The relationship between individual root anatomy and fine root system development in blueberry seedlings: dominance of diarch roots in initial root systems

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Abstract: Heterogeneity of individual root traits (heterorhizy) is thought to be one of the most important mechanisms that maintain the functional gradients in fine root systems. It is necessary to know heterorhizy and its relation to the root system architecture in seedlings, because it is the initial form of fine root development. Herein, we investigated individual root traits and the root system architecture of blueberry seedlings by using the protoxylem grouping. Most individual roots were diarch but some laterals were monarch. Compared to the monarch, the diarch roots had significantly larger diameters and tissues, and over half of them exhibited secondary growth. In other words, frameworks of the blueberry seedlings were diarch, although those of cuttings had been at least triarch as seen in our previous study. The employment of diarch frameworks may optimize investment for root system expansion of blueberry seedlings. Our results imply ontogenetic dynamics of heterorhizy in blueberries.

Keywords: blueberry, heterorhizy, individual root, primary root, protoxylem, seedling

Abbreviations: PG, protoxylem group; PRS, percent of roots exhibiting secondary growth

Introduction

Fine roots are relatively thin and young portions of a plant root systems being essential to absorb water and nutrients. While it has often been defined based on a diameter of less than 1 or 2 mm, many researchers have propounded that, especially in woody plants, there are functional gradients even within the same diameter ranges (e.g., Pregitzer et al. 2002). These characteristic shifts have been comprehended by appropriate topological classifications established during last few decades (Hishi 2007, McCormack et al. 2015). For example, root-order based classification is a frequently used method and it was first developed by Fitter (1982) who subdivided fine root systems into certain branching units (see Materials and Methods). The root-order clearly and universally explains how root traits change with branching structures from the most distal ends toward the coarse mother roots in diverse tree species (Doi et al. 2017, Guo et al. 2008, Ouimette et al. 2013, Pregitzer et al. 2002, Valenzuela-Estrada et al. 2008). However, the application of root-orders often breaks both minimum construction units and partial characteristic continuums, namely individual roots derived from each apical meristem. There is heterogeneity of individual root traits in morphology, anatomy, and growing ability (heterorhizy) in various plant species.
Heterogeneous individual roots are commonly classified into absorptive and framework roots, such as fibrous and pioneer roots (e.g., Zadworny and Eissenstat 2011). Ontogenetically heterogeneous individual roots are regularly arranged by branching hierarchy, which is a vital factor to cause the functional gradients (Hishi 2007). Therefore, another key is also needed to clarify fine root system architectures related to the developmental mechanisms.

Protoxylem grouping is an anatomical classification that distinguishes individual roots by the number of protoxylem poles. Depending on their number, each protoxylem group (PG) is classified into monarch (group with one protoxylem pole), diarch (group with two protoxylem poles), and so on. This strong indicator of heterorhizy corresponds to the functional gradients and developmental processes of fine root system architectures, meaning that number of protoxylem poles increases with growing ability and lifespan of individual roots. Additionally, there is a regular distribution pattern which PGs taper from larger mother roots to smaller daughter roots (Baba et al. 2018, Hishi and Takeda 2005). In particular, the tapering pattern of PG distribution is known to occur in primary root systems of young seedlings in various tree species. For instance, in Ginkgo biloba L. (Ginkgoaceae) seedlings less than a year old, primary roots are diarch or triarch but secondary laterals are always diarch (Bonacorsi and Seago 2016). In our study, these ordering of roots in seedlings, primary or secondary, does not represent growing status but positional hierarchies related to radicles. See also Materials and Methods. In the case of Pseudotsuga menziesii (Mirb.) Franco (Pinaceae) up to three years old, primary roots are commonly triarch and sometimes tetrarch, secondary laterals are usually diarch and occasionally triarch, and further laterals are always diarch (Bogar and Smith 1965). Larger PGs and wide PG variation can be seen in citrus (several genotypes in Rutaceae) seedlings (Eissenstat and Achor 1999, Hayward and Long 1942). Hayward and Long (1942) reported that Valencia orange (Citrus sinensis (L.) Osbeck) and other strains of sweet orange seedlings less than 2-months old has primary roots that are commonly octarch and occasionally heptarch to nonarch, and lateral roots ranged from triarch to octarch, with tetrarch and pentarch being the most common. Unlike these tapering patterns, it is possible that there are other PG distribution patterns which larger PGs appear with root system development. Horsley and Wilson (1971) reported that Betula papyrifera Marsh. seedlings have diarch primary roots, and lateral roots are diarch at first but become successively triarch, tetrarch, pentarch, and hexarch as the root tip enlarges. Considering the temporal and spatial growth of woody root systems, qualitative changes of heterorhizy with plant growth can be important in root ecology to adapt to a changeable internal and external environment.

