Production of small molecular weight catalysts and the mechanism of trinitrotoluene degradation by several *Gloeophyllum* species

David Newcombe, Andrzej Paszczynski*, Wioletta Gajewska, Mario Kröger, Gregor Feis, Ronald Crawford

**Abstract**

The ability of *Gloeophyllum* species to produce dimethoxybenzoquinones (DMBQ), particularly 2,5-dimethoxyhydroquinone (2,5-DMHQ), and oxalic acid was investigated. The involvement of these compounds, along with hydrogen peroxide and Fe(III), in 2,4,6-trinitrotoluene (TNT) degradation was examined. Salicylic acid (SA) and phenol (PH) were used as probes to trap Fenton process-produced hydroxyl radicals in several of the investigated species. All the cultures degraded SA and PH readily. A low concentration of 2,3-dihydroxy benzoic acids was detected in cultures of only two *Gloeophyllum* species. TNT was rapidly transformed by *G. trabeum*, but ring-UL-14C-TNT was not converted to 14CO₂. Mass balance studies indicated that about 74% of the radioactivity from TNT remained in the culture supernatant. Analysis of culture extracts revealed several aromatic nitro-amines and nitro-aldehydes and their oligomeric coupling products formed by Schiff base reaction mechanism. 2,2,6,6-Tetramethyl-1-piperidynoloxide (TEMPO), a stable free radical, was used as a trap in *in vitro* reactions containing hydrogen peroxide, 2,5-DMHQ, and TNT. The coupling products of TEMPO and 2,5-DMHQ were detected, indicating semi-dimethoxy quinone radical formation. The *in vitro* Fenton-like reaction containing all above reactants except methoxyquinones produced degradation products from TNT similar to those extracted from *G. trabeum* cultures, suggesting that reduced oxygen species produced by Fenton-like reactions are involved in the transformation of TNT by brown-rot fungi such as *G. trabeum*. © 2002 Elsevier Science Inc. All rights reserved.

1. Introduction

Several recent advances have been made in our knowledge of the mechanisms that brown rot fungi use to degrade wood and other organic compounds. Paszczynski et al. [1] found 2,5-dimethoxyhydroquinone and 4,5-dimethoxycatechol (4,5-DMC) in a *Gloeophyllum trabeum* culture extract. They suggested that these hydroquinones might be involved in Fenton-like reactions that could participate in the depolymerization of lignin. Dimethoxyhydroquinones (DMHQ) have been isolated previously and studied as antimicrobial agents. 2,5-DMHQ was isolated from *Polyporus fumosus* [2] and from *G. sepiarium* [3]. However, the strong antifungal activity of *G. sepiarium* was related to production of oospolactone 3,4-dimethyl-8-hydroxyisocoumarin, rather than to 2,5-DMHQ. 2,5-DMHQ was also isolated from two higher plants, *Delbergia melanoxylon* (Leguminoseae) [4] and *Acorus calamus* (Araceae) [5], well known for their medicinal value in Africa and India, respectively. 2,5-DMBQ was isolated from *Lenzites thermophilla* in 1950 by Burton [6] during investigations of the antibiotic thermophilin secreted by this fungus. The structure of thermophilin (2,5-DMBQ) was not known at the time. Its structure, along with that of 3,4-dimethoxy-8-hydroxyisocoumarin, was elucidated many years later by LeBlanc and Babineau [7].

Other researchers have reported that liquid cultures of *G. trabeum* accumulate low molecular weight aromatic compounds in significant concentrations [8,9]. In related chemical studies, Parra et al. [10] demonstrated that a mixture of 2,3-dihydroxybenzoic acid (2,3-DA) and 3,4-dihydroxycinnamic acid (3,4-DB) together with Fe(III) degraded di-oxygen-extracted lignin and bleaching effluent to up to 80%, confirming the ability of dihydroxybenzenes to participate in degradation of soluble modified lignin. In another laboratory, 2,5-DMHQ was also isolated from *G. trabeum* and was used as the source of electrons for reduction of Fe(III) and oxygen in an extracellular, hydroquinone-driven Fenton-like reaction.
ton-like reaction. Polyethylene glycol-4000 (PEG) was used to examine the depolymerization potential of DMHQ/FeIII/O2 in vivo and in vitro [11]. The substantial depolymerization of PEG was demonstrated; however, PEG as a simple aliphatic homopolymer did not truly represent the native lignocellulose heteropolymer, which is far more resistant to degradation.

