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BIOTRANSFORMATION OF 2,4,6-TRINITROTOLUENE (TNT) BY ECTOMYCORRHIZAL BASIDIOMYCETES

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ABSTRACT

The ability of four ectomycorrhizal basidiomycetes to biotransform 2,4,6-trinitrotoluene (TNT) in axenic culture was tested. All species were capable of TNT biotransformation to a greater or lesser extent. When biotransformation was expressed on a biomass basis 4 out of the 5 isolates tested were equally efficient at transforming TNT. The factors regulating TNT biotransformation were investigated in detail for one fungus, *Suillus variegatus*. When the fungus was grown under nitrogen limiting conditions the rate of biotransformation decreased relative to nitrogen sufficient conditions, but no decrease was observed under short term carbon starvation. Extracellular enzymes of *S. variegatus* could transform TNT, but transformation was greater in intact cells. The mycelial cell wall fraction did not degrade TNT. The TNT concentration that caused 50 % reduction in biomass (EC_{50}) for *S. variegatus* was within the range observed for other basidiomycete fungi, being between 2-10 $\mu g\ mL^{-1}$. The potential use of ectomycorrhizal basidiomycetes as *in-situ* bioremediation agents for TNT contaminated soils is discussed. © 1997 Elsevier Science Ltd

KEYWORDS: basidiomycetes, ectomycorrhizal fungi, microbial degradation, trinitrotoluene

INTRODUCTION

White rot basidiomycetes are prolific at degrading persistent organic chemicals containing aryl rings [1, 2], and have been used as *in-situ* bioremediation agents [3]. Many organic compounds are broken down by lignolytic enzymes that are induced by white rots under carbon and nitrogen limiting conditions [1, 2]. However, white rot fungi have a major limitation as bioremediation agents, in that for establishment of the mycelium in soil for bioremediation purposes, the soil must be inoculated with the fungus growing on an organic substrate (usually wood chips) [3]. Once the carbon source has been depleted they will become ineffective in bioremediation and will require further amendments with woody substrates.

Ectomycorrhizal basidiomycetes have considerable potential as biological agents to facilitate degradation of persistent organic pollutants [4-6]. Given that ectomycorrhizal basidiomycetes obtain carbon from their host

plants, there is sustainable establishment of ectomycorrhizal mycelium in soils in the presence of the host [7]. Virtually all tree species are infected by ectomycorrhizal symbionts and in forest soil ectomycorrhizal mycelia are extensive and in some instances constitute about half the soil microbial biomass [8, 9]. There are increasing indications that some ectomycorrhizal fungi produce a number of extracellular enzymes typical of white rot basidiomycetes [8, 10] and in some cases these may be effective at degrading organic pollutants [4]. The ability of a range of ectomycorrhizal basidiomycetes to degrade recalcitrant organic pollutants such as the pesticides 2,4-D and atrazine [6], PCBs [5] and 2,4 dichlorophenol [11] has already been demonstrated.

The ability of white rot fungi to degrade 2,4,6- trinitrotoluene (TNT) has been widely investigated [12-16] and the white rots may prove an effective way to degrade TNT in contaminated soils *in-situ*. TNT contamination of old munitions manufacturing and storage sites have been targeted for bioremediation with the white rot fungus *Phanerochaete chrysosporium* [16, 17]. To determine if ectomycorrhizal basidiomycetes have potential to bioremediate TNT contaminated soils we tested the ability of a range of ectomycorrhizal species to biotransform TNT. The physiological regulation of TNT biotransformation was investigated in one species that was highly efficient at degrading TNT.

