RESEARCH PAPER

# Comparative branching order and root anatomy of clonal and seed-grown avocado trees (*Persea americana* Mill.).

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## Abstract

**C. Fassio, R. Cautin, A. G. Perez-Donoso, M. Castro, and C. Bonomelli. Comparative branching order and root anatomy of clonal and seed-grown avocado trees (*Persea americana* Mill.). Int. J. Agric. Nat. Resour. 134-144.** Characterizing roots according to their branching order and anatomy is a useful approach for identifying functional differences within and among root systems. In this study, the root branching order and the anatomy of each root order (stele and cortex area) were examined in two-year-old "Duke 7" avocado (*Persea americana* Mill.) trees propagated by seed and by clonal techniques. The root systems were found to have three different root orders that exhibited differences in the occurrence of secondary xylem. Fine roots (first- and second-order roots) presented only primary growth, whereas the main roots (third-order roots) exhibited secondary growth. Transverse sections of roots from the different orders showed pentarch, hexarch or heptarch tracheal element distributions. Newly emerged, long, unbranched pioneer roots were observed only in the clonal trees and showed particular anatomical features, such as a larger diameter and a proportionally greater cortex area than other roots, as well as primary growth. Additionally, significant differences were found between clonal and seedling trees in the stele area of third-order roots; clonal propagation resulted in larger stele areas in this type of root. Our results suggest that propagation methods influence the presence of new pioneer roots and the anatomy of third-order roots; clonal root systems branch more extensively than seed-grown root systems and develop a vascular system with a larger transport capacity.

**Key words**: Branching order, cortex, propagation technique, root anatomy, stele.

## Introduction

Recent research in avocado (*Persea americana* Mill.) has shown that propagation techniques modify root morphological traits and biomass allocation

patterns in avocado (Fassio *et al*., 2016). The root system of clonal avocado trees shows the typical morphology of rooted cuttings, with a crown of roots originating from a relatively short stem, resulting in a shallow root system with more fine roots (first- and second-order roots) and increased root length density. In contrast, seedling rootstocks have a main tap root and lateral roots with nar-

rower angles that penetrate more deeply into the soil. Studies of many cultivated plants suggest the importance of researching the anatomical traits of different root system components in order to improve the understanding of their functions in terms of nutrient uptake and transport (Du & Wei, 2018). These characteristics are currently receiving increased attention as selection criteria for plant breeding due to their crucial role in improving plant performance (Wissuwa *et al*., 2016; Carvalho & Foulkes, 2018).

Water absorption and transport to above-ground tissues are possible because perennial plants are able to develop a variety of root types. This process, which is termed heterorhizy (Hishi, 2007; Baba *et al.,* 2018), enables the specialization of roots to perform specific biological functions (Bagniewska-Zadworna *et al*., 2014). Primary roots may be classified into short and thin roots (usually up to 1 mm in diameter), which are called fibrous, fine or feeder roots, and long, fast-growing pioneer roots, which form the framework of the root system and are also called coarse, framework or skeleton roots (Lyford, 1980; Sutton & Tinus, 1983; Zadworny & Einssestat, 2011). Phenotypic plasticity allows root systems to react quickly and adapt to changing environmental conditions (Hodge, 2009).

Recent studies have shown that fine roots, or lower-order roots, are characterized by the presence of a living parenchymatous cortex and commonly have a smaller diameter and length (Pregitzer *et al*., 2002; Wang *et al.,* 2019), higher specific root area and specific root length (Wang *et al*., 2006), lower stele to root diameter ratio (Gu *et al*., 2014), higher mycorrhizal colonization rate (White *et al*., 2013; Chen *et al.,* 2016), higher N concentration (Pregitzer *et al*., 2002; Bowsher *et al.,* 2016), lower C (also cellulose) concentration (Guo *et al*., 2004), and greater absorption ability (Rewald *et al*., 2011; McCormack *et al*., 2015) than other root types. For many species, the major anatomical difference between branching orders is the presence or absence of secondary xylem, which is tightly linked to the root absorption and transport functions (Esau,

1977; Eissenstat & Anchor, 1999; Hishi, 2007). Fine roots that grow at the distal position of the root system show primary growth mainly in the first three orders of roots (especially first-order roots), while second- and third-order roots can show both primary and secondary development (Guo *et al.,* 2008).

