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Changing directions: tree hydraulic redistribution from canopy soil

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Abstract

Changing directions: tree hydraulic redistribution from canopy soil

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One of the most important and noticeable components of the canopy environment is the large biomass of epiphytes composed of vascular and non-vascular plants (e.g., mosses, lichens and ferns) which accumulates and decompose over time forming what we know as canopy soils. These arboreal soils are important because they provide habitat and can retain water and nutrients for epiphytes and their associated biota. While it is true that large trees can access water from deep in the ground during dry periods, the physical difficulty of moving water to a high crown is thought to be a major limitation to height in trees. Thus, a reservoir accessible to the crown without fighting gravity may allow stomata to remain open for photosynthesis while reducing cavitation risk and regulating plant temperature to sustain positive carbon balance under dry conditions. A wide range of tree species in temperate and tropical rainforests have the ability to sprout adventitious roots from branches under these water reservoirs; however, water uptake from canopy soil has not been confirmed or measured. This study approach how water from canopy soil can be redistributed to other organs along the tree body by reverse or bi-directional flow mitigating the effects of drought conditions.

To answer this enigma, this research assessed canopy root anatomy, water redistribution and physiological performance of trees with canopy soil under adverse conditions of water availability. Three approaches were used to accomplish these objectives: lab, greenhouse and field experiments. Initially, microscopy anatomical comparisons were made between canopy and terrestrial roots, to ensure that these were functional and could potentially facilitate water absorption from canopy soil. If the canopy roots vascular system is directly connected to the host tree, aboveground water can be then reallocated to other organs of the host tree. In order to assess canopy water uptake and redistribution, greenhouse and field trials were designed, using poplar trees (*Populus trichocarpa*) and applying air-layering technique to develop canopy soil systems and roots. Dye technique, stable isotope (deuterium- δD) and sap flow sensor methods were used to track water movement from aboveground pools to other tree organs. Finally, greenhouse trials were designed to simulate plant water relations (in a simplified system) between canopy soils and host trees under low water availability conditions, to evaluate the advantages and significance of canopy soil, as an extra-water source that could mitigate the effects of drought.

Data show that canopy and terrestrial roots are anatomically similar and present all necessary features to be hydraulically functional. Dye-experiments and sap flow data confirmed that there is water uptake by canopy roots in this poplar system and suggest potential bi-directional water flow (i.e. up- and downwards sap flow) within the tree. This is supported by the isotopic data that confirmed that water from canopy soil is reallocated not only at the canopy level but also could reach belowground pools. Data also suggest that trees with canopy roots can mitigate drought conditions by taking up water from canopy soils and maintain their water and carbon balance for a longer period than plants without them. It is expected that temperatures in the Pacific Northwest will rise, and drought will become more severe. Such conditions are predicted to increase heat stress-related tree mortality in the western US. The role of canopy soils and roots may be crucial to plant biodiversity in critical habitats, and likely account for an important but understudied source of water and nutrients in trees, especially under conditions where trees become heat stressed.

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DEDICATION

To my parents and brother,
to Marcia Baker and
to Gerardo

CHAPTER 1

Introduction

Tree canopies harbor a dynamic ecosystem of diverse species and complex trophic interactions that have been an enigma of the treetops for a long time. The large accumulation and decomposition of organic material such as dead epiphyte detritus and canopy litter form “canopy soils” (Benzing, 1998; Haristoy et al., 2014; Nadkarni et al., 2004; Paulian, 1951; Wolf, 1993) on top of tree branches that promote formation of adventitious “canopy roots” (Hertel & Köhler, 2010; Pérez et al., 2005; Sillett & Bailey, 2003; Vance & Nadkarni, 1992). The development of this extensive root system underneath arboreal soils has been widely reported in many tree species found in temperate and tropical rainforests (Herwitz, 1991; Leary et al., 2004; Nadkarni, 1981; Nadkarni & Primack, 1989; Sillett & Bailey, 2003). Such phenomena have been explained as convergent evolution to increase nutrient uptake of the host trees by providing access to an extra source of nutrients at the canopy level in addition to that found in the soil underground (Moore, 1989; Nadkarni, 1981; Sanford, 1987). Canopy soils are not only considered a significant nutrient source, but also an extensive water store that may be available to the host trees via canopy roots (Gotsch et al., 2016; Haristoy et al., 2014; Hertel & Köhler, 2010). While field observations confirm presence of canopy soil and roots routinely, their functional contribution to the whole-tree physiology remains elusive, especially in relation to the ability to take up water and nutrients.

An important and noticeable component of the canopy environment is the large biomass of epiphytes composed of vascular and non-vascular plants including mosses, lichens, bromeliads, and ferns. These organisms can retain and accumulate water and nutrients from rainfall and dry precipitation - accounting for ~92% and 365% of canopy soil dry weight during dry and wet periods respectively- (Köhler et al., 2007). While it is true that large trees can access water from deep in the ground during dry periods, the physical difficulty of moving water to a high crown is thought to be a major limitation to growth in trees (Koch & Fredeen, 2005). Thus, a reservoir accessible to the crown without fighting gravity may maintain the tree water potential

for photosynthesis, reduce risk of cavitation, and regulate plant temperature to sustain positive carbon balance during hot, dry conditions.

Canopy soil studies have been focused primarily on nutrition, rather than on the possibility that these systems can provide benefits to a tree's water-budget. Based on the quantitative amount of a tree's total fine root system, Hertel (2010) considered canopy roots' nutrient contribution negligible, thus, suggesting that the actual ecological benefit from these roots is minimal for the host tree (Hertel & Köhler, 2010). However, other studies have revealed that canopy roots have the ability to take up minerals (in the form of radionuclides) from the canopy soil and transfer them at least to adjacent branches and leaves, as in *Salix syringiana* saplings (Nadkarni & Primack, 1989). In the case of *Ceratopetalum virchowii*, roots do not take up nutrients directly from the canopy soil but they absorb dissolved nutrients from rainwater draining from the stem (Herwitz, 1991). Moreover, evidence shows that canopy roots associate with nitrogen-fixing microorganisms as in *Acacia koa* or with arbuscular mycorrhizal fungal in *Acer macrophyllum* (big leaf maple), suggesting an enhanced nutritive benefit for the canopy via canopy roots (Kennedy et al., 2010; Leary et al., 2004). Even though it is well known that nutrient absorption by roots requires water and that canopy soils accumulate a high proportion of water, no studies exist in the literature addressing water uptake from canopy soil. The present study pioneers this uncharted area by investigating the impacts of canopy soil on the water budget of the host tree. First, results provide evidence that canopy roots developed into canopy soil facilitate water uptake from the canopy, and second, demonstrate that water absorbed from the canopy can be redistributed throughout the tree and mitigates the effects of drought stress.

It is expected that temperatures in the Pacific Northwest of North America will rise, and drought will become more severe in the future climate (Snover et al., 2019). Such conditions are predicted to increase heat stress-related tree mortality in the western US. At least four species important to Washington forests, *Picea sitchensis* (Sitka spruce), *Acer macrophyllum* (bigleaf maple), *Acer circinatum* (vine maple) and *Alnus rubra* (red alder), have been found to sprout adventitious roots from branches into canopy soil mats (Nadkarni, 1981; Sillett & Bailey, 2003). The role of canopy roots and epiphytes that constitute canopy soils may be crucial to plant

biodiversity in critical habitats, and likely account for an important but understudied source of water and nutrients in trees, especially under conditions where trees become heat stressed.

In order to understand how canopy soils influence water dynamics in the host tree through the functions of canopy roots, this study addressed three main questions starting from the organ level 1) assessing canopy root activity, and scaling up to an individual level, 2) testing canopy water redistribution and 3) its physiological and ecological role under specific scenarios, in this case water deficit in the soil. Three approaches were used to accomplish each goal: field, lab and greenhouse experiments. In most of the experiments, canopy roots were manually induced using an air-layering technique (Hartmann et al., 2011) on mature poplar trees (*Populus sp.*), simulating canopy soils in nature but here, grown in a controlled environment. Species of this woody genus grow rapidly and are relatively easy to propagate clonally using stem cuttings or by air-layering. In contrast, in the field study, naturally occurring canopy roots were obtained directly from mature big leaf maple trees (*Acer macrophyllum*) growing in the Olympic peninsula, Washington State.

In Chapter 1, I illustrate the activity of canopy roots based on their morphology and anatomy compared with terrestrial roots. Root water uptake can be influenced by changes in anatomy, suberization and hydraulic physiology along the root length (i.e. abundance and activity of aquaporins). In this chapter, I report microscopic observations of canopy roots from different sources (greenhouse, nursery and field) and compare them with terrestrial roots, identifying specific features that may influence their function, particularly those traits involved in root hydraulic conductance and water absorption.

If canopy water uptake has some impact on tree water budget, it must be reallocated from the canopy to other tree organs through the vascular system. Chapter 2 shows the hydraulic redistribution of water absorbed from canopy soil throughout the tree (canopy- and lower levels) by multi-directional water flows (i.e., reverse and bi-directional flow). For this study I use stable isotope and sap flow methods to test whether the canopy roots function to channel water movement from the canopy to other parts of the tree in more than one direction.

Finally, Chapter 3 is focused on how plants can utilize water from the canopy soils to mitigate physiological stress in water deficit conditions. I test the hypothesis that plants with canopy soils will take up water from aboveground and redistribute it through the plant, decoupling the tree-canopy water status from the soil water content. This would delay the onset of drought stress by channeling water from the canopy soils to maintain hydraulic conductivity, water potential, stomatal conductance, and eventually photosynthesis and keep the whole-tree functional in stressful environmental conditions.

Understanding water relations of canopy soils and trees is the first step toward a greater comprehension of the overall crown microclimate and the physiological implications of epiphytes on tree growth and survival under heat stress. Understanding the functions of canopy roots will provide insights on an underexplored reservoir of water and nutrients available in crowns of tall trees. This reservoir may function as a source of water that prevents or repairs embolisms in the tree's vascular system. Physiological and ecological data describing the conditions and benefits of the functions of canopy soils will allow for a new understanding of how we define hydraulic redistribution in trees. This understanding may reach beyond a single tree and lead to a recognition of reverse flow of water within the soil-plant-atmosphere continuum. Expansion of our understanding of water movement within forests will have significant implications for ecological studies and atmospheric models of effects of climate change.

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CHAPTER 2

Anatomy and morphology of adventitious canopy roots: A comparative study with terrestrial roots

Abstract

One of the most interesting effects of canopy soils is the promotion of aerial adventitious roots, also called “canopy roots,” from tree branches into epiphyte mats. Specific conditions of darkness, moisture and inorganic matter under the canopy soils could explain the development of canopy roots, however their morphology, anatomy and functionality are largely unknown. This qualitative study compared anatomical and morphological features of terrestrial with canopy roots to better understand their functions.

As effects of drought stress, stem hydraulic capacitance, transpiration and root hydraulic conductance decline, and as soil water content diminishes, roots have less access to water. In response to this, plants allocate more resources (carbon, nitrogen, etc.) to develop new or deeper roots to reach groundwater supplies or other water sources that help overcome the water deficit. Another alternative could be an investment in canopy roots. These organs are located at the canopy level and have a different water source (canopy soil), therefore may not be affected by soil water deficit as much as terrestrial roots. The current study made comparisons of canopy roots from three different growth locations (greenhouse, nursery and field) with terrestrial roots, using microscopy and qualitative visual observations. We identified specific features correlated to their functioning, particularly traits related to root hydraulic conductance and water uptake. Overall, we failed to observe any anatomical or morphological differences between canopy and terrestrial roots, even though they develop from different organs of the tree. However, the presence of woody tissue in terrestrial roots suggests a decrease in absorption compared to canopy roots, implying a higher absorption and hydraulic conductance of the latter.

1. Introduction

The soil-plant-atmosphere continuum model (SPAC) represents the entire plant hydraulic path, from the root hairs in the soil to the stomata at the interface of plant and atmosphere (Sperry et al., 2002). Variations in soil water content or water demand will be followed by rapid changes in transpiration. However, if the changes are maintained for a longer period, the plant will slowly modify its hydraulic system to adjust to the new environment (hydraulic acclimation). These changes not only occur at the canopy level with the reduction of stomatal conductance (Mori & Schroeder, 2004; Wilkinson & Davies, 2002), but also belowground, particularly in the capacity of roots to take up water (Javot & Maurel, 2002; Luu & Maurel, 2005; Siemens & Zwiazek, 2004).

Root hydraulic conductance is one of the main parameters that influence the capacity of the root to take up water, conferring upon the root the ability to adjust and respond rapidly to different environmental conditions. Even though most of the recent studies attribute root hydraulic conductivity to the abundance and modifications of aquaporins (water channels) (Aroca et al., 2012; Helen Bramley et al., 2009; Javot & Maurel, 2002; Laur & Hacke, 2014; Saliendra N.Z. & Meinzer F.C, 2003; Steudle, 2001), there are other parameters equally important that provide root plasticity, including root architecture and metabolism. Thus, anatomical and morphological features of roots, such as diameter or length, number of cell layers, degree of suberisation and the radial and axial water conductance pathways influence hydraulic conductivity. Therefore, not only the influence of aquaporins is relevant to understand root functionality (not the scope of this study) but also it is very important considering root hydraulic properties (Helen Bramley et al., 2009; Carmen Martinez-Ballesta et al., 2011).

There are different types of roots across plant species. All plants develop a primary root (derived from an embryonic radicle) and a variety of lateral roots. There are also adventitious roots that display similar functions as lateral roots but are developed from aerial tissues (Bellini et al., 2014). Canopy Roots (CR) are adventitious roots that grow in temperate and tropical forests, particularly from tree branches that have accumulated a large biomass of epiphytes, invertebrates and litter on their surfaces. Decomposition of this material over time generates

inorganic matter very similar to underground soil. This “soil” in the canopy, also called “canopy soil” has a composition and properties that promote root development from the branches, penetrating the canopy soil (Fig. 1). Previous studies have suggested that canopy roots (CR) can take up water (and nutrients) from canopy soil and redistribute it throughout the tree (Cantillo et al., 2020; Haristoy et al., 2014; Hertel & Köhler, 2010; Kennedy et al., 2010; Nadkarni, 1981a; Nadkarni & Primack, 1989). However, this activity of CR and their physical and hydraulic properties have not yet been confirmed.

Although canopy and terrestrial soils have similar composition, environmental factors and seasonality influence each one distinctly. Under dry conditions, concentration of roots in dry soils where plants cannot cope with transpiration stress is risky. An alternative strategy is to allocate more resources to develop new or deeper roots that reach groundwater supplies or external water sources that help overcome the water deficit (Hill et al., 2013; Rosado et al., 2011; Vervoort & van der Zee, 2012). One of the possible hypotheses about CR formation is that these roots grow in response to adverse conditions when present in combination with hormones signaling induction of root development (Cavelier et al., 1997; Danin, 1997; Haissig et al., 1994; Hartmann & Kester, 1988; Hartmann & Trumbore, 2016; Herwitz, 1991; Nadkarni, 1981b).

For this study, we hypothesized that canopy roots have similar structural, morphological and anatomical traits as terrestrial roots, which suggest that they are actively functional roots. On the other hand, since CR do not experience the same stress conditions during the dry season as terrestrial roots, we expected to find anatomical differences in the traits related to hydraulic conductance. This would suggest that the presence of CR and canopy soil will be advantageous when water supply from belowground soil is insufficient to replenish water loss through transpiration. To investigate this, we made qualitative comparisons using microscopy to identify anatomical attributes correlated to root hydraulic conductance between canopy roots and their corresponding terrestrial roots in two different plant species *Populus sp.* (poplar) and *Acer macrophyllum* (big leaf maple).

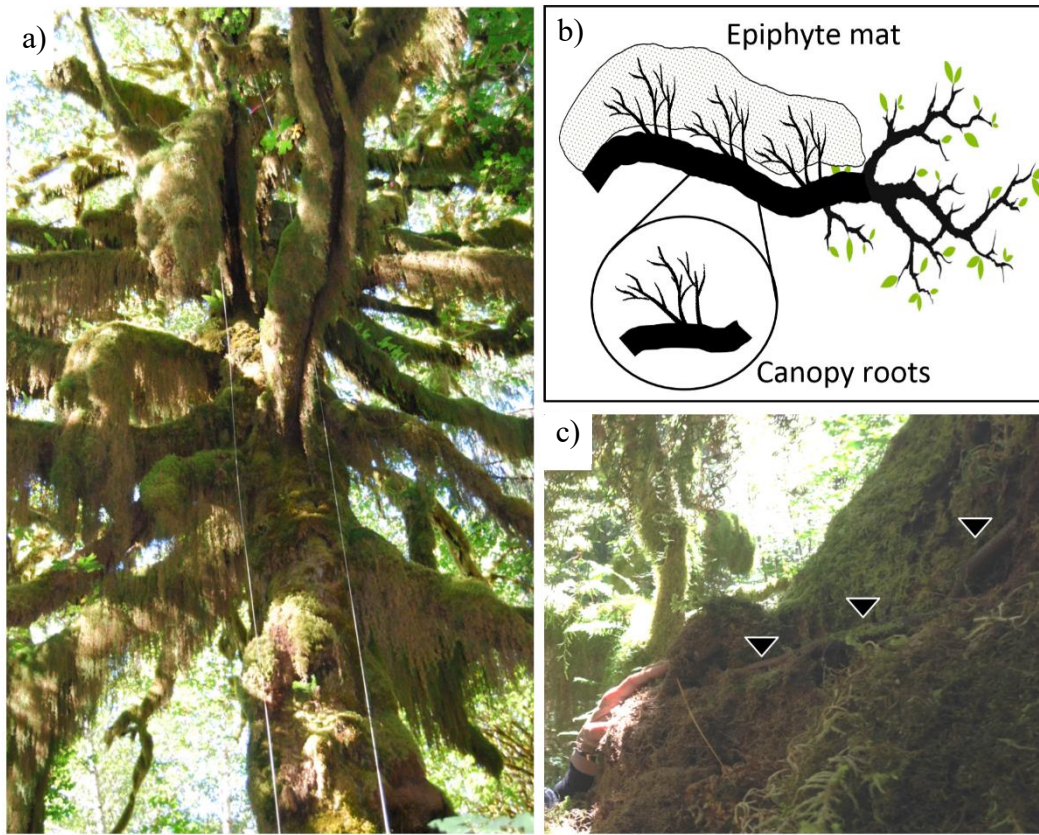


Fig. 1 Canopy soil in an old-temperate rainforest (Olympic Peninsula, WA-USA). A) *Acer macrophyllum* (bigleaf maple) completely covered from trunk to branches with canopy soil 15-30cm thick and composed mostly of mosses and lichens. B) Diagram of a branch with canopy roots growing out of the branch and into canopy soil. C) Canopy roots after removal of canopy soil on a branch of a bigleaf maple tree (black arrows).