MATERIALS AND METHODS

Fungal isolates and culture conditions

The ectomycorrhizal basidiomycetes *Paxillus involutus, Pisolithus tinctorius* (2 isolates), *Suillus variegatus* and *Tylospora fibrillosa* were maintained in 90 mm diam. Petri dishes containing modified Melin-Norkrans agar medium (MMN) of the following composition $(NH_4)_2HPO_4$ (0.5 g), KH_2PO_4 (0.3 g), $MgSO_4.7H_2O$ (0.14 g), $CaCl_2.6H_2O$ (0.05 g), NaCl (0.025 g), $ZnSO_4$ (0.003 g), Fe EDTA (0.0125 mg), citric acid (0.0125 mg), glucose (10 g), thiamine (0.01 mg), agar (13.3 g) and distilled H_2O (1.0 L), with the media adjusted to pH 5.5.

For liquid culture studies agar plugs (5.0 mm diam.) of each fungus were removed from the leading edge of a colony growing on MMN and transferred aseptically into 90 mm diam. Petri dishes containing 20 mL of MMN minus agar. The cultures were allowed to grow until a suitable biomass developed (7-10 d) when mats of mycelium were transferred to amber glass bottles (125 mL) containing 5.0 mL of liquid MMN. The bottles were sealed with steel crown-cap lids (with a 5.0 mm hole in the centre) fitted with sterilized teflon-faced liners. The required concentration of TNT was added to the culture vessels by injecting the 10 µL of TNT in an acetone carrier through the teflon septa using a Hamilton syringe to give a TNT concentration of 5.0 µg mL^{-1}. Sterile controls with TNT and autoclaved cultures of each fungus were included in this and all subsequent experiments. Throughout all experiments fungal cultures were maintained at 20°C in the dark.

TNT synthesis

TNT was prepared using a 2-step nitration of toluene [13]. Toluene (100 mL) was added to a mixture of concentrated HNO_3 (130 mL) and concentrated H_2SO_4 (240 mL), and refluxed at 80°C for 1 h. The mixture was then heated at 100°C for a further 2 h, and then poured over ice. The crude 2,4-dinitrotoluene (DNT) was recystallised from ethanol to give faintly yellow crystals (mp 70.6°C, lit. 70.5_C). DNT (50 g) was added to a mixture of concentrated HNO_3 (130 mL) and concentrated H_2SO_4 (240 mL), refluxed at 110°C for 3 h and the mixture poured onto ice. The crude 2,4,6-trinitrotoluene was recrystallised from ethanol to give yellow, needle-

like crystals (mp 80.5°C, lit. 80.9°C). The infrared spectrum and mass spectrum of the TNT reproduced library spectra. The purity of the TNT was found to be 99% from GC-MS (Hewlett Packard GC model 5890 interfaced with a Hewlett Packard MS detector 5972), the remainder being residual DNT.

Assay for TNT biotransformation

After 3 d incubation the crown cap was removed and TNT remaining in solution was determined using a colourimetric assay adapted from the method of Spiker *et al.* [16]. The method involved adding 5.0 mL of acetone to the culture vessel, followed by thorough mixing. Acetone-culture medium (2.0 mL) was then pipetted into a 4 mL quartz cuvette containing 10 µL of a saturated solution of Na_2S and 50 µL KOH (1.0 M). The solution was mixed for 5.0 min. to allow colour development and the absorbance read at 462 nm. Fungal mycelia were dried overnight at 70°C and biomass determined gravimetrically.

