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# Experimentally reduced root–microbe interactions reveal limited plasticity in functional root traits in *Acer* and *Quercus*

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• *Background and Aims* Interactions between roots and soil microbes are critical components of below-ground ecology. It is essential to quantify the magnitude of root trait variation both among and within species, including variation due to plasticity. In addition to contextualizing the magnitude of plasticity relative to differences between species, studies of plasticity can ascertain if plasticity is predictable and whether an environmental factor elicits changes in traits that are functionally advantageous.
• *Methods* To compare functional traits and trait plasticities in fine root tissues with natural and reduced levels of colonization by microbial symbionts, trimmed and surface-sterilized root segments of 2-year-old *Acer rubrum* and *Quercus rubra* seedlings were manipulated. Segments were then replanted into satellite pots filled with control or heat-treated soil, both originally derived from a natural forest. Mycorrhizal colonization was near zero in roots grown in heat-treated soil; roots grown in control soil matched the higher colonization levels observed in unmanipulated root samples collected from field locations.
• *Key Results* Between-treatment comparisons revealed negligible plasticity for root diameter, branching intensity and nitrogen concentration across both species. Roots from treated soils had decreased tissue density (approx. 10–20 %) and increased specific root length (approx. 10–30 %). In contrast, species differences were significant and greater than treatment effects in traits other than tissue density. Interspecific trait differences were also significant in field samples, which generally resembled greenhouse samples.
• *Conclusions* The combination of experimental and field approaches was useful for contextualizing trait plasticity in comparison with inter- and intra-specific trait variation. Findings that root traits are largely species dependent, with the exception of root tissue density, are discussed in the context of current literature on root trait variation, interactions with symbionts and recent progress in standardization of methods for quantifying root traits.

**Key words:** Trait plasticity, root morphology, root architecture, specific root length, branching intensity, mycorrhizal colonization, *Quercus rubra*, *Acer rubrum*, oak, maple.

## INTRODUCTION

Plant biology is renewing its interest in trait-based ecology, explicitly examining among- and within-species variation and how variation affects plants' resource acquisition, community structure and ecosystem function (Violle *et al.*, 2012). Phenotypic plasticity elicited by abiotic or biotic factors is an important part of this variation, with consequences for ecological interactions, species coexistence and biodiversity (Clark, 2010). Similar to the above-ground leaf and wood traits that exhibit complex 'spectra' of inter- and intra-specific variation (Wright *et al.*, 2004; Chave *et al.*, 2009), traits affecting the form and function of fine roots in woody plants also show complex phenotypic variation. The consequences of this variation may include shifts in the strategies used by plants for soil resource acquisition directly and indirectly, via root–symbiont interactions (Fitter, 1985; Smith and Read, 2008; Comas and Eissenstat, 2009; Espeleta *et al.*, 2009; Hodge, 2009).

One basic question is whether the presence or absence of natural symbionts in fine roots elicits phenotypic plasticity in one or more functional traits. Another is whether such phenotypic plasticity is functionally advantageous, potentially compensating for inferior resource acquisition when beneficial symbionts are scarce or absent. Questions about plasticity in plant functional traits are generally relevant because they may influence plant species coexistence and competition (McGill *et al.*, 2006; Violle *et al.*, 2012). Furthermore, the traits of fine root systems play potentially critical roles in nutrient uptake, and in carbon cycling (Eissenstat and Yanai, 1997; Burton *et al.*, 2000; Gill and Jackson, 2000). An improved, integrative understanding of root functional traits, including trait plasticity, may aid in forecasting how forest communities and ecosystems will be affected by climate change and other disturbances (Norby *et al.*, 2004).

Phenotypic plasticity is defined as the capacity of a given species or genotype to exhibit a predictably variable phenotype

in response to variation in the environment (Pigliucci, 2001). In combination with heritable variation, phenotypic plasticity modulates the ecological ranges of generalist and specialist taxa or genotypes (Bell and Sultan, 1999; Sultan, 2010), as well as taxa's contributions to ecosystem function (Bell *et al.*, 2000; Richmond *et al.*, 2005; Sultan, 2010). Although plasticity is widespread, not every trait is plastic to every environmental signal. Moreover, species or genotypes may vary in their capacity to respond plastically. Gauging whether plasticity is adaptive (i.e. allows an organism to match its phenotype to a given environmental context) is challenging. One approach is to demonstrate relationships between particular syndromes of plasticity across environments, and differences in relative fitness across those environments (Schmitt *et al.*, 2003), with fitness quantified as lifetime Darwinian fitness (number and/or quality of offspring) or by a component or proxy of fitness. Another approach is to ask whether a plastic shift in response to stress or resource scarcity changes a trait in a manner consistent with a functional advantage to obviate that stress or scarcity (Sultan, 2010).

