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Research

Contrasting the morphology, anatomy and fungal colonization of new pioneer and fibrous roots

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Summary

- Not all roots born as first-order branches are the same and this has important consequences for overall function. We hypothesized that, compared with fibrous roots, pioneer roots are built to live longer at the expense of absorptive capacity.
- We tested this hypothesis by investigating pioneer and fibrous roots in their first 14 d of life in the arbuscular mycorrhizal tree species: *Acer negundo*, *Acer saccharum*, *Juglans nigra*, *Liriodendron tulipifera* and *Populus tremuloides*. Root observations were made with root-access boxes that allowed roots to be sampled at known ages in field-grown trees.
- Compared to fibrous roots, pioneer roots had larger diameter, lower specific root length, greater average length and a lack of mycorrhizal or nonmycorrhizal fungal colonization. Pioneer roots < 14 d old had more layers of hypodermis with a lower percentage of putative passage cells and more protoxylem groups than similar age fibrous roots.
- Our results suggest that pioneer roots are constructed for defense against biotic and abiotic challenges, exploration of soil distal to the stem, high fibrous root branching and secondary development with high axial hydraulic conductivity at the expense of mycorrhizal colonization and high absorptive capacity for water and nutrients.

Introduction

Root systems have a complex architecture that has important consequences for overall function. Diameter and positions in the branching hierarchy are important traits defining root functional status (Pregitzer *et al.*, 2002; Guo *et al.*, 2008; Valenzuela-Estrada *et al.*, 2008). While branching order has strong utility in identifying roots of different function, not all roots are born the same. Diversity over individual roots of a given order has been called heterorhizy (Noelle, 1910; Hishi *et al.*, 2006). In woody plants, researchers have commonly distinguished between the fibrous roots (= feeder, short or absorptive roots) and pioneer roots (= long, framework or skeletal roots), where the former are thought to be the principal roots for water and nutrient absorption and the latter to be the main exploratory roots that eventually develop the framework of the root system (Liang, 1932; Wilcox, 1964; Horsley & Wilson, 1971; Kolesnikov, 1971; Sutton & Tinus, 1983). A similar classification was described by Lyford & Wilson (1964) and Lyford (1980), who revealed that pioneer roots tend to branch intensively and develop into multiple pinnate branched fans, often undergoing radial expansion into woody structural roots, whereas fibrous roots may not branch at all and never undergo secondary thickening (also see Eissenstat & Achor, 1999).

Pioneer roots are characterized by high growth potential (Wilcox, 1968; Pages, 1995). In *Quercus rubra*, pioneer roots may elongate at a rate of 5–10 mm d^{-1} in contrast to smaller diameter, fibrous roots which have a maximum elongation rate of 2 mm d^{-1} (Lyford, 1980). Additionally, fast-growing pioneer roots have a high initial growth rate that may be maintained for up to 10 d, compared to the relatively brief growth period (2 d) of fibrous roots. Pioneer roots of seedlings of *Quercus robur* were also characterized by a strong correlation between growth patterns and size of the apical diameter (Pages, 1995). Roots with an apical diameter > 1 mm often develop secondary xylem, whereas smaller roots are more likely to stay nonwoody roots (Lyford & Wilson, 1964; Lyford, 1980), and die sooner

(Wells & Eissenstat, 2001; Anderson *et al.*, 2003). According to Hishi (2007), longevity may be mediated by the number of protoxylem poles: tetrarch roots with a higher predisposition for secondary development tend to live longer than roots with a diarch stele. Because the number of protoxylem poles may also reflect lateral primordia formation (Esau, 1965; Hejnowicz, 2002), these fundamental differences in vascular anatomy may influence not only longevity but the potential for lateral branching.

Regulation of root penetration by symbiotic microbes may differ distinctly among pioneer and fibrous roots. It is poorly understood why certain symbionts preferentially infect young fibrous roots but not young pioneer roots (Lyford & Wilson, 1964). We hypothesize that pioneer roots, in contrast to fibrous roots, are better defended with both mobile and immobile defenses, including fewer passage cells and a more prominent hypodermis. Despite the significance of such variation, little systematic investigation has been conducted of patterns of colonization of mycorrhizal and nonmycorrhizal fungi in relation to root anatomy in these two types of roots.

In this study, we made a detailed investigation of pioneer and fibrous roots in their first days and weeks of life from trees planted in a common garden where environmental variation could be minimized. We also examined how these newly formed roots compared with a mixed-age population of roots. We chose five arbuscular mycorrhizal tree species that varied widely in fibrous root morphology (e.g. diameter). We purposely avoided ectomycorrhizal trees because of the known strong effects of this type of fungus on root morphology. We tested the general hypothesis that, compared to new fibrous roots, newly emerged pioneer roots are built to live longer at the expense of absorptive capacity, as indicated by a more pronounced hypodermis with fewer passage cells, more numerous protoxylem poles and lower mycorrhizal and nonmycorrhizal fungal colonization.

