borohydride (0.5 M), then 1 ml of aqueous (1:1 v/v) ethyl alcohol, 1 ml of 1 mM acetic acid solution and 2 ml of 20 mM sodium phosphate buffer pH 6.5 were added. The solution was further incubated for 15 min at 40°C with 2 units of β -glucanase enzyme (Sigma) and 50 mM sodium acetate buffer (5 ml), pH 4.0, was added and then centrifuged at 8,000 rpm for 10 min. The supernatants were collected and an aliquot (100 μ l) was incubated with 100 μ l of β -glucosidase (Sigma) (20 units ml^{-1}) at 40°C for 15 min. Afterwards, 5 ml of 30 mM solution of *para*-hydroxybenzoic acid hydrazide in alkaline diluent (0.25 mol l^{-1} sodium hydroxide, 0.025 mol l^{-1} trisodium citrate and 0.005 mol l^{-1} calcium chloride in water) were added. The control was represented by 200 μ l of 1 g l^{-1} solution of glucose. The solution was heated in boiling water bath for 5 min and then the absorbance of the samples was measured at 415 nm. The percentage of β -glucans present in the samples was also calculated on the quantity of total carbohydrates, which were determined by anthrone reaction.

Assays of different filtrates fractions on aflatoxin production

The lyophilised fractions were added (final concentration of EM 0.8% w/v, EPSt 0.7% w/v) to 25 ml PDB. These concentrations were calculated in relation to the yield ratio of EM, EPSt and lyophilised filtrates. The flasks were inoculated with *A. parasiticus* conidia and incubated at 30°C. The fungal growth and aflatoxins production were assayed after 3, 6 and 9 days of incubation.

Determination of *A. parasiticus* conidia germination

The conidia germination was carried out on Czapek Dox Broth (Difco) agarised with 0.2% w/v of Bacto Agar (Difco), supplemented with 2% w/v of the LF of the different isolates, and with 0.8% w/v EM and 0.7% w/v EPSt extracted from CF42, inoculated with 1×10^6 *A. parasiticus* conidia and incubated at 30°C. After 16 and 24 h of incubation, the conidia germination was evaluated.

Analysis of the aflatoxins

The aflatoxins ($B_1+B_2+G_1+G_2$) analyses were performed as previously reported (Fanelli et al. 2000), by extracting culture filtrates and mycelia in chloroform/methanol (2:1 v/v) three times. The extracts were collected after filtration on anhydrous Na_2SO_4 , concentrated under a N_2 stream and quantified by HPLC.

Experiments with lyophilised filtrates, extracellular material and esopolysaccharides trypsinated without *A. parasiticus* inoculum

PDB (25 ml) was inoculated with 1×10^6 *A. parasiticus* conidia and incubated for 6 days at 30°C to induce aflatoxin production. After filtration (Millipore 0.2 μ m) of the mycelium, the filtrates were sterilised and then aflatoxins produced by *A. parasiticus* were quantified (as noted in "Analysis of the aflatoxins"). These filtrates were supplemented with LF (2% w/v), EM (0.8% w/v) and EPSt (0.7% w/v) without *A. parasiticus* inoculum and incubated at 30°C. To verify the possible interaction of filtrates and fractions with the mycotoxins already present in the culture media, the aflatoxins were detected after 3, 6 and 9 days of incubation and compared with those present in the control (sample without the addition of the lyophilised filtrates and fractions).

Assay of the anti-oxidant activity of the different lyophilised and fractionated filtrates

The anti-oxidant activity of the lyophilised filtrates and their fractions was tested via the crocin bleaching test as reported by Tubaro et al. (1998). Crocin was extracted twice from saffron using methanol. The amount of crocin was measured in methanol, by spectrophotometric assay, at 443 nm and calculated using the absorption coefficient ($\epsilon=1.33 \times 10^5$ mol $^{-1}$ cm $^{-1}$ at 443 nm). The reactions were carried out at 40°C using distilled water as solvent and 4-azidophenacyl bromide (APAB) as oxidant. The bleaching rate of crocin was monitored for 10 min, after the addition of APAB. The final volume was 2 ml and the reaction solution contained 40 mM APAB and 0.24 mM crocin water solution. Each sample was tested at five different concentrations and compared with a kinetic crocin bleaching test using only 40 mM APAB and 0.24 mM crocin water solution. Blanks without crocin were run to rule out spectral interferences between each compound and crocin.

