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ORIGINAL PAPER

Decomposition of tobacco roots with modified phenylpropanoid content by fungi with contrasting lignocellulose degradation strategies

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Abstract The decomposition of tobacco roots with genetic modifications to lignin biosynthesis by the ligninolytic fungus *Phanerochaete chrysosporium*, by the cellulolytic fungus *Chaetomium globosum*, and by microbial communities in soil were examined to determine whether the rates of decomposition of the modified and unmodified roots decomposed at different rates, whether the order of colonization by *P. chrysosporium* and *C. globosum* facilitated decomposition, and whether the microbial community in soil was conditioned by exposure to roots subsequently so that the subsequent decomposition of the roots was increased. Both *P. chrysosporium* and *C. globosum* decomposed the modified roots more rapidly, at least initially, than the unmodified roots. Colonization by *P. chrysosporium* facilitated the subsequent decomposition by *C. globosum*, presumably because by degrading lignin, *P. chrysosporium* increased the susceptibility of the polysaccharide component of root material to attack by *C. globosum*. Selection of the soil microbial community by exposure to the modified residues accelerated subsequent decomposition of the root modified. Although demonstrating effects of the lignin modification on decomposition, they are relatively subtle and in most cases short-lived (less than 40 days) ones to which the microbial community is able to adapt, and therefore, we conclude that there are unlikely to be any persistent effects of the modified lignin on the soil decomposer community.

Keywords *Chaetomium globosum* · Genetic modification · Lignin · Tobacco · *Phanerochaete chrysosporium*

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Introduction

Plant material is a structurally complex mixture of biochemicals including polysaccharides and lignin, which vary in solubility and resistance to microbial attack. Generally, the initial colonization of dead plant material is by bacteria and fungi, which utilize the more labile components, i.e. starches, sugars and other soluble compounds of low molecular weight. Invertebrates facilitate further degradation by micro-organisms by comminuting and physically conditioning the plant material, and through the activity of their microbial symbionts which utilize polysaccharides (Dix and Webster 1995). The cellulose and hemicelluloses are also selectively utilized by free-living ascomycete 'soft rot' fungi (Dix and Webster 1995). At a later stage of decomposition, the decaying plant material may be colonized by lignolytic basidiomycetes, the 'white rot' fungi (Hammel 1997).

Lignin is a major structural component of plants. It is a high molecular weight, three-dimensional aromatic heteropolymer, associated with cellulose and hemicellulose (Jeffries 1990). It offers physical protection to the polysaccharide components from microbial and enzymatic attack (Blanchette et al. 1997). The insolubility of lignin leads to low bioavailability, and its

relative chemical stability means that oxidative, rather than hydrolytic, degradation systems are required for its breakdown (Hammel 1997). *Phanerochaete chrysosporium* is one of the few fungal species able to produce the low specificity exoenzymes needed to degrade lignin (Webster and Weber 2007). In addition to many isozymes of lignin and manganese peroxidases, a range of dehydrogenases, reductases and transferases (Cullen and Kersten 2004) are also produced and further extend the range of utilizable substrates (Tuor et al. 1995).

The relative complexity of lignin means that it is a major determinant of the decomposition of plant material in soil, although recent studies have pointed to greater importance of physical protection and accessibility to microbial and enzymic attack in soil than has previously been considered (Ladd et al. 1996; Dungait et al. 2010, 2012). In addition, the complexity of lignin affects the digestibility of forage by herbivores and the ease with which cellulose is extracted during of paper pulping. The monolignol branch of the phenylpropanoid pathway leading to lignin biosynthesis in plants has, therefore, been targeted for genetic modification both with improving understanding of lignin biochemistry and biosynthesis and altering the properties of plant material to facilitate paper pulping and digestibility as objectives (Atanassova et al. 1995; Chen et al. 2004; Halpin et al. 1994; O'Connell et al. 2002; Vailhé et al. 1998). Altering the properties of lignin has implications also for the subsequent decomposition of plant materials in the natural environment and has been discussed in the context of altering C sequestration in soil as a regulator in the global biogeochemical cycle of C (Hopkins et al. 2007). We have shown that down regulation of cinnamyl alcohol dehydrogenase (CAD) or caffeic acid O-methyltransferase (COMT) in both tobacco (*Nicotiana tabacum* L.) and poplar (*Populus tremula* × *Populus alba*) affects the short-term decomposition in soil of the plant material (Hopkins et al. 2001, 2005, 2006; Pilate et al. 2002; Tilston et al. 2004). For example, increased initial rates of decomposition of stem material is because of increased solubility and accessibility of the polysaccharide components in modified tobacco to microbial attack (Hopkins et al. 2006; Webster et al. 2005). Such differences in the substrate quality of plant material for decomposer organisms also contribute to differences in the composition of microbial communities in rhizospheres and on decomposing residues from modified plants (Halpin et al. 2006; Hénault et al. 2006).

