

Anatomical traits associated with absorption and mycorrhizal colonization are linked to root branch order in twenty-three Chinese temperate tree species

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Summary

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- Different portions of tree root systems play distinct functional roles, yet precisely how to distinguish roots of different functions within the branching fine-root system is unclear.

- Here, anatomy and mycorrhizal colonization was examined by branch order in 23 Chinese temperate tree species of both angiosperms and gymnosperms forming ectomycorrhizal and arbuscular–mycorrhizal associations.

- Different branch orders showed marked differences in anatomy. First-order roots exhibited primary development with an intact cortex, a high mycorrhizal colonization rate and a low stele proportion, thus serving absorptive functions. Second and third orders had both primary and secondary development. Fourth and higher orders showed mostly secondary development with no cortex or mycorrhizal colonization, and thus have limited role in absorption. Based on anatomical traits, it was estimated that c. 75% of the fine-root length was absorptive, and 68% was mycorrhizal, averaged across species.

- These results showed that: order predicted differences in root anatomy in a relatively consistent manner across species; anatomical traits associated with absorption and mycorrhizal colonization occurred mainly in the first three orders; the single diameter class approach may have overestimated absorptive root length by 25% in temperate forests.

Key words: anatomy, arbuscular mycorrhizas, ectomycorrhizas, fine roots, root architecture, root diameter, root function, root length.

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Introduction

In mature trees, woody roots extend 10 m or more from the tree trunk to support the shoot system, and to explore a large volume of soil, whereas small nonwoody roots arising from the woody root framework provide a large surface area and intimate contact with soil to ensure effective resource acquisition (Pregitzer, 2002; Robinson *et al.*, 2003). Multiple root functions are performed simultaneously by different portions of the root system (Robinson *et al.*, 2003).

The recognition of the functional differentiation in the tree root system has led to the efforts of separating roots with

different functions. For example, roots with the primary function of resource acquisition needed be defined so that root uptake could be better understood (Jackson *et al.*, 1997). From an ecosystem perspective, it is necessary to distinguish the rapidly cycling portion within the root system (McClagherty *et al.*, 1982; Jackson *et al.*, 1997; Joslin *et al.*, 2006). Consequently the entire root system was divided into different parts and, often more conveniently, into two parts (Jackson *et al.*, 1997). One is termed fine roots (e.g. all roots < 2 mm diameter), which are considered to be nonwoody, ephemeral absorptive roots, another is termed coarse roots (e.g. all roots > 2 mm diameter), or the perennial roots serving

mainly transport, anchorage, and storage functions (Pregitzer, 2002).

Research in the past decade, however, has begun to question the validity of such a simple division between fine and coarse roots, and the view that all roots of a given size class function in the same way (Majdi *et al.*, 2001; Wells & Eissenstat, 2001; Pregitzer *et al.*, 2002; Wells *et al.*, 2002; Joslin *et al.*, 2006; Guo *et al.*, 2008a,b). Increasing evidence suggests that tree fine roots are complex branching structures composed of a large number of individual root segments differing in morphology, chemistry, and physiology (Pregitzer *et al.*, 1998, 2002; Majdi *et al.*, 2001; Wells *et al.*, 2002; Guo *et al.*, 2004). Moreover, these functionally distinct root segments may be grouped according to their position on the branching root system such that the most distal root tips (or first-order roots) are thinner, richer in nitrogen and have higher respiration rates than more basal roots, implying that they are more active in nutrient uptake (Pregitzer *et al.*, 1998, 2002; Pregitzer, 2002). In addition, the majority of the root length was found to concentrate on the distal branches consisting of first- and second-order roots, suggesting that these lower roots provide most of the root surface for resource uptake whereas higher orders may serve mainly transport functions (Pregitzer *et al.*, 2002). Branching order of individual roots seems important for understanding root functions in the fine root systems of trees.

However, no study so far has determined whether branch order effectively distinguishes roots with absorptive capacity in the entire root system. This task is challenging because root functions, such as resource uptake, are difficult to measure directly (Lucash *et al.*, 2007). A common method, measuring nutrient uptake on excised roots, is questionable because excision significantly alters nutrient uptake processes, especially the energy-intensive processes such as nitrate uptake, leading to errors in uptake estimates (Bloom & Caldwell, 1988; Volder *et al.*, 2005; Lucash *et al.*, 2007). Another method, the intact root method, avoids root excision, but measures uptake of the entire root branching system, and thus can not quantify the differences in uptake capacity among individual roots differing in branching position, chemistry and physiology (Pregitzer *et al.*, 2002; Hishi, 2007).

Indirect methods, particularly anatomical methods, may be useful because anatomy and physiology are tightly linked (Esau, 1977; McKenzie & Peterson, 1995a,b; Eissenstat & Achor, 1999; Hishi, 2007). In trees, root systems can be separated into the root segments with primary development and those that undergo secondary development and eventually become structural portion of the root system (Esau, 1977; Peterson *et al.*, 1999; Pregitzer *et al.*, 2002). It is generally considered that roots with primary development have a living cortex and mycorrhizal association and are mainly responsible for water and nutrient absorption (Esau, 1977; Peterson *et al.*, 1999; Brundrett, 2002). Losing cortex greatly curtails root uptake capacity because of the loss of absorptive surface area and preclusion of mycorrhizal colonization (Enstone *et al.*,

2001; Brundrett, 2002; Wells & Eissenstat, 2003; Kumar *et al.*, 2007).

By contrast, roots that undergo secondary development may have limited capacity for uptake. When secondary growth occurs, pericycle forms cork cambium, which produces cork tissue with hydrophobic suberized cells and eventually develops into a continuous cork layer. This cork layer serves as a barrier to water and nutrient absorption (Peterson *et al.*, 1999; Taylor & Peterson, 2000) so that a root with a continuous cork layer may lack absorptivity (Wells & Eissenstat, 2003; Hishi, 2007). Moreover, development of secondary xylem increases root transport capacity (Kumar *et al.*, 2007). Therefore, secondary development may represent a major shift of root function from absorption to transport (Wells & Eissenstat, 2003; Hishi, 2007). It appears that the anatomical features reveal critical differences in resource uptake, mycorrhizal colonization, and transport capacity among individual roots.

