Short report

Which is the best indicator for distinguishing between fine roots with primary and secondary development in *Cryptomeria japonica* D. Don: Diameter, branching order, or protoxylem groups?

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Abstract: Fine roots of *Cryptomeria japonica* were separated into two functional groups: primary roots that serve as the principal agent for water and nutrient absorption and secondary roots that have transport capacity and protect the plant from environmental stress. Individual roots can also be categorized by three characteristics: diameter, branching order, and number of protoxylem groups. We investigated the relationships of these two functional groups with the three categories and evaluated which category was a better index for distinguishing primary from secondary roots by using the Pianka overlap index. Primary and secondary roots showed no exact correspondence to any of the three categories and had overlap in each category. Therefore neither was a useful indicator to distinguish primary from secondary roots. However, in the case of *Cryptomeria japonica*, we can roughly distinguish primary from secondary roots on the basis of whether root diameter is less than or greater than 0.6 mm.

Keywords: *Cryptomeria japonica*, diameter, fine root anatomy, overlap degree

Introduction

Individual roots in fine root systems consist of two functional groups, roots with primary development and roots with secondary developments (Pregitzer 2002, Guo et al. 2008c). Roots with primary developments have a living cortex, develop symbiotic associations with soil fungi, and are responsible for water and nutrient absorption (Hishi 2007, Guo et al. 2008c). Roots with secondary developments have a cork layer and secondary xylem that provides protection from environmental stresses and carries out transport, anchorage, and storage functions (Brundrett 2002, Guo et al. 2008a,b, Valenzuela-Estrada et al. 2008). Roots with primary and secondary developments have been distinguished based on their branching order, number of protoxylem groups, and diameter within the fine root system (e.g., Hishi and Takeda 2005a,b, Guo et al. 2008c).

The branching order of individual roots is important for understanding root functions in fine root systems (Guo et al. 2008c). First-order roots have relatively smaller diameter, higher specific root length (Pregitzer et al. 2002), higher nitrogen concentrations, higher respiration rates (Pregitzer et al. 1998, 2002), and a shorter lifespan (Wells et al. 2002). Therefore, branching order has been reported as a useful indicator to characterize individual roots as primary or secondary development (Pregitzer 2002, Guo et al. 2008c).

Individual roots in the fine root system also show different anatomical characteristics. The number of protoxylem groups, first formed in the xylem, often reflects life-cycle differences of individual roots within the same root system (Hishi and Takeda 2005a,b). Therefore, the number of protoxylem groups within the fine root system is an indicator of whether the fine root will progress to secondary growth or not, because the number of protoxylem groups does not change throughout the life cycle of individual roots (Noelle 1910, Hishi and Takeda 2005a). Hishi and Takeda (2005a) studied root anatomy in *Chamaecyparis obtusa* and found that roots with two strands of protoxylem (diarch roots) tend to die before secondary development, while

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roots with four strands of protoxylem (tetrarch roots) usually advance to secondary development before they die; thus, diarch roots tend to be ephemeral and have nutrient uptake capacity, whereas tetrarch roots tend to be perennial and have transport capacity. Therefore, the number of protoxylem groups can be an indicator to characterize individual roots as primary or secondary development.

Diameter is also a useful index to distinguish individual roots with primary and secondary developments. Roots with a high number of protoxylem groups are larger in diameter and usually become secondary development, while roots with a low number of protoxylem groups are smaller in diameter and few roots become secondary development (Hishi and Takeda 2005a, Zadworny and Eissenstat 2011).

Cryptomeria japonica is the dominant tree species in most Japanese plantations, and this species covers half of the plantation area in Japan (Japan FAO Association 1997). Karizumi (2010) showed that most roots of *C. japonica* usually had five strands of protoxylem (pentarch), but there is little information on anatomical characteristics of individual roots of the fine root systems of *C. japonica*.