Like the branches of a tree, higher-order or pioneer roots primarily perform support, transport, and storage functions, and these roots have relatively long average life expectancies (Pregitzer *et al*., 2002). In the first stage of development, pioneer roots undergo primary growth; they have a higher initial growth rate and a larger diameter than the small absorptive roots and exhibit extended elongation before branching (Polverigiani *et al*., 2011). There is no clearly defined age at which those roots initiate secondary growth. The acquisition of transport capacity in pioneer roots is facilitated by the initiation of secondary growth and the formation of tracheal elements that are larger and more numerous than those found in fine roots (Bagniewska-Zadworna *et al*., 2014). Furthermore, these root branching orders exhibit stele development and lignification, cortical cell suberization, and exodermal cork layer accumulation (Hishi, 2007). Stele development inhibits nutrient and water uptake and fungal symbioses but improves transport and stress tolerance, indicating a transition from absorptive to structural functions (Comas & Eissenstat, 2009).

In woody plants, pioneer roots form the framework of the root system (Sutton & Tinus, 1983; Bagniewska-Zadworna *et al*., 2012) and act as the progenitors of fibrous root branches in an expanding root system. Newly emerged pioneer roots are designed to live longer at the expense of their absorptive capacity, as indicated by their more pronounced hypodermis with fewer passage cells, more numerous protoxylem poles and lower colonization by mycorrhizal and nonmycorrhizal fungi than those of fine roots. Despite the very different functions and the importance of pioneer roots, there has been limited research, especially

in fruit crops, into the morphological traits and functions of this type of root.

The objective of this research was to characterize the root systems of trees from the same genotype propagated by different methods, sexual (seedling) and asexual (clonal), and to determine the differences in root orders and root anatomy under the different propagation methods.

## Materials and Methods

### Site and plant material

The study was carried out at the experimental station of Pontificia Universidad Católica de Valparaiso in the province of Quillota (32° 50 S; 71° 13 W), Valparaíso Region, Chile. The soil type was loamy clay of lacustrine origin and moderate depth. The plant material for Duke 7 clonal trees was hardwood cuttings 10 cm long and 0.5 cm in diameter. These cuttings were taken from mature shoots from stock trees, which were maintained under commercial management practices for fertility, irrigation and pest control in the avocado germplasm bank at the experimental station. Additionally, seeds were collected from uniform fruits at the physiological maturity stage from the same stock plants. Duke 7 cuttings and seeds were both collected in late winter from the same healthy and vigorous mother tree (10 years old). The plants were propagated in a climatized and shaded greenhouse at 25 to 30 °C, 50 to 100% RH, and an average photosynthetic photon flux (PPF) at midday of 200 and 250 $\mu$mol m$^{-2}$ s$^{-1}$.

### Propagation treatments

Clonal propagation was performed using the Brokaw method based on the double grafting technique described by Frolich and Platt (1971). Vigorous one-month-old seedlings of *P. americana* 'Esther' were used as a temporary nurse-root system and were apically wedge-grafted with *P. americana*

'Duke 7' budwood (clone). A single, stronger bud was retained when the 'Duke 7' graft began to grow. The plants were transferred to a dark growing chamber at 25–30 °C when they reached 2 cm height. When the etiolated shoots reached a height of 8 to 10 cm, they were removed from the dark conditions and drenched with rooting hormone IBA (1600 ppm), and a metal ring was clamped near the base of the shoot. Nutrients were provided by incorporating 15N–4.05P–9.96K controlled-release fertilizer (Osmocote Plus, 8–9-month slow release) into 2.5 kg m$^{-3}$ of substrate (Scotts-Sierra Horticultural Products, Marysville, OH). For seedling propagation, 'Duke 7' seeds were placed in 1 L polyethylene bags. Sphagnum peat-based substrate (Sunshine Mix 1; Sun Gro Horticulture, Bellevue, WA) was used for both propagation techniques, and after seven months of growth, the trees were planted in an open field at a spacing of 3×3 m, where they grew for one year.

