

***Trametes versicolor*: potential for atrazine bioremediation in calcareous clay soil, under low water availability conditions**

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This manuscript includes 2 figures and 3 tables.

Scientific relevance: We investigated the feasibility of *T. versicolor* for actively degrading atrazine (at usual field application rates) in non-sterile calcareous clay soil of South Portugal, under low water availability (-0.7 and 2.8 MPa) and with scarce organic matter content. Results strongly suggested that this species could potentially be used for bioremediation of soil treated with triazine herbicides in semi-arid and Mediterranean-like ecosystems. As far as we are aware, very little work has looked at the influence of soil water potential on triazine biodegradation rates by white rot fungi in non-sterile soil, *T. versicolor* in particular. We therefore consider our work to be an important contribution in the field of applied environmental microbiology.

Abstract

This study has examined the feasibility of *Trametes versicolor* for actively degrading atrazine ($0.5 \mu\text{g g}^{-1}$) in non-sterile calcareous clay soil (Algarve, Portugal) microcosms for up to 24 weeks (20°C), under low water availability (soil water potentials of -0.7 and -2.8 MPa). Soil respiration, enzymatic (dehydrogenase and laccase) activities and atrazine quantification by high-performance-liquid-chromatography (HPLC) were assessed.

Respiration and dehydrogenase activity (DHA) were significantly ($p < 0.05$) enhanced in soil containing the inoculant, particularly in the presence of atrazine, indicating that it remained metabolically active throughout the study. Furthermore, up to 98 and 85% (at -0.7 and -2.8 MPa respectively) of atrazine was degraded in soil containing both atrazine and the inoculant, compared to 96 and 50% in soil containing atrazine only. The contribution of *T. versicolor* to atrazine degradation was only significant ($p < 0.005$) under the driest soil treatment conditions. The strategies used for enhancing colonisation and biodegradation capabilities of the inoculant, as well as the selection of sawdust as carrier were thus effective. However, there were no differences ($p > 0.05$) in quantified laccase activity in soil containing the inoculant and the control. Overall, this study demonstrates that *T. versicolor* is a strong candidate for atrazine bioremediation in soil with low moisture and organic matter contents, such as that found in semi-arid and Mediterranean-like ecosystems.

Keywords: *Trametes versicolor*; Biodegradation; Atrazine; Soil microcosms; Water potential; Soil respiration; Enzymatic activity.

1. Introduction

The widespread incorporation of herbicides into soil every year is of major concern, since they potentially can pose a threat to our health as well as to the quality of soil, surface water and groundwater resources (Häggbloom, 1992; Kearney and Roberts, 1998; Kuo and Regan, 1999; Ashman and Puri, 2002). Atrazine is a chlorinated aromatic herbicide heavily used worldwide for control of broad-leaved weeds in agricultural produce (Ghani *et al.*, 1996; Houot *et al.*, 1998; Ralebitso *et al.*, 2002), as well as in urban and recreational areas (Gadd, 2001).

Atrazine and related triazines are moderately persistent in soil (Pointing, 2001) with reported half-life values ranging from 35 to 50 days, depending largely on soil environmental conditions (Topp, 2001; Rhine *et al.*, 2003). Microbial metabolism has long been regarded as the most important mechanism of atrazine degradation in soil (Armstrong *et al.*, 1967; Gravilescu, 2005). Nevertheless, in conditions of low moisture and nutrient contents, microbial metabolism is compromised and atrazine persistence may increase (Weber *et al.*, 1993). Soil water potential has been widely recognised as a determinant factor controlling soil microbial growth and activity rates. Yet, very little research has looked at atrazine biodegradation in soil under low moisture regimes (Moreno *et al.*, 2007).

The application of white-rot fungi for bioremediation of common environmental contaminants looks promising. Similar to other white-rot species, *T. versicolor* has shown to be able to metabolise a wide range of organic compounds (Bumpus *et al.*, 1985; Gadd, 2001). This ability is generally attributed to the production of extracellular ligninolytic enzymes such as laccase, which is non-specific in regard to its substrate (Thurston, 1994; Youn *et al.*, 1995; Pointing, 2001; Šašek *et al.*, 2003; Baldrian, 2004). White rot species can also tolerate a broad range of environmental conditions, including temperature, nutrient and moisture contents (Maloney, 2001; Magan, 2007). In previous studies, *T. versicolor* was shown to exhibit good tolerance to water stress conditions (Mswaka and Magan, 1999; Fragoeiro and Magan, 2005) as well as to triazine pesticides (Gadd, 2001; Šašek *et al.*, 2003; Fragoeiro and Magan, 2005). Further, the mycelial growth habit and hyphal extension allow rapid substrate colonisation and

utilization of compounds that are not otherwise readily available to the wider microbial community (Reddy and Mathew, 2001; Magan, 2007).

So far, most studies involving the use of ligninolytic fungi for bioremediation purposes have been carried out in liquid media (e.g. Ryan and Bumpus, 1989), often in bioreactors (Novotný, 2004). *T. versicolor* has been seldom studied in the soil environment although there are reports of its successful application in sterile soil (Lamar, 1993) and soil extract broth (Fragoeiro and Magan, 2005). In non-sterile soil, knowledge is limited on other factors which can influence pesticide degradation, such as competitive interactions between the introduced fungi and native microbial populations (Šašek *et al.*, 2003).

