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Short communication

Growth responses of indigenous *Frankia* populations to edaphic factors in actinorhizal rhizospheres

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ABSTRACT

Quantitative PCR (*q*PCR) was used to follow population dynamics of indigenous *Frankia* populations in bulk soil, in leaf-litter-amended soil and in the rhizosphere of *Alnus glutinosa* or *Casuarina equisetifolia* at 2 matric potentials representing dry and wet conditions in soil microcosms. Analyses revealed between 10- and 100-fold increases of *Frankia* populations within the incubation period of 12 weeks independent of treatment. Numbers were generally higher under dry conditions and in the rhizosphere, with that of *C. equisetifolia* supporting highest abundance. Frankiae detected at any time and treatment belonged to either subgroup I of the *Alnus* host infection group or the *Elaeagnus* host infection group, with those of the *Elaeagnus* host infection group largely representing the genus in all samples under wet conditions, and in bulk and leaf litter amended soil under dry conditions. Subgroup I of the *Alnus* host infection group was most prominent in the rhizosphere of both plant species where it represented up to 95% of the genus with higher percentages in that of *C. equisetifolia*.

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Members of the bacterial genus *Frankia* are generally characterized as nitrogen-fixing actinomycetes that form root nodules in symbiosis with a variety of woody plants [4,5]. While a considerable amount of information is available on nodule-forming populations, and the interaction of frankiae with host plant species [5,9], information on frankiae in soil, its second ecological niche, is relatively scarce [8]. Most of the information on soil *Frankia* populations focuses on their potential to form root nodules [19,35,36,39], diversity as a function of host plant species [1,2,23,35] and the effects of environmental characteristics on nodule-forming capacity [16,31,37,40]. Environmental characteristics such as soil organic matter and matric potential, for example, have been shown to affect the development of specific *Frankia* populations [17,18,20,21], with organic matter such as leaf litter supporting growth of one specific population, i.e., subgroup II of the *Alnus* host infection group represented by strain Ag45/Mut15 only [17]. Leaf litter amendments to soils consequently resulted in shifts of nodule-forming *Frankia* populations from subgroup I to subgroup II, both for introduced and indigenous populations [21]. Effects of matric potential are less well established even though drier conditions seem to favor root nodule formation of frankiae representing subgroup I over those of subgroup II [20]. While these effects focus on nodule-forming frankiae, their impact on population dynamics of frankiae in soil is still unknown.

The aim of this study was to take advantage of *q*PCR tools recently developed for the quantification of specific *Frankia* populations in soils [27,29] in order to assess abundance and growth of indigenous populations in microcosms over time as a function of plant species, potential carbon resource supply and matric potential. Microcosms were established in 50-ml falcon tubes using 40 g of soil (dry wt.) per tube at a density of approximately 1 g cm^{-3}. The soil was a Xenia silt loam, a mesic Aquic Hapludalf with about 1% organic material from a naturalized stand of exotic Autumn Olive (*Elaeagnus umbellata*) in Champaign County, Illinois (40.150014 N, 88.13002 W). This soil was collected in early spring 2013, and kept at 4 °C for about half a year before use. Half of the microcosms were adjusted to and maintained at a matric potential of −0.001 MPa (hereafter referred to as "wet"), the other half at a matric potential of −0.005 MPa (hereafter referred to as "dry") using round-bottom ceramic suction tubes (8 cm long with an outer diameter of 6 mm [#0652X02-B01M1], Soilmoisture Equipment Corp., Santa Barbara, CA) and 10 or 50 cm water columns, respectively [20].