Materials and Methods

The common garden was located at the Russell E. Larson Agricultural Research Center near State College, Pennsylvania, USA (40.8°N, 77.9°W). The garden consists of 16 species of trees that were planted in a randomized complete block design with eight blocks in 1996. Plants were obtained as 1-yr-old liners from local native-plant nurseries, except for *Acer negundo* plants, which were collected in early spring from seedlings around State College. In each block, each species was planted in groups of six trees in a double row of three trees with a spacing of 3 m between trees within the row and 3 m between the double rows. There was a 9-m spacing between the six-tree species plots. Thus each species had a total of 48 trees. Soils were relatively fertile Hagerstown silt loam, well-drained, with a pH ranging from 6.1 to 6.5 and with some areas high in calcium. Before 1996, the site was used as a grass hayfield. The entire area was fenced to keep out deer. Blocking was used to control variation in soil characteristics (primarily depth of the A and B horizons). For this study, we only examined the roots of *Acer negundo* L., *Acer saccharum* March., *Juglans nigra* L., *Liriodendron tulipifera* L. and *Populus tremuloides* Michx., species that are known to vary widely in the diameter of their fibrous roots.

Root observations were made with root-access boxes (rhizotrons) with a clear transparent window that allows a root to be followed from birth and then sampled at a known age (Comas *et al.*, 2000; Bouma *et al.*, 2001; Resendes *et al.*, 2008; Volder *et al.*, 2009). Root boxes ($0.6 \times 0.5 \times 0.4$ m deep) were installed in a pit dug to the size of the box in April 2008, with the transparent window facing the tree, *c.* 1 m from the trunk and with all but the top 5 cm below the soil surface (Fig. 1a). Soil from that location was used to provide good contact of the window with the surrounding soil. Eight root boxes with 16 root windows per species, in eight blocks, were used with one root box in each species plot and each root box containing two windows on opposite sides of the box. The observation windows (0.55×0.3 m) were made of acetate film. Observations began 2 months after the boxes were installed, permitting the soil to settle and growth of new roots against the windows to occur. Initial roots appearing in the window were traced with a black permanent marker. New roots and new root growth visible on the acetate were then traced every day with a different-colored marker similar to that described previously (Resendes *et al.*, 2008). Growth of each individual root was inspected daily over 14 d and roots were harvested on the last day of inspection, giving a range of ages, with the oldest roots *c.* 2 wk old. Roots at this age were unpigmented or only lightly pigmented. Fibrous roots were distinguished qualitatively from pioneer roots based on their relative diameters. Additional characteristics, such as a prominent root tip and faster root extension, were also cues that could be used to recognize pioneer roots. Roots were sampled by cutting through the acetate film and excising the root at the branching point, so that only first-order roots (roots with tips and no branches) were analyzed.

In August 2010, 2 yr after root box installation, we made a second harvest of fibrous and pioneer roots of unknown age to examine root structure and fungal colonization under soil conditions where disturbance was minimized. Pioneer roots were pigmented and all fibrous roots of *L. tulipifera* and *J. nigra* were pigmented but, in the other species, some fibrous roots were unpigmented. Roots were collected from the same root boxes but, because first-order pioneer roots were relative rare under undisturbed conditions, we dug into the soil behind the window to collect more root branches than collected previously in 2008 until we had acquired at least six first-order pioneer roots of each species. Some of the *Populus* root branches in these collections were ectomycorrhizal.

