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β -Glucan synthase induction in mushrooms grown on olive mill wastewaters

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Abstract β -1,3-Glucan synthase activity and its induction by olive mill wastewaters (OMW) was studied in ten fungal strains (*Auricularia auricula-judae*, *Lentinula edodes*, *Pleurotus eryngii*, *Stropharia aeruginosa*, *Agrocybe aegerita*, *P. pulmonarius*, *Armillaria mellea*, *P. ferulae*, *P. ostreatus*, *P. nebrodensis*). A microtiter-based enzymatic assay on β -1,3-glucan synthase activity was carried out on all mycelia growth both on the control medium and on OMW. Among the fungi assayed, *L. edodes* β -1,3-glucan synthase was highly enhanced in OMW. The main components of OMW, i.e. phenols and lipids, were added separately to the control medium, to highlight the mechanism of *L. edodes* β -1,3-glucan synthase induction. A Southern blot analysis and PCR with degenerated primers were carried out to detect the presence of *fks1*-like genes in these Basidiomycetes. The sequences obtained from the ten Basidiomycota were remarkably similar to *fks1* from *Filobasidiella neoformans*. Spectrofluorimetric and RT-PCR analyses of β -1,3-glucan synthase were performed on the mycelia of *L. edodes*. In this fungus, a strong stimulation of β -1,3-glucan synthase mRNA and protein was recorded in the presence of OMW and phenols.

Introduction

Over the past decade, interest in β -1,3-glucans and their biological activities has grown. Glucans, together with chitin, represent the greatest quantity of polysaccharides in fungal cell walls (Ruiz-Herrera and Sentandreu 1991). β -Glucans are a very heterogeneous class of glucose-derived polysaccharides, most of them containing β -1,3 and β -1,6 linkages. The main chain is represented by β -D-glucopyr-

anosile monomers 1,3-linked with lateral 1,6-branching. The degree of branching, the molecular weight and the secondary structure often determine the biological activity of these compounds. The biological role played by β -glucans depends on the structure of the polysaccharides and their cellular displacement. β -Glucans located on the cell surface seem to have different physiological functions with respect to others secreted outside the cell wall (Reshetnikov et al. 2000). Several studies have focused their attention on the medicinal properties of these polysaccharides. According to a growing number of authors (Furue 1986; Yadomae 1992; Bohn and Bemiller 1995), β -1,3-glucans can stimulate the immune system by enhancing host resistance in response to several diseases. Moreover, they seem to be involved in the inhibition of some types of tumours, although it is not yet clear how. For these features, fungal glucans are pharmacologically classified as biological response modifiers (BRM). Most BRM immune-modulator β -1,3-glucans have been isolated from Basidiomycota, such as *Lentinula edodes* (lentinan) and *Schizophyllum commune* (schizophyllane; Yadomae and Ohno 1996; Wasser and Weiss 1999). The immune response is determined by the length of the β -1,3-glucan, its frequency and the kind of branching (Yadomae 1992).

β -1,3-Glucans biosynthesis has been extensively studied in *Phytophthora cinnamomi*, *Cochliobolus miyabeanus*, *Saccharomyces cerevisiae*, *Aspergillus nidulans*, *Saprolegnia monoica*, *Achlya ambisexualis*, *Filobasidiella neoformans* and *Candida albicans* (Zevenhuizen and Bartnicki-Garcia 1968; Reiskind and Mullins 1981; Kopecka and Kreger 1986; Surarit et al. 1988; Sanchez-Hernandez et al. 1990). These glucans are synthesised by a membrane protein complex, β -glucan synthase (UDP-glucose 1,3- β -D-glucan 3- β -D-glucosyl transferase, EC 2.4.1.34), starting from UDP-glucose. The β -1,3-glucan synthase complex is composed of a catalytic subunit (FKS) and a regulatory subunit (RHO). FKS synthesises the polymer, starting from glucose monomers, whereas RHO is a G protein that activates FKS by means of GTP-dephosphorylation. RHO activity is then regulated by

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Table 1 Bank code numbers (International Bank of Edible Saprophytic Mushrooms, CNR) and names of the fungal species analysed

Bank code	Fungal species
Smr 054	<i>Auricularia auricula-judae</i>
Smr 090	<i>Lentinula edodes</i>
Smr 171	<i>Pleurotus eryngii</i>
Smr 181	<i>Stropharia aeruginosa</i>
Smr 200	<i>Agrocybe aegerita</i>
Smr 230	<i>P. pulmonarius</i>
Smr 423	<i>Armillaria mellea</i>
Smr 504	<i>P. ferulae</i>
Smr 684	<i>P. ostreatus</i>
Smr 710	<i>P. nebrodensis</i>

ROM (a wall GDP-GTP exchange factor protein which is activated by environmental stresses) and causes alterations in the fungal cell wall (Ruiz-Herrera 1991; Bickle et al. 1998). This study is part of a research project aimed at recycling agro-industrial wastes, particularly olive mill wastewaters (OMW), that pose environmental problems in Mediterranean areas where over 2 millions tons of this waste are produced in a short harvesting season (November–February). OMW were found to be a good substrate for *L. edodes* biomass production, as they are rich in lipids (about 1.5%) that stimulate mycelial growth (Song et al. 1989, 1990; Zjalic et al. 2002). Moreover, an increased yield of β -glucans in *L. edodes* grown on OMW was recorded by Belardinelli et al. (2000).

The aim of this work was to assay the β -glucan synthase in saprophytic mushrooms and to study the effect of OMW on β -glucan synthase activity. Biochemical and molecular aspects of the enzyme stimulation were studied in the medicinal mushroom *L. edodes*.

Materials and methods

Microorganisms and culture media preparation

The fungal strains (Table 1) were collected from the International Bank of Edible Saprophytic Mushrooms (CNR, Monterotondo Scalo, Rome, Italy). All isolates were grown for 41 days at 25°C, with stirring at 150 rpm, in 25 ml of MEP (control medium: 30 g l⁻¹ malt extract, 1.5 g l⁻¹ Peptone) or in OMW (4 g organic matter l⁻¹ dry weight; d.w.). *L. edodes* was also grown in MEP to which were added phenols (PHE; 7.5 mg ml⁻¹) or lipids (LIP; 13 mg ml⁻¹) extracted from OMW, as reported below. Mycelial biomass from both media were collected on days 7, 14, 21, 28, 34 and 41 and used for further experiments.

Phenols and lipids extraction

Phenols were extracted three times from 20 ml of OMW, using ethylacetate (v/v). The lipid fraction was also extracted three times from 20 ml of OMW, with hexane (v/v). Both extracts were dried under a N₂ flow.