In this study, we attempted to distinguish roots of different functions by examining anatomical features of different branch orders in the branching fine root systems of 23 Chinese temperate tree species differing in taxonomic rank (angiosperms vs gymnosperms) and mycorrhizal type (ectomycorrhizas (EM), and arbuscular mycorrhizas (AM)). We predicted that root anatomy associated with resource absorption and transport would vary consistently with root branch order despite the wide differences in root diameter among species.

Materials and Methods

Study site and species selection

The study sites were located in four different temperate regions of China. These four sites represented four points on a national-scale sampling of root architecture of Chinese tree species. Our main sampling site (Site 1) was located in Maoershan research station (45°21'–45°25'N, 127°30'–127°34'E) of Northeast Forestry University, in Heilongjiang, China. This site had a continental temperate monsoon climate with mean January, July and annual temperatures of –19.6°C, 20.9°C, and 2.8°C, respectively. The mean annual precipitation was 723 mm, with 477 mm distributed from June to August (Zhou, 1994). Soils were Hap-Boric Luvisols (Gong *et al.*, 1999) with high organic matter, and were well drained.

At this site, three forest types were chosen for root sampling in mid August of 2007. From a naturally regenerated secondary deciduous hardwood forest *c.* 50 yr old (Site 1a) four AM angiosperm species, five EM angiosperm species and six angiosperm species capable of supporting both AM and/or EM were sampled. Two EM gymnosperms were sampled from a pine plantation *c.* 40 yr old (Site 1b) and a deciduous EM conifer was sampled from a larch plantation *c.* 50 yr old (Site 1c). All species sampled were listed in Table 1. All trees sampled had an age of 20–50 yr.

Table 1 Taxonomic list, species abbreviation, dominant mycorrhizal (MYC) type and sampling site of the 23 tree species in this study

| | Abbreviation | MYC type ^a | Site ^b |
|--|--------------|-----------------------|-------------------|
| Gymnosperm | | | |
| Pinaceae | | | |
| <i>Larix gmelinii</i> | Lagm | EM | Site 1c |
| <i>Pinus sylvestris</i> var. <i>mongolica</i> | Pisy | EM | Site 1b |
| <i>Pinus koraiensis</i> | Piko | EM | Site 1b |
| <i>Pinus tabulaeformis</i> | Pita | EM | Site 3 |
| Angiosperm | | | |
| Aceraceae | | | |
| <i>Acer davidii</i> | Acda | AM | Site 1a |
| <i>Acer ginnala</i> | Acgi | AM & EM | Site 1a |
| <i>Acer ukurunduense</i> | Acuk | AM & EM | Site 1a |
| Betulaceae | | | |
| <i>Alnus mandshurica</i> | Alma | AM & EM | Site 1a |
| <i>Alnus hirsute</i> | Alhi | AM & EM | Site 1a |
| <i>Betula platyphylla</i> | BepI | EM | Site 1a |
| <i>Betula costata</i> | Beco | EM | Site 1a |
| Cornaceae | | | |
| <i>Cornus officinalis</i> | Coof | AM & EM | Site 4 |
| Fagaceae | | | |
| <i>Quercus acutissima</i> | Quac | EM | Site 1a |
| <i>Quercus dentate</i> | Qude | EM | Site 1a |
| <i>Quercus mongolica</i> | Qumo | EM | Site 1a |
| Juglandaceae | | | |
| <i>Juglans mandshurica</i> | Juma | AM | Site 1a |
| Oleaceae | | | |
| <i>Fraxinus mandshurica</i> | Frma | AM | Site 1a |
| <i>Fraxinus rhynchophylla</i> | Frrh | AM | Site 2 |
| <i>Syringa pekinensis</i> | Sype | AM & EM | Site 2 |
| Rutaceae | | | |
| <i>Phellodendron amurense</i> | Pham | AM | Site 1a |
| Tiliaceae | | | |
| <i>Tilia mandshurica</i> | Tima | AM | Site 2 |
| Ulmaceae | | | |
| <i>Ulmus laciniata</i> | Ulla | AM & EM | Site 1a |
| <i>Ulmus japonica</i> | Ulja | AM & EM | Site 1a |

^aAM, arbuscular mycorrhizas; EM, ectomycorrhizas.

^bSee detailed site information in the Materials and Methods section.

To complement the root sampling at site 1, species sampled earlier at three other sites (Sites 2, 3 and 4) were included. At Site 2, three angiosperm species were sampled in late July 2006, including two AM species and one AM and EM species from a naturally regenerated forest *c.* 50 yr old. This site was located in Baihua Mountain of Northwest Beijing (39°49′–39°53′N, 115°30′–115°38′E). The site had a warm-temperature continental climate with mean January, July, and annual temperatures of –7.1°C, 18.3°C and 4.8°C and mean annual precipitation of 595 mm with > 75% distributed from June to August (Land Environmental Protection Department of Beijing Municipal Planning Committee, 1988). Soils were Haplic Luvisols (Gong *et al.*, 1999) with high organic matter and were well drained.

At Site 3, an EM gymnosperm from naturally regenerated forest *c.* 80 yr old (Table 1) was sampled in early July 2006.

This site was located in the Guandishan Pangquangou Nature Reserve in the middle range of LuLiang Mountain in Shanxi Province (37°20′–38°20′N, 110°18′–111°18′E), with mean January, July and annual temperatures of –7.7°C, 23.5°C and 8.8°C, respectively. The mean annual precipitation was 400–600 mm with 65% mm occurring from July to September. Soils were Haplic Luvisols (Gong *et al.*, 1999) with medium organic matter content, and were well drained.

