



Effect of co-cultivation of two *Pleurotus* species on lignocellulolytic enzyme production and mushroom fructification

Maira Carabajal^a, Laura Levin^a, Edgardo Albertó^b, Bernardo Lechner^{a,*}

^aLaboratorio de Micología Experimental, Departamento de Biodiversidad y Biología Experimental (DBBE), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, PROPLAME-PRHIDEB-CONICET, 1428 CABA Buenos Aires, Argentina

^bInstituto de Investigaciones Biotecnológicas (IIB-INTECH), CONICET-UNSAM, C. C. 164, 7130 Chascomús, Buenos Aires, Argentina

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ABSTRACT

Co-cultivation of mushroom species produce changes on fungal enzyme production and fructification that were not in-depth studied hitherto. This work was conducted with the aim of studying how the interactions between *Pleurotus ostreatus* and *Pleurotus citrinopileatus* in a dual culture affect the lignocellulolytic enzymes production, influence yield values and modify the morphological properties of basidiomata obtained. With this purpose monocultures of both species in a straw-based substrate were investigated, as well as dual cultures with 3 different spatial patterns (one fungus in the upper portion of the bag and the second in the bottom, the opposite spatial pattern, and a mix of spawn from both, scattered in each bag). Co-cultivation did not increase basidiomata production, moreover, it inhibited carpophore development of *P. ostreatus* in the mix, and decreased basidiomata stem's length significantly. Biological efficiencies achieved in monocultures of *P. ostreatus* and *P. citrinopileatus* were 95.3% and 67.6% respectively. Different spatial patterns affected not only enzyme production but also mycelial yield. Cellulolytic and xylanolytic activities detected increased significantly when *P. ostreatus* was inoculated in the upper portion of the bag and *P. citrinopileatus* in the bottom part, but co-cultivation did not stimulate ligninolytic enzyme production. The highest laccase (8.2 U g^{-1}) and Mn-peroxidase (7.5 U g^{-1}) activities were detected in monocultures of *P. ostreatus*, in coincidence with maximum lignin degradation (28%).

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1. Introduction

Species of the genus *Pleurotus* (Fr.) P. Kumm known as oyster mushrooms, rank third place in worldwide production of edible mushrooms after *Agaricus bisporus* and *Lentinula edodes*, and comprise various edible species with medical, biotechnological and environmental applications (Cohen et al., 2002). *Pleurotus* species present high adaptability to produce basidiomata within a wide variety of agro-industrial lignocellulosic wastes due to their production of ligninolytic and hydrolytic enzymes (Mikiashvili et al., 2006).

The current knowledge available on the effects of solid-state co-cultivation of two white rot fungi on the production of lignin-degrading enzyme activities is limited. Co-cultivation of fungi could imply oxidative stress on both fungal partners, and it may accelerate a fungal metabolic switch to secondary metabolism, thus

stimulating wood decay and production of lignin-degrading enzymes (Chi et al., 2007). Mixed fungal cultures could lead to a higher enzyme production through synergistic interactions, but the final result seems to depend on many factors such as the particular species combination, the mode of interaction between species and the microenvironmental or nutritional conditions in the substrate under colonization (Gutierrez-Correa and Tengerdy, 1997; Chi et al., 2007). Nevertheless, experimental evidence suggests that the competition for space and nutrients may result in enhanced degradation of lignin (Sundman and Näse, 1972; Asiegbu et al., 1996; Watanabe et al., 2003; Chi et al., 2007) and in elevated production of lignin modifying enzymes, such as laccase (Freitag and Morrell, 1992; White and Boddy, 1992; Score et al., 1997; Iakovlev and Stenlid, 2000; Baldrian, 2004; Chi et al., 2007).

Interactions with *Pleurotus ostreatus* appeared to be especially valuable regarding wood dry weight loss and lignin loss. In a study published by Sundman and Näse (1972), mycelial interactions with *P. ostreatus* resulted in synergistic effects during the degradation of lignin and lignosulfonates with most of the twenty fungal species studied. Higher enzyme activities have been reported for

* Corresponding author. Tel.: +54 11 47872706; fax: +54 11 45763384.

E-mail address: bernardolechner@gmail.com (B. Lechner).

co-cultures of *Phanerochaete chrysosporium* and *P. ostreatus* when compared to the respective monocultures (Verma and Madamwar, 2002). Thus, *P. ostreatus* appears to possess specific potential to be used in co-cultures (Chi et al., 2007).

Environmental factors such as water availability, temperature, pH, and their interactions, have significant impact on mycelial colonization and fruiting potential (Magan, 2008). Fungi have to compete for space and nutrients during co-cultivation, and the onset of fruiting is observable after a stimulus, such as physiological stress (Lechner and Papinutti, 2006). Not only this source of stress, but also the change in environmental conditions, may decrease the mushroom production period. Mushroom farmers are not only interested in obtaining increasing yields, but also in offering high quality products, where the morphology of basidiomata is important. Colour and size of pileus and stem are features highly appreciated by farmers (Lechner and Albertó, 2011). Co-cultivation could allow producing basidiomata of two different species in the same bag simultaneously, saving time to mushrooms growers. Additionally, the stress caused by competition between two different species on a same substrate could reduce the length of stages of mushroom production or increase yields of one or both species, rendering a clear economical benefit. To the best of our knowledge the effect of co-cultivation on basidiomata yield and lignocellulolytic enzymes production has not been tested up to now. The results achieved in this investigation will contribute to expand the knowledge on this area.