In the research reported here we used the model compound TNT, which is more difficult to degrade than PEG. TNT-transforming organisms, TNT degradation pathways, and the fate of TNT and other organonitro compounds in the environment have been well studied [12–17] and standards of various expected metabolites are readily available.

Some of the most extensive mineralization of TNT by a biological system has been observed in cultures of the white rot fungus P. chrysosporium growing under lignolytic conditions [18]. The non-specific lignin-degrading enzymes produced by P. chrysosporium catalyze the oxidation of many xenobiotic compounds [19]. The mechanisms of TNT transformation by P. chrysosporium may be similar to those of brown rotters since both types of fungi exist in nature in similar environments and both degrade wood. P. chrysosporium initiates TNT degradation by reduction of a nitro group, followed by oxidation and subsequent degradation of the aromatic ring to CO2 [20–22]. Reductive steps are thought to be catalyzed in a stepwise fashion by an aromatic nitroreductase [21,22]. This activity in P. chrysosporium was found to be membrane-bound in two studies [23,24] and soluble in another [20]. Mineralization of TNT by P. chrysosporium has been found to be correlated with expression of lignolytic activity, including production of various peroxidases.

Recent research points toward the importance of manganese peroxidase in the oxidation of reduced TNT metabolites. Scheibner and Hofrichter [25,26] showed that cell-free preparations of manganese peroxidase in the oxidation of reduced TNT metabo-
lites, TNT metabolites 2ADNT, 4ADNT, 4-amino-2,6-dinitrotoluene (2-ADNT), and 2,6-diamino-4-nitrotoluene (2,6-DANT) to unknown metabolites. Furthermore, these researchers noted that the presence of reduced thiols like glutathione or the amino acid cysteine considerably enhanced the rate and extent of TNT mineralization. In similar experiments with manganese peroxidase purified from Phlebia radiata, Van Aken et al. [27] described the ability of these enzymes to oxidatively attack and mineralize TNT through a free radical mechanism. This observation is relevant to our research because brown rotters are believed to employ extracellular Fenton-like radical-based reactions as their main extracellular degradation process.

This paper examines the ability of the brown rot fungus Gloeophyllum trabeum to transform TNT. The roles of dimethoxyquinones, oxalic acid, Fe(III), and hydrogen peroxide in the transformation of TNT are examined. We studied eight Gloeophyllum species for production of methoxyquinones and oxalic acid. All strains produced oxalic acid, and five produced methoxyquinones, especially 2,5-DHQ, when grown on malt extract medium or mineral media. New complexes of oxalic acid and iron were identified using electrospray ionization tandem mass spectrometer (ESI-MS/MS). In vitro Fenton-like reactions produced degradation products from TNT similar to those found in fungal cultures.

2. Materials and methods

2.1. Fungal strains

The fungal strains used in this research and their sources are listed in Table 1.

2.2. Reagents

All chemicals, unless specified otherwise, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) at the highest purity available. Ring-UL-14C-TNT was prepared by two-step nitration from ring-UL-14C-toluene (specific activity, 58.9 µCi/mmol; radiochemical purity, >99%) measured by high-performance liquid chromatography (HPLC) and liquid scintillation counting [28]. Non-labeled TNT was prepared from 2,4-dinitrotoluene as described by Lewis, et al. [28]. The TNT metabolites 2ADNT, 4ADNT, 4,5-dimethoxy-1,2-benzenediol were synthesized from 2,5-dimethoxy-1,4-benzenediol and 4,5-dimethoxy-1,2-benzoquinone obtained from TCI (Tokyo, Japan). Typically, 200 mg of the benzoquinone was placed in a 20-ml glass vial and 3 ml of 95% ethanol added. The solution was stirred, 100 mg sodium borohydride was added, and the vial was capped. The cap was opened briefly at short intervals to release accumulated hydrogen. When the solution became white and effervescence ceased, the reaction was considered complete. Next, 5 ml of deoxygenated water was added to the reaction mix-