We have followed up these investigations linking lignin modifications to differences in rhizosphere communities by examining the roles of specific decomposer fungi which have different lignin decomposition strategies. In this study, we have investigated the decomposition of roots from reduced CAD and reduced COMT tobacco plants by *P. chrysosporium* (Burds.; a white rot fungus) and *Chaetomium globosum* (Kunze; a soft rot fungus), and by microbial communities selected in soil by previous exposure to the modified plant material. Our aim was to explore interactions between specific members of the decomposer community and modifications to lignin biosynthesis in tobacco. Our hypotheses were: (1) that both white rot and soft rot fungi will decompose the genetically modified plant materials faster than unmodified plant material, (2) that colonization by *P. chrysosporium* before *C. globosum* will facilitate decomposition compared to colonization in the opposite order and (3) that selection of the soil microbial community by exposure to the modified residues will accelerate subsequent decomposition of the correspondingly modified root modified. We have used a model peat/sand mixture rather than natural soil to reduce the confounding factors of physical accessibility in which soil mineral colloids restrict access by micro-organisms to plant residues and to facilitate sterilization of the matrices.

Materials and methods

Root material

Triplicate tobacco (*N. tabacum* cv. 'Samsun') plants with downregulated CAD, COMT or both CAD and COMT simultaneously (Atanassova et al. 1995; Abbott et al. 2002; Halpin et al. 1994) and isogenic unmodified plants were grown in a horticultural peat medium for 10 weeks in a heated greenhouse. At harvest, root material was collected, washed and dried to constant mass at 40 °C and chopped to 5-mm lengths. Carbon and N contents were determined for ground sub-samples using a CHN analyser (Carlo-Erba, Milan, Italy).

Preparation of fungal inocula

Isolates of *P. chrysosporium* (IMI 232175, ATCC 34540) and *C. globosum* (IMI 185462) were obtained from CABI Bioscience (Egham, UK), freeze-dried spores were reconstituted in sterile water and grown on potato dextrose agar plates incubated for 2 weeks at 20 °C. Four 1-cm diameter discs were cut from the leading edge of the mycelia, added to 100 mL of conical flasks filled with sand cornmeal medium (Butler 1953) and then incubated for 3 weeks at 20 °C during which time the flasks were shaken periodically to distribute mycelium.

Effect of inoculation with fungi on root decomposition

The effects of two species of fungi and plant genotype on decomposition of root materials were investigated in a two-phase inoculation experiment. Horticultural peat (5 g of dry

weight, 932 mg Cg^{-1}, 275 mg Ng^{-1}, pH 5.7) was mixed with either 2 % sand cornmeal medium containing fungus (inoculated treatments) or autoclaved damp sand (uninoculated treatments). One hundred milligrams of root material was added to the peat–sand mixtures, and there were corresponding treatments without root material which served as a control for the root addition. The water content of the peat was maintained at 70 % of water holding capacity and the microcosms were incubated at 20 °C. After 62 days, another 2 % aliquot of sand cornmeal medium containing cultured fungi was added to the microcosms: To half of the microcosms, the same fungus was added (i.e. *C. globosum* followed *C. globosum*, and *P. chrysosporium* followed *P. chrysosporium*), and to the other half of the microcosms the fungus was changed (i.e. *C. globosum* followed *P. chrysosporium*, and *P. chrysosporium* followed *C. globosum*). Uninoculated treatments also had a further 2 % aliquot of autoclaved damp sand added after 62 days. Treatments were replicated six times in the first phase of incubation, declining to three replicates for the second incubation phase. Root decomposition was estimated by comparing CO_2 evolution from root-amended with unamended peat/sand mixtures (i.e. net CO_2 production) for a total of 109 days using a Respicond IV respirometer (Nordgren Innovations AB, Umeå, Sweden; Nordgren 1988). At the end of the experiment, the soil microbial biomass N was determined using the fumigation and extraction method of Amato and Ladd (1988) and was used as an index of microbial growth on the different root materials. This is not an ideal index of biomass in the current context because assimilation of N during decomposing C-rich residues because the major plants components of interest in this study, lignin and polysaccharides, do not contain N. For this reason, we can only use it as an indirect indicator of biomass change rather than as a direct measure of root C assimilation into microbial biomass.