Anti-oxidant capacity was calculated as the linear regression plot [supposed anti-oxidant] / [crocin] vs $A_{\text{crocin}}/A_{\text{supposed anti-oxidant}}$, where A is the bleaching rate. The anti-oxidant activity of the complex mixture was calculated by dividing the slope obtained for each sample by the slope obtained for 1 mM of BHT.

Analysis of anti-oxidant enzyme activities in *A. parasiticus* treated with lyophilised filtrates of CF42

Analyses of the activities of the different anti-oxidant enzymes were performed according to the method of Kim et al. (2004a,b). Superoxide dismutase (SOD; EC 1.15.1.1) activity was evaluated by spectrophotometric assay. In this competitive inhibition assay, superoxide generated by xanthine-xanthine oxidase is detected by monitoring the reduction of nitrobleu tetrazolium at 505 nm. Total SOD activity was measured at pH 7.8 in Tris-HCl 0.2 M and pH 10.0 in sodium carbonate buffer 50 mM. One unit of activity was defined as the amount of enzyme that yields 50% of maximal inhibition of nitrobleu tetrazolium reduction by superoxide.

Catalase (CAT, EC 1.11.1.6) activity was measured by monitoring the decomposition of H₂O₂ that was followed by decrease in absorbance at 240 nm. The difference in absorbance at 240 nm per unit per time is a measure of the catalase activity.

Glutathione peroxidase (GPX, EC 1.11.1.9) was assayed by monitoring at 340 nm the decrease in absorbance due to the oxidation of NADPH at 37°C. The final concentration in the reaction mixture was 50 mM potassium phosphate buffer, pH 7.0, 0.5 mM EDTA, 1 mM reduced glutathione, 0.5 mM NADPH, 0.5 mM sodium azide, 0.24 U ml⁻¹ glutathione reductase and 150 μM H₂O₂. One unit of the enzyme oxidises 1 μmol of NADPH min⁻¹.

Cloning and sequencing of *A. parasiticus* *hsf2*-like

DNA extracted from *A. parasiticus* NRLL 2999 strain was amplified in a thermal Eppendorf Mastercycler gradient following amplification steps [94°C×2 min; (94°C×30 s, 56°C×45 s, 72°C×1 min)×35 times; 72°C×8 min] using primers *hsf*-deg (forward 5'-GAGTTCGCCAAGACSYTRATC-3'; reverse 5'-GCGTTGATCGARTTCTCGTGAC-3'), designed on the consensus conserved nucleotides region of *hsf1-2* in several fungal species found in GenBank. The amplification under high stringency conditions gave a unique band for *hsf2*-like (542 bp, GenBank accession number AY850227). The fragment was cloned in pGEMT easy vector (Promega) sequenced and aligned with TBLASTX 2.1 software of the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST>). Blast results indicate a high homology [aminoacids identity of 28–73% with *Schizosaccharomyces pombe* heat shock transcription factor (HSF) and of 39–58% with *Homo sapiens* heat shock transcription factor 2-Hsf2] of the conceptual aminoacidic translation of *hsf2*-like with the heat shock transcription factor Hsf2 of several animal and fungal species. New specific primers Ap-*hsf* (forward 5' ACGCTAATCCCCGA ACTTTT 3'; reverse 5' TCTCTCCGCAGCTTGGTAAT 3'), were designed and used for the subsequent RT-PCR analysis. The couple of 18S primers used as internal standard Ap-18S (forward 5'-ATGGCCGTTCTTAGTTGGTG-3'; reverse 5'-GTACAAAGGGGCAGGGACGTA-3') originated a single fragment of 500 bp, whilst the primer

chosen for the amplification of *hsf2*-like originated a single fragment of 350 bp.

aflR, *norA* and *hsf2*-like semi quantitative RT-PCR analysis

Total RNA from 100 mg of freeze-dried mycelia was extracted using Tri-Reagent protocol (Sigma) and was spectrophotometrically quantified by determining the optical density at 260 nm. RNA was treated with RNase-free Dnase I and then resuspended in 20 μl of DEPC-treated water. RNA was extracted 40, 48, 60, 72, 90 and 96 h (three tubes each day) after the addition of lyophilised filtrate to *A. parasiticus* culture, and was used to develop an *aflR*, *norA* and *hsf2*-like RT-PCR assay. RT was performed using 500 ng of total RNA and 200 U of Superscript reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The RT reaction mixture (0.5 μl) was used for *aflR*, *norA* and *hsf2*-like specific PCR amplification together with 10 pmol of *A. parasiticus* *aflR*, *norA* and *hsf2*-like specific primers. The programme included 30 cycles consisting of 95°C for 30 s, 58°C for 20 s and 72°C for 1 min. RT-PCR control reaction mixtures contained either water or 10 ng of *A. parasiticus* DNA. The constitutive expression of ribosomal 18S RNA was tested by using RNA extracts from *A. parasiticus* in each time point. A semi-quantitative *aflR*,

