Research Article   **Open Access**

Rapid Response by the Ectomycorrhizal (ECM) Community of a Lodgepole Pine Forest to Diesel Application as Indicated by Laccase Gene Fragment Diversity

Ken Cullings* and **Julia DeSimone**

NASA-Ames Research Center, MS 239-11, Moffett Field, CA 94035-1000, USA

***Corresponding author:** Ken Cullings, NASA-Ames Research Center, MS 239-11, Moffett Field, CA 94035-1000, USA, Tel: 805-698-1610; Fax: 650-604-3954; E-mail: cullings1@earthlink.net

Abstract

A combination of field baiting and molecular methods was used to identify basidiomycete fungi that are able to withstand conditions within diesel contamination in a pine forest soil. Diesel was applied to 3, 1 meter x 1 meter blocks, which penetrated to a depth of 4 cm. After two weeks fungi that exhibited positive growth responses were identified by amplifying laccase (phenol oxidase) gene fragments using primers specific to basidiomycete laccase genes. The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA) gene repeat was amplified as well to provide a broad community screen, to indicate the pool of basidiomycete fungi that could respond to diesel application. Results indicated that ectomycorrhizal (ECM) fungi in the genera *Russula*, *Piloderma* and the ECM species *Tricholoma saponaceum* (rather than wood-rotting basidiomycetes) responded rapidly to diesel application. These results support recent hypotheses regarding the ecological role of ECM fungi in carbon cycling via exploitation of short chain phenolic carbon compounds. Further the ECM fungi that responded correspond roughly to classification of ECM exploration type, a criterion that describes ECM fungi on the basis of their hyphal growth patterns and potential for enzymatic activity. Finally, because it is apparent that these ECM fungi can survive and express laccase genes under conditions created by diesel contamination, these data provide new models for phytoremediation and site restoration strategies that utilize ECM fungi and pines.

Keywords: Bioremediation; Ectomycorrhizae; Laccase; Piloderma; Pinus contorta; Russula; Tricholoma

Introduction

Wood rotting and ectomycorrhizal (ECM) fungi naturally possess the enzymes necessary to break down the carbon chains found in many types of anthropogenic contamination [1-4]. Studies of model white rot fungi (e.g., *Pleurotus ostreatus*) indicate that lignin-degrading fungi will do just that [5] and can reduce hydrocarbon contamination in soils by as much as 40% in as little as one month [6]. Much of the work in these reductions is performed by fungal laccases, polyphenol oxidases that utilize a wide array of phenolic substrates that include lignin in wood and soil humic compounds, and also polycyclic aromatic hydrocarbons (PAH) and polychlorinated byphenols (PCB's) [1]. The latter are widespread soil and water contaminants of anthropogenic origin that can accumulate via several sources, including diesel spills [7].

Laccases are easily inducible, are involved in lignin beak-down, utilize a broad range of substrates are often extracellular and reduce toxicity of these compounds via immobilization to humic substances, thus lowering their bioavailability [1]. Laccases are easily inducible, utilize a broad range of substrates, and are often extracellular. Phenolic-based substrates that are utilized by laccases include polycyclic aromatic hydrocarbons (PAH) and polychlorinated by phenols (PCB's), both of which are widespread soil and water contaminants of anthropogenic origin. Because of these utilities, the range of industrial uses is wide and includes enzymatic remediation of soil and water contaminants, polymer synthesis, pulp bleaching, lignolytic-cellulosic biofuels production, and textile dye bleaching [1].

Hence, fungal laccases are considered excellent candidates for use in enzyme-based remediation strategies [1,8-10].

Petroleum hydrocarbon contamination of boreal and forest soils is a persistent problem with several sources including leaking transfer pipes and storage tanks, and surface spills [2]. While there are indications that microbial processes can aid in the remediation of spills in these ecosystems, our understanding of the microbial communities and how they function under spill conditions are not completely understood [2]. In this study, we "baited" soils in a lodgepole pine (*Pinus contorta*) forest with diesel, and used molecular-genetic methods to assess the effects of contamination on basidiomycete laccase gene diversity. We performed this work in a field setting rather than in the lab so that all processes governing and impacting fungal function and diversity would be intact. Laccases catalyze the reduction of oxygen to water, and in so doing oxidize phenolic-based substrates [1].