At Site 4, an AM and EM angiosperm from planted trees *c.* 20 yr old (Table 1) was sampled in early July 2006. The sampling location for Coof was at the southern part of Taihang Mountain in Shanxi Province (35°52′–37°20′N, 110°24′–111°45′E), with mean January, July and annual temperatures of –4.6°C, 21°C, and 9.9°C, respectively. The mean annual precipitation was 670 mm with 47% mm distributed from June to August. Soils were Haplic Luvisols (Gong *et al.*, 1999) with medium organic matter content, and were well drained.

Root excavation and preliminary processing

For each species, three root samples (one sample from each of the three chosen trees) were collected from 0–20 cm of soil following three steps. A 1 × 1 m plot was first identified within a 2-m distance of the tree stem. A specially constructed fork (with three teeth, each 20 cm long and 1 cm diameter, and having a pointed tip) was then used to loosen the soil in the sampling area. Root branches were followed to the tree stem and cut from the main lateral woody roots. When necessary, more plots were established to ensure that each root sample contained at least eight intact distal root branches including more than five orders.

Once collected, each root sample was divided into two subsamples: one was gently washed in deionized water and immediately fixed in Formalin-Aceto-Alcohol (FAA) solution (90 ml 50% ethanol, 5 ml 100% glacial acetic acid, 5 ml 37% methanol); the other was immediately put on ice and transported to the laboratory within 4 h and frozen for dissection and morphological analysis at a later date.

Anatomical assessments

More than 15 root branches of each species fixed in FAA solution were dissected into different orders as described in Fitter (1982, 1987), Berntson (1997), and Pregitzer *et al.* (2002) with the most distal root tips labeled as first order. Our order designation strictly followed Strahler's stream ordering system (described in detail by Pregitzer *et al.*, 2002). We therefore did not differentiate between the finest first-order roots located at distal ends of a well-branched fine root system and the root tips of pioneer roots (often of larger diameters) that are rapidly developing and may be destined to become higher-order roots (Wells & Eissenstat, 2003).

For each species, 20 segments were randomly chosen per order for first to third order, and fifteen segments per order for

fourth and fifth order. The sample size differed among branch orders because of the limited number of high-order roots obtained in the field sampling for some species. In total, 2070 individual root segments across 23 species were dissected for anatomical analysis.

After the dissection, individual root segments were stained with safranin-fast green, dehydrated in a set of alcohol solutions, embedded in paraffin, and sections 8 μm thick were prepared (de Neergaard *et al.*, 2000). These sections were measured for anatomical features, and photographed under a compound microscope (BH1; Olympus). For each root segment, three cross-sections were chosen. For root segments < 1 cm long, three sections near the root base were chosen. For root segments > 1 cm long, three sections evenly distributed between 1 cm from root tip (for the first-order roots) or the branching point (for higher-order roots) to root base were chosen.

For each root cross-section, root diameter, cortex thickness, and stele diameter were measured to the nearest 1 μm , and the presence of mycorrhizal colonization, secondary xylem (SX) and continuous cork layer (CCL) were recorded (see the Supporting Information, Fig. S1). For root diameter, cortex thickness, and stele diameter of each root segment, the average values of the three sections were calculated. In addition, the ratio of stele diameter to root diameter (or stele : root diameter ratio) was calculated to indicate the proportion of root diameter devoted for resource transport. For mycorrhizal colonization and features of secondary development (SX and CCL), their appearance on any of the three sections was regarded as the presence on the entire root segment. For AM, appearance of coils (or arbuscules) was accepted as evidence of colonization (Brundrett, 2004; Vierheilig *et al.*, 2005). For EM, fungal sheath and/or Hartig net in sections were considered evidence for colonization (de Neergaard *et al.*, 2000).

Morphological analyses

More than five intact root branches for each species were dissected for morphology as described in Pregitzer *et al.* (2002). The length of relatively short root segments were measured using a 40 \times stereomicroscope with an ocular micrometer (± 0.025 mm), while the length of relatively long root sections (e.g. fourth- and fifth-order roots) was assessed using a measuring tape to the nearest 0.5 mm (Guo *et al.*, 2004). The total root length for the first five orders was calculated for each species.

Data analysis

Root diameter, cortex thickness, stele diameter, stele : root diameter ratio were rank-transformed to satisfy the assumption of normality (Zar, 1999). Differences in these variables among root order, diameter class (0.25-mm intervals), and species were analysed by a mixed-level ($5 \times 11 \times 23$) three-way

factorial ANOVA with Tukey's HSD for unequal sample size. The Spearman's rank correlation coefficients were calculated for order, diameter class of 0.25-mm intervals, root diameter, cortex thickness, stele diameter, stele : root diameter ratio, mycorrhizal colonization presence, SX presence and CCL presence.

Stepwise discrimination analysis was conducted to examine the groupings of species based on morphological and anatomical traits of first-order roots. Root traits used in the analysis included mean root diameter, mean cortex thickness, mean stele diameter, and mean stele : root diameter ratio of each species. In each step one variable was selected on the basis of its significance.

The proportion of the total root length with uptake capacity (i.e. no CCL) and mycorrhizal colonization was calculated as the ratio of the cumulative absorptive or mycorrhizal root length to the total root length of the first five orders. All statistical analyses were performed using SPSS software (2001, ver. 13.0, SPSS Inc., Chicago, IL, USA).

Results

Variations in root traits by branch order

Across all species, root diameter, cortex thickness, stele diameter, and stele : root diameter ratio (i.e. the ratio of stele diameter to root diameter) differed by branch order (all P values < 0.01) (see the Supporting Information, Table S1). In addition, root diameter, stele diameter, and stele : root diameter ratio differed significantly between each order, whereas cortex thickness did not show significant differences between first and second order but did among other orders (see the Supporting Information, Table S2).