Effect of previous exposure of soil to modified root material on subsequent decomposition

The effect of exposure of soil to tobacco root material with the different genotypes subsequent decomposition of root material was tested. The soil was a brown forest soil with sandy loam texture of the Sourhope series (Tilston et al. 2004). For the exposure phase, 120 mg of root material samples contained in 10×20 mm nylon mesh (0.25-mm^2 mesh) bags were buried in 12 g (dry weight equivalent) aliquots of soil and were incubated at 20 °C for 225 days. During this incubation, the soils were regularly ventilated and mixed, and the water content of the soil maintained at 50 % water holding capacity. After the exposure phase, the mesh bags were removed and 10 g (dry weight equivalent) samples of soil were amended with 100 mg of root material and incubated at 20 °C for 42 days in miniaturized respirometric chambers (Heilmann and Beese 1992). Root decomposition was estimated by comparing CO_2 evolution from soils that had received a second amendment with root material with those that had not received a second amendment (Webster et al. 2000). The microbial biomass N content of the soils was determined as described above after the decomposition phase.

Statistical analyses

Data were analysed by either the two-way analyses of variance or the repeated measures analysis of variance procedures of Genstat release 6.1 (VSN International Ltd., Hemel Hempstead, UK). Means were separated according to Fisher's least significant difference test or Tukey's honestly significant difference test.

Results

Decomposition by fungi with contrasting lignocellulose degradation strategies

During the first inoculation and incubation phase, both the plant genotype and the fungal inoculum had significant effects on root decomposition (Fig. 1). In the uninoculated treatment, reduced CAD–COMT roots decomposed more slowly than all the other roots, which did not differ significantly from each other, over the first 20 days (Fig. 1). Inoculation with either fungus significantly increased root decomposition compared with the uninoculated control (Fig. 1). Downregulation of CAD significantly increased the initial decomposition of root material by *C. globosum* for both the reduced CAD and the reduced CAD–COMT genotypes (Fig. 1). Downregulation of CAD and COMT significantly increased the initial decomposition *P. chrysosporium* (Fig. 1). The effects of different plant genotypes on decomposition were, however, only short-lived so that after 62 days there were no significant differences (Fig. 1). The effects of inoculation with fungi did, however, persist with all the inoculated treatments having decomposed significantly at 62 days compared to the uninoculated treatments (Fig. 1).

After the second fungal inoculation and incubation phase, no significant effects of plant genotype were detected (Fig. 2). The only fungal inoculum treatment that had a significant effect was the succession of *C. globosum* after *P. chrysosporium*, in which decomposition was significantly greater than that of the other inoculum treatments for all plant genotypes (Fig. 2).

The microbial biomass was greater for all the treatments that included root addition compared to the without-root

