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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-

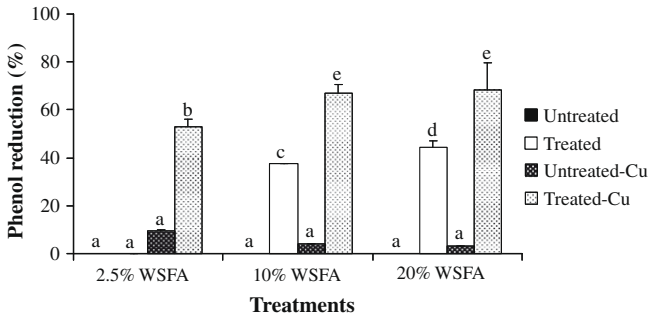

Fig. 1. Percentage of phenol reduction from a basal glucose–peptone liquid medium supplemented with WSFA at 2.5, 10 and 20% (v/v) treated with *C. rigida* in the absence (□) or presence of additional Cu(II) ions (▒), either un-treated in the absence (■) or presence of additional Cu(II) ions (▓). The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

tration might have an influence on the activation of physiological mechanisms leading to fungal transformation and that phenol concentration would determine *per se* the efficiency of the process. When cultures were supplemented with $CuSO_4$ (300 μM), a higher reduction of phenol content (66%) was achieved in cultures with 10 and 20% of the WSFA and laccase activity was dramatically increased (Fig. 2). This suggests a link between phenol reduction and laccase activity since its ability to efficiently oxidize these compounds is well known. Moreover, in the case of cultures supplemented with 2.5% of WSFA, very low levels of laccase were detected (0.03 U mL^{-1}) and no phenol reduction occurred, which would also lend support to the role of laccase in phenol reduction. However, no significant differences in laccase activity were obtained in cultures with different WSFA concentrations and no copper supplementation. Since laccase activity was only determined after 15 d, different profiles on laccase activity throughout the whole incubation period could, at least partially, explain the higher phenol reduction at 10 and 20% WSFA. However, the action of other concomitant fungal mechanisms could also be involved in phenol content reduction, as previously suggested (de la Rubia et al., 2008).

The beneficial effect of copper on residue transformation seems to be related to its influence on enzyme activity and stability, resulting in an increase in the catalytic activity of the enzyme (Baldrian and Gabriel, 2002). In addition, copper could also induce laccase gene transcription, as has been shown in the case of *Pleurotus ostreatus* and *Trametes versicolor* (Colins and Dobson, 1997; Faraco et al., 2003). Opposite results were observed for *Ganoderma applanatum*, *Poria subvermispora* and *Pleurotus pulmonaris* after the addition of copper in culture media supplemented with another type of olive-mill waste (Matos et al., 2007; de la Rubia et al., 2008). This could be explained by the different fungal regulatory mechanisms involved in laccase biosynthesis. In fact, several patterns and mechanisms for laccase regulation and production have already been described (Crowe and Olsson, 2001), including both constitutive and inductive forms (Muñoz et al., 1997). For this reason it is important to analyse the environmental conditions leading to detoxification, which can be different depending on each fungus and the culture conditions. A better understanding of detoxification mechanisms as well as clarifying the regulation of laccase synthesis seems to be essential to optimize results.

Chromophore levels of culture supernatant were also determined (Fig. 3), since differences in chemical parameters of the oxidized functional groups and other product molecules in the WSFA, which are indicative of transformation reactions, could be affected by fungal treatment. Colour increased in cultures grown on medium at 20% WSFA, but remained at the same level for media at 2.5 and 10% WSFA when compared with their respective controls with heat-killed mycelium. Similar results regarding increases in absorbance have been reported for *Lentinus tigrinus* and *Trametes hirsuta* grown on green olive wastewater (Aggelis et al., 2002). The increase in absorbance might be related to polymerization reactions of free soluble phenols present in WSFA, whose concentration was reduced, and would be particularly evident in samples containing high phenol concentrations, as was the case for cultures containing 20% of the soluble fraction of "alperujo". Polymerization of phenolic compounds present in olive-mill wastewater by *P. coccineus* laccase has already been described, suggesting that this enzyme plays an important role in the transformation of these industrial effluents (Jaouani et al., 2005).