2. Materials & Methods

2.1 Plant material

Canopy roots (CR) samples were collected from : 1) *Populus spp.* (poplar) grown in the greenhouse and in an outdoor tree nursery, and 2) *Acer macrophyllum* (bigleaf maple) collected in the field on the Olympic Peninsula (Quilcene and Chimacum Villages, WA – 47° 53' 40.8984" N, 122° 49' 25.953"W). Poplar trees in the greenhouse were *Populus x canescens* (grey poplar), a hybrid between *Populus tremula* (common aspen) X *Populus alba* (white poplar). This rapidly growing woody species was relatively easy to propagate using stem cuttings. Cuttings (30-40cm) were initially grown at the R. L. Goss Farm at the WSU Puyallup Research, WA and later transported to the Douglas Research Conservatory greenhouse at the Center for Urban Horticulture, University of Washington (47° 39' 27''N, 122° 17'21''W; 10 m elevation), Seattle, WA. They were transplanted into potting soil (Sunshine Mix #4 – Sun Gro Canada Ltd) using 37.8L blow-molded plastic pots and were grown over a two-year period (1.5m height) in the greenhouse. The average air temperature was 22/19C, 16/8h day/night. The average daily light integral (DLI) was 9.5mol m⁻² d⁻¹ of photosynthetically active radiation (PAR). Relative humidity was 60-80% on average. For nursery samples, we used trees of *Populus trichocarpa* (Torr. & A. Gray) clone Nisqually-1 that were ~10-12 years old, growing in the outdoor nursery area at the Center for Urban Horticulture (University of Washington, Seattle-WA).

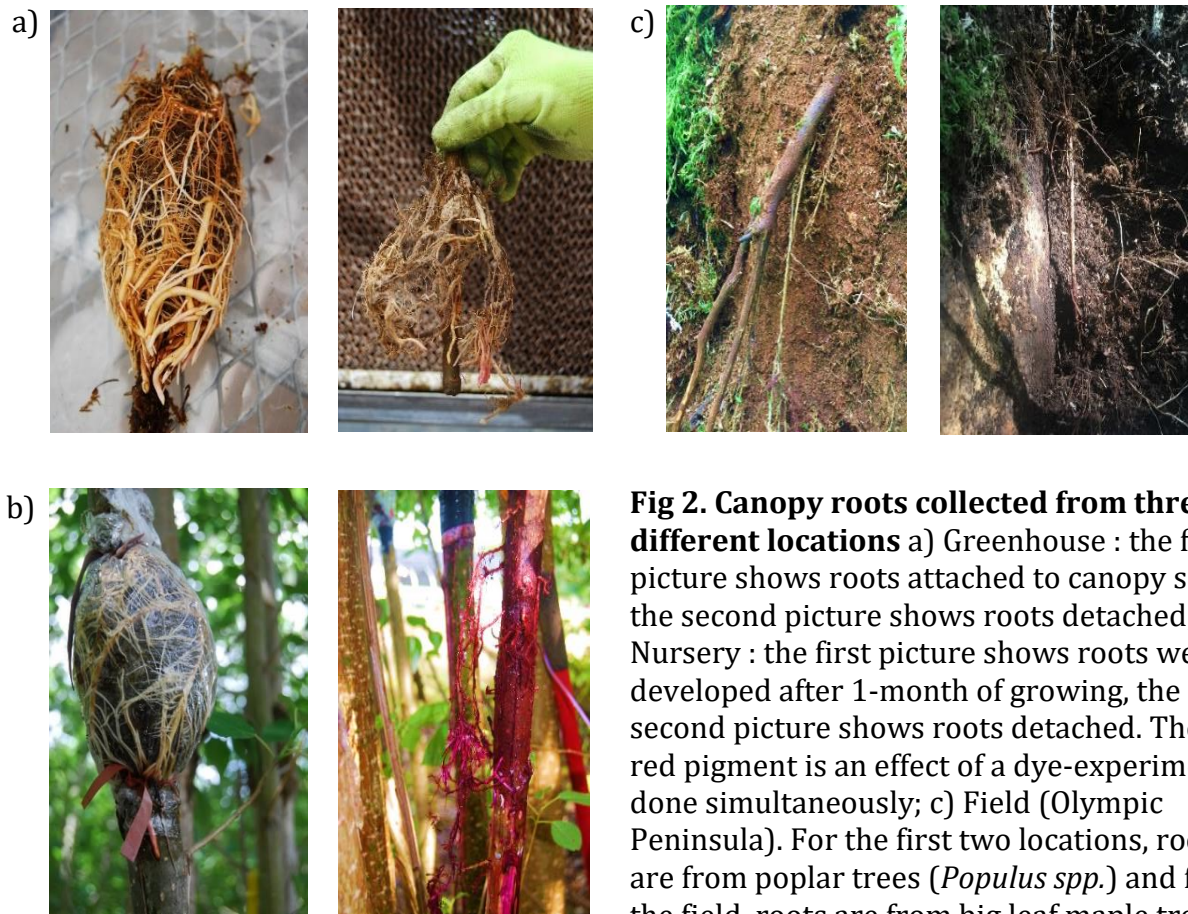


Fig 2. Canopy roots collected from three different locations a) Greenhouse : the first picture shows roots attached to canopy soil, the second picture shows roots detached; b) Nursery : the first picture shows roots well-developed after 1-month of growing, the second picture shows roots detached. The red pigment is an effect of a dye-experiment done simultaneously; c) Field (Olympic Peninsula). For the first two locations, roots are from poplar trees (*Populus spp.*) and for the field, roots are from big leaf maple trees (*Acer macrophyllum*).

2.2 Experimental design

2.2.1 Sample collection

To promote formation of adventitious roots from both *Populus* species used in the greenhouse and nursery experiments, we applied an air-layering technique (Hartmann et al., 2014). This consists of removing with a knife a ~4-5cm section of the bark (20 to 30cm from the shoot tip) about 3-4mm depth on the branch and exposing completely the primary xylem, including removal of bark and soft (cambial) tissue. Then, rooting liquid (Wood's rooting

compound, OBC Northwest Inc, Canby, OR) consisting of indole-3 butyric acid (IBA) and naphthalene acetic acid (NAA) in a concentration of 1:10 dilution was applied to the exposed surface, followed by encasing the area with moist sphagnum or tree moss and finally covering it with plastic and aluminum foil. Roots were collected after ~1.5 months (Fig. 2a-b). Roots were separated carefully out of the peat moss, keeping their structure intact as much as possible and stored submerged in 10% ethanol in plastic tubes. Samples were maintained cooled and stored in a refrigerator (4°C) until analysis.

Canopy and terrestrial root samples in the greenhouse were collected from trees subjected to one of the treatments of an experiment that spanned 15-days during the summer of 2018. There were three different treatments ($n = 11$ per treatment): 1) Well-watered; 2) Dry trees; and 3) Dry-wet trees. This latter was from which root samples for the current study were collected. These trees were water-restricted at the pot level but watered through the canopy root systems. Each system (three canopy root systems per plant) was connected to a 1000ml IV-bag that was refilled every other day. To maintain the soil completely dry, we used a heavy-gauge (4 mil) plastic sheet to cover each pot, from the stem to the greenhouse floor.

In the field on the Olympic Peninsula, we randomly selected eight bigleaf maple trees that held canopy soil of ~10-15cm thickness (Fig. 2c). We climbed three trees to reach branches (~10-20m height) with epiphytic material on each tree using single rope climbing techniques. Canopy soil was removed discreetly, and roots were separated from the branch, trying to keep root structure intact. Small root samples were stored in plastic tubes with ethanol (10%) and big samples were wrapped in humid paper towels and stored in plastic bags. Samples were transported in coolers to the lab and stored in a refrigerator (4°C) until analysis.

Terrestrial roots (TR) were collected only for poplar samples in the greenhouse and nursery simultaneously with canopy roots. Samples were collected within 50cm of the trunk; they were separated from the main tree and cleaned carefully, removing all soil debris, and trying to keep their structure intact. Samples were stored as for canopy roots (mentioned above).

2.2.2 Microscopy analysis

Samples were sorted by branch developmental order (centrifugal) with respect to the whole root system and developmental stages within the root segment sectioned (Fig. 3). We analyzed serial cross-sections (~10-20 μm thick) of two categories of root tips: a) 30-50mm and b) 60-70mm from the root apex. Sectioning was made with a vibratome (Model 1000, TPI, Inc., St Louis, MO, USA). Sections were mounted in distilled water and examined using a microscope attachment camera (Motic Moticam 1SP CMOS 1.3 MP, Motic, BC, Canada) to photograph leaf cross-sections at 40- and 200-fold magnification (Nikon model Optiphot). Some of the samples were stained with toluidine-blue and acid-fuchsin for better resolution of vascular structures. Measurements of specific traits were recorded using ImageJ (NIH) software: diameter of xylem, phloem, stele and cortex; developmental stage (number of developmental poles), visibility of Casparian strip and endodermis thickness. Comparisons were made between canopy and terrestrial root samples collected from the same tree. All anatomical traits were ultimately averaged to the root level; three 300- μm -long sections per root were analyzed and then averaged from three root samples per plant for CR and TR treatments (greenhouse: $n = 11$; nursery: $n = 8$).

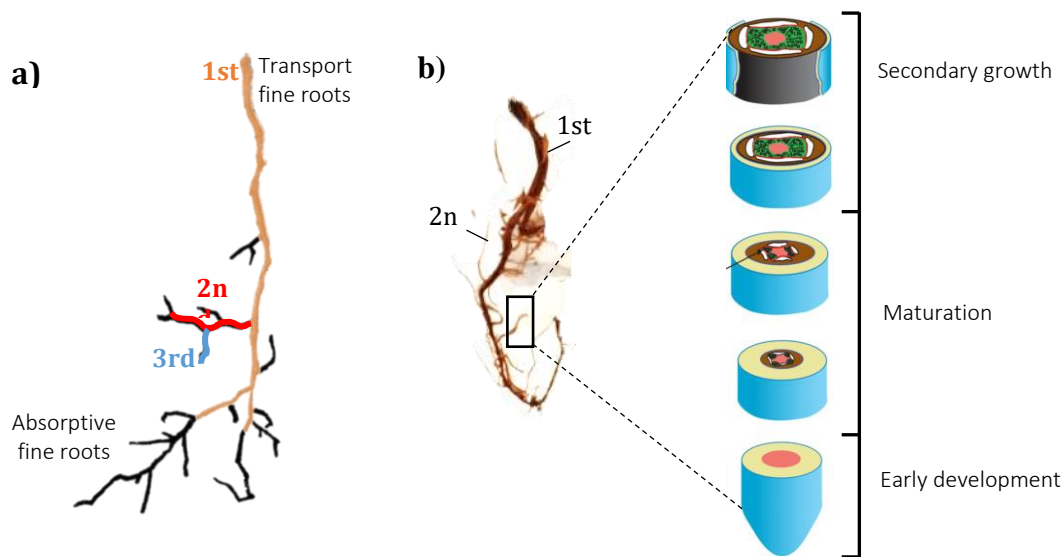


Fig 3. Root developmental orders. a) Diagram illustrating developmental order, with 1st order (brown) being the principal root system (older developmental stage), and 2nd (red) the first branch from the main root and 3rd order (blue), a branch of a 2nd order root - the higher the order, the younger in developmental stage. Usually the principal root system is suberized and works for transport, whilst higher order fine roots are less suberized and work for most of the absorption. b) 1st and 2nd order in a canopy root and developmental stages within a 2nd order root. Roots are divided in three segments: The tip represents the earliest development; moving back from the tip tissue is more mature (second segment) leading to secondary growth (third segment).

2.3 Data Analysis

Measurements of microscopic features were averaged and tested for significant treatment effects, contrasting CR against TR values using paired T-tests by distance from the root tip: a) 30-50mm and b) 60-70mm from the root apex. Significance levels were of 5, 1, and 0.1%. (*P<0.05, **P<0.01, ***P<0.001).

3. Results

3.1 Structure and morphology

Overall, at a macroscopic level there were no significant differences of canopy and terrestrial roots with respect to their structure and morphology. However, we did find differences in size and developmental stages between CR and TR roots of the same plant (Fig. 4). Qualitative measurements showed that terrestrial roots have a larger cortical diameter, approximately two to three times more than canopy roots; they were larger, more developed, thicker and showed presence of cork (suberized regions mainly at the primary root) compared to CR, that appear to be earlier in their developmental stages, without evidence of secondary growth, thinner and softer. For both, we were able to observe lateral roots from the outer epidermal layers, more abundant in CR and only evident in 2nd and 3rd order branches of TR (i.e., non-suberized roots = no evidence of cork tissue) (Fig. 4b).

For TR, there was evidence of a primary root with a woody surface (well-developed bark) from which numerous lateral, suberized fine roots developed. In CR there was no significant primary root, instead there was mostly a conglomerate of seminal roots with multiple lateral roots, all non-woody; the latter structure could be the result of the limited medium in which CR roots develop (peat moss boles) that promoted a clumped growth of roots in a restricted space (Fig. 4a). In general, TR showed approximately three times higher density compared to canopy roots, with similar abundance of root tips. However, fine root tips in CR have a higher diameter than terrestrial ones.

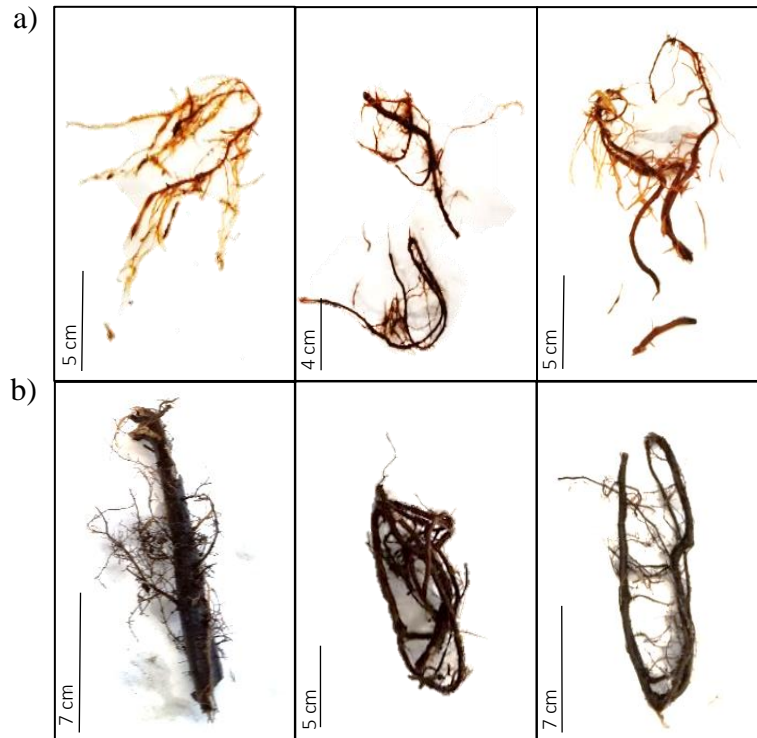


Fig. 4 Morphological and structural differences between canopy (a-top) and terrestrial (b-bottom) roots collected from *Populus spp.* (poplar trees) from nursery and greenhouse. CR in the greenhouse and nursery were collected after ~1.5 months of induction. Terrestrial roots were removed from within 50cm of the trunk).

3.2 Anatomical observations

Basic anatomical features of the cortex were mostly similar in both root types. All the studied samples have similar structure, with parenchymatic cortex involved by a variable number of xylem and phloem strands, endodermis with or without evident Casparian strips. However, stele diameter in TR was about one-half that of the entire root, compared to CR for which the stele was around one-third of the cortical area (Fig. 5; 1 to 4). Supporting the morphological observations, we observed a single epidermis (ep) and exodermis (ex) layer, respectively for CR, while in TR there were around three to four layers of outer epidermal layers (darker layers on the Fig. 5; 5 to 8). These correspond to the cork formation (phellem) consisting of alternating layers

of suberized cells and sclerenchyma. The inner and outer cortical cells were similar in both root types.

The inner boundary of the cortex, the endodermis, consisted of cells that develop the Casparian strip. This layer was well-developed in TR, but not in CR. At the stele level, protoxylem or primary xylem strands, containing mostly protoxylem (px) cells alternated with strands of protophloem (primary phloem) (pp) in CR, while TR presented both protoxylem and larger metaxylem (secondary xylem) vessels alternating with internal metaphloem strands (Fig. 4; 9 to 12). A varying number of xylem strands occurred among each type and was also dependent on the distance from the tip of each root. Cross sections made closer to the root tip had fewer protoxylem strands than mature portions of the same root. CR contained generally four pronounced protoxylem strands (tetrarch), while for terrestrial roots there were above six strands (Fig. 4; 9 & 11).

CR sections farther from the root apex (oldest root portions) showed features of a more advanced state of development, for both types of roots. Some CR presented an increase in their epidermal thickness and their endodermal layers with a fully developed Casparian strip, as well as protoxylem strands. Also, there was evidence of both primary and secondary xylem and phloem. A similar scenario was seen for TR at the stele level, but the arrangement of xylem strands was lost showing multiple strands alternating with protophloem. Also observed was a well-noted and possibly suberized endodermis with periderm formed from the outer layers of the pericycle, epidermis with cork, and much more suberization than samples collected closer to the apex (Fig. 4; 11 & 12).

Anatomically, CR had greater root and stele diameter (38.5% and 15.6% higher, respectively), narrower xylem vessels (25% smaller average lumen diameter), more cortical cell layers and 16.7% thicker endodermis compared to TR. Their epidermal cells were smaller, and as seen previously, some of the roots collected (samples collected closer to the apex) did not show an evident Casparian band. On the other hand, TR samples showed a marked endodermis and Casparian strip but thinner (12.5 μ m), and greater and more developed xylem vessels (25%

bigger than CR). Except for endodermis thickness, all the traits evaluated were significantly different between CR and TR (Fig. 5).