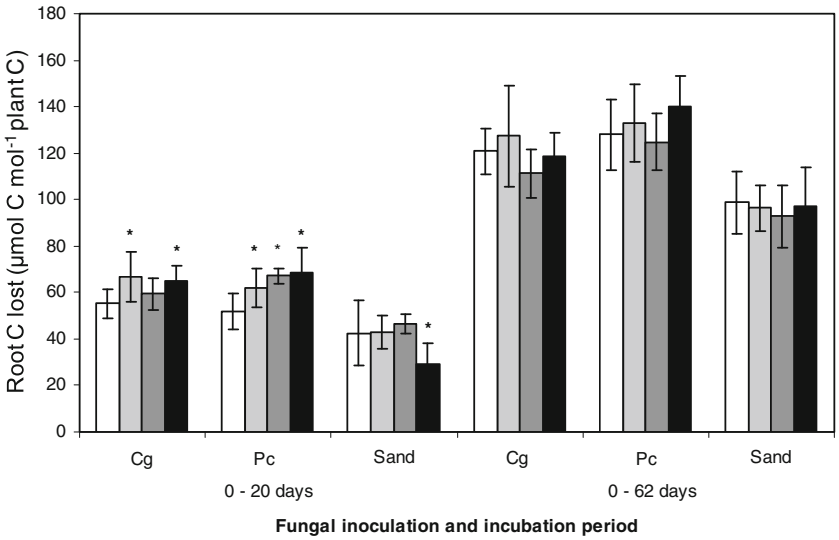

Fig. 1 Root C lost during 20 and 62 days of decomposition of unmodified root material (*unshaded columns*) and roots from reduced CAD (*lightly shaded columns*), reduced COMT (*medium-shaded columns*) or reduced CAD-COMT (*fully shaded columns*) plants. Inoculation with *C. globosum* or *P. chrysosporium* is indicated by *Cg* or *Pc*, respectively. Each value is the mean of three replicates, and the *error bars* represent standard deviations. Columns for genetically modified genotypes identified with an *asterisk* are significantly different from the unmodified control incubated under the same conditions according to Tukey's HSD ($P<0.050$)

controls, but there were no significant effects of either plant genotype or inoculation regime (Fig. 3).

Decomposition in soils previously exposed to material from plants with the same genotype

Previous exposure to plant material had a significant effect on the subsequent decomposition of added root material (Fig. 4). Initially, the rate of decomposition of root material was significantly greater in soil that had not been previously exposed to root material compared to soil that had been previously exposed to root material of the same genotype. After 21 days, decomposition rates in pre-exposed soil were significantly greater than in unexposed soil and by the end of the experiment (42 days), the equivalent of 15–20 % more root C had been lost from the exposed than from unexposed treatments irrespective of root genotype (Fig. 5). However,

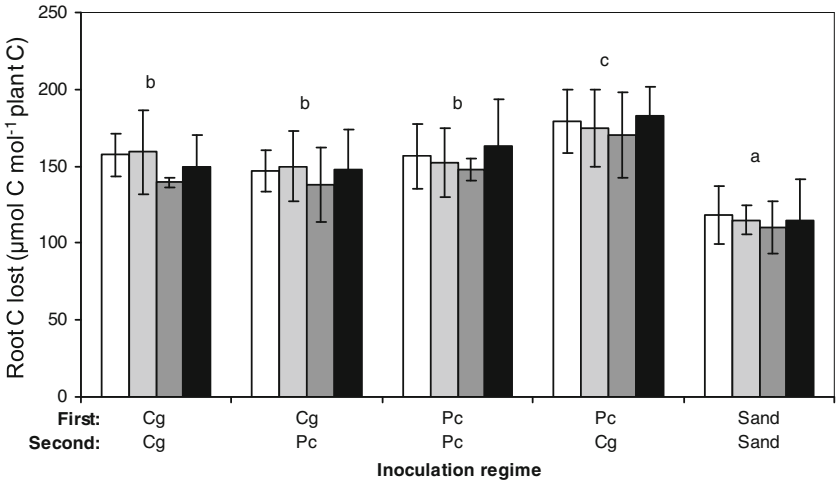

Fig. 2 Root C lost during 109 days of decomposition of unmodified root material (*unshaded columns*) and roots from reduced CAD (*lightly shaded columns*), reduced COMT (*medium-shaded columns*) or reduced CAD-COMT (*fully shaded columns*) plants. The order of inoculation with *C. globosum* (*Cg*) and *P. chrysosporium* (*Pc*) is indicated. Each value is the mean of three replicates, and the *error bars* represent standard deviations. Inoculation regimes identified with common *letters* are not significantly different from each other according to Fisher's least significant difference ($P<0.001$)