The study reported here examined five tissue-level traits: root diameter, root tissue density, specific root length (SRL, length per unit biomass), branching intensity and nitrogen (N) concentration. Morphological traits such as root diameter, tissue density and SRL are functionally important because surface area and especially length affect both foraging and uptake (Fitter, 1991; Robinson *et al.*, 1999). Root diameter and tissue density potentially govern SRL by controlling the amount of root length deployed per unit root biomass (Fitter, 1991). All three of these traits show extensive interspecies variation (e.g. Comas and Eissenstat, 2009), and are potentially subject to tradeoffs – thinner or less dense roots have greater surface area and length for direct nutrient uptake but less cortical tissue to support mycorrhizal colonization (e.g. Brundrett, 2002). Additionally, we examine what is often termed an architectural trait: the root branching intensity of fine root clusters (Comas and Eissenstat, 2009). Like the topology of whole root systems (Fitter, 1991), root branching intensity of fine root clusters (e.g. short-lived annual roots that do not become woody) can vary widely among and potentially within species, and also affects soil exploration by root systems. Finally, a physiological aspect of fine roots relevant to mycorrhizal symbionts is the concentration of tissue N, because it is correlated with respiration (i.e. metabolism) (e.g. Reich *et al.*, 1998). Although plants benefit from microbial symbionts when soil nutrients are limited, symbionts can be metabolically costly to support (Peng *et al.*, 1993) and different symbionts may require different levels of support (Trocha *et al.*, 2010). We acknowledge that other root traits such as density and length of root hairs may also affect soil resource mining by individual roots, especially for less mobile nutrients such as phosphorus (P) (Ma *et al.*, 2001; Reymond *et al.*, 2006). We note, however, that root trait plasticity in response to lack of colonization is of central interest independent of P status (Hetrick *et al.*, 1988) or other aspects of plant mineral nutrition.

In the limited studies that have explored plasticity in tissue-level root traits, a key focus tends to be reduced nutrient influx from the absence of mycorrhizal partners. A possible compensatory syndrome of plasticity in uncolonized roots is increased SRL (Berta *et al.*, 1995; Espelata *et al.*, 1999), whether achieved via reductions of diameter, tissue density or both. Some studies, however, have either not found a difference in SRL (Hetrick *et al.*, 1988) or only temporarily found a difference when plants were small (Eissenstat *et al.*, 1993). Lack of arbuscular mycorrhizal (AM) colonization has occasionally decreased root diameter but only when uncolonized roots were compared with roots colonized with particular AM fungi and not others (Berta *et al.*, 1995). Counter to hypotheses of compensatory plasticity, root branching intensity in several studies decreased when mycorrhizal symbionts were absent, potentially due to plants missing signals that stimulate root branching (Balestrini *et al.*, 1992; Berta *et al.*, 1995; Karabaghli-Degron *et al.*, 1998; Hodge, 2009; and references therein). In other cases, root branching either did not differ (Vigo *et al.*, 2000) or actually increased when symbionts were absent (Hetrick *et al.*, 1988; Forbes *et al.*, 1996). A review examining root branching in the presence and absence of symbionts concluded that the response is confined to coarse-rooted species (e.g. magnoliids, grasses) and taxa with high mycorrhizal dependence (Hetrick, 1991).

We aimed to shift the focus toward the fine roots of woody plants, specifically two common and frequently coexisting trees, one an AM-forming taxon (*Acer rubrum*) and the other ectomycorrhizal (EM)-forming (*Quercus rubra*). We also wanted to look beyond the effect of symbiotic interactions on plant performance via shifts in total root growth, overall root system architecture, root proliferation or root allocation (e.g. Bell and Sultan, 1999; Hodge, 2009). This motivated the design of our study, using manipulative treatments imposed on isolated segments of much larger root systems established by a 2-year-old potted tree seedling (*sensu* Espelata *et al.*, 1999). We trained segments of intact tree seedling root systems into satellite pots (Fig. 1) filled with natural soils collected from a forest in the native range of these plant taxa. In half of those satellite pots, soils were heat-treated to greatly reduce the microbial community's ability to colonize newly grown fine roots. This experimental set-up allowed us to quantify functional traits on colonized and uncolonized roots from the same set of individuals. In addition to documenting whether host root traits display significant phenotypic plasticity, we were able to

FIG. 1. Root segments were trained into smaller satellite pots from main pots (3·8 L) containing 2-year-old seedlings of *Acer* or *Quercus*. Two to four satellites were installed per main pot. See text for details of root manipulations and soil treatments.

evaluate the hypothesis that plasticity, if it occurs, is consistent with compensating for reduction in soil resource acquisition that would result when symbionts are absent.

By working with two dominant co-occurring plant host taxa that form two different mycorrhizas, we represent two out of seven major types (Smith and Read, 2008): *Quercus rubra* (EM-forming) and *Acer rubrum* (AM-forming). The two taxa are native to the temperate deciduous forests of north-eastern North America (Runkle, 1982; Abrams, 1992). Employing more than one species (e.g. Zangaro *et al.*, 2007) allows us to contextualize the relative magnitude of plasticity, examining whether it is smaller or larger than between-species differences. Finally, to enhance comparisons in the greenhouse, we also compared our experimental roots with undisturbed roots collected during a field survey.