Soils in half of the tubes were planted with seedlings ($n = 3$ per tube) of either *Alnus glutinosa* or *Casuarina equisetifolia*. Both plant species are host plants for different *Frankia* populations, with the exotic *C. equisetifolia* usually not forming nodules in soils from

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temperate regions. Seeds of these plant species had been surface sterilized in 3% H_2O_2 for 10 min, been washed twice with sterilized distilled water for 10 min each, and then been germinated on sterile water agar. Plantlets were transferred to microcosms 2 weeks after germination. The remaining half of tubes stayed non-vegetated, but the soil was mixed with 2% (wt/wt) leaf litter of either *A. glutinosa* or *C. equisetifolia* ground to a particle size of about 0.1 mm. This setup resulted in soil microcosms with 4 treatments per plant species: treatment 1 (plants, dry), treatment 2 (plants, wet), treatment 3 (organic matter, dry), and treatment 4 (organic matter, wet). All tubes were kept at 23 °C with a photoperiod of 16/8 h (day/night, respectively) for 3 months. Microcosms were sampled destructively at 3 times: directly after planting (t_0), and 6 or 12 weeks after planting (t_6 and t_{12}). For each time and treatment combination, 2 microcosms were analyzed. In vegetated microcosms, samples were separated into bulk and rhizosphere soil (except for t_0). Rhizosphere soil was defined as the soil adhering to plant roots when the plants were being removed from the remaining soil (i.e. bulk soil). DNA was extracted from homogenized bulk and rhizosphere samples as well as from leaf litter-amended soil samples in triplicate 250 mg soil samples using the SurePrep™ Soil DNA Isolation Kit (Fisher Scientific, Houston, TX) with small modifications as described before [29].

Ten-fold dilutions of the DNA extracts were used as a template in SybrGreen-based quantitative PCR (*q*PCR) analyses for nitrogen-fixing members of the genus *Frankia* using 23S rRNA gene sequences as target [27]. More specific analyses focused on four subgroups of the genus, i.e. subgroups I and II of the *Alnus* host infection group (cluster 1), *Casuarina*-infective strains and members of the *Elaeagnus* host infection group (cluster 3), that were represented by *Frankia* strains ArI3, Ag45/Mut15, CcI3 and EAN1pec, respectively [27,28]. *q*PCR quantification of frankiae was performed for each sample in triplicate in a total volume of 10 μl containing 5 μl of SsoADV SybrGreen Mix (BioRad, Hercules, CA), 0.2 μl of each primer (100 nM each) and 1 μl of template in an Eco Real-time PCR system (Illumina, San Diego, CA) using an initial denaturation at 95 °C for 5 minutes, and 40 cycles of denaturation at 95 °C, annealing at 64 or 66 °C depending on the primer combination, and extension at 72 °C, each for 30 s [27,28]. The amplification was followed by a melting curve analysis. Quantification was based on standard curves generated from purified PCR products of 23S rRNA gene fragments of strains ArI3, Ag45/Mut15, CcI3 and EAN1pec depending on the primer combination. Amplicons were generated using genus-specific primers 23Fra1533f/23Fra1769r, and concentrations measured with a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, USA). Copy numbers were calculated from concentrations (http://www.uri.edu/research/gsc/resources/cndna.html). Copy numbers were divided by copy numbers of the 23S rRNA gene per genome (2.5 for unknown populations) to relate copy numbers to *Frankia* cell numbers [27]. Results of all analyses were corrected for extraction efficiencies. These were determined as the ratio of inoculated *Salmonella typhimurium* (ATCC14028) cells detected by *q*PCR-based quantification of a 268-bp *inv*A gene fragment before and after extraction as described previously [29]. Two way ANOVAs followed by TukeyHSD test were performed in R to compare abundance of *Frankia* and subgroups in bulk or amended soils and rhizospheres of *Alnus* and *Casuarina* plants.

Microcosms analyzed for *Frankia* at t_0, revealed *Frankia* cell densities of $3 \pm 1 \times 10^4$ cells (g soil)$^{-1}$ which was at the detection limit of our current quantification method (Fig. 1). These numbers were about 2 orders of magnitude lower than those previously encountered in natural soils using *q*PCR-based analyses [27,29]. These low numbers are likely a function of sample collection after winter and subsequent storage of soils at 4 °C for half a year, even though short-term storage at 4 °C has been shown to not significantly influence diversity and relative abundance of bacteria in DNA-based microbial community analysis [15]. Incubation for 6 and 12 weeks increased *Frankia* populations between 10- and 100-fold (i.e. 2 and 81×10^5 cells (g soil)$^{-1}$) in all samples, i.e. bulk soil, the rhizosphere or after the addition of leaf litter, independent of plant species and matric potential ($P < 0.001$) (Fig. 1).