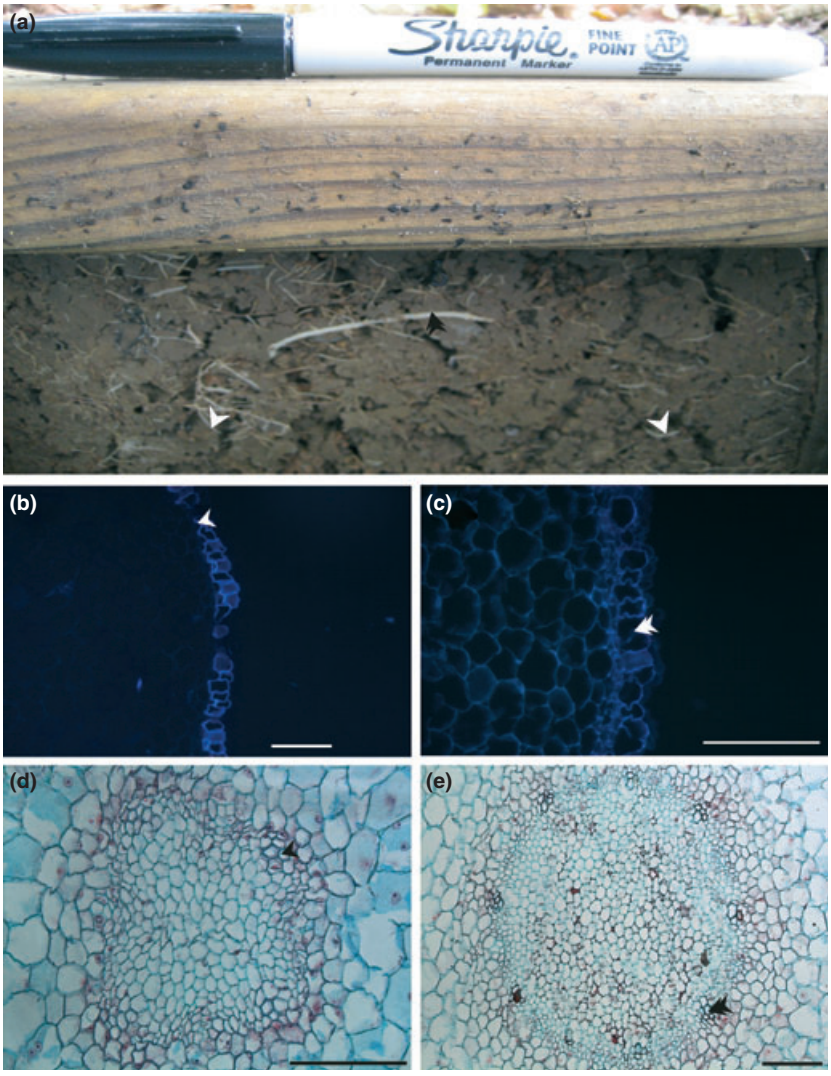

Fig. 1 Morphology and anatomy of fibrous and pioneer roots. (a) View through the root-access window showing fibrous (single arrowheads) and pioneer (double arrowhead) roots of *Acer saccharum*. (b, c) Hypodermis of fibrous (b) and pioneer (c) roots of *Liriodendron tulipifera*. Note one (single arrowhead) hypodermal layer in fibrous roots and multiple (double arrowheads) hypodermal layers in pioneer roots. Hypodermal wall suberization was observed using fluorescence over root cross-sections stained with toluidine blue O. (d, e) Anatomy of the stelar region in fibrous (d) and pioneer (e) roots in *L. tulipifera* examined under light microscopy. Note the protoxylem poles in fibrous (single arrowhead) and pioneer roots (double arrowhead) over cross-sections stained with fast green and safranine. Bars: (b, c, d) 100 μm; (e) 200 μm.

For only the 2008 root collection, roots were scanned with a desktop scanner in grayscale at 600 dpi. Images of individual roots were analyzed for length and diameter using WhinRhizo software (Regent Instruments Inc. Quebec, Canada). Scanned individual roots were then dried and weighed. Specific root length (SRL) was determined by dividing root length by mass.

Fungal colonization

After SRL estimation for the 2008 roots, dried roots were placed individually in glass tubes (20 ml) in 10% KOH overnight, then bleached in an ammonium-hydrogen peroxide solution for 10 min and stained with 0.05% trypan blue (w/v) in glycerine, lactic acid and distilled water in a 1 : 1 : 1 ratio by volume at 80°C for 15 min (Brundrett *et al.*, 1996). Stained roots were washed in water acidified with a few drops of lactic acid and placed in destaining solution. To avoid destruction of fragile root samples, whole roots were placed on microscope slides without cutting. Observations were performed under an Olympus light microscope (Olympus, Tokyo, Japan). Roots were carefully inspected for the presence of mycorrhizal and nonmycorrhizal fungi. Septate hyphae and nonmycorrhizal fungal structures were indicative of the presence of nonmycorrhizal fungi. Arbuscules, vesicles and hyphal coils indicated the presence of mycorrhizal fungi. No ectomycorrhizal fungi were observed in any of the tree species in the 2008 collection. The fungal colonization intensity of each individual root was assessed according to Trouvelot *et al.* (1986), by measuring the number of fungal infection structures longitudinally along the root in similar size root fragments relative to the number of examined cells. To estimate the intensity of colonization within individual roots, the frequency of particular intersection intervals were analyzed using the MYCOCALC program (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

For the 2010 roots, roots were placed directly in FAA (5 ml formaldehyde + 5 ml acetic acid + 90 ml 70% ethanol,

after washing, and then cleared and stained as described in the previous paragraph. In the case of those *Populus* root branches that exhibited ectomycorrhizal colonization, the percentage of total root tips colonized was assessed by determining the number of roots showing anatomical and morphological characteristics of ectomycorrhizas relative to the total number of tips examined. Roots were classified as ectomycorrhizal on the basis of the features of the colonized roots (Peterson *et al.*, 2004).

Root anatomical studies

Harvested root samples were fixed immediately in the FAA and, after 48 h, dehydrated in an alcohol series. Then material was infiltrated and embedded in Paraplast Plus (Sigma-Aldrich Inc., St. Louis, USA). Cross-sections 10 μm thick were obtained using an American Optics microtome and stained with either fast green and safranine or 0.5% toluidine blue O in 1% sodium tetra borate. Root sections stained using toluidine blue O were also examined under fluorescent microscopy (filter 02; excitation wavelength 365 nm). Observations were performed under a Carl Zeiss Axioscop 20 (Jena, Germany) light and fluorescence microscope.