## MATERIAL AND METHODS

### *Soil collection and preparation*

To grow root material in soil derived from a natural forest community containing abundant and diverse spores of AM and EM fungi, we used inoculum gathered directly from forest soil and indirectly from trap culture. To produce trap culture inoculum, we gathered O and A horizon soil, leaf litter and fine root fragments from Black Rock Forest (BRF, see description below) in late autumn 2007 below canopies of *Q. rubra* and *A. rubrum* or *A. saccharum* trees. This material was chopped with scissors and mixed with sand (1 : 1, v/v) and used to grow sorghum seedlings in 650-mL Deepots (Stuewe, Corvallis, OR, USA) (trap culture method, INVAM 2007 http://invam.caf.wvu.edu/). Because forest soil may not provide especially active inoculum for AM, we employed trap cultures in an effort to ensure the presence of fresh healthy spores of AM fungi and thus treatment differences in colonization. Awareness that the trap culture method can favour certain AM taxa over others motivated us to obtain additional fresh O and A horizon material from BRF in mid-March 2008 to use as direct inoculum. Fresh soil and trap culture soils were mixed in a 2 : 1 ratio (v/v) and then divided into two batches. One batch was used to create a control treatment fostering root colonization. The other, aiming to restrict colonization, was lightly moistened and heated at 90 °C for 1·5 h in a soil sterilizer (SS-5; Pro-Grow Supply, Brookfield, WI, USA). Similar soil heat treatments have been used in a variety of contexts to reduce viability of fungal spores (e.g. Sylvia and Schenk, 1984). Tests of these two soil treatments on separate samples of field soil did not show significant differences in nitrate and phosphate concentration between heat-treated and non-treated soil ($P = 0{\cdot}91$ and 0·08, respectively; $n = 5$). Before filling satellite pots, both types of soil were mixed with washed sand (1 : 2, v/v) to ensure infiltration of water through pots and to facilitate root harvesting. A dilute liquid fertilizer was added weekly (described below) to ensure that nutrient concentrations would remain similar between treatments, and high enough to maintain seedling vigour.

While our soil preparation methods are more naturalistic than use of a single fungal taxon or inoculum (or commercial mixes), caution in interpreting results is warranted as it was beyond the scope of our study to characterize the microbial communities of soil and rhizosphere with DNA- or RNA-based methods. As described below, we did assess overall AM and EM colonization.

### *Greenhouse experiment*

The greenhouse experiment was carried out at Barnard College's Ross Greenhouse in New York City between March and June 2008 with 11–15 h day length and 21–25 °C (day) and 16–20 °C (night) mean greenhouse temperature. Experimental pots were installed on 2-year-old seedlings of 16 *A. rubrum* and 15 *Q. rubra*, growing in 3·8-L pots and originally germinated from seeds collected in remnant forests of the New York metropolitan area. *Quercus* predominately associates with EM fungi (Dickie *et al.*, 2001) while *Acer* is commonly associated with AM fungi (Helgason *et al.*, 2002). For each potted seedling, woody root segments were excavated from the main pot and trimmed of all existing fine roots to aid in sterilization and to ensure that only newly grown and newly colonized fine roots would be sampled at the end of the experiment. Prior to replanting, root segments were surface-sterilized with dilute bleach then thoroughly rinsed. Seedlings were further divided into 25 or 50 % bleach surface-sterilization treatments to compare the adequacy of sterilization. Root segments were gently bent and replanted in 'satellite pots' (650-mL Deepots); multiple satellite pots were set up on each main pot, and securely fastened them to the main pot (Fig. 1). Any exposed root segments were coated in petroleum jelly to reduce drying. The larger 'parent' plants that furnished experimental root segments remained attached and continued to grow in their original 3·8-L nursery pots (modified from Espelata *et al.*, 1999). Seedlings in the main pot leafed out and thereafter both satellite and main pots were monitored, kept well-watered (semi-weekly by greenhouse staff) and were several times given commercial liquid fertilizer (Peters Professional 20–20–20) at a rate of 50 p.p.m. N during the experiment. There were eight seedlings in each treatment except seven seedlings of *Q. rubra* in 50 % bleach. The experiment ran for 70 d. While less than a typical growing season, and shorter than recently reported root longevities for *Quercus* and *Acer* (McCormack *et al.*, 2012), assessment of mycorrhizal colonization (described below) revealed that fine roots of both *Q. rubra* and *A. rubrum* had become colonized with EM and AM mycorrhiza, respectively.

Fine-root growth was successful in 60 of the 99 satellite pots in the greenhouse experiment. Failures were often due to the experimental set-up, with physical breakage observed on the root segment interconnecting a satellite pot to the seedling in the main pot. Success rates were significantly lower in *Q. rubra* (22 of 47 pots, 47 %) than *A. rubrum* (38 of 52 pots, 73 %) ($\chi^2 = 7{\cdot}09$, d.f. = 1, $P < 0{\cdot}01$) but we found no effect on success rate due to soil treatment ($\chi^2 = 0{\cdot}42$, d.f. = 1, $P > 0{\cdot}1$) or the strength of bleach ($\chi^2 = 0{\cdot}68$, d.f. = 1, $P > 0{\cdot}1$). Similarly, colonization as well as root traits did not differ significantly between bleach levels, although a strictly additive effect of bleach indicated that N concentrations were lower in the 25 % bleach treatment ($F_{1,31} = 7{\cdot}75$, $P < 0{\cdot}05$).

### *Root harvesting and trait quantification*

To harvest materials, trained root segments were cut, removed from the pots and washed. Terminal root clusters (clusters of

first- and second-order fine roots, all <1 mm in diameter) were collected. Sampling of terminal roots to include only first- and second-order roots standardized interspecific comparisons and also limited analyses to roots that function in nutrient absorption and form associations with mycorrhizal fungi (Comas *et al.*, 2002; Guo *et al.*, 2008; McCormack *et al.*, 2012). Sampled material was divided into two sub-samples, one for morphological and mycorrhizal colonization assessments, and the other to assess N concentration.

