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Early pine root anatomy and primary and lateral root formation are affected by container size: implications in dry-summer climates

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Abstract

Although the presence of root anatomical structures of young *Pinus ponderosa* seedlings grown in containers of contrasting volume (164 vs. 7000 cm^3) was similar, seedlings reared 60 days in the large container had more vascular cambium although the xylem thickness was similar. In addition, seedlings in large containers had nearly twice as many resin ducts within the vascular cambium as their cohorts in small containers. Taproot length closely matched container depth. Though lateral root emission rates were similar between container sizes, large container seedlings had more than 2X the number of lateral roots as those from small containers. These differences in morphophysiological characteristics may be important to seedling establishment on sites that experience dry summer conditions, or for seedlings destined to drier, harsher sites. Further work to elucidate the ramifications of these morphophysiological differences on seedling establishment is warranted.

Keywords

Container; Lateral roots; Meristematic zone; Nursery; Resin duct; Root anatomy; Root collar; Seedling quality; Taproot

Contents

1 Introduction

Around the world, forests have adapted to areas with prolonged summer drought, as occurs, for example, in the Mediterranean Basin and portions of the western United States. Beside the amplification of water stress during the dry season, increased frequency and intensity of wildfire and other disturbances is predicted to negatively affect the geographic distribution, growth, sustainability, biodiversity, and function of forests (Dale et al. 2001; Kurz et al. 2008; Sturrock et al. 2011; Anderegg et al. 2015; Seidl et al. 2017; Pureswaran et al. 2018).

Pinus ponderosa Lawson & C. Lawson provides an example of a species adapted to prolonged summer drought but challenged by changes in climate and disturbance patterns. This pine is a widespread species throughout the western United States (Oliver and Ryker 1990). For much of its range, cold, moist winters are followed by prolonged warm-to-hot summer temperatures and drought during which available soil moisture declines dramatically (Warren et al. 2005). Adapted to this climate, the natural regeneration strategy of *P. ponderosa* focuses on root development. During their first year, taproots of naturally regenerating *P. ponderosa* develop rapidly (up to 5 cm week^{-1}) and can exceed 60 cm the first year, accompanied by vigorous formation (i.e., 75+) of lateral roots (Larson 1963). This rapid root growth provides seedlings access to moisture in the lower soil profiles below the competitive effects of other vegetation, sometimes within a month of germination (Kolb and Robberecht 1996a). Access to water allows transpiration to continue, a necessary function to maintain sublethal stem temperatures in seedlings at the soil surface (Kolb and Robberecht 1996b). Even with such adaptations, natural regeneration success can be low (Keyes et al. 2007).

Fire frequency and intensity are challenging and changing natural regeneration patterns of *P. ponderosa*. Indeed, while *P. ponderosa* has typically regenerated after fire and progeny present more growth on burned than unburned sites (Owen et al. 2020), the spatial magnitude of recent fires, which dramatically increases distances between areas with viable seed sources, reduces natural regeneration potential (Korb et al. 2019). Moreover, changing climate, with warmer temperatures at lower elevations, is shifting lower treelines upward in elevation (Urza et al. 2020), witnessed by more *P. ponderosa* regeneration at higher elevations than lower elevations (Korb et al. 2019). The combination of warmer, drier climate with continued uncharacteristically large and intense fires is projected to decrease post-fire natural regeneration (Rodman et al. 2020).

Given the changes in climate and their synergistic effects on disturbances, the need for forest restoration will continue to increase. Planting is expected to play a significant role (Haase and Davis 2017). In addition to desired forest rehabilitation (e.g., Kolb et al. 2019), planting of forest tree species, including *P. ponderosa*, is likely necessary to maintain adapted populations across the range as climate continues to change (i.e., assisted migration) (Williams and Dumroese 2013; Rehfeld et al. 2014). Successful survival and growth of reforestation seedlings after outplanting will necessitate that seedlings have high morphophysiological quality (Grossnickle and MacDonald 2018), especially where planting environments become harsher (Jacobs et al. 2005; Villar-Salvador et al. 2010, 2012; Oliet and Jacobs 2012). Of the morphological quality attributes, root collar diameter is considered the most important (Wakeley 1935; Ritchie et al. 2010; Grossnickle 2012). Larger root collar

diameter is generally associated with larger seedlings that perform better on dry sites than seedlings of smaller diameter (Andivia et al. 2021); improved growth leads to more resource mobilization (Villar-Salvador et al. 2012) that supports drought stress avoidance (Grossnickle 2005). In particular, larger *P. ponderosa* seedlings can withstand first-season declines in photosynthesis and transpiration as soil moisture availability wanes, and promptly increase critical physiological processes once precipitation events resume in autumn (Pinto et al. 2016).