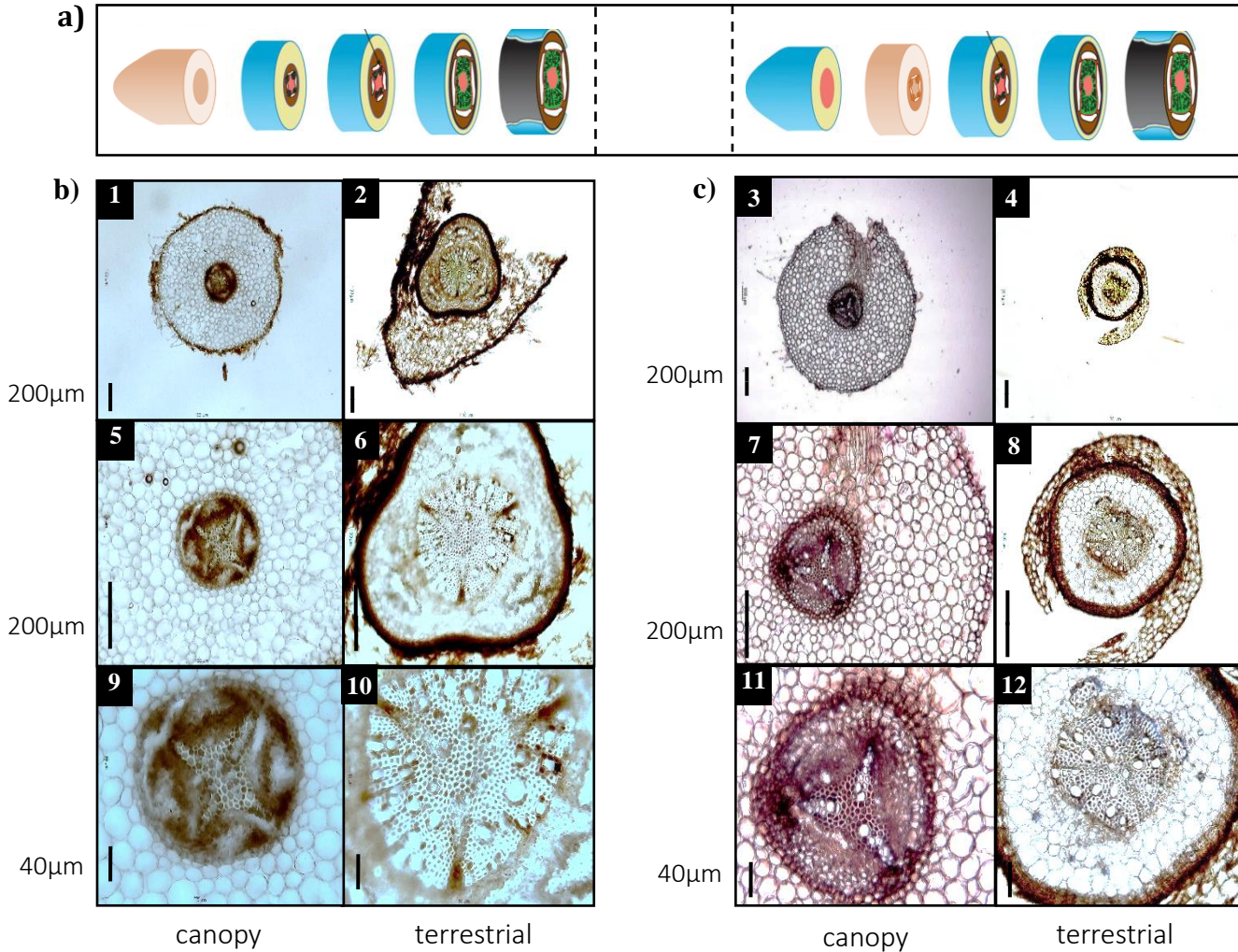


Fig. 5 Microscopy observations of CR vs. TR collected from poplar trees (*Populus spp.*) in the greenhouse and nursery. All root samples are 2nd order and were sectioned in two different locations shown in orange in **a) left panel showing tip segment (30-50mm from root apex); right panel showing maturation segment (60-70mm from root apex). **b**) microscopic images from tip segment. **c**) Microscopic images from mature segment. Each row corresponds to a different magnification: 40X (top), 100X (middle), 200X (bottom). ep=epidermis, ex=exodermis, ck = cork, px = protoxylem, pp= protophloem, mx= metaxylem and mp = metaphloem, cs = Casparian strip, pc= pericycle, pd= periderm**

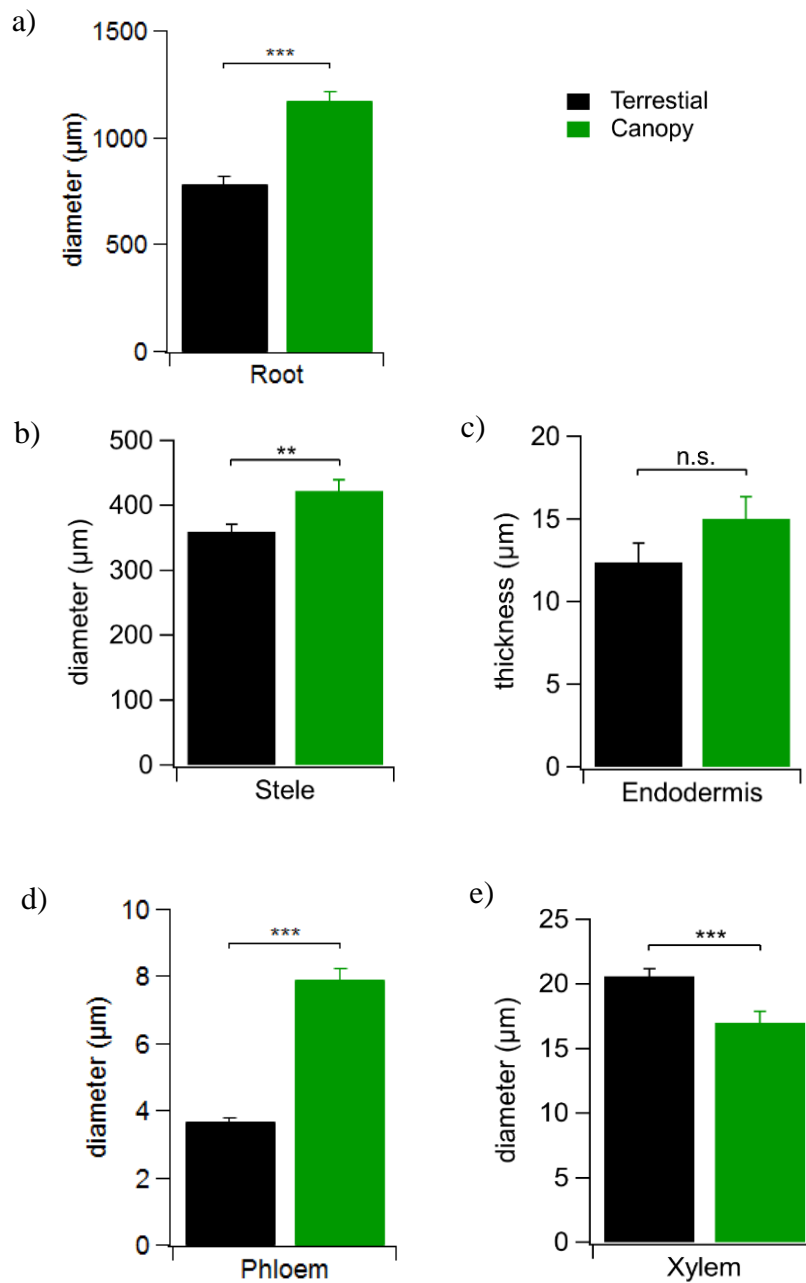


Fig 6. Measurement of anatomical traits in Canopy Roots (CR) and Terrestrial Roots (TR). All measurements are in µm (micrometers) and are the average of three visual fields per sample. Root samples are 2nd order and were sectioned in two different locations: 30-50mm and 60-70mm from root apex. T-tests significance p-values: *P<0.05, **P<0.01, ***P<0.001.

4. Discussion

We compared for the first time the structure, morphology and anatomy of CR versus TR, identifying traits that could give us insight into their root hydraulic conductance and overall functionality. An important observation from this study was that CR have similar morphology and present all anatomical traits necessary for root water transport, which suggests that CR are functional (Fig. 5) and can be used as additional organs to absorb water or nutrients.

All the studied samples have similar root structure, with cortical cells involved by a variable number of xylem and phloem strands, endodermis with or without evident Casparian strips, exodermis and a simple epidermis. However, distinct tissue distribution, cell shapes and number of cell layers was noted (Fig. 5, S1 & S2). There were two main anatomical differences between canopy and terrestrial roots; root and stele diameter were much larger for CR than TR, however diameters of xylem vessels were significantly higher in TR compared to CR. On the other hand, secondary growth such as suberized cells of the epidermis and possibly cells of the endodermis were evident in TR but not in CR, whose epidermal cells were smaller and not suberized. Based on these last observations, we suggest that CR are hydraulically more efficient due to the lack of suberized cell-walls (more water permeability). On the other hand, TR had higher xylem vessel diameter, which indicates that TR may have a higher root hydraulic conductance because of the higher volume of their conduits. This TR trait suggests a possible trade-off between suberization and xylem vessel diameter to increase root hydraulic conductance.

4.1 Root hydraulic conductance and xylem development

Anatomy plays a major role in root hydraulics by determining the radial pathway the water absorbed must pass through living tissue before reaching the xylem vessels. In the axial plane, the flow of water occurs along the xylem vessels and tracheids. The relationship between radial and axial resistances determines the resistance of the whole root and distribution of water uptake (Zwieniecki et al., 2003).

Previous studies have shown that one of the main factors that affect root hydraulic conductance is the diameter of the xylem vessels (i.e., average lumen diameter). Bramley et al. (2009) and Gallardo (1996) demonstrated that hydraulic conductance in lupin roots is at least two-fold greater than the wheat root system due to their increased xylem development (Bramley et al., 2009; Gallardo et al., 1996). Also, it has been recorded in citrus, that roots with small xylem vessels generate greater resistance to water movement (Olmstead et al., 2006). There is a positive correlation between diameter of xylem conduits elements and hydraulic conductivities of roots, this means that larger vessels have a greater potential for water flow. According to the Hagen-Poiseuille equation, total hydraulic conductance varies to the fourth power of the vessel radius (Steudle & Peterson, 1998). As we move away from the root apex, the abundance and diameter of xylem vessels increases, resulting in axial conductance increasing in a similar pattern (Bramley et al., 2007).

Drought conditions inhibit root growth, and root structure could be affected as well as suberization, all of which may alter water flow pathways. Therefore, the intensity and stress duration could be critical for root water flow properties (Siemens & Zwiazek, 2004). In this study, most of TR experienced mild to severe drought stress; plants in the greenhouse were completely water restricted at the pot level (Greenhouse experiment explained in Materials & Methods section) and plants at the nursery were exposed to dry conditions before collection (summer season) for ~2-months. Despite TR being in a drier environment than CR, results showed that TR had larger xylem vessel diameters (~25% larger) than CR. This suggests that TR have a greater hydraulic conductance than CR which contradicts our hypothesis that CR are hydraulically more efficient than TR in terms of axial water flow. Axial hydraulic conductance may accumulate with distance along the root which implies that axial conductance probably is several orders higher than the total hydraulic conductance of a root. Therefore, the greatest limitations to water flow must be given by the radial pathways (Steudle & Peterson, 1998). Further studies of hydraulic conductance measurements at different distances from the root tip are required to confirm that TR have a greater capacity for water flow than CR.

4.2 Suberization as a response to drought stress and its impacts on hydraulic conductance

Physical root properties related to their anatomy are highly variable; major differences are seen among species, habitats and even along the length of an individual root. Examples of these differences are formation of aerenchyma, the development of the endo- and exodermis (with Casparian bands, suberin and thickened, modified walls), death of the epidermis and in some cases the entire central cortex, development of lateral roots, and production of bark. The complex anatomical structure of roots across various tissues (epidermis, cortex, stele) must be considered during radial passage across the root cylinder as well as a longitudinal flow component (already mentioned above). Water deficit conditions induce the development of apoplastic barriers for water and ion flow as strategies to survive an adverse environment (Bramley et al., 2009; North & Nobel, 1992; Steudle, 2000).

Drought can increase suberization in cell layers outside the stele, thereby preventing desiccation of meristematic tissues in the pericycle and the root tip (North & Nobel, 1992). As expected in this study, the environment where roots were developed influenced the anatomy of some of the structures making up the apoplastic pathway. Compared to CR that developed and remained in a humid environment, TR sampled from the greenhouse that had been exposed to dry soil showed suberization and thickening of the exo- and endodermis (Fig. 5). Development of a suberized interface between living tissue and rhizosphere could be expected to minimize water losses. Previous research has found that in both, woody and herbaceous species growing under conditions of water deficit, roots develop a suberized exodermis that helped them to retain water but also exerted some negative influence on their overall hydraulic conductance. (Riegerl & Litvin, 1999; Taleisnik et al., 1999; Zimmermann & Steudle, 1998).

Suberization reduces the permeability of cell walls to water and thus may also decrease root hydraulic conductance, generating overall a greater resistance to the passage of water from the soil into the vascular system than the resistances generated by the axial pathway (Siemens & Zwiazek, 2004). From this, we can infer that TR possibly have a lower hydraulic conductance than the CR, even though they showed larger xylem diameters. Given that there are no measurements of hydraulic conductance per se, we can only speculate that TR are less

hydraulically effective than CR due to the presence of suberin in the apoplast. Further studies are needed to better identify suberized structures and to determine which structural and anatomical changes are most influential for root hydraulic conductance. Another important apoplastic barrier within the radial pathway is the Casparian strip at the endodermis. Studies have been able to separate the Casparian bands from the endodermis and identify the different components, aliphatic and aromatic suberin, which increase their accumulation under water deficit conditions creating an additional resistance for water flow. It is expected that suberin laid down in the tangential, radial and transverse walls of the exodermis and endodermis under water deficit, thickening both layers, which could result in localized high resistances for water and ion flow in the root apoplast (North & Nobel, 1992; Taleisnik et al., 1999).

Our results also show that TR had secondary growth (bark) in their exodermis. Although there are studies that have revealed water uptake in woody roots through suberized and lignified surfaces (bark), the significance and amount of water that is absorbed is minimal and the surface must be soaked or hydrated for a long period for water to be absorbed by the woody tissues (Cuneo et al., 2018; Earles et al., 2016). Most studies correlate the thickening of cell walls (suberized) and secondary growth (woody) with low hydraulic conductance. On the other hand, CR did not show suberized cell walls and they presented a greater number of lateral roots, which possibly contribute to a greater water absorption (Fig. 3).

4.3 Root structure, morphology and allocation hypothesis in response to water deficit

Hertel & Kochler (2010) reported that CR comprise a low absolute amount of fine root biomass of a tree and low density in the canopy soil compared to TR. Furthermore, they found that CR have fine roots with larger diameter, which provides them a prolonged lifespan but a simultaneous reduction in water and nutrient uptake ability than smaller fine roots. There are also reports that an increase in root diameter corresponds to a drought stress response in tree species for temperate and tropical rainforest (Hertel & Köhler, 2010; Metcalfe et al., 2008). Experiments by Hertel et al. (2010) were carried out in the field, where canopy soil was drier than ground soil,

thus their structure are well in accordance with the differences in microclimatic conditions they found (Hertel & Köhler, 2010). However, in this study CR were grown under regular wetting conditions, which was reflected in their morphology: larger diameters and more lateral roots compared to TR.

One way for plants to adapt to a limiting belowground resource is to increase the fine root area, for example decreasing the root diameter (RD) and increasing the specific root length (SRL) (Cortina et al., 2008; Lopez-Iglesias et al., 2014). These traits are advantageous under drought stress because smaller RD provides higher resistance to formation of embolisms through the smaller xylem vessel diameter, and a higher SRL increases extensional and deeper growth (Alameda & Villar, 2012). The unusual results in this study (TR with smaller RD and larger xylem diameter) suggest that under dry soil TR had fewer cortical cell layers but maintained their xylem vessel diameter to transport water, which could have caused embolisms in the last days of the experiment. Moreover, thicker fine roots in TR probably allowed them to penetrate into more compacted soils created by soil drying (Bengough et al., 2016; Ye et al., 2019).

On the other hand, when water is limiting, plants should shift allocation of resources (carbon, nitrogen and nutrients) toward roots where photosynthates can be used to create structures for increasing water uptake. This shift in allocation should increase root mass growth as soil moisture declines. Root architecture is a determinant trait with respect to resource-uptake efficiency (Lynch, 2005). In the present study, the lateral root branching density for TR was reduced under drought conditions compared to CR (Fig. 3). According to Ye et. al (2019), seedlings of *Populus euphratica* seem to conserve the high metabolic cost of root construction and maintenance by reducing their branch density under water deficit conditions, in addition to reducing competition for water among the roots of an individual plant (Ye et al., 2019). In this study, CR ended with a dense branching of lateral roots, which could suggest that even though plants were subjected to stress conditions belowground, the presence of wet canopy soil aboveground induced biomass allocation towards the CR to increase their root surface area and maximize water absorption from the canopy soils. Friend (1994) reported some aspects of regulation of transport from sources and sinks related to the development of adventitious roots

(Friend et al., 1994). This study suggested that the main control of biomass allocation between roots and shoots lies in sink activity. Thus, factors affecting sink activity are probably the most important for regulating allocation and partitioning. Also, proximity between the source to sink and vascular connections between them is very important. Success or failure of rooting depends upon the establishment of a vascular link between source and the new sink (i.e., root). In this case, CR are more proximal to the leaves (source) than TR, which could provide them more advantages and chances to obtain the resources needed to build roots when there is more water available.

4.4. Conclusions and future directions

In summary, in this study first we confirmed that CR featured all the basic attributes of an active root using the TR as a basis for comparison, which suggests that these adventitious roots are functional and are used for water (and nutrient) uptake. Also, we found that even though TR and CR are developed from the same individual, the environmental conditions influenced their structure, morphology and anatomy, which could influence directly their root hydraulic conductance. The absence of suberization and the greater density of fine roots (lateral roots) suggest a greater conductance for CR, however their xylem vessel diameter is smaller than TR vessels, which indicates higher resistance to water transport. On the other hand, TR showed thickening and suberized cell walls of the endodermis and exodermis, probably as a response of the dry conditions. These results do not reject our hypothesis that CR are hydraulically more efficient than TR. However, since the radial water pathway from soil to the stele is considered a larger resistance under drought conditions, we speculate that because CR are in well-watered environment compared to TR, they must have a higher hydraulic conductance. Further studies of root hydraulic conductance combined with measurements of multiple anatomical and structural parameters are necessary to fully confirm that canopy roots are hydraulically more efficient than terrestrial ones under stress conditions.

Demand and supply of different resources required for the growth, physiology and functioning of plants are determined by environmental factors, thus seasonality (Mei et al., 2015). To understand the ecological and physiological role of the CR in plant water relations, it

may be necessary to also consider how seasonal variations affect adventitious root development and functionality. Root water uptake can be influenced by changes in anatomy, suberization and hydraulic physiology along the root length (i.e., abundance and activity of aquaporins). When root tips are small, the proportion of water absorbed through the tip decreases rapidly with root length. Uptake through the root tips predominates when tips are relatively large, unsuberized and permeable. This relationship could change dramatically under conditions that promote suberization (e.g. water deficit or salinity). This explains how phenological (i.e., root tip growth) and developmental (i.e., formation of suberized cell layers) changes can alter patterns of water uptake across a growing season.

Finally, resource allocation is of key importance to the biology of adventitious roots because it may help explain the rooting capacity of some species, as well as the successful field performance of adventitiously rooted species (Bloom et al., 1985; Friend et al., 1994). Beyond all possible hypotheses about adventitious root formation, it is evident that these roots grow in response to adverse or limiting conditions, either by water, nutrients, oxygen, light or temperature. These conditions, in combination with hormones signaling induction, the presence of specific factors (e.g., moisture, darkness, organic matter) and the availability of carbon allocation stimulate the formation of adventitious roots. Canopy roots could be considered a convergent adaptation that occurs when plants of different origins evolve under similar environmental pressures.