### Treatment and experimental design

Nine nursery trees from each propagation method ('Duke 7' clonal and seedling plants) were planted in the field at a spacing of 3×3 m. After one year, they were completely harvested to characterize their root systems. During the growth year, the plants were watered and fertilized through Microjet (20 L h$^{-1}$) sprinklers with a Hoagland and Arnon nutrient solution (Hoagland & Arnon, 1938) modified according to Maas & Poss (1989) to provide optimal nutrient availability conditions. This modified solution was prepared by adding 1 mL-L of a micronutrient solution containing 46.3 mM $H_3BO_3$, 9.10 mM $MnCl_2$ 4 $H_2O$, 0.77 mM $ZnSO_4$ 7 $H_2O$, 0.32 mM $CuSO_4$ 5 $H_2O$ and 0.12 mV $NaMoO_4$ 2 $H_2O$ to a macronutrient solution consisting of 1 mM $NH_4H_2PO_4$, 6.0 mM $KNO_3$, 5.8 mM $Ca(NO_3)_2$, 4$H_2O$, 2.0 mM $MgSO_4$ 7 $H_2O$, and 0.4 mM EDTA monosodium salt. A Diviner 2000 frequency domain reflectometry (FDR) probe (Sentek PTY, Australia) was used to determine the timing and amount of irrigation to maintain

the soil water content at close to field capacity (25 mm at 30 cm of soil depth).

One year after planting, the trees were extracted with the whole-tree excavation method for the entire root system as described by Atkinson (2000). Soil blocks (1 m × 1 m × 1 m) were excavated at a distance of 0.5 m from each tree. Each plant was completely harvested, and every whole root system was placed in containers (100 L) and soaked in tap water for 12 hours. Fifteen roots of each order per tree were sampled to characterize their anatomical traits (diameters of the stele and cortex). The effects of the propagation system on the root traits of each root order were tested by one-way factorial analysis of variance (ANOVA), and Fisher's LSD test (P=0.05) was used to identify differences in the means of the diameters of the stele and cortex between propagation systems. All statistical analyses were performed using the statistics program R (version 3.2.5) (R Foundation for Statistical Computing, Vienna, Austria).

*Root morphology and anatomy*

Before dissection, root segments were rinsed and cleaned with deionized (DI) water at 4 °C, and the root components were grouped according to their hierarchical order (first-, second- or third-order roots) using the approach developed by Valenzuela-Estrada *et al*. (2008). For each treatment, the number, diameter and length of the first- and second-order roots were scanned and analyzed in WinRhizo Pro version 2007d (Regent Instruments, QC, Canada), and the third-order roots were measured using a digital caliper (Mitutoyo Absolut Digimatic, Japan). Once the roots in the root systems of each treatment were classified and measured, the roots were dissected and maintained in an FAA solution (10 formalin: 5 glacial acetic acid: 50 ethanol). Histological cuts were performed at the Botany Laboratory of Pontificia Universidad Católica de Chile. The protocol for preparing the samples for microscopy consisted of progressive alcohol dehydration (50%,

70%, 95% and 100% for 30 minutes each). The tissue was then embedded in water-soluble wax, and a 5-µm-thick section was cut with a rotary microtome (Spencer 820 microtome, American Optical Co, Buffalo, NY, USA). The sections were stained with Safranin and Fast Green.

The samples were observed using an optical Olympus Vanox BX40 compound microscope attached to a computer and a camera (Lumix Panasonic, model DMC-ZS25). For each histological preparation, photographs were taken with a 10x target at a resolution of 640 x 480 pixels, and the images were analyzed using Scion Image for Windows Beta 4.02 (Scion Corporation, Frederick, MA, USA) to determine the area occupied by the stele and cortex. The stele is the vascular cylinder in the center of the root where the xylem and phloem are found, and the cortex is the area of parenchymatic tissue that surrounds the stele and is between the endodermis (the internal part in contact with the stele) and the epidermis.

## Results

In the first year after planting, the avocado root branch network in the clonal trees (Figure 1A) consisted of four root orders as classified by the morphometric approach of Valenzuela-Estrada *et al*. (2008). Third-order roots were the main framework roots that branched into secondary roots (second-order roots) and then into first-order (lateral) roots of different lengths and thicknesses (Table 1). White feeder roots (first- and second-order roots) were the most important components of the clonal root system. A different kind of unbranched root with a large diameter emerged from the main axis of the root system (etiolated stem); this kind of root was similar to pioneer roots reported in other species.

