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Technical Note

# Transformation of the water soluble fraction from "alpeorujo" by *Coriolopsis rigida*: The role of laccase in the process and its impact on *Azospirillum brasiliense* survival

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ABSTRACT

The objective of this work was to evaluate the ability of the white rot basidiomycete *Coriolopsis rigida* to detoxify the water soluble fraction from "alpeorujo" (WSFA), a solid by-product produced by the olive oil extraction industry and characterized by a high concentration of phenols which limits its use as fertilizer and/or amendment. *C. rigida* reduced the phenol content in the liquid media supplemented with WSFA at 10 and 20% (v/v) after 15 d of incubation. The analysis of WSFA toxicity after fungal treatment showed that *C. rigida* was responsible for a significant increase in the survival rate of *Azospirillum brasiliense*, a $N_2$ fixing soil rhizobacterium which promotes plant growth.

Supplementation of culture medium with $CuSO_4$ (300 μM) resulted in strong laccase induction thus facilitating higher phenol reduction and detoxification of WSFA. *In vitro* reactions using a crude extracellular preparation from laccase-active *C. rigida* showed phenol removal as well as detoxification of the WSFA at 20%. These results suggest that *C. rigida* reduces the phenol content of the WSFA through the effect of laccase on free phenolic compounds consequently decreasing the toxic effect on *A. brasiliense*, which suggests that the enzyme plays an important role in the process. These findings have implications in the management and revalorization of olive-mill residues treated with laccase-producing fungi and their potential impact on integrative agricultural systems including organic residues and the co-inoculation with microorganisms which can facilitate the growth of plants of agricultural interest.



## 1. Introduction

The use of the two-phase centrifugation system for olive oil extraction generates high amounts of a solid by-product called "alpeorujo" (approximately 800 kg $t^{-1}$ of processed olives) (Alburquerque et al., 2004). The water soluble fraction of "alpeorujo" (WSFA) contains polyphenols and simple aromatic compounds, which are structurally heterogeneous and inhibit microorganism and plant growth such as the main monomeric phenols tyrosol and hydroxityrosol (Aranda et al., 2006; de la Rubia et al., 2008). Proper handling and detoxification practices are therefore required if "alpeorujo" is to be revalorized as a potential fertilizer or amendment (Sampedro et al., 2005; Alburquerque et al., 2009; Federici et al., 2009). Different methods have been developed to reduce the phytotoxic compounds in this residue such as biological treatments with lignin-degrading fungi (Aranda et al., 2006; Sampedro et al., 2007; de la Rubia et al., 2008). Several of these fungi, including the white rot basidiomycete *Coriolopsis rigida,* are currently being analysed as bioremediation agents of agro-industrial residues and their wastewater, since they can be used as an ecological alternative for residue management with effective options for recovery and reuse of by-products (Alburquerque et al., 2009; Federici et al., 2009). However, further research is needed to understand the transformation mechanisms of the residue and the implications of its use for agricultural purposes.

Laccase is the only ligninolytic enzyme secreted by *C. rigida* growing on "alpeorujo" (Aranda et al., 2006) or glucose–peptone medium (Saparrat et al., 2002). This enzyme catalyzes the oxidation of phenolic compounds and aromatic amines to radicals using molecular oxygen as an electron acceptor. Thus, it could play an important role in the transformation and detoxification of these compounds present in "alpeorujo" according to results reported by other authors (D'Annibale et al., 2004; Jaouani et al., 2005). It is also essential to explore the effect of fungal-treated olive-mill wastes on soil microflora (Sampedro et al., 2005; de la Rubia et al., 2008). This point is particularly important since the nutritional status of the soils can be improved by the use of bioinoculants such as plant growth-promoting rhizobacteria (PGPR) (Gadagi et al., 2003). These bacteria such as *Azospirillum brasiliense*

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stimulate plant potentialities, facilitating the acquisition of nutrients and resistance to stress conditions, being able to accelerate plant growth, especially roots, in heavily contaminated, disturbed and/or nutrient-poor soils (Kamnev et al., 2005; del Amor et al., 2008). The presence of these bacteria in the soil can help to minimize the effect of toxic compounds and other adverse factors which affect plant growth by improving plant fitness and the quality of soil or substrate used (Barea et al., 2002; Huang, 2004).

The aim of this work was to better understand the ability of *C. rigida* to transform the WSFA as well as to analyse its impact on *A. brasiliense* survival. The analysis of this fraction is particularly relevant since it contains the majority of the toxic compounds from "alpeorujo". The role of laccase on WSFA transformation, as well as the effect of Cu(II) in the medium, which is a cofactor of this enzyme, is also discussed.

## 2. Materials and methods

### 2.1. The water soluble fraction from "alpeorujo" (WSFA)

Olive-mill residue "alpeorujo" was collected from an "orujo" olive-oil manufacturer (Sierra Sur S.L., Granada, Spain). The WSFA from the residue was obtained by Soxhlet extraction with water in a 1:8 (w/v) proportion for 16 h (Aranda et al., 2006) and then analysed for pH (4.7), phenols (4.2 g $L^{-1}$) according to the Folin–Ciocalteau method (Osono and Takeda, 2001), for colour (abs 395 nm at pH 7.0, 38.9; colour units, 47 790) employing the Ergül method (Ergül et al., 2009), for protein (4.7 mg $mL^{-1}$) according to the Bradford method (1976) and for reducing sugars (37.5 mg $L^{-1}$) by the Somogy–Nelson method (Somogyi, 1945). The WSFA was autoclaved and conserved at 4 °C until use.