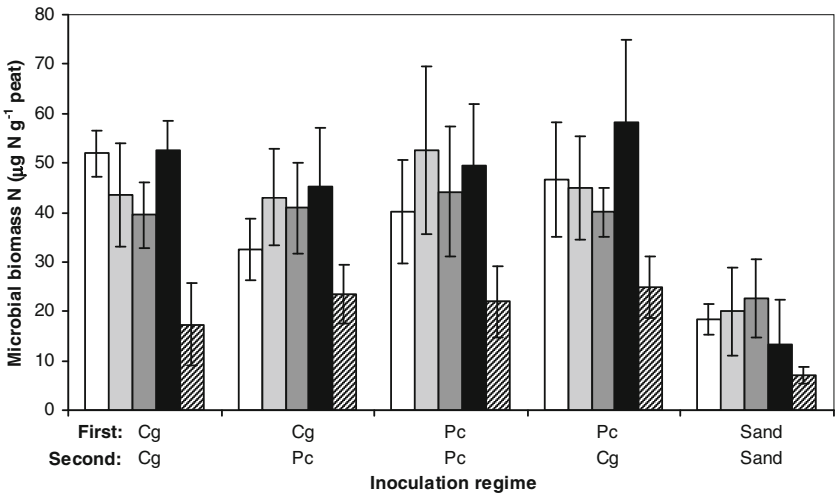

Fig. 3 Microbial biomass N contents of peat either amended with root material from unmodified tobacco plants (*unshaded columns*), from reduced CAD plants (*lightly shaded columns*), reduced COMT plants (*medium-shaded columns*), reduced CAD-COMT plants (*fully shaded columns*), or not amended with root material (*hatched columns*). The order of inoculation with *C. globosum* (*Cg*) and *P. chrysosporium* (*Pc*) is indicated. Each value is the mean of three replicates and the *error bars* represent standard deviations

there were no significant effects of plant genotype on decomposition or microbial biomass N at the end of the experiment (Figs. 4 and 5).

Discussion

Considering the initial fungal inoculation phase, our main observations are, first, that initially, roots with modified lignin decomposed in peat either at the same rate or more slightly more slowly than unmodified roots. This is in contrast with the decomposition of tobacco stems in which the modified material decomposed consistently faster than the unmodified stems in soil (Hopkins et al. 2001; Webster et al. 2005). Second, however, when the decomposer community was augmented with fungi, the modified roots decomposed initially more rapidly than the unmodified roots. This is consistent with the evidence that the plant contains more labile C or that labile, polysaccharide components of the modified plant materials were more accessible to microbial attack than in the unmodified plants (Hopkins et al. 2006; Webster et al. 2005). Furthermore, the effect of the genetic

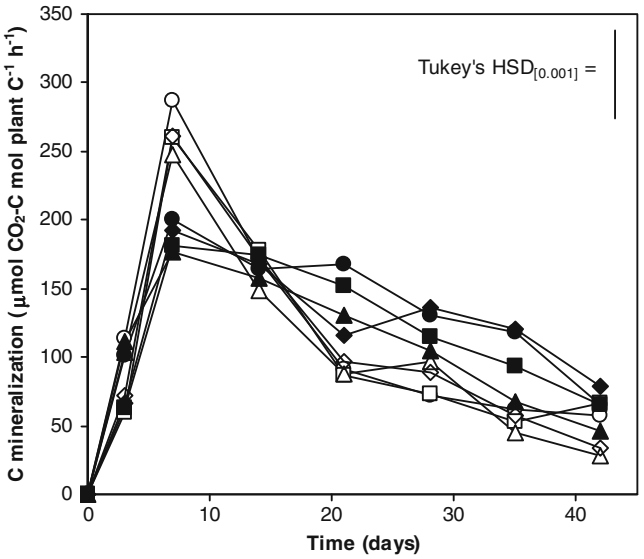

Fig. 4 Net CO_2 production over 42 days during the decomposition of unmodified root material (*filled circle*, *open circle*) and roots from reduced CAD (*filled square*, *open square*), reduced COMT (*filled triangle*, *open triangle*) or reduced CAD-COMT (*filled diamond*, *open diamond*) plants in soil either unexposed (*open symbols*) or pre-exposed (*filled symbols*) to dead root material of the same genotype. Net CO_2 production was estimated as the difference in CO_2 production between soil amended with root material and control, unamended soil. Each value is the mean of three replicates, and *error bars* are excluded for clarity, but more than 70 % are less than 20 % of the mean. Tukey's HSD bar is valid for comparisons between unexposed and pre-exposed treatments only