This study aimed to (1) assess the potential of *T. versicolor* for actively degrading atrazine at 0.5 µg g⁻¹ (usual field application rates) in non-sterile calcareous clay soil, under low water availability conditions (-0.7 and -2.8 MPa). Soil respiration, dehydrogenase and laccase activities were determined in combination with atrazine quantification by HPLC, under the study conditions. The selection of soil water potentials had the water availability range for microorganisms and plants (i.e. -0.03 MPa, field capacity, to -1.5 MPa, wilting point) as reference.

2. Materials and methods

2.1. Pre-incubation of *T. versicolor* (R26)

Pre-incubation of the fungal inoculum involved growing the isolate in sterile jars on wet sterile sawdust (50% water content, used as carrier) at 25°C for up to 3 weeks, until the substrate was colonised by mycelium. The jar had a vented cap (polypropylene membrane 0.22 µm pore size) allowing adequate aeration. In order to avoid desiccation, the jars were placed inside a polyethylene box, where the equilibrium relative humidity was maintained by a glycerol/water solution (400 ml).

2.2 Soil preparation, conditions and treatments

The soil used was a calcareous clay soil (top-soil, 0-20 cm) and was collected from an arable field plot in Lagoa, Algarve, Portugal. The soil had the following main characteristics, analysed by the National Soil Resources Institute (NSRI, Cranfield University) and given as mg g⁻¹ oven dried soil: soil organic carbon (SOC), 12.1; water, 353; sand, 320; clay, 470; silt, 210; pH 6.8; annual average values of precipitation (mm) and temperature (°C) on site were 400 and 17 respectively; there is no history of pesticide inputs in the last 4 years. Plant residues and stones were removed manually at the time of collection and soil was sieved (2 mm) and air-dried at 20°C for 7 days prior to use. Air-dried soil samples (10 g) were weighed into Universal (25 ml) bottles and target soil water potentials of -0.7 and -2.8 MPa were set by reference to a soil adsorption curve and the addition of sterile reverse osmosis (RO) water (Bastos, 2008).

i) Atrazine addition to soil

Atrazine was dissolved in RO water and the solution was sonicated for 1 min until complete dissolution of the herbicide. The amount of water used for dissolution was the same as that required for setting the target soil water potential treatment, calculated by reference to the soil water adsorption curve (Bastos, 2008). The solution was then added to soil (5 g), in order to obtain a final concentration of atrazine of 0.5 µg g⁻¹. This concentration corresponds to usual field application rates of the herbicide (Ghani *et al.*, 1996; Abdelhafid, 2000). The fortified soils were thoroughly homogenised and kept for 1 day at 4°C allowing microbial activity to stabilise at the required water potential levels, before incubation and analysis.

ii) Soil supplemented with sterile sawdust

Wet (50% w w⁻¹) finely chopped sterile sawdust was kept overnight at 4°C. It was then added to air-dried soil (5 g) in order to obtain a concentration of 5% (w w⁻¹) and samples were left equilibrating overnight at 4°C. Conditioning of the treated soil to the required water potentials was then done by reference to a soil-sawdust calibration curve (Bastos, 2008). The procedure followed that described in *i*).

iii) Soil supplemented with sterile sawdust + atrazine

Soil was amended with sterile sawdust as described in *ii*). Conditioning of the soil to the treatment water potentials was done by reference to a soil-sawdust adsorption curve and the addition of sterile RO water supplemented with atrazine, in order to obtain a final

concentration of atrazine in soil of $0.5 \mu\text{g g}^{-1}$. The procedure followed that described in i).

iv) *Inoculation of T. versicolor into soil*

Sawdust colonised by the test isolate (0.5 g) was added to air-dried soil (5 g) in order to obtain a concentration of $5\% (\text{w w}^{-1})$ and mixed until a homogeneous mixture was obtained. The procedure followed that described in ii).

v) *Incorporation of T. versicolor + atrazine in soil*

The preparation of the homogeneous mixture of *T. versicolor* with sawdust ($5\% \text{ w w}^{-1}$) and its incorporation into air-dried soil (5 g) was described previously. The procedure followed that described in iii).

2.3 Incubation of soil microcosms

Treated soil samples and non-treated controls were incubated at 20°C for up to 24 weeks within polyethylene boxes previously thoroughly cleaned. Each box also contained a glycerol/water solution (400 ml), in order to maintain the equilibrium relative humidity within each microcosms the same as that of the soil treatments. All treatments involved in this work are summarised in Table 1.

2.4 Temporal evaluation of soil respiration

CO_2 evolved from total soil microbial respiration was determined by gas-chromatography (GC) through static sampling. Following incubation, Universal bottles containing soil samples were sealed and left for 3 h at 20°C prior to analysis, thus ensuring detectable volumes of CO_2 in the soil headspace. Headspace (5 ml) was then injected into a gas chromatographer equipped with a packed column (Porapak Q packed glass column) and a thermal conductivity detector (Carlo Erba Instruments, GC 8000 Series MFC 800). Five replicates of each treatment were sampled. The GC settings were the following: column and injector temperatures, 100°C ; detector temperature, 180°C ; carrier gas (Helium) at a flow rate of 36 ml min^{-1} ; the calibration gas consisting of a standard mixture ($10.01\% \text{ v v}^{-1} \text{ CO}_2$ in N_2) was injected three times at the beginning and after each set of 15 samples. Soil respiration rate was expressed as $\mu\text{g CO}_2 \text{ g}^{-1} \text{ soil h}^{-1}$.