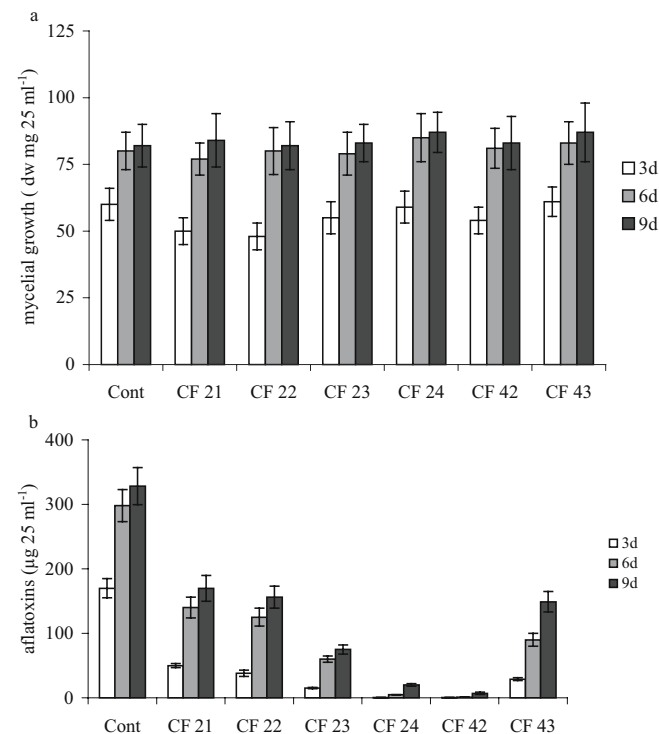


Fig. 1 Effect of lyophilised culture filtrates (2% w/v) from six isolates of *L. edodes*, added to the PDB media inoculated with *A. parasiticus*, on the **a** mycelial growth [as dry weight (*dw*)] and **b** aflatoxin production ($B_1+B_2+G_1+G_2$) in the culture filtrates after 3, 6 and 9 days of incubation at 30°C. The data represent the mean ± SEM from $n=3$ samples for each time point from three separate experiments

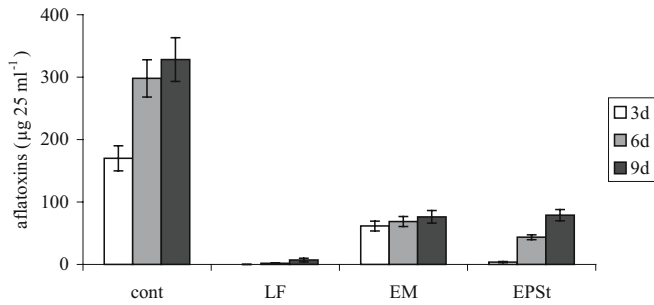


Fig. 2 Effect of the lyophilised filtrates (*LF*) (2% w/v), extracellular materials (*EM*) (0.8% w/v), esopolysaccharides trypsinated (*EPSt*) (0.7% w/v) from the isolate *Lentinula edodes* CF 42, added to the PDB media inoculated with *Aspergillus parasiticus*, on aflatoxins ($B_1+B_2+G_1+G_2$) formation in the culture filtrates after 3, 6 and 9 days of incubation at 30°C. The data represent the mean \pm SEM from $n=3$ samples for each time point from three separate experiments

norA and *hsf2*-like RT-PCR was used to study *A. parasiticus aflR*, *norA* and *hsf2*-like expression in each time point. The ratio of *aflR*, *norA* and *hsf2*-like/18S PCR products was determined using Molecular Analyst Software (Bio-Rad) and this ratio was used as an index of the relative *aflR*, *norA* and *hsf2*-like mRNA expression in samples.

Statistical analysis

All the data presented are mean value (\pm SEM) of three determinations. In all experiments, mean values were compared using Student's *t*-test.