Enzyme assays

β -Glucan synthase activity was assayed on 4 ml of homogenised mycelia for 15 min at 30°C and calculated on the basis of nanomoles of glucose incorporated (GI), as reported by Shedletzky et al. (1997). Enzyme activity was assayed on all isolates grown on MEP and OMW. Only in the case of *L. edodes* was the assay also carried out on mycelium grown on PHE and LIP. Paraquat (PQ; 250 μ M, 500 μ M), an oxidative stress inducer, was added to *L. edodes* grown on MEP for 7 days and the activity of the enzyme was monitored after 30 min and 24 h.

Superoxidedismutase (SOD) activity in fungal cultures filtrates was evaluated spectrophotometrically 7 days after inoculation (O'Neil et al. 1988). SOD activity was measured on 50 μ l of culture filtrates at pH 7.8 in 2 ml of Tris-HCl (0.2 M). A standard curve was produced using bovine SOD (Sigma) at different concentrations (0.1, 0.25, 0.5, 1.0, 2.0 units ml⁻¹). The amount of protein that yields in 1 min 50% of maximal inhibition of nitroblue tetrazolium reduction by superoxide was defined as a unit of activity.

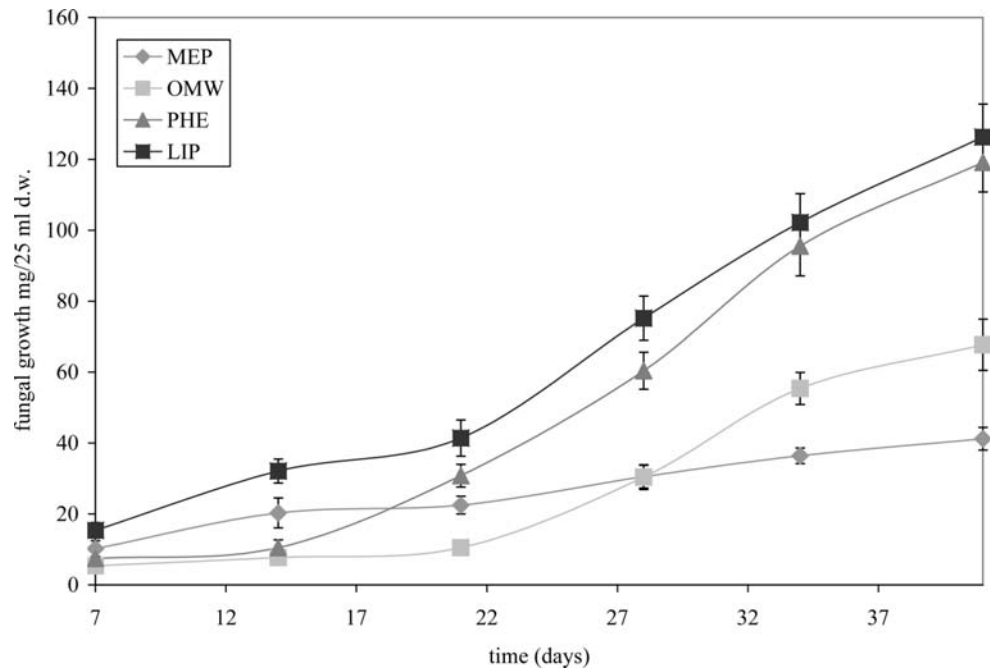
Catalase (CAT) activity was determined in fungal culture filtrates (50 μ l) 7 days after inoculation, using 2 ml of phosphate buffer (0.2 M) and H₂O₂ (10 mM) at pH 7.4. The rate of H₂O₂ disappearance was measured spectrophotometrically at 240 nm (O'Neil et al. 1988). One unit of CAT was defined as the amount of enzyme that degraded 1 μ mol H₂O₂ min⁻¹.

Laccase activity was assayed 7 days after inoculation, according to Hargin and Obst (1973). Laccase activity was measured on 50 μ l of culture filtrates at pH 4.5 in 3.4 ml of sodium acetate buffer (50 mM) and syringaldazine (0.06 M), using a Beckman DU 530 spectrophotometer at 525 nm. Protein concentration was assayed according to Bradford (1976; Sigma Chemical Co., St Louis, Mo.).

Cloning and sequencing of the *F. neoformans fks1* homologue

DNA was extracted from mycelia of all isolates (Table 1) and amplified in a thermal cycler (Perkin Elmer 9700), using degenerate fks1deg primers (forward 5'-ATY-GATGCBAAYCARGACAAAYTA-3', reverse 5'-CA-TYTGTTCDCCCATAACCAG-3') designed on the consensus conserved nucleotides region of *fks1* in several fungal species. The PCR amplification steps were: 94°C for 2 min and then 35 cycles of 94°C for 30 s, 56°C for 45 s and 72°C for 1 min, followed by 72°C for 8 min. Amplification in high stringency conditions gave a unique

Fig. 1 *L. edodes* growth (mg d. w. in 25 ml) at different times (7, 14, 21, 28, 34, 41 days) in different media (*MEP* control). Data represent the mean \pm SEM for each time-point for mycelia ($n=3$) from three different experiments



420-bp band for all the fungi assayed. These fragments were cloned in pGEMT easy vector (Promega), sequenced and aligned with TBLASTX ver. 2.1 software at the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST>).

The accession numbers for the *fksI*-like gene fragments assigned by GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/>) were AY157842 for *L. edodes* and AY254574–AY254583 for all the other fungi analysed.

All the sequences obtained by amplification using degenerated primers on all the fungi studied were aligned using Multalin software (Corpet 1988) and their theoretical translation was compared with a fragment of FKS1 (1002–1108) sequence from *F. neoformans*.

New specific primers *lfks1f* (5'-TACCTCGAG-GAATGTCTAAA-3') and *lfks1r* (5'-ATTGGTAA-TACTCCGAATGTT-3') were designed based on the *L. edodes fksI*-like sequence and used for the subsequent RT-PCR analysis.

Southern blot analysis with *fksI*-like probe

DNA from each mycelium (Table 1) was digested with *EcoRI*, transferred to Nylon membranes and hybridised with the probe for the gene encoding β -1,3-glucan synthase. The probe to detect the *fksI*-like gene in all the mycelia considered (Table 1) was a 420-bp fragment of a *fksI*-like gene derived from amplification of *L. edodes* genomic DNA with the PCR digoxigenin (DIG)-labelling system (Roche). Digested DNA was separated by 6 h of electrophoresis in 0.8% agarose gel at 30 V and transferred to a N⁺ Nylon membrane (positive-charged Nylon membrane; Roche Diagnostics, Mannheim, Germany). Hybridisation was carried out according to Roche DIG hybridisation manual.