Branch order and root traits correlated strongly but the direction of correlation differed by root trait. Correlation was positive and strong between order and stele diameter ($r = 0.862$), stele : root diameter ratio ($r = 0.833$), SX presence ($r = 0.802$), CCL presence ($r = 0.792$) and root diameter ($r = 0.613$), but was negative and strong between order and mycorrhizal colonization ($r = -0.733$) and cortex thickness ($r = -0.597$) (Table 2). Similar patterns were found across species of the same mycorrhizal type and taxonomy rank, or within each species (detailed results not shown).

The species differed in their relationship between order and various root traits. Average diameter generally increased with order, but the specific patterns differed among species (Fig. 1): diameter showed no significant differences in at least three consecutive orders and then increased dramatically in the higher orders in 13 species; diameter was constant across five orders in two species (i.e. *Acer ukurunduense* and *Betula costata*); and diameter increased steadily across five branch orders in eight species.

Stele diameter increased gradually with order in most species and significant differences frequently were found between the

Table 2 Spearman's rank correlation coefficient matrix for order, diameter class, root diameter, cortex thickness, stele diameter, stele : root diameter ratio, mycorrhizal (MYC) colonization presence, secondary xylem (SX) presence and continuous cork layer (CCL) presence across 2070 root segments from 23 tree species

| | Order | Diameter class | Root diameter | Cortex thickness | Stele diameter | Stele : root diameter ratio | MYC presence | SX presence |
|-----------------------------|----------|----------------|---------------|------------------|----------------|-----------------------------|--------------|-------------|
| Diameter class | 0.615** | | | | | | | |
| Root diameter | 0.613** | 0.951** | | | | | | |
| Cortex thickness | -0.597** | -0.156* | -0.166* | | | | | |
| Stele diameter | 0.862** | 0.794** | 0.828** | -0.458** | | | | |
| Stele : root diameter ratio | 0.833** | 0.464** | 0.741** | -0.742** | 0.862** | | | |
| MYC presence | -0.733** | -0.392** | -0.399** | 0.615** | -0.664** | -0.718** | | |
| SX presence | 0.802** | 0.374** | 0.375** | -0.731** | 0.717** | 0.806** | -0.807** | |
| CCL presence | 0.792** | 0.378** | 0.383** | -0.771** | 0.717** | 0.812** | -0.845** | 0.934** |

** , * , Correlation was significant at $P = 0.01$ and $P = 0.05$, respectively.

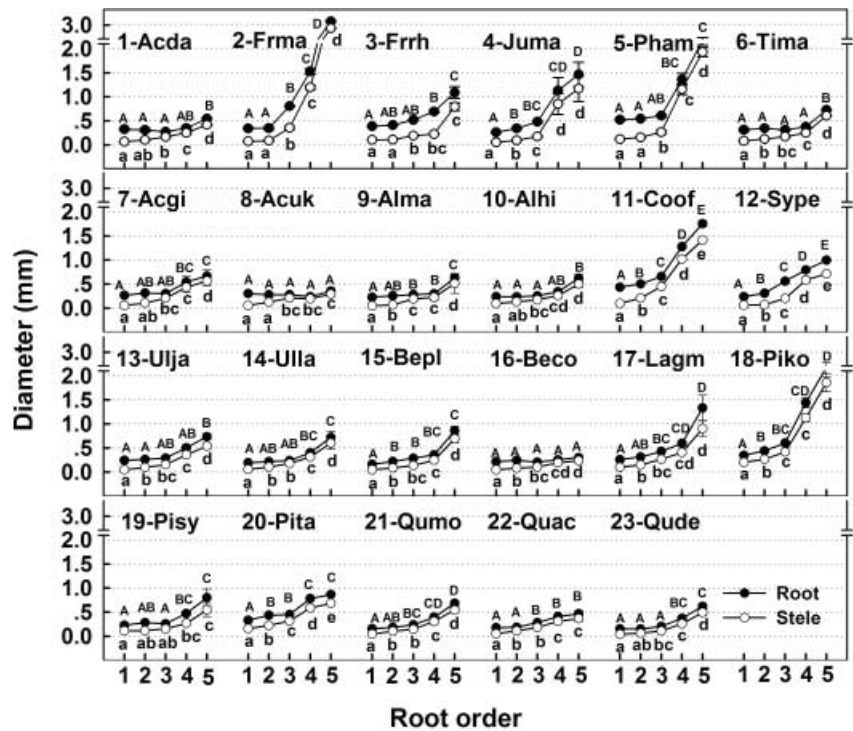


Fig. 1 Root diameter and stele diameter by branch order in 23 Chinese temperate tree species. Arbuscular mycorrhizal (AM) species, 1–6; AM and ectomycorrhizal (EM), 7–14; EM species, 15–23. See Table 1 for species abbreviations. Error bars represent 1 SE of the mean. Upper case letters that differ within a species indicate significant ($P < 0.05$) differences in root diameter among root orders, whereas lower case letters that differ within a species indicate significant ($P < 0.05$) differences in stele diameter among root orders.

successive orders (Fig. 1). Even in species whose root diameter remained constant in the first four or five orders, the stele diameter increased steadily and significantly with order, as in *Acer davidii*, *Acer ukurunduense*, *Alnus hirsuta*, *Betula costata*, *Tilia mandshurica*, and *Ulmus japonica* (Fig. 1).

Stele : root diameter ratio increased dramatically in the first three or four orders, but leveled off in higher orders in most species (Fig. 2). The magnitude of increase became much smaller from fourth to fifth order in 12 species, and from third to fourth order in eight species. The exceptions were *T. mandshurica*, *Quercus dentate*, and *Pinus koraiensis* whose stele : root diameter ratio increased constantly (almost linearly) with root order (Fig. 2). Overall, the greatest increase in

stele : root diameter ratio occurred most frequently between second and third order, or third and fourth order (Fig. 2).