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SUPPLEMENTARY MATERIAL

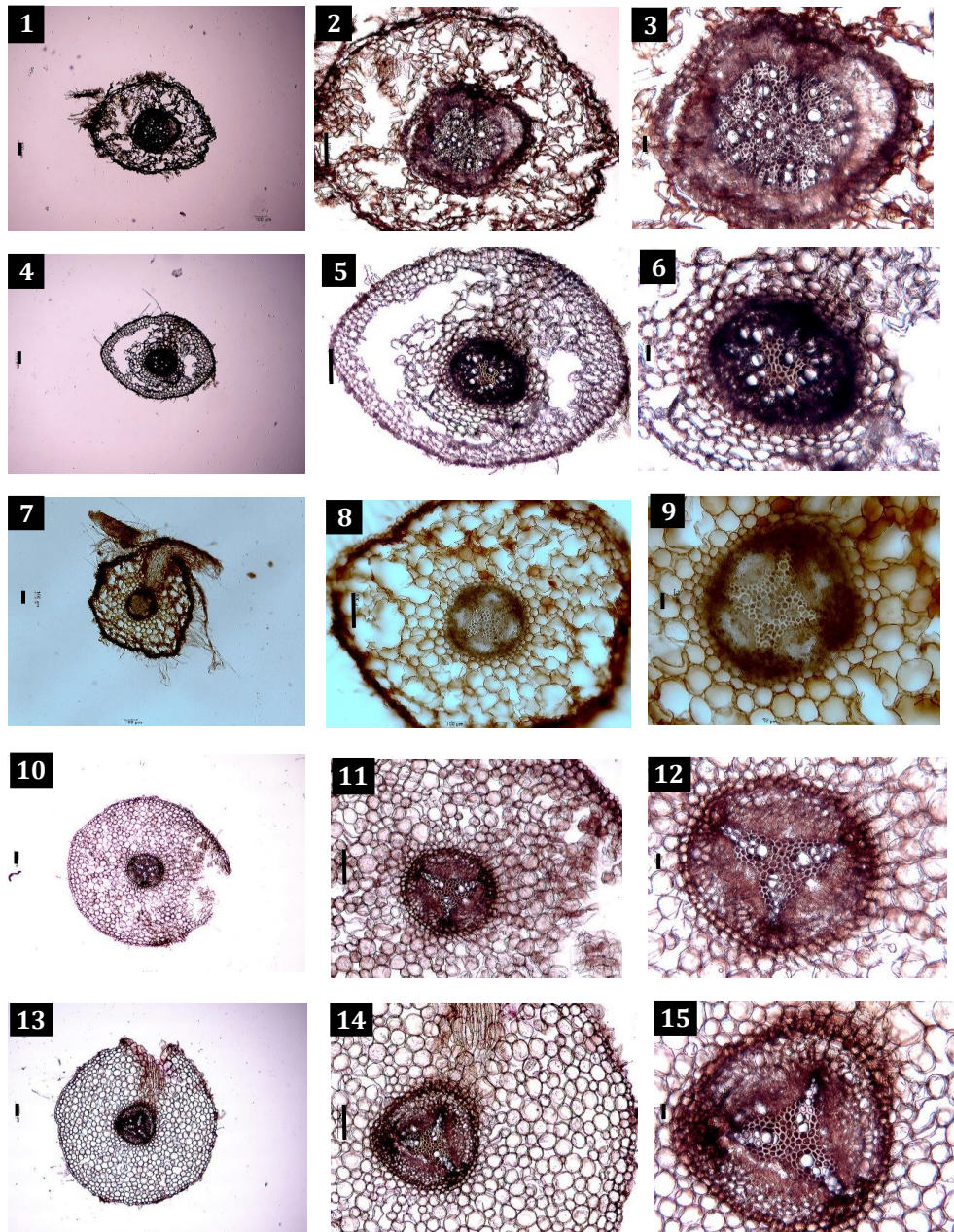


Fig. S1 Microscopy observations of canopy roots collected from poplar trees (*Populus spp.*) in the greenhouse and nursery. All root samples are 2nd order and were sectioned 60-70mm from root apex. Each column corresponds to a different magnification: 40X (left), 100X (middle), 200X (right).

SUPPLEMENTARY MATERIAL

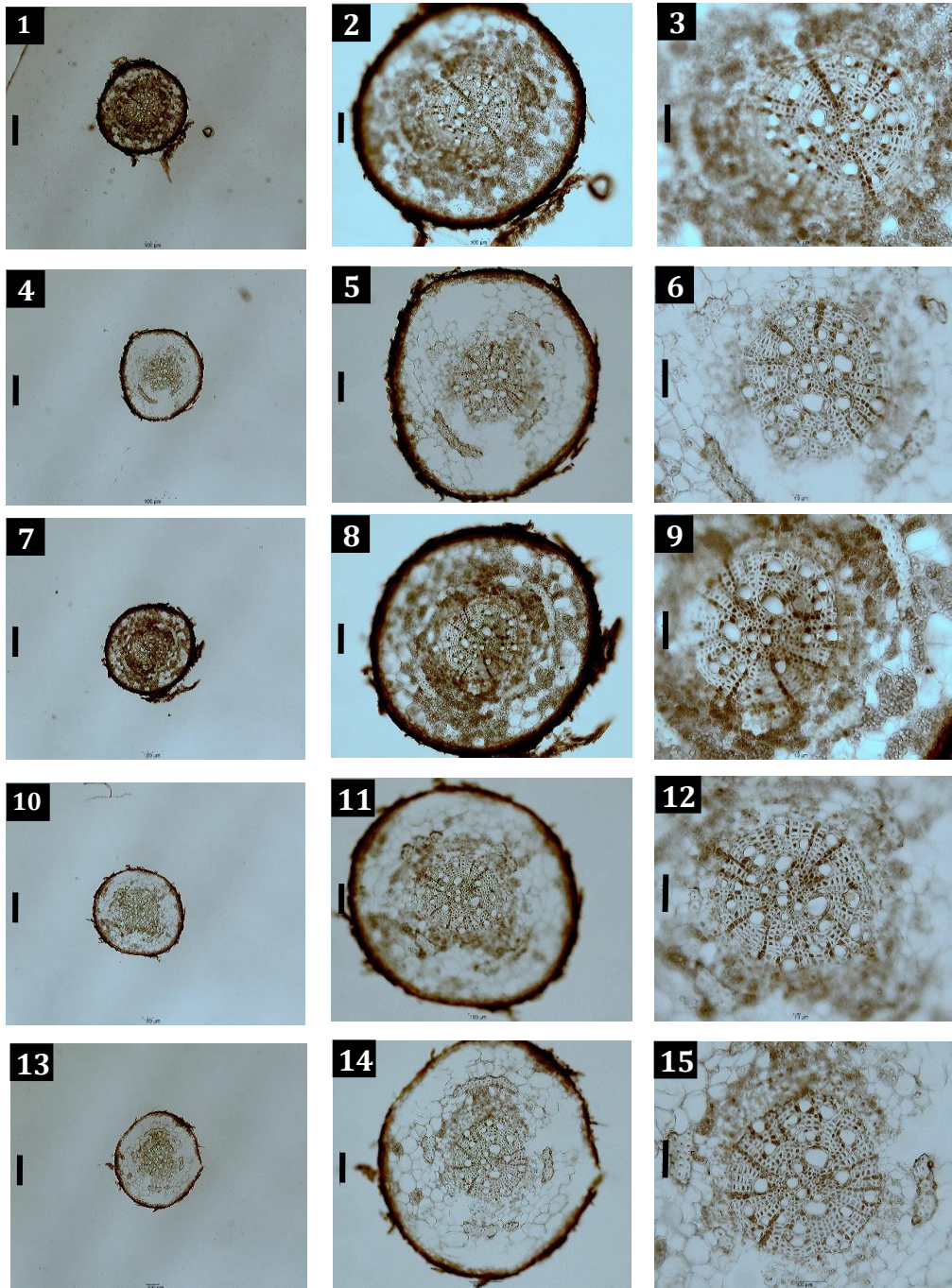


Fig. S2 Microscopy observations of terrestrial roots collected from poplar trees (*Populus spp.*) in the greenhouse and nursery. All root samples are 2nd order and were sectioned 60-70mm from root apex. Each column corresponds to a different magnification: 40X (left), 100X (middle), 200X (right).

CHAPTER 3

Canopy hydraulic redistribution – water from canopy soil to other plant tissues

Abstract

A growing number of studies have demonstrated that atmospheric water (i.e., fog, rain, dew) can be absorbed in the canopy, including directly through leaves or bark, and transported to lower plant tissues in the opposite direction of the SPAC (soil-plant-atmosphere continuum). A noticeable component of the canopy environment is the large biomass of epiphytes composed of vascular and non-vascular plants (e.g., mosses, lichens and ferns), which can retain and accumulate water and nutrients from rainfall and dry precipitation. While it is true that large trees can access water from deep in the ground during dry periods, the physical difficulty of moving water to a high crown is thought to be a major limitation to height in trees. Thus, a reservoir accessible to the crown without fighting gravity for long-distance water transport may allow stomata to remain open for photosynthesis while reducing cavitation risk and regulating plant temperature to sustain positive carbon balance.

The current study examined if water from canopy soil can be absorbed and redistributed throughout the tree body by reverse or bi-directional flow. Field and greenhouse trials were designed using poplar trees (*Populus sp.*) as model organisms to test water redistribution from canopy soil to the host tree. We applied different techniques - dye (acid-fuchsin), stable isotopes (deuterium - δD) and sap flow sensors (Heat Ratio Method) - to trace water movement from canopy roots to different organs: branches, leaves, trunk up- and downstream and belowground roots. Data indicated that: First, canopy roots are able to uptake water from the canopy soil; and second, water absorbed from the canopy can be reallocated to other plant organs (mostly at the canopy level) by reverse and bi-directional flow. Our findings suggest a new water uptake mechanism at the canopy level that challenges the unidirectional SPAC model, showing that belowground soil is not a unique water source, but canopy soil could be used as a potential subsidiary water supply probably under conditions of water deficit.

1. Introduction

Water movement from soil to different plant organs is determined by energy gradients, often described in terms of “water potential.” It is assumed that the intercellular airspaces inside a leaf are nearly or completely saturated with water vapor (Cernusak et al., 2018) relative to the atmosphere, that is typically unsaturated, driving a net efflux of water (i.e transpiration). Similar gradients in water potential influence water movement across the entire plant body, known as SPAC (soil-plant-atmosphere continuum), where water moves along the gradient of water potential from the soil to the leaf-to-air interface. Across this pathway, the soil water potential (Ψ_{soil}) is usually highest (least negative) followed by the water potential of root (Ψ_{root}), stem (Ψ_{stem}), and leaf (Ψ_{leaf}), resulting in movement of water from areas of high (less negative) to low (more negative) water potential (i.e from soil to leaves) (Berry et al., 2019; R. Goldsmith, 2013). However, there are several reports of downward movement of water (i.e. reverse flow), when Ψ_{soil} drops below Ψ_{leaf} . Under these circumstances, water can move in any direction in the SPAC through the vascular system of roots and stems towards the lowest water potential (Burgess & Dawson, 2004; R. Goldsmith, 2013; Nadezhdina et al., 2010). This type of hydraulic redistribution (HR) depends on the orientation of the water potential gradient across the SPAC and can be measured by different experimental approaches that detect bi-directional and reverse flow (Nadezhdina et al., 2010) .

There are two fundamental challenges that plants must face as they grow taller: First, total water potential and the energy required to move water from the soil to the leaves are affected gradually as the force of gravity increases. Second, the total resistance to water movement from sources to sinks will increase as vascular pathways increase in length (Koch & Freden, 2005). To deal with these physical constraints, plants have developed different strategies that not only increase efficiency of water transport, but also alleviate the effects of soil water deficit. Several studies have revealed plants’ ability to take up water from alternative sources, in addition to their main source of water from soil. Foliar and bark water absorption from fog, cloud-borne mist, and dew has been discovered to be new pathways of water uptake that can occur under conditions of soil water deficit, when the Ψ_{atm} is higher compared to Ψ_{plant} or Ψ_{soil} ; this results in a driving gradient for water to move in reverse or bi-directional flow across the

plant, thus mitigating the effect of water stress on the general plant water budget (Breshears et al., 2008; Kerr & Beardsell, 1975); Boucher et al., 1995; Burgess & Dawson, 2004; Ewing et al., 2009; Limm et al., 2009).

Therefore, precipitation occurring as rain or snow is not the only source of water contributing to the terrestrial ecosystem water balance and therefore to growth and survival of plants; water supply for plants depends on several components of the water budget, including interception, runoff, soil water storage, and evaporation (Breshears et al., 2008). For example, Eller et al. (2013) reported for the first time that fog water absorbed by foliage, also known as foliar water uptake (FWU) can be transported through the xylem to above and belowground pools (i.e. stem, branches, roots), suggesting that water uptake at the canopy level can be redistributed through the plant by reverse flow. This provides a means by which water movement is not always unidirectional or bidirectional; rather, it is possible that it can be moving in more than one direction at once (when water moves from higher Ψ_{atm} (during a leaf wetting event) to lower Ψ_{stem} by foliar water uptake, while also simultaneously moving from higher Ψ_{soil} to lower Ψ_{stem} , thus refilling the plant from two directions - Fig. 1) (Eller et al., 2015; Goldsmith, 2013; Simonin et al., 2009; Limm et al. 2009).

Besides water in the atmosphere (i.e., fog, dew, drizzle), there is also evidence of a potential secondary water source at the canopy level that resembles soils on the ground, also formed by the accumulation and decomposition of epiphytic plants, foliage, and debris from the host tree. This canopy soil (CS), also called arboreal soil, accumulates great quantities of water and nutrients that not only can be used by epiphytes, but also by host trees themselves via adventitious canopy roots (Aubrey et al., 2013; Haristoy et al., 2014; Kennedy et al., 2010; Nadkarni, 1981; Nadkarni & Primack, 1989). The presence of canopy soil, as a novel source of water attached to the host tree, generates an additional component to the SPAC that has not been studied before in the context of plant water relations. This new source will likely affect water gradients and therefore water movement and transport. Aboveground water uptake (foliar or bark water absorption) occurs when soil water supply is limited, therefore we can expect that CS water uptake takes place similarly and that water absorbed from the canopy (either by canopy roots or bark) could be reallocated to other plant organs where there is lower water potential.

Recent studies have used stable isotopes of hydrogen/oxygen ($\delta^2\text{H}/\delta^{18}\text{O}$) tracers to detect the presence of water exchanged between the atmosphere and leaves as it moves into the soil. Using this technique, Eller (2013) and Cassana (2016) observed that water absorbed by foliage was transported to soil in seedlings of *Drimys brasiliensis* and *Araucaria angustifolia* in Brazil (Cassana et al., 2016; C. Eller et al., 2013). On the other hand, water movement and direction has been observed using sap flow heat ratio methods (Burgess et al., 2001) which confirm that water from foliage is incorporated into plant vasculature and transport throughout the whole plant (Burgess & Dawson, 2004; C. Eller et al., 2013; Gotsch et al., 2014; Nadezhdina et al., 2010; Steppe et al., 2010). These studies suggested that foliar water uptake may also serve to recharge wood capacitance, and clearly demonstrate reversed water movement through stems and trunks during leaf wetting periods. Although both techniques apply tracers to determine sap flow, they have important differences regarding the scales at which they work; heat pulse measurements are generally made over lengths of 15mm and are integrated over several minutes, whereas the isotope tracing method operates on a scale of meters and is integrated over several days (Kalma et al., 1998).

The present study combined both tracking methods to investigate the possibility of water flow from canopy soil to other organs and therefore demonstrate that 1) canopy soil water uptake occurs in the same context as foliar or bark absorption (i.e., when $\Psi_{\text{soil}} < \Psi_{\text{plant}}$) and that 2) water absorbed is redistributed through reverse or bi-directional flow to other organs of the plant. This hydraulic redistribution may reduce water deficit in the plant and the length of the transport pathway length from soil to leaves, mitigating the detrimental effects of water stress on hydraulic conductivity.

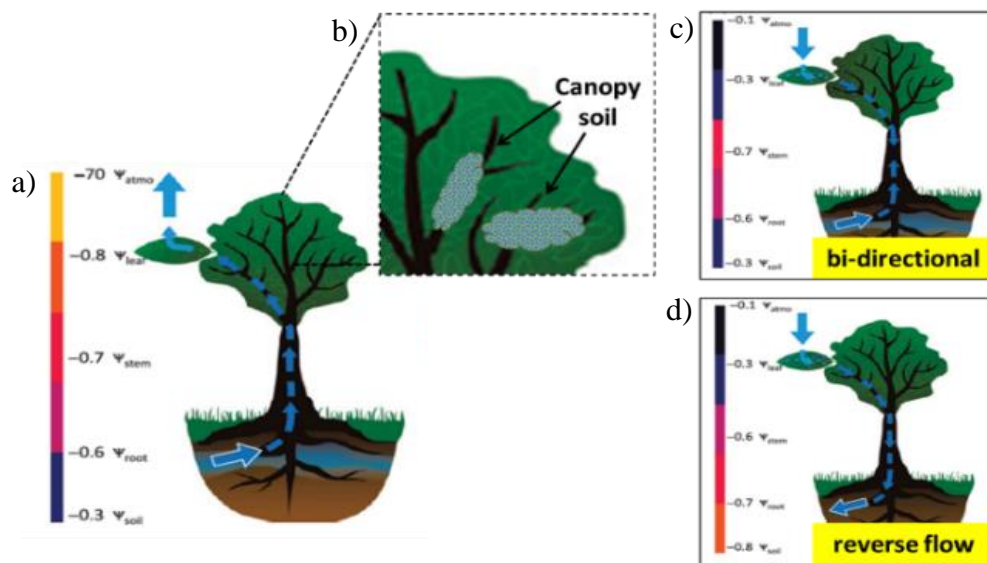


Fig 2. Potential variations of water movement when canopy soil is present. a) Upward water movement (against gravity). **b)** Location of canopy soils on trees as potential reservoirs of water. **c)** Bidirectional water movement (upward and inward) – water supply coming from underground soil and a secondary water supply coming from canopy soil. **d)** Only reverse flow of water movement coming from canopy soil or aboveground.

2. Materials & Methods

2.1 Field experiments

2.1.1 Dye-tracking experiment

For dye-tracking experiments, *Populus trichocarpa* (Torr. & A. Gray) clone Nisqually-1 growing in the outdoor nursery area at the Center for Urban Horticulture (University of Washington, Seattle-WA) was used as plant material for promoting adventitious roots from their branches applying the air-layering technique (Hartmann et al., 2011). This consists of removing with a knife a ~4-5cm section of the bark (20 to 30cm from the shoot tip) on the branch and completely exposing the primary tissue, including removal of all the bark and periderm around the stem. Then, rooting liquid (IBA- Indole-3 butyric Acid and NAA- Naphthalene Acetic Acid, 1:10) was applied to the exposed surface followed by encasing the area with moist sphagnum or tree moss and finally covering it with plastic and aluminum foil.