In this study, individual roots of *Cryptomeria japonica* were characterized based on their diameter, branching order, and number of protoxylem groups. The objectives of this study were (1) to describe the anatomy of fine roots with primary and secondary developments and (2) to determine an indicator (diameter, branching order, and/or number of protoxylem groups) for classifying individual roots as roots with primary or secondary development.

Materials and Methods

Site description

The study site was in Oharano Forest Park, Kyoto City, Japan (34°5N, 135°37E, 400 m above sea level). The mean annual temperature was 16.8°C and the annual precipitation was 1,581 mm (measured at the Kyoto Weather Station, about 12 km from the study site). The study plot of 10 × 15 m was created in a 44-year-old *C. japonica* plantation. The plot was divided into 6 subplots of 5 × 5 m that were used for root sampling. In the study plot, the organic soil layer has a moder humus layer about 2-4 cm thick. The soil in the study plot is brown forest soil and is classified as category B_D (Forest Soil Division 1976).

Anatomical traits of fine root systems

Samples of fine root systems were collected in May, August, and November 2012. Six soil blocks (15 × 15 cm) were excavated at a 10-cm depth on each sampling occasion. Four root systems were carefully gathered from each soil block, and a total of 72 fine root systems (6 subplots × 4 root systems × 3 sampling times) were collected. Root samples were placed in plastic bags and transported to the laboratory. Soil samples were stored in a refrigerator at 4°C. All root samples were gently washed with tap water to remove organic matter and soil minerals, and each root sample was stored in 70% ethanol.

In the analysis, we included only fine roots from the first three branching orders because most of the fourth-order root segments were incompletely excavated in the samples. Individual roots of fine root systems were separated into different branching orders following the procedure described by Pregitzer et al. (2002). The most distal root tips were labeled as first-order roots; roots with two first-order roots joined together were labeled as second-order roots; and two second-order roots joined together were labeled as third-order roots. Sixty root segments were randomly selected from the first and second order, and 24 root segments were selected from third order at each sampling time. A total of 432 root segments were selected for anatomical observation. Root segments were kept in a Petri dish in 70% ethanol, and cross-sections of individual roots were dissected manually at the center of each root segment under a dissecting microscope. The root diameter, the number of protoxylem groups, and the presence of secondary xylem were recorded. These observation methods are detailed in McKenzie and Peterson (1995a,b) and Hishi and Takeda (2005a). Roots with secondary xylem were classified as secondary roots; those without secondary xylem but with passage cells were classified as primary roots in this study. All anatomical observations were performed with a Nikon Eclipse 80i microscope equipped with a 130-W mercury light. UV illumination was achieved with a UV-1A filter. The proportions of primary to secondary roots showed no significant differences between seasons ($P > 0.05$, contingency table analysis).

Calculation of overlap degree for primary and secondary roots

The degree of overlap between primary and secondary roots was calculated for diameter, branching order, and protoxylem group categories using the Pianka overlap index (Pianka 1973). This index was originally used for estimating niche overlap for two species within one resource category. In this study, we used this index for estimating the overlap degree for primary and secondary roots in each category. Overlap degree, $\alpha_{ps,}$ was calculated by

following equation:

$$\alpha_{ps} = \frac{\sum_{i=1} p_{pi} p_{si}}{\sqrt{\sum_{i=1} (p_{pi})^2} \sqrt{\sum_{i=1} (p_{si})^2}}$$

where p_{pi} and p_{si} are the proportions of the category *i* of *p*, primary root, and *s*, secondary root, respectively. The index is symmetrical and assumes values between 0 and 1. Zero indicates that primary and secondary roots show exact correspondence to the categories, 1 indicates complete overlap, and intermediate values represent partial overlap in the categories.

Statistical analysis

Student's *t*-test was used to test the differences in root diameter between primary and secondary roots. One-way analysis of variance was used to test the differences in root diameter of roots with different branching orders and numbers of protoxylem groups. Statistical analyses were performed using SPSS v.20 (IBM Inc., USA).