Cortex thickness was either stable (fourteen species) or increased (seven species) in lower orders in most species. Cortex disappeared at a certain higher order, primarily at fourth order (18 species) (Fig. 3).

Mycorrhizal colonization was confined primarily to the first three orders (Table 3). Colonization rate generally declined with order in the first three orders. First order had the highest colonization rate, reaching 100% in eight out of nine EM species, six out of eight AM and EM species, and two out of six AM species. In comparison, third order had a colonization rate $< 50\%$ in 15 species with five species devoid of colonization.

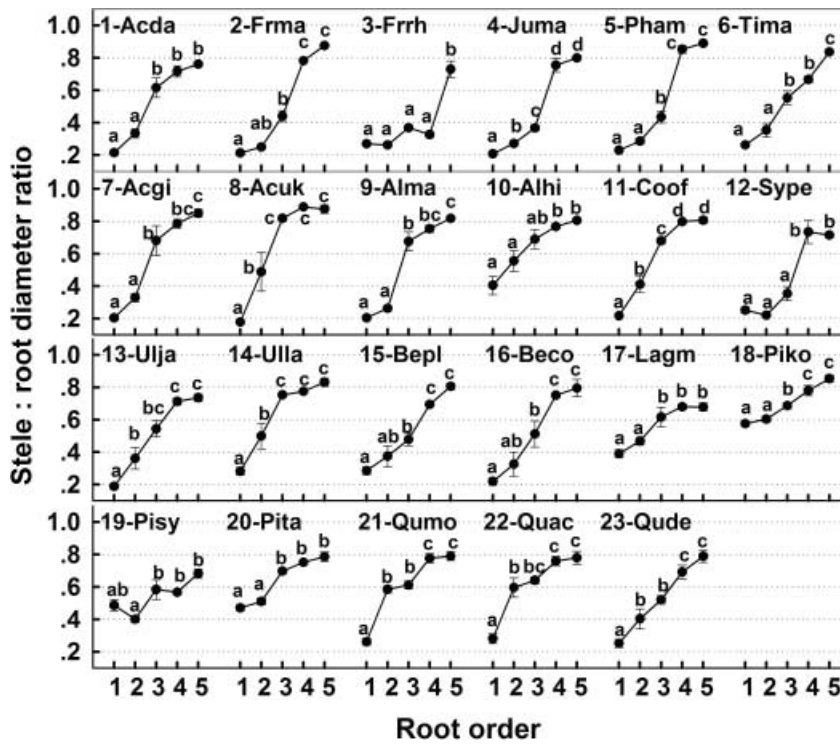


Fig. 2 Stele : root diameter ratio by branch order in 23 Chinese temperate tree species. Arbuscular mycorrhizal (AM) species, 1–6; AM and ectomycorrhizas (EM), 7–14; EM species, 15–23. See Table 1 for species abbreviations. Error bars represent 1 SE. Lower case letters that differ within a species indicate significant ($P < 0.05$) differences among root orders.

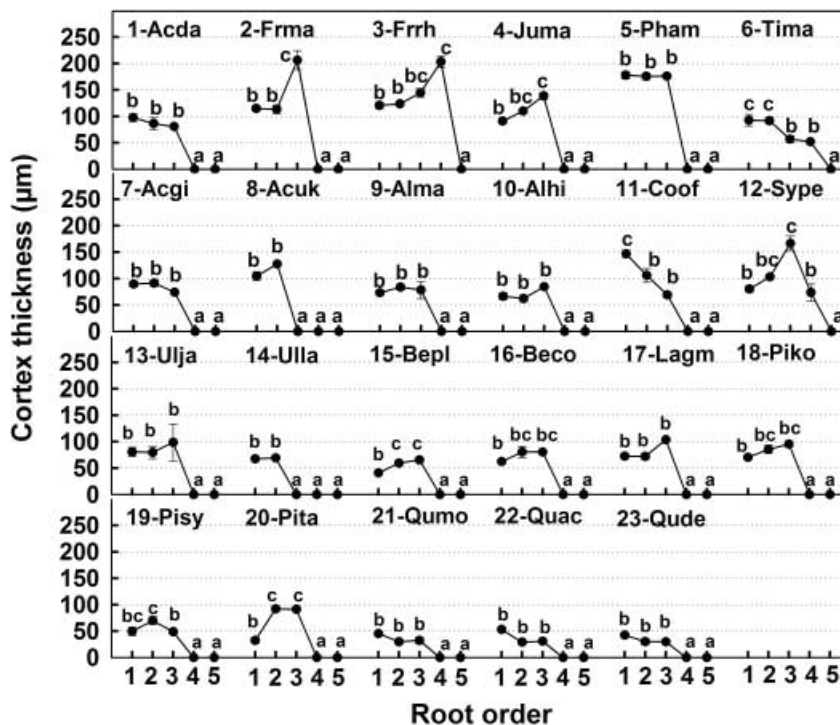


Fig. 3 Cortex thickness by branch order in 23 Chinese temperate species. Arbuscular mycorrhizal (AM) species, 1–6; AM and ectomycorrhizas (EM), 7–14; EM species, 15–23. See Table 1 for species abbreviations. Error bars represent 1 SE of the mean. Lower case letters that differ within a species indicate significant ($P < 0.05$) differences among root orders.

No mycorrhizal colonization was found in fourth and higher orders, with the only exception of *Fraxinus rhynchophylla* (Table 3).