<table>
<thead>
<tr>
<th>Fungal strains used in this research</th>
<th>ATCC no.</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gloeophyllum trabeum</td>
<td>12678</td>
<td>Univ. of Maine Collection</td>
</tr>
<tr>
<td>Gloeophyllum sepiarium</td>
<td>32892</td>
<td>Univ. of Maine Collection</td>
</tr>
<tr>
<td>Gloeophyllum abietinum</td>
<td>*</td>
<td>USDA Forest Products Lab</td>
</tr>
<tr>
<td>Gloeophyllum mexicanum</td>
<td>64431</td>
<td>ATCC</td>
</tr>
<tr>
<td>Gloeophyllum protractum</td>
<td>*</td>
<td>USDA Forest Products Lab</td>
</tr>
<tr>
<td>Gloeophyllum subferruginum</td>
<td>*</td>
<td>USDA Forest Products Lab</td>
</tr>
<tr>
<td>Gloeophyllum striatum</td>
<td>24597</td>
<td>ATCC</td>
</tr>
<tr>
<td>Gloeophyllum carbonarium</td>
<td>64503</td>
<td>ATCC</td>
</tr>
<tr>
<td>Postia placenta</td>
<td>11538</td>
<td>Univ. of Maine Collection</td>
</tr>
</tbody>
</table>

* No accession number found.
ture, and the pH was adjusted to 7 with HCl. The sample was extracted five times with equal volumes of ether under a stream of N₂. The N₂-saturated ether fraction was then dried at room temperature under a vacuum in a Brinkman (Westbury, NY) model Rotovapor R rotating evaporator. After all the solvent was removed, the flask was transferred to a lyophilizer (Labconco Lyph Lock 4.5, Labconco Corp, Kansas City, MO) to remove traces of water. The 2,4-DMHQ was weighed and dissolved under anaerobic conditions (10% H₂, 90% N₂) in acetonitrile for analysis of purity by HPLC and gas chromatography/mass spectrometry (GC-MS) (see below for methods).

2.3. Culture conditions

All fungal strains were grown in malt extract broth (2–3% w/v) or in a defined medium originally developed for P. chrysosporium [29] and containing 1% glucose, 0.5 mM NH₄NO₃, and 0.1 mM MnCl₂. This medium was modified for growth of brown rot fungi by including 0.5 mM asparagine and 0.5 mM arginine [30]. It was buffered with 10 mM 2,2'-dimethylsuccinic acid sodium salt at a pH of 4.5. Malt medium was prepared by adding 25–30 g malt extract (Difco Laboratories, Detroit, MI) to 1000 ml of water. Static cultures were established at 24°C or 30°C in the dark by inoculating a 150-ml Erlenmeyer flask containing 50 ml of medium or a 1-liter Roux flask containing 150 ml of medium. Two methods were used to inoculate flasks. The first consisted of removing approximately 2 cm² of mycelial mat from a 6-week-old liquid culture. After this material was added, it was broken into small pieces by shaking the flasks for about 20 s. The second method utilized a Brinkman model PT 10/35 homogenizer (Brinkman Instruments, Westbury, NY) to liquefy the mycelial mat from a 50-ml 4-week-old culture, and 200 µl of the suspension were transferred to flasks containing 50 ml of medium.

2.4. Extraction and analysis of fungal supernatant

Mature fungal cultures and non-inoculated controls were extracted and analyzed for TNT metabolites and other organic compounds. Typically, a culture was filtered through a glass wool filter to remove mycelia, and the filtrate was extracted twice with equal volumes of methylene chloride by shaking for 5 min. Prior to the second extraction 1 ml of 4N H₂SO₄ was added to the aqueous fraction. The organic fractions were combined, and approximately 5 g of Na₂SO₄ were added. The suspension was passed through a Whatman #2 filter (Whatman, Clifton, NJ), and the filtrate dried under a gentle stream of nitrogen. During this step care was taken to minimize exposure to moisture. Once completely dry, the concentrate was resuspended in 500 µl of acetonitrile for GC-MS or ESI-MS and ESI-MS/MS analysis.