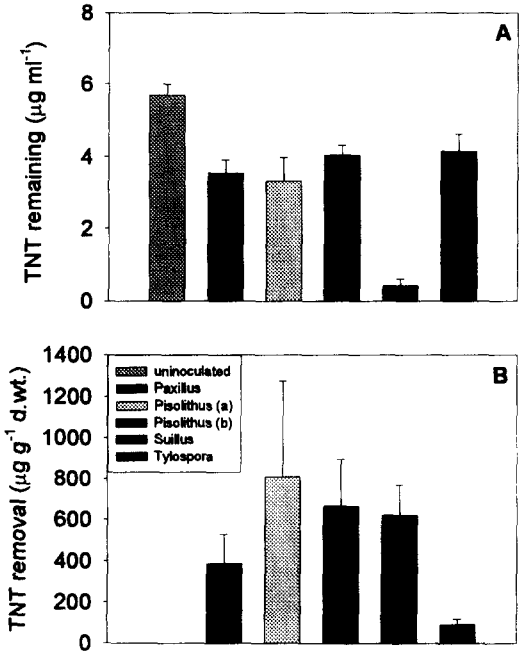
Experiments to investigate the effect of nutrient status of the medium on biotransformation by *S. variegatus* were conducted by altering the glucose and ammonium content of the MMN medium. Normal levels of glucose and ammonium were those in the standard MMN. For low glucose and low nitrogen 0.1 g L^{-1} glucose and 5.0 mg L^{-1} $(NH_4)_2HPO_4$ were used. Fungal cultures were set up in a factorial design with standard glucose and ammonium, standard glucose and low ammonium, low glucose and standard ammonium, and low glucose and low ammonium. The concentration of TNT added to the cultures was 5.0 µg mL^{-1}. TNT biotransformation was assayed at 16 h and 40 h.

To determine if degradation was occurring via extracellular enzymes or by cell wall bound enzymes, TNT was incubated with culture filtrate and cell wall preparations of the most prolific TNT degrading fungus, *S. variegatus*. To obtain culture filtrate the fungus was grown in liquid MMN (4 plugs per 20 mL of solution) for 10 d. Culture filtrate (5.0 mL) was placed in amber culture vessels with a cocktail of protease inhibitors [phenyl methyl sulphonyl fluoride (0.1 mM), Pepstatin B (1.0 M), EDTA (1.0 mM)] and the metabolic inhibitor sodium azide (16 mM) (to prevent microbial growth) were added immediately. The cell wall component was obtained by freeze fracturing mycelium mats with liquid nitrogen and suspending the fractured mycelium in MMN containing the protease inhibitor cocktail and sodium azide. The suspension was then centrifuged at 36,000 g for 10 min. The resulting pellet was washed three times in the same medium and transferred to amber bottles where it was suspended in 5.0 mL of the same medium. The amount of cell wall added to the vessels was approximately equivalent to that present in live mycelial inoculum. Live mycelium in the absence of inhibitors was also included in this experiment. All treatments, including sterile controls, had TNT added to them to give a concentration of 5.0 µg mL^{-1}. Degradation was then determined after three d.

Determining EC_{50} value for TNT

A dose response curve for TNT for *S. variegatus* was obtained by placing a 5.0 mm plug of the leading edge of the hyphae directly into the amber culture vessels containing 5 mL of MMN and fitted with teflon-lined crimp-top lids. The required concentration of TNT (ranging from 2-100 µg mL^{-1}) was injected through the teflon septa in 10 µL of acetone as the carrier. The zero TNT concentration only received 10 µL of acetone. The bottles were incubated at 20°C for 10 d and then the fungal dry wt. was determined after 24 h drying in an oven at 70°C.

Fig. 1. Removal of TNT from a 5 μg mL^{-1} solution by *Paxillus involutus, Pisolithus tinctorius, Suillus variegatus* and *Tylospora fibrillosa* after 36 h incubation expressed as (a) TNT remaining in solution and (b) as TNT removal per gram of biomass dry wt.. Each figure is the mean of 8 replicates and bars are the standard error of the mean.