The largest increases were observed in the rhizosphere of both plant species, with plant species and matric potential affecting overall abundance. In the rhizosphere of *C. equisetifolia* about four times as many frankiae were observed than in the rhizosphere of *A. glutinosa* ($P < 0.001$), and about twice as many in dry microcosms compared to numbers of frankiae in wet microcosms ($P = 0.004$) though differences were not statistically significant for alders ($P = 0.46$) (Fig. 1). Numbers in bulk soil ($P = 0.03$) or soils amended with leaf litter ($P = 0.06$) were about half of those in the rhizosphere (Fig. 1). The increase in *Frankia* populations was usually greatest between t_0 and t_6 ($P = 0.01$) with about 10-fold increases representing 3–5 generations, assuming exponential growth and doubling times between 8 and 15 days, with faster doubling times generally observed under dry conditions and in the rhizosphere. Except for treatments with *Casuarina* plants (i.e. bulk soil and rhizosphere), where additional 10-fold increases were obtained, numbers of *Frankia* remained similar or increased only slightly between t_6 and t_{12} ($P = 0.3$) with 0–2 generations and doubling times of about 25 days and higher (Fig. 1). These data indicate the possibility of density-dependent population dynamics of *Frankia* in bulk soil, the rhizosphere and leaf litter amended soils, with lower, though statistically not significantly different ($P = 0.24$) densities in soils with *A. glutinosa* compared to those with *C. equisetifolia*, and under wet conditions compared to dry soils ($P < 0.001$) (Fig. 1). Thus, numbers of frankiae previously encountered in natural soils using *q*PCR-based analyses [27,29] might actually represent density limited populations similar to those in stationary phase conditions of pure cultures.

Although many soils contain significant amounts of organic material, carbon resources are usually not meant to be easily available. Considering that microbial growth in bulk soil might therefore be significantly affected by carbon limitation [7,11], the 10- to 100-fold increase in indigenous *Frankia* populations in bulk soil during the 6–12 week incubation period is quite surprising. However, densities at the beginning of the incubation were very low with about 10^4 cells (g soil)$^{-1}$ and increased to final abundance values of about 10^6 cells (g soil)$^{-1}$ which represented numbers normally encountered in soils in our previous studies using *q*PCR-based analyses [27,29]. The increase might therefore reflect changing environmental conditions in our experimental set up, with low numbers obtained after storage of soils at 4 °C, and increasing numbers as a function of increasing temperature (23 °C), enhanced water availability and consequently better nutrient availability [30].

At t_0, *Frankia* cells belonged to subgroup I of the *Alnus* host infection group represented by strain ArI3 ($1 \pm 0 \times 10^4$ cells (g soil)$^{-1}$), and the *Elaeagnus* host infection group represented by strain EAN1pec ($1 \pm 0 \times 10^4$ cells (g soil)$^{-1}$). Cells of subgroup II of the *Alnus* host infection group represented by strain Ag45/Mut15 and of *Casuarina*-infective strains represented by strain CcI3 were not detected at t_0, nor after incubation for 6 (t_6) or 12 (t_{12}) weeks. In bulk soil, abundance patters of frankiae of subgroup I of the *Alnus* host infection group and those of the *Elaeagnus* host infection group followed patterns obtained for the genus, i.e. with largest increases usually obtained between t_0 and t_6, and little or no increases in cell numbers between t_6 and t_{12}, except again for treatments with *Casuarina* plants only (Figs. 2 and 3). Growth of frankiae representing the *Elaeagnus* host infection group was usually faster between t_0 and t_6 than that of frankiae of *Alnus* subgroup I with doubling times between 6 and 11 days compared to 10 and 15 days, and thus frankiae of the *Elaeagnus* host infection group represented higher

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