GC-MS analysis of the extracts was performed with a Hewlett-Packard (HP) series II 5890 gas chromatograph equipped with a capillary fused-silica column (30 m × 0.25 mm) coated with CP-SIL 8CB MS (Chrompack, Middelburg, The Netherlands). The injector temperature was set at 250°C, and the GC-MS interface was set at 280°C. Chromatographic separations were achieved under a linear temperature gradient from 100 to 200°C at a rate of 5°C/min and then from 200 to 300°C at 20°C/min. Samples (1 to 5 µl) were injected via an automatic injector (HP-7673). A HP quadrupole MS (5989A) controlled by HP MS Chemstation software (PC version) was used for MS and analysis under the following conditions: repeller, 7 V; emission, 300 V; electron energy, 70 eV. The source temperature was 250°C, and the quadrupole temperature was 125°C. The scan parameters were 30 to 350 or 30 to 750 m/z. Interpretation of the MS spectrum was aided by the Wiley and National Institute of Standards and Technology library of mass spectra stored in the Chemstation database (approximately 200,000 spectra).

Fungal culture filtrates were analyzed for TNT, metabolites, and methoxyquinones directly on an HP 1090 HPLC equipped with a 150 mm × 1.00 mm phenyl-hexyl column (Phenomenex, Torrance, CA) and an ultraviolet-visible (UV/Vis) diode array detector. Samples were eluted using a gradient mobile phase containing 18 mΩ water (A) and 0.1% formic acid in methanol (B). The method was as follows: flow rate, 0.65 ml/min; injection volume 20 µl; 30% B (0 min) to 100% B (at 20 min, held for 10 min) followed by a return to initial conditions (at 30 min) for 5 min to rinse and equilibrate the column; peak detection was at 254 nm, 260 nm, 285 nm, and 299 nm, with peak-actuated scanning from 200–600 nm. Salicylic acid, phenol, catechol, hydroquinone, and isomers of dihydroxybenzoic acid were also detected in the fungal culture supernatants with HPLC. The same phenyl-hexyl column was used, and the samples were eluted using an isocratic gradient containing 75% phosphate (A): 6.80 g KH₂PO₄, 1 ml H₂PO₄ in 1000 ml of deionized water (resistivity > 18 megaohm-centimeter), and 25% acetonitrile in water (B) at
a flow rate of 0.65 ml/min; injection volume 20 μl. Absorption detection was at 230 nm with peak-actuated scanning from 190–600 nm.

2.5. ESI-MS and ESI-MS/MS mass spectrometry

Analyses of TNT, TNT degradation products, methoxy quinones, oxalic acid, oxalic acid metal complexes, and radical trap products were completed using a Micromass Quattro II mass spectrometer (Altrincham, England) equipped with an electrospray ionization probe, two quadrupole analyzers, and a hexapole collision cell. The ion source was operated in the negative- or positive-ion mode. Samples were delivered into the source at a flow rate of 5 μl/min using a syringe pump (Harvard Apparatus, South Natick, MA). A potential of 2.5 kV was applied to the electrospray needle. The sample cone was kept at an average of 15 V and the counter electrode, skimmer, and RF lens potentials were tuned to maximize the ion beam for a given solvent. Argon was used as a collision gas during daughter analysis. The source temperature was kept constant at 80°C. The instrument was calibrated using a polyethylene glycol solution. All spectra were an average of 10–15 scans.