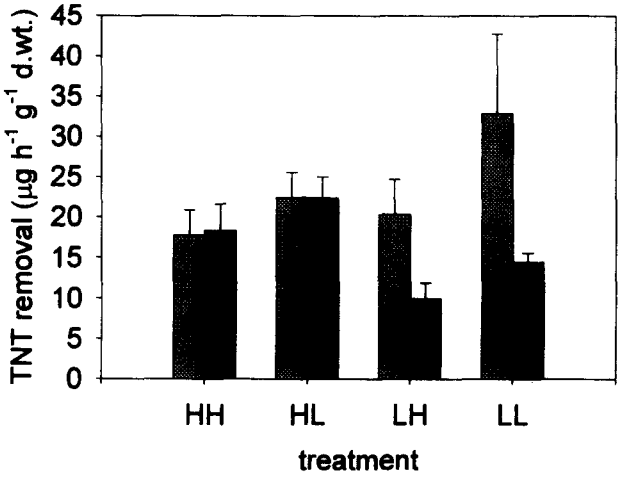


RESULTS

All ectomycorrhizal fungi investigated were able to biotransform TNT to some extent as shown by the removal of TNT from solution after three d. incubation. Control treatments containing either autoclaved mycelia or medium alone showed no detectable loss of TNT (data not shown), confirming that loss of TNT was via biotransfromation rather than adsorption onto myceial surfaces of auto-degradation in the culture medium. The large difference between *S. variegatus* and the other species observed in Fig. 1a was due principally to its much larger biomass. When the data were corrected to units of biotransformation per unit biomass, there was little difference in the ability to degrade TNT with the exception of *T. fibrillosa* which was clearly less effective at TNT degradation (Fig. 1b). As both *P. involutus* and the two *P. tinctorius* isolates produced highly coloured metabolites in the external medium which interfered in the absorbance readings, because *T. fibrillosa* was much less effective than the other species, and since *S. variegatus* biomass production was the greatest, *S. variegatus* was selected for further experimentation.

Lignolytic enzymes are known to be involved in the degradation of a wide range of organic chemicals by *P. chrysosporium* [1, 2] and some ectomycorrhizal basidiomycetes are also known to induce lignolytic enzymes *in vitro* [8]. Low nitrogen and carbon supply initiates the production of lignolytic enzymes in white rots [1, 2] and it is likely that this will also be the case for ectomycorrhizal basidiomycetes [8, 10]. Low nitrogen and carbon supply did not stimulate TNT biotransformation by *S. variegatus* (Fig. 2). Conversely low nitrogen supply inhibited biotransformation by 40 h, but not low carbon. The fact that low nitrogen inhibits biotransformation suggests that the fungus is nitrogen stressed. As it is under nitrogen stressed conditions that lignolytic enzymes are produced it is unlikely that lignolytic enzymes are involved in initial stages of TNT degradation. The rate of biotransformation of TNT was consistent at the two harvests in the nitrogen replete treatments (Fig. 2), indicating that substrate had not become limited after 40 h.

Fig. 2. Removal of TNT from a 5 μg mL^{-1} solution by *Suillus variegatus* after 16 (light shaded histogram bars) and 40 (dark shaded histogram bars) h incubation under different nitrogen and carbon nutritional regimes expressed as TNT removal per gram of biomass dry wt. per hour. Labelling on the X-axis refers to nutritional regime:- HH is high nitrogen and high carbon, HL is high nitrogen and low carbon, LH is low nitrogen and high carbon, and LL is low carbon and low nitrogen. The concentrations of nitrogen and carbon used in this experiment are given in the text. Each figure is the mean of 8 replicates and bars are the standard error of the mean.



The experiment to determine if cell walls and culture filtrate were capable of biotransforming the TNT showed that cell walls had no biotransforming capacity. (Fig. 3). Culture filtrate was capable of TNT biotransformation suggesting that extracellular enzymes can degrade TNT. However, this biotransformation capacity was considerably less than intact mycelium. Intact mycelium degraded 600% more TNT than the culture filtrate (Fig. 3). The concentration of enzymes in the culture filtrates should be at least as high as in the media containing intact mycelium since the culture filtrate was harvested from cultures of similar mycelia density to the intact fungal

cultures. This may therefore suggest that while biotransformation can occur via extracellular enzymes the intact fungus utilises a number of pathways to effect the biotransformation of TNT.