Results

Mycelial growth and aflatoxin production by *A. parasiticus* cultures incubated in the presence of lyophilised filtrates from different *L. edodes* isolates

The growth of the toxigenic fungus was not significantly affected by the presence of the lyophilised filtrates of the different *L. edodes* isolates in the medium (Fig. 1a). The conidia germination of *A. parasiticus* was not influenced by the addition of *L. edodes* raw filtrates (control 92%, LF 88–90% germination).

Aflatoxin production was otherwise inhibited, although at a different extent (ranging from 48 to 98% of inhibition), by all the tested isolates (Fig. 1b). In general, toxin control was more evident during the first 3 days of incubation and the inhibition trend decreased during the 9-day period. In particular, *L. edodes* CF42 demonstrated the best inhibiting effect, which exceeded 90% during the experiment; this isolate was chosen to carry out further analyses.

The EM and EPSt fractions from lyophilised filtrates of CF42 added to the substrates inoculated with *A. parasiticus* showed a less efficient inhibiting effect on aflatoxin formation in comparison to raw filtrates of the same isolate (Fig. 2). However, an inhibiting effect (about 75%) is maintained up to 9 days of incubation by EM and EPSt. Up to 3 days, EPSt showed the same inhibiting effect as LF filtrate, without affecting fungal growth and conidia germination.

Anti-oxidant activity and β -glucans quantity of different *L. edodes* culture filtrates and fractions

All the filtrates demonstrated some anti-oxidant ability, in the following order: CF21>CF23>CF43>CF24>CF22>CF42 (Table 1). The isolate CF42 showed the best inhibiting effect on aflatoxin formation (Fig. 1b), but the lowest anti-oxidant activity as measured by crocin test. The anti-oxidant activity of all the different filtrates is low in comparison with 1 mM BHT (28.71 \pm 2.5), a well known anti-oxidant used as reference. A close correlation between anti-oxidant activity and aflatoxin inhibition has not been observed even if a slight effect can not be excluded. On the contrary, a highly significant direct relationship between the quantity of β -glucans and the inhibition of aflatoxin production ($R=0.84$, $p<0.01$) is shown in Table 1. The filtrates showing the highest quantity of β -glucans (CF42 180.50 \pm 18.83 mg l⁻¹; CF24 131.88 \pm 17.65 mg l⁻¹; CF23 116.44 \pm 12.71 mg l⁻¹) were also the best inhibitors of aflatoxin production. In the lyophilised filtrates of CF21, CF22 and CF43, which are less efficient in aflatoxin inhibition, the presence of β -glucans was about 30% lower in comparison with the other strains.

The esopolysaccharides fractions from *L. edodes* CF42 (Table 2), EM and EPSt, presented lower anti-oxidant activity (0.12 \pm 0.03 and 0.016 \pm 0.004, respectively) and

Table 1 Antioxidant capacity, β -glucans content, percentage (%) of β -glucans/total sugar present in the lyophilised filtrates (LF) and percentage (%) of aflatoxin inhibition (after 9 days of incubation)

LF	Antioxidant capacity (mM BHT)	β -Glucans (mg l ⁻¹)	Percent of β -Glucans/total sugar	Aflatoxin inhibition (%)
CF 21	1.28 \pm 0.15	108.38 \pm 11.52	0.90 \pm 0.08	48.22 \pm 4.60
CF 22	0.40 \pm 0.09	105.02 \pm 14.22	0.82 \pm 0.05	47.51 \pm 5.21
CF 23	0.92 \pm 0.09	116.44 \pm 12.71	0.98 \pm 0.08	77.15 \pm 6.70
CF 24	0.48 \pm 0.07	131.88 \pm 17.65	1.21 \pm 0.09	93.90 \pm 3.23
CF 42	0.36 \pm 0.08	180.50 \pm 18.83	1.81 \pm 0.09	98.10 \pm 1.50
CF 43	0.72 \pm 0.08	98.98 \pm 11.22	0.72 \pm 0.06	54.61 \pm 4.55

showed by different lyophilised filtrates from *L. edodes* cultures added to PDB media inoculated with *A. parasiticus* and incubated at 30°C

Table 2 Antioxidant capacity, β -glucans content, percentage (%) of β -glucans/total sugar present in lyophilised filtrates (LF), extracellular material (EM), esopolysaccharides trypsinated (EPSt) and

