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Enzymatic mechanisms and detoxification of dry olive-mill residue by *Cyclocybe aegerita*, *Mycetinis alliaceus* and *Chondrostereum purpureum*

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ABSTRACT

The detoxification of dry olive-mill residue (DOR), a lignocellulosic by-product, was investigated using three agaric fungi: *Cyclocybe aegerita*, *Mycetinis alliaceus* and *Chondrostereum purpureum*. The lignin-modifying enzymes (LME) secretion pattern of the above-mentioned fungi such as DyP-type peroxidase (DyP), laccase (Lac), unspecific peroxygenase (UPO), and manganese peroxidase (MnP) was determined in presence and absence of DOR. In *C. aegerita*, a laccase (Lac) and unspecific peroxygenase (UPO) induction was found when DOR was present in the medium. Thus, these enzymes appeared to be responsible for DOR detoxification and, indirectly, its plant-growth-promoting effect. In the experiment performed with *M. alliaceus*, no differences were found in DyP-type peroxidase (DyP) secretion when the basal barley medium was supplemented with DOR. However, MnP and Lac activities in DOR-barley reached a maximum after 5 weeks of incubation with a concomitant decline in DOR phytotoxicity. *C. purpureum* completely eliminated DOR phytotoxicity but no significant production of LME was detected in soy or barley fungal media. Other enzymatic mechanisms were also investigated, in relation to hydrolytic enzymes and the intracellular system cytochrome P450 monooxygenase (CYPs). Our results suggest the participation of a complex enzymatic system (intra and extracellular) in *C. purpureum* for the biotransformation of DOR.

1. Introduction

Olive-oil production is centred mostly in Mediterranean Basin countries (Mateo and Maicas, 2015), with Spain topping the list of producers (1.1 million metric tonnes in 2013) (FAOSTAT, 2013). The current predominant method for the olive-oil extraction is a two-phase system of centrifugation that separates the oil from a semi-solid waste known as “alpeorujo” or two-phase olive-mill waste (TPOMW) (Morillo et al., 2009). TPOMW is dried and subsequently submitted to extraction with organic solvents (e.g. hexane) to generate “orujo” oil and a new lignocellulosic by-product called dry olive residue (DOR) (Arjona et al., 1999). DOR can be considered a phytotoxic agro-industrial waste due to its high organic load, polyphenol content and low-molecular-weight organic acids (Vlyssides et al., 2004). This by-product can also be considered a source of phenolic compounds for technological and pharmaceutical products, but the raw material would have to be submitted to a complex purification process to recover these compounds with the required quality (Forbes-Hernández et al., 2014). Despite their antioxidant properties, phenolic compounds have been identified as being responsible for the high phytotoxicity of olive-mill wastes (OMWs). Their negative effects are due to the generation of reactive oxygen species, some of which undergo an auto-oxidation reaction (García-Sánchez et al., 2012). Therefore, the improper disposal of olive-mill residues can cause environmental problems (Dermeche et al., 2013).

To ensure a more environmentally friendly and sustainable industry, proposals have been made to use agricultural wastes as raw materials for new products or applications. Several physicochemical approaches have been employed to treat OMWs such as ozonization, electrocoagulation, and physical fractionation (Chedeville et al., 2009; Hanafi et al., 2010; Aranda et al., 2012). They are, however, difficult to apply in practice due to several

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complications related to large-scale implementation, such as cost-efficiency and environmental or technical concerns, since physicochemical treatments can produce toxic sludge or air pollutants (Kapellakis et al., 2008).

The phytotoxic and antimicrobial properties of OMWs impede their direct application to the soil. However, the high content in organic matter and plant nutrients makes them potentially useful as fertilizers and/or soil amendments (Dermeche et al., 2013; Siles et al., 2013). The biological treatment of OMWs is an alternative approach to their detoxification. Composting is one of the most widespread techniques for phytotoxicity reduction, phenolic removal and fertilizer production from such wastes (Monetta et al., 2014). Nonetheless, composting of the residue requires a longer incubation time to reach maturity compared to monoculture fermentations with specific microorganisms. The bioconversion of DOR by white-rot fungi (WRF) in monoculture fermentations represents one of the most efficient treatment methods (Reina et al., 2013).