Results and Discussion

Anatomical characteristics of fine roots in C. japonica

Among of the observed roots, 251 were primary roots and 181 were secondary roots. Primary and secondary roots had different anatomical traits. Passage cells, which are epidermal cells that lack secondary walls (Hishi and Takeda 2005a), were found in primary roots (Fig. 1a, b, and d). These cells have low resistance to water (Peterson and Enstone 1996) and therefore absorb water and nutrients (Taylor and Peterson 2000). Secondary roots formed mature central metaxylem in a convex curve and developed secondary xylem around metaxylem (Fig. 1c, e, and f). Intact cortical cells with phi-thickenings were observed mainly in primary roots and in early stages of secondary development (Fig. 1a, b, d, and e). Phi-thickenings are found on the radial and tangential walls of root cortical cells (Gerrath et al. 2002, 2005) and are considered to be supportive tissues (Weerdenburg and Peterson 1983). In secondary roots, the vascular system expanded as the cortical cells shrunk (Fig. 1c and f). These results showed that cortical cells and phi-thickenings collapsed and endodermis was compressed as the secondary vascular system developed in the secondary roots. Hishi and Takeda (2005a) and Hishi (2007) also suggested that the diameters of most primary and secondary roots showed no clear increments during their growth periods. The results suggested that the diameter does not change during the transition from primary to secondary growth in the fine roots because secondary vascular tissues expand into the cortical layer.

Individual roots were separated into six size categories based on 0.10-mm diameter-intervals (Fig. 2a). The mode of primary roots was 0.51-0.60 mm and that of secondary roots was 0.6- 0.70 mm. The mean diameters were significantly different between the primary (0.52 mm) and secondary roots (0.65 mm; $P < 0.001$). In the branching order category, the proportion of primary to secondary roots was highest in first-order roots, and third-order roots consisted entirely of secondary roots (Fig. 2b). Second-order roots consisted of both primary and secondary roots. These trends are similar to those found in previous studies (Hishi and Takeda 2005a, Guo et al. 2008c). Diarch roots were mainly primary roots, while pentarch roots were mainly secondary roots (Fig. 1a and f, Fig. 2c). Triarch and tetrarch roots were either primary or secondary (Fig. 1b, c, d, and e), but the proportions of primary roots were higher in the triarch than in the tetrarch roots (Fig. 2c). Roots with a high number of protoxylem groups have large diameters at early developmental stages and proceed to secondary growth with diameter growth (Zadworny and Eissenstat 2011). These big roots (e.g., >1.0 mm) were rarely found in the samples in this study.

Which is the best indicator to distinguish primary from secondary roots in C. japonica?

The proportion of primary to secondary roots decreased as roots increased in diameter from <0.4 mm to >0.8 mm. In the >0.8 mm diameter class, all roots were secondary roots. The overlap degree of primary and secondary root groups, α_{ps}, was 0.635 in the diameter category. Individual roots were separated into first-, second-, and third-order roots (Fig. 2b). The mean diameter of first- (0.52 mm), second- (0.58 mm), and third-order roots (0.71 mm) significantly differed among all three categories ($P < 0.001$). In the branching order category, the proportion of primary to secondary roots decreased from first- to third-order roots, and the overlap degree, α_{ps}, was 0.577. Individual roots were separated into diarch, triarch, tetrarch, and pentarch roots based on the number of protoxylem groups (Fig. 2c). The mean diameters of diarch (0.48 mm), triarch (0.54 mm), tetrarch (0.65 mm), and pentarch (0.80 mm) roots were significantly different ($P < 0.001$). The proportion of primary to secondary roots decreased moving from diarch to pentarch roots. The overlap degree, α_{ps}, was 0.719 in protoxylem group category.