In container nurseries, larger root collar diameter is generally achieved by growing seedlings in larger containers that can provide more resources to individual plants along with lower production densities that favour radial growth (Landis et al. 1990, 2010). Moreover, larger containers are often longer (i.e., deeper) and considered better suited for taprooted species because the extra rooting depth can enable seedlings to reach soil moisture deeper in the soil profile, especially in Mediterranean-like climates with summer drought (e.g., Chirino et al. 2008; Pinto et al. 2012). In addition, the taproot and shallow first order lateral roots have been shown to be a crucial component for tree stability (Yang et al. 2017; Dumroese et al. 2019; Montagnoli et al. 2020). This stability is the primary function of coarse roots because it contributes to plant survival, whereas instability and low survival are related to the poor architectural characteristics of the root system (Haywood et al. 2012; Yang et al. 2017). Thus, a seedling having a root system with a deeper taproot and a more developed network of first order lateral roots would also have enhance stability.

Although root collar diameter has long been touted as the critical morphological attribute associated with seedling performance, the mechanism has remained elusive. Larger diameter may infer more hydrological tissues that enable seedlings to maintain physiological processes and avoid deleterious effects of cavitation (Kavanagh and Zaerr 1997). Understanding this nexus of morphological and physiological features would inform the production of nursery stock better able to withstand establishment pressures after outplanting, and to the best of our knowledge, the effect of container size upon the anatomical, cytological, and architectural organization of the root system has yet to be examined. Here we report a study using *P. ponderosa* that begins to examine the nexus of root collar diameter, seedling hydrology, and taproot and lateral root development toward a better understanding of how nursery culture may be leveraged to produce seedlings of higher quality.

2 Material and method

The experiment was conducted in a greenhouse (U.S. Department of Agriculture Forest Service, Rocky Mountain Research Station, Moscow, ID, USA; 46.7232°N, 117.0029°W) under natural day-length. In early April we sowed a northern Idaho source of stratified (28 days at 2–3 °C) *P. ponderosa* seeds (Clearwater County, Idaho, USA; 46.5338, -116.1307; 963 m) into containers filled with a peat:vermiculite (1:1, v:v) substrate (Sunshine Special Blend Forestry #1, Sun Gro Horticulture, Bellevue, WA, USA). We grew seedlings in 30 small (Ray Leach Cone-tainer Super Cells (164 cm^3, 3.8 cm top diameter, 21-cm depth; Stuewe and Sons, Inc, Tangent, OR, USA) and 30 large (7000 cm^3, 15 cm top diameter, 50-cm depth) containers. Twelve days after sowing, we noted onset of germination (i.e., cotyledons became visible above

the substrate surface). Containers were thinned to one seedling 9 days after onset of germination (hereafter "DAG"). Seedlings were irrigated as needed when mass of the water in the container reached 75–80% of the total water mass at container capacity (Dumroese et al. 2015). Beginning 28 DAG and continuing once each week for 4 weeks (until 60 DAG), irrigation water contained Peters Professional 20-20-20 General Purpose Fertilizer (N:P_2O_5:K_2O) that delivered 1 mg seedling^{-1} nitrogen each application (Peters Professional, The Scotts Company, Marysville, OH, USA). Containers were rotated about every 14 days. Day:night temperatures averaged 28:16 °C.

A random sample of 10 seedlings for each container size was harvested 14, 28, and 60 DAG. At each harvest we gently rinsed the substrate from the roots and measured taproot length. At 60 DAG and for each seedling, we also measured height and counted the number of first order lateral roots. In addition, we collected a 1-cm-long sample of taproot from three zones that represented different developmental stages: (i) the meristematic zone represented the primary root structure including the root apical meristem and root cap, (ii) the middle zone corresponding to the middle segment of the taproot axis, and (iii) the collar zone that included the root collar). In particular, the middle and collar zones represented the secondary root structure having tissues produced by vascular cambium activity, whereas the collar zone represented a transition zone between the root and the shoot.