2.5 Temporal evaluation of dehydrogenase activity

The method for DHA quantification was adapted from von Mersi and Schinner (1991). It is based on the incubation of soil with the substrate INT (2 (p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride), followed by the extraction and colorimetric estimation of the reduction product INF (iodonitrotetrazolium formazan). The calibration curve ($Y = 1.0657x + 0.0061$) which was firstly produced using a standard INF solution ($100 \mu\text{g INF ml}^{-1}$), showed a good correlation ($r^2 = 0.998$) between the concentration of INF and the optical density of the INF solution.

The INT solution was prepared by dissolving 500 mg of INT into 2 ml of N,N-dimethylformamide, followed by the addition of 50 ml of RO water. The solution was sonicated (2 min) and the volume was brought up to 100 ml using RO water. The final concentration of the substrate solution was 0.5% (w v⁻¹). For every analysis, fresh INT solution was prepared and stored in the dark until use.

Soil (0.5 g) at the treatment water potentials was weighed into sterile test tubes and mixed with 740 μl of Tris-HCl buffer (1 M, pH 7.0) and 1 ml of the substrate solution. Test tubes were sealed with sterile sponge stoppers and incubated in the dark at 40°C for 2 h. Following incubation, 5 ml of extraction solution (N,N-dimethylformamide: ethanol in a 1:1 ratio) were added to the mixture and samples were kept in the dark for 1 h. During this time, every sample was vigorously shaken (using the vortex) at 20 min intervals, ensuring an efficient extraction of the product INF. Aliquots of 2 ml were then transferred to Eppendorf tubes and centrifuged for 2 min. The supernatant (200 μl) was introduced into microplate wells and the INF was determined spectrophotometrically at 450 nm using a Microplate reader (Dynex Technologies Ltd., UK).

Controls were also prepared for estimating the chemical reduction of INT under the same conditions. For each treatment, controls were prepared using autoclaved soil (121°C, 20 minutes) and were treated like samples. Five replicates of each treatment (including respective controls) were sampled. The INT reduction of the control was then subtracted to that of the samples and DHA was expressed as ng INF g⁻¹ soil 2 h⁻¹.

2.6 Temporal evaluation of laccase activity

Estimating soil laccase activity involved 2 steps: i) extraction of laccase from soil; ii) quantification of enzymatic activity based on the oxidation of the redox substrate ABTS

(2,2-azino-bis-ethylbenthiazoline-6-sulphonic acid). The procedure described below was firstly calibrated and optimised using purified commercial laccase from *Rhus vernificera* in crude acetone powder (50 Units mg⁻¹ solid, minimum) as standard. The calibration curve obtained ($Y = 26.33x + 1.643$) showed a good correlation ($r^2 = 0.971$) between the concentration of commercial purified laccase (mg ml⁻¹) and laccase activity (U).

i) Laccase extraction from soil

The extraction method employed was based on the protocol described by Criquet *et al.* (1999) with adaptations by Fragoeiro and Magan (2005). Sub-samples (2 g) of treated soil and non-treated controls were weighed into sterile test tubes and 8 ml of phosphate buffer in water (10 mM, pH 6.0) were added. The suspension was kept under agitation (incubator shaker, 250 rpm) at 4°C for approximately 1 h. Aliquots of 1 ml were then placed into 1.5 ml Eppendorfs and centrifuged (3800 rpm) for 6 min at room temperature. The supernatant containing the enzyme was stored at -18°C until analyses.

ii) Quantification of laccase activity

The method for determining laccase activity using an enzyme extract was based on the protocol described by Buswell *et al.* (1995) with adaptations by Fragoeiro and Magan (2005). The reaction mixture performing a total of 300 µl was contained into a 96 well microtitre plate. It was prepared with 150 µl sodium acetate buffer (0.1 M, pH 5), 50 µl ABTS (0.55 mM) and 100 µl enzyme extract. The procedure was carried out at ambient temperature, although the substrate ABTS and the buffer were at 40°C when added to the reaction mixture. The incubation was performed at 40°C for 1 h. Positive laccase activity was indicated by a green colourisation of the reaction mixture, characteristic of the ABTS oxidised form. The product was determined spectrophotometrically at 405 nm using a Microplate reader set in the Endpoint reading mode, with 5 seconds of agitation at the beginning. Control samples were prepared using boiled enzyme (15 min). Five replicates of each treatment including the respective controls were used. One enzyme activity unit (U) was defined as the amount of enzyme required for producing a 0.001 increase in the optical density of the reaction mixture per minute, under the conditions of the assay. Results were expressed as U g⁻¹ soil.