The goal of this endeavor was to determine 1) whether fungal communities would respond rapidly to the addition of this substrate in terms of detection of laccase genes by PCR and also in terms of measurable changes in laccase diversity following 2 weeks of exposure, 2) whether wood rotting or ECM fungi would dominate the taxa that do respond and 3) to provide new candidate fungal species that could be explored as potential enzyme sources and also as specific plant/fungal combinations for phytoremediation and/or habitat restoration strategies.

Methods

Experiment Design

Diesel (750 ml/block) was applied to a depth of 4 centimeters (CM) corresponding to the depth of the thin organic layer to soils using a watering can, in three replicate 1 meter (M) x1 M blocks in an old growth lodgepole pine stand near Yellowstone. The study site was comprised of 100-150 year old lodgepole pine with 300.0 square feet/ acre basal area. Several cores were taken in the area to ensure that ECM roots present in the soil. Treatment plots were paired with adjacent control plots, into which no diesel was added. This arrangement ensured that treatment and control blocks were in closer proximity to each other than treatments to treatments/controls to controls, minimizing the likelihood that any patterns measured were due to random distribution of fungal hyphae in the soil rather than response to treatment.

Two weeks following application, 9 soil samples (3 samples from within each of 3 plots within each block) 1 centimeter (cm) diameter X 4 cm in depth) were taken from each paired treatment and control plot, for a total of 27 from both treatment and control, and a grand total of 54 soil samples.

Molecular Analysis

Soils were sifted to remove root and ECM material, DNA was prepped from soils using Mo-Bio PowerSoil DNA isolation kit, and DNA was amplified from each of the 54 cores by PCR using the basidiomycete-specific laccase primers Cu1F/2r, according to the method of Luis et al. [11]. In addition to amplifying, cloning and sequencing laccase fragments, we also amplified cloned and sequenced basidiomycete nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) amplicons using the basidiomycete-specific primer combination ITS1/4b using methods previously described [12]. This was done for 2 reasons. First, in order to identify the broad pool of basidiomycete fungi that might respond to diesel contamination. Second, for finer scale identifications to accompany what we hypothesized would be genus- or family-level identifications from the laccase fragments.

Polymerase Chain Reaction products were cloned using the Qiagen PCR Cloning plus Kit, plating with Kanamycin selection and blue/white screening, and modified by the protocol by adding 5 ul of sample to the ligation as described by Cullings and Hanely [12]. Thirty sequences from each plot were considered for species identification, providing a target total of 270 sequences for sequence analysis. Sequences were subjected to BLAST analysis to determine closest taxonomic affinities.

Results

Gels of PCR amplification of laccase DNA fragments two weeks following diesel amplification revealed laccase PCR product in 5 of 9 treatment plots, but none in any of the untreated plots, indicating rapid growth of basidiomycete fungi into diesel treated soils (Figure 1). The 5 plots were distributed amongst all 3 treatment blocks, rather than concentrated in only one indicating that the pattern was due to response rather than a concentrated distribution of fungal hyphae. The study was designed with N=3 replication in order to allow for statistical comparison of species richness/diversity between controls and treatments and to compare reactions by the ECM vs. wood rotting