2.6. Analysis of 14C radiolabeled compounds

Radiolabeled CO2 was captured in culture flasks equipped with hanging cups containing 1 ml 1N NaOH [31]. At regular intervals the NaOH was removed and the cup rinsed twice with equal volumes of deionized water. The NaOH and rinses were combined and added to a 20-ml scintillation vial containing 15 ml of Biosafe scintillation cocktail (Research Products International, Inc., Mount Prospect, IL). Supernatant samples (1 ml) were removed from the flasks and added directly to 15 ml of scintillation cocktail. Fungal hyphae were removed by filtration, rinsed with water, and added to an equal volume (approximately 40 ml) of tissue solubilizer (NEN Research Products, Boston, MA) and incubated for 24 h at 50°C. Aliquots (1 ml) of the solubilized tissue solution were then added to 15 ml of Ready Organic scintillation cocktail (Beckman Instruments, Fullerton, CA). All samples were analyzed with a Tri-Carb

![Graph showing concentration of various compounds over time](image-url)
Table 2
Degradation of TNT and acetophenone by G. trabeum and P. chrysosporium

<table>
<thead>
<tr>
<th></th>
<th>(% of 14C-TNT)</th>
<th>(% of 14C-Acetophenone)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Filtrate</td>
<td>14C2</td>
</tr>
<tr>
<td>P. chrysosporium</td>
<td>30 ± 1.2†</td>
<td>10 ± 1.2</td>
</tr>
<tr>
<td>G. trabeum</td>
<td>74.1 ± 2.8</td>
<td>2.7 ± 0.3</td>
</tr>
</tbody>
</table>

* Data not collected.
† Data were collected after four weeks of incubation and are represented as the percentage of total radionuclide added ± standard deviation. Heat-killed and non-inoculated cultures were run in tandem as controls (data not shown). The amount of total TNT added to cultures of P. chrysosporium (4 mg/l) was less than the amount added to cultures of G. trabeum (20 mg/l).

2100TR scintillation counter (Packard Instrument company, Inc., Downers Grove, IL).

3. Results

3.1. Transformation of TNT

G. trabeum (Table 1) was able to grow in minimal medium augmented with TNT and to tolerate fairly high (35 mg/l) concentrations of TNT (Fig. 1). However, concentrations of TNT at 40 mg/l significantly inhibited the hyphal growth. Comparisons of cultures grown in the presence of 30 mg/l TNT to control cultures without TNT revealed an enhancement of hyphal growth by this amount of TNT (Fig. 1).

G. trabeum cultures grown for 2 weeks in the minimal salts medium and then spiked with TNT transformed the TNT within 2 days (Fig. 2). Different concentrations of nitrogen in the culture had no effect on TNT degradation (Fig. 2A, B, C, D). The transient accumulation of 2-amino-4-aminodinitrotoluenes was observed during growth in all four media (Fig. 2). Small amounts of 2,4-DANT were observed during growth in mineral media, but not in malt extract medium. This metabolite was not always detectable by HPLC in the culture supernatant, but was detectable by GC/MS after extraction and concentration.

In order to follow the fate of TNT in the cultures, similar experiments were run by growing G. trabeum for 2 weeks in a nitrogen-limited (1.2 mM) minimal medium with subsequent addition of ring-UL-14C-TNT (Table 2). After four weeks of incubation, less than 3% of added TNT was mineralized to 14CO2 and about 15% of the 14C was associated with the hyphal fraction. Furthermore, most (>70%) of the radioactivity was found in the aqueous fraction of the culture (Table 2). In the same incubation period, P. chrysosporium was able to mineralize about 10% of TNT, and about 60% of the 14C was associated with hyphae. Both cultures were also incubated with ring-UL-14C-acetophenone. Four percent of the added radioactivity was recovered from cultures of G. trabeum as 14CO2. In a similar experiment P. chrysosporium removed more than 85% of the radioactivity from the medium and released about 15% of the label as 14CO2. The comparison between these two fungi showed that G. trabeum has limited ability compared to P. chrysosporium to metabolize the aromatic ring, and ring substituents have little effect on this process.

3.2. Radical trapping with TEMPO

The compound 2,2,6,6-tetramethyl-1-piperidynyloxide (TEMPO), a stable free radical, was employed as a trap in in vitro reactions containing Fe(II), 2,5-DMHQ, and TNT. The coupling product of TEMPO and 2,5-DMHQ was detected (Fig. 3). The known biological transformation products of TNT were never observed in this reaction mixture (data not shown). The presence or absence of oxygen did not influence this reaction.