Taproot samples were fixed in formalin–acetic acid–alcohol (FAA, 5:5:90 v:v:v), dehydrated in an ethanol series (25%, 50%, 75% and absolute) at 4°C, and embedded in Technovit 7100 (Bio-Optica, Milan, Italy) before cutting. Fixed material was immersed in a progression of Technovit–ethanol solutions (30, 60, 100%; 24 h each). After polymerization we used a rotative microtome (Leica RM 2125RT, Leica Biosystems Nussloch GmbH, Germany) to produce cross-sections with an 8-µm thickness that were then stained for 1 minute with toluidine blue (0.04%) and photographed in wet conditions using an Olympus BX63 light microscope equipped with an Olympus DP72 camera. Images were analysed by ImageJ 1.41o software (Wayne Rasbanb, National Institute of Health, USA) to measure the ray length of the cross section (mm), the thickness of secondary xylem (mm), and the number of vascular cambium cells, medullary rays, and resin ducts.

We compared the container size effect (small and large) for each measured variable using an independent T-test (SPSS 20.0; SPSS Inc., Chicago IL, USA). Differences were considered significant at $p \leq 0.05$.

3 Results

3.1 Tap root length, shoot height, and lateral root number

At the first (14 DAG) and second (28 DAG) sampling points, taproot length was unaffected by container size (Figure 1a, b; Figure 2). At 60 DAG, container size had a significant effect ($p < 0.001$) on taproot length, with seedlings in large containers having longer taproots than seedlings grown in small containers (Figure 1c; Figure 2). At 60 DAG, container size was also significant for shoot height ($p = 0.003$) and lateral root number ($p = 0.006$). Seedlings grown in large containers were nearly twice as tall as their cohorts in small containers (59 mm ± 11 SD vs. 31 mm ± 3 SD). Seedlings from large containers had more than 2X the number of lateral roots as those from small

containers (59 ± 15 SD vs. 26 ± 4 SD), but the rate of lateral root formation per cm of taproot was similar (1.2 ± 0.3 SD vs. 1.3 ± 0.3 SD; p = 0.660).
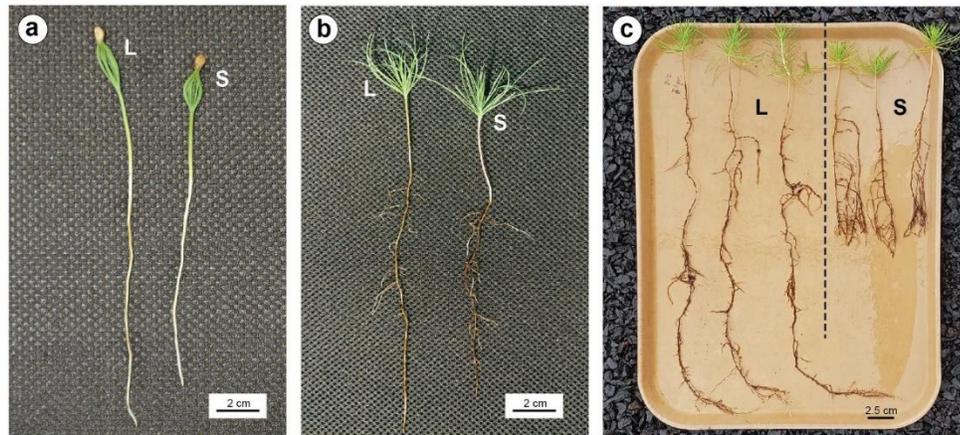
Figure 1. Morphological differences between seedlings of *Pinus ponderosa* grown in small (S; 164 cm^3) and large (L; 7000 cm^3) containers at 14 (a), 28 (b), and 60 (c) days after germination.
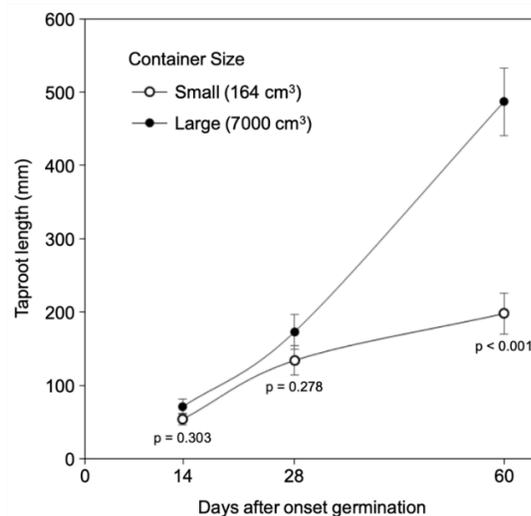