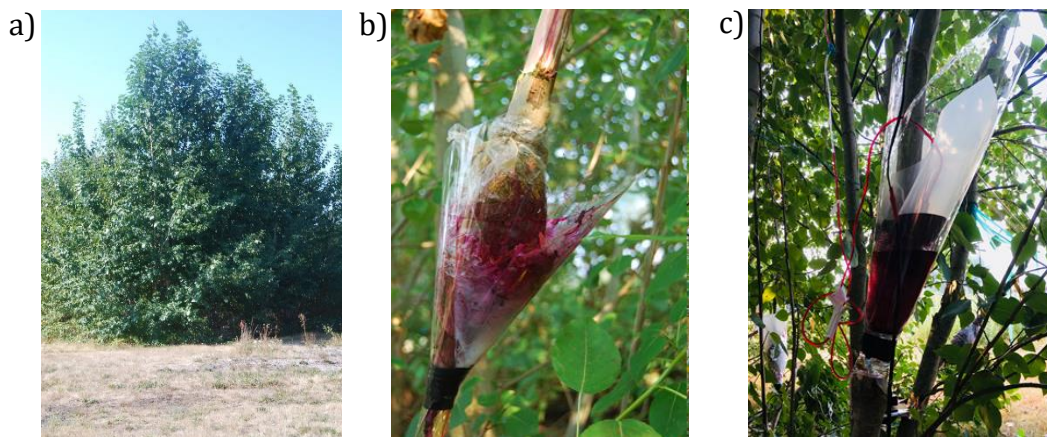


Fig 2. Dye-tracking experimental setup a) Poplar tree nursery located at the Center for Urban Horticulture (CUH), University of Washington, Seattle; b) Canopy-soil system surrounded by plastic cone setup; c) Canopy soil immersed in dye (acid-fuchsin) added via IV- bag tubing.

After roots were developed (after ~1 month), a system built with acetate-sheets in the shape of cones was installed around each branch holding canopy soil; each cone was located immediately below the system and was completely sealed at the bottom with silicone and

electric-tape around the trunk to prevent leaking (Fig. 2). Acid-fuchsin dye solution (0.05% w/v; molecular weight = 585.52 g/mol) was added slowly to canopy soil via IV-bag tubing system until saturated (~500ml). Dye movement was tracked *in vivo*, removing the bark at intervals and recording the rate of the dye movement for around 5-hrs after the dye was applied. Experiments started around 900-1000hr in the morning. We peeled the branch (i.e. removing all the bark and periderm around the stem, leaving exposed the cambium) starting as close as possible to the canopy soil (up – and downstream) along the branch until evidence of dye-traces faded, then waited for ~15-20mins and repeated the same, starting from where it was stripped the last time. This experiment was repeated four times (from 2014 to 2018, one experiment per year) in summer (26-30C) on 8-10 trees each time. Besides rate of dye movement, data collected were entirely observational, counting and noticing only presence or absence of dye traces, down and up-stream of the branch holding canopy soil and its trunk.

2.2 Greenhouse experiments

To investigate reverse flow and canopy hydraulic redistribution we assessed the direction and the velocity of sap flow within the tree using two methods: 1) isotopic labeling, measuring the isotope concentration of water - deuterium to hydrogen (δD) in vascular tissue as it changed with distance, direction and time from irrigation, complemented with 2) sap flow sensors applying the Heat Ratio Method (HRM) to measure direction and velocity of sap flow from canopy to other plant organs.

2.2.1 Experimental design

This study was performed on *Populus x canescens* (grey poplar) trees, a hybrid between *Populus tremula* (white poplar) X *Populus alba* (common aspen). This rapidly growing, woody species was induced to develop adventitious roots on its branches and was relatively easy to propagate using stem cuttings. Cuttings (30-40cm) were initially produced at the R. L. Goss Farm at the WSU Puyallup Research, WA and later transported to the Douglas Research Conservatory greenhouse at the Center for Urban Horticulture, University of Washington (47°

39° 27' N, 122° 17' 21" W; 10 m elevation), Seattle, WA. They were transplanted into potting soil (Sunshine Mix #4 – Sun Gro Canada Ltd) using 37.8l blow-molded plastic pots and were grown over a two-year period (1.5m height) in the greenhouse. The average air temperature was 22/19C, 16/8h day/night, supplemented with high pressure sodium light (400 W single phase bulbs, Phillips Electronics North America Corp., Andover, MA, USA) to compensate for the lack of sufficient sunlight during the winter-time. The average daily light integral (DLI) was 9.5mol m⁻² d⁻¹ of photosynthetically active radiation (PAR). The air temperature and light intensity were recorded by pendant type data loggers (UA-002-08, Onset Computer Corporation, Bourne, MA, USA) at 30-min intervals during the experiment period. Relative humidity was 48/54% day/night in average.

This experiment spanned 15 days and was repeated twice, in 2017 and 2018 during the summer season (end of August – beginning of September). Thirty-three trees were randomly selected and subjected to two different watering regimes: **1)** Well-watered trees (referred as *WW*) were watered to saturation point every-other day ($n=2$ for isotope and sap flow experiment), and **2)** Dry-wet trees (referred as *DW*) were water-restricted at the pot level but were water supplied at the canopy level through canopy root systems ($n=8$ for isotope experiment; $n=7$ for sap flow experiment); each system (three systems per plant) was connected to a 1000ml IV-bag that was refilled every other day. To maintain the soil completely dry, we used heavy-gauge (4mm) plastic sheeting that fully covered the pot from the stem to the ground. Soil irrigation was suppressed at the pot level for the entire experiment, and tap water was added every-other day through IV-bags connected to canopy soil. Each poplar tree had three different canopy soil systems connected to individual IV-bags, that later were also used to supply isotope solution (Fig. 3).

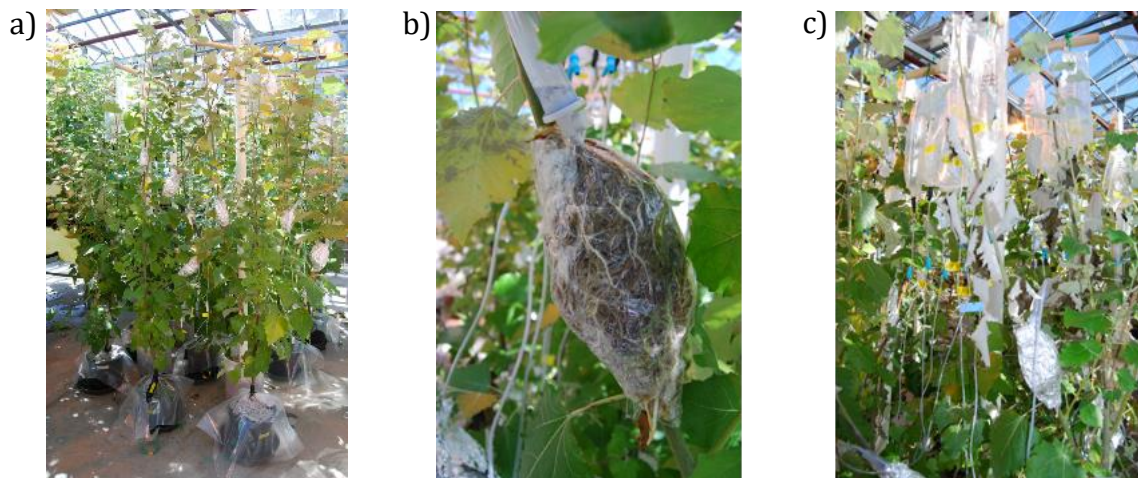
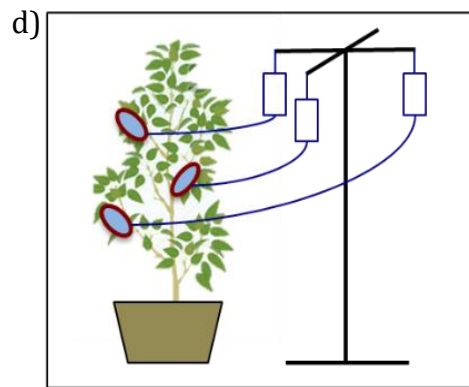


Fig 3. Canopy soil and Experimental design.

a) 2-yo poplar trees (*Populus* spp.) in the greenhouse, pots are covered with a plastic film to avoid soil wetting from dripping canopy systems and to keep soil underground completely dry; **b)** Canopy soil systems with canopy roots developed; **c)** IV-bags connected to each canopy soil system; **d)** Diagram of canopy soil systems connected to IV- bags as water supply. Each tree had three different canopy soil systems on separate branches with individual IV-bags.



2.2.2 Isotope labeling

Stable isotopes of hydrogen were used to track water movement from canopy soil to the host tree. Water labelled with deuterium (heavy water) was applied directly to canopy soil of DW trees ($n = 8$) via IV- bags tubing. The isotopic composition is expressed in delta notation (δD) relative to the V-SMOW standard. Labeled water (-1000 ‰) applied via canopy soil was composed of a mixture of tap water (-60 ‰) and water enriched in deuterium oxides (99.96%; Sigma-Aldrich, St. Louis, MO, USA). Samples were collected after two days of deuterium addition – on day 15. Within each DW tree, samples were collected from eight different areas

(Fig. 4): Canopy roots (R1), branch-upstream (B1: samples were collected 5 cm apart from CS), leaves from the same branch that hold canopy soil (L1), samples of trunk - upstream (T1) and downstream (T2), leaves from a neighbor branch (L2), neighbor branch sample (B2) and ground soil roots (R2). All branch samples collected were first-order and fully suberized with no green (photosynthetic) tissue, to avoid any possible transpiration effects. Samples of lower-height pools (i.e., ground roots and trunk down-stream) were collected to test whether deuterium from canopy soil was transported to lower parts of the tree by reverse flow. Control samples were collected from WW treatment ($n=2$), from the same locations as DW, except for the canopy soil samples (they did not have canopy soil developed). Deuterium was supplied around 1700h on day-13, making sure that all three-canopy soil systems were completely saturated while avoiding dripping or leaks that could reach pot-soil.

Comparisons of isotopic enrichment were done between DW as treatment and WW as control for each sample in each location ($n= 8$). Every-other day, WW plants were irrigated directly from the pot with tap water until saturation (1000-1500ml), meanwhile DW plants were irrigated only via IV-bags connected to canopy soil systems (1000ml), except for the last two-days of experiment when tap water was replaced by labeled water (δD). Because DW plants were water restricted at the pot level (they were completely covered with plastic), isotopic enrichment found in other organs was considered evidence of canopy soil water redistributed throughout the tree. Ground roots were collected randomly from different spots in the pot, from the surface to the bottom of the pot (c. 20cm). All samples were stored in falcon tubes, sealed with parafilm and kept frozen at -80C before analysis.

Water from plant tissues was extracted by cryogenic vacuum distillation (Ehleringer et al. 2000, West et al. 2006). Samples in extraction vials were heated to 100°C and evaporated water was trapped in U-tubes submerged in liquid nitrogen. In this study, we used 1h of extraction time to ensure a fully unfractionated water sample. Finally, samples were sealed, placed in a bath and thawed prior to analysis. Analysis was carried out using an Isotope Mass Spectrometer (IMS) at the Center for Stable Isotope Biogeochemistry (CSIB), (University of California, Berkeley, USA). Results values (δD) obtained are expressed in ‰ on a V-SMOW scale.

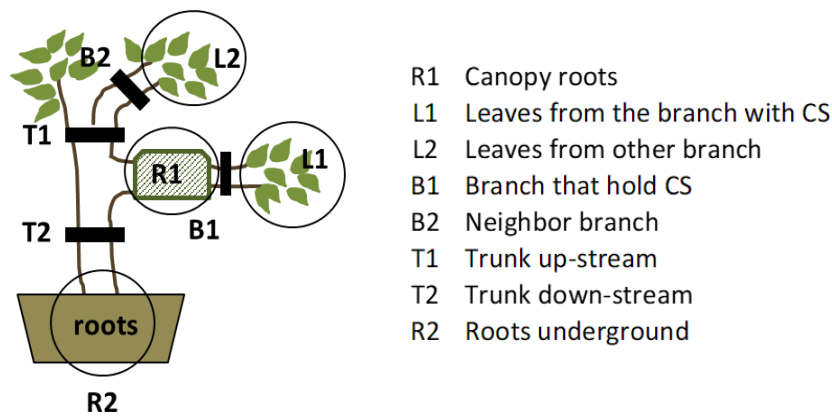


Fig 4. Isotope labeling enrichment. Six different samples along the tree body were collected 3-days after adding deuterium (δD) to canopy roots (R1). Samples were collected up- and downstream of canopy soil (including ground soil roots) to test whether canopy water could be transported to belowground regions (reverse flow). Samples were collected twice (2017 & 2018) for two treatments –WW and DW plants. Labeled water (-1000 ‰) applied to canopy soil was composed of a mixture of tap water (c.60 δD) and water enriched in deuterium oxides (99.96%; Sigma-Aldrich, St. Louis, MO, USA).

2.2.3 Sap flow measurements

For this experiment, a new sap flow system based on a heat-ratio method (HRM) (Burgess et al., 2001) was manufactured for the first time by Dotmote Labs, WA. This system is based on Arduino, which relies on an external heat pulse method (Clearwater *et al.* 2009) that has been validated previously in the greenhouse and outdoors settings. Details of the system are reviewed in (John et al. 2020).

Sap flow gauges were connected to a stand-alone miniature, Arduino-based data logger with wireless capabilities. The measurement protocol was based on the heat-ratio method as described in (Skelton et al., 2015). The heat ratio method entailed measuring the temperature of two equidistant sensors placed on either side of a heating element that transmits heat pulses lasting 10 seconds to the branch every thirty minutes. Each heat pulse heated the branch by

approximately 2-3°C. As the heat pulse decayed, the heat ratio was quantified as the change in temperature of the downstream sensor (Δt_1) divided by the change in temperature of the upstream sensor (Δt_2), known as the heat ratio (Fig. 5, a-b). A mean heat ratio was quantified at a point when the heat ratios were stable, which for our setup was the dissipation period between 55s and 75s, consistent with previous studies (James et al., 2002; Skelton et al., 2015).

Eleven sap flow gauges were set-up over a period of six days (day 6 – 11) of the 15-day drought experiment explained above. We installed one sensor per plant: Seven DW plants ($n=7$); two well-watered plants ($n=2$, WW) and two completely dry plants ($n=2$, DD). Sensors were installed externally in direct contact with the bark of the branch (diameters of <1 cm), immediately below the canopy soil systems (CS) proximal to the trunk (Fig. 5, c-d); for trees without CS, sensors were installed on random branches (<1 cm) at the same level of trees with CS. To reduce noise from electrical and temperature input, gauges were covered with plastic and aluminum foil to maintain them isolated and dry. Continuous measurements of sap flow were made daily (every 30-minutes) until the end of the experiment, as mentioned above. Some of the nodes experienced loss of data for few hours of measurements because of loose wiring and technical issues in the greenhouse (days 8-10 of the experiment).

We calculated the reference, zero flow value for these plants using the greenhouse environmental data collected over 5 days with air humidity and temperature sensors (Gemlink, QCom Control Systems, USA). We assumed that the sap flow was zero during nights between 2400h -0200h; mean heat ratio value during this period was used as a baseline for zero sap flow, individually for each plant. Sap flow velocity was then calculated according to Burgess et al (2001) and is expressed as Vh (cm/s), that represent the changes of velocity reached by each sensor during experiment. Thermal diffusivity (k) was set to 2.5×10^{-3} cm²/s (Marshall, 1958).

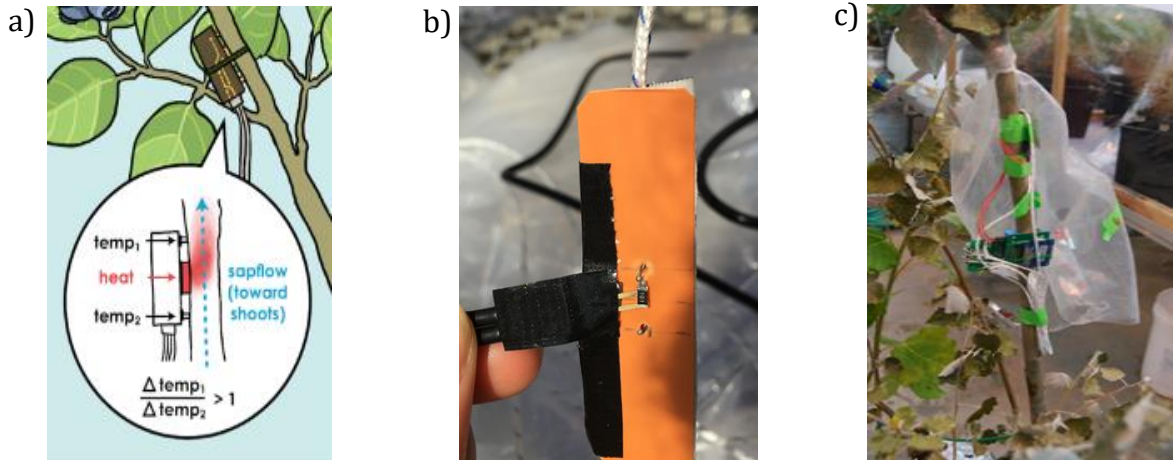


Fig 5. Sap flow sensor method. **a)** Diagram of miniature external sap flow gauges for capturing plant water movement (John et al. 2020, *in prep.*). **b)** Upside down view of one of the sapflow gauges – temperature probes and heater are visible. **c)** Sensor installed on a poplar tree branch. Sensors were covered with plastic for thermal isolation and water protection. **d)** Location where sensors were installed – directly below each canopy soil system (red arrow corresponds to sensor).



2.3 Data Analysis

Dye experiment data were mostly observational, counting and noticing only presence or absence of dye, down- and upstream of the branch and trunk. Mean percentage of number of trees that present evidence of dye per year was calculated as evidence to show canopy water absorption.

For isotopic labeling, δD values were expressed in ‰ on a V-SMOW scale. We also used the *IsoError* mixing model (Phillips & Gregg, 2001) to estimate the contribution in % of canopy soil water in different organs. These calculations consider the variability of both mixture and source: Water from canopy soil (-1000 ‰) and water from belowground soil or tap water (-

70‰). *IsoError* data was expressed in % with their respective standard error (SE) for each organ [Refer to Phillip and Gregg (2001) for detailed explanation of the calculations]. Since data did not meet a normal distribution, a Mann-Whitney U test (non-parametric) was used to determine if the isotopic composition of samples from DW plants were significantly different from WW plants, considering each collection point on the tree separately ($p < 0.05$).

Sap flow data based on HRM was first standardized over 5-days (for DW) and 2-days (for WW and DD) for each 24-hour period to data collected from 0000 to 0200, when we assumed zero sap flow. Then sap flow for each hour interval was calculated for each tree, and these values were used for descriptive statistics (mean, SE) across all trees in each treatment: $n=7$ (DW) and $n=2$ (WW and DD). This time series was plotted for days 6-11 of the experiment for DW trees, but only days 9-11 for WW trees because of technical issues during the experimental period.