Statistical analysis

The effects of species and root type (fibrous or pioneer) were analyzed using a two-way ANOVA. For traits expressed as percentages the Bliss angular transformation was used. The significance of differences among means was assessed using Duncan's mean comparisons, at $P = 0.05$. Significance of the relative frequency of roots exhibited by some incidence of mycorrhizal colonization was determined using a two-sample binomial probability test.

Results

Occurrence

To estimate root parameters, we collected lateral roots at the end of June 2008. Roots were categorized into two types, fibrous and pioneer roots, based on the root diameter (Fig. 1a). Observation of tip-ended roots of first order permitted us to exclude diameter and anatomical changes associated with branching order. Of the new lateral roots examined for morphology, growth rate and fungal colonization 2 months after disturbance, the percentage that were pioneer roots was 12.5% for *P. tremuloides*, 17.1% for *A. negundo*, 26.5% for *L. tulipifera*, 19.3% for *J. nigra* and 37.4% for *A. saccharum*. Species that produced fibrous roots more slowly (e.g. *A. saccharum*, *J. nigra* and *L. tulipifera*) had a higher proportion of pioneer roots than species with faster fibrous root production (*P. tremuloides* and *A. negundo*) ($r = -0.98$, $P < 0.01$).

In the 2010 collection, > 2 yr after root box installation, first-order pioneer roots, except in *L. tulipifera*, were difficult to locate and represented only a small fraction of the total first-order roots observed. The vast majority were fibrous roots. The high percentage of the pioneer root observed in 2008 was probably a result of cutting higher order roots when installing the root boxes.

Morphology

Species differed significantly in the diameter of young (< 2 wk) fibrous roots and pioneer roots ($P < 0.001$), with *L. tulipifera* having the coarsest first-order fibrous roots and *P. tremuloides* exhibiting the finest first-order fibrous roots (Table 1; Fig. 1a). SRL varied significantly across species ($P < 0.001$) as well as root types ($P < 0.001$) but the interaction of species × root type was not significant ($P < 0.614$). The SRL of the pioneer roots was highest (smallest diameter) in *P. tremuloides* and lowest in *L. tulipifera* (coarsest diameter). A similar pattern was observed for the fibrous roots across species. Morphological traits always differed significantly between fibrous and pioneer roots within a species. As expected, compared with fibrous roots, pioneer roots had larger diameter, lower SRL and greater average length (length visible on the windows from tip to first branching point). At the tree species level, there was evidence that roots of larger-diameter species tended to be longer ($r = 0.63$, $P = 0.048$).

Anatomy

The hypodermis was identified by secondary wall thickening or fluorescence under UV light of outer tangential walls in cell layers below the epidermis. The number of hypodermal cell layers varied significantly between the newly emerged pioneer and fibrous roots (root type: $P = 0.01$) but not among tree species ($P > 0.27$). In contrast to a well-developed multilayered hypodermis in pioneer roots, the hypodermis of fibrous roots was composed of only one or two layers of cells (Table 1; Fig. 1b,c). The relative frequency of passage cells also varied significantly between root types, with pioneer roots exhibiting a lower percentage than fibrous roots of putative passage cells (cells with no evidence of secondary wall thickening or fluorescence under UV light; $P < 0.01$). Across tree species, pioneer roots of *P. tremuloides* and *A. negundo* exhibited the lowest number of hypodermal layers whereas *A. saccharum*, *J. nigra* and *L. tulipifera* represented species with a multilayered hypodermis. In contrast, hypodermal layers in fibrous roots were generally similar across species. Overall, species with pioneer or fibrous roots of coarser diameter tended to have more hypodermal layers ($r = 0.89$, $P < 0.001$) than species with roots of finer diameter. The number of cortical cell layers also differed between root types, with more layers of cortical cells among pioneer roots than fibrous roots across

Table 1 Morphological, anatomical and growth characteristics of young, first-order fibrous and pioneer roots (< 14 d old) of the tree species *Populus tremuloides*, *Acer negundo*, *Acer saccharum*, *Juglans nigra* and *Liriodendron tulipifera*