communities, if the data warranted. However, because no taxa amplified from control soils using laccase-specific PCR primers, and because the only fungi that responded positively to diesel addition were ECM fungi, these comparisons could not be performed. Hence, we report a survey of ECM species that did respond to diesel.
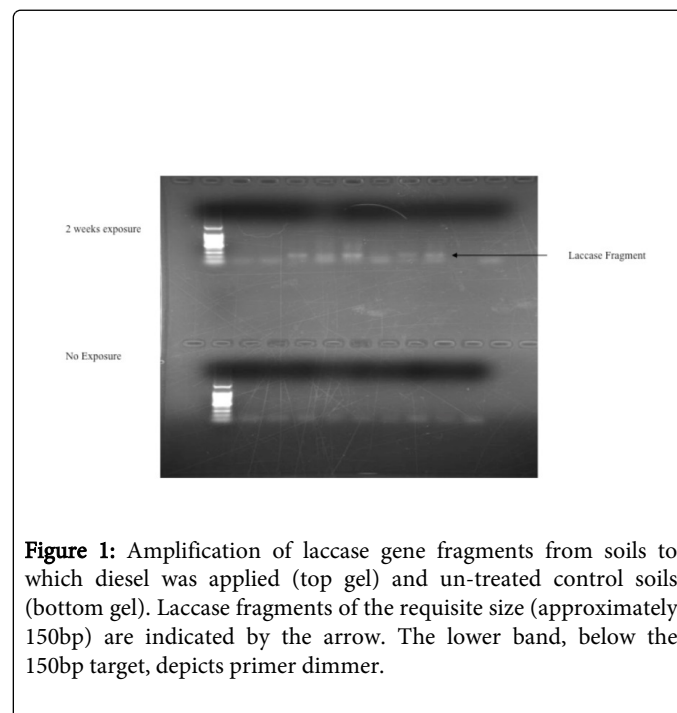

Figure 1: Amplification of laccase gene fragments from soils to which diesel was applied (top gel) and un-treated control soils (bottom gel). Laccase fragments of the requisite size (approximately 150bp) are indicated by the arrow. The lower band, below the 150bp target, depicts primer dimmer.

Sequencing of 150 clones from diesel-contaminated blocks (30 from each of the 5 of 9 plots that amplified) revealed 12 unique laccase fragments (Table 1) belonging to ECM fungi in the Russulaceae, Atheliaceae, Tricholomataceae and also one individual taxon in the Agaricaceae. None of the 6 sequences sharing homology with those in the Tricholomataceae or Agaricaceae shared sufficient homology with a species in any genus to allow identification beyond the family level. The 4 sequences with homology to the Russulacae and Athelaceae shared homology with the genera *Russula* and *Piloderma* respectively, but none in Genbank matched with sufficient homology to identify below the genus-level.

Family	# Unique Sequences
Russulaceae	2
Athelaceae	3
Tricholomataceae	6
Agaricaeae	1

Table 1: Identifications of Fungal Laccases by Family, via BLAST Search (70% or greater match to a genus in each family)

Sequencing of ITS clones provided additional resolution. In keeping with the laccase fragments, 2 species of the genus *Russula* were detected, as were 4 *Piloderma* taxa. One definitive identification by greater than 95% BLAST search match was obtained in the Tricholomataceae, *Tricholoma saponaceum* (Table 2). In addition, the ITS screen indicated the presence of ECM genera in several prominent EM fungal groups (Suilloid group of fungi, the Cortinariaceae,

Cantharellaceae, Hydnaceae, Mycenaceae and Thelephoraceae) (Table 3) that were absent from the laccase gene pool.

Russulaceae		
	Russula nigiricans	
	Russula turci	
Atheliaceae		
	Piloderma olivaceum	
	Piloderma lanatum	
	Uncultured *Piloderma* (2)	
Tricholomataceae		
	Tricholoma saponaceum	
	Uncultured *Tricholoma*	

Table 2: ITS Taxa Corresponding to Laccase Families (95% or greater Genbank match)

Family	Number of ITS "Taxa"
Cortinariaceae	15
Suilloid Group	5
Thelephorales	5
Hygrophoraceae	1
Mycenaceae	1
Cantherellaceae	1
Polyporaceae	1
Hydnaceae	1

Table 3: Fungal Families Present Indicated By ITS But Absent From Laccase Gene Pool

Discussion

Our data indicate that only ectomycorrhizal (ECM) fungi (and not wood rotting fungi) exhibited rapid responses to the application of diesel to a forest soil as evidenced by sequence and BLAST analysis of laccase gene copies. We hypothesize that laccase, being a single copy gene, is more difficult to detect relative to nrDNA gene copies which are part of a tandem repeat unit that can be repeated up to 10,000 times in a single genome. Hence, it is through positive growth responses to an added substrate that laccase genes reached detection levels in DNA extracts from forest these soils.