In order to explore the effects of fungal treatment on residue toxicity, which limits its potential use as a fertilizer or amendment, we analysed the survival rate of *A. brasiliense* in un-treated and fungal-treated WSFA at concentrations of 2.5, 10 and 20% in the absence or presence of additional Cu(II) (Fig. 4). In the case of untreated samples, bacterium survival decreased as the phenol concentration increased. Sayadi and Ellouz (1993) reported that the inhibitory effect of olive-mill wastewater on microorganisms, including fungi, was concentration-dependent. After fungal treatment, the growth of bacteria was enhanced in cultures containing 10 and 20% WSFA supplemented with copper. The increase in *A. brasiliense* survival after phenol concentration reduction by fungal treatment demonstrates the inhibitory effect of these compounds. This increase in *A. brasiliense* survival may therefore explain the

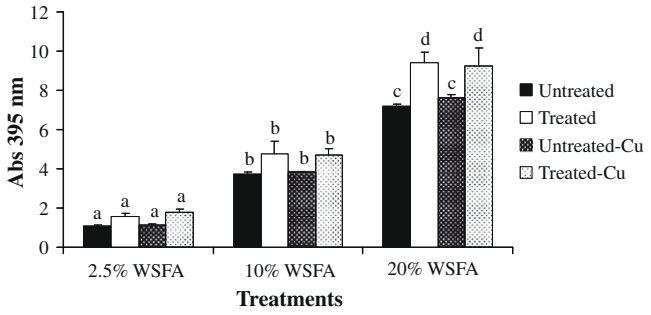

Fig. 3. Absorbance level at 395 nm at pH 7.0 from a basal glucose–peptone liquid medium supplemented with WSFA at 2.5, 10 and 20% (v/v) treated with *C. rigida* in the absence (□) or presence of additional Cu(II) ions (▒), either un-treated in the absence (■) or presence of additional Cu(II) ions (▒) for 15 d of incubation. The data are means of three replicates ±SD (bars). Means followed by the same letter are not significantly different (Tukey test, $p < 0.05$).

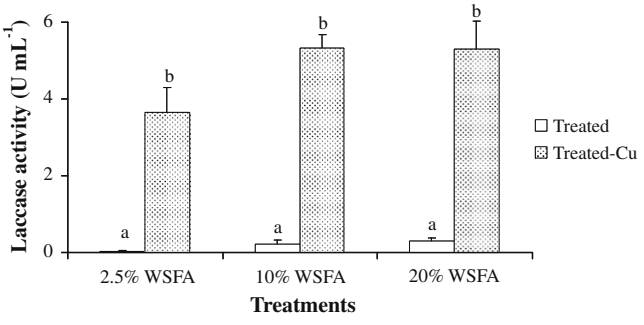

Fig. 2. Extracellular laccase activity of *C. rigida* cultures grown on a basal glucose–peptone liquid medium supplemented with WSFA at 2.5, 10 and 20% (v/v) in the absence (□) or presence (▒) of additional Cu(II) ions for 15 d of incubation. The data are means of three replicates ±SD (bars). Means followed by the same letter are not significantly different (Tukey test, $p < 0.05$).

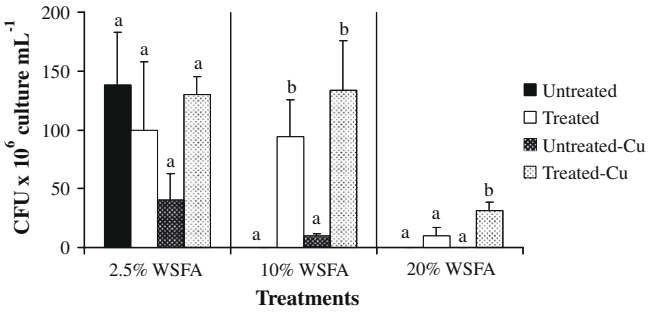

Fig. 4. *A. brasiliense* growth (CFU mL^{-1}) after 24 h of incubation in a basal glucose–peptone liquid medium supplemented with WSFA) at 2.5, 10 and 20% (v/v) in the absence (□) or presence of additional Cu(II) ions (▒), either un-treated in the absence (■) or presence of additional Cu(II) ions (▒) for 15 d of incubation. The data are means of three replicates ±SD (bars). Means followed by the same letter are not significantly different (Tukey test, $p < 0.05$).