The root system of the seedlings was composed of a long main root (tap root) that divided into secondary branches (third-order roots) and then into second- and first-order (lateral) roots that were similar in length and thickness to those in the clonal root system (Figure 1B).
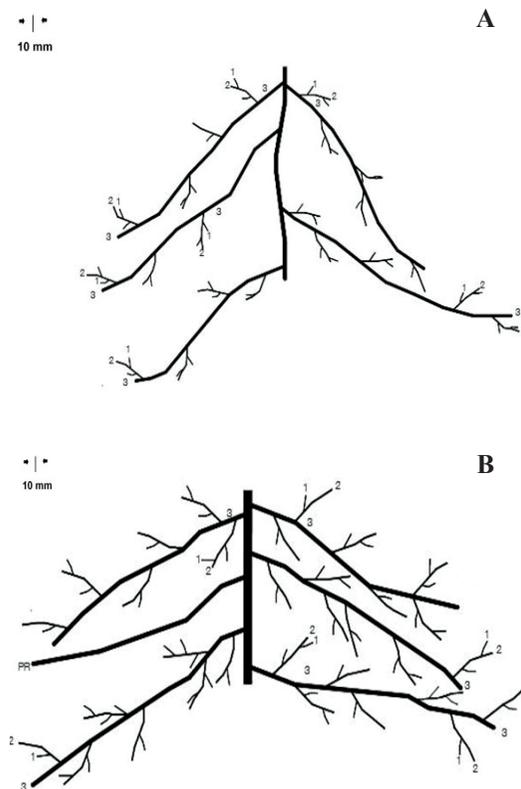
**Figure 1.** Schematic diagram of root branching order in *P. americana* 'Duke 7' trees. A. Clonal trees: (1) first-order roots, (2) second-order roots, (3) third-order roots or main roots and (4) pioneer roots (PR). B. Seedling trees: (1) First-order roots, (2) second-order roots and (3) third-order roots or main roots.

**Table 1.** Morphological characteristics of roots from different branching orders in clonal and seed propagated trees of *P. americana* 'Duke 7'.

| Hierarchical root order | Propagation technique | |
|---|---|---|
| | Clonal plants | Seedlings |
| First-order | | |
| Length (cm) | 3.0 ± 0.2 a | 3.0 ± 0.1 a |
| Thickness (mm) | 1.1 ± 1.3 a | 1.2 ± 0.4 a |
| Second-order | | |
| Length (cm) | 10.5 ± 1.5 a | 10.6 ± 0.8 a |
| Thickness (mm) | 2.3 ± 1.1 a | 2.1 ± 0.9 a |
| Third-order | | |
| Length (cm) | 60.6 ± 2.6 a | 70.4 ± 1.9 a |
| Thickness (mm) | 8.2 ± 0.7 a | 7.2 ± 0.4 a |
| Pioneer | | |
| Length (cm) | 30.3 ± 1.4 | |
| Thickness (mm) | 7.1 ± 1.8 | |
| Tap | | |
| Length (cm) | | 60.4 ± 2.5 |
| Thickness (m) | | 12.5 ± 2.9 |

The data represent the mean ± standard deviation. Different letters indicate significant differences between columns.

The anatomical analysis of the roots from both clonal and seedling rootstocks showed the presence of a cortex formed by parenchymatic cells, but as the root diameter increased and secondary growth was observed, the cortex made up a smaller proportion of the root.
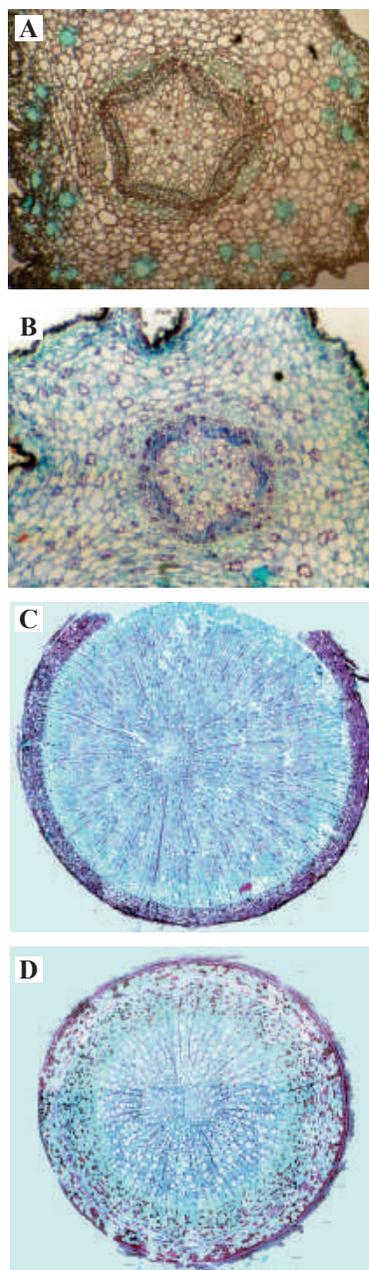


**Figure 2.** Cross-sections (10X) of a first-order root (a), a second-order root (b), and a third-order root (c) of a *P. americana* 'Duke 7' clonal tree; third-order root of a seedling tree (d).