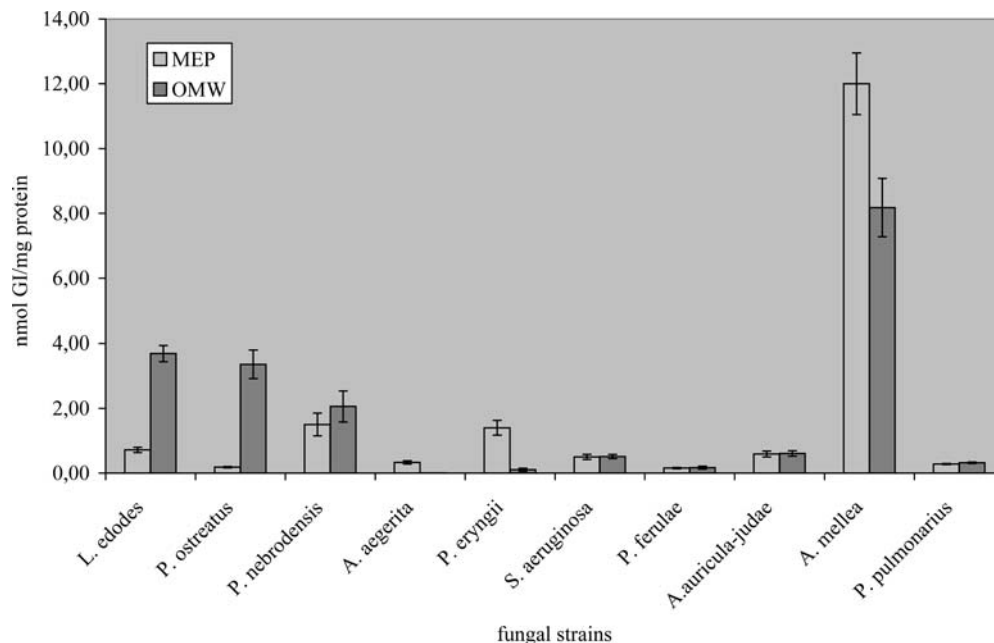
FksI-like semi-quantitative RT-PCR analysis

Total RNA from 100 mg of freeze-dried mycelia was extracted using the 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 μ l of diethylpyrocarbonate-treated water. RNA was extracted after days 7, 14, 21, 28, 34 and 41 (three tubes each day) and was used to develop a *fksI*-like RT-PCR assay. RT was performed using 500 ng of total RNA and 200 units of Superscript reverse transcriptase (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. The RT reaction mixture (0.5 μ l) was used for *fksI*-like specific PCR amplification together with 10 pmol of *L. edodes fksI*-like specific primers. The program included 30 cycles 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 *L. edodes* DNA. Constitutive expression of ribosomal 18S RNA was tested using RNA extracts from *L. edodes* each day and from the media. A semi-quantitative *fksI*-like RT-PCR was used to study *L. edodes fksI*-like expression each day and in each medium. The ratio of *fksI*-like/18S PCR products was determined using Molecular analyst software (Bio-Rad Laboratories, Hercules, Calif.) and this ratio was used as an index of the relative *fksI*-like mRNA expression in samples (Doohan et al. 1999).

Statistical analysis

All experiments were performed three times. Mean values were compared using Student's *t*-test.

Fig. 2 Determination of β -glucan synthase activity, expressed as nmol GI mg^{-1} protein, in different fungal strains after 41 days of growth on MEP and OMW. Data represent the mean \pm SEM for each time point for mycelia ($n=3$) from three different experiments



Results

Fungal growth

All the fungi grown on OMW yielded a higher biomass after 41 days, compared with the control medium (values ranging between 50–100 mg and 30–60 mg in 25 ml d.w., respectively). Fungal growth on PHE and LIP fractions was determined on the days mentioned above only for *L. edodes*. Compared with the control medium, OMW (67.7 ± 7.2 mg d.w. in 25 ml), LIP (126.3 ± 9.5 mg d.w. in 25 ml) and PHE (119.1 ± 8.3 mg d.w. in 25 ml) stimulated the growth of *L. edodes* after 41 days of incubation (Fig. 1). A slight decrease in growth was initially recorded when *L. edodes* grew on OMW and PHE (Fig. 1).

β -1,3-Glucan synthase activity

The β -1,3-glucan synthase activity of fungal isolates was assayed 41 days after inoculation. The results are summarised in Fig. 2. In the control medium, all fungal strains displayed β -1,3-glucan synthase activity, although to a different extent. *Armillaria mellea*, a mushroom pathogenic to trees, showed the highest activity. β -Glucan synthase activity behaved differently when the isolates grew on OMW. Looking at them separately, an increase of about 12-fold was evident for *L. edodes* and *P. ostreatus*. No differences were recorded for *Agrocybe aegerita*, *Stropharia aeruginosa*, *P. ferulae*, *Auricularia auricularia-judae*, *P. pulmonarius* and *P. nebrodensis*, whereas a reduction in the activity was recorded for *A. mellea* and *P. eryngii*. In another series of experiments, the induction of β -1,3-glucan synthase activity in *L. edodes* was also monitored in the presence of PHE and LIP during mycelial growth (Fig. 3a). In comparison with the other substrates, the highest enzyme activity was reached after 14 days in

the presence of PHE and after 21 days in the presence of OMW. Afterwards, β -glucan synthase activity decreased and values were similar in all treatments after 41 days. When LIP was added to the control medium, the inhibitory effect was slight or not existent. The addition of PQ (both concentrations) enhanced β -glucan synthase activity. In particular, an increase of 5.3-fold after 30 min and 4.6-fold after 24 h was recorded when 250 μM PQ was added to the control medium. A slight decrease in β -glucan synthase activity was initially observed when 500 μM PQ was added. Afterwards, enzyme activity increased by 5.7-fold after 24 h (Fig. 3b).

Sequence alignment analysis

Using degenerated primers fks1deg forward and reverse, fragments were amplified for all fungi tested; and the TBLASTX results indicated a high homology of these amplicons with the catalytic subunit of the *F. neoformans* *fks1* gene (GenBank accession number AAD11794). The sequences obtained were aligned using Multalin software (Corpet 1988; Fig. 4), which showed a certain degree of genetic diversity among the analysed fungi, according to the different restriction profiles resulting from the Southern analysis as reported in the next section.