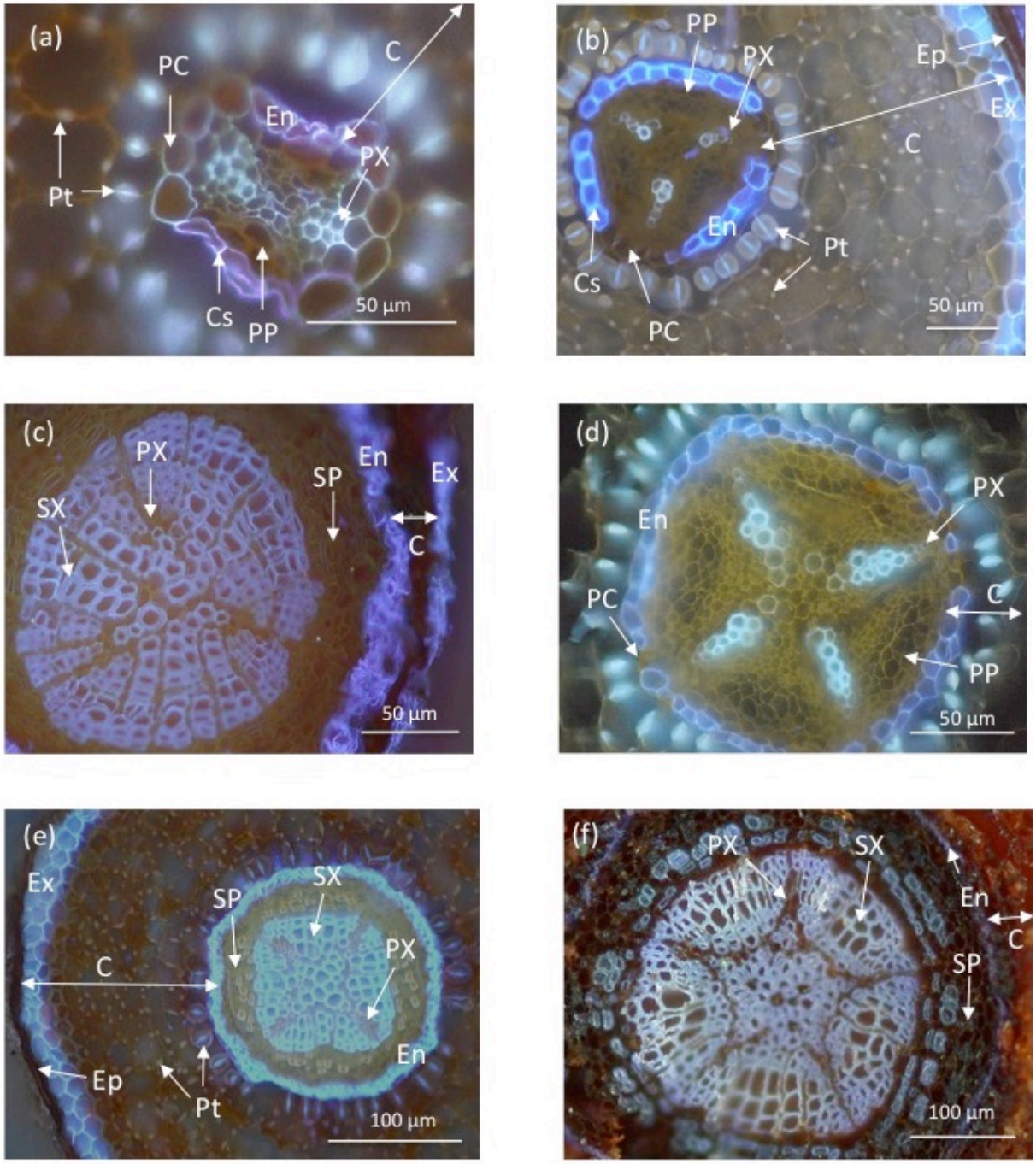

Fig. 1. Light micrograph of root cross sections. (a) Diarch root with primary development. (b) Triarch root with primary development. (c) Triarch root with secondary development. (d) Tetrarch root with primary development. (e) Tetrarch root with secondary development. (f) Pentarch root with secondary development. PX, protoxylem; SX, secondary xylem; PP, protophloem; SP, secondary phloem; En, endodermis; Ex, exodermis; Ep, epidermis; PC, passage cell; C, cortex; Cs, casparian strip; Pt, phi-thickenings

Guo et al. (2008c) studied anatomical traits in roots of 15 tree species and showed that branching order was a better indicator than diameter for distinguishing secondary roots because root diameter varied among tree species. However, in one species, *C. japonica*, the overlap degree of primary and secondary roots was over 50% in the three categories in our study. Therefore, these results suggest that it is difficult to exactly distinguish primary from secondary roots in *C. japonica* by using either diameter, branching order, or protoxylem group categories.