Morphology tissue samples were stained with neutral red and imaged on a flat-bed scanner. Samples were then dried at 60 °C for 72 h, weighed with a microbalance and saved for mycorrhizal staining (see below). Images were analysed with Delta-T SCAN software (Cambridge, UK), using the 'length sin θ' algorithm to produce raw data for the sample's diameter distribution, length and volume. Data from the 'tip count' algorithm were used to account for the point of origin of root clusters and calculate branching density (root tips per unit length; Comas and Eissenstat, 2009). The mode of diameters quantified along the entire length of the sample was used as the diameter estimate for each sample. Tissue density was calculated by dividing root biomass by estimated tissue volume. SRL was calculated from root length and biomass.

The same root samples used for morphological assessment were also examined for mycorrhizal colonization, which required rehydration as well as species-specific protocols for clearing and staining. Roots were re-hydrated concurrently with clearing either by soaking in 10 % KOH for 16–36 h at room temperature or boiling at 90 °C for 10 min. Roots were then rinsed with 85 % ethanol followed by an 85 % ethanol soak for 5 min to remove neutral red. *Quercus* samples were soaked for 15 min in 3 % $H_2O_2$ and *Acer* in 30 % $H_2O_2$ to remove plant pigments, then washed with water and checked under the dissecting microscope for cortical cell layer visibility. Roots were acidified with 5 % hydrochloric acid (HCl) for 5 min and incubated in 0·05 % trypan blue stain in a 90 °C water bath for 20 min. The roots were rinsed with water and stored in lactoglycerol (1 : 1 : 1 lactic acid, glycerol and water) (Grace and Stribley, 1991). Each sample was mounted on slides with glycerin jelly (Widden, 2001). In *Acer* samples, AM colonization was scored using the magnified intersections method (Giovannetti and Mosse, 1980; McGonigle *et al.*, 1990) with a magnification of 200imes;. Arbuscules, vesicles and non-septate hyphae within roots were scored as colonization, and then expressed as per cent of total segments scored. In *Quercus* samples, EM colonization was scored if a tip appeared sheath-covered under a dissecting microscope (15×), expressed as per cent of total root tips scored.

The other fine root subsamples were analySed for N concentration. After storing at −20 °C, these roots were ground in liquid $N_2$, which transformed samples into a dry powder; recalcitrant segments of vascular stele were chopped with dissecting scissors. Total N concentration was measured on a homogenized subsample with an elemental analySer (Model EA 1108; Fisons Instruments, Beverly, MA, USA).

### *Field root traits and colonization*

To corroborate the greenhouse measurements, root traits and mycorrhizal colonization were quantified for root samples collected from BRF in Orange County, New York, USA (41 °N, 74 °W) and the Penn State Experimental Forest (PSU) in Barree Township, Pennsylvania, USA (41 °N, 78 °W). Mean air temperature was −2·8 °C (January) and 23·6 °C (July) with mean annual precipitation of 1285 mm in BRF, and −3·7 °C (January) and 21·8 °C (July) with mean annual precipitation of 1010 mm in PSU (Turnbull *et al.*, 2003). Elevation at BRF was 143–167 m and at PSU was 282–294 m above sea level. Soil at BRF was a Swartswood and Mardin very stony soil, characteristically gravelly loam to gravelly silt loam to only about 15 cm. There was an average of 734 trees $ha^{-1}$ and an average basal area of 21·0 $m^2$ $ha^{-1}$ with *Q. rubra* as the dominant species along with *Q. prinus* (chestnut oak) (approx. 66 % of the basal area; Turnbull *et al.*, 2002). Soil at PSU was Atkins or Andover fine loam to a depth of about 40 cm. The average tree density was 479 trees $ha^{-1}$ and an average basal area of 422·5$m^2$ $ha^{-1}$ with *Tsuga canadensis* as the dominant species, followed by *A. rubrum*, *A. saccharum* and *Q. alba* (J. Harding, PSU Director of Forest Lands, pers. comm., 2010).

In both forests, root segments bearing visible fine roots were excavated from undisturbed soil. Roots were traced back to a mature tree and processed similarly to the greenhouse material, including clusters of only first- and second-order root segments (<1 mm in diameter). Tissues were of unknown age, in contrast to those harvested from the greenhouse, which were known to be new growth. Morphological and architectural traits were determined as described for the greenhouse samples. Tissue N was not analysed. Mycorrhizal colonization of field samples was quantified using the methods described above except that root pigments were bleached using a solution of household $NH_3$ and $H_2O_2$ (approx. 20 % $NH_3$ and 3 % $H_2O_2$, 1 : 10, v/v).

### *Statistical methods*

Five continuous response variables – diameter, tissue density, SRL, branching intensity and N content – were analysed using factorial ANOVA models implemented in SAS Version 9·1.3 (SAS Institute, 2002–2004) to estimate means and test the significance of experimental factors. There were two levels of each fixed-effect experimental factor: strength of bleach pretreatment (25 %, 50 %), soil treatment (natural control, heat-treated) and taxon (*Acer*, *Quercus*). A paired analysis of treatments on the same seedling was not used so that all the data produced could be used, including cases were only one of the two satellite pots installed on a seedling produced roots. Mycorrhizal colonization between heat-treated and control pots was analysed with similar models to validate that treatment resulted in differences in colonization.