2.7 Monitoring atrazine concentration in soil

A two-step procedure was involved in assessing the rate of atrazine degradation in soil microcosms: i) pesticide extraction from soil and ii) HPLC analysis of soil extracts. The method employed for atrazine extraction and quantification was adapted from that of Elyassi (1997) and Fragoeiro and Magan (2005).

i) Atrazine extraction from soil

Soil samples corresponding to the SA and SAT treatments (at -0.7 and -2.8 MPa) were weighed (2 g) into test tubes. Aliquots (3 ml) of methanol (100%) were added to soil, the tubes were sealed and shaken at 300 rpm in a circular motion shaker for 24 hours in the dark at room temperature. Following agitation soil was allowed to settle until a clear supernatant was obtained (30 min aprox.). Aliquots of supernatant (extract) was then withdrawn with a syringe and filtered using a nylon 0.22 µm syringe filter.

ii) HPLC analysis of soil extracts

Extracts were diluted with acetonitrile (75% sample: 25% acetonitrile). A volume of 50 µl was injected into a Gilson HPLC system equipped with a Gilson 117 UV detector operating at 215 nm, a Gilson 231XL sampling injector, Gilson 306 pump, Gilson 811 C dynamic mixer and an Altima C18 5 mm column (4 mm x 250 mm x 4.6 mm). The column operated at ambient temperature with a flow rate of 1.5 ml min⁻¹. An isocratic mobile phase system was established using acetonitrile:water at a ratio of 70:30. Atrazine eluted at approximately 9.8 min. The limit of detection

2.8 Data handling and statistical treatment

For comparison between means of treatments in respect to respiration, enzymatic activities and atrazine quantification, analysis of variance (ANOVA) was performed using STATISTICA (Version 7) at a significance level $p = 0.05$. Standard error of means are shown as vertical bars in figures and indicated in Tables as $\pm$ SE.

3. Results

3.1. Temporal evaluation of microbial respiration

Figure 1 shows the respiration rate for the clay soil under different soil treatments incubated at (A) -0.7 MPa and (B) -2.8 MPa (20°C) for up to 24 weeks. Generally, soil

treated with atrazine was shown to produce over 40% more CO₂ than non-treated soil (SS). Throughout the study, soil containing the inoculum alone (ST) showed a significant ($p<0.01$) increase in respiratory rates compared to un-inoculated soil (S). Surprisingly, differences between respiration rates of soil treated with atrazine (SA), sawdust (SS) and *T. versicolor* (ST) individually, were often not significant ($0.05<p<0.16$) under the treatment soil conditions. Overall, the highest CO₂ evolution rates ($0.01<p<0.04$) were achieved by soil containing both atrazine and the inoculum (SAT). These were at least 20% higher than in the absence of the inoculant. Maximal respiration rates occurred generally after week 6, and were followed by a slow but consistent decrease. Under drier conditions and throughout the study, respiration rates were generally not statistically different ($0.05<p<0.27$) between treatments.

3.2. Temporal evaluation of dehydrogenase activity

Figure 2 shows the DHA for the clay soil under different soil treatments incubated at (A) -0.7 MPa and (B) -2.8 MPa (20°C) for up to 24 weeks. Regardless of soil treatment, the highest DHA levels were achieved under the wettest conditions ($p<0.001$). Non-treated soil had the lowest DHA but sawdust and atrazine supplements (individually or combined) enhanced this enzymatic activity by over 40% at both water potentials ($p<0.001$) over the first 6 weeks. However, after 12 weeks and from then onwards, soil carrying the inoculum alone (ST) showed over 20% higher DHA compared to sawdust-treated soil whether atrazine was present or not. Activity rates peaked after 6 weeks but overall, they remained high throughout the study, even under the driest soil conditions. Nevertheless, irrespective of water potential, there was no significant ($p>0.09$) difference between soil inoculated with *T. versicolor* in the presence (SAT) and absence (ST) of atrazine from week 6 onwards.

3.3. Temporal evaluation of fungal laccase activity

Table 2 shows ABTS oxidation levels in the clay soil under different soil treatments incubated at (A) -0.7 MPa and (B) -2.8 MPa (20°C) for up to 24 weeks. Interestingly, substrate oxidation was found to occur in non-treated clay soil in the absence of the fungus at -0.7 MPa. Further, there was enhanced substrate oxidation ($p<0.001$) as a response to atrazine (SA) and sawdust (SS) alone under both water regimes. Differences between laccase activity in soil containing sawdust only and that carrying the inoculant were generally only significant ($p<0.03$) at -2.8 MPa, with the second treatment having

over 96% higher laccase production than the first. Under the wettest conditions, the SAT treatment had only minimal levels of this enzyme, when comparing to the remaining treatments, including that of soil carrying the inoculum alone (ST). This was most evident after 6 weeks. Surprisingly, the opposite was observed under drier soil conditions, with the SAT treatment having nearly 40% higher ($p < 0.0004$) laccase activity than that of ST, although differences between SAT and SA were not statistically significant ($p > 0.06$). Very little activity was found after 24 weeks, independent of the treatment conditions.

3.4. Monitoring atrazine concentration in soil microcosms

Table 3 shows the remaining atrazine ($\mu\text{g g}^{-1}$) in clay soil incubated for up to 24 weeks at (A) -0.7 and (B) -2.8 MPa (20°C) in the absence (SA, SSA) and in the presence (SAT) of *T. versicolor*. Two controls were used in order to reduce bias in respect to the contribution of the sawdust supplement for atrazine degradation under the study conditions. The amount of atrazine present in the soil decreased with the incubation period in all treatments, and this was more rapid during the first 6 weeks.