Southern analysis

Southern analysis of the fungal DNA with the probe for the *fks1*-like gene is shown in Fig. 5. Among the ten fungal DNA, positive hybridisation signals appeared in all strains (Fig. 5). Analysis of the sequences indicated a restriction site for *EcoRI* in two of the samples tested. This suggests that the hybridisation profile of *P. eryngii* and *P. ostreatus* could be the result of *fks1*-like gene separation in

Fig. 3 a,b β -Glucan synthase activity. Data represent the mean \pm SEM for each time point for mycelia ($n=3$) from three different experiments. **a** Determination of β -glucan synthase activity in *L. edodes* smr 090 at different times (7, 14, 21, 28, 34, 41 days) in different media. **b** Determination of β -glucan synthase activity, after 7 days of incubation of *L. edodes* 30 min and 24 h after the addition of 250 μ M PQ and 500 μ M PQ. cont MEP

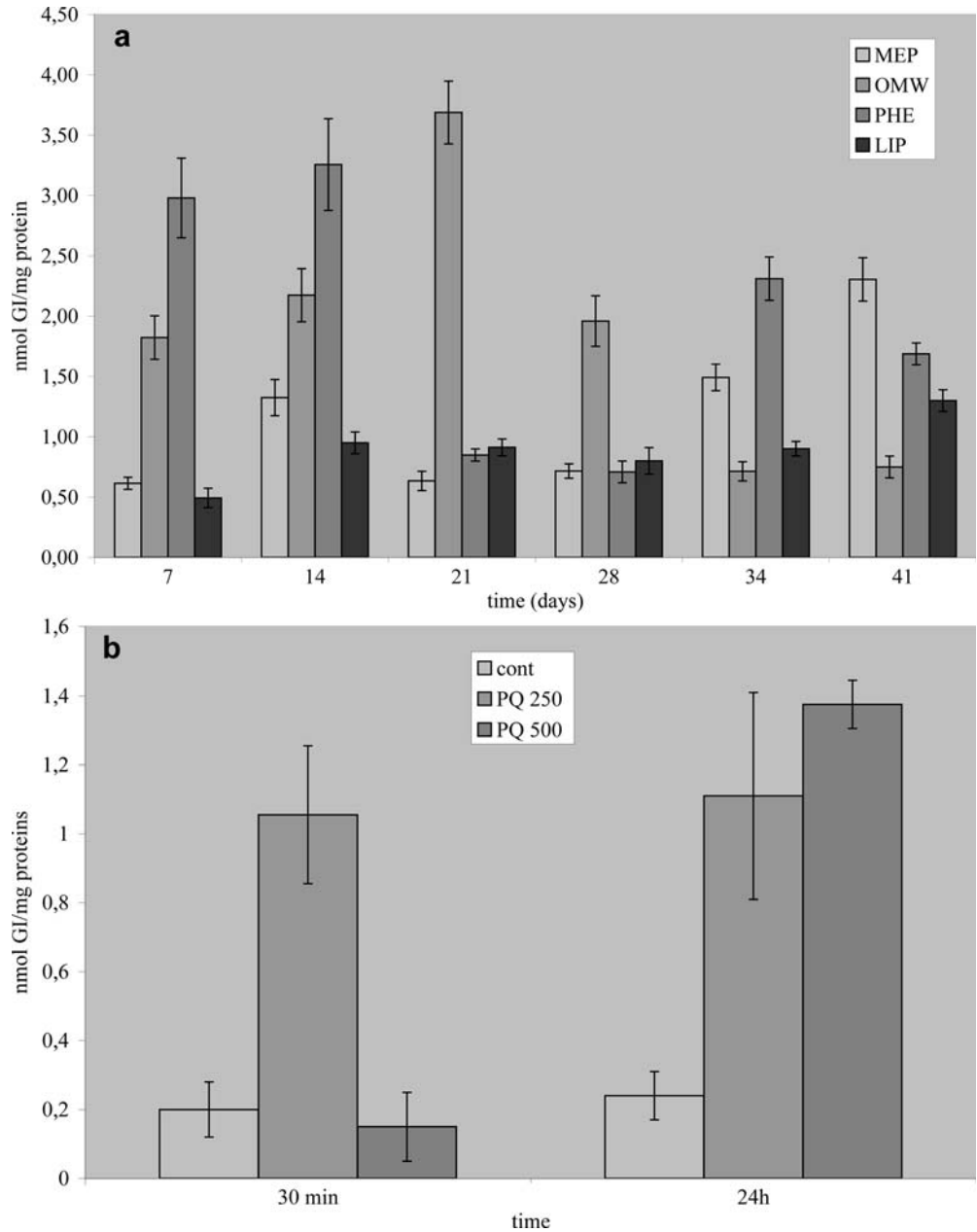
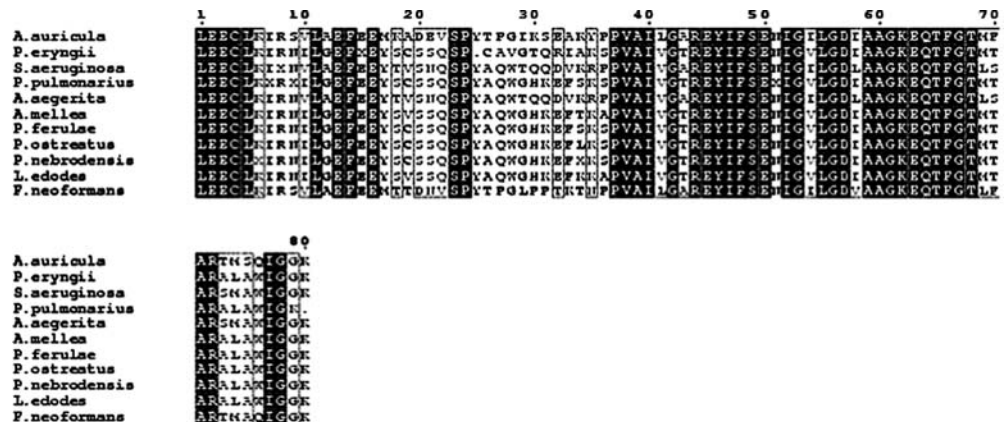


Fig. 4 Alignment analysis of the *fksl*-like fragments of the analysed fungal species, translated into the corresponding amino acid sequences (translation generated through TBLASTX ver. 2.1 software). These fragments were compared with part of the conserved glucan synthase domain of *F. neoformans* (GenBank accession number AAD11794)