After a full three-way ANOVA found the effects of bleach pretreatment to be non-significant (or, for one trait, strictly additive), samples from the two bleach treatments were pooled. A two-way ANOVA examined effects of soil treatment and taxon, both considered fixed effects. Factors were considered statistically significant if $P \leq 0{\cdot}05$. Although there was some imbalance in the final data sets (i.e. more maple than oak samples), most variables and residuals were normally distributed with the exception of per cent colonization, which was arcsine square-root transformed, and diameter and N concentration, which were logarithmically transformed. Transformations significantly improved normality and homoscedasticity, and results with transformed

and untransformed variables were consistent. For simplicity and clarity, results using untransformed data are presented in tables and figures. We corroborate our results by drawing on prior published papers that measure these traits in oak and maple and by examining variation in a sample of field-collected roots. Specifically, we tested whether colonized roots from the greenhouse study had similar colonization and traits (except for N concentration) to undisturbed field roots, using a two-way ANOVA model that included fixed effects of taxon and two locations: 'greenhouse', which consisted of all greenhouse pots, and 'field', which consisted of field specimens gathered at the PSU and BRF sites. Despite imbalance in our data sets (e.g. more maples than oaks, more greenhouse samples than field samples), statistical meta-analyses suggest that imbalance does not inflate Type 1 error rates when testing fixed effects using one-way and two-way ANOVA models (Harwell *et al.*, 1992). Finally, we also calculated correlation coefficients between all pairs of traits, using combined data from both taxa and from the heat-treated and control soil treatments.

## RESULTS

### *Efficacy of manipulative treatments*

Colonization was close to zero in heat-treated pots in the greenhouse (*A. rubrum* approx. 2 % colonization; *Q. rubra* approx. 1 % colonization), significantly higher in control samples, and differed between the AM- and EM-forming taxa (*A. rubrum* approx. 12 %; *Q. rubra* approx. 23 %; taxon-by-treatment interaction $F_{1,56} = 4{\cdot}59$, $P < 0{\cdot}05$; treatment effect $F_{1,56} = 30{\cdot}4$, $P << 0{\cdot}05$). Average levels of colonization for *Q. rubra* were intermediate between the two rather different levels found in our two field locations (<5 % at BRF versus nearly 40 % in Pennsylvania) while colonization of greenhouse and field samples of *Acer* was relatively more consistent (approx. 12 % in greenhouse, approx. 18 % in both field locations) (location-by-taxon interaction term in an ANOVA, $F_{2,39} = 4{\cdot}71$, $P < 0{\cdot}05$).

### *Root trait plasticity and variation between taxa*

Root diameter was not significantly different between roots grown in control or heat-treated soils for either *Quercus* and *Acer* but taxon differences were statistically significant (Table 1, Fig. 2A). Also, there was no significant difference in branching intensity in response to the two soil treatments, although branching intensity was significantly greater in *Q. rubra* than in *A. rubrum* (Table 1; Fig. 3). In contrast, there was an overall pattern across both taxa with tissue density significantly lower in roots grown in heat-treated soil and no difference in tissue density between the taxa (Table 1, Fig. 2B). There was an overall pattern in SRL across both taxa of longer SRL when grown in heat-treated soils and shorter SRL in control/natural soils; SRL also differed between *Quercus* and *Acer* and treatment differences were much smaller than the difference between taxa (Table 1, Fig. 2C). We did not find differences in N concentration between taxa, or plasticity in response to the two treatments (Table 1; Fig. 4). While this may reflect the small numbers of samples available for estimating N concentration in *Quercus*, the estimates are similar to a previous study that included these taxa (Comas and Eissenstat, 2009).

Because there was sample-to-sample variation in mycorhizal colonization, we could examine its correlation with functional traits, an alternative way to examine trait phenotypic plasticity. Tissue density was positively correlated with per cent mycorrhizal colonization in *Quercus*, with correlations between other root traits and colonization in *Quercus* being non-significant (Table 2). In *Acer*, there were no relationships between any root traits and per cent mycorrhizal colonization (Table 2).

### *Trait variation in field samples*

Comparing root traits between those grown in the greenhouse treatments (combined) and in field-collected samples, *Acer* had overall thicker roots than *Quercus* (mean $\pm$ 1 s.e.: $0{\cdot}21 \pm 0{\cdot}05$ vs. $0{\cdot}33 \pm 0{\cdot}01$ mm, respectively; main effect of taxon, $F_{1,40} = 68{\cdot}6$, $P < 0{\cdot}001$) but taxon differences in root diameter were greater in the greenhouse where *Q. rubra* was contrasted against *A. rubrum* compared with the field where *Q. rubra* was contrasted against *A. saccharum* (location effect, $F_{1,40} = 10{\cdot}4$, $P < 0{\cdot}01$, interaction $F_{1,40} = 4{\cdot}5$, $P < 0{\cdot}05$). There was similar tissue density for *Q. rubra* (mean $\pm$ 1 s.e. $0{\cdot}135 \pm 0{\cdot}013$ g cm$^{-3}$) and for greenhouse-grown *A. rubrum* (mean $\pm$ 1 s.e. $0{\cdot}134 \pm 0{\cdot}007$ g cm$^{-3}$) but the density of field-grown *A. saccharum* was significantly greater (mean $\pm$ 1 s.e. $0{\cdot}346 \pm 0{\cdot}076$ g cm$^{-3}$; interaction effect, $F_{1,40} = 8{\cdot}9$, $P < 0{\cdot}01$). SRL, potentially affected by changes in either diameter or tissue density, differed between species with maple having