In the absence of the fungus, $0.071 \mu\text{g g}^{-1}$ of atrazine was recovered from sawdust supplemented soil after 6 weeks, corresponding to around 14% of its initial concentration. For the same time period, only $0.023 \mu\text{g g}^{-1}$ of atrazine (i.e. 4% of the initial concentration) was extracted from soil containing the inoculum. By the end of the study, residues of the herbicide in soil were decreased to 0.019 and 0.011 in the absence (SSA) and presence (SAT) of the inoculum, corresponding to 96 and 98% degradation respectively. At -0.7 MPa, the impact of *T. versicolor* on atrazine breakdown in soil was only significant ($p < 0.003$) within the first 12 weeks. In contrast, at -2.8 MPa, there was still a significant ($p < 0.01$) difference between both treatments at the end of the study.

4. Discussion

In this study, *T. versicolor* was inoculated into non-sterile soil containing atrazine at usual field application rates for up to 24 weeks under low water regimes. Atrazine quantification by HPLC, combined with the assessment of soil microbial respiration and dehydrogenase activity allowed estimating the feasibility of this white-rot species to

remain metabolically active and degrade atrazine under the study conditions. Laccase activity was also determined as an indicator of *T. versicolor* relative activity, in order to evaluate the contribution of this enzyme in the degradation process. An optimal performance of *T. versicolor* in terms of growth and enzymatic activity is dependent on its capability to compete with native microflora in contaminated soil (Bumpus, 1993; Levanon, 1993; Baldrian, 2004). According to Šašek *et al.* (2003), this is an important aspect since the interaction between both parts can result in either inhibition or cooperation in the degradation process. In order to enhance *T. versicolor* colonisation and activity under such conditions, two strategies were employed: pre-incubation of the fungus on a ligninolytic substrate (wet sawdust) prior to inoculation into soil; use of sawdust as carrier (5 g inoculant to 95 g soil) but also as nutrient source selective for the fungus. Other authors have used different carriers and inoculant/soil ratios, ranging from 5% woodchips-based *T. versicolor* inoculum (Fragoeiro and Magan, 2005) to 50% straw-based inoculum (Novotný *et al.*, 2003).

Temporal soil respiration

Soil respiration was used as an indicator of overall microbial activity and pesticide breakdown. Increased respiratory activity following incorporation of sawdust and atrazine (individually or combined) was not surprising as they provide nutrient sources suitable for native microorganisms (Mandelbaum *et al.*, 1993; Haney *et al.*, 2002; Moreno *et al.*, 2007). Our results were thus comparable to those in other studies which used atrazine at similar concentrations (Dzantor and Felsot, 1991; Moreno *et al.*, 2007). Further, sawdust addition may have also improved aeration throughout soil, favouring microbial activity in ways equivalent to that reported by Boyle (1995) using alfalfa and bran.

Respiratory activity was also enhanced in soil containing the inoculum, indicating that the test isolate was able to remain metabolically active throughout the study. However, few significant differences were found between that and soil containing sawdust alone, which suggests competitive interactions between the inoculant and native microflora. The highest CO₂ evolution rates were obtained from soil containing both atrazine and the inoculant, indicating atrazine breakdown by the test isolate, even under limiting water potentials of -2.8 MPa. Comparable results were obtained by Fragoeiro and Magan (2008), who employed *T. versicolor* for bioremediation of a pesticide mixture

(simazine, dieldrin and trifluraline at 5 ppm) in soil microcosms. Using a sandy loam soil under similar water potentials, they have also reported maximal CO₂ evolution from soil containing both the pesticide mixture and *T. versicolor*.

Overall, respiration rates peaked at around 6 weeks, followed by a consistent decrease towards the end of the study due to nutrient exhaustion (Balba et al., 1998). There was evidence that water potential was the limiting factor for soil respiration, as respiratory activity between treatments was generally not statistically different under the driest soil conditions. This is consistent with Conant et al. (2004), who found that drier (-1.0 and -1.5 MPa) soils have substantially lower respiration rates than those moist (-0.03 to -0.05 MPa), partially due to severely restricted bacterial activity.

Temporal dehydrogenase activity

Biological dehydrogenation (oxidation) of organic matter under aerobic conditions is ultimately linked to the respiratory chain and the synthesis of adenosine triphosphate (ATP) (Trevors, 1982; von Mersi and Schinner, 1991) and is catalysed by dehydrogenases (Harris and Steer, 2003; Nannipieri *et al.*, 2002, 2003). Besides organic matter decomposition, intracellular dehydrogenase activity has also been associated with other key soil functions such as xenobiotic degradation (Min *et al.*, 2001; Acosta-Martinez *et al.*, 2003). This enzymatic activity has been widely recognised as a good indicator of microbial activity, since it is linked to viable cells only and has shown to be positively correlated to respiration under different soil conditions (e.g. von Mersi and Schinner, 1991; Garcia et al., 1994; Jimenez et al., 2002). In this study, DHA was determined in order to assess the overall soil oxidative status and this enzymatic activity in relation to atrazine biodegradation.

Regardless of soil treatment, the highest DHA levels were achieved under the wettest conditions, which is supported by previous studies (Quilchano and Maranon, 2002). Further, supplement addition to soil (sawdust and atrazine, individually or combined) generally enhanced DHA regardless of soil water potential. In contrast, the low DHA levels in soil containing the inoculum alone over the first 6 weeks, is likely to reflect competitive interactions between the inoculant and native microorganisms, agreeing with respiration data. However, increased DHA from then onwards indicated that the inoculum remained metabolically active, even under the driest soil conditions.