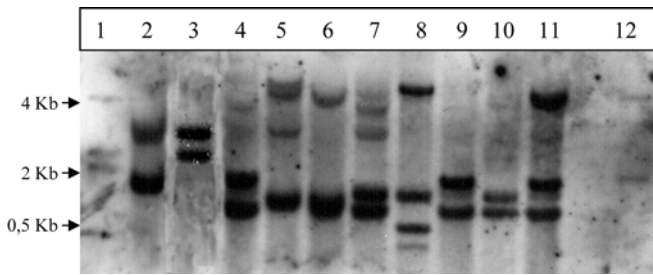


Fig. 5 Southern blot analysis of the genomic organisation of the *fks1*-like gene in several fungal species. Lane 1 Molecular markers (λ_{HindIII}), lanes 2–11 fungal DNA *EcoRI*-restricted. Lane 2 *A. auricula-judae*, lane 3 *L. edodes*, lane 4 *P. eryngii*, lane 5 *S. aeruginosa*, lane 6 *A. aegerita*, lane 7 *P. pulmonarius*, lane 8 *A. mellea*, lane 9 *P. ferulae*, lane 10 *P. ostreatus*, lane 11 *P. nebrodensis*, lane 12 positive control (pGEM-T::Lefks1-like; Lefks1-like statement for *L. edodes fks1*-like)

Table 2 Antioxidant and phenol-degrading enzymes expressed as units mg^{-1} protein in different media (MEP, OMW, PHE, LIP) in *L. edodes* smr 090 7 days after inoculation. Data represent the mean \pm SEM for each time point for mycelia ($n=3$) from three different experiments

Enzyme	Medium			
	MEP	OMW	PHE	LIP
Laccase	0.9 \pm 0.01	8 \pm 0.8**	70 \pm 5**	0.74 \pm 0.01
SOD	60 \pm 5	250 \pm 10**	100 \pm 0.5*	20 \pm 0.6
CAT	140 \pm 10	350 \pm 20**	300 \pm 5**	50 \pm 4

* $P < 0.01$

** $P < 0.001$

two positive signals and thus could indicate the presence of a single-copy *fks1*-like gene in the genome of these mushrooms. The patterns originating from *EcoRI* restriction could indicate the presence of one *fks1*-like gene in *P. ostreatus* and *P. eryngii* and at least two in *L. edodes*, *A. auricula-judae* and *P. ferulae*; and other patterns suggest

Fig. 6 a,b RT-PCR analyses of *L. edodes fks1*-like gene. Data represent the mean \pm SEM for each time point for mycelia ($n=3$) from three different experiments. **a** RT-PCR analysis of the *fks1*-like gene in *L. edodes* grown on MEP (control), OMW, PHE and LIP at 7, 14, 21, 28, 34 and 41 days. Arrowheads A, B Position of 420-bp *fks1*-like mRNA and 250-bp 18S rRNA products, respectively. **b** Gene transcript quantity was measured by relative RT-PCR using the internal standard 18S RT-PCR signal as the denominator, as described in the Materials and methods. CONT MEP

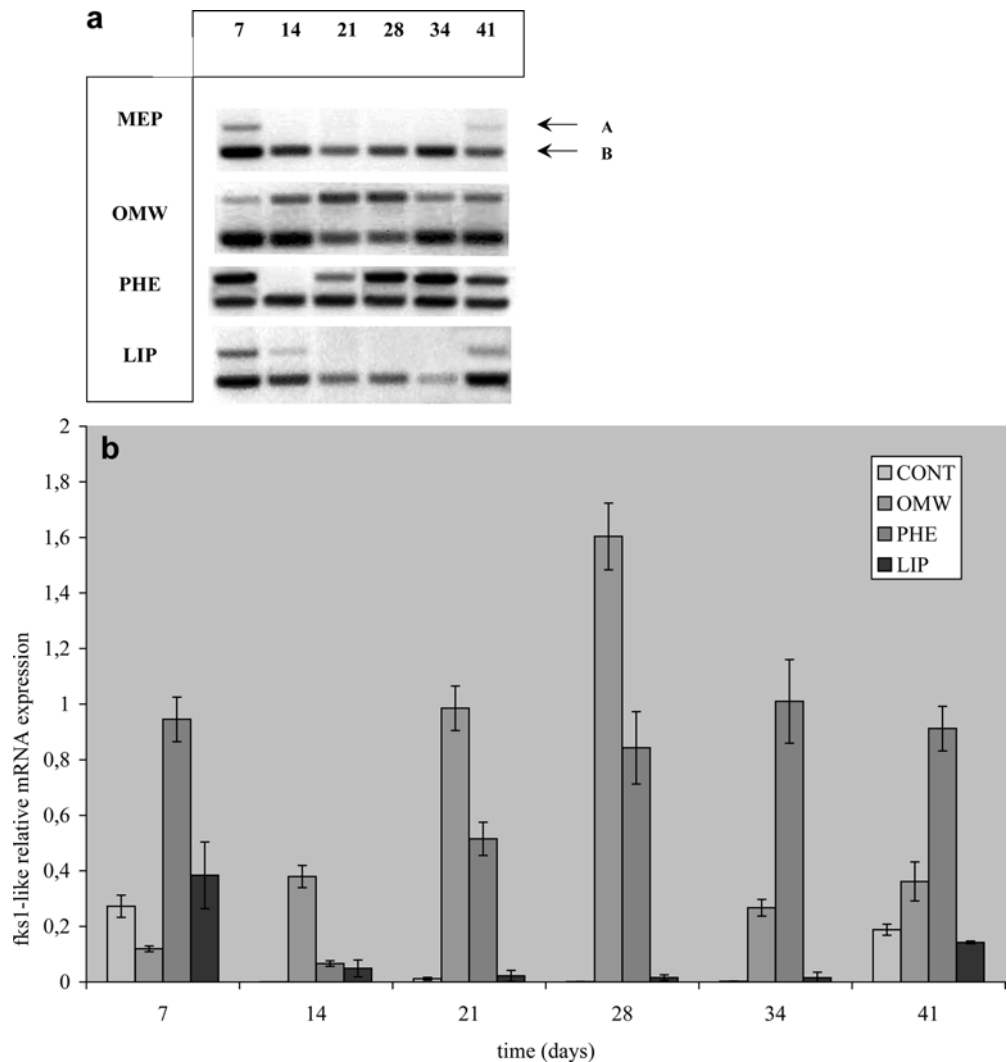
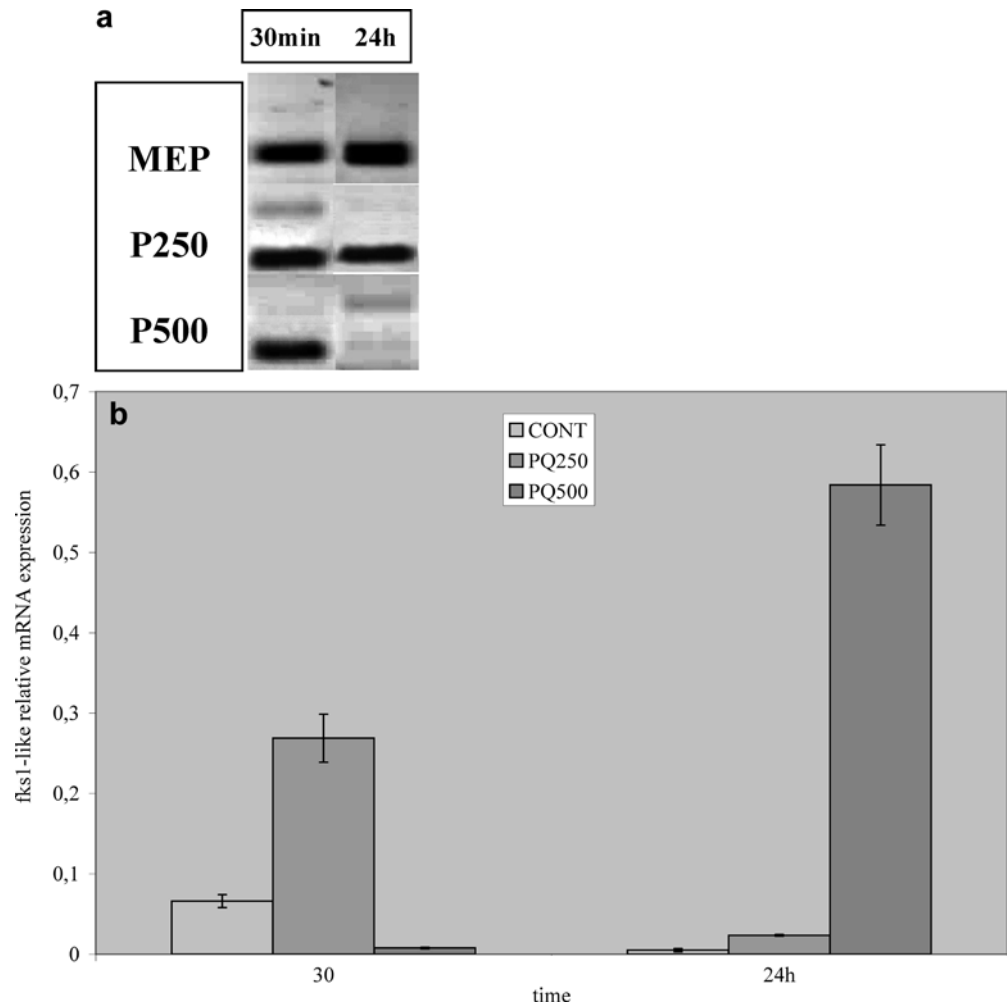