Table 3
Production of 2,5-dimethoxybenzoxine and 4,5-dimethoxy-1,2-benzoxine (DMBQs) by Gloeophyllum strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Concentration of DMBQ (mg/l)</th>
<th>Production confirmation by GC/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPLC analysis</td>
<td>Day 14</td>
</tr>
<tr>
<td>G. abietinum</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G. carbonarium</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G. subferrugineum</td>
<td>3.5–8.5†</td>
<td>5.3–13.0</td>
</tr>
<tr>
<td>G. striatum</td>
<td>3.3–7.4</td>
<td>2–3.5</td>
</tr>
<tr>
<td>G. mexicanum</td>
<td>0</td>
<td>1.0–1.1</td>
</tr>
<tr>
<td>G. protractum</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>G. trabeum</td>
<td>2.4–10.0</td>
<td>9.4–20.0</td>
</tr>
<tr>
<td>G. sepiarium</td>
<td>1.0</td>
<td>1.0–3.4</td>
</tr>
</tbody>
</table>

† The data are given as ranges due to wide fluctuations of quinone concentration observed in cultures.

Fig. 3. Structures of a semi-quinone radical, TEMPO, and their coupling product observed during in vitro experiments employing TEMPO as a radical trap.
3.3. Production of DMBQ by cultures

In our HPLC analyses both quinones, 2,5-dimethoxy-1,4-benzoquinone and 4,5-dimethoxy-1,2-benzoquinone, were eluted in a single peak; this fraction was called DMBQ. When the level of DMBQ was observed over time in cultures of *G. trabeum* with and without added TNT, the DMBQ concentration initially dropped significantly in cultures with TNT added (data not shown). After an incubation time of 10 days the levels of DMBQ in both cultures were...
not significantly different. However, when cultures were spiked with 20 mg/l TNT again, the level of DMBQ was significantly greater than in non-spiked controls (data not shown). Six of eight strains produced DMBQs when grown on malt extract media (Table 3). The concentrations ranged from 1 mg/l for G. mexicanum to 13 mg/l for G. subferrugineum and 21 mg/l for G. trabeum.

3.4. Determination of hydroxyl radical in fungal cultures

Solutions of salicylic acid were added to 2-week-old cultures to detect the presence of hydroxyl radicals. HPLC analysis showed that all of the cultures examined degraded salicylic acid (Fig. 4A). In addition, the presence of 2,3-dihydroxy benzoic acid was observed in G. striatum and G. protactum (Table 4). The presence of this hydroxylation product is an indication of hydroxyl radical in solution [32]. In a similar experiment phenol was used as the hydroxyl radical trap (Fig. 4). The four cultures of the Gloeophyllum species examined readily degraded phenol, but hydroquinone and catechol that might be expected as products of hydroxyl radical reactions [33] were not observed in direct analysis of culture filtrates with HPLC.

3.5. Chelation of iron by oxalic acid

The complexes formed by oxalic acid with FeCl₃ were examined at two different pHs using negative ESI-MS/MS. The solutions contained 3 mM oxalic acid and 1 mM FeCl₃; the pH was adjusted with NaOH. At pH 1.5 the expected hexacoordinated octahedral complex was observed with two carboxylic groups of oxalic acid protonated. The other complex observed contained two oxalate molecules and a single ferric ion. In the second complex iron was most likely additionally coordinated by two molecules of water. Reduced iron complexes have not been observed under these conditions (Fig. 5A). At pH 4.5 ferric and ferrous complexes were clearly visible (Fig. 5B). In addition, a double charged peak of Fe(IV)O, coordinated by two oxalates (m/z = 148), was observed. The structures of complexes were confirmed using MS/MS daughter analyses (data not shown).