Surprisingly, unlike that observed in the remaining treatments, DHA in the SAT treatment was the highest over 24 weeks, which is inconsistent with respiratory activity. This adding to the fact that the SAT treatment did not show improved levels of this enzyme (when comparing to ST), suggested that atrazine degradation by this white-rot species may not be coupled to the oxidative metabolism of the fungus. It is possible that under the treatment environmental conditions, atrazine was not being used by *T. versicolor* for generation of ATP (Haney *et al.*, 2002). This enzymatic activity has been scarcely studied in relation to atrazine biodegradation by white-rot fungi in soil.

Previous work has linked single pesticide degradation and DHA activity in soil (e.g. Min *et al.*, 2001; Moreno *et al.*, 2007). Moreno *et al.* (2007) reported enhanced DHA in soil containing atrazine in the range of 0.2 to 1000 mg kg⁻¹ at 28°C. In contrast, McGrath and Singleton (2000) monitored pentachlorophenol (PCP) biodegradation in a clay loam. While PCP concentration was found to decrease (from 250 to 2 mg kg⁻¹) in just 6 weeks, levels of DHA remained minimal throughout the study. They suggested that the generation of toxic PCP biodegradation products may have been inhibitory to DHA (McGrath and Singleton, 2000). However, it is unlikely for that to explain the low DHA obtained in this study in soil containing the inoculant. Previous work has shown that very few toxic simazine breakdown products were originated by this inoculant in soil extract broth at -0.7 and -2.8 MPa, using the luminescent bacterium *Vibrio fischerie* (standard toxicity assay) (Fragoeiro, 2005).

Temporal laccase activity

Since it is difficult to directly assess fungal growth in soil (Novotný *et al.*, 1999, 2004), colonisation of white-rot fungi is usually determined indirectly through enzymatic activity. The ability of such fungi to degrade pesticides has been largely associated with the production of the glycoprotein laccase (polyphenol oxidase) in the presence of adequate ligninolytic substrates (Hägglom, 1992; Paszczynski and Crawford, 2000; Novotný *et al.*, 1999, 2004). Such enzymes have broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups. ABTS is considered to be a primary mediator for laccase and therefore its oxidation is generally regarded as an indication of laccase activity (Youn *et al.*, 1995; Podgornik *et al.*, 2001).

ABTS oxidation did occur in non-treated clay soil under the study water potentials, contrary to that found by Fragoeiro and Magan (2008) using a sandy loam. This may be because other genera of fungi (e.g. *Aspergillus*, *Rhizopus*), actinomycetes (e.g. *Streptomyces*) and also some bacteria (e.g. *Pseudomonas*, *Bacillus*) are known to express laccase activity at some extent (Kearney and Roberts, 1998). It suggests that this enzymatic activity may not be suitable for assessing *T. versicolor* relative activity in non-sterile soil. The incorporation of sawdust (individually or combined with atrazine) has shown to stimulate LAC production, which might be a reflection of an active fungal and actinomycete communities in such soil types (Brown, 1979; Wilson and Griffin, 1975; Harris, 1981; Magan, 1988, 1997; Halverson *et al.*, 2000).

Very little research has looked at the implications of soil water potential on LAC activity. In this study, whereas the incorporation of *T. versicolor* into soil did not resulted in enhanced laccase activity at -0.7 MPa, that enhancement was obtained under drier soil conditions. This indicates that *T. versicolor* had ligninolytic activity under -2.8 MPa, similarly to that reported by Boyle (1995) and later by Fragoeiro and Magan (2008). Further, that result suggests that LAC production by the inoculant may be influenced by competitive interactions with native microflora (White and Boddy, 1992) at -0.7 MPa, when the wider fraction of the microbial community was metabolically active. For example, there is evidence of total inhibition of ligninolytic activity in *T. versicolor* when co-inoculated in soil with species of the genus *Trichoderma* (Freitag and Morrell, 1992). According to Novotný (1999), such interactions may explain why *T. versicolor* generally produces relatively low levels of laccase in non-sterile soil.

Under wetter soil conditions, soil containing both atrazine and the inoculant has shown minimal levels of laccase, compared to soil containing sawdust and atrazine. In contrast, the opposite was found at -2.8 MPa. Overall, evidence suggests that atrazine degradation in this soil by *T. versicolor* may have had little or no contribution of laccase activity under the conditions studied. It is therefore likely that other enzymes may have been involved at a larger scale. For example, Podgornik and co-workers (2001) defended that ABTS is also a good substrate for manganese peroxidase (MnP) in *P. chrysosporium* and therefore there is the possibility of this ligninolytic enzyme to have been equally responsible for ABTS oxidation to a certain degree. Additionally, those same authors confirmed that Mn(III) complex formation during cultivation of *P.*

chrysosporium can provide a false-positive for laccase, when ABTS is used as substrate. Further research is needed on the link between ABTS oxidation and laccase activity from *T. versicolor*, as well as between such enzymatic activity and pesticide degradation by this species in non-sterile soil. Similarly, although it is known that *T. versicolor* produces both MnP and lignin peroxidase (LiP) in culture (reviewed by Tuor *et al.*, 1995), much remains to be done in order to evaluate the contribution of these enzymes in atrazine biodegradation by *T. versicolor* in the soil environment.