These results are in keeping with recent data that indicate that some ECM fungi (*Gomphidius viscidus* and *Laccaria bicolor*) can grow with diesel as their sole nutrient source [13]. This is perhaps not surprising. ECM fungi are recently evolved from wood-rotting basidiomycetes [14], and are obligate mutualists that form intimate associations with the roots of host trees. However, despite being dependent upon a mutualistic interaction with a host tree for carbon uptake and survival, many ECM fungi demonstrate the ability to utilize lignin as a nutrient source [15,16]. Further, many produce laccases and other enzymes involved in carbon cycling in natural ecosystems [1,2,7,9,10,12,17,18]. Indeed, recent evidence suggests that many EM fungi possess the ability to adopt at least a partial saprophytic habit in times of low host carbon availability [17,19-22], or when carbon substrate is added [21]. Because of these findings, it has been hypothesized that they may derive at least part of their carbon nutrition by enzymatic means [16,19,20,23], though their capacity to break down wood is the topic of considerable debate [1]. However, while they may not possess the capacity to fully break down the complex lignin polymers in wood, it has been hypothesized that ECM may play a significant role in carbon cycling via metabolism of the shorter chain phenolic breakdown products created by the metabolism of lignin by other microbes [20]. These smaller compounds are analogous to components of many anthropogenic contaminants, represented here by diesel.

Our data revealed genera in ECM fungal 3 families that exhibited positive responses to diesel application; the Russulaceae (2 *Russula* species), Atheliaceae (3 *Piloderma* species), and the Trichoomataceae. Russuloid fungi are known to have laccase activity [23], and species within the Russulaceae can exhibit significant oxidative enzyme activities [24]. However, culture-based experiments involving measurement of potential remediation function of specific *Russula* species obtained from non-contaminated soils indicate that these fungi are often poor performers [23]. Despite this, it was noted that fungi are likely to perform differently in a natural setting and in symbiosis with their host trees than they do in pure culture, particularly if these fungi are obtained from contaminated soils which would presumably screen for more active isolates.

ECM fungi can often be classified by hyphal "exploration type", which is based on their foraging distance in the soil, which can often be correlated with potential to produce oxidizing enzymes [25]. *Russula* species are classified by this system as a type that possesses high phenol oxidizing potential, and hence potentially have a high ability to utilize substrate via lignolysis. In keeping with this classification, the results of our study indicate that some *Russula* species exhibit rapid, positive growth responses when exposed to diesel, supporting the hypothesis that at least some ECM fungi could fulfill a role in carbon cycling in forest ecosystems through the utilization of less recalcitrant phenolic compounds, if not from complete breakdown of woody substrate [20].

The data also indicated positive responses by fungi in the family Tricholomataceae. However, genetic matches to members of the Tricholomataeae were low, 70% or less, indicating gaps in our knowledge regarding the diversity of these taxa. Indeed, molecular evidence indicates that this family is paraphyletic with subgroups that are comprised of several other families [26]. Hence, conclusions regarding these taxa are less certain. Despite this, our studies of effects of decreased host photosynthetic potential on ECM communities indicate that both groups can be prevalent members of the soil hyphal community in pine forests [12]. Further, *Tricholoma* species possess laccase and proteinase activity [27,28] and significant lignin-degrading potential has been demonstrated in several *Tricholoma* species [16,24]. Indeed, *T. aurantium* may utilize low molecular weight lignin-derived substrates more efficiently than some traditional wood-rotting fungi [16]. Though the laccase data indicate more species in this family than our ITS screen(most likely due to the paraphyletic nature of the Tricholomataceae) our data support the notion that members of this ECM genus could take part in carbon cycling in forest soils and could provide useful models for ectomycorrhiza-mediated restoration strategies in diesel contaminated soils.