Secondary development, as indicated by the presence of both SX and CCL occurred primarily in the fourth and higher

orders (Table 3). Secondary development did not occur in the first order roots of any species except *A. hirsuta*, and occurred in a portion of second- and third-order roots: 17–71% of second-order roots in eight species, 7–88% of third-order roots in 14 species, and all third order roots in five species

Table 3 Mycorrhizal colonization and secondary development of different root orders in 23 tree species

| | Order 1 | | | Order 2 | | | Order 3 | | | Order 4 | | | Order 5 | | |
|--|---------|----|-----|---------|----|-----|---------|----|-----|---------|----|-----|---------|----|-----|
| | MC | SX | CCL | MC | SX | CCL | MC | SX | CCL | MC | SX | CCL | MC | SX | CCL |
| AM | | | | | | | | | | | | | | | |
| <i>Acer davidii</i> | + | | | ++++ | | | + | ■ | ○ | | ■ | ● | | ■ | ● |
| <i>Fraxinus mandschurica</i> | + | | | ++ | | | + | □ | ○ | | ■ | ● | | ■ | ● |
| <i>Fraxinus rhynchophylla</i> | ++++ | | | ++++ | | | ++++ | | | ++++ | | | | ■ | ● |
| <i>Juglans Mandshurica</i> | + | | | + | | | + | □ | | | ■ | ● | | ■ | ● |
| <i>Phellodendron amurense</i> | +++ | | | ++++ | | | +++ | □ | ○ | | ■ | ● | | ■ | ● |
| <i>Tilia mandshurica</i> | ++++ | | | ++++ | | | | ■ | ● | | ■ | ● | | ■ | ● |
| AM and EM | | | | | | | | | | | | | | | |
| <i>Acer ginnala</i> | ++++ | | | ++++ | | | + | □ | ○ | | ■ | ● | | ■ | ● |
| <i>Acer ukurunduense</i> | ++++ | | | + | □ | ○ | | ■ | ● | | ■ | ● | | ■ | ● |
| <i>Alnus mandshurica</i> | ++++ | | | ++++ | | | + | □ | ○ | | ■ | ● | | ■ | ● |
| <i>Alnus hirsuta</i> | +++ | □ | ○ | + | □ | ○ | + | □ | ○ | | ■ | ● | | ■ | ● |
| <i>Cornus officinalis</i> | ++++ | | | ++ | □ | ○ | | ■ | ● | | ■ | ● | | ■ | ● |
| <i>Ulmus laciniata</i> | ++++ | | | + | □ | ○ | | ■ | ● | | ■ | ● | | ■ | ● |
| <i>Ulmus japonica</i> | ++++ | | | +++ | □ | ○ | + | □ | ○ | | ■ | ● | | ■ | ● |
| <i>Syringa pekinensis</i> | +++ | | | ++++ | | | ++ | □ | | | ■ | ● | | ■ | ● |
| EM | | | | | | | | | | | | | | | |
| <i>Betula costata</i> | ++++ | | | +++ | □ | ○ | + | □ | ○ | | ■ | ● | | ■ | ● |
| <i>Betula platyphylla</i> | ++++ | | | ++ | □ | ○ | ++ | ■ | ○ | | ■ | ● | | ■ | ● |
| <i>Larix gmelinii</i> | ++++ | | | ++++ | | | + | □ | ○ | | ■ | ● | | ■ | ● |
| <i>Pinus koraiensis</i> | ++++ | | | ++++ | | | ++++ | | | | ■ | ● | | ■ | ● |
| <i>Pinus sylvestris var. mongolica</i> | ++++ | | | ++++ | | | ++ | □ | ○ | | ■ | ● | | ■ | ● |
| <i>Pinus tabulaeformis</i> | ++++ | | | ++++ | | | +++ | □ | ○ | | ■ | ● | | ■ | ● |
| <i>Quercus acutissima</i> | +++ | | | ++ | □ | ○ | + | □ | ○ | | ■ | ● | | ■ | ● |
| <i>Quercus dentata</i> | ++++ | | | ++++ | | | +++ | □ | ○ | | ■ | ● | | ■ | ● |
| <i>Quercus mongolica</i> | ++++ | | | +++ | | | | ■ | ● | | ■ | ● | | ■ | ● |

AM, arbuscular mycorrhizas; EM, ectomycorrhizas; MC, mycorrhizal colonization; SX, secondary xylem; CCL, continuous cork layer.
 □, A portion of root segments observed had secondary xylem; ■, 100% roots observed had secondary xylem.
 ○, A portion of root segments observed had continuous cork layers; ●, 100% roots observed had secondary xylem.
 +, ++, +++, +++++, colonization rates of 1–49%, 50–75%, 76–99%, and 100%, respectively.
 Blanks mean that mycorrhizal colonization, secondary xylem, or cork layer were not observed in any root segments.

(*A. ukurunduense*, *Cornus officinalis*, *T. mandshurica*, *Ulmus laciniata* and *Quercus mongolica*). Secondary development occurred in all individual roots of fourth and fifth order in all but one species (i.e. *F. rhynchophylla*, Table 3).

Variations in root traits by diameter and diameter class

Root diameter, stele diameter, stele : root diameter ratio differed significantly by diameter class of 0.25-mm intervals whereas cortex thickness did not differ significantly (Table S1). In addition, Tukey's HSD tests found no significant differences in stele : root diameter ratio and cortex thickness among first three diameter classes (i.e. 0–0.25 mm, 0.25–0.5 mm and 0.5–0.75 mm, Table S2).

Across 23 species, significant correlations were found between diameter and most root traits. However, these correlations were weaker than those between root order and the same root traits (Table 2), and the same pattern was found across species of the same mycorrhizal type and taxonomy rank, or within each species (detailed results not shown).

Variations in root traits by mycorrhizal type and taxonomy rank

Stele : root diameter ratio and root diameter were the two key variables to separate all species into four groups along two function axes ($P < 0.05$, Fig. 4). Function 1 was mainly related to stele : root diameter ratio and distinguished angiosperms from gymnosperms, whereas function 2 was mainly related to root diameter and distinguished different mycorrhizal types in angiosperm species (Fig. 4). The discrimination rate was 74% with six mistakes out of 23 training samples, resulted from the incorrect categorization between AM and EM angiosperms and other angiosperms (Fig. 4).