	P. tremuloides		*A. negundo*		*A. saccharum*		*J. nigra*		*L. tulipifera*	
	Fibrous	Pioneer	Fibrous	Pioneer	Fibrous	Pioneer	Fibrous	Pioneer	Fibrous	Pioneer
Morphology										
Root diameter (mm)	0.3^{a} (0.01)	1^{b} (0.1)	0.4^{p} (0.03)	1.1^{q} (0.06)	0.5^{a} (0.04)	1^{b} (0.04)	0.6^{p} (0.04)	2.1^{q} (0.4)	0.9^{a} (0.05)	2^{b} (0.2)
Specific root length (m g^{-1})	3^{a} (0.4)	1.3^{b} (0.3)	1.3^{p} (0.1)	0.4^{q} (0.08)	1.2^{a} (0.1)	0.5^{b} (0.09)	1.3^{p} (0.07)	0.5^{q} (0.1)	0.8^{a} 0.09	0.3^{b} (0.06)
Average root length (mm)	11.7^{a} (0.7)	16.4^{b} (3.5)	13.4^{p} (0.9)	30.3^{q} (5.6)	6.8^{a} (0.5)	16^{b} (1.3)	10.9^{p} (0.5)	42.9^{q} (4.3)	9.3^{a} (0.6)	12.6^{b} (1.2)
Anatomy										
Mean number of hypodermal layers	1^{a}	2.9^{b}	1^{p}	2^{q}	2^{a}	4.8^{b}	1^{p}	5.9^{q}	1.8^{a}	5.9^{b}
Passage cells (%)	32^{a}	5^{b}	23^{p}	5^{q}	18^{a}	2.5^{b}	15^{p}	0^{q}	17^{a}	2.5^{b}
Mean number of cortical cell layers	5.4^{a}	15.9^{b}	4.6^{p}	12.4^{q}	6^{a}	10^{b}	7.3^{p}	24.4^{q}	9.5^{a}	27.5^{b}
Archic structure[1]	Di-tri^{a}	Tri-pent^{b}	Di^{p}	Tri^{q}	Di^{a}	Tetr^{b}	Tetr^{p}	Poly^{q}	Tetr^{a}	Poly^{b}
Number of roots examined	6	8	8	9	6	9	7	9	6	10
Growth rate and fungal colonization										
Mycorrhizal fungal colonization[2] (%)	5.0^{a} (0.08)	0^{b}	9.5^{p} (0.7)	0^{q}	6.8^{a} (0.4)	0^{b}	21^{p} (1.7)	0^{q}	17.2^{a} (2.4)	0^{b}
Nonmycorrhizal fungal colonization[2] (%)	12.4^{a} (1.5)	0^{b}	15.2^{p} (0.4)	5.3^{q} (0.9)	3.1^{a} (0.6)	0.4^{b} (0.1)	5.8^{p} (0.5)	0.9^{q} (0.08)	6.7^{a} (1.2)	1^{b} (0.2)
Days of root growth	2.9^{a} (0.2)	5.8^{b} (0.6)	3.0^{p} (0.3)	6.3^{q} (0.7)	2.0^{a} (0.1)	4.9^{b} (0.4)	2.5^{p} (0.1)	5.4^{q} (0.7)	2.6^{a} (0.2)	3.3^{a} (0.6)
Root growth rate[3] (mm d^{-1})	7.0^{a} (0.4)	5.2^{a} (1.0)	6.8^{p} (0.4)	11.5^{q} (1.9)	4.1^{a} (0.2)	6.2^{b} (0.7)	6.1^{p} (0.2)	7.0^{p} (0.7)	5.3^{a} (0.3)	8.2^{b} (0.8)
Number of roots examined[4]	70	10	87	18	57	34	117	28	50	18

Values are mean (SE) unless otherwise indicated.
Within a species, fibrous and pioneer roots are denoted by a different letter significant at $P < 0.05$.
[1]Number of protoxylem groups in the stele.
[2]Intensity of colonization by arbuscular mycorrhizal or nonmycorrhizal fungi 14 d after root appearance.
[3]Growth on the first day of appearance.
[4]Number of roots examined in the 'Morphology' and 'Growth rate and fungal colonization' sections.

species. For species with the finest roots (*P. tremuloides* and *A. negundo*) the number of cortical cell layers across fibrous or pioneer roots was lower than for species with coarser roots (*J. nigra* and *L. tulipifera*) (Table 1).

Vascular anatomy was also distinctly different in the two root types in pioneer and fibrous roots < 14 d old. Across species, the pioneer roots exhibited a higher number of protoxylem groups than fibrous roots (Table 1; Fig. 1d,e; $P < 0.05$). There was a general pattern of increasing numbers of protoxylem groups with increasing fibrous and pioneer root diameter ($r = 0.65$, $P > 0.2$ and $r = 0.95$, $P < 0.001$, respectively). Species with the finest fibrous roots (*P. tremuloides* and *A. negundo*) had about half as many protoxylem groups as species with coarser fibrous roots (*J. nigra* and *L. tulipifera*).

Growth rate and fungal colonization

A complete lack of mycorrhizal colonization in roots < 14 d old was a characteristic feature of pioneer roots across all species and in contrast to the colonization observed in fibrous roots (Table 1) ($P < 0.05$). The relative frequency that a 14-d-old fibrous root had some incidence of mycorrhizal colonization among the total fibrous roots of this age class examined was 15% ($n = 70$; $P < 0.001$ of difference with pioneer roots) for *P. tremuloides*, 24% ($n = 87$, $P < 0.001$) for *A. negundo*, 24% ($n = 50$, $P < 0.001$) for *L. tulipifera*, 15% ($n = 117$, $P < 0.001$) for *J. nigra* and 14% ($n = 57$, $P < 0.001$) for *A. saccharum*. Mycorrhizal colonization of fibrous roots varied across species from 5% (*P. tremuloides*) to 21% (*J. nigra*). The differences between pioneer and fibrous roots in the number of layers of the hypodermis and in the frequency of passage cells were generally consistent with their differences in fungal colonization (Fig. 2).