From the above perspective, heterorhizy in the primary root system of seedlings must be investigated as the starting state of the root system lifespan. Such information is lacking in the blueberry and its relatives. Their distal fine roots are known as “hair roots” with the following specific properties: the hair roots have extremely fine diameter less than 100 µm, a simple anatomy with one or two xylem poles (monarch or diarch in PG), ericoid mycorrhizal association, and no root hairs (Allaway and Ashfold 1996, Briggs and Ashfold 2001, Read 1996, Valenzuela-Estrada et al. 2008). Previously, we showed the regular tapering pattern of PGs in the rabbiteye blueberry (Vaccinium virgatum Ait.) cuttings (Baba et al. 2018). Aside from hair root-like individual roots, they have large framework individual roots with triarch to heptarch, whose diameters often exceed 100 µm even near the apices and sometimes reached 300 or 400 µm with increasing protoxylem poles. This is similar in adult bushes growing in the field (Baba et al. unpublished data). Young seedlings need to quickly expand their root systems as well as cuttings. Because large framework roots have a higher initial growth rate than the small absorptive roots (Zadworny and Eissenstat 2011), such framework roots with larger PGs may help to rapidly expand root systems in blueberry seedlings. However, unlike the cuttings or adults of blueberries, their seeds and initial fine root systems seem to be too small to form such large roots, which will require excessive resource investments. It was expected that young seedlings would show specific heterorhizy that anatomical traits, especially number of protoxylem poles, of frameworks are minimized. Therefore, we hypothesized that framework roots including primary roots of blueberry seedlings are triarch (the minimum PG with frequent secondary growth in the cuttings).

The aim of this study is to show heterorhizic properties of young blueberry seedlings using PGs, and to provide fundamental information about fine root ecology in blueberries.

Materials and Methods

Plant materials and sampling

Plant materials were prepared at the Faculty of Agriculture Field Science Center, Tokyo University
of Agriculture and Technology, Fuchu, Tokyo, Japan (35°41′00″N 139°29′12″E). Fully ripened fruits of the open-pollinated highbush blueberry (*V. corymbosum* L.) ‘Bluecrop’ and the rabbiteye blueberry (*V. virgatum* Ait.) ‘Tifblue’ were harvested in mid-July 2016. Approximately 100 seeds were collected from each cultivar and immediately sown in nursery boxes filled with mixtures of peat moss and Kanuma soil (3:1, v:v). The boxes were put on an open bench and irrigated daily so that the soil surface was moist. The seeds started to germinate from one month after the sowing. For each cultivar, six seedlings whose cotyledons were completely extended were carefully pulled off in December of the same year. The seedlings were gently washed with tap water to remove the media and taken an image using a scanner (600 dpi resolution, 8-bit grayscale, TIFF format; GT-9800F, EPSON, Japan). After scanning, they were stored in 70% ethanol solution until further investigations.

**Root observation and measurement**

In this study, the methods for morphological and anatomical observation were generally based on Baba et al. (2018). We sorted individual roots into three branching hierarchies defined as follows: P as primary roots (elongated directly from seeds), L1 as lateral roots of P, and L2 as lateral roots of L1. One individual root of each branching hierarchy per seedling was selected. Length, lateral root number (branching number), and maximum root orders of the individual roots were measured using the scanned images. The individual root length was measured with a segmented line tool of ImageJ (National Institutes of Health, USA). Branching number and maximum root order were visually counted. The root order is defined following the methods of Fitter (1982): the lowest 1st-order ranges from a root apex with a meristem to a joining point with another root, and a higher order root (nth) extends from the junction of two of the same lower order (n-1th) roots to the joining point with another same or higher order root.