CF42	Antioxidant capacity (mM BHT)	β -Glucans (mg l ⁻¹)	Percent of β -glucans/total sugar	Aflatoxin inhibition (%)
LF	0.36±0.08	180.50±18.83	1.81±0.09	98.10±1.50
EM	0.12±0.03	104.01±15.25	7.00±1.61	76.77±2.44
EPSt	0.016±0.004	114.10±11.71	7.10±0.90	75.96±3.22

β -glucans quantity (104.01±15.25 and 114.10±11.71 mg l⁻¹, respectively) in comparison with the LF fraction. The percentage of β -glucans in relation to the total sugar content in the filtrate was shown to be higher, showing that the simple sugar, oligo- and small polysaccharides (<10,000 Da) lost during the precipitation procedures may have likely contained a lower quantity of β -glucans in comparison with the precipitated fraction.

Aflatoxin production by *A. parasiticus* analysed in the mycelium and culture filtrate in the presence of lyophilised filtrates from the isolate CF42

Aflatoxin analyses were carried out in the mycelia and in the culture filtrate of *A. parasiticus* untreated (Fig. 3a) and treated with LF from CF42 (Fig. 3b), at different times (24, 36, 48, 60, 72, 96 h and 7 days), to exclude the possibility that the presence of lyophilised filtrates in the substrate could hamper the output of the toxins. The results obtained showed that both in the control and in the treated samples, a similar mean percentage of aflatoxins was detected in the mycelia (control 1.0±0.2; treated 1.5±0.3 $\mu\text{g mg}^{-1}$ dw) in comparison with those present in the filtrate. In the samples treated with lyophilised filtrates, the aflatoxins were not detected up to 48 h of incubation both in the mycelium and in the filtrate. After this period, the level of toxins increased up to 7 days but always remained significantly ($p<0.001$) lower than that detected in the control. Furthermore, the amount of toxins in the experiments carried out in the presence of aflatoxins in the PDB media, without *A. parasiticus* inoculums, supplemented with different lyophilised filtrates, was roughly similar in all examined samples (control 260.1±25.6; treated samples from 247.2±21.6 to 271.4±29.1 $\mu\text{g 25 ml}^{-1}$).

Anti-oxidant enzymes activities in the mycelium of *A. parasiticus* in the presence of lyophilised filtrate of CF42

The activities of SOD, CAT and GPX (during the time course) in the mycelium of *A. parasiticus* from cultures treated and not treated with lyophilised filtrates is presented in Fig. 4a–c.

SOD activity (Fig. 4a) both at pH 7.8 and 10.0 was significantly ($p<0.001$ at 60 h) stimulated by the addition of lyophilised filtrates in the substrate in comparison to con-

percentage (%) of aflatoxin inhibition (after 9 days of incubation) showed by the fractions (from *L. edodes* CF 42) added to PDB media inoculated with *A. parasiticus* and incubated at 30°C

trol, and a peak was evident at 60 h of incubation. Even if a decrease appeared after this time, the activity of the SOD enzymes remained higher than that in control up to 7 days of incubation. CAT and GPX activities in the presence of lyophilised filtrates of isolate CF42 were not significantly different compared to those detected in the control. Both enzymes showed the same trend during the time course and between 48 and 96 h, then decreased to levels lower than that observed in the control. CAT and GPX activities were not significantly stimulated by the presence of lyophilised filtrates.