WRF comprise numerous fungi with the ability to secrete class II peroxidases (PODs, EC 1.11.1.-), including lignin peroxidases (LiPs, EC 1.11.1.14), manganese peroxidases (MnPs, EC 1.11.1.13), and versatile peroxidases (VPs, EC 1.11.1.16) (Hatakka, 1994). These fungi can transform lignocellulosic wastes into valuable nutritional ingredients for plants by degrading lignin and converting complex polysaccharides into simple sugars (Sharma and Arora, 2015). Among the oxidative enzymes involved are: PODs, heme-thiolate peroxidases (HTP, EC 1.11.2-), dye-decolorizing peroxidases (DyPs, EC 1.11.1.19), and laccases (Lacs, EC 1.10.3.2). Heme-thiolate peroxidases are a different class of peroxidase enzymes, which catalyse the transfer of peroxide-oxygen to substrate molecules. The unspecific peroxygenases (UPOs, EC 1.11.2.1) belong to this group. DyPs are able to oxidize a wide range of organic substrates, some of which are difficult to transform by the typical peroxidases (e.g. dyes derived from anthraquinone). Lacs have a low oxidation potential (compared to peroxidases), but they are able to oxidize chains of non-phenolic aromatic rings with the aid of a small diffusible mediator which they previously activate (Areskogh et al., 2010). There is broad agreement about the participation of PODs and Lacs in lignin degradation (Hammel and Cullen, 2008; Munk et al., 2015). However, the role of DyPs and UPO in ligninolysis requires further research, although UPOs seem to participate in the polymerization of aromatic water soluble compounds of lignocellulose residue extracts (Reina et al., 2013). These biocatalysts are also able to oxidize non-phenolic lignin-model compounds and they seem to be involved in the bioconversion of methoxylated compounds that can be derived from lignin (Kinne et al., 2011). In addition to the secreted enzymes, intracellular enzymes included in the microsomal fractions can also take part in the ligninolytic process by metabolizing the numerous potential toxic compounds derived from wood and organic matter transformation (Morel et al., 2013). This intracellular enzymatic system, represented by multigenic families, includes cytochrome P450 monooxygenases (CYPs, EC 1.14.14.1). CYPs are also heme-thiolate proteins which are involved in different xenobiotic detoxification pathways, the most important of which is the hydroxylation of substrates. In basidiomycetes, these groups of enzymes can break down various xenobiotics and lignin metabolites released by the action of extracellular peroxidases (Ide et al., 2012; Doddapaneni et al., 2013). In addition, non-enzymatic mechanisms participate in the conversion of lignocellulose, which can be achieved with the aid of reactive oxygen species generated via Fenton reactions (Hammel et al., 2002).

The agaric fungus *Cyclocybe aegerita* (formerly *Agrocybe aegerita*) has been extensively studied in the last decade for its ability to secrete a stable UPO (Ullrich et al., 2004), as have been species of the genus *Mycetinis* (formerly *Marasmius*), which is well known for its DyP secretion (Ferreira Gregorio et al., 2006; Scheibner et al., 2008). *Chondrostereum purpureum* is a ligninolytic fungus with the ability to colonize hardwoods and produce oxidative enzymes (Vartiamäki et al., 2008; Bellgard et al., 2014). Although extensive research has been performed on the role of *C. purpureum* as a biocontrol agent with potential for biodegrading lignin components, its ligninocellulolytic enzyme secretion pattern has not yet been studied.

2. Materials and methods

2.1. Dry olive mill residue (DOR)

DOR was obtained from Sierra Sur Olive Oil Company in Granada, Spain (2010–2011 harvest). It was passed through 8-mm sieves, autoclaved in three cycles, and stored at 4 °C before use.

2.2. Organisms and culture conditions

The agaric fungi *M. alliaceus* (private collection of IHIZ, Germany) and *C. aegerita* (DSMZ22459) as well as *C. purpureum* (DSMZ4894) were pre-cultured at 24 °C on 2% MEA for 2 weeks to obtain fresh inoculum.

Solid-state fermentation (SSF) was performed in 250-ml Erlenmeyer flasks. Support media were barley grain for all the fungi except for *C. purpureum*, for which barley or soy seeds (18 g) were used, and 30 ml of distilled water was added prior to autoclaving. These media were inoculated with 9 ml (50% v/w) of a fungal homogenized suspension of 4 fully covered fungal agar plates (2 weeks old) added to 80 ml of sterile water and blended with an Ultra-Turrax (IKA T25). After 1 week of incubation at 28 °C, half of the flasks were mixed with 18 g of DOR (50% w/w) while the others were kept without DOR, as barley or soy control samples. The same treatments were set up with heat-inactivated mycelium in order to detect any possible phenol adsorption. Flasks were sampled after 0, 1, 2, 3, 4, and 5 weeks. DOR was separated manually from the support media barley or soy and part of the waste was dried for 1 week in an oven at 40 °C in order to perform phytotoxicity experiments (Reina et al., 2013). The rest of the fermented solids was suspended in water (1:5 w/v) and the mixture was shaken 2 h on a rotatory shaker at 120 rpm a room temperature, centrifuged and filtered. Filtrates were used to measure extracellular enzyme activities, pH, total phenol content, and the molecular-mass distribution of water-soluble aromatic fragments released from DOR.

As a means of obtaining mycelia for *C. purpureum* intracellular enzymes determination, a submerged fermentation (SF) was performed with a suspension of soy liquid medium (2% of soybean flour in water) and an aqueous extract of DOR (ADOR) (Aranda et al., 2004). The composition of soybean flour was (per 100 g): Fats (21 g), carbohydrates (23 g), proteins (40 g), Na (1 mg), fibre (3 g), Ca (210 mg), Fe (69 mg), K (1670 mg) vitamins: B1 (0.581 g); B2 (1.16 g), B6 (0.461 g). Non-inoculated soy and ADOR-soy media were used as controls. Each flask was inoculated using 4 ml of a homogenized fungal suspension obtained as described above. ADOR was added after 7 days of incubation and, at the same time, 1-aminobenotriazole (ABT) was added to the ADOR-soy media in a 1-mM concentration. ABT, an inhibitor of CYP, has been extensively used as an inhibitor of oxidative drug metabolism (Mico et al., 1988). The *C. purpureum* cultures were stirred (100 rpm) at 25 °C for 15 days with sampling at 5 and 15 days. Samples were filtered and fungal biomass was used for intracellular determination of CYPs.

SSF and SF experiments were performed in triplicates for each treatment and week of incubation, and the data were reported as the mean values with standard deviation of triplicates.