The first- and second-order fine roots in both root systems presented only primary growth, and a higher percentage of their area was occupied by the cortex (greater than 85%) compared with that in the third-order roots. In the stele of these roots, the initial development of a vascular cylinder composed of protoxylem and protophloem was observed (Figure 2). According to the number of protoxylem poles, the roots generally showed pentarch, hexarch, heptarch, and octarch patterns in their steles.

As the roots increased in diameter, the proportion of the roots made up of the stele increased. Third-order main roots exhibited secondary growth with completely developed vascular tissue, and the periderm formed in the external layers of the pericycle. The cortex was completely displaced, and its proportion of the total area was minimal. Lignification and an increase in the amount of epidermis were also clearly visible.

The statistical analysis of the stele area of the first-, second- and third-order roots from all treatments revealed that the stele area in third-order roots was affected by the propagation method; larger stele areas were observed in clonal trees than in seedling trees (Figure 3).

Another distinctive feature of the clonal root system was the presence of long pioneer roots that developed from the main stem. Anatomically, this type of root had a larger diameter than the other roots and showed primary growth with a large proportion of the area occupied by the cortex (greater than 80%) (Figure 4). The epidermis tended to be thinner and not lignified, and a hypodermis composed of two layers of cells was found below the epidermis. The cortex was larger than the stele and appeared as a group of unspecialized parenchymatic cells. The cortex was found to be limited by an endodermis in the form of a single layer of compact cells separating the cortex from the central cylinder. The vascular tissue appeared inside the stele as seven poles of xylem vessels (heptarch pattern), and red-stained particles that could correspond to storage substances, such as tannins and starch granules, were observed in the cortex (Figure 5).
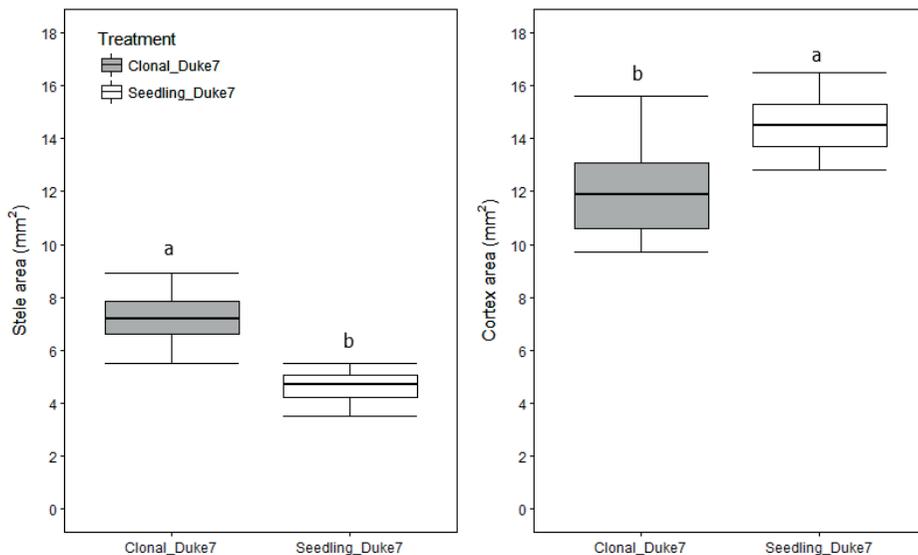