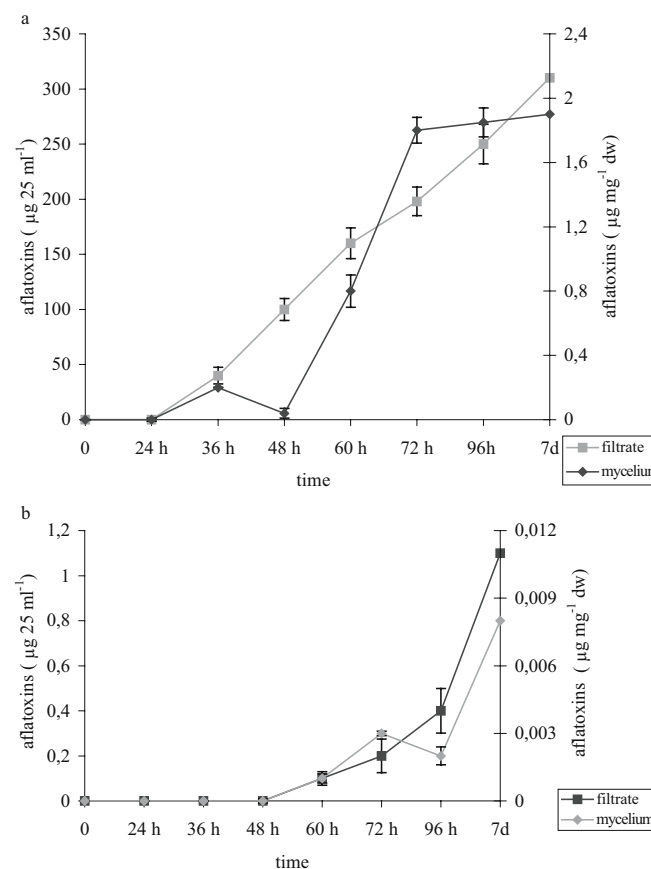


Fig. 3 Aflatoxins (B1+B2+G1+G2) production **a** in the mycelium and filtrate by *A. parasiticus* inoculated in PDB medium and **b** in the mycelium and filtrate by *A. parasiticus* grown in PDB supplemented with 2% w/v of lyophilised filtrate from isolate CF 42 of *L. edodes*. The data represent the mean±SEM from $n=3$ samples for each time point from three separate experiments

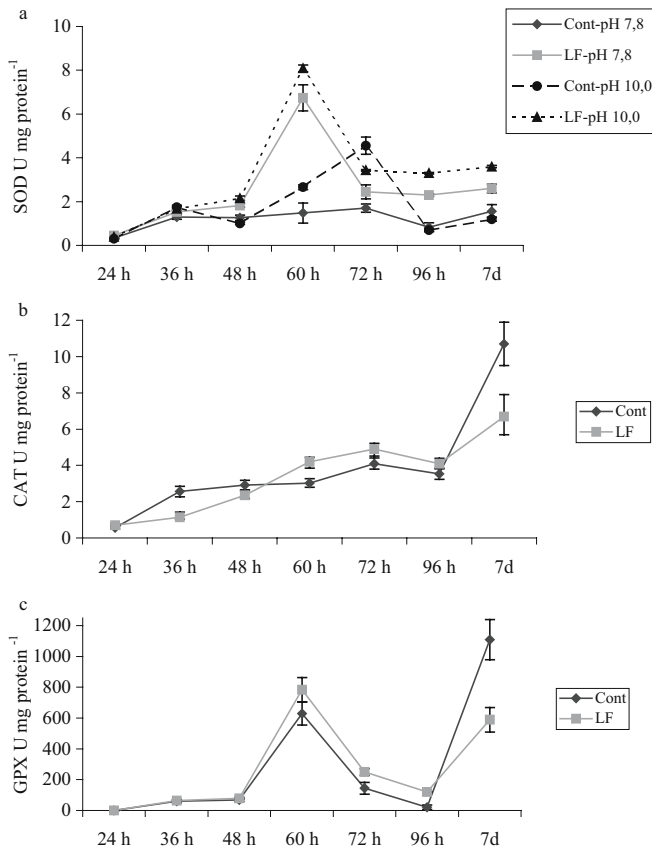


Fig. 4 Activity of different anti-oxidant enzymes, **a** superoxide dismutase (SOD) pH 7.8 and 10.0, **b** catalase (CAT), **c** glutathione peroxidase (GPX), at different times of incubation, showed by *A. parasiticus* mycelia in the absence (Cont) and in the presence of lyophilised filtrate (LF, 2% w/v) from cultures of *L. edodes* CF 42. The data represent the mean \pm SEM from $n=3$ samples for each time point from three separate experiments

Analyses of the expression of *aflR*, *norA* and *hsf2*-like in the mycelium of *A. parasiticus* treated with lyophilised filtrate of CF42

RT-PCR analyses (Fig. 5a,b) carried out on the mycelia of *A. parasiticus* grown in the presence of lyophilised filtrates of *L. edodes* CF42 showed that the filtrate is able to delay the activation of aflatoxin gene cluster in comparison with the control. In fact, *aflR* and *norA* mRNA relative expression were significantly ($p<0.001$) inhibited at 40–60 h, remained similar or lower at 72 h, and only after 90 h they were similar or higher in comparison with the control. The levels of *aflR* and *norA* mRNA in the treated samples never reached the highest values of the control.