Contradictory evidence has led to the role of laccase production in the co-metabolism of pesticides with lignin by white-rot fungi not yet to be well understood (Youn *et al.*, 1995). In this study, laccase production has shown to be highly impacted by soil treatment, particularly by soil water potential. Bending *et al.* (2002), who used *T. versicolor* for biodegradation of atrazine in liquid culture for up to 42 days, have reached similar conclusions. Similarly, Mougín *et al.* (1996) have also reported that the degradation of lindane in soil by *Phanerochaete chrysosporium* was independent of laccase production by the fungus. In contrast, Fragoeiro and Magan (2008) reported extremely high laccase activity (797.8 units ml⁻¹) by *T. versicolor* in a sandy loam soil (25°C, -0.7 MPa) treated with a pesticide mixture (simazine, dieldrin and trifluralin, 5-30 ppm). Besides having been associated also with interspecific interactions of the fungus (White and Boddy, 1992), Novotný (1999) has further suggested that laccase production may be influenced by the nature and concentration of the potential contaminant and soil environmental conditions (Tuor *et al.*, 1995). It is also likely to vary with the white-rot strain and perhaps with the carrier used (Mougín *et al.*, 1996; Boyle, 1997) or be dependent on the combination of the aforementioned factors.

Monitoring atrazine concentration in soil microcosms

Pesticide degradation in soil was estimated by determining the amount of herbicide extracted from soil after 6-24 weeks (20°C, -0.7 and -2.8 MPa), compared to its initial concentration. The decrease in recovered atrazine in the SA and SSA treatments can be explained by the presence of active native microbial populations, capable of degrading the herbicide under the study conditions. This was consistent with the enhanced microbial metabolic activity found for the same time period. Other studies (Haney *et al.*, 2002; Moreno *et al.*, 2007) have reported similar results on the capability of native soil populations to degrade atrazine added at low concentrations. For example, Moreno *et al.*

(2007) have recently demonstrated that 50% of the atrazine added (5 ppm) to clay loam with freely available water had been degraded by day 16 (28°C) and that no herbicide was recovered after 45 days.

However, herbicide breakdown was substantially enhanced in soil containing the inoculum, particularly within the first 12 weeks. It provides evidence that *T. versicolor* was able to grow and actively degrade atrazine in non-sterile soil under low water availability conditions. It also suggests that the pre-incubation of the test isolate and the use of sawdust as carrier were effective for this species. In this case, it is likely that the relationship established between the inoculum and native degraders was mainly cooperative, agreeing with earlier findings by Boyle (1995). In contrast, Tornberg *et al.* (2003) reported that this species failed to remain viable once inoculated in non-sterile soil.

Over 24 weeks, the contribution of *T. versicolor* was found to be no longer significant at -0.7 MPa. In contrast, surprisingly, its contribution was still significant at -2.8 MPa, when the remaining fraction of the microbial community had their metabolic activity limited by water restriction. This clearly shows that this white-rot species is able to actively biodegrade potential contaminants under environmental conditions, which do not promote biodegradation by soil native microflora. It is likely to be partially explained by the mycelial growth habit, allowing rapid and efficient colonisation of soil while maximising interactions between extracellular enzymatic activity and the surrounding environment (Maloney, 2001; Reddy and Mathew, 2001). Atrazine breakdown was less pronounced towards the end of the study, whether *T. versicolor* was present or not, due to nutrient exhaustion.

5. Conclusion

This study has shown that *T. versicolor* has the potential to be used as a bioremediation agent for atrazine and related triazine compounds in non-sterile calcareous clay soil, under low water availability conditions. This may be particularly relevant for bioremediation strategies in semi-arid and Mediterranean-like ecosystems.

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Table 1. Summary of the soil treatments involved in this work. Key to treatments: WP, water potential; SD, sawdust; Atra, atrazine., Tv, *T.versicolor*.

	Ref	WP (-MPa)	Atra (ug)	SD (%)	SD+Tv
Soil	S	-0.7; -2.8	-	-	-
Soil + Atra	SA	-0.7; -2.8	0.5	-	-
Soil + SD	SS	-0.7; -2.8	-	0.5	-
Soil + SD + Atra	SSA	-0.7; -2.8	0.5	0.5	-
Soil + Tv	ST	-0.7; -2.8	-	-	0.5
Soil + Atra + Tv	SAT	-0.7; -2.8	0.5	0.5	0.5

Table 2. Temporal laccase activity (U g/soil) based on ABTS oxidation in clay soil incubated for up to 24 weeks at 20oC at (A) -0.7 and (B) -2.8 MPa as a response to different soil amendments.