Multiple laccase-like genes have been detected in *Piloderma* (Atheliaceae) species [29]. Because of this, and because they are often detected in organic soil layers, it has been hypothesized that they may have some role in lignin degradation processes [29]. Our data indicate that *Piloderma* taxa exhibit positive growth responses to diesel application, suggesting that they could be utilizing short chain phenolic compounds as nutrient sources. Unfortunately, over 3 fruiting seasons we found no *Piloderma* fruiting bodies. Hence, identifications cannot be made to the species level at this time.

The majority of fungi in the soil pool did not exhibit a positive PCR response to diesel application. Most notable in this group of fungi are those in the Cortinariaceae and the Suilloid group (e.g., *Suillus* and *Rhizopogon* species). Suilloid fungi tend to be long distance exploration types that in general lack lignin-degrading abilities [25]. However, some *Suillus* species can exhibit significant phenol-oxidation activity in field settings in which all factors that influence function are present and intact [12,19] and in some remediation settings [23]. *Rhizopogon* sp, however, exhibit little if any remediation potential [23], a finding that would seem to be supported by the lack of growth response we measured in our study. Cortinarioid fungi tend to be medium distance types, also generally lacking in phenol-oxidizing activity. Thus, in addition to being an indicator of physiological potential, Agerer's exploration type may provide a rough indicator of fungi for potential screening for ectomycorrhizal-mediated phytoremediation of phenolic-based contaminants and as enzyme sources for industrial use.

In conclusion, our data indicate that the ECM community can respond rapidly to application of diesel as a potential nutrient source. Further, our results provide new information as to the range of environmental conditions that can be tolerated by some Russuloid and Athelioid fungi, and fungi in the Tricholomataceae. Finally, even if the ECM fungi in this system are playing no role in diesel degradation, ECM fungi can protect the host from damage when growing in harsh environments [30] thereby enhancing their survivability in extreme habitats. Thus, our data provide new ECM models for further studies into phytoremediation or habitat restoration strategies that utilize specific host/fungal combinations.

References

1. Baldrian P (2006) Fungal laccases - occurrence and properties. FEMS Microbiol Rev 30: 215-242.

2. Robertson SJ1, McGill WB, Massicotte HB, Rutherford PM (2007) Petroleum hydrocarbon contamination in boreal forest soils: a mycorrhizal ecosystems perspective. Biol Rev Camb Philos Soc 82: 213-240.

3. Saparrat MC1, Guillén F, Arambarri AM, Martínez AT, Martínez MJ (2002) Induction, isolation, and characterization of two laccases from the white rot basidiomycete Coriolopsis rigida. Appl Environ Microbiol 68: 1534-1540.

4. Taprab Y1, Johjima T, Maeda Y, Moriya S, Trakulnaleamsai S, et al. (2005) Symbiotic fungi produce laccases potentially involved in phenol degradation in fungus combs of fungus-growing termites in Thailand. Appl Environ Microbiol 71: 7696-7704.

5. Kerem Z1, Friesem D, Hadar Y (1992) Lignocellulose Degradation during Solid-State Fermentation: Pleurotus ostreatus versus Phanerochaete chrysosporium. Appl Environ Microbiol 58: 1121-1127.

6. Eggen T, Majcherczyk A (1998) Removal of polycyclic aromatic hydrocarbons (PAH) in contaminated soil by white rot fungus Pleurotus ostreatus. International Biodet and Biodeg 41: 111-117.

7. Bamforth S, Singleton I (2004) Review: Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. J Chem Tech Biotech 80: 723-736.

8. Ang E, Zhao H, Obbard JP (2005) Recent advances in the bioremdiation of persistent organic pollutants via biomolecular engineering. Enz Micro Tech 37: 487-496.

9. Camarero S1, Ibarra D, Martínez MJ, Martínez AT (2005) Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. Appl Environ Microbiol 71: 1775-1784.

10. Soden DM1, Dobson AD (2001) Differential regulation of laccase gene expression in Pleurotus sajor-caju. Microbiology 147: 1755-1763.

11. Luis P, Walther G, Kellner H, Martin F, Buscot F (2004) Diversity of laccase genes from basidiomycetes in a forest soil. Soil Bio Biochem 36: 1025-1036.