Fig. 3. Removal of 5 μg mL^{-1} TNT from a 5 μg mL^{-1} solution by *Suillus variegatus* cell walls, culture filtrate and intact mycelium after 36 h incubation expressed as TNT remaining in solution. Each figure is the mean of 8 replicates and bars are the standard error of the mean.

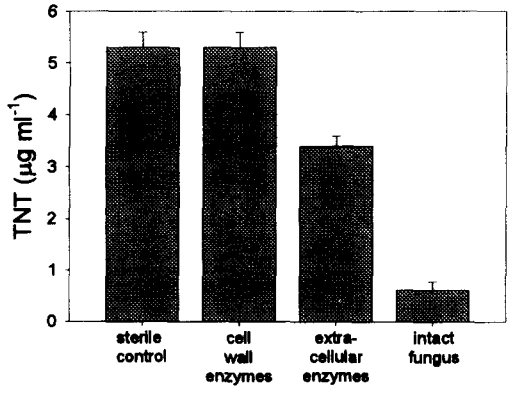
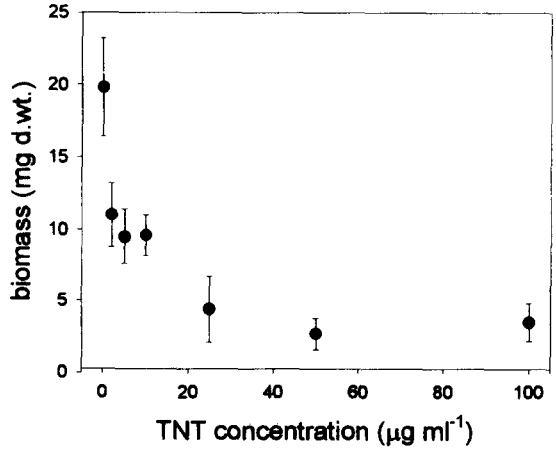


Fig. 4. Dose response curve for *Suillus variegatus* biomass production in the presence of TNT. Cultures were grown for 10 d. Each point is the mean of 8 replicate and bars are the standard error of the mean.



The dose response for the toxicity of TNT to *S. variegatus* shows that fungal growth is sensitive to TNT. The 50% effect concentration (EC_{50}) of TNT for *S. variegatus* was between 2-10 µg m

DISCUSSION

A wide range of bacteria [18, 19] and fungi including ascomycetes [20], deutoromycetes [21] and basidiomycetes [12-16] are capable of degrading TNT. Within the basidiomycetes only white rot fungi have been shown to degrade TNT. This study has shown that four ectomycorrhizal basidiomycetes are also capable of degrading TNT (Fig. 1). It appears that the mechanisms of initial degradation of TNT are common throughout all microorganisms tested. This mechanism is reduction of one of the nitro groups on the aryl ring to form hydroxylamino-dinitrotoluenes and further reduction resulting in the formation of amino- dinitrotoluenes [13, 18, 19]. Although *P. chrysosporium* effects the degradation of many organic chemicals through lignolytic enzymes [2], lignolytic enzymes are not involved in the initial degradation of TNT [13, 14]. Initial attack on TNT by *P. chrysosporium* is also through reduction of the nitro groups on the aryl ring [13, 14]. However, once initial reduction to give amino- dinitrotoluenes occurs, lignolytic enzymes of *P. chrysosporium* have been shown to further degrade amino- dinitrotoluenes through oxidative attack, eventually leading to mineralization [13, 14]. Hydroxylamino-dinitrotoluene, an early intermediate of TNT reduction, inhibits the *P. chryosporium* lignases, preventing further degradation [13].