TABLE 1. *Summary of two-way ANOVAs from the greenhouse experiment, for the fixed effects of soil treatments (heat-treated or natural controls) and between taxa (*Acer *or* Quercus*)*

| | Root diameter (cm) | | Root tissue density (g cm$^{-3}$) | | Specific root length (m g$^{-1}$) | | Branching intensity (tips cm$^{-1}$) | | Root N concentration (%) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | *F* | *P* | *F* | *P* | *F* | *P* | *F* | *P* | *F* | *P* |
| Soil treatment | 0·06 | 0·81 | **7·57** | **< 0·05** | **6·71** | **< 0·05** | 0·80 | 0·38 | 0·34 | 0·35 |
| Taxon | **189·9** | **< 0·001** | 0·38 | 0·54 | **228·4** | **< 0·001** | **8·45** | **< 0·01** | 1·97 | 0·17 |
| Treatmentimes × Taxon | 1·16 | 0·29 | 0·66 | 0·42 | 2·20 | 0·14 | 0·63 | 0·43 | 0·59 | 0·45 |

Error d.f. = 56, except for N concentration for which d.f. = 34. Sample sizes within combination of the two treatments are presented in Figs 2–4. Significant results are shown in bold type.

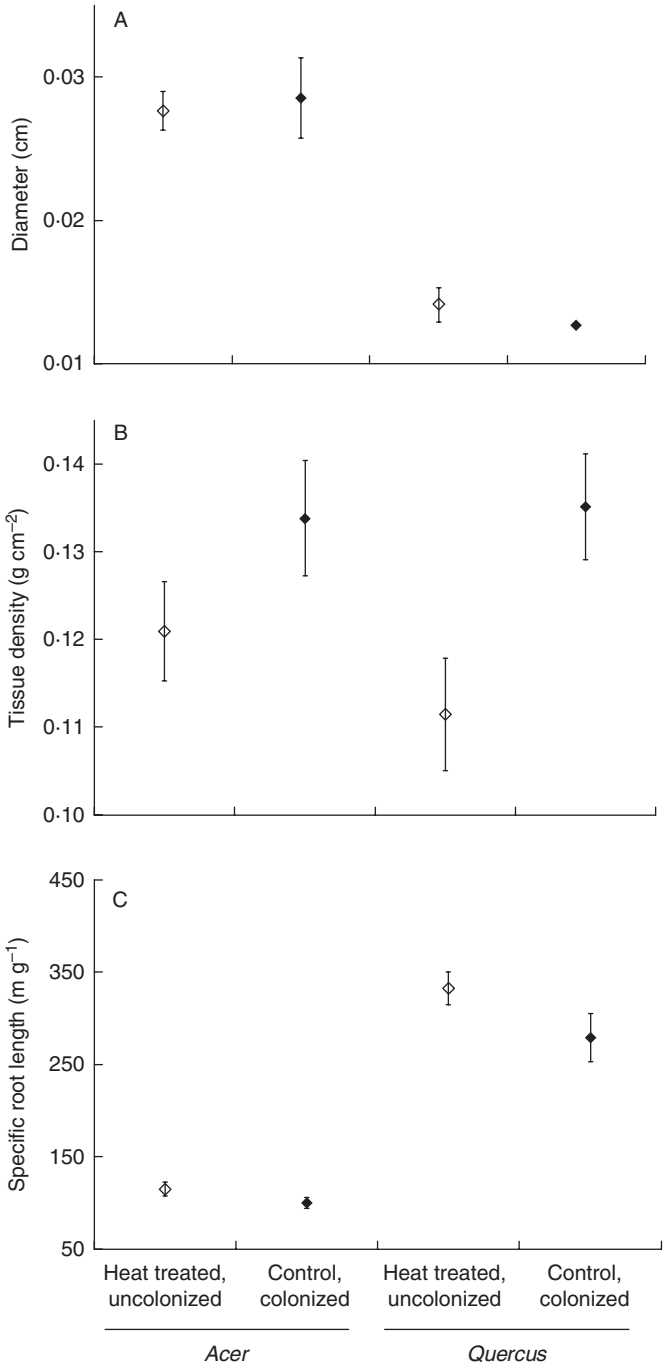


FIG. 2. Means ( ± s.e.) for (A) diameter, (B) tissue density and (C) specific root length assessed on four combinations of taxon and soil treatments: *Acer rubrum* heat treated and uncolonized ($n = 19$); *A. rubrum* control, colonized ($n = 19$); *Quercus rubra* heat treated, uncolonized ($n = 12$); *Q. rubra* control, colonized ($n = 10$).