12. Cullings K, Hanely J (2010) Dwarf Mistletoe Effects on Soil Basidiomycete Community Structure, Soil Fungal Functional Diversity, and Soil Enzyme Function: Implications for Climate Change. Soil Bio Biochem 42:1176-1178.

13. Li X1, Huang Y, Wei ZC (2008) [Degradation effect of two pure cultured ectomycorrhizal fungi on diesel]. Ying Yong Sheng Tai Xue Bao 19: 1579-1584.

14. Hibbett DS1, Gilbert LB, Donoghue MJ (2000) Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. Nature 407: 506-508.

15. Haselwandter K, Bobleter O, Read DJ (1990) Degradation of 14C-labelled lignin and dehydropolymer of coniferyl alcohol by ericoid and ectomycorrhizal fungi. Arch Micro 153: 52-354.

16. Trojanowski J, Haider K, Hu¨tterman A (1984) Decomposition of 14C-labelled lignin holocellulose and lignocellulose by mycorrhizal fungi. Arch Micro 139: 202-206.

17. Courty PE, Bréda N, Garbaye J (2007 Relation between oak tree phenology and the secretion of 130. organic matter degrading enzymes by Lactarius quietus ectomycorrhizas before and during bud break. Soil Bio Biochem 39: 1655-1663.

18. Pritsch KS, Raidl E, Markensteiner H, Blashcke, Agerer R, et al. (2004) A rapid and highly sensitive method for measuring enzyme activities in single mycorrhizal tips using 4-methylumbelliferone-labeled fluorogenic substrates in a mciroplate system. J Micro Met 58: 233-241.

19. Cullings K1, Ishkhanova G, Henson J (2008) Defoliation effects on enzyme activities of the ectomycorrhizal fungus Suillus granulatus in a Pinus contorta (lodgepole pine) stand in Yellowstone National Park. Oecologia 158: 77-83.

20. Cullings K1, Courty PE (2009) Saprotrophic capabilities as functional traits to study functional diversity and resilience of ectomycorrhizal community. Oecologia 161: 661-664.

21. Cullings K, Ishkhanova G, Ishkhanov G (2010) Induction of saphophytic behavior in the ectomycorrhizal fungus Suillus granulatus by litter addition in a Pinus controta (Lodgepole pine) stand in Yellowstone. Soil Bio Biochem 42: 1976-1981.

22. Tedersoo L, Kõljalg U, Hallenberg N, Larsson KH (2003) Fine scale distribution of ectomycorrhizal fungi and roots across substrate layers including coarse woody debris in a mixed forest. New Phytol 159:153-165.

23. Mehard A, Cairney JWG (2000) Ectomycorrhizas-Extending the capabilities of rhizosphere remediation? Soil Bio Biochem 32: 1475-1484.

24. Gramss G, Gunther TH, Fritsche W (1998) Spot tests for oxidative enzymes in ectomycorrhizal, wood and litter decaying fungi. Myco Res 102: 67-72.

25. Agerer R (2001) Exploration types of ectomycorrhizae: A proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. Mycorrhiza 11: 107-114.

26. Moncalvo JM1, Lutzoni FM, Rehner SA, Johnson J, Vilgalys R (2000) Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. Syst Biol 49: 278-305.

27. Kim JH1, Kim YS (2001) Characterization of a metalloenzyme from a wild mushroom, Tricholoma saponaceum. Biosci Biotechnol Biochem 65: 356-362.

28. Wang HX1, Ng TB (2004) Purification of a novel low-molecular-mass laccase with HIV-1 reverse transcriptase inhibitory activity from the mushroom Tricholoma giganteum. Biochem Biophys Res Commun 315: 450-454.

29. Chen DM, Bastias BA, Taylor AFS, Cairney JWG (2003) Identification of laccase-like genes in ectomycorrhizal basidiomycetes and transcriptional regulation by nitrogen in Pisolithus byssium. New Phyto 157: 547-554.

30. Cullings K1, Makhija S (2001) Ectomycorrhizal fungal associates of Pinus contorta in soils associated with a hot spring in Norris Geyser Basin, Yellowstone National Park, Wyoming. Appl Environ Microbiol 67: 5538-5543.