The expression of *hsf2*-like (heat shock transcription factor 2-like) was markedly expressed at 48 and 96 h in the treated samples in comparison with the control ($p<0.001$). At 60 h the highest *hsf2*-like mRNA level in the control was reported. The higher activities of SOD (from 48 to 72 h), CAT and GPX (from 48 to 96 h) were also reported in these periods (Fig. 4a–c).

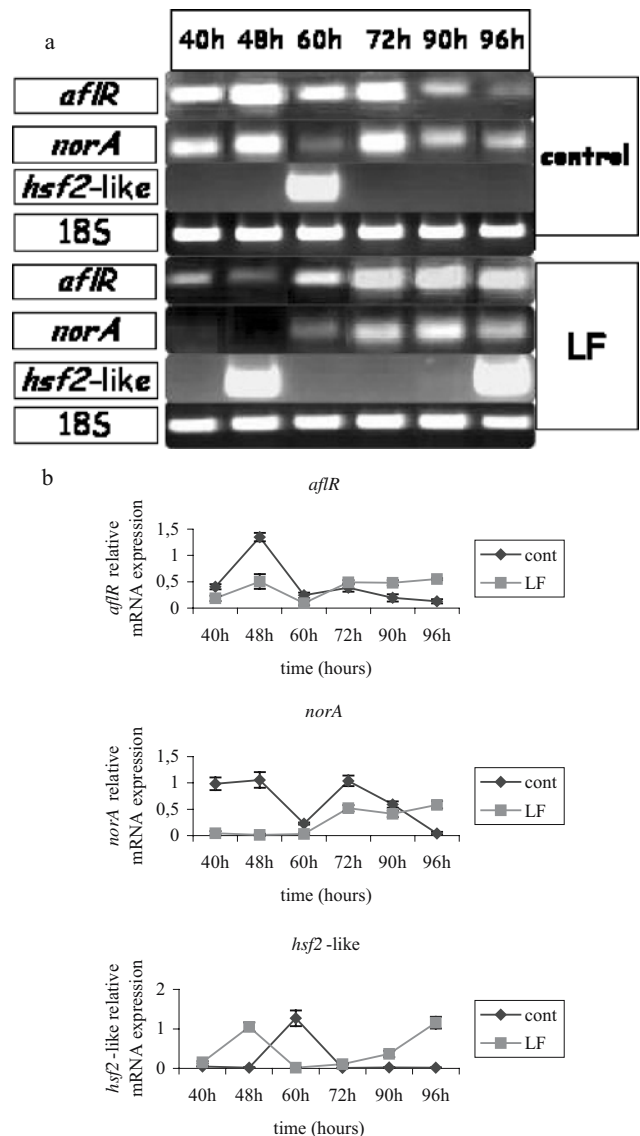


Fig. 5 RT-PCR analysis (**a**) of *aflR*, *norA*, *hsf2*-like in the mycelia of *A. parasiticus* treated with lyophilised filtrate (LF) (2% w/v) of *L. edodes* CF 42 and untreated, control (cont), at different times of incubation. In the graph below (**b**), the mean \pm SEM of the relative *aflR*, *norA*, *hsf2*-like mRNA expression normalised on rRNA 18S levels are reported. Each assay represents RNA from $n=5$ mycelia from three separate experiments

Discussion

The assayed *L. edodes* isolates are able to inhibit aflatoxin production and the isolate CF42 shows the best inhibiting results. This effect could probably be due to the different compounds present in the extracts with different inhibiting strategies. This can be evidenced by the use of the fractions obtained by *L. edodes* CF42 filtrates, which leads to the decrease in the inhibiting effect but not to its complete loss.

Results obtained in this study lead us to suggest that inhibition of aflatoxins has to be ascribed to an interference exerted by CF42 filtrates on the regulation of toxin formation. Although several studies have been carried out on

aflatoxin biosynthesis, to date there has been no report on how external stimuli, as pro- or anti-oxidant treatments, lead to aflatoxin stimulation or inhibition.