For the anatomical observation, each individual root was cut into 18-μm-thick transverse sections on a freezing stage using a sliding microtome (MC-802C and TU-213, Yamatokohki, Japan). Preliminarily, we confirmed how the individual root anatomy was developed using an extra seedling derived from ‘Bluecrop’. A P-root with 2nd-order was cut at 0–2 mm (the apex of 1st-order), 6–8 mm (the base of 1st-order), and 12–14 mm (the most basal portion of the root) from the apex (Fig. 3a, b, c), and an L1-root with 1st-order was cut at 0–0.8 mm (the apex of the root) and 1.6–2.4 mm (the base of the root) from the apex (Fig. 3d, e). Two transverse sections were obtained from each portion. One of the two sections was stained with 0.1% (w/v) berberine hydrochloride solution for 1 h and counterstained with 0.5% (w/v) safranin O dissolved in 50% ethanol solution. This staining defines Casparian bands in the cortex (Lux et al. 2005). The other section was not stained. All the sections were observed under a fluorescence microscope (BX50, Olympus, Japan) equipped with a U-MWU filter (excitation, 330–385 nm; dichroic mirror, 400 nm; barrier, 420 nm, Olympus), and taken an image using a digital camera (DP72, Olympus). In this observation, some protoxylem poles did not develop enough to fluoresce in the apical parts of 1st-orders (see results and discussion, Fig. 3a, d). Therefore, root sections of the six seedlings were obtained from “apical” (a base of 1st-order or an apex of 2nd-order) and “basal” (the most basal point of a root) parts. For roots with only 1st-order, only one section per root was cut because these two points overlap. The observation and imaging methods are described above but no staining was conducted. The root diameter, epidermis thickness, cortex layer number, cortex thickness, spreads of exodermal and endodermal fluorescence, stele diameter, and number of protoxylem poles were measured on the apical sections. The spreads of exodermal and endodermal fluorescence were visually classified into five degrees as follows: 0 (no fluorescence), 1 (only Casparian bands, <25% of the total cell number), 2 (25% to <50% of total cell number), 3 (50% to <75% of the total cell number), 4 (≥75% of the total cell number). Presence or absence of secondary xylems were confirmed on the basal sections and PRS (percent of roots exhibiting secondary growth) was calculated.

**Statistical analysis**

Considering small sample sizes and possible genetic heterogeneity of the seedlings, we pooled the data regarding to the parental cultivars before statistical analysis. The composition of PGs was compared between each of the branching hierarchies using Fisher’s exact test and the Benjamini and Hochberg correction. The means of each root trait except PRS were compared between different PGs using the permuted Brunner-Munzel test (Neubert and Brunner 2007). Average PRS was compared with Fisher’s exact test. All the statistical analyses were performed with R 3.3.3 (R Core Team 2017).
Results and Discussion

Anatomical features of individual roots

Anatomical features in individual roots of blueberry seedlings were basically consistent with those of the cuttings in our previous study (Fig. 1; Baba et al. 2018). The apical sections consisted of a one-layered epidermis without root hairs, a two- or three-layered cortex with thick walled exodermis and endodermis, and relatively thin steles. Epidermal cells of P-roots often shrunk or were sloughed off, while those of L1- or L2-roots were more intact. We successfully distinguished individual root PGs from the apical sections, which are located between basal 1st- and apical 2nd-orders. In contrast to our hypothesis, the PGs of all individual roots were classified into diarch or monarch (Fig. 2, discussed below). Changes of internal structures with the distance from root apices are also shown in Fig. 3. On the most apical sections of 1st-orders, fluorescence of protoxylem and endodermis were not or weakly detected, but the exodermis and its Casparian bands showed strong fluorescence (Fig. 3a, d). On the basal sections of 1st-order, the exodermis and protoxylem developed completely and the endodermis was partly formed (Fig. 3b, e).

Fig. 1. Fluorescent images of root transverse sections with different branching hierarchies. (a, b) apical and basal section of P-root with 3rd-order, (c) apical (basal) section of L1-root with 1st-order, (d) apical (basal) section of L2-root with 1st-order of ‘Bluescrop’ seedlings, (e, f) apical and basal section of P-root with 1st-order of ‘Bluescrop’ seedlings, (g, h) apical (basal) section of L2-root with 1st-order of ‘Tifblue’ seedlings. Arrowheads, asterisks, and arrows indicate exodermis, endodermis, and one pole of protoxylem, respectively.
Fig. 2. Frequency of individual roots with different protoxylem groups (PGs) among each of the branching hierarchies (n = 6 in seedlings of ‘Bluecrop’ and ‘Tifblue’). The branching hierarchies are defined as follows: P as primary roots, L1 as lateral roots of P, and L2 as lateral roots of L1. Significant difference of PG composition is detected only between P and L2 with pooled data regarding to parental cultivars (P < 0.05).