Fig. 7 a,b Effect of PQ on *L. edodes fks1*-like gene. Data represent the mean \pm SEM for each time point for mycelia ($n=3$) from three different experiments. **a** RT-PCR analysis of the *fks1*-like gene in *L. edodes* grown for 7 days on MEP (control), PQ250 (MEP + 250 μ M PQ) and PQ500 (MEP + 500 μ M PQ) at 30 min and 24 h after the addition of PQ. *Arrowheads A, B* Positions of 420-bp *fks1*-like mRNA and 250-bp 18S rRNA products, respectively. **b** Gene transcript quantity was measured by relative RT-PCR using the internal standard 18S RT-PCR signal as the denominator, as described in the Materials and methods



the presence of multiple *fks1*-like genes in the other mushrooms.

Fks1-like specific semi-quantitative RT-PCR analysis

RNA was isolated from another set of cultures and RT-PCR analysis of *fks1*-mRNA in *L. edodes* was carried out on days 7, 14, 21, 28, 34 and 41. No homology was found between the first 100 bp of the *L. edodes fks1*-like 420-bp fragment and the complete *fks2* sequence of *S. cerevisiae*. *L. edodes* *fks1*-specific primers were drawn to the not-conserved region between these two genes, in order to most likely avoid amplicons from this alternative *fks2* β -glucan synthase in the RT-PCR assay, whose induction was not considered in this present work. A single amplification fragment (420 bp) was detected, following *fks1*-like specific RT-PCR amplification of *L. edodes* (Fig. 6a). In the case of *L. edodes*, the inclusion of primers 18S forward and reverse in the *fks1*-like RT reaction mixtures generated two bands: the 420-bp *fks1*-like fragment and the 250-bp 18S fragment (Fig. 6a). In *L. edodes*, OMW and PHE enhanced the transcription of *fks1*-like mRNA, although at different times. In particular, OMW stimulated *fks1*-like expression starting from

14 days, compared with the control medium, while PHE had a greater effect on this induction at almost all time-points (Fig. 6a,b). When 250 μ M PQ and 500 μ M PQ were added to *L. edodes* grown on MEP for 7 days, the *fks1*-like mRNA transcription was, respectively, enhanced of about 5-fold and 100-fold after 24 h (Fig. 7a,b).

Analysis of laccase, SOD and CAT

Both OMW and PHE media enhanced enzymatic activities in *L. edodes* (Table 2). With respect to the control medium, a 9-fold increase in laccase activity ($P<0.001$) was recorded in the case of *L. edodes* grown on OMW; and an increase of 78-fold was assayed for the same fungus when grown on PHE. *L. edodes* antioxidant enzymes SOD and CAT were stimulated by OMW and PHE. In particular, in comparison with the control, the antioxidant system expressed as SOD and CAT showed an increase of 3-fold and 2-fold when *L. edodes* was grown on OMW and PHE, respectively (Table 2).

Discussion

The mycelia of saprophytic mushrooms were able to grow on OMW, although initially depressed and/or delayed in the presence of inhibitory compounds, such as phenols (Tomati and Galli 1992; Elisashvili et al. 2002). This delay may be due to mechanisms developed by the organism to overcome unfavourable environmental conditions created by the presence of toxic compounds. The increase of CAT, SOD and laccase activity, as reported in the case of *L. edodes* during the early stage of growth on OMW and PHE, would suggest that the antioxidant and degrading activity of those enzymes probably prepared more suitable conditions for the growing organism (Zjalic et al. 2002).