3. Results

3.1 Field experiments

3.1.1 Dye-tracking experiment

By tracking acid-fuchsin dye over a period of time after it was added to canopy soils, we observed clear evidence of dye movement within the tree towards the leaves and the trunk, suggesting water uptake from canopy soil (either by bark or canopy roots) and showing that there could be bi-directional water movement (i.e. up- and downwards) within the tree (Fig. 6). We noticed dye movement over the branches in every trial (from 2014 to 2018, four total trials). However, dye-traces differed from each other in each trial, and the appearance time of traces highly varied between 2hrs to 4hrs among years; this could be correlated to the dye dissipation pace through the branch and vascular system and the effects of daily transpiration rate.

More than half of the trees tested in 2014 (66.6%) showed evidence of the dye moving upstream towards the distal end such as the tips of the branches and leaves, while in later years (2017 and 2018), the evidence of dye movement was lower (about 45-50%, respectively), and

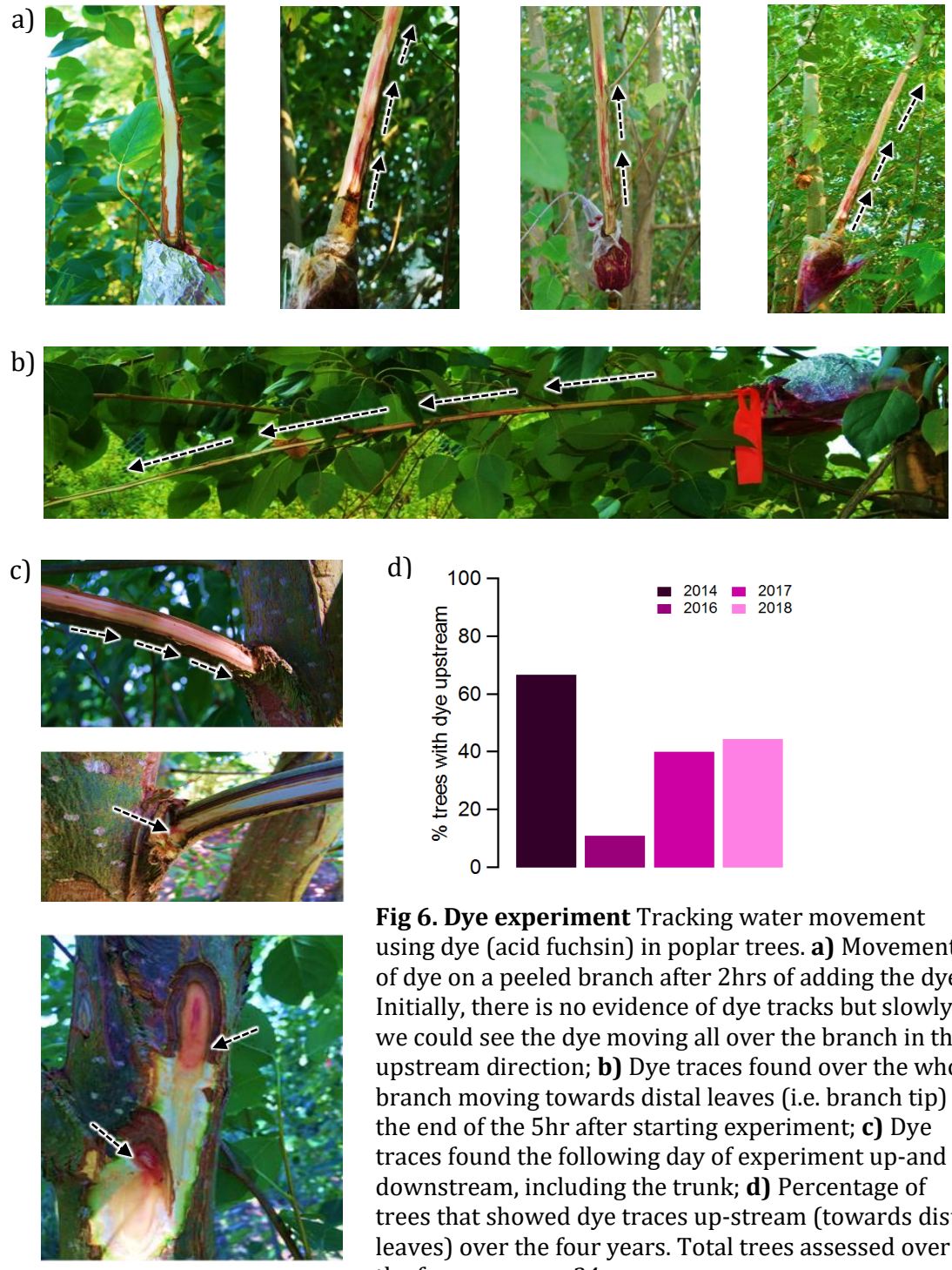


Fig 6. Dye experiment Tracking water movement using dye (acid fuchsin) in poplar trees. **a)** Movement of dye on a peeled branch after 2hrs of adding the dye. Initially, there is no evidence of dye tracks but slowly we could see the dye moving all over the branch in the upstream direction; **b)** Dye traces found over the whole branch moving towards distal leaves (i.e. branch tip) at the end of the 5hr after starting experiment; **c)** Dye traces found the following day of experiment up-and downstream, including the trunk; **d)** Percentage of trees that showed dye traces up-stream (towards distal leaves) over the four years. Total trees assessed over the four years $n = 34$.

was the lowest in 2016 with only 15% of the total trees tested (Fig.6d). The evidence of downstream dye movements (i.e. towards the proximal end of the trunk) signifying the reverse flow was present but scarcer; a total of five trees of 34 in the four trials showed traces of the downstream dye movement on the same or the next day after applying the dye (~14.7%) (Fig.6c).

3.2 Greenhouse Experiments

3.2.1 Isotope labeling

Isotopic labeling ratios varied between years, showing similar enrichment responses higher than tap water (-60‰) at the aboveground level in branches holding the canopy soil system (B1), leaves from the same branch (L1) and leaves and neighbor branch (B2 and L2, respectively). Since deuterium solution was directly added to the canopy soil, canopy root samples showed the highest enrichment values as expected, between -10 to -15‰ for both years, as they were the first organs to come into contact with heavy water. In this order, for 2017 the samples that showed the greatest enrichment after the canopy roots were the leaves (L1 and L2) with -20 to -30‰ and branch with CS (B1) with -22‰. Meanwhile, no enrichment was observed in the trunk downstream (T2) and belowground roots (R2), ranging between -58 to -62‰ respectively. There was no evidence of enrichment in control plants (WW), except for higher ratios in leaves (L1 & L2) around -40 to -45‰; this could be attributed to transpiration and fractionation of water by leaves (Fig. 7). Even though most of the enrichment values were higher in plants with CS (DW) than control plants (WW) in 2017, the only significant difference was found from the samples taken at the distal end of the branch with CS, that is B1 ($p < 0.05$) (Table 1).

Applying the two-source mixing model (Phillips & Gregg, 2001) using enriched water from canopy soil and tap water as the two different sources, we found that neighbor branches (B2) for DW plants in 2017 had around 9.6% of canopy soil water, meanwhile leaves from other branches (L2) ~35-36% and belowground components (roots underground and rhizosphere -R2) around 7% (Table 1).

In 2018, the mobilization and redistribution of enriched water was less evident, reaching leaves (L1 & L2) and branches (B1 & B2) ratios of -40 to -53‰ (aboveground level), and lower levels (i.e. T1, T2 & R2) values that indicated no enrichment ($< -60‰$). Control plants (WW) presented similar values as the previous trial, with no enrichment except for leaves (-40 to -45‰). According to the two-source mixing model, leaves and branch with CS were between 23% and 18%, respectively; and neighbor leaves (L2) and branches (B2) with 21% and 15%, respectively. Meanwhile, lower level organs had lower percentages of heavy water $\sim 2-5\%$. These results suggest a water distribution restricted to the canopy, however there is not statistical difference between DW and WW plants for 2018 for any of the samples (Fig. 7).

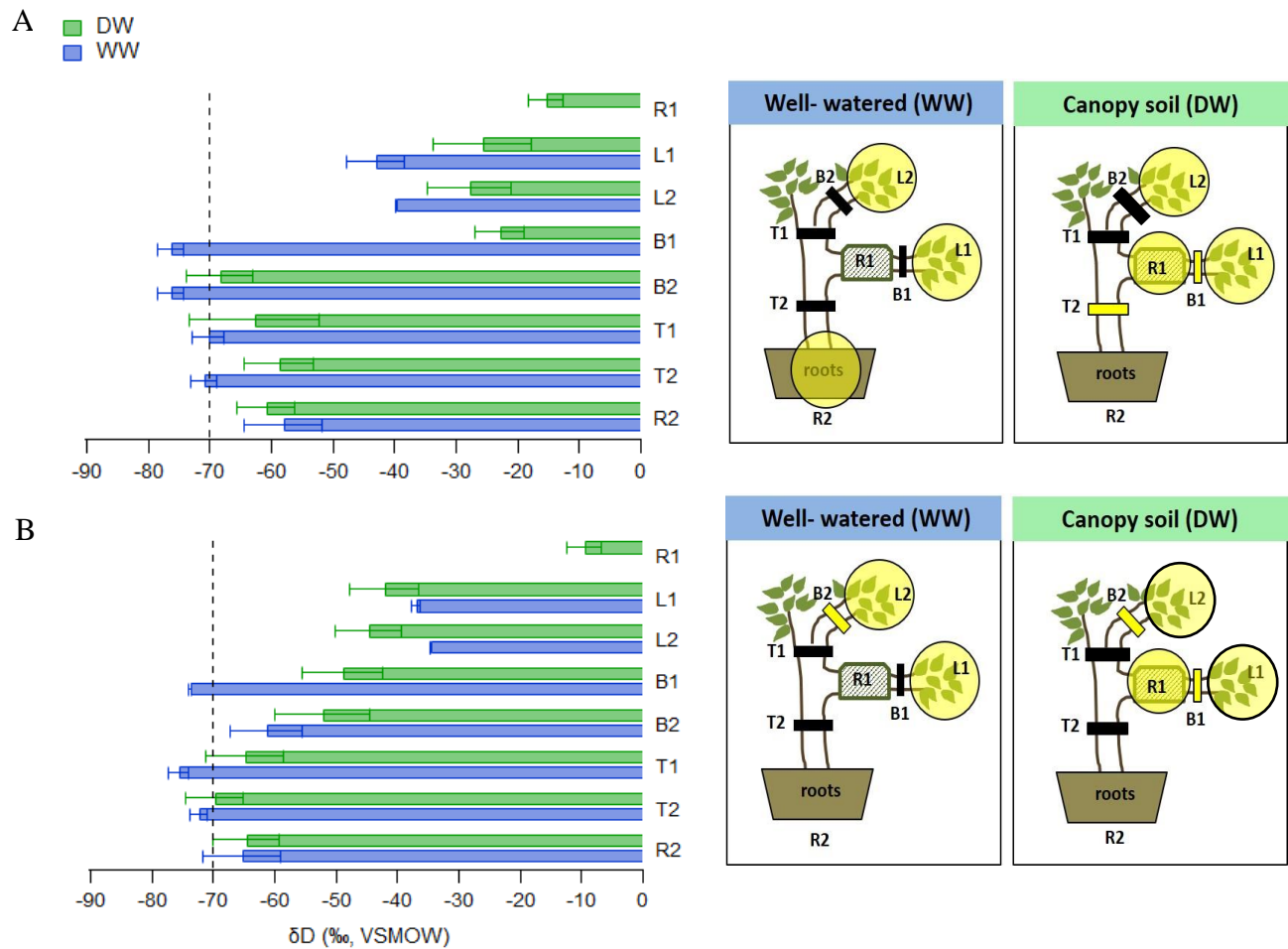


Fig 7. Isotopic labeling Deuterium (δD) enrichment in DW trees, directly added to canopy soil, compared to control plants (WW) that were not enriched and were watered every other day. Samples were collected at the end of the 15-day drought experiment, after trees were exposed to enrichment for three days. The experiment was repeated twice: A) 2017- top; B) 2018- bottom. To the right, diagrams correspond to the location on trees where enrichment was detected (yellow areas). Values are average of DW (n=8), and WW (n=2), and are expressed in ‰ on a V-SMOW scale. Enrichment was considered present for all samples that had a value above the standard (70‰) and their respective control (i.e., comparisons were made between DW and WW for each sample). Tap water (standard) = *dashed line*.

Year	Location	δD (‰)	SD	fA	sfA	p-
2017	R1	-15.4	6.26	0.45	0.01	0.026*
	L1	-25.8	17.91	0.36	0.04	0.304
	L2	-28.0	15.19	0.35	0.03	0.635
	B1	-22.9	8.82	0.39	0.02	0.05*
	B2	-68.5	12.28	0.09	0.03	0.179
	T1	-62.7	23.74	0.06	0.05	0.831
	T2	-58.9	12.52	0.09	0.03	0.887
	R2	-61.0	10.47	0.07	0.02	0.777
2018	R1	-9.6	7.85	0.50	0.02	0.0929
	L1	-42.3	15.83	0.23	0.04	0.513
	L2	-44.8	15.05	0.21	0.04	0.231
	B1	-48.9	18.42	0.18	0.05	0.231
	B2	-52.3	21.59	0.15	0.05	0.769
	T1	-64.9	17.96	0.04	0.05	1
	T2	-69.8	12.94	0.02	0.03	0.641
	R2	-64.6	14.98	0.05	0.04	0.769

Table 2. Isotopic labeling proportions Calculated proportions of two different sources: Tap water and deuterium (δD) in each sample collected at different locations from the DW trees after adding deuterium solution at the end of the 15-day experiment period. Locations correspond to the same used on Fig.4. Values of enrichment labeling (δD) are expressed in ‰ on a V-SMOW scale and tap water (standard) = -70‰; SD corresponds to standard deviation of $n=8$ samples for DW. **fA** is the proportion of deuterium water (%) in a sample relative to tap water and **sfA** its standard error (Calculations were obtained by *IsoError* two-source mixing model). Stars correspond to significant values relative to control treatment WW (**$p \leq 0.05$**)

3.2.2 Sap-flow measurements

Trees with canopy soil in DW treatment showed a different direction of sap flow (values below zero level) by day-8 and day10-11 compared to the control plants (WW), suggesting a possible reverse flow; this reversal pattern could be likely caused by water uptake from canopy soil, as DW plants were completely water restricted at the ground soil level, and their only water

supply was at the canopy soil. Plants in the other treatments (i.e., WW and DD) did not show noticeable evidence of reverse sap flow; well-watered (WW) plants reached the highest magnitudes of sap flow at noon (0.10 cm/s) and a relative constant flow within 0-0.025 cm/s the rest of the day (i.e. morning and afternoon). On the other hand, DD plants showed a brief but not prominent reverse flow (~ -0.025 cm/s) for a few hours and followed a mostly constant pattern the remaining days, reaching the lowest positive values of all the treatments around noon (max. 0.05cm/s) (Fig. 8).

By contrast, DW plants show a variable flow pattern reaching values of ~ 0.075 cm/s at noon (maximum) and possible reverse flow ~ -0.025 cm/s at nighttime (day8 – day 9). At the end of the experiment (day-10 to day-11), reverse flow is more pronounced and frequent compared to other days in the experiment (Fig. 8). The magnitude of sap flow reversals was $\sim 33.3\%$ of its own diurnal sap velocity.

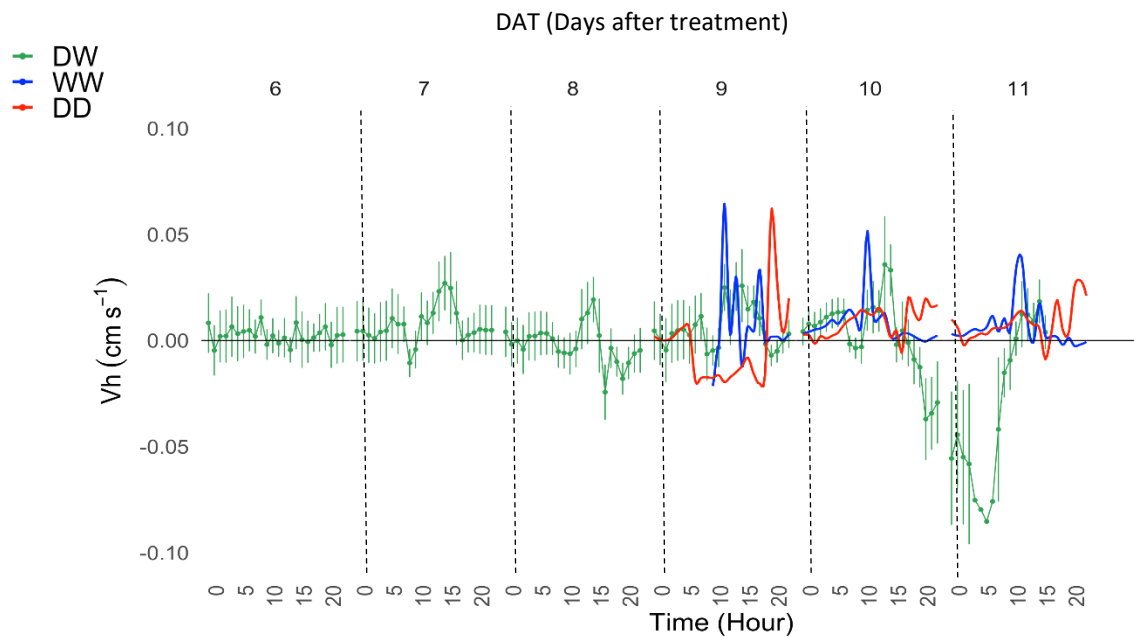


Fig 8. Sap flow patterns in three different treatments. DW (green line) corresponds to poplar trees with canopy soil and dry soil. Water was only added from canopy soil via the IV-bag system; WW (blue line) corresponds to poplar trees that were well-watered every other day during the entire experiment; DD (red) corresponds to poplar trees that were completely dry during the 15-day experiment. Data were normalized to flow magnitudes to allow comparisons among individuals of different treatments and different parts of the trees. These data correspond to the average of $n=8$ (DW) and $n=2$ (WW) treatment. The speed at which sap moves through entire sapwood area is referred as sap velocity (V_h cm/s). Reverse flow is indicated when V_h reach values below zero (red lines).