Figure 2. Time-course measurement of taproot length of *Pinus ponderosa* seedlings grown in small (164 cm^3) and large (7000 cm^3) containers at 14, 28, and 60 days after germination (n = 10). Error bars represent standard deviation.

Table 1. Microscopic parameters (means ± SD) obtained 60 days after germination from a cross-section of taproot in the collar zone of *Pinus ponderosa* seedlings (n = 10) grown in small (164 cm^3) and large (7000 cm^3) containers.

Container size	Ray length (mm)	Secondary xylem thickness (mm)	Vascular cambium cells (n)	Medullary rays (n)	Resin ducts (n)
Small	0.66 ± 0.02	0.7 ± 0.05	3.8 ± 0.6	5.5 ± 0.5	6.2 ± 0.8
Large	0.70 ± 0.02	0.8 ± 0.03	8.8 ± 1.0	6.0 ± 0.0	10.0 ± 1.1
p value	0.076	0.052	< 0.001	0.062	< 0.001

3.2 Root anatomical traits

3.2.1 Meristematic zone

Container size had no effect on the root anatomical organization (Figure 3a, b). From the histological perspective, the primary structure was characterized by an actinostele-type configuration with the vascular tissue organization having an alternation of 4 xylem and 4 phloem bundles, all nestled in the pith tissue (Figure 3). The central cylinder was surrounded by the pericycle further surrounded by endodermis. External to the endodermis, the cortex preceded the epidermis (Figure 3).
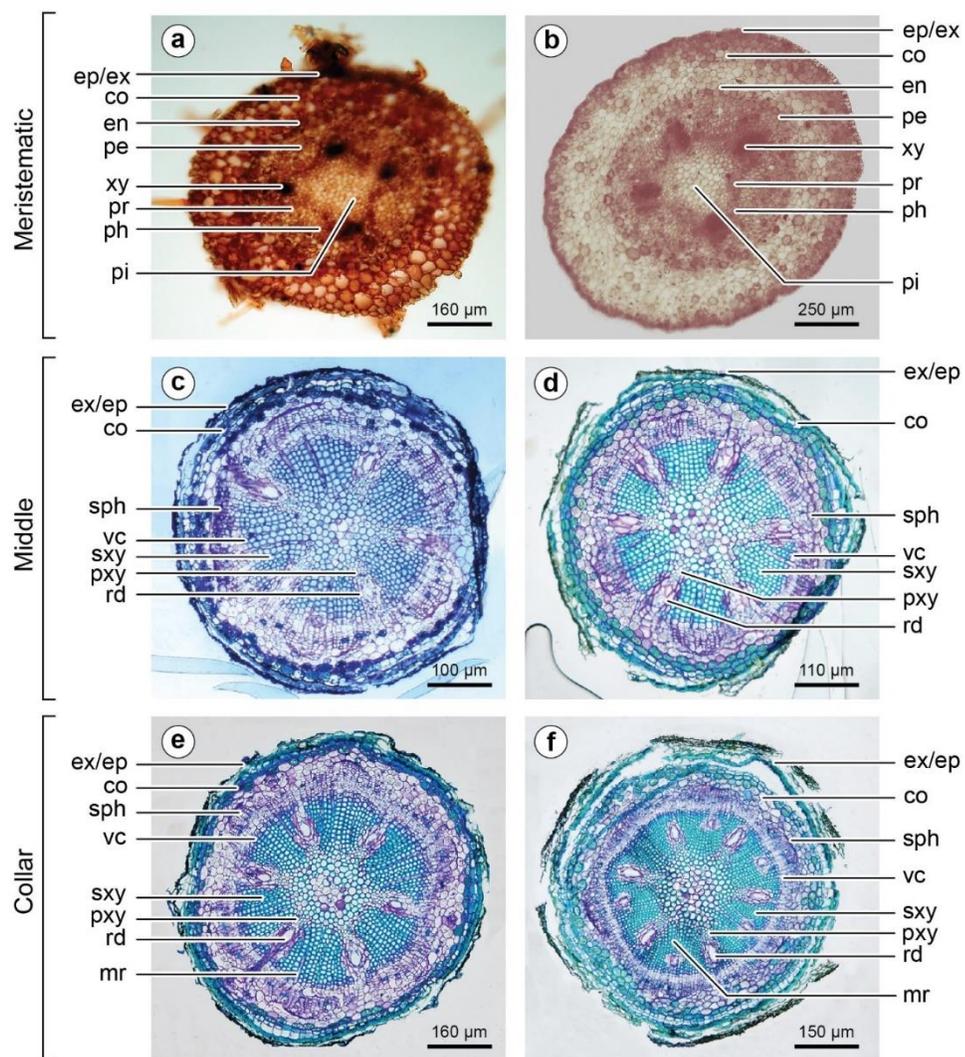