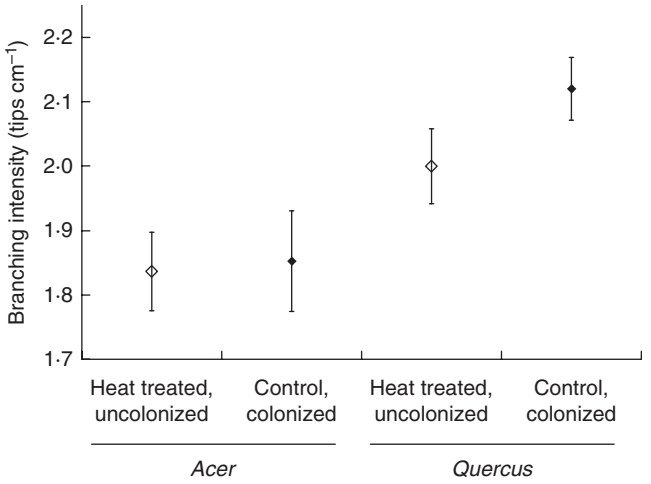


FIG. 3. Means ( ± 1 s.e.) for branching frequency, a root architecture trait. Sample sizes as in Fig. 2.

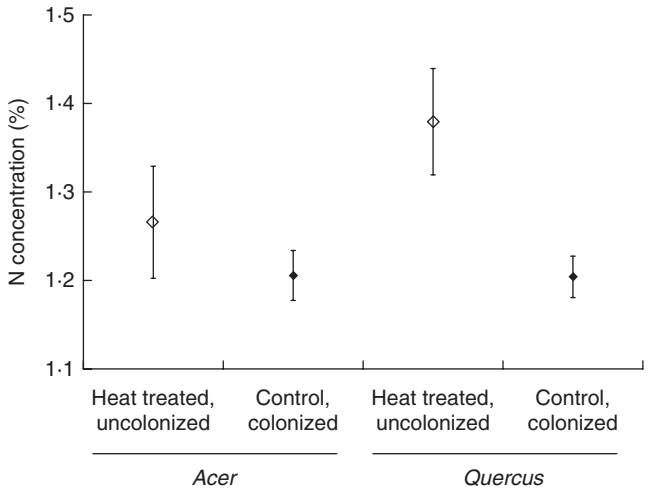


FIG. 4. Means ( ± s.e.) for root N concentration, a physiological trait. Samples sizes: *A. rubrum* heat-treated, uncolonized, $n = 15$; *A. rubrum* control, colonized, $n = 16$; *Q. rubra* heat-treated, uncolonized, $n = 4$; *Quercus*, control, colonized, $n = 3$.

greater SRL as expected, but no significant differences across greenhouse versus field conditions (taxon effect: $F_{1,40} = 4{\cdot}62$, $P < 0{\cdot}05$; location effect, $F_{1,40} = 0{\cdot}944$, n.s., interaction $F_{1,40} = 0{\cdot}22$, n.s.). Branching intensity did not differ significantly between taxa or growing location (taxon effect, $F_{1,40} = 0{\cdot}167$; location effect, $F_{1,40} = 1{\cdot}52$, interaction $F_{1,40} = 2{\cdot}70$, all n.s.).

TABLE 2. *Within-species root trait correlations of greenhouse-grown roots*

| | Diameter | Tissue density | SRL | Branching intensity | % Colonization |
|---|---|---|---|---|---|
| Diameter | | **−0·57** | 0·11 | **−0·53** | −0·28 |
| Tissue density | **−0·49** | | **−0·68** | **0·62** | **0·54** |
| SRL | −0·07 | **−0·66** | | −0·20 | −0·28 |
| Branching | −0·31$^{+}$ | **0·51** | **−0·38** | | 0·32 |
| % Colonization | 0·08 | 0·00 | −0·09 | 0·08 | |

*Q. rubra* coefficients are above the diagonal, and *A. rubrum* coefficients below. Bold type indicates coefficients significant at a level of $P < 0{\cdot}05$, $^{+}P < 0{\cdot}10$.

## DISCUSSION

Manipulation of soil biota in this study had few significant effects on root traits that could be linked to compensatory mechanisms of plants to acquire soil resources without assistance from soil micro-organisms. Thus, there is limited support for the idea that plants deploy compensatory mechanisms when they grow roots that lack access to the partnerships normally formed with soil and rhizosphere biota. Indeed, limited or even negligible plasticity was evident in both an AM- and an EM-forming species. The decreased tissue density observed in uncolonized roots may have partially resulted from the absence of filled intercellular spaces by colonizing fungi, possibly an inevitable and passive plastic response (*sensu* van Kleunen and Fischer, 2005). The absence of soil micro-organisms may have also decreased root tissue density through promotion of a less dense cortex, potentially via lack of chemical signalling needed to produce dense cortical cells with carbohydrates to support mycorrhizal associations. SRL – a trait that can be altered by changes in diameter, tissue density or a combination of the two – was significantly decreased by the presence of soil micro-organisms and such a decrease may have been connected, in part, to our observed increase in tissue density. Branching was generally similar between colonized roots, especially in *Acer*. In contrast, differences in branching between taxa were statistically significant.