The colonization of fibrous and pioneer roots < 14 d old by nonmycorrhizal fungi varied significantly ($P < 0.05$; Table 1). The intensity of nonmycorrhizal structures present within pioneer roots was usually much lower than that within fibrous roots. Nonmycorrhizal fungi were observed more frequently in fibrous roots of fast-growing *P. tremuloides* and *A. negundo*, whereas fibrous roots of *A. saccharum, L. tulipifera* and *J. nigra* were only weakly colonized.

Average root length and root growth rate varied among species, but the parameters were species specific, resulting in statistically significant species × root type interactions ($P < 0.001$ for root length; $P < 0.001$ for root growth rate). Except for *L. tulipifera,* pioneer roots generally were twice as long as fibrous roots (Table 1). There was a tendency for pioneer roots to have a higher initial growth rate than fibrous roots, which was significant in three of the five species.

Comparisons of mixed-age first-order pioneer roots with similarly mixed-age first-order fibrous roots more than 2 yr after root box installation revealed results similar to that observed in roots < 14 d old – very low intensities of mycorrhizal and nonmycorrhizal fungal colonization in the pioneer roots, whereas the fibrous roots were well colonized with mycorrhizal fungi (Table 2). In three of the five species, all the pioneer roots had developed secondary tissues. In a fourth species (*J. nigra*), 33% of pioneer roots exhibited secondary development, and in *L. tulipifera* all pioneer roots still showed primary development. None of the first-order fibrous roots examined exhibited signs of secondary development. Mixed-age fibrous roots were similar to fibrous roots < 14 d old in archic structure and number of hypodermal layers (compare Table 2 with Table 1). The relatively high percentage of cells classified as passage cells was lower in the mixed-age fibrous roots than in the roots < 14 d old, presumably because the secondary walls and lignin and suberin deposition in the largely pigmented mixed-age fibrous roots had further developed. Similarly, mycorrhizal colonization was higher in the mixed-age roots than in the roots that were < 14 d old.

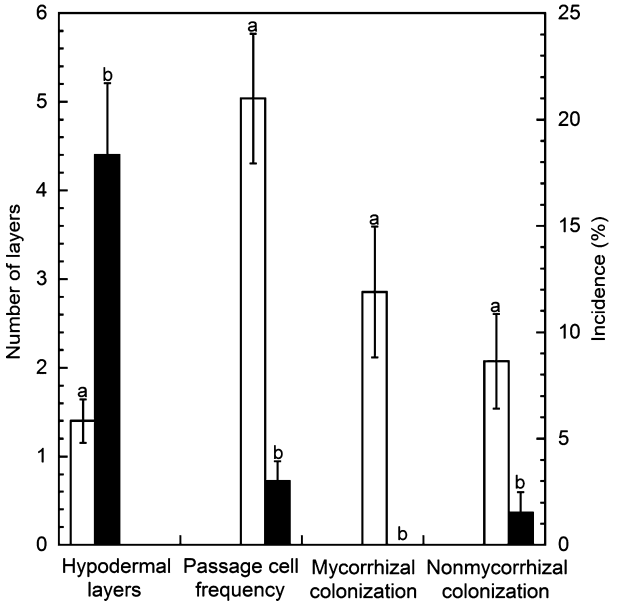

Fig. 2 General patterns of fungal colonization and hypodermal anatomy in fibrous (open bars) and pioneer (closed bars) roots < 14 d old, averaged across five tree species. Means followed by a different letter are significantly different ($P < 0.05$). No mycorrhizal colonization was observed in any pioneer roots in any tree species. Error bars indicate SE.

Discussion

We hypothesized that, compared with fibrous roots, pioneer roots are constructed at birth with structural features that allow the roots to live longer, branch more extensively and provide for a vascular system with a larger transport capacity, but at the expense of lower absorptive potential. By comparing pioneer and fibrous roots in the field in their first 2 wk of development, we found strong support for this hypothesis across a diverse group of arbuscular mycorrhizal

Table 2 Anatomical characteristics and fungal colonization (%) of first-order fibrous and first-order pioneer roots of unknown age harvested 2 yr after installation of the root windows of the tree species *Populus tremuloides*, *Acer negundo*, *Acer saccharum*, *Juglans nigra* and *Liriodendron tulipifera*