The objectives of this work were: (i) to study if the interactions between two *Pleurotus* species affect the production of lignocellulolytic enzymes and their ability to degrade the substrate; (ii) to evaluate if co-cultivation influences yield values and morphological properties of basidiomata obtained. To achieve these aims monocultures of *P. ostreatus* and *Pleurotus citrinopileatus* in a straw-based substrate were investigated, as well as dual cultures with different spatial patterns.

2. Materials and methods

2.1. Strains

Pleurotus sapidus, BRAZIL, EMBRAPA, commercial strain, Leg. A. Fontes Urben. ICFC 631/05 (ICFC = IIB-INTECH Collection of Fungal Cultures); *Pleurotus sajor-caju*, ARGENTINA, CHASCOMÚS, commercial strain, isolated in 2005, ICFC 632/05; *P. ostreatus*, BRAZIL, SÃO PAULO, Molle das Cruces, Leg. E. de Souza, isolated in 2006, ICFC 688/06; *Pleurotus djamor*, AUSTRIA, isolated in 1993, Leg. Hilber. incorporated in 2000, ICFC 376/00; *P. ostreatus*, ARGENTINA, CHASCOMÚS, Leg. E. Albertó, isolated in 1999 from a commercial mushroom farm, ICFC 153/99; *P. citrinopileatus*, ARGENTINA, SANTA FE, Leg. E. Albertó, commercial strain, 2006, ICFC 707/06.

2.2. Strain selection

Agar plates with Nobles' medium (Nobles, 1948) were inoculated with an agar plug (0.7 cm diam.) cut from the advancing margin of a 5-d-old colony grown on MEA (1.2% malt extract, glucose 1%, agar 2%). Incubation was carried out at 25 °C. Radial growth was measured.

2.3. Spawn production

Fifty ml glass jars filled with boiled wheat grains and 1% w/w CaCO₃ were sterilized for 1.5 h at 121 °C, cooled and inoculated with an agar plug (1 cm diam.) cut from the advancing margin of a 5-d-old colony grown on Nobles' medium. Bottles were incubated in the dark, at 25 °C, with periodical shaking.

2.4. Substrate preparation and co-cultivation patterns

To obtain basidiomata, standard methods for fruiting species of *Pleurotus* were applied (Zadrazil, 1974; Lechner and Albertó, 2011). Polypropylene bags of 20 × 40 cm were filled with 150 g (dry weight) of substrate (97% wheat straw, 3% CaCO₃); humidity was adjusted (w/w) to 70%. Bags were stopped with cotton plugs held by PVC (polyvinyl chloride) cylinders and autoclaved at 121 °C, for 2 h. After cooling, bags were inoculated with 3% (wet weight) of spawn. Monocultures of *P. ostreatus* (PO) and *P. citrinopileatus* (PC) were assayed as well as dual cultures with three different spatial patterns: Pattern 1) co-cultivation of *P. ostreatus* in the upper portion of the bag and *P. citrinopileatus* in the bottom part of the bag (PO/PC); Pattern 2) co-cultivation of *P. citrinopileatus* in the upper portion of the bag and *P. ostreatus* in the bottom part of the bag (PC/PO); Pattern 3) a mix of spawn from *P. ostreatus* and *P. citrinopileatus* scattered in each bag (PC + PO). For spawning practice, the sterilized bag's content was emptied into a plastic tray in a laminar flow chamber. Then bags were filled again, stopped and incubated at 25 °C in the dark until total substrate colonization. Each treatment consisted of ten replicates.

2.5. Fruiting conditions

After spawning run, six small cuts (20 mm long) were regularly made on the bag surface. Bags were transferred to a culture room (2.5 × 4.5 m) for basidiomata induction. Room conditions were controlled: temperature was kept at 18–20 °C with 9 h light /15 h dark photoperiod (20 W fluorescent light), humidity levels were kept between 75% and 85%; watering by spray (fog type) was automatically provided (5 min every 4 h).

2.6. Cropping period, crop yield and quality traits assessment

Two flushes were collected during the cropping period [defined as the time elapsed between the induction day and the last harvest day 120 days]. Mature basidiomata were collected and the following production and quality traits were registered: A) Production (harvested basidiomata); B) Primordia initiation (in days from the start of incubation); C) Biological efficiency (BE): the ratio of kg of fresh mushrooms harvested per kg of dry substrate and expressed as a percentage; D) Morphological quality traits: i) Pileus width and length; ii) Stem length and diameter.

2.7. Sample preparation

Samples of substrates colonized by mycelium were collected at three different stages of the solid-state fermentation: T1 (30 d), after complete colonization of the substrate; T2 (43–64 d), after the end of first flush; T3 (120 d), spent substrate after the last mushroom harvest. Entire solid cultures were dried at 90 °C, until constant weight, ground in a mortar, and stored until they were used for chitin and lignin determination. The fungal biomass content of dry-solid cultures was determined by N-acetyl-D-glucosamine (NAG) released from chitin after hydrolysis with 6 N HCl. Analytical grade NAG served as reference (Plassard et al., 1982). Lignin in the dried samples was determined by the TAPPI method (Tappi Methods, 1983). Crude extracts were obtained by adding 50 mM sodium acetate buffer pH 4.8 to the samples from each freshly harvested culture (5:1, w/w), stirring for 20 min, followed by filtration and centrifugation. The supernatants were stored at –20 °C until needed.

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