Jayashree and Subramanyam (2000), who compared toxigenic and non-toxigenic *A. parasiticus* isolates, concluded that intracellular oxidative stress can be considered as a “prerequisite” for aflatoxin production. The aflatoxin-inhibiting effect of the *L. edodes* filtrates seems to be correlated to anti-oxidant activity per se, but also overall to the stimulation of the fungal anti-oxidant system. In fact, all the tested mushroom filtrates and purified fractions demonstrated anti-oxidant activity against peroxidation (by Crocin test); however, this could only partially explain the inhibiting effect on aflatoxin production because no close and direct correlation was observed between the extent of aflatoxin inhibition ability and anti-oxidant activity in the different filtrates analysed. Another aspect to be considered is the impact of lyophilised filtrates on the oxidative status of *A. parasiticus* mycelia. It has been shown that they positively affect the anti-oxidant system of the *A. parasiticus* cells, in particular the SOD activity, and this can probably influence the production of aflatoxins. This result suggests that the enhancement of anti-oxidant enzymatic activity between 48 and 96 h in the LF-treated samples could hamper aflatoxin synthesis. In this period, the rate of aflatoxin increase in the treated samples is extremely low compared to that in untreated ones.

L. edodes is able to accumulate and release in the culture media lentinans and other β -glucans (Wasser and Weis 1999), as shown in Tables 1 and 2. A direct relation between aflatoxin inhibition and β -glucan content of *L. edodes* lyophilised filtrates is reported in this study. β -Glucans could be amongst the factors responsible for the inhibiting effect on aflatoxins. The ability of fungal β -glucans to act as free radical scavengers was recently shown (Slamenova et al. 2003), and in animal models glucans and glycoproteins extracted from fungi protect the macrophages from the damages caused by lipoperoxide accumulation, mainly by activating the transcription of genes related to the macrophage anti-oxidant system (Yuan et al. 1996). Since the molecular analyses carried out on *A. parasiticus* mycelia treated with CF42 filtrates show a significant activation of *hsf2*-like, a similar effect (i.e. an enhancement of fungal anti-oxidant defences) could be hypothesised. Hsf1–2 are transcription factors present in *Saccharomyces cerevisiae* activated by reactive species (as superoxide anion), that in turn, while interacting with other factors such as Yap-1 and Skn7, modulate the expression of the anti-oxidant system (Estruch 2000). This is the first detection of *hsf2*-like in *Aspergillus parasiticus*. Even if its involvement is not conclusive, it is possible that there is an oxidative stress response mechanism in *A. parasiticus* that plays, as in yeast, a pivotal role in the regulation of ROS levels in the cell, as also shown by Kim et al. (2004a,b). In fact, the contemporaneous activation of *hsf2*-like and of anti-oxidant enzymes could represent a regulation of anti-oxidant pattern by oxidative stress-related transcription factors that, however, must be fully investigated.

In a seemingly synchronised way, lyophilised filtrates treatment triggers the cell anti-oxidant system, delays *aflR* and *norA* transcriptional activation and inhibits toxin production. This delaying and lowering effect could be crucial for determining the final amount of aflatoxins released in the culture media, which, as previously shown, represents 2% in comparison with the control.

It could be suggested that, besides showing an anti-oxidant activity per se, lyophilised filtrates interfere with the cascade of signals that allows aflatoxin biosynthesis even if we cannot indicate at which step of the pathway this event occurs. However, in this picture, it could be hypothesised that β -glucans accumulated in the culture filtrates of *L. edodes* could be able to inhibit aflatoxin production by *A. parasiticus* through an enhancement of the internal anti-oxidant system. This result could also be achieved by the stimulation of oxidative stress-related transcription factors.

Fungal extracts could represent a promising tool in mycotoxin control in comparison with chemicals or natural products as plant extracts (Mahoney and Molyneux 2004; Sanchez et al. 2005) because of their low toxicity when released in the environment and for their facilities under growth conditions and extraction procedures. Furthermore, *L. edodes* can also grow on waste materials as olive mill wastewaters (Zjalic et al. 2002; Reverberi et al. 2004), which allows the reduction of the release of these pollutants in the environment. Lyophilised filtrates could be applied on the seeds alone, or in association with other food grade compounds, to prevent aflatoxin formation in feeds by using extracts that could strengthen the animal immune system (Guo et al. 2004). The polysaccharides of this basidiomycete, aside from their healing effects, have a low cytotoxicity on animal cells (Vickers 2002) and could contribute to the “functional value” of the feed supplied with these extracts (Xu 2001).

This research indicates some novel insights about the relation amongst oxidative stress, anti-oxidant fungal enzymes and aflatoxins production in *A. parasiticus*. An exhausting profile of these mechanisms could allow us to set the right strategy to control aflatoxins. Furthermore, extracts from the edible basidiomycete *L. edodes* could represent a “food grade” tool against aflatoxins.

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