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Ectomycorrhizal exudates and pre-exposure to elevated CO_2 affects soil bacterial growth and community structure

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ABSTRACT

Ectomycorrhizal fungi produce low molecular weight organic compounds, supporting diverse microbial communities. To link mycorrhizal root exudation directly to bacterial responses, we used Scots pine exudates with (*Suillus variegatus* and *Piloderma fallax*) and without mycorrhiza as substrata for forest soil bacteria. Bacterial growth and vitality was monitored, and community composition determined using T-RFLP, cloning and sequencing. We investigated if the amount of organic acids in exudates explained bacterial growth, and whether bacterial communities were influenced by pre-exposure to elevated atmospheric CO_2. We demonstrated functional differences in bacterial growth rates related to CO_2. There was a shift in the bacterial community (e.g. *Burkholderia* sp. and gamma-proteobacteria) toward organisms better able to rapidly utilize exudates when pine microcosms were pre-exposed to elevated CO_2. Soil bacteria from all treatments tended to grow more abundantly and rapidly in exudates from *Piloderma*-colonized seedlings, suggesting that the organic acids and/or unidentified compounds present supported greater growth.

1. Introduction

Soil microorganisms are an essential part of carbon (C) and nutrient cycling. Ectomycorrhizal (EM) fungi are a group of microorganisms of particular interest in terms of C flow since they are symbiotically associated with woody plant roots, ubiquitously present in boreal forests and have access to a ready supply of photosynthate from their the host plants (Hobbie, 2006). EM fungi thereby link plant roots to the surrounding soil environment and other microorganisms via their extensive extraradical mycelia (Agerer, 2001; Wallander et al., 2001). Mycorrhiza-associated bacterial communities represent a third part of the mycorrhizal symbiosis (Bonfante and Anca, 2009). Common mycorrhiza-associated bacteria are taxa such as *Burkholderia* and *Bacillus*, but tree species, soil conditions and fungal species affect what taxonomical groups will associate with the mycorrhiza. Bacteria are known to variously colonise the hyphal mantle and Hartig net of EM root tips, along with extraradical mycelium (Cairney and Meharg, 2002), and they stimulate mycorrhiza formation (Garbaye, 1994). One of the primary factors affecting microorganisms is the quantity and quality of exudates released by the mycorrhizal association (Heinonsalo et al., 2000; Cairney and Meharg, 2002; Dennis et al., 2010). The exudates, especially low molecular weight (LMW) organic compounds such as organic acids and amino acids, support diverse microbial communities, and enhance mineral weathering and nutrient uptake (Jones and Darrah, 1994; Jones, 1998; Bomberg et al., 2003; Bais et al., 2006). There is emerging evidence that tree species and associated fungi select for specific microbes through exudates (Frey-Klett et al., 2005b; Prescott and Grayston, 2013; Uroz et al., 2013), for

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example under elevated CO_2 (Drigo et al., 2010). The presence of a fungus may also reduce soil bacterial activity (Christensen and Jakobsen, 1993; Olsson et al., 1996). Although exudation may constitute a significant part of the overall C budget of mycorrhiza (Grayston and Campbell, 1996; van Hees et al., 2005; Fransson et al., 2007), field collected data on exudation is largely absent from the literature due to difficulties involved in collecting and measuring these compounds in natural systems, and a rapid turnover of LMW organic compounds (van Hees et al., 2005; Neumann et al., 2009).

Given their fundamental role in C cycling soil microorganisms are also central in ecosystem responses to environmental perturbations. Increasing anthropogenic inputs of atmospheric CO_2 have significant impacts on C cycling via altered plant photosynthate (Hyvönen et al., 2007; van Groenigen et al., 2014), and effects on the belowground soil compartment are thus indirect. Carbon input into the soil could increase and may consist of a greater C allocation through for example root exudation, turnover and respiration (Pregitzer et al., 2008; Phillips et al., 2011, 2012; Nie et al., 2013), and this increase has been shown to affect soil microorganisms (e.g. Fransson et al., 2001; Finzi et al., 2006; Ross et al., 2006; Lesaulnier et al., 2008), although no response to CO_2 has been reported as well (Larson et al., 2002; Janus et al., 2005). Root and fungal exudation can specifically increase, and/or chemical composition can be altered (Johansson et al., 2009; Fransson and Johansson, 2010; Phillips et al., 2011). In a recent study, Phillips et al. (2011) showed that rates of root exudation from intact *Pinus taeda* roots increased by an average of 50% in response to elevated CO_2, providing the first field-based empirical support suggesting enhanced rates of microbial activity and nitrogen cycling to be driven by root-derived C input. Microbial community composition has been shown to change in response to CO_2 (Ross et al., 2006; Carney et al., 2007; Lesaulnier et al., 2008; Feng et al., 2010; He et al., 2012), and microbial activity under increased CO_2 has been targeted in a number of studies (Larson et al., 2002; Finzi et al., 2006; Carney et al., 2007; Hofmockel and Schlesinger, 2007; Zheng et al., 2010; Phillips et al., 2011; He et al., 2014; Staddon et al., 2014). However, no general tendency in the CO_2 response has become clear, and observations range from undetectable or small effects to large effects. This is probably explained by differences in experimental treatments, environmental conditions (e.g. soil nutrients), microbial communities and type of ecosystem studied. Very few studies have tried to directly link the effects of specific root (Marschner et al., 2002; Weisskopf et al., 2008; Micallef et al., 2009; Badri et al., 2013) and mycorrhizal exudates (Phillips et al., 2011; Shi et al., 2012; Kaiser et al., 2014) to soil microbial communities. Despite the central role of EM fungi in mediating soil responses to belowground C fluxes and environmental perturbations such as elevated CO_2, empirical data on exudation and the impact of those exudates on microbial community composition and function is still largely lacking for the boreal forest system.