Changes in fungal metabolism and the composition of cell wall polysaccharides occur under stress or adverse conditions (Smits et al. 2001). In response to some stress signals, fungi have mechanisms to preserve cell wall integrity. The main fungal responses to cell wall stress are: the synthesis of several cell wall components, the activation of the glucan synthase regulatory subunit RHO that interacts with FKS1 or FKS2 and the up-regulation of β -1,3-glucans synthesis by transcriptional activation of the alternative synthase, FKS2 (Bickle et al. 1998; Smits et al. 2001). Alteration of the cell wall structure could increase glucan synthesis, as reported in the case of *L. edodes* grown on OMW (Belardinelli et al. 2000).

OMW greatly stimulated the β -1,3-glucan synthase activity of *L. edodes* and *P. ostreatus*. Inhibition, although to a different extent, was recorded in *A. aegerita*, *P. eryngii* and *A. mellea*. However, these displayed the highest glucan synthase activity in both media, compared with all other fungi. No effect was recorded for the other mushrooms. It is known that β -glucan synthases represent a group of enzymes that share, in the fungi, only a few common features that determine a different specificity for the substrate (GDPG, UDPG, ADPG), optimal pH (5.8–7.8), temperature (20–37°C) and activation or inactivation by different divalent metal ions (Mg, Ca, Fe; Ruiz-Herrera 1991).

To highlight the differences in β -1,3 glucan synthase activity, an alignment of the theoretical translation of the amplicons generated through PCR with degenerated primers was performed. The primers were chosen in the highly conserved glucan-synthase domain of *F. neoformans* FKS1. The peptide alignment showed that all the fragments analysed present a degree of homology between 60% and 82.5% when compared with *F. neoformans* FKS1. Only 54% of the amino acids present in the fragments taken into consideration is common to all samples (Fig. 4). These differences found in a relatively short and highly conserved region were highlighted by a Southern blot analysis, using as probe the *fks1*-like of *L. edodes*. All the fungi showed at least a single copy (range 1–4 copies) of the *fks1*-like gene (Fig. 5). The polymorphism of the restricted fragments showed a different genomic organisation of the *fks1*-like gene in the mycelia analysed. This molecular approach seems to indicate that the variation in enzymatic activity could be in part

ascribed to the diversity of the *fks1*-like gene found in the fungi analysed. Nevertheless, it cannot be excluded that the different enzymatic activities may be due to the particular environmental conditions in which the fungi grown. In fact stress-inducing molecules, such as phenols, can alter the cell wall, stimulating the biosynthesis of its components (Soleas et al. 1997; Schouten et al. 2002).

To gather further information about the OMW action, PHE and LIP fractions were added separately to the control medium, since the organic fraction of OMW is mainly composed of lipids and phenols (Di Giovacchino et al. 1988; Zjalic et al. 2002). The effect of these fractions in *L. edodes* was studied in order to evaluate their ability to induce enzymatic activity (Fig. 3a). The spectrofluorimetric assay showed an enhanced β -1,3-glucan synthase activity in the presence of both PHE and OMW, the values being comparable for the two substrates. This was not the case for LIP. The enzyme seemed temporally regulated, behaviour similar to other inducible fungal enzymes, such as chitin synthase (Wang and Szaniszló 1999) and laccase (Dekker et al. 2002; Chen et al. 2003). To elucidate the way in which the enzyme was stimulated (release from an inactive form or mRNA neotranscription), a RT-PCR analysis was carried out on mycelia grown on the different substrates. The stimulation observed in the OMW and PHE media was connected with a transcriptional regulation of *fks1*-like mRNA (Fig. 6a,b).

The enhancement of β -glucan synthase activity did not seem correlated with an increase in fungal growth. In fact the LIP treatment highly enhanced fungal growth without significantly affecting enzyme activity and *fks1*-like transcription.

The transcriptional regulation of *fks1* in stress conditions was studied on the yeast model (Smits et al. 2001; Bickle et al. 1998). Those authors suggested that β -1,3-glucan synthase activation was due to a modulation of the RHO regulatory subunit using a GDP-GTP exchange factor protein that is stimulated by stress-inducing compounds present in the media. Oxidative stress modulates cell wall biosynthesis in yeast, activating RHO that subsequently regulates FKS activity, both at a transcriptional and enzymatic level. A similar mechanism could act as a switch for the β -glucan biosynthesis in *L. edodes*. In fact, conditions of oxidative stress may occur in the presence of OMW, due to either the presence of oxidising phenol compounds or the reactive oxygen species (ROS) formed during the action of laccase, particularly stimulated by OMW and PHE (Schultz et al. 1990; Giovannozzi-Sermanni et al. 1991; Hammel et al. 2002). The release of oxidative products during fungal growth could alter oxidative homeostasis and stimulated the secretion of extracellular antioxidant enzymes or the accumulation in the cell wall of antioxidant compounds, such as β -glucans (Popov and Lewin 1999). In fact, many fungal β -glucans show an antioxidant activity, as reported for gluconoxylomannan in *Tremella* spp (Reshetnikov et al. 2000) and lentinan in *L. edodes* (Fanelli et al. 2000). Thus, the presence of ROS, highly reactive and potentially toxic in high concentrations, could be an external stimulus

for modifying the cell wall composition, to prevent penetration by toxic compounds. This hypothesis could be confirmed by the use of the radical-generator paraquat in the growth media (Fig. 7a,b). In fact, this compound enhanced the activity of β -glucan synthase and the transcription of the *fksI*-like gene in the conditions analysed. The increased β -glucan synthase and β -glucans production could be considered as a fungal defence mechanism for overcoming stress conditions. Fungal growth on a potentially pollutant substrate could have a double effect: the degradation of agricultural wastes and the production of pharmacologically active compounds.