**Figure 3.** Boxplot of the mean stele and cortical area (mm²) of the third-order roots of *P. americana 'Duke 7'* clonal and seedling trees. Different lowercase letters within the clusters of bars indicate significant differences (P<0.05) in root traits between the propagation systems according to Fisher's LSD test.
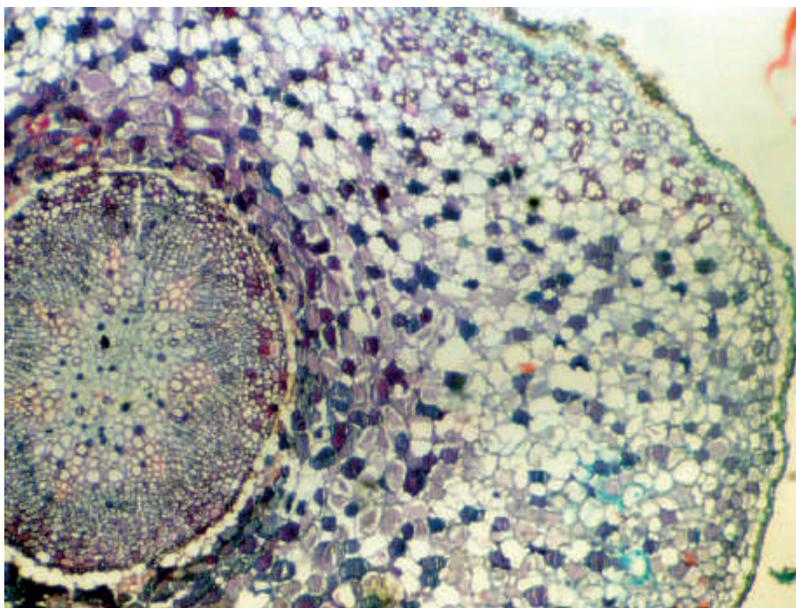
**Figure 4.** Cross-section of a pioneer root of a clonal avocado plant (10X).
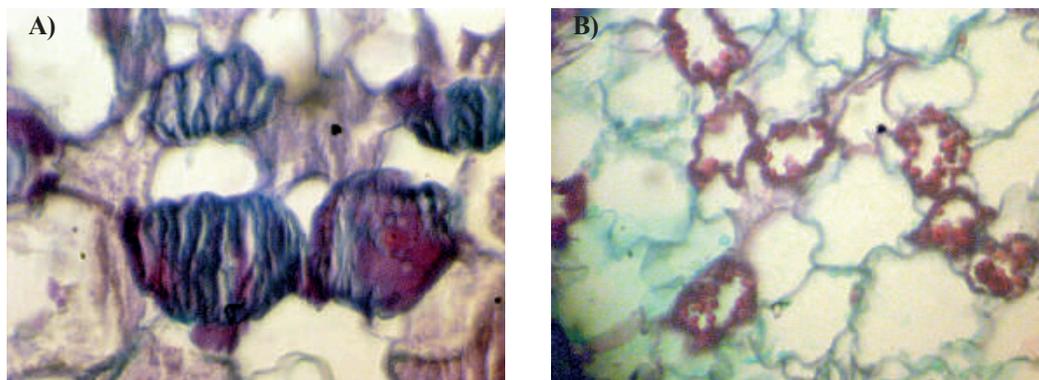


**Figure 5.** Cross-section of a pioneer root from a clonal avocado plant showing storage substances including tannins (a) and starch granules (b) (40X).

## Discussions

The different root orders present in the root system of avocado plants differed anatomically.

The first- and second-order roots in the clonal and seedling root systems exhibited primary growth. Their root areas are mainly occupied by cortical tissue, which is closely associated with nutrient and water absorption from the soil (Pregitzer, 2002; Guo *et al.,* 2008; Wang *et al.,*

2019). Similar results were found by Baba *et al*., 2019, in the fine roots of blueberry seedlings and cuttings.

The third-order roots showed secondary growth, with greater development of the vascular cylinder (stele) and lignification. In woody plants, secondary growth is an important factor in increasing transport capacity, and it implies the transition from the absorptive function to effective xylem transport. Therefore, traits related to the transport

function (stele diameter and xylem area) increased with increasing root order.

The differences between propagation systems observed in the stele area of third-order roots, which develop near the principal axis of each root system (i.e., a tap root system in seedling propagation and an adventitious system in clonal propagation), may have arisen because the roots originate from different tissues. In tap roots (derived from an embryonic radicle), lateral roots normally originate from the poorly developed vascular cylinder of the parental root (Bellini *et al.*, 2014). In the adventitious avocado roots that develop under clonal propagation, roots emerge from an etiolated stem and are induced to grow artificially by wounding and hormone application. According to Estay *et al.* (2016), clonal avocado rootstocks develop new roots from the abundant phloem parenchyma surrounding well-defined and isolated groups of phloem fibers in which secondary growth has been initiated.