4. Discussion

4.1 First evidence: bi-directional water movement

Overall, dye experiments confirmed that water from canopy soil can be absorbed and transported not only to the distal branches but also there is back flow towards the trunk/stem suggesting that the dye movement can take place in more than one direction. Although results were variable each year, on average ~15% of the trees showed evidence of dye moving in both directions (distal ends and towards trunk), which supports reversal and possibly bidirectional flow from CS (Fig. 6). Dye-tracking experiments have been commonly used to evaluate hydraulic conductance from roots/trunk to canopy *in-vivo* (Choat et al., 2003; Demason et al., 2006; Salguero-Gómez & Casper, 2011), radial xylem flow in stems or atmospheric water uptake on excised leaves or branches (Eller et al., 2013; Goldsmith et al., 2017; Mayr et al., 2014), however it has never been before applied *in-vivo* in water uptake from adventitious roots or canopy soil. In the present study, this method presented high variability among years and individual trees, possible due to: first, the sap flow rate is determined by the rate of transpiration and the availability of water in the soil; most experiments were performed during the summer when the temperature ranged between 25 and 27°C, relative humidity varied from 50% to 80% and trees were not irrigated for a long period of time (~ 2-months). Although environmental factors were not too extreme to affect plant performance in a severe way, in many cases, the recorded stomatal conductance reached very low values ~34-77 mmol/m²s to maximum ~108-213 mmol/m²s, which on average indicates a very low transpiration rate, that could have affected sap flow overall and therefore the time of dye appearance.

Secondly, there was a chance of losing traces if the dye was transported through superficial vasculature (i.e., upper layers of the bark) while removing the bark during the tracking process and counted it as negative evidence. Previous dye-tracking experiments have reported that in hardwood species, due to their wood sectoriality, water travels preferentially through the outermost xylem vessels (Demason et al., 2006; Salguero-Gómez & Casper, 2011). Despite the variability and possible experimental errors, our dye experiment strongly suggests that there was bi-directional sap flow from the canopy soil evidenced by dye traces both on branches and on stems of multiple trees assessed.

4.2 Canopy internal hydraulic redistribution: from canopy soil to neighboring branches and leaves

Plant water movement usually is unidirectional and determined by water potentials gradients, from higher Ψ in the soil to lower Ψ the leaves. However, in this study we show that canopy soil water accounted for a proportion of the water in poplar trees not only at the canopy level, but also in lower-level organs, ranging from ~50% to ~10% throughout the whole plant. Also, we provide further evidence of the redistribution of CS water to different plant organs, most likely by reverse flow, as sap flow data suggested (Fig. 8).

Previous studies of foliar water uptake (FWU) have found that fog water contributed ~7.2% to 28% of the water source used by adult trees species in a tropical forest during the dry season (Fu et al., 2016; Liu et al., 2014). In the present study, the contribution of canopy soil water at the canopy level was estimated to range between 10% to 36% (Table 1) which is even higher than the estimates of the studies on FWU. There are two potential reasons for these higher estimates. First, the very low water availability at the ground soil level, which forced trees to use much more water from other sources available, in this case, water from canopy soil. Second, while FWU is determined by leaf absorption, CS water can be absorbed via canopy roots that are connected directly to the tree vascular system or directly from the bark, which is most of the time exposed to canopy soil moisture, hence facilitates more water diffusion.

Deuterium enrichment in distant leaves (L2) and branches (B2) from canopy soil, although not significant, suggest that trees can redistribute water at the canopy level to other organs under stressful conditions. It is noteworthy that water redistribution is not only restricted to the canopy (although most of it), but also in a lesser extent to lower-level organs proximal to the trunk (T2) and roots (R2) via downstream flux (Table 1). Previous studies have also demonstrated this type of water transport from the canopy to the ground and belowground pools (even moisten the soil close to the roots) (Burgess & Dawson, 2004; Eller et al., 2013; Goldsmith, 2013). This suggests that in addition to atmospheric water (e.g., fog, rain or dew) that can contribute to the plant hydraulic potential through FWU, there is a new mechanism to

redistribute water, either through canopy roots or through bark absorption from the canopy soil water, a novel source of water, especially for those trees that develop massive epiphytic mats.

Overall, we were able to see a similar trend between trials regarding the distribution of the isotopic enrichment - same ratio of individuals (~70%) tested each year showed enrichment in most of the samples collected, including canopy and belowground parts, while the other (~30%) showed enrichment restricted to the canopy level. There were significant differences on branches that hold CS (B1), although not for the remain organs sampled. Our observations showed a pattern of enrichment in multiple samples collected for DW around the canopy level, which suggests internal hydraulic redistribution to other branches in the canopy but further studies with a larger sample size and a higher enrichment level will be required to fully confirm it.

On a final note, surprisingly control plants (WW) that were not treated with deuterium showed certain degree of enrichment on their leaves and terrestrial roots. Both organs (i.e., leaves and roots) have naturally some level of fractionation; on leaves this is determined by the transpiration rate, where lighter molecules tend to be released faster over the heavier ones. In roots, the mechanism of fractionation is not well-understood yet, but hypotheses are related to root water diffusion pathways (not water uptake), symplastic movement through the endodermis and vascular bundle, and to some intrinsic metabolism in living cells (Dawson & Ehleringer, 1993; Ellsworth & Williams, 2007; Zhao et al., 2016).

4.3 Canopy soil water redistribution occurs by reversal flow

Dye experiments showed that there is not only water movement towards the distal ends of the branch, but also dye was moving towards the trunk; this qualitative observation suggested that there is not only one-direction movement, as SPAC establishes, but also that reverse flow may occur in the presence of canopy soils. This was further corroborated by the sap flow measurements. That is, our sap flow observations showed that there was “reverse flow” on DW plants (i.e., plants with canopy soil) by the last days of the experiment compared to WW plants

(i.e., control plants) (Fig. 8), supporting in this way the dye experiment observations of dye traces moving backwards.

It is known that water movement through plants is driven by water potential gradients - from high to low water potential; usually, underground soil has a higher Ψ_{soil} compared to the rest of the plant organs and the atmosphere, forcing water to physically move in one-direction. However, in this study, water potential gradients have been manipulated to simulate a natural scenario when there is lower water availability in the ground compared to aboveground, this means when soil water potential Ψ_{soil} is lower than the plant or atmosphere water potential, causing water to move in the opposite direction (i.e., reverse flow) overnight. This reverse flow was seen mostly in the last days (day-10-day15 after treatment) of the experiment when ground soil was ~80-90% drier than at the beginning of the experiment (day1-day5) and water deficit symptoms were more noticeable, however simultaneously to reverse flow, we could observe also positive sap flow during the daylight, indicating that water movement was also happening in a bi-directional way (i.e. up-and downstream) (Fig. 8). This pattern of reverse flow synchronized with the nighttime has been also observed in a couple of studies. Sap flow can travel downwards along the trunk or roots (Burgess et al., 2000; Forster, 2014; Nadezhdina et al., 2009, 2010) or as water absorption by foliage that can rehydrate stems and soils (Eller et al., 2013).

Differences in the timing of water uptake is determined by how wet the soil is and the atmosphere. Simonin (2013) reported that concurrent water uptake by the crown and the roots is most likely to occur when transpiration is interrupted by a crown wetting event (i.e. FWU). Under this scenario, stem water potential would be initially less than Ψ_{leaf} and Ψ_{root} as both leaves and roots would have direct access to water sources (i.e., atmospheric water and soil water content, respectively). In our study, humidity in the atmosphere was not considered as effective as the amount of water stored in the canopy soil. As the experiment proceeded, ground soil in DW trees continued drying (i.e. by soil evaporation and root water uptake), while the canopy soil always remained with a high-water content (i.e. fully saturated). Therefore, it was expected that at the beginning of the experiment, the trees depend entirely on ground soil water content to refill water lost by transpiration, but as time went by, the water available in the ground soil decreased

and thus, the resistance and tension required to uptake water became higher and water transport towards the leaves was more difficult.

In contrast, the water movement from the canopy soil to other branches required less energy and became more noticeable (Fig. 8), which explains the reverse flow into the last stage of the experiment. It is important to underline that the distance from the water source to the sink is also a decisive factor in water transport. Energetically, the fact that the canopy soil is closer to the leaves, facilitates the movement of water in the opposite direction to where it is most needed, initially towards the leaves to maintain carbon assimilation, and later, to the stem and ground roots. This is consistent with Berry et al. (2018) and Steppe et al. (2018), who determined that net water flow is governed by the water potential gradients and capacitance of each individual tissues. Thus, the flow rate is higher to tissues with larger capacitance and lower water potential. Steppe et al. (2018) demonstrated that FWU induce radial stem growth driven by changes in cell turgor pressure, suggesting that water sources different from ground soil may also serve to recharge wood capacitance, confirming reversal flow through stems and trunks. In some cases, water absorbed from foliage can be released into the soil (given a sufficient water potential), it is possible a complete reversal of the SPAC (Berry et al., 2019; R. Goldsmith, 2013; Simonin et al., 2009; Steppe et al., 2018).

Even though foliar water absorption and canopy soil water uptake are based on the same concept and probably occur under similar conditions, environmental factors affect them in distinct ways. Usually, severe drought will cause stomata closing on leaves, reducing conductance and therefore altering mesophyll conductance, which eventually will limit water movement within the leaf (Zhou et al.; 2013). On the other hand, high vapor pressure deficit (VPD) and long periods without rainfall will contribute to the rapid drying of canopy soils (20%-40% water content) due to its high fiber content and absence of mineral soils. However, canopy soil is also made of extensive amounts of live mosses, non-vascular plants and decaying organic material that allows it to hold up to 20 times its weight throughout the wet and misty seasons (over 70% water content)(Enloe et al., 2006; Forest et al., 1995; Tejo, 2013).

We measured sap flow velocity as an indicator of water movement direction and frequency of water uptake, but not the magnitude. Based on FWU, the ratio of water absorbed depends on the atmospheric VPD, when water potential gradients into leaf/soil can be satisfied during high humidity periods (i.e low VPD, high $\Psi_{\text{atmosphere}}$). This occurs in nature during dry periods between rain events, during dry seasons or in water limited ecosystems. In the experimental approach used here, the magnitude of water absorbed may have relied on two aspects: first, water availability in the canopy soil to refill the amount of water lost by transpiration, when ground soil water availability was critical and the plant switched to the alternative water source; and second, the ability and amount of canopy roots developed and the bark diffusion rate to uptake water from aboveground. Dry-biomass observations showed that on average canopy soils retained approximately 81.92% water of their dry-weight (data not shown).

Finally, according to the sap flow observations we can suggest that there is a new type of hydraulic redistribution occurring in trees that presented canopy soil – the difference in this case is that canopy soil water potential ($\Psi_{\text{canopy soil}}$) becomes higher than Ψ_{soil} (ground soil), not Ψ_{leaf} , this generated a sufficient water potential gradient that we noticed as a reversal signal by day-14 and day-15 (Fig. 8).

4.4 Conclusions

In summary, in this study it was possible to confirm that water can be absorbed from CS, either by roots or bark (Fig. 6). Furthermore, we found a similar deuterium enrichment pattern in both years of experiment, suggesting that the water stored in the CS is internally redistributed through the xylem to different organs throughout the tree, mostly at the canopy level (i.e. branches with canopy soil, leaves and neighbor branches) (Fig. 7). Finally, this hydraulic redistribution was confirmed and supported by reverse flow happening over night by the last stage of the experiment when plants were experiencing the most severe effects of drought (Fig. 8). In alignment with FWU studies, this implies that the SPAC model is not unidirectional and that the water column within the xylem can move both reverse and bidirectional.

Canopy soil water uptake has been reported indirectly in a study about mineral uptake and translocation above-and belowground in *Salix syringiana* (Nadkarni & Primack, 1988) and as far we know, this is the first evidence of water absorbed from canopy soil, facilitated by canopy roots and redistributed throughout the tree. The observed sap flow reversal from canopy soil, qualitatively with the dye experiments and quantitatively with the sap flow sensors demonstrates the usefulness of these techniques to track water movement *in-vivo*. Even though dye-tracking experiments are commonly used to explore hydraulic pathways (Demason et al., 2006), this was the first-time using this technique to track water movement at a canopy level from canopy soil. Although we could confirm that the dye was transported to different regions of the tree, it is still necessary to implement a more controlled and precise system that reduces the degree of experimental errors (i.e. leak risks or removal of dye-evidence) and allows a more quantitative approach.

On the other hand, reversal and bidirectional flow has been shown previously using HRM. In this study we used a new sap flow technology based on John et al. 2020; this sensor has multiple advantages, it is inexpensive to manufacture, low-cost and minimally invasive, proper for the size of poplar branches that we used, however this was the first time they were applied into a study, therefore many experimental errors still need to be repaired and considered for future experiments.

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CHAPTER 4

Canopy soil – an additional water source for plants under drought stress

Abstract

Multiple plant species can absorb water through their leaves and/or through bark under specific circumstances (e.g. rainfall or fog), that could locally relieve water tension in branches. Some tropical and temperate trees accumulate living and dead plants over branches that eventually develop into a large mat of organic matter in the canopy, known as canopy soil (CS). These trees may sprout adventitious roots underneath these canopy soil mats; however, water uptake from canopy soil, either via canopy roots or bark absorption has not been confirmed or measured.

This study examined the effect of CS on water relations and plant performance of trees under drought conditions. To determine whether canopy soil provides functional benefits such as alleviation of water deficit in trees, a model system was developed using young poplar trees (*Populus spp.*). Air-layering technique was used to induce canopy roots developing into root media wrapped around branches, which mock canopy soil. A series of greenhouse experiments performed over three years tested the contribution of this canopy system under three water status treatments – well-watered, dry, and dry with wet canopy soil over a 15-day experimental period. Plant water relations and photosynthetic parameters were measured. We observed that trees with canopy soils were able to take up water from the aerial root medium and redistribute it through the plant, mitigating the effect of water loss via transpiration and reduced water potential. This in turn sustained photosynthetic activity in droughted trees with a canopy water supply. We infer that canopy soil and roots facilitated an additional water source that helped decouple the dependence of canopy water status on belowground soil water content. Development of this

model system provides the first evidence that water uptake via canopy soil can mitigate the deleterious effects of soil water deficits in trees.

1. Introduction

Plants, especially tall trees, not only faced with physical constraints throughout their lives, they must also overcome numerous environmental limitations, one of the most important of which is soil water availability. Variations in soil water content affect many aspects of plant growth and physiology (Chaves et al., 2003; Sperry et al., 2012). Not only are plant water relations parameters (e.g. leaf water potential - Ψ_{leaf} , hydraulic conductivity, turgor pressure) determined by soil water availability, but also gas exchange, carbon assimilation, growth and plant distribution are dependent on the water content (Prior & Eamus, 2000; Stephenson, 1990; Tyree & Zimmermann, 2002b). Water relations of plants are understood generally through a widely accepted conceptual framework that describes unidirectional movement of water through a plant, with soil water as the only source. Water is transported through the soil-plant-atmosphere continuum (SPAC), from roots through stems to leaves (Berry et al., 2014; Eller et al., 2013; Goldsmith, 2013). Thus, over the lifetime of a plant, water uptake by roots is equal to water loss from leaves, with the result that plant water potential (Ψ_{plant}) equilibrates with soil water content (Snyder et al., 2003; Tyree & Zimmermann, 2002a).

While it is true that large trees can access water from deep in the ground during dry periods, the physical difficulty of moving water to a high crown is thought to be a major limitation to growth in trees (Koch & Fredeen, 2005). Thus, a water reservoir accessible to the crown without fighting gravity could help alleviate the hydraulic constraints. Although SPAC assumes mass balance between root water uptake and leaf water loss, multiple studies suggest that at the canopy level, plants can also take up water from the atmosphere [e.g. foliar water uptake (FWU) or water bark absorption] (Berry et al., 2019; Breshears et al., 2008; Burgess & Dawson, 2004; Earles et al., 2016; Limm et al., 2009; Mayr et al., 2014a; Oliveira et al., 2005; Simonin et al., 2009). This new pathway contradicts the general perception of the SPAC by establishing water movement from the leaves to other tissues including belowground pools (roots) in an opposite direction (reverse or bidirectional flow). If plants possess mechanisms to

obtain water from sources other than the underground soil, then leaf water potential will no longer be constrained solely by the soil water moisture.

An important and noticeable component of tree canopy environments in many forest ecosystems is the large accumulation of epiphytic biomass composed of vascular and non-vascular plants (e.g. mosses, lichens, bromeliads and ferns) on top of the tree branches. These organisms can retain and store water and nutrients from rainfall and dry precipitation, accounting for ~92% to 365% dry weight of the epiphyte mats, also called canopy soil (CS), during dry and wet periods respectively (Köhler et al., 2007). Additionally, it has been reported that these large epiphyte masses ameliorate temperature fluctuation and moisture loss in tropical and temperate tree crowns through evaporative cooling (Freiberg, 2001; Gotsch et al., 2016; Stuntz et al., 2002). Due to their composition, abundance and potential role in ameliorating climate impacts, the canopy soils as an additional source of moisture and nutrient resources may be important to be included in predicting the climate impacts on forest ecosystems (Díaz et al., 2010; Forest et al., 1995).

As epiphyte communities colonize, grow, and accumulate dead organic material, more canopy soils are formed, and with them the initiation and elongation of adventitious roots from the branches. This natural aerial rooting is the basis of a well-known propagation technique “air-layering” used by horticulturists to propagate rooted cuttings, generally consisting of peat moss to serve as “soil” to stimulate root growth along stems or branches (Hartmann et al., 2011). Nadkarni (1994) used this experimental approach in *Senecio spp.* to identify the specific stimuli that promote canopy roots. This study suggested that moisture and darkness alone are not sufficient to induce aerial roots, but also it is necessary the inorganic nutrients released by the epiphytes (canopy soil). The development of roots over the bare bark provides a better substrate for epiphytic colonization and accumulation. Eventually these plants will decay and generate canopy soil, thus creating a positive feedback between the host trees and their epiphytes (Nadkarni, 1994).

Multiple aboveground uptake mechanisms of aerial water sources have been observed for at least 300 years (Hales et al., 1727). Foliar water uptake (FWU) has been one of the most

studied and recognized as an additional water source that could mitigate the effects of water deficit (Berry et al., 2014; Cassana et al., 2016; Eller et al., 2013; Nguyen et al., 2017; Simonin et al., 2009; Steppe et al., 2018). Even though canopy soil could be a potentially significant extra-water source, this has not yet been documented. To evaluate the effect CS has on tree physiology, we used poplar trees (*Populus spp.*) as a model species by applying an air-layering technique to induce canopy roots and assess the extent to which plant water and carbon relations may be decoupled from the soil water availability when canopy soil is present as an additional water source. *Populus* species in general have high transpirational demand and are among the most sensitive woody plants to water stress. On the other hand, they are particularly productive (i.e. large leaf and root production) and easy to root for propagating vegetatively (Marron et al., 2003; Rhodenbaugh & Pallardy, 1993). Further, due to their water requirements, they are excellent subjects for studies of water balance, particularly for determining adaptive responses by which they avoid severe water deficits (Pallardy & Kozlowski, 1981).

In the present study, we assessed the ecophysiological effects of canopy soils as an alternative water source in *Populus sp.* We hypothesized that water uptake from the canopy soil is a viable water acquisition mechanism which can improve water status and maintain carbon balance under water deficit conditions. We conducted multiple ecophysiological measurements under greenhouse conditions to investigate how canopy soil influences plant water relations and overall plant performance. We demonstrated for the first time that water from canopy soils is absorbed via the canopy root system, and then internally redistributed to maintain water status and plant physiological functions under water deficit conditions.