Root length with uptake capacity and mycorrhizal colonization

A large proportion of the root length in the first five orders had root anatomical features associated with resource acquisition (e.g. large proportion of root cortex, low stele : root diameter ratio, no continuous cork layer) and mycorrhizal fungal

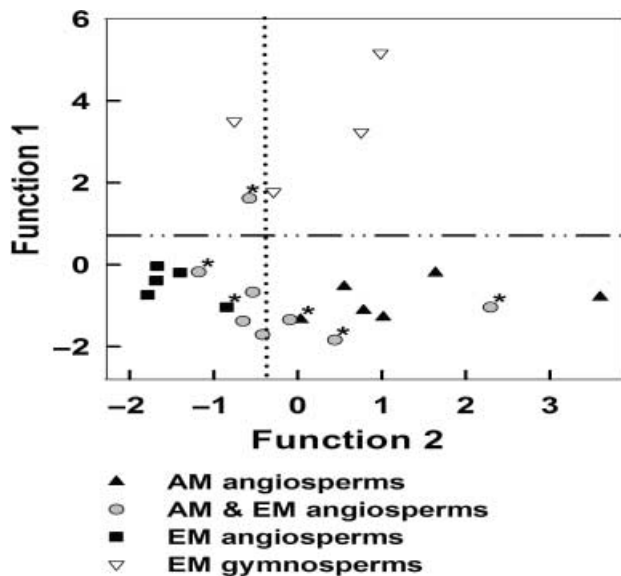


Fig. 4 Localization of 23 species of four groups defined by two discriminant functions. Function 1 was mainly related to stele : root diameter ratio; function 2 was mainly related to root diameter. AM, arbuscular mycorrhizal; EM, ectomycorrhizal. *, Species were categorized mistakenly by the discriminant analysis.

appearance. Averaged across species, the potentially absorptive length accounted for 75% of the total root length, ranging from 43% in *A. hirsuta* to 96% in *F. rhynchophylla* and *Syringa pekinensis*. Mycorrhizal length accounted for 68% of total root length, averaged across species, with a range of 22% in *Juglans mandshurica* to 96% in *F. rhynchophylla* (detailed results not shown).

Discussion

Anatomical features and functional roles of different branch orders

Anatomical traits differed significantly by root branch order within each species sampled in this study. As order increased, root diameter (Fig. 1), stele diameter (Fig. 1), stele : root diameter ratio (used to indicate transport capacity here) (Fig. 2) and the degree of secondary development such as presence of SX and CCL increased significantly (Table 3), whereas cortex thickness (Fig. 3) and mycorrhizal colonization rate declined significantly (Table 3).

Changes in root anatomy by branch order suggest that root segments of different branch order play different roles in root functioning. Based on the existence of the cortex, stele : root diameter ratio, mycorrhizal colonization rate, and CCL presence rate, five branch orders may be separated into two groups: roots with the primary function of resource uptake and roots serving functions other than absorption (Figs 1–3, Table 3). First-order roots had an intact cortex, a high mycorrhizal colonization rate, a low stele : root diameter ratio, and

showed no signs of secondary growth in all but one species, thus representing absorptive roots. By contrast, fourth- and higher-order roots had SX and CCL in all individual roots across all species except *F. rhynchophylla* (which lacked CCL in the fourth order). Moreover, the roots of these two orders had no cortex and mycorrhizal occurrence in all species, but a high stele : root diameter ratio. Therefore, they may have limited uptake capacity and primarily serve functions such as transport, anchorage, and storage. Second and third order showed some signs of secondary development, according to species (Table 3). Overall, the distal two or three orders seem to be the ones that are responsible for resource uptake in most species.

We recognized that anatomical traits, although tightly linked to root functions, are not direct measures of them. Therefore, our conclusions about functional roles of different branch orders need be validated by direct measures of root uptake and transport capacity in future studies. Nonetheless, the findings of this study represent a significant step forward in understanding the relationship between root form and root function.

The anatomical differences among branch orders found in this study might explain the systematic variations in root chemistry and lifespan with increasing order (Pregitzer *et al.*, 2002; Guo *et al.*, 2004, 2008a), and the role of different root orders in ecosystem C and nutrient cycling (Guo *et al.*, 2008b). Our results showed that first-order roots comprised mostly cortical cells (Figs 1–3). Cortical cells generally have high metabolic rate and require higher concentrations of nitrogen and phosphorus to support their physiological activity (Lux *et al.*, 2004). Thus, it is hardly surprising to find the highest concentrations of nitrogen (Pregitzer *et al.*, 2002; Guo *et al.*, 2004) and respiration rates (Pregitzer *et al.*, 1998; D.L. Guo, unpublished) in first-order roots. In addition, cortical cells are easily damaged or lost under stress (Brundrett, 2002; Wells & Eissenstat, 2003; Soukup *et al.*, 2004), which renders first-order roots the most ephemeral of all orders. Their short lifespan, combined with their considerable biomass, make the first-order roots the biggest contributor to root turnover among all branch orders (Guo *et al.*, 2008a,b). By contrast, fourth and fifth order were mainly composed of secondary vascular tissues which are known to have low physiological activity because of the large proportion of dead cells (Pregitzer *et al.*, 2002), and thus require low concentrations of nitrogen (Guo *et al.*, 2004), and probably have low respiration rates. Moreover, the well-developed cork layer and secondary xylem can protect roots from environmental stresses and herbivore pressure (Brundrett, 2002), ensuring high-order roots long life spans, and thus, low turnover rates (Wells & Eissenstat, 2003; Guo *et al.*, 2008a,b).

Our results also revealed which roots in the tree fine-root systems might be colonized by mycorrhizal fungi. Mycorrhizal colonization was found in the first to third order in 18 species, but was absent in the fourth and fifth orders in all species except *F. rhynchophylla* (Table 3). Our results confirm the general view that first-order roots are preferentially colonized

(Pregitzer, 2002), but show that at least two more branch orders can be colonized (Fig. 3, Table 3). Therefore, quantifying mycorrhizas in roots must go beyond first-order root tips.