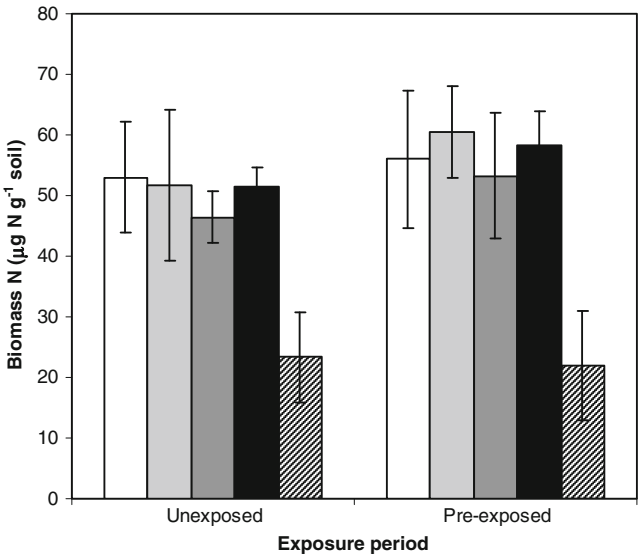

Fig. 5 Microbial biomass N contents of soil either unexposed or pre-exposed to root material from unmodified tobacco plants (*unshaded columns*), from reduced CAD plants (*lightly shaded columns*), reduced COMT plants (*medium-shaded columns*), reduced CAD-COMT plants (*fully shaded columns*), or not amended with root material (*hatched columns*). Each value is the mean of three replicates, and the *error bars* represent standard deviations

modifications on decomposition by *P. chrysosporium* is consistent with the action of lignin peroxidase preferentially cleaving aldehyde moieties (Faix et al. 1985) which are more abundant in reduced CAD plants and the relative depletion of recalcitrant syringyl moieties in reduced COMT plants (Bahri et al. 2006). Third, we observed that after a short-lived, relatively rapid phase of decomposition, roots with modified lignin and unmodified roots decomposed at the same rate irrespective of the inoculum treatments. Considering the second fungal inoculation phase, when *C. globosum* followed *P. chrysosporium* decomposition was promoted possibly because the ligninolytic action of *P. chrysosporium* exposed previously protected cellulose and hemicellulose (Bao and Reganathan 1992), which was subsequently exploited by *C. globosum.*

Although there is apparently no experimental consensus regarding the hypothesis that previous exposure to either living plants or plant litter from the same species results in functional adaptation of the decomposer community (Ayres et al. 2006), our results show greater decomposition occurred in soil exposed to plant material than from unexposed treatments irrespective of plant genotype. Reports of adaptation resulting in faster decomposition are frequently associated with material from inherently more recalcitrant plant species such as *Quercus rubra* compared with *Acer saccharum* and *Betula alleghaniensis* (Hansen 1999) or more recalcitrant organs such as stems rather than leaves (Cookson et al. 1998). Roots tend to be more recalcitrant than stems or leaves because they contain more lignin, and the lignin has a more condensed structure than is found in other parts of the plant (Bertrand et al. 2006), thus our results support the tendency for microbial decomposer communities to become adapted to more recalcitrant plant debris.

Conclusions

The data from these experiments have shown first that *P. chrysosporium* and *C. globosum* decomposed the modified roots more rapidly, at least initially, than the unmodified roots. This observation is similar to that for stems, for which the effects of plant genotype on decomposition was also short lived. Second, that *P. chrysosporium* facilitates the subsequent decomposition by *C. globosum*. This was presumably because by degrading lignin, *P. chrysosporium* led to an increase in the subsequent susceptibility of the polysaccharide component of root material to attack by *C. globosum*. This observation was, however, independent of the plant genotypes. Third, selection of the soil microbial community by exposure to the modified residues did accelerate subsequent decomposition of the root modified. This observation was also independent of the plant genotype. Thus, we demonstrate effects of the lignin modification on decomposition, but they are relatively subtle and in most cases short-lived to which the microbial community is able to adapt and therefore we conclude that there are unlikely to be any persistent effects of the modified lignin on the soil decomposer community.

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