### 2.2. Coriolopsis rigida cultures

*C. rigida* LPSC (Culture collection of the La Plata Spegazzini Institute) strain No. 232 (Spanish Type Culture Collection, CECT 20449) was grown on a basal glucose–peptone medium (Saparrat et al., 2002) and supplemented with the WSFA at 2.5, 10 and 20% (v/v). The addition of $CuSO_4$ (300 μM) to this medium with the WSFA, as an inducer of laccase activity (Saparrat et al., 2002), was also tested. Homogenized pellets from 7-d-old shaken cultures were used as inoculum according to Saparrat et al. (2002). Three replicate cultures per treatment were grown at 150 rpm and 28 ± 1.5 °C for 15 d. Since the aromatic compounds can be adsorbed by the mycelium and/or be transformed by non-biological processes, controls inoculated with a heat-killed mycelial suspension were incubated under identical conditions. The mycelium was removed from the liquid cultures by centrifugation at 20 000 *g* for 10 min at 4 °C. The supernatant was collected to measure phenols according to the Folin–Ciocalteau method (Osono and Takeda, 2001), and optical density was determined at 395-nm using McIlvaine buffer pH 7.0 (Ergül et al., 2009). Laccase activity was determined by using 2,6-dimethoxyphenol as substrate and expressed as international enzymatic units (μmol $min^{-1}$) (Saparrat et al., 2002). The toxicity of the supernatant on *A. brasiliense* was evaluated as described below.

### 2.3. In vitro reaction on the WSFA using a C. rigida crude laccase preparation

An aliquot of a crude laccase preparation from *C. rigida* liquid cultures (at a dosage of 20 U per reaction) obtained according to Saparrat et al. (2002) was used to treat the WSFA at 20%. The mixture was incubated under agitation (150 rpm) at 28 ± 1.5 °C for 24 h and analysed for residual phenols. The mixture was also evaluated for its toxicity on *A. brasiliense*. Two controls were also run under identical conditions, one with the WSFA and heat-inactived crude laccase preparation and another containing only the WSFA.

### 2.4. Azospirillum brasiliense survival

The relative toxicity of the WSFA samples after fungal treatment was evaluated by analysing the effect on the survival of an *A. brasiliense* CECT 590 T isolate, assessed by counting colony-forming units (CFU $mL^{-1}$) by a dilution and plating method using selective Congo red-medium (Rodríguez-Cáceres, 1982). Inoculum was grown on Luria–Bertani medium under agitation (200 rpm) at 37 ± 1.5 °C for 24 h, then centrifuged for 20 min at 4000 rpm and resuspended in saline solution to reach a 0.5 OD at 540 nm (Weber et al., 2001). An aliquot (100 μL) was inoculated into 900 μL of both untransformed WSFA and transformed by either the fungus (2.5, 10 or 20%) or the crude laccase preparation (20%). The WSFA, previously sterilized by filtration, was treated at 37 °C and 500 rpm for 24 h after inoculation. The resultant cultures, which were carried out in triplicate, were then centrifuged for 20 min at 4000 rpm and resuspended in 1 mL of saline solution, diluted appropriately and spread onto selective Congo red-medium. The plates were incubated overnight at 37 °C and growth was measured by counting CFU.

### 2.5. Statistical analysis

The data were analysed by a one-way ANOVA and means were contrasted by Tukeýs test at $p < 0.05$ using the SPSS 17.0 software for Windows.

## 3. Results and discussion

Previous studies have already reported the beneficial effects of fungi treated "alpeorujo" on plant growth, with the laccase-producing fungus, *C. rigida* demonstrating that it has highly promising potential in this regard (Aranda et al., 2006). This fungus significantly reduced the phenols in media supplemented with the WSFA at 10 and 20% (v/v) compared with those inoculated with heat-killed mycelium (Fig. 1), the reduction becoming greater as the WSFA increased (38 and 44%, respectively), but no significant reduction was obtained at 2.5% WSFA. These results are consistent with those reported by Aranda et al. (2006), who found a phenol reduction of 73% for cultures of this fungal strain on non-diluted WSFA, suggesting that the phenol concen-

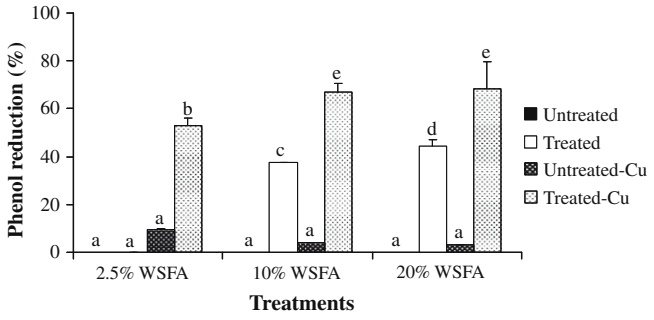


**Fig. 1.** Percentage of phenol reduction from a basal glucose–peptone liquid medium supplemented with WSFA at 2.5, 10 and 20% (v/v) treated with *C. rigida* in the absence (□) or presence of additional Cu(II) ions (▒), either un-treated in the absence (■) or presence of additional Cu(II) ions (▓). The data are means of three replicates ±SD (bars). Means followed by the same letter are not significantly different (Tukey test, $p < 0.05$).