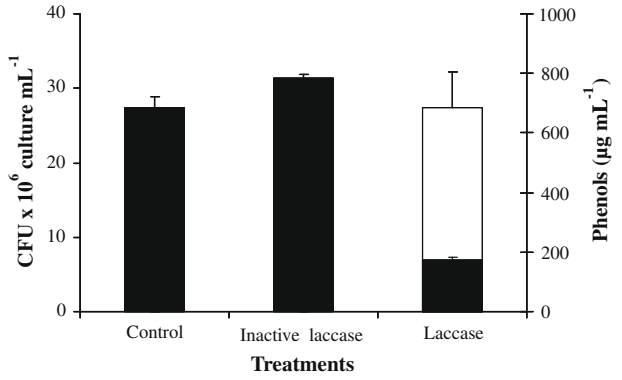

Fig. 5. *A. brasiliense* growth (CFU mL^{-1}) (□) on reaction mixtures containing WSFA at 20% (v/v) either un-treated (control) or treated with active (laccase) or inactive (inactive laccase) laccase, after 24 h of incubation, and their phenol content (■). The data are means of three replicates ±SD (bars). Means for phenols followed by the same letter are not significantly different (Tukey test, $p < 0.05$).

detoxifying effect of *C. rigida* on WSFA and could provide an additional reason for using fungal-treated "alpeorujo" as an agronomic fertilizer or amendment. Thus, *C. rigida* might detoxify the compounds from the WSFA, but the efficiency of the process is affected by either the initial WSFA concentration or Cu(II) ion supplementation or a combination of both. It should also be taken into account, that longer periods of fungal treatment might probably improve the results obtained in this work.

In order to analyse the role played by *C. rigida* laccase in detoxifying "alpeorujo", *in vitro* assays were carried out using an active crude laccase preparation (Fig. 5). The reaction-mixture with the enzyme decreased the phenol content (75% with respect to untreated WSFA) and toxicity against *A. brasiliense* after 24 h of incubation. No bacterial growth occurred in samples where the enzyme was either inactive or absent. Contrary to previous reports (de la Rubia et al., 2008), our results showed that laccase participates in the transformation of free soluble phenols from "alpeorujo" into compounds with less toxicity and/or biostatic effect on soil bacteria. This agrees with studies carried out with pure laccase on olive-mill wastewater which showed an increase in wheat germinability, suggesting that the phenolic fraction was detoxified by the enzyme (Casa et al., 2003). Although reduction of phenols clearly increased bacteria survival, the growth of *A. brasiliense* was not as high as could have been expected in samples treated with fungus at higher WSFA concentrations. This is particularly clear when comparing survival in cultures at 20% WSFA, with a slightly higher phenol concentration than in 2.5% WSFA cultures, but with a low bacterial growth. The absence of a strict correlation between bacterial growth and phenol concentration, as well as an apparent higher toxicity in samples in cultures with 20% WSFA suggest that other toxic compounds could also be involved in the toxicity of the residue.

4. Conclusions

This study highlights that WSFA transformation and detoxification by *C. rigida* liquid cultures is dependent on their concentration in the medium. The fungal laccase oxidizes free phenols present in this agricultural residue, involved in its plant and microbial toxicity, through the formation of radicals leading to polymerization. The presence of Cu(II) could be an important factor to take into account since increase laccase secretion and, as consequence, produce the higher reduction of phenols. It contributes to detoxify the residue promoting the growth of *A. brasiliense*, microorganism which could facilitate the growth of plants of agricultural interest. Further experiments are in progress to evaluate the combined effect of "alpeorujo" treated with laccase-producing fungi and *A. brasiliense* on plant growth.

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