Figure 3. Anatomy of *Pinus ponderosa* seedlings from three taproot zones (meristematic, a, b; middle, c, d; and collar, e, f) grown 60 days after germination in small (a, c, e; 164 cm^3) and large (b, d, f; 7000 cm^3) containers. Abbreviations: co: cortex; en: endodermis; ep/ex: epidermis/exodermis; ex/ep: exodermis/epidermis; mr: medullary ray; pe: pericycle; ph: phloem; pi: pith; pr: procambium; pxy: primary xylem; rd: resin duct; sph: secondary phloem; sxy: secondary xylem; vc: vascular cambium; xy: xylem.

3.2.2 Middle zone

The anatomical organization of secondary structures in seedlings from small and large containers were similar (Figure 3c, d). Regardless of container size, cross-sections presented a thick layer of secondary xylem extending outwardly from the central zone where the old xylem arcs of the primary structure remained visible (Figure 3c, d). This secondary xylem tissue was characterized by medullary rays arising in correspondence to the xylem arcs, with each ray containing a single resin channel. On the edge of the secondary xylem, the vascular cambium formed a single layer of initial cells that separated the secondary xylem from the secondary phloem that extended externally. More peripheral to the secondary phloem, a degenerating cortex tissue and several fragments of the dead epidermis remained visible (Figure 3c, d).

3.2.3 Collar zone

Cross sections revealed anatomical differences between seedlings grown in small (Figure 3e) and large (Figure 3f) containers. Although ray length, the number of medullary rays, and the thickness of the secondary xylem were the same regardless of container size (Figure 3e, f; Table 1), seedlings in large containers had more than twice the number of undifferentiated vascular cambium cells than did seedlings in small containers (Figure 3e, f; Figure 4a, b; Table 1). In addition, the number and position of resin ducts was affected by container size. Resin ducts in seedlings grown in small containers formed within the medullary rays (Figure 3e) and thus the number of ducts was similar to the number of rays (Table 1). Resin ducts also formed within the medullary rays of seedlings grown in large containers, but additional resin ducts formed in the secondary xylem between the medullary rays, resulting in nearly 2X the number of resin ducts observed in seedlings from small containers (Figure 3f; Table 1).
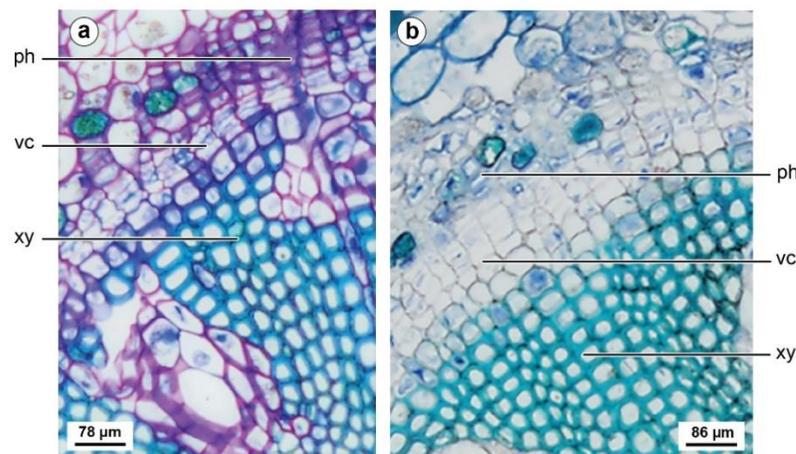

Figure 4. Vascular cambium in the collar zone of *Pinus ponderosa* seedlings grown 60 days after germination in small (a; 164 cm^3) and large (b; 7000 cm^3) containers. Abbreviations: ph: phloem; vc: vascular zone; xy: xylem.