Moreover, studies of the phenotypic plasticity of grass root anatomy in response to light intensity found that shading by up to 30% of normal daylight led to a higher stele proportion, larger stelar cells and larger xylem vessels in the root due to an increased need for high transport capacity in the shade, where leaf area is larger (Wahl *et al.*, 2001). Furthermore, previous researchers have found that a high stele cross-sectional area causes high root axial conductivity (Wang *et al.*, 2006; Fassio *et al.*, 2009; Phoura *et al.,* 2020). In this study, seedling trees were exposed to sunlight during the last step of propagation in the nursery, but clonal trees grew in an etiolation area (dark room) for one month and in a shaded glasshouse area to avoid light stress. Therefore, this condition may have resulted in the larger stele areas in the clonal trees than in the seedling trees.

Previous studies on temperate hardwood species (Bengough, 2003; Wang *et al.*, 2006) have found

that the cortical cell diameter and tissue density of roots increase so that the roots can more easily penetrate deeper soil layers, which have higher mechanical impedance. It is important to highlight that the root systems of seedling trees tend to extend to greater depths than those of clonal trees, so their anatomical structure is probably adapted to this condition.

The presence of new pioneer roots emerging from the root system and their anatomical characteristics were distinctive to the clonal root system and allowed those plants to expand their root growth. Furthermore, the presence of tannins and starch granules in those roots could be involved in defense against pathogens such as *Phytophthora cinnamomi* (Cahill & Mccomb, 1992) and others. According to Zadworny & Einsenstat (2011), pioneer roots are constructed to defend against biotic and abiotic challenges and to explore the soil distal to the stem. However, no descriptive information is available on this type of root in avocado, and few studies on the anatomy of avocado roots in general have been published. Therefore, continuing this line of investigation will be very important in determining the importance of this type of root and its quantity and appearance frequency in clonal avocado root systems.

Our results suggest that propagation methods influence the presence of special features in avocado root systems. The root systems of clonal plants generate third-order roots with larger stele areas than their seedling counterparts. Moreover, new pioneer roots appear to be generated more frequently in clonal root systems. These pioneer roots have a larger diameter and a proportionally greater cortex area than other roots and exhibit primary growth. These morphological traits allow clonal root systems to have more extensive branching than seedling root systems, which increases the volume of soil available as a source of water and mineral nutrients and results in a vascular system with a larger transport capacity.

**Resumen**

**C. Fassio, R. Cautin, A.G. Perez-Donoso, M. Castro, y C. Bonomelli. Análisis comparativo del orden de ramificación y la anatomía de raíces de paltos (*Persea americana* Mill.) propagados en forma clonal y por semilla. Int. J. Agric. Nat. Resour. 134-144.** La caracterización de raíces de acuerdo con su orden de ramificación y la anatomía es una herramienta útil para identificar diferencias funcionales entre y dentro de un sistema radicular. En este estudio el orden de ramificación y la anatomía de cada orden de raíces (área de estela y corteza) fueron determinados en plantas de 2 años de la variedad Duke 7 propagadas mediante las técnicas de propagación clonal y a través de semillas. Se encontraron raíces de tres diferentes órdenes, las que presentaron diferencias en términos de la presencia de xilema secundario. Las raíces finas de primer y segundo orden presentaron solo crecimiento primario mientras que las raíces principales de tercer orden exhibieron crecimiento secundario. Secciones transversales de las raíces mostraron una distribución de los elementos del xilema: pentarca, hexarca y heptarca. Nuevas raíces pioneras emergidas, estaban presentes sólo en las plantas clonales y presentaron características particulares de su anatomía tales como diámetro grande, crecimiento primario y una mayor proporción de área de corteza que de estela. Adicionalmente, se encontraron diferencias significativas entre el área de estela de las raíces de tercer orden entre plantas propagadas en forma clonal y por semilla. Lo cual revela que la propagación clonal genera áreas de estela más grandes en este tipo de raíces. Estos resultados sugieren que el método de propagación ejerce un importante efecto en la presencia de nuevas raíces pioneras y en la anatomía de raíces de tercer orden, que permitirían a los sistemas radicales clonales ramificarse más extensivamente y proveer de un sistema vascular con una mayor capacidad de transporte de agua y nutrientes desde el suelo.

**Palabras clave:** Anatomía de raíces, corteza, estela, orden de ramificación, técnica de propagación.

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