3.6. Analysis of TNT degradation products by Fenton-like reactions containing oxalic acid

Solid TNT (50 mg/l) was added to the reaction mixture of 3 mM oxalic acid, 1 mM FeCl₃, and 10 mM H₂O₂ the 50. The pH of the solution was adjusted to 4.5 with NaOH. After 24 hours the reaction mixture was analyzed using ESI-MS/MS. Numerous organic products were tentatively identified as shown in Fig. 6 using daughter analyses.

4. Discussion

The complex structure and chemical composition of lignocellulosic biopolymers have significant influences on their degradation in the environment. Fungi are the only organisms known capable of complete degradation of fully lignified wood. The mechanisms by which these microorganisms initiate their attack on wood is not fully understood. The present leading hypothesis is that ligninolytic fungal peroxidases and oxidases are too large to penetrate into intact wood, [34,35] so both classes, white rot and brown rot fungi, use low molecular weight redox compounds (mediators) to launch their initial attack on intact wood cell walls. Research on brown rot decay mechanisms became focused on the involvement of reduced species of oxygen generated from hydrogen peroxide by chelated transition metal molecules after Koenigs proposed in 1974 the involvement H₂O₂-Fe(II) as a radical generating system in brown rot.
decay [36]. Several groups have proposed experimental
evidence that supports an oxygen radical-based degradation
mechanism whereby brown rot fungi might attack wood
[1,9,11,37].

In research reported here we used TNT as a model
recalcitrant compound to study brown rot degradative
abilities. TNT and related munitions compounds can cause liver
damage, react with hemoglobin, and also uncouple oxidative
phosphorylation in mammalian mitochondria. Their
mutagenicity is also well documented [38,39]. The electro-
philic nature of the nitro group causes TNT to readily
oxidize biological reductants, causing toxicity directly or by
formation of other reactive products such as nitroarene
radicals [40]. In addition, the nitro groups draw electrons
from the aromatic \( \pi \) bonds, effectively reducing the electron
density of the conjugated aromatic system. As a result, TNT
is resistant to degradation via electrophilic attack by oxy-
genases [41–43]. In order for TNT to be mineralized, orga-
nisms must first remove or transform the nitro groups. Its
abundance, persistence, and resistance to degradation make

Fig. 5. Mass spectra of oxalic acid chelated Fe(III) at pH 1.5 (A) and pH 4.5 (B). The insets are the proposed structures, and the arrows point to their corresponding m/z fragment.

TNT one of the most intensely studied hazardous organo-nitro compounds with respect to bioremediation. The ability of white rot fungi to degrade nitroaromatics has been well documented [44, 45]. In cultures of *P. chrysosporium*, approximately 35% of the radioactivity from ring-labeled 14C-TNT added at a concentration of 1.3 mg/l was trapped as 14CO₂. Of the remaining radioactivity, 25% was water-soluble material, and 16% was soluble in methylene chloride and shown by HPLC analysis to be more polar than TNT [46]. Higher concentrations of TNT appear to inhibit TNT mineralization in this fungus. Spiker et al. [47] found that TNT was inhibitory to spore germination of *P. chrysosporium* at concentrations greater than 5 mg/l. This toxicity could be related to the

Fig. 6. Mass spectra of compounds present during *in vitro* experiments containing oxalic acid (3 mM), FeCl₃ (1 mM), H₂O₂ (10 mM), with solid TNT added to a final concentration of 50 ppm. Structures of detected compounds are presented near their m/z ions.

Fig. 7. Proposed dimer formation by the spontaneous Schiff-base reaction between an amine and aldehyde derivative of TNT degradation in Fenton-like reactions.
activity of TNT as an oxidant, since reduction to 2- or 4-aminodinitrotoluene relieved toxicity.