Fig. 3. Anatomical development in the roots of a ‘Bluecrop’ seedling. Letters near each root tip indicate their branching hierarchies (P as primary roots, L1 as lateral roots of P). Each two images of transverse sections are derived from the following portions: (a) 0–2 mm (the apex of the 1st-order), (b) 6–8 mm (the base of the 1st-order), and (c) 12–14 mm (the most basal portion of root) from the apex in the P-root with 2nd-order, (d) 0–0.8 mm (the apex of root) and (e) 1.6–2.4 mm (the base of the root) from the apex in the L1-root with 1st-order. The upper or right sections were stained with 0.1% (w/v) berberine hydrochloride solution and counterstained with 0.5% (w/v) safranin O dissolved in 50% ethanol solution. Lower case letters indicate the following structures: en, Casparian band of endodermis; ex, Casparian band of exodermis; m, metaxylem; p, protoxylem; s, secondary xylem.
In the P-root, the metaxylem also started to develop. On the most basal sections of P-root, metaxylem was surrounded by a secondary xylem (Fig. 3c).

The exodermis is widely seen in angiosperm roots (e.g. Perumalla et al. 1990), the endodermis matures within a few millimeters of the root tip whereas the exodermis matures several centimeters from the tip in most species (Enstone et al. 2003). However, we observed the development of exodermis preceded that of endodermis (Figs. 1, 3a, b, d, e). Allaway and Ashfold (1996) reported that Lysinema ciliatum R. Br. (Ericaceae) hair roots possessed both an exodermis and endodermis, and the exodermis had more developed walls than the endodermis. We also confirmed that the spread of exodermal fluorescence in all the apical sections was 4-degrees, namely they covered almost all of the hypodermal cells and the passage cells were hardly observed (Figs. 1, 3). On the other hand, the degrees of spread for endodermal fluorescence varied and were comparatively low; those of diarch and monarch roots were 2.5 ± 0.3 (mean ± SE) and 0.2 ± 0.2 in ‘Bluecrop’ seedlings, 1.7 ± 0.3 and 1.0 ± 0 in ‘Tifblue’ seedlings. Previously, we also found and illustrated that the exodermal fluorescence was stronger than the endodermis (Baba et al. 2018). The precocity and intensification will mean that the exodermis is more important functional roles than the endodermis in Ericaceae. In particular, mature exodermis resists the penetration of pathogenic and mycorrhizal fungal hyphae (Meyer and Peterson 2013). As ericoid mycorrhizal fungi are ubiquitous and dominant symbiotic partners of ericoid plants, they are mainly constrained within epidermal cells even though they maintain saprotrophic capacity (e.g., Read 1996, Martino et al. 2018). Thus, it is possible that ericaceous exodermis works as an essential barrier to impede hyphal invasion of thin endodermis and steles.

**Heterorhizic specificity in blueberry seedlings**

The root systems of blueberry seedlings consisted of only diarch and monarch roots (Fig. 2). All the P-roots were diarch while monarch roots appeared as branching hierarchies progressed. As a result, the PG compositions of P- and L2-roots in pooled data were significantly different. The measured root traits except the cortex layer number, were significantly larger in diarch roots than in monarch roots (Table 1). Both the tapering pattern of PGs with progress of branching hierarchies and the characteristic differences between PGs are broadly similar with the root system of the cuttings and with several studies investigating primary root systems of tree species (Baba et al. 2018, Bogar and Smith 1965, Bonacorsi and Seago 2016, Eissenstat and Achor 1999, Hayward and Long 1942). Therefore, it seems that individual roots with larger PGs produce roots with same or smaller PGs in branching architectures of many woody species, including blueberry seedlings.

The rabbiteye blueberry cuttings and adult bushes of ‘Bluecrop’ and ‘Tifblue’ possessed framework individual roots with triarch and larger PGs, and those roots tended to progress to secondary growth, while their diarch and monarch roots hardly developed a secondary xylem (Baba et al. 2018, Baba et al. unpublished data). However, only diarch and monarch roots were detected and secondary growth of diarch roots was frequently observed in seedlings of this study (Fig. 2, Table 1). This is an important feature in the root system of blueberry seedlings, which suggests the following two points: (1) diarch roots are distinctively adaptable to the construction of young primary root systems, especially as frameworks, and (2) larger PG roots will appear during plant growth like in Betula papyrifera Marsh. (Horsley and Wilson 1971).