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References

- Belardinelli M, Galli E, Tomati U (2000) Lentinan production by *Lentinula edodes* grown in oil mill wastewaters. Proc World Conf Biomass Energy Indp 1:251
- Bickle M, Delley PA, Schmidt A, Hall MN (1998) Cell wall integrity modulates RHO1 activity via exchange factor ROM2. EMBO J 17:2235–2245
- Bohn JA, Bemiller JH (1995) (1 \rightarrow 3)- β -D-glucans as biological response modifiers: a review of structure–functional activity relationships. Carbohydr Polym 28:3–14
- Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein–dye binding. Anal Biochem 72:248–254
- Corpet F (1988) Multiple sequence alignment with hierarchical clustering. Nucleic Acid Res 16:10881–10890
- Chen S, Dengo M, Ge W, Buswell JA (2003) Induction of laccase activity in the edible straw mushroom, *Volvariella volvacea*. FEMS Microbiol Lett 218:143–148
- Di Giovacchino L, Mascolo A, Seghetti L (1988) Sulle caratteristiche delle acque di vegetazione delle olive. Riv Ital Sost Grasse 65:481–488
- Dekker RFH, Barbosa AM, Sargent K (2002) The effect of lignin-related compounds on the growth and production of laccases by the ascomycete, *Botryosphaeria* sp. Enzym Microb Technol 30:374–380
- Doohan FM, Weston G, Rezanoor HN, Parry DW, Nicholson P (1999) Development and use of a reverse transcription-PCR assay to study expression of Tri 5 by *Fusarium* species in vitro and in planta. Appl Environ Microbiol 65:3850–3854
- Elisashvili V, Kachlishvili E, Tsiklauri N, Khardziani T, Bakradze M (2002) Physiological regulation of edible and medicinal higher Basidiomycetes lignocellulolytic enzyme activity. Int J Med Mushroom 4:159–166
- Fanelli C, Tasca V, Ricelli A, Reverberi M, Zjalic S, Finotti E, Fabbri AA (2000) Inhibiting effect of medicinal mushroom *Lentinula edodes* (Berk.) Sing. (Agaricomycetideae) on aflatoxin production by *Aspergillus parasiticus* Speare. Int J Med Mushroom 2:229–236
- Furue H (1986) Biological characteristic of schizophyllan (SPG) and its clinical effect. Med Immunol 12:65–77
- Giovannozzi-Sermanni G, Perani C, Porri A, De Angelis F, Barbaruto MV, Mendola D, Nicoletti R (1991) Plant cell wall degradation by means of *L. edodes* and chemical characterization of produced soluble lignocellulose. Agrochimica 35:174–189
- Hammel KE, Kapich AN, Jensen KA, Ryan ZC (2002) Reactive oxygen species as agents of wood decay by fungi. Enzym Microb Technol 30:445–453
- Hargin JM, Obst JR (1973) Syringaldazine as effective reagent for detecting laccase and peroxidase in fungi. Experientia 29:381–386
- Kopecka M, Kreger DR (1986) Assembly of microfibrils in vivo and in vitro from (1 \rightarrow 3)- β -D-glucan synthesised by protoplasts of *Saccharomyces cerevisiae*. Arch Microbiol 143:387–395
- O'Neil P, Davies S, Fielden EM (1988) The effect of pH and various salts upon the activity of a series of superoxide dismutases. Biochem J 241:41–46
- Popov I, Lewin G (1999) Antioxidant homeostasis: characterization by means of chemiluminescent technique. Methods Enzymol 300:437–456
- Reiskind JB, Mullins JT (1981) Molecular architecture of the hyphal wall of *Achlya ambisexualis* Raper. I. Chemical analyses. Can J Microbiol 27:1092–1099
- Reshetnikov SV, Wasser SP, Nevo E, Duckman I, Tsukor K (2000) Medicinal value of the genus *Tremella* Pers. (Heterobasidiomycetes). Int J Med Mushroom 2:169–193
- Ruiz-Herrera J (1991) Biosynthesis of β -glucans in fungi. Int J Gen Mol Microbiol 60:73–81
- Ruiz-Herrera J, Sentandreu R (1991) Fungal cell wall synthesis and assembly. In: McGinnis MR, Borgers M (eds) Current topics in medical mycology, vol 3. Springer, Berlin Heidelberg New York, pp 169–217
- Sanchez-Hernandez E, Garcia-Mendoza C, Novaes-Ledieu M (1990) Chemical characterization of the hyphal walls of the basidiomycete *Armillaria mellea*. Exp Mycol 14:178–183
- Schouten A, Wagemakers L, Stefanato FL, Van Der Kaaj R, Van Kan JAL (2002) Resveratrol acts as a natural profungicide and induces self-intoxication by a specific laccase. Mol Microbiol 43:883–894
- Schultz TP, Hubbard TF, Jin L, Fisher TH, Nicholas DD (1990) Role of stilbenes in the natural durability of wood: fungicidal structure–activity relationships. Phytochemistry 29:1501–1507
- Shedletzky E, Unger C, Delmer DP (1997) A microtiter-based fluorescence assay for (1,3)- β -glucan synthases. Anal Biochem 249:88–93
- Smits GJ, Van der Ende H, Klis FM (2001) Differential regulation of cell wall biogenesis during growth and development in yeast. Microbiology 147:781–794
- Soleas GJ, Diamandis EP, Goldberg DM (1997) Resveratrol: a molecule whose time has come? And gone? Clin Biochem 30:91–113
- Song CH, Cho KY, Nair NG, Vine J (1989) Growth stimulation and lipids synthesis in *Lentinula edodes*. Mycology 81:514–522
- Song CH, Cho KY, Nair NG (1990) The incorporation of labeled precursor into lipids of *Lentinula edodes* and the effect of lipids on the uptake of nutrients. Mushroom J Trop 11:1–12
- Surarit R, Gopal PK, Shepherd MG (1988) Evidence for a glycosidic linkage between chitin and glucan in the cell wall of *Candida albicans*. J Gen Microbiol 134:1723–1730
- Tomati U, Galli E (1992) The fertilizing value of waste waters from the olive processing industry. In: Kubat J (ed) Humus, its structure and role in agriculture and environment. (Proceedings of the tenth symposium humus et planta). Prague, pp 117–126
- Wang Z, Szaniszló PJ (1999) *WdCHS3*, a gene that encode a class III chitin synthase in *Wangiella (Exophiala) dermatitidis*, is expressed differentially under stress conditions. J Bacteriol 182:874–881
- Wasser SP, Weiss AL (1999) Medicinal properties of substances occurring in higher Basidiomycetes mushrooms: current perspectives (review). Int J Med Mushroom 1:31–62
- Yadomae T (1992) Immunopharmacological activity of β -glucan: structure–activity relationship in relation to various conformations. Jpn J Med Mycol 33:67–277
- Yadomae T, Ohno N (1996) Structure–activity relationship of immunomodulating (1 \rightarrow 3)- β -D-glucan. Recent Results Dev Chem Pharm Sci 1:23–33
- Zevenhuizen LPTM, Bartnicki-Garcia S (1968) Chemical structure of the insoluble hyphal wall glucan of *Phytophthora cinnamomi*. Biochemistry 8:1496–1502
- Zjalic S, Fabbri AA, Ricelli A, Reverberi M, Galli E, Fanelli C (2002) Effect of olive oil mill waste waters on the edible and medicinal mushroom *Lentinula edodes* (Berk.) Sing. Growth and lignin degrading enzymes. Int J Med Mushroom 4:85–94