4 Discussion

Despite larger containers being 2.4X deeper and having 40X the volume, we observed no differences in the anatomical organization of seedling tissues; at 60 DAG

the middle and collar zones presented secondary tissues. We did, however, observe increases in the amounts of vascular cambium cells. This may reflect an earlier onset of vascular cambium activity in large containers (data not shown) that led to more of this tissue at 60 DAG, or be associated with an overall higher cambial activity (Montagnoli et al. 2019). Regardless, the thickness of the xylem area was, however, similar among the two container sizes; rays followed the same pattern. Interestingly, the additional cambial activity in seedlings in large containers led to a two-fold greater number of resin ducts compared to seedlings in small containers.

Exogenous and indigenous factors readily influence resin duct formation by the cambium (Werker and Fahn 1969). Resin ducts are present constitutively (i.e., in the absence of any biotic stress) and are induced in response to damage signalling (i.e., traumatic) (Vázquez-González et al. 2019, 2020). Although a large intraspecific genetic variation in resin duct attributes occurs among populations within species (Vázquez-González et al. 2020), a greater number of resin ducts in tree species, including *P. ponderosa* (Kane and Kolb 2010), infers a higher resin flow and a wider anatomical barrier (i.e., more resistance) to entry by insects and diseases (Lombardero et al. 2000; Boucher et al. 2001; Rodriguez-Garcia et al. 2014; Hood and Sala 2015). Abiotic stress that exerts more limiting conditions and reduces carbon accumulation affects resource allocation to this defence (Herms and Mattson 1992; Sampedro et al. 2011; Moreira et al. 2015; Vázquez-González et al. 2019). Indeed, *Pinus nigra* provenances from colder environments produce more and larger radial resin ducts than those from warmer environments (Esteban et al. 2012) and differences in resin duct characteristics of *Picea* were positively correlated with aridity (O'Neill et al. 2002). Evidence suggests that while conditions that improve tree vigor (i.e., increased availability of water, nutrients, and/or light) can increase resin duct number or size, so too can conditions that limit carbon accumulation (Vázquez-González et al. 2020). Eventually, however, limited carbon accumulation as a result of stress can lead to a decrease in resin duct abundance and size (Vázquez-González et al. 2020). Our results suggest that the large containers may present an improved environment for *P. ponderosa* seedling growth, presented as an increase in resin duct number. Given that nursery nutrient supply and irrigation were not confounded, and that irrigation was supplied at non-stressful levels for *P. ponderosa* (Dumroese et al. 2011b), it is unclear what the advantage may be. We assert that seedlings of *P. ponderosa* growing in large containers during the very first growth stage devote more resources toward formation of their defence system than do seedlings in small containers.

Taproot length was, not surprising, a function of container depth. Taproot elongation was arrested at the bottom of the containers by air pruning. Although lateral root initiation is under hormonal (i.e., genetic) control within the main root axis meristem (Sutton 1980 and references therein), nursery practices, such as root pruning can induce emergence of supernumerary lateral roots (Nelson 1989 and references therein). In the short duration of our study, we observed no difference in the number of lateral roots per cm of taproot. This suggests that container size had no immediate effect on emission, despite new laterals appearing on woody plants within a few days of root pruning (Carlson and Larson 1977) and air pruning of the *P. ponderosa* taproot likely occurring in the small (shorter) containers weeks earlier than in the large (taller) containers (Figure 2). Two months after germination, we did observe, however, that first-order lateral roots in small containers, having much less

distance to the container wall than in large containers (1.9 vs. 7.5 cm), were being deflected downward along the interior of the container (Figure 1).