In any study, the choice to fertilize (or not) inevitably results in uncertainty about plants' nutrient status, growth and biotic interactions. Our choice of periodically applying N–P–K fertilizer to both control and heat-treated satellite pots was motivated by a concern that low colonization in heat-treated pots would result in nutrient scarcity and indirect changes in root growth. In control/natural pots, this practice may have enhanced the nutrient status of plants to a point where colonization by mycorrhizas was reduced (Graham *et al.*, 1991). Despite these sources of uncertainty, we found a statistically significant disparity in overall colonization between treatments, for both taxa. Additionally, colonization in *Q. rubra* in manipulated greenhouse treatments was comparable not only to colonization in field-collected samples, but also to colonization observed in undisturbed field roots in several prior studies (Beckjord and Mcintosh, 1983; Dickie *et al.*, 2002; Lunt and Hedger, 2003), although higher average colonization levels in field roots have been reported by others – $35 \pm 5$ % (Bainard *et al.*, 2011) and $35 \pm 6$ % (Karpati *et al.*, 2011). Similarly, colonization levels of *A. rubrum* were comparable to similar root material collected from our field sites, but were lower than some reports in the literature (22–50 %: Helgason *et al.*, 2002; Lawrence *et al.*, 2003).

Limiting root sampling in the field to fresh terminal roots of first and second branching orders and limiting the experiment to just 70 d may have excluded aged fine roots, as root longevity of *Acer* and *Quercus* can exceed 200 d (McCormack *et al.*, 2012). With time and age it is possible that roots may become colonized with more or different mycorhiza. While such temporal shifts in colonization might alter root physiology or tissue density, it seems unlikely that they could retroactively alter morphological characteristics that formed earlier in root development, or root branching intensity.

Indeed, multiple factors contribute to variation within an individual, not just phenotypic plasticity evoked by a single environmental factor. Plasticity is itself a heritable attribute, as specific genes are used for receiving and transducing specific environmental signals, but some changes characterized as plasticity are, in fact, related to development or ageing rather than elicited by the environment directly (Pigliucci, 2001). In fine roots of woody species, selecting for only the first and second branching orders standardizes comparisons among taxa and also helps to separate tissue-level root trait plasticity elicited by the environment from developmental trait shifts (e.g. Berta *et al.*, 1995). Similarly, our protocol of trimming roots prior to initiating our greenhouse experiment, necessary for initial sterilization of root segments, also helped to reduce disparities in root age, which is known to affect colonization (Resendes *et al.*, 2008). (Although it is possible that this root trimming might have influenced both root traits and colonization, trimming and surface-sterilization were nevertheless imposed consistently across taxon and soil treatment combinations.) A failure to distinguish between root-age effects and environmentally induced effects per se may be partly responsible for the mixed results in the research published to date. Moreover, in woody taxa, there has been recent convergence on root selection methods with the identification that clusters of first- and second-order fine roots define the ephemeral fine root system, which is the portion of the fine root system that functions in the acquisition of soil resources (Guo *et al.*, 2008; Xia *et al.*, 2010). Such techniques are essential for comparative approaches.

In terms of interspecific variation, our results corroborate previous reports that root diameter drives variation in SRL among taxa. *Acer* had consistently lower SRL than *Quercus*, attributable to the larger diameter of *Acer* roots and consistent with prior studies (Comas and Eissenstat, 2004; Withington *et al.*, 2006). Broader surveys of interspecific variation in SRL among woody species found variation in SRL to reflect variation in root diameter rather than tissue density (Comas and Eissenstat, 2009). More generally, comparisons between greenhouse-grown and field samples support the idea that among-species differences in SRL and related traits are large in comparison with plasticity of these traits. Other studies comparing pot-grown and field plants have found consistent species rankings in a variety of tissue- and organ-level functional traits (including SRL and its determinants; Mokany and Ash, 2008). For temperate woody species, the consistency of among-species disparities in morphological root traits across studies and growing seasons supports the conclusion that there is somewhat limited plasticity in these traits (e.g. Pregitzer *et al.*, 2002; Comas and Eissenstat, 2004). This result contrasts with often-cited reviews about the plasticity of roots to colonization, which focused largely on species that are coarse-rooted and highly dependent on mycorrhizas and on root traits different from those that we investigated (Hetrick, 1991).

Despite finding significant differences in mycorrhizal colonization between treatments, responses cannot be associated exclusively with mycorrhizal fungi because our experiment's soil treatments potentially differed in bacterial or invertebrate communities as well as mycorrhizal fungi. Moreover, characterizing soil or rhizosphere microbial communities with classical or DNA-based methods was beyond the scope of our study, and the success or failure of a biotic factor in eliciting plasticity may be particular to individual species of mycorrhizal fungi (e.g. Berta *et al.*, 1995), especially when using a soil mix

combining fresh field-collected material with material from trap cultures. Nonetheless, the treatments were clearly effective as well as more naturalistic than use of a single fungal taxon or inoculum, which may not be representative of a natural community (Maherali and Klironomos, 2007). Indeed, given the diversity of soil factors that potentially elicit root trait plasticity, our finding of limited plasticity is yet more striking.

In conclusion, observing limited plasticity in combination with consistent differences between taxa implies that a hierarchy of factors governs root trait variation. Our study and a handful of others suggest that root trait variations due to plasticity are smaller than interspecific differences. If common, such a result would greatly simplify and strengthen ecosystem- and community-level investigations that require information about the costs and benefits of constructing and maintaining fine root tissues, and especially comparisons of functional traits and their associated benefits and costs across coexisting species in forest communities (Westoby and Wrights, 2006).

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