Moreover, we found that mycorrhizal colonization rate was generally higher in EM than AM species (Table 3). In particular, first-order root tips had 100% colonization rate in all but one EM but not in AM species (Table 3), supporting the notion that EM species rely heavily on mycorrhizal fungi for resource uptake (Read & Perez-Moreno, 2003; Chapman *et al.*, 2006).

The correspondence between root anatomy and branch order suggest that branch order can be used as a tool to separate roots of different functions. However, our results also support the possibility that several orders might form a functional module as proposed by Pregitzer *et al.* (2002) because first two or three order roots were similar in anatomy. We noted that the number of branch orders that were confined to primary development differed markedly by species (Table 3) so that the functional module, if exists, may have different architecture in different species. Future studies linking root anatomy, physiology, and demography on the branching fine root systems may better resolve the precise definition of functional root modules in trees.

Diameter classes and anatomical features

Our results support the proposition that a single diameter class cannot be used to define functional root module (or 'fine roots' in the traditional sense) across multiple species (see Pregitzer, 2002) because the diameter of the same branch order varied markedly across species (Fig. 1). Given the relatively consistent linkage between order and anatomy across species (i.e. first-order roots had primary development in all but one species, and fourth-order roots had secondary development in all but one species, Table 3), wide variations in diameter of the same order make a single diameter class unreliable in comparing roots of same functions across species (Fig. 1). For example, *Phellodendron amurense* had a first-order root diameter of > 0.5 mm, which is similar to the fifth order root diameter in *A. davidii*, *A. ukurunduense*, *B. costata*, and *Quercus acutissima* (Fig. 1).

Even within species, root diameter (a continuous variable) or diameter class fails as a reliable tool for root functional classification because in some species diameter was relatively constant yet root anatomy clearly changed. For example, diameter did not change significantly in the first four or five orders in *A. davidii*, *A. ukurunduense*, *A. hirsuta*, *B. costata*, *T. mandshurica*, and *U. japonica* (Fig. 1), but shifts of root anatomy occurred as order increased (Table 3). Therefore, in these species, diameter obscures critical changes such as the loss of cortex and mycorrhizal colonization, and the appearance of SX and CCL as order increased. The generally weak correlation between diameter (or diameter classes of 0.25 mm intervals) and root anatomical indices (Table 2) supports the notion that diameter was an inferior predictor of root anatomical change than branch order.

Anatomical differences by taxonomic rank and mycorrhizal type

Stele : root diameter ratio of first-order roots was the primary trait distinguishing gymnosperms from angiosperms (Fig. 4). Gymnosperms had higher stele : root diameter ratios (Figs 2 and 4), and thus a higher stele and lower cortex proportion than angiosperms, which probably reflects the inherent differences between these two groups. The gymnosperms studied here are all conifers, which are known to have xeromorphic foliage that reduces water loss (Richardson & Berlyn, 2002). The greater stele proportion in first-order roots of these conifers may also be a xeromorphic feature that facilitates water uptake and transport. Another explanation is that tracheids in conifers have lower conductivity than vessels in angiosperms so that more of them are needed by conifers to achieve a transport capacity comparable to that of angiosperms (Sperry *et al.*, 2006). Notably, the difference in stele : root diameter ratio between gymnosperms and angiosperms disappeared in the fourth- and higher-order roots (Fig. 2).

By contrast, the diameter of first-order roots was the major trait separating different mycorrhizal types in angiosperm species. The AM angiosperms had greater diameter and cortex area than EM and AM & EM angiosperms (Figs 1–3), which probably reflects the advantage of AM fungi in roots that have greater cortex area (Brundrett, 2002).

Root length with uptake capacity and mycorrhizal colonization

Our study provided estimates of the absorptive and mycorrhizal length proportions in the first five orders based on anatomical evidence. Averaged across species, the absorptive length accounted for 75%, and the mycorrhizal length for 68% of the total root length in the first five orders. Even though these estimates were based on one-time sampling, a limited number of species and indirect methods, they represent an improvement from the total absorptive root length estimates based solely on 0–2 mm diameter class, which considered all fine root length as being equally absorptive (Jackson *et al.*, 1997). Assuming that the total fine root length for the 0–2 mm diameter class included only the first five orders (a conservative assumption, as indicated by our data showing that 18 out of 23 species contained at least five orders within the < 1.5 mm diameter class, Fig. 1), the absorptive length estimated based on root anatomy would be 25% less than the estimates based on diameter class in temperate forests.

Conclusions

For the past several decades, roots have been one of the least understood components of plant functioning and ecosystem material cycling, largely owing to the inability of linking root structure and function (Wells & Eissenstat, 2001; Pregitzer

et al., 2002; Withington *et al.*, 2006). Our results showed that branch order relatively consistently predicted anatomical changes in the branching fine root system across 23 temperate tree species. Therefore, branch order may serve as a useful tool to distinguish the roots that are confined to primary development, and are mainly absorptive and mycorrhizal, from the roots that have lost their cortex and undergone secondary development, and thus perform mainly transport, anchorage and storage functions. Specifically, we showed that first two orders were primarily absorptive and mycorrhizal roots, and that shifts of root function from uptake to transport appeared to occur at the third or fourth order in most species.

The correspondence between root anatomy and branch order reported here, along with the growing appreciation that distal two or three branch orders have much shorter life spans than higher orders (Withington *et al.*, 2006; Guo *et al.*, 2008 a,b) show that these smallest distal roots (possibly as modular units) play a disproportionately important role in resource acquisition and ecosystem carbon and nutrient flux. Future studies linking anatomy, demography and physiology on the branching fine root network should lead to a better understanding of functional modules in plant root systems.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Light micrograph of root cross-sections.

Table S1 Results of mixed-level three-way factorial ANOVA

Table S2 Root diameter, cortex thickness, stele diameter, and stele : root ratio by order and diameter class of 0.25-mm intervals

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