Results of our mineralization experiment (Table 2) confirmed the superiority of P. chrysosporium over G. trabeum for both TNT and acetophenone degradation. However, freshly inoculated cultures of G. trabeum were almost ten times more resistant to high concentrations of TNT than a similar culture of P. chrysosporium. We observed the typical biotransformation products that are indicative of reductive degradation (Fig. 2), suggesting that the pathway through 4-ADNT is the most favorable initial reduction reaction. However, 2,4-DANT did not accumulate, and the 2,4-DANT concentrations observed were much lower than those of the mono-amino products. This result implies that there are reactions that compete with the reduction of the second nitro group to an amino group, or that 2,4-DANT is rapidly further transformed. It has been shown in previous studies that oligomers can form by the reaction of hydroxy-laminio and nitroso groups in the presence of oxygen, forming 4,4',6,6'-tetra-nitro-2,2'-azoxytoluene or 2,2',6,6'-tetranitro-4,4'-azoxytolueno [12,22,48,49]. Cultures of G. trabeum incubated with TNT maintained a dark brown color, which is consistent with polymer formation. However, standards of the azoxy compounds did not match any compounds observed in the culture supernatants and identified by GC/MS.

In an effort to determine the fate of the metabolites, other polymerization and condensation reactions were considered. A Schiff-base formation occurs spontaneously between an aldehyde and an amine and is a well-known reaction in biological systems [50]. In the current study the key metabolites for such a reaction would be trinitrobenzaldehyde (TNBA) or 4-amino-2,6-dinitrobenzaldehyde (4-ADNBA). These compounds could undergo reactions at either the amino or the aldehyde position, forming oligomers or even larger polymers (Fig. 7). Since we observed aminodinitrobenzaldehyde in culture supernatants of G. trabeum, such reactions are presumably likely to occur. Analyses of G. trabeum cultures (Fig. 2, Table 4) and in vitro reactions (Fig. 6) revealed several aromatic nitro-amines and nitro-aldehydes that could form the expected polymers. Any heteropolymer formed would be particularly difficult to analyze, because there are numerous possibilities for the formation of condensed metabolites with 2,3,4 or more benzene ring cores, with different combinations of nitro- and amino-substitutions. The small peaks (m/z 390–440) (Fig. 6) revealed daughter fragmentation patterns consistent with dimer structures. The similarities between TNT degradation products in these experiments and compounds found in fungal cultures suggest the involvement of reduced oxygen species produced by Fenton-like reactions in fungal cultures.

The in vitro experiments with 2,5-dimethoxy-1,4-hydroquinone, FeCl2 and TNT in reaction mixtures did not produce any of the detectable degradation products found in the G. trabeum cultures (data not shown). Moreover, six out of eight Gloeophyllum strains examined produced methoxylated quinone isomers (Table 3). We did not find a correlation between quinone production and TNT transformation in G. trabeum, but secretion of methoxylated quinones seems to be a common feature of the Gloeophyllum genus.

Salicylic acid and phenol were used as probes to trap hydroxyl radicals in cultures of several of the investigated species. All of the cultures examined readily degraded salicylic acid and phenol. Low concentrations of 2,3-dihydroxy benzoic acid was observed in the media extracts of cultures of G. striatum and G. prostromptum. This product is indicative of the presence of hydroxyl radicals, but the low concentration of this compound and its absence from cultures of other Gloeophyllum species suggest the involvement of mechanisms other than the hydroxyl radicals in these reactions. ESI-MS/MS results (Fig. 6) showed a peak at m/z = 260. Daughter fragmentation analyses revealed a fragmentation pattern consistent with TNT (mass 227) substituted with OOH* (mass 33). The pKa for hydroperoxy radical is 4.8, [51] and should permit the existence of this species in our reaction, which had a pH = 4.5.

The coupling products of TEMPO and 2,5-dimethoxyquinoline (2,5-DMHQ) were detected and are indicative of semi-dimethoxyquinone radical formation. This observation suggests that the presence of semiquinone radicals in fungal cultures, but subsequent data do not support their direct involvement in TNT degradation.

The research presented supports the involvement of reduced oxygen species, generated by Fenton-like reactions in the degradation of TNT by brown rot fungi. We are now conducting additional experiments to characterize the reduced oxygen species involved in redox reactions in brown rot cultures. We are using specifically labeled 2,4,6-15NO2, -CD3, and -ringD1 TNT, specific spin trap compounds, hydrogen peroxide and tert-butyl hydroperoxide, and 18O-labeled water and oxygen to address this fundamental question.

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References