Among the three categories, root diameter is

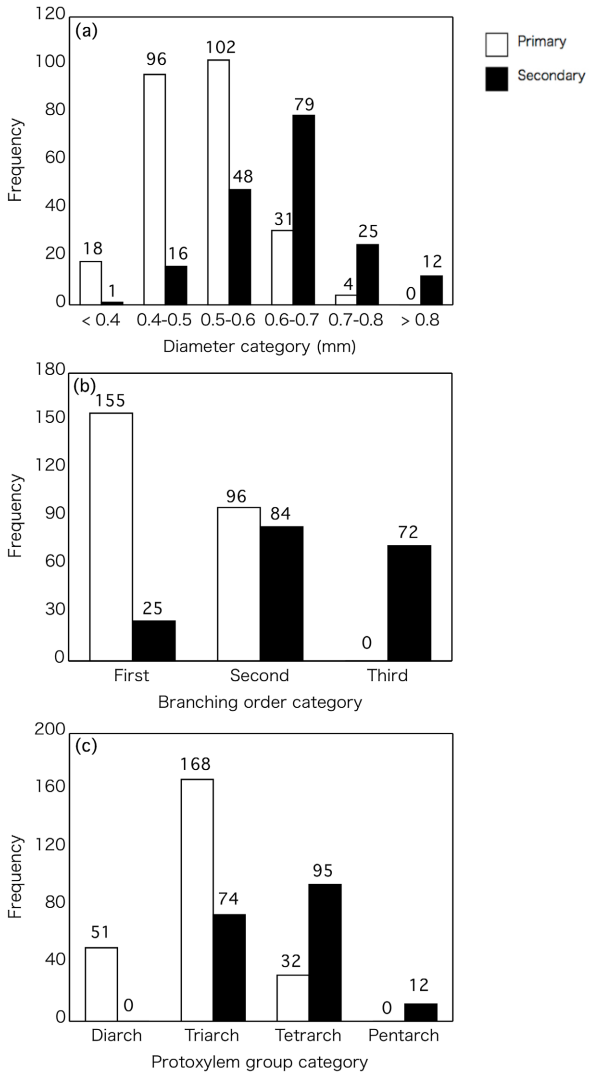

Fig. 2. Distribution of primary and secondary roots at (a) diameter category, (b) branching order category and (c) protoxylem group category. Number in this figure means the number of roots in each category.

easily measured by image analysis and is also an important parameter for quantitative estimation of factors such as root biomass. In this study, most of the roots <0.5 mm in diameter were primary roots and most of the roots >0.7 mm in diameter were secondary roots. The mode of primary roots was 0.51–0.60 mm and that of secondary roots was 0.61-0.70 mm. We then used a value of 0.6 mm diameter to distinguish primary from secondary roots. We observed 216 primary roots and 65 secondary roots of <0.6 mm in diameter (Fig. 2a), and then secondary roots were overestimated at 23.1% against total number of roots (< 0.6 mm). While we observed 35 primary roots and 116 secondary roots of >0.6 mm in diameter, and primary roots were overestimated at 23.2% against total number of roots (> 0.6 mm). We can roughly distinguish primary from secondary roots using a criterion of 0.6 mm root diameter with about 20% error. In this study, only 72 root systems were observed for anatomical study. Enumerating root systems is important in many ecological studies. Therefore, diameter may be useful in distinguishing primary from secondary roots.

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