	P. tremuloides		*A. negundo*		*A. saccharum*		*J. nigra*		*L. tulipifera*	
	Fibrous	Pioneer	Fibrous	Pioneer	Fibrous	Pioneer	Fibrous	Pioneer	Fibrous	Pioneer
Anatomy										
Mean number of hypodermal layers	1	–	1	–	1.9	–	1^{p}	4.5^{q}	1.6^{a}	4.6^{b}
Passage cells (%)	4.5^{a}	0^{b}	18.5^{p}	0^{q}	5^{a}	0^{b}	10^{p}	0^{q}	6.6^{a}	1.3^{b}
Archic structure[1]	Di-tri	S	Di	S	Di	S	$Tetr^{p}$	$Poly^{q}$ or S	$Tetr^{a}$	$Poly^{b}$
Number of roots examined	6	6	6	6	6	6	6	6	6	6
Fungal colonization										
Mycorrhizal fungal colonization[2] (%)	15.4^{a} (4.1) [31.2]	0^{b}	6.1^{p} (1.2)	0^{q}	27.2^{a} (3.4)	0^{b}	34.5^{p} (5.7)	0^{q}	45.9^{a} (7.2)	0.09^{b} (0.1)
Nonmycorrhizal fungal colonization[2] (%)	8.5^{a} (0.2)	2.3^{b} (0.5)	8.1^{p} (1.3)	1.3^{q} (0.05)	2.8^{a} (0.8)	0.1^{a} (0.09)	4.9^{p} (0.6)	1.2^{q} (0.1)	5.1^{a} (0.4)	0.7^{b} (0.01)
Number of roots examined[3]	32	6	32	6	32	6	32	6	15	6

Values are mean (SE) unless otherwise indicated.
Within a species, fibrous and pioneer roots are denoted by a different letter significant at $P < 0.05$.
[1]Number of protoxylem groups in the stele of roots with primary development. Roots with secondary development are denoted by 'S'. The percentage of primary and secondary development of *J. nigra* pioneer roots was 67% and 33%, respectively.
[2]Intensity of colonization by arbuscular mycorrhizal or nonmycorrhizal fungi. The colonization (%) of *P. tremuloides* root tips of ectomycorrhizal root branches is given in square brackets.
[3]Number of roots examined in the 'Fungal colonization' section.

temperate tree species. When contrasting mixed-age, first-order pioneer and fibrous roots (Table 2), most pioneer roots had undergone secondary development whereas fibrous roots had retained their primary tissues, also supporting our hypothesis. Lack of secondary development in fibrous roots, an indicator of the ephemeral nature of this portion of the root system, has been reported previously in other species (Eissenstat & Achor, 1999; Guo *et al.*, 2008).

To our knowledge, no one has previously compared the anatomy of young pioneer roots before they had undergone secondary development with fibrous roots of similar age under field conditions. The hypodermis (or exodermis) in roots is a key structural feature that has been linked to both protection from desiccation (Cruz *et al.*, 1992) and pathogenic fungal colonization (Kamula *et al.*, 1994) and as a way to regulate nutrient and water absorption (Peterson & Enstone, 1996; Krishnamurthy *et al.*, 2009) including mycorrhizal colonization (Sharda & Koide, 2008). In our study, newly emerged pioneer roots had a multilayered hypodermis with a lower percentage of putative passage cells than the fibrous roots, which had only a weakly developed, generally single-layer hypodermis with a much higher percentage of passage cells. A higher frequency of passage cells is associated with increased root mycorrhizal fungal colonization (Sharda & Koide, 2008), pathogenic fungal colonization (Kamula *et al.*, 1994), nutrient absorption (Taylor & Peterson, 2000) and water absorption (Huang & Eissenstat, 2000). The existence of a multi-layer hypodermis has been noted by Shishkoff (1987) and Esau (1965). The strong differences between young pioneer and fibrous roots in the number of layers of the hypodermis and in the frequency of passage cells are consistent with their differences in colonization by both mycorrhizal and nonmycorrhizal fungi (Fig. 2). Although such a correlation between the frequency of passage cells and fungal colonization is in accordance with results obtained by others (Sharda & Koide, 2008), we indicate for the first time that it occurs differentially in fibrous and pioneer roots in the early stages of development in endomycorrhizal trees under field conditions. Pioneer roots are presumably constructed to enhance the likelihood that they are long-lived, which apparently leads to a sacrifice in their potential for rapid colonization by mycorrhizal fungi and high absorptive capacity associated with numerous passage cells in order to limit their vulnerability to biotic and abiotic stressors. This pattern of root colonization and passage cell occurrence was evident in young roots (Table 1) as well as mixed-age roots (Table 2). Moreover, while soil disturbance and root age might influence absolute root colonization by mycorrhizal fungi, these factors did not contribute to the relative differences in colonization between pioneer and fibrous roots.

Previously investigators observed in ectomycorrhizal tree species that pioneer roots frequently are not colonized by mycorrhizal fungi, in contrast to fibrous roots (e.g. Woodroof, 1934; Lyford, 1980). Rarely have authors focused on

arbuscular mycorrhizal colonization as in this study. This work supports previous conclusions that mycorrhizal colonization is sparse in pioneer roots, both in roots < 14 d old and in older mixed-age roots.