To link root and EM exudation directly to bacterial community responses we used exudates from mycorrhizal and nonmycorrhizal Scots pine (*Pinus sylvestris*) seedlings as growth substrates for forest soil bacteria. Based on our previous work (Johansson et al., 2009; Fransson and Johansson, 2010) we expected exudates from different EM fungal species to differ in total amounts and composition of LMW organic acids, thus potentially driving bacterial community responses. Bacteria were extracted from soil microcosms with mycorrhizal pine seedlings pre-incubated either in ambient or elevated CO_2 levels. We also expected that pre-exposure to elevated CO_2 levels had already impacted soil bacterial communities via belowground C flow, resulting in community differences. Bacterial growth and vitality was monitored over 144 h, and community composition was determined using T-RFLP, cloning and sequencing. We tested if 1) the amount of exudates and LMW organic acids explained bacterial growth and proportion of live cells; 2) exudates associated with a given mycorrhizal fungus (*Suillus*, *Piloderma*) enhanced the growth of bacteria associated with that fungus; and 3) the pre-exposure of mycorrhizal plants in soil microcosms to elevated CO_2 affected bacterial community composition and/or community function, in that the bacteria were better able to rapidly utilize exudate C sources, compared to bacterial communities pre-incubated at ambient CO_2 levels.

2. Materials and methods

2.1. Fungal strains, soil microcosms and pre-exposure to elevated CO_2

The overall experimental design is summarized in Table 1. Two common EM fungal species, *Piloderma fallax* (isolate code UP113, GeneBank accession number **DQ179125**) and *Suillus variegatus* (UP597, **EF493256**), with different growth rates and growth form, were used to produce mycorrhizal Scots pine (*P. sylvestris*) seedlings according to Fransson and Johansson (2010). Soil microcosms were prepared and maintained according to Fransson and Rosling (2014); details are given in Fig. S1. Eight replicate soil microcosms per fungal treatment and three replicate soil microcosms without seedlings or fungus (nonplanted controls) were prepared for each CO_2 treatment, and incubated in growth facilities with controlled CO_2 levels. Initially incubation was done at ambient CO_2 levels (360 ppm), until the extraradical mycelium started growing in the soil and/or growth of seedlings was apparent (two weeks for *S. variegatus*, one month for *P. fallax*, two months for nonplanted controls). This approach was chosen since the two fungal species differ in growth rate and we wanted the elevated CO_2 incubation to start as soon as extraradical mycelia were established. After starting the incubation at ambient levels, half of the replicates were moved to a second chamber with elevated CO_2 levels (710 ppm), and microcosms were pre-exposed to elevated CO_2 levels for a total of five months before bacteria were extracted from the soil (see below). The *Suillus* elevated CO_2 treatment was excluded from further analysis since the mycelia growth was fast and covered the microcosms after only 8 weeks of growth.

2.2. Extraction of bacteria from pre-exposed soil microcosms

Soil samples (10 g fresh weight) were collected from each of four randomly chosen microcosms per fungal x CO_2 treatment combination (*Suillus* ambient, *Piloderma* ambient and *Piloderma* elevated) and from three replicates each for nonplanted control ambient and nonplanted control elevated, giving a total of 18 samples. Soil bacteria were extracted using Nycodenz (Medinor AB, Stockholm, Sweden), allowing density-gradient separation of prokaryotic cells from soil material and eukaryotic cells (Rickwood et al., 1982; Lindahl and Bakken, 1995), and each of the replicates within treatments were handled separately. Extracted bacteria were suspended in 2 x potassium phosphate buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 5.5). The pH of the buffer was similar to that of the exudates and soil from which the bacteria originated. The bacterial cell concentration was measured in a Bürker chamber using an Axioplan fluorescence microscope (Zeiss, Oberkochem, Germany). Bacteria were immediately placed in exudate treatments as described below.

2.3. Production and analysis of exudates

Exudates were produced in liquid culture under axenic conditions and ambient CO_2 levels, and collected during an earlier study