Our study on *S. variegatus* also suggests that lignolytic enzymes are not involved in the initial stage of TNT degradation. Indeed, conditions that are known to stimulate lignolytic enzymes in other basidiomycetes (low nitrogen and carbon status of the external medium) inhibited TNT degradation (Fig. 2). A number of studies have investigated the capability of ectomycorrhizal fungi to degrade persistent organic pollutants (2,4-D, PCBs and atrazine) under nitrogen sufficient and limiting conditions. In all studies nitrogen limitation had an adverse or negligible effect on degradation [5, 6], again implying that lignolytic enzymes are not important for at least the initial stages of organic pollutant degradation by ectomycorrhizal basidiomycetes. The ability of ectomycorrhizal fungi to degrade persistent organic pollutants under nitrogen and carbon replete conditions is important in the context of soil remediation since the bulk of ectomycorrhizae mycelia in symbiosis would generally be expected to be carbon and nitrogen sufficient [9].

Stahl and Aust [15] argue that the initial reduction of TNT by *P. chryosporium* occurs via redox potential driven reduction at the plasmalemma. This conclusion was based on experiments where cell walls, lysed cell contents, culture filtrate and intact cells were investigated to see if they could reduce TNT. Only intact *P. chryosporium* mycelium reduced TNT and Stahl and Aust [15] concluded, therefore, that it was the plasmalemma redox potential that caused reduction. Our results differ from those of Stahl and Aust [15] in that we found that the culture filtrate of *S. variegatus* could biotransform (presumably via reduction) TNT, though not as effectively as intact cells (Fig. 3). A major difference in our experimental protocol to that of Stahl and Aust [15] is that we included a cocktail of protease inhibitors in the *S. variegatus* culture filtrates. Ectomycorrhizal basidiomycetes may release considerable amounts of non-specific extracellular proteases when grown in liquid culture [8], as does *P. chryosporium* [22]. Therefore, in experiments with *P. chryosporium* where protease inhibitors are not added there remains a possibility that other enzymes present may be degraded. In a study on protease enzyme production in *P. chryosporium* produces a number of proteases under different nutritional regimes, each having different abilities to degrade

extracellular enzymes [22]. The protease inhibitors were specific for certain classes of proteases [22]. Although we added a protease inhibiting cocktail to the culture filtrate it is possible that not all proteases were effectively inhibited and, therefore, the TNT biotransformation capacity of the culture filtrate must be viewed as a minimum as some residual proteolytic activity could have been present in the filtrates. The fact that extracellular enzymes are capable of degrading TNT does not preclude the involvement of plasmalemma redox mediated reduction or cytosolic biotransformation, particularly as intact cells were much more efficient at biotransfromation than the culture media. The fact that ectomycorrhizal basidiomycetes appear to biotransform TNT via extracellular enzymes has considerable importance in the potential use of ectomycorrhizal fungi as agents of bioremediation. The volume of soil that would be influenced by ectomycorrhizal fungi with respect to biodegradation would be greatly enhanced in the presence of extracellular enzymes. Also, extracellular enzymes may diffuse into regions of the soil matrix that are inaccessible to fungal hyphae, possibly leading to more effective bioremediation.

The toxicity of TNT to *S. variegatus* (Fig. 4) is similar to that of *P. chrysosporium* [16] with EC_{50} within the range of 2-10 $\mu g\ mL^{-1}$. The concentrations of TNT in contaminated soils has been reported to be up to 12 $mg\ g^{-1}$ soil dry wt. [16]. Such high soil concentrations have obvious implications for the bioremediation potential of both *P. chrysosporium* and *S. variegatus*. However, it is likely that on contaminated sites that the concentration of TNT in soil solution will be considerably lower than 12 $mg\ g^{-1}$. Also, most contaminated sites have considerable heterogeneity with respect to soil pollutant concentrations.

We have demonstrated that ectomycorrhizal fungi have the capacity to biotransform TNT. This biotransformation potential is exhibited by both intact hyphae and by extra-cellular enzymes. Biotransformation occurs under carbon replete conditions which are likely to be experienced by the bulk of the mycelium in symbiosis with the host plant.

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