Root longevity is likely longer in pioneer than in fibrous roots. Developmentally, pioneer roots are built with the ultimate function of becoming the framework roots of the tree. The multiple layers of hypodermis, few passage cells and frequent occurrence of secondary development are all consistent with a root function that prioritizes longevity at the expense of absorptive capacity.

Numerous protoxylem poles have been linked not only to extended root longevity (Hishi & Takeda, 2005), but also to increased potential to form lateral root branches (Esau, 1965; Hejnowicz, 2002), thus allowing pioneer roots to rapidly develop into higher order roots. Protoxylem pole anatomy in pioneer roots has been examined both in seedlings and in forest trees (Hatch & Doak, 1933; Horsley & Wilson, 1971; Pages, 1995). Within the pioneer root, moreover, stelar anatomy may differ depending on the position in the root, with a higher number of protoxylem poles in the basal than apical parts of the root system (Wilcox, 1962). Thus, pioneer roots may be constructed with a high ability to explore the soil, and when they reach a nutrient-rich patch, readily release lateral fibrous root branches that can capture the localized nutrients. By readily developing secondary vascular tissue, pioneer roots increase their potential to transport water and nutrients from the highly absorptive fibrous root laterals.

In studies attempting to understand root function, attention has recently been paid to the importance of identifying small-diameter roots in the context of their different branching orders, as roots of different order can vary widely in root function (Pregitzer *et al.*, 2002; Guo *et al.*, 2008; Valenzuela-Estrada *et al.*, 2008). This branching order approach does not negate the importance of distinguishing between pioneer and fibrous roots, even if they are of the same order. If the pioneer roots, which are typically coarser, longer and have a more pronounced tip than the fibrous roots, are lumped with the fibrous roots of a similar branching order, then the branching-order approach will do little to improve the characterization of roots that are functionally equivalent. In undisturbed soil of more mature forests, this is not likely to be a serious problem because new pioneer roots of first or second order are probably rare. However, in young developing trees or where disturbance occurs (e.g. use of ingrowth cores for understanding carbon and nitrogen dynamics), pioneer root formation is much more frequent. Under these conditions, distinguishing the pioneer from the fibrous roots becomes much more important if the goal is to understand the function or stoichiometry of the ephemeral or absorptive fraction of the root system.

One of the differences observed between species with high SRL and those of low SRL were the proportion of pioneer roots, with low SRL species with relatively coarse fibrous roots producing a higher proportion of pioneer roots. Similar results were found among citrus rootstocks using ingrowth cores (Eissenstat, 1991). It seems that those species that produce more pioneer roots in response to disturbance may be less able to readily produce fibrous roots, but may rely more on mycorrhizal hyphae for nutrient acquisition (Graham *et al.*, 1991). It also should be recognized that the relatively high proportion of pioneer roots observed in 2008 was probably the result of cutting existing higher order roots, which we have observed promotes pioneer root formation. Other approaches of observing roots, including the use of ingrowth cores, rhizotrons and minirhizotrons, would also favor pioneer root production, at least in the first several months after installation, compared with undisturbed soil conditions.

Two of our species, *A. negundo* and *P. tremuloides*, are noted for establishment in old fields and other large openings rapid growth and prolific seed production, but shade intolerance. In contrast to these early successional species, *A. saccharum* is noted for its shade tolerance and dominance in late successional forests. *Juglans nigra* and *L. tulipifera* are intermediate between these two extremes. It is noteworthy that, in contrasts across species, *A. negundo* and *P. tremuloides* tended to have fewer hypodermal layers and a higher percentage of passage cells in their fibrous and pioneer roots than the later successional species as well as a lower percentage of pioneer root production soon after soil disturbance. In addition, in the mixed-age root collection, these two early successional species also tended to have lower mycorrhizal colonization of their fibrous roots but higher nonmycorrhizal fungal colonization of both their fibrous and pioneer roots. While only preliminary, these patterns collectively suggest that the fibrous and pioneer roots of early successional tree species such as *A. negundo* and *P. tremuloides* might be constructed more for high absorptive capacity and rapid proliferation with less dependence on mycorrhizal fungi, at the expense of greater vulnerability compared to later successional species. More research is needed to determine if these patterns are indeed consistent across a wider range of species.

In summary, we found that fibrous and pioneer roots clearly differ in structure, fungal colonization and growth responses from the first days of development. Pioneer roots are built to be the eventual framework of the tree, expand the root system both horizontally and vertically in the soil, and provide a more exploratory nature for nutrient acquisition. In contrast, fibrous roots have a much greater role in water and nutrient absorption. Because of these different roles, new pioneer roots, besides having a prominent root tip and much larger diameter (lower SRL), have more layers of hypodermis with fewer passage cells and more protoxylem poles than fibrous roots. This allows pioneer roots to more readily defend against biotic and abiotic challenges, explore soil with distance, enable the potential for extensive lateral fibrous root branching and a high axial hydraulic

conductivity at the expense of absorptivity of water and nutrients and encouragement of mycorrhizal colonization.

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