Comparing our present and former studies, the diarch roots of seedlings possess the smaller diameter and thinner tissues (i.e. epidermis, cortex, and steles) than the triarch and larger PGs of cuttings, although the diarch roots of seedling were slightly thicker than those of cuttings and maintained the ability to develop secondary xylems (Table 1, Baba et al. 2018). These morphological features of diarch indicate its benefit to young blueberry seedlings that have to rapidly expand their root systems under substantial resource limitation. One of the most possible advantages of diarch is smaller costs of construction and maintenance than the larger PGs. Because of the reduced tissues, required costs for root construction should be smaller for diarch than for the larger PGs. The reduction of extra-layers of cortex other than exo- and endodermis in diarch roots may economize even metabolic costs of seedling root systems. The thinner steles and simple primary xylem structures, which probably serve enough water transport ability, may also cooperate on such economization. Furthermore, the thinner epidermal and cortical layers will indirectly support the construction of root systems by making it easier for their laterals to emerge from steles. These aspects of diarch roots are expected comprehensively to minimize carbon investments required for root system expansion in blueberry seedlings. From another viewpoint, the smaller diameter and more simple structures of diarch roots will reinforce absorption efficiency via increase specific root length and specific root surface area (Comas et al. 2013, Eissenstat and Achor...
Thereby, the advantages of diarch in blueberry seedlings are thought to be related with optimization of absorption capacity. In contrast, absorptive fine roots with smaller diameters and lower root-orders have high respiration rates (Jia et al. 2013, Makita et al. 2009). Due to the dominance of such diarch roots (Fig. 2; Table 1), whole root system respiration rates and metabolic activity of young blueberry seedlings may tend to be excess. The rapid and high frequency of secondary growth and loss of the outermost layers (Fig. 1 b, f, Table 1) is not related only with root-system development itself but possibly also with suppress excess increment of absorption capacity as a risk of metabolic activity.

In conclusion, we found that the young seedlings of blueberries employ diarch roots as the main components of their fine root systems. Our results hint strategy of blueberry seedlings, under the size and resource limitations, to harmonize functions of absorption and construction on the diarch roots. During ontogeny of blueberries, this strategy will change to another one that different individual roots specialize in each function by the appearances of large framework roots with triarch and larger PGs. In the future, to understand significance of the heterorhizic dynamics within lifecycle of blueberry root systems, it should be elucidated how triarch and larger PGs appear with root system development in relation to other ontogenetic changes. In addition, the relationship between PGs and more physiological traits such as specific root length or respiration rate should be investigated not only in blueberries but also in various species.

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### Table 1. Traits of each protoxylem group (PG) root in blueberry seedlings with different parental cultivars

| Trait                  | ‘Bluecrop’ | ‘Tifblue’ | Significance of difference between PGs<sup>a</sup> |
|------------------------|------------|-----------|--------------------------------------------------|
|                        | Diarch     | Monarch   | Diarch | Monarch |                                     |
| Root number<sup>b</sup> | 13         | 5         | 16     | 2        |                                     |
| Root length (mm)       | 10.8±2.6   | 1.0±0.4   | 6.2±1.6 | 1.4±0.4  | *                                  |
| Branching number       | 9.9±2.7    | 0±0       | 7.1±2.1 | 0±0      | *                                  |
| Maximum root order     | 2.15±0.30  | 1±0       | 2.19±0.25 | 1±0     | *                                  |
| Root diameter (μm)     | 135.4±7.5  | 84.4±6.0  | 140.2±7.2 | 87.2±5.9 * |
| Epidermis thickness (μm)<sup>c</sup> | 29.0±1.6 | 19.9±2.1 | 29.4±1.6 | 23.8±4.0 | *                                  |
| Cortex layer number    | 2.15±0.10  | 2±0       | 2.13±0.09 | 2±0     | NS                                  |
| Cortex thickness (μm)  | 28.2±2.0   | 16.3±2.7  | 27.3±2.0 | 14.7±1.2 | *                                  |
| Stele diameter (μm)    | 39.4±2.8   | 23.0±2.0  | 47.4±2.9 | 19.6±1.8 | *                                  |
| PRS (%)<sup>d</sup>    | 61.5       | 0         | 50.0    | 0        | *                                  |

<sup>a</sup>Pooled data regarding to parental cultivars were used for statistics. Asterisks or NS show significant or nonsignificant differences between PGs by the permuted Brunner-Munzel test except for PRS or Fisher’s exact test in PRS (n depends on each sampled root number. P < 0.05).

<sup>b</sup>Number of sampled roots.

<sup>c</sup>Replications of diarch and monarch roots in ‘Bluecrop’ are 11 and 4, respectively, because epidermal cells of two diarch and one monarch root were completely sloughed off.

<sup>d</sup>Percent of roots exhibiting secondary growth.

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