A)

	Incubation time (weeks)			
	0	6	12	24
S	0.30 ± 0.29	2.55 ± 1.06	1.88 ± 0.88	0.67 ± 0.41
SA	9.04 ± 1.41	12.0 ± 3.53	7.92 ± 1.57	0
SS	9.50 ± 0.77	24.5 ± 9.60	16.4 ± 3.04	0
SSA	10.3 ± 1.12	28.7 ± 0.79	19.9 ± 0.32	0
ST	8.49 ± 3.53	21.2 ± 9.11	13.3 ± 2.89	0
SAT	18.5 ± 5.66	0.51 ± 0.22	0.50 ± 0.51	0

B)

	Incubation time (weeks)			
	0	6	12	24
S	0.72 ± 0.11	1.97 ± 0.80	0.86 ± 0.23	0
SA	0	21.5 ± 4.32	15.7 ± 2.03	0
SS	6.50 ± 0.51	11.1 ± 3.88	0	0
SSA	8.57 ± 1.01	13.4 ± 0.90	6.20 ± 0.56	0
ST	10.0 ± 1.06	14.6 ± 3.63	9.88 ± 1.54	0
SAT	14.2 ± 1.90	23.1 ± 5.69	13.4 ± 2.01	0

Table 3. Remaining atrazine (ug/g) in clay soil at (A) -0.7 and (b) -2.8 MPa at 20oC in the absence (SA, SSA) and presence (SAT) of *T.versicolor*.

(A)

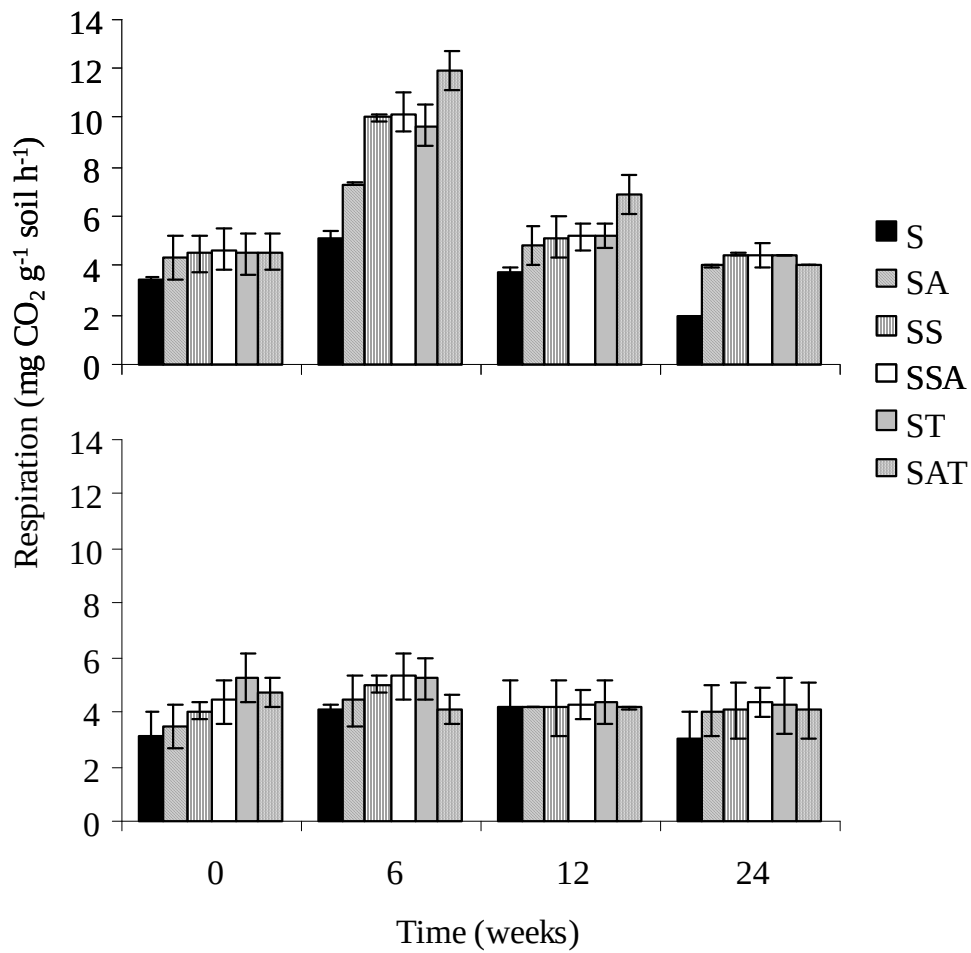
Incubation (weeks)	Treatment	Remaining atrazine ($\mu\text{g g}^{-1}$)
0	SA	0.495 ± 0.01
	SSA	0.500 ± 0.03
	SAT	0.500 ± 0.02
6	SA	0.080 ± 0.01
	SSA	0.071 ± 0.02
	SAT	$0.023^* \pm 0.02$
12	SA	0.036 ± 0.03
	SSA	0.034 ± 0.02
	SAT	$0.016^* \pm 0.03$
24	SA	0.022 ± 0.06
	SSA	0.019 ± 0.04
	SAT	0.011 ± 0.05

B)

Incubation (weeks)	Treatment	Remaining atrazine ($\mu\text{g g}^{-1}$)
0	SA	0.480 ± 0.03
	SSA	0.495 ± 0.03
	SAT	0.490 ± 0.01
6	SA	0.262 ± 0.04
	SSA	0.255 ± 0.01
	SAT	$0.102^* \pm 0.03$
12	SA	0.258 ± 0.02
	SSA	0.242 ± 0.02
	SAT	$0.084^* \pm 0.02$
24	SA	0.254 ± 0.06
	SSA	0.237 ± 0.04
	SAT	$0.076^* \pm 0.03$

* Statistically different from both controls (SA and SSA) at $p < 0.05$.

Figure 1. Respiration rates for the clay soil under different soil treatments for up to 24 weeks at 20oC under (A) -0.7 and (B) -2.8 MPA. For key to treatments see Table 1.





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