In the United States, typical containers for usual reforestation efforts are usually ≤ 175 cm^3 and 15 to 25 cm deep. Selection of an appropriate container size is a function of the Target Plant Concept (Dumroese et al. 2016), where other factors associated with, for example, site, species, objectives, and costs, are also considered. The literature on forest seedling production in container nurseries is, however, replete with reports that large containers can be used to grow large seedlings. Increasing container size increases production costs (Bowden 1993) and therefore is only justifiable when seedling performance benefits from the additional resource investment (Wenny 1995). In general, on harsher sites with limited available soil moisture, large seedlings are justified because they outperform small seedlings (Andivia et al. 2021). While many seedling quality attributes contribute to this performance, root volume is a critical factor (Ritchie et al. 2010). Container conifer seedlings, in particular, show an increment of root system volume related to the increment of container volume (Carlson and Endean 1976; Romero et al. 1986; Oliet et al. 2009; Pinto et al. 2011; Dumroese et al. 2013) with root biomass correlating positively with plant growth potential (Larsen et al. 1986; Williams et al. 1988). This relationship is present in hardwoods, too (e.g., Aphalo and Rikala 2003; Dumroese et al. 2011a; Mariotti et al. 2015; Sun et al. 2018).

The generally accepted paradigm is that longer taproots enable formation of a deeper root system able to access moisture lower in the soil profile (South et al. 2005; Pemán et al. 2006; Chirino et al. 2008). This hypothesis is supported by observations that *P. ponderosa* seedlings characterized by a shorter root system show water stress symptoms even when a sufficient amount of water remains deeper in the soil profile (Pinto et al. 2012). In addition, *P. ponderosa*, seedlings with longer taproots produced in larger containers showed no advantage on mesic sites in northern Idaho USA compared to those with shorter taproots from smaller containers, but did improve performance on drier, rockier sites (Pinto et al. 2016). In addition, in the presence of high amounts of competition, *P. ponderosa* with longer taproots had improved survival compared to those with shorter taproots (Pinto et al. 2012). Interestingly, the stocktypes used by Pinto et al. (2012, 2016) showed similar rates of photosynthesis and transpiration, and these rates were influenced more by seasonal soil moisture trends rather than seedling root collar diameter, root mass, or container size. During the year of establishment, autumnal resumption of higher levels of photosynthesis as soil moisture increases is key to survival; *P. ponderosa* seedlings with longer initial taproots showed improved physiological status compared to seedlings with shorter taproots (Pinto et al. 2016). In the strongly taproot species *Pinus palustris*, seedlings with air-pruned taproots rapidly generate geotropic roots that appear and function like taproots (South et al. 2001). In *P. ponderosa*, 30-year-old trees established as seedlings with air-pruned taproots developed a dominant taproot (Dumroese et al. 2019), demonstrating the on-going plasticity of root system architecture.

We noted more vascular cambium development in large containers versus small containers. Though xylem thickness was unaffected, resin duct occurrence increased with larger container size, suggesting high plasticity in cell development by the vascular cambium. Although not fully explored by this study, xylem attributes may also be affected by resource (i.e., water, nutrient) supply afforded by rooting volume. Thus, is the accepted paradigm that seedlings with greater stem diameters have

enhanced performance (Wakeley 1935; Ritchie et al. 2010; Grossnickle 2012 and references therein) related to vascular cambium activity? This seems likely given that diameter of individual xylem cells is associated with resistance to cavitation (smaller diameters are more resistant; e.g., Davis et al. 1999; Pittermann and Sperry 2003) and/or abundance of xylem relative to other vascular cambium attributes that could afford additional buffering against cavitation events. More research to improve our understanding of the nexus of overall seedling size, root collar diameter, xylem development (individual cell characteristics as well as overall abundance), and resistance to cavitation is needed, especially as these traits are, or are not, affected by nursery practices.

5 Conclusions

From this study, we conclude that container size exerts an effect on early seedling growth, with large containers yielding seedlings having more vascular tissue within a few weeks of growth than small containers. Although the presence of anatomical structures was similar among container sizes, but more resin ducts were produced in seedlings in large containers. Given that other work with *P. ponderosa* has failed to demonstrate a difference in photosynthesis and transpiration rates among container sizes, and short-term nursery effects are mitigated by long-term plasticity, more work to elucidate the mechanism(s) surrounding seedling anatomy, stem diameter, hydrology, physiological processes toward improving performance of planted seedlings, particularly on sites with dry-summer droughts is required.

6 Acknowledgments

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