2. Material & Methods

2.1 Plant Material

This study was performed on *Populus x canescens* (grey poplar) trees, a hybrid between *Populus tremula* (white poplar) X *Populus alba* (common aspen). Stem cuttings (30-40cm) were initially grown at the R. L. Goss Farm at the Washington State University Puyallup Research &

Extension Center, WA and later transported to the Douglas Research Conservatory greenhouse at the Center for Urban Horticulture, University of Washington (47° 39' 27''N, 122° 17'21''W; 10 m elevation), Seattle, WA. They were transplanted into potting mix (Sunshine Mix #4, Sun Gro Canada Ltd) using 37.8l blow-moulded plastic pots and were grown over two-year period (1.5m height) in the greenhouse. The average day/night air temperatures were 22/19C with supplemental lighting to maintain a photoperiod of 16/8h day/night. The average daily light integral (DLI) was 9.5mol m⁻² d⁻¹ of photosynthetically active radiation (PAR). Relative humidity was 60-80% on average.

To develop aerial canopy roots on branches, three small branches per tree (3 to 4 cm in diameter) were randomly selected from ~ 50 to 100 cm above ground. The selected branches were about 60-80cm long with leaves along and at the tips. To induce formation of canopy roots, we used an air-layering technique. We first removed bark of a 2 to 5cm section length (2-5mm depth) on a branch to expose the cambium, applied liquid rooting compound (1:10) [Indole-3 butyric Acid (IBA) and Naphthalene Acetic Acid (NAA) -Wood's Rooting Compound, OBC Northwest, Inc., Canby, OR] using a brush, and encased the cut surface with moist sphagnum peat moss, and finally covered with plastic wrap and aluminum foil to create a dark and moist environment. Adventitious roots were fully developed after 1.5months.

2.2 Experimental design

Each experiment spanned 15 days and was repeated three times - during the fall (2015) and during the summer (2017 and 2018). Fifteen trees in 2015 and thirty-three trees in 2017 and 2018 were randomly selected and subjected to three different watering regimes ($n=5$ per treatment in 2015; $n = 11$ per treatment in 2017 and 2018): 1) Well-watered trees (referred as *WW*) were watered to saturation every-other day, 2) Dry trees (referred as *DD*) were water-restricted over the entire experiment period. Lastly, and 3) Dry-wet trees (referred as *DW*) were water-restricted at the pot but watered through the canopy root systems. Each system (three canopy root systems per plant) was connected to a 1000ml IV-bag that was refilled every other day (Fig. 1). To maintain the soil completely dry, we used a heavy-gauge (4 mil) plastic sheet to cover each pot,

from the stem to the greenhouse floor. The following parameters were measured daily or every-other day: soil moisture tension (kPa), stomatal conductance (g_s), chlorophyll fluorescence (F_v/F_m), leaf water potential (Ψ_{lw}) and carbon assimilation ratio (A).

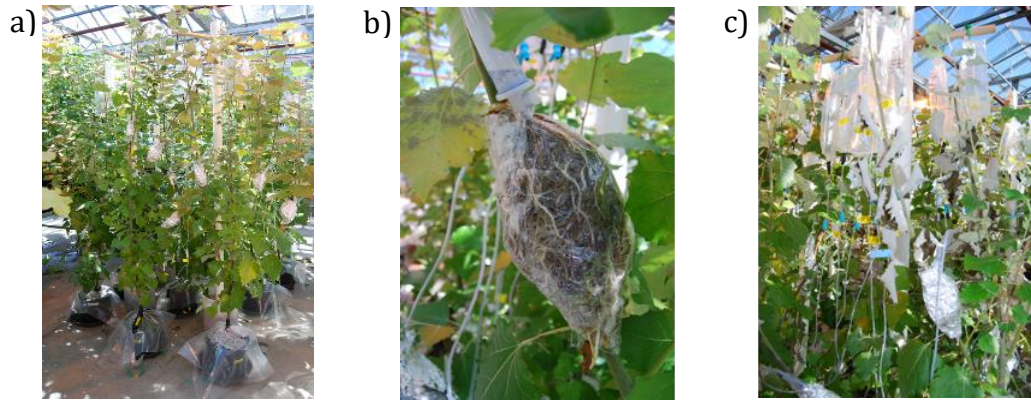
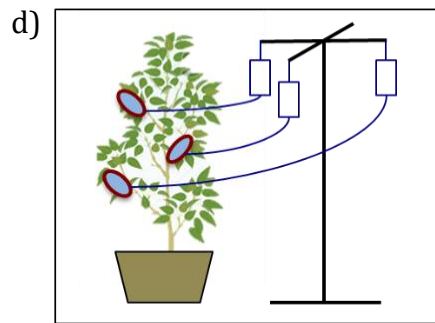


Fig 1. Canopy soil and Experimental design. **a)** 2-yo poplar trees (*Populus* spp.) in the greenhouse; pots are covered with plastic to avoid dripping from canopy systems and to keep soil underground completely dry **b)** Canopy soil systems with canopy roots developed **c)** IV-bags connected to each canopy soil system **d)** Diagram of canopy soil systems connected to IV- bags as water supply. Each tree had three different canopy soil systems on separate branches and each one corresponded to an IV-bag individually.



2.3 Plant water budget

2.3.1 Soil moisture tension

To understand the impact of canopy soil on the water status of trees under drought conditions, we assessed water-related parameters in different compartments of a tree. To estimate water status at the soil level, we measured soil water tension (kPa) for each tree every morning (0900-1000 hours) using soil moisture sensors (Watermark, Irrrometer, Riverside, CA). Sensors were permanently placed into the soil at 15-20cm depth from $d=1$ to $d=15$ of the experiment.

Data are given in tension units of kPa, where higher values (less negative) correspond to less resistance of water absorption, thus higher soil water content. Lower (more negative) values correspond to greater resistance between the soil particles and the roots, thus lower soil water content.

2.3.2 Leaf water potential (Ψ_{lw})

Every two days, we measured midday (1200-1400hours) leaf water potential (Ψ_{leaf} , MPa) of three leaves from each tree, using a Scholander pressure chamber (model 1000: PMS Instruments, Albany, OR). Midday Ψ_{leaf} is thought to represent the water status of trees at the time of the day of greatest water stress (Bhaskar & Ackerly, 2006). Fully mature leaves were selected randomly from different branches of the tree and water potential values were averaged. Water potential values of trees under *DW* treatment were averages of leaves samples collected on branches with and without canopy soil.

2.3.3 Stomatal conductance (g_s)

Measurements of stomatal conductance and transpiration rate were made daily between 800-1200 hours using a steady state leaf porometer (SC-1, METER Group, Inc. USA). Youngest, fully expanded leaves were selected randomly (two leaves per tree), one each from the lower and upper canopy.

2.4 Photosynthetic performance parameters

2.4.1 CO_2 assimilation rate (A)

We measured leaf net CO_2 assimilation rate (A) in 2017 and 2018 (every other day) during the experimental period with a portable gas analyzer system (LI6400XT, Li-Cor, Inc., Lincoln, NE, United States) between 800 and 1300 hrs. We chose from each pot, one fully expanded, non-shaded leaf from the canopy (at ~1m height). CO_2 assimilation rate (A) measurements were taken from 2 to 5 trees per treatment, with settings of $1500\mu\text{mol m}^{-2}\text{s}^{-1}$ PFD for saturating light intensity, 400 mmol mol^{-1} of $[CO_2]$ as reference, $300\mu\text{mol s}^{-1}$ flow rate. Leaf

temperature and relative humidity of the chamber were maintained at 20°C and 40-60%, respectively. The leaf samples were clamped onto the chamber head and exposed to the environmental settings above for 10 mins before measurements.

2.4.2 Photochemical efficiency of PSII (F_v/F_m)

As a parameter to assess photosynthetic stress experienced by the poplar trees, we used the maximal photochemical efficiency as an indirect measure of the amount of damage in the photosystem II (PSII). We performed these measurements using a portable fluorometer OS-30P+ (Opti-Sciences, Inc., Hudson, NH, USA). We selected randomly three subsamples per tree (2015: $n=5$ trees per treatment; 2017 and 2018: $n=11$), each one from a different height of the body of the tree (lower, middle and upper canopy) and averaged them per replicate. The samples were dark-adapted for 60-min before taking minimal fluorescence (F_o) followed by a saturated pulse of light flash to obtain maximal fluorescence (F_m). Variable fluorescence, $F_v = (F_m - F_o)$, was calculated by a built-in program to estimate maximal photochemical efficiency of PSII (F_v/F_m).

2.5 Data analysis

Each of the five physiological parameters was averaged by tree and by day in each experimental trial. In each trial, trees were clustered rather than interspersed (potential environmental gradient along a greenhouse was not considered), so we first took the following approach to see if position in the greenhouse influenced response variables. First, we considered that the grid of tree placement consisted of three areas, so each tree in each trial was categorized according to its position (one of the three categories). Then we tested whether any of the response variables differed by position, in comparisons of DD and DW trees; specifically, testing for fixed effects of canopy water, position, and their interaction. In no case was position significant, so we dropped it from additional tests. Due to the treatment-clumped design, we used a mixed effect model to assess positioning of the treatments effect on variation; this was not found to be significant ($\alpha = 0.05$ level). Analysis of variance (ANOVA) was applied to each

response variable based on a two-way factorial design. For each variable, we compared three different treatments (i.e. DD, DW and WW) on each day of the experimental period when the variable was measured, treating trial as a random factor, and fixed effects of treatment, time, and their interaction. Although the trials were analyzed in aggregate, we display the results separately to evaluate consistency among trials. Additionally, statistical tests were carried out for each trial-day separately, comparing only DD and DW treatments from *Day 8 –Day 15* (when we started to see differences among treatments) to determine at what point (if any) canopy water delivery modified the response of plants growing in dry soil. Analyses were carried out with RStudio version 3.2.2 (R Development Core Team 2015).

3. Results

3.1 Effect of canopy soil on plant water budget

3.1.1 Soil moisture tension (kPa)

Throughout the measurement period, soil moisture tension values followed similar trajectories in the three years (Fig. 2). Soil moisture tension remained high for the WW treatment between 0 and -25 kPa over the experimental period. For both DD and DW treatments, soil moisture tension had a similar variation pattern as WW treatment in the first 4 to 5 days after treatment (DAT) but began decreasing by ~6-7 DAT reaching values between -40 and -80 kPa. The main differences among treatments were seen after 7 DAT, where soil moisture tension in DD and DW decreased considerably compared to WW for all years. For the first two years (2015 & 2017), DW had less negative soil moisture tension compared to DD during the entire experiment –meaning higher soil water content than DD. This could be due to water redistributed from canopy soils to the ground (i.e. reverse hydraulic redistribution) or a reduced water uptake from the soil given the presence of an extra water source at the canopy. However, in 2018, DW and DD treatment did not show significant difference during the experimental period.

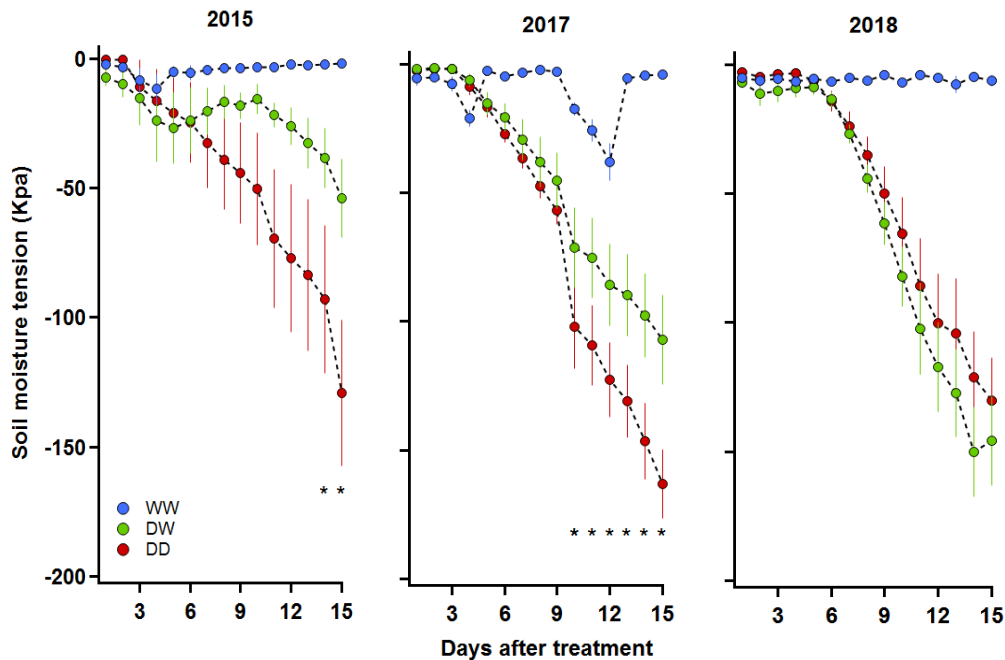


Fig. 2 Soil moisture tension (kPa) during 15-days experiment repeated three different years (2015: $n=5$ trees per treatment; 2017 and 2018: $n=11$). Points represent the means \pm SE. High soil moisture tension (higher values) represents more resistance or tension for plants to uptake water (i.e. lower soil water availability). Plants treatments were well-watered (WW), water withheld from the soil (DD) and water restricted at pot-level but provided via canopy soil (DW). Stars represent the level of significant difference between DW and DD per day: $P \leq 0.05$.

3.1.2 Stomatal conductance (g_s)

For all the three trials, trees with (DW) and without canopy soil (WW & DD) had consistent trajectories for stomatal conductance over the experiment period (Fig. 3). Stomatal conductance (g_s) values were similar for the first 6 DAT, except in 2018 where plants in all treatments behaved similarly until 8-DAT. After ~ 7 -8 DAT, considerable differences were evident among treatments; overall WW leaves had higher values of stomatal conductance, reaching the highest g_s ($> 600 \text{ mmol m}^{-2} \text{ s}^{-1}$) in 2015, compared to the other two years. On the other hand, DW and DD leaves showed similar variations of g_s for the three years, with a greater difference in 2015. Stomatal conductance for DW remained higher than DD in 2015 and 2017,

suggesting both: sustained transpiration rate for DW leaves despite having similar drought conditions as DD plants, and a positive effect on plant water relations due to the canopy soils. However, in 2018, as shown for soil moisture tension, the differences between treatments were reduced, reaching similar g_s at the end of the experiment. The greatest significant differences among treatments were seen in 2017 (10-DAT to 15-DAT), while 2015 and 2018 had lesser differences.

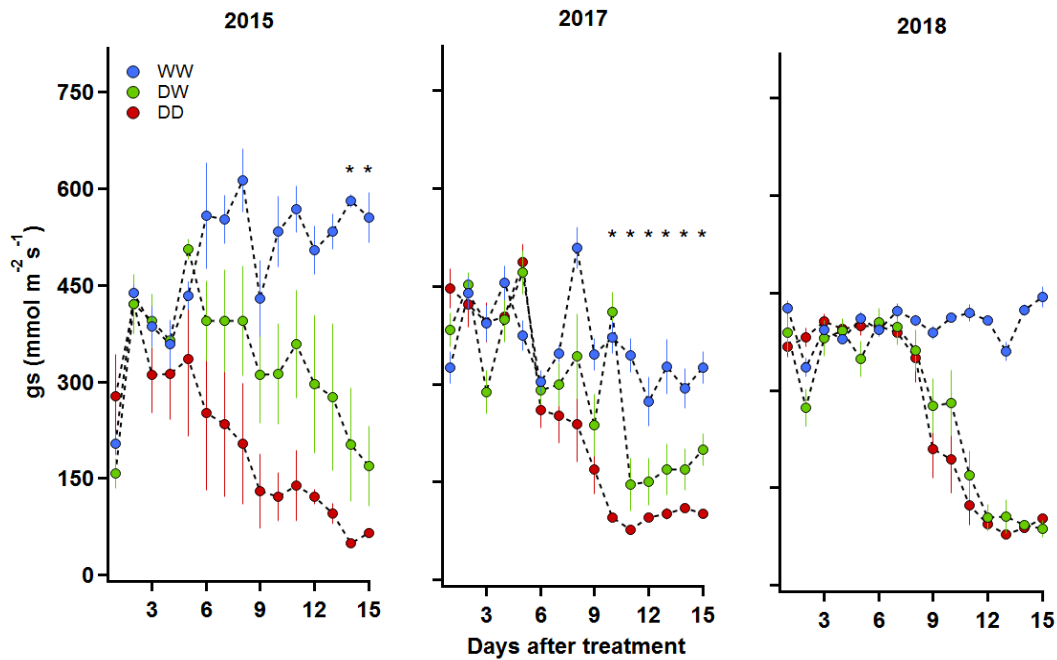


Fig. 3 Stomatal conductance (g_s) performance during a 15-days experiment repeated three different years (2015: $n=5$ trees per treatment; 2017 and 2018: $n=11$). Points represent the means \pm SE. Plants treatments were well-watered (WW), water withheld from the soil (DD) and water restricted at pot-level but provided via canopy soil (DW). Stars represent the level of significant difference between DD and DW each day: $P \leq 0.05$

3.1.3 Leaf water potential (ψ_{leaf})

The effect of canopy soil was more noticeable for the ψ_{leaf} values than for g_s or soil moisture tensions. There were significant differences between DW and DD treatments during most of the experimental period for each of the three years, with DW plants maintaining a higher ψ_{leaf} than DD treatment (Fig. 4). This result not only is consistent with the results found in soil moisture tension and stomatal conductance, it further highlights a direct and beneficial effect of providing water from the canopy soil.

For ψ_{leaf} values, more variability was seen in 2015 and 2017 compared to 2018. At the beginning of the experiment (2-5 DAT), there were no significant differences among treatments as expected and coinciding with soil moisture tension data, suggesting that there was still enough underground soil water available to sustain ψ_{leaf} . However, after 8-DAT, the effect of water deficit started to be more noticeable in 2015 and 2017, but not 2018. Significant differences among treatments were seen after 10-11 DAT, with DD reaching the lowest values of ψ_{leaf} (-2.5MPa) followed by DW that remained lower than WW (-1.0 to -1.5MPa), but not as low as the DD treatment. The greatest differences of ψ_{leaf} among treatments were seen at the end of the experiment (14-15 DAT) for all the three years. Leaves in the WW treatment had the highest values of ψ_{leaf} , followed by DW with lower values compared to WW, except in 2015 were they reached similar ψ_{leaf} . Leaves in the DD treatment reached the lowest values of ψ_{leaf} at the end of the experiment. The sustained elevated ψ_{leaf} of DW (compared to DD) during the experimental period, implies that the extra water source from canopy soil alleviated effects of soil water deficit. This canopy-supplied water is likely absorbed through canopy roots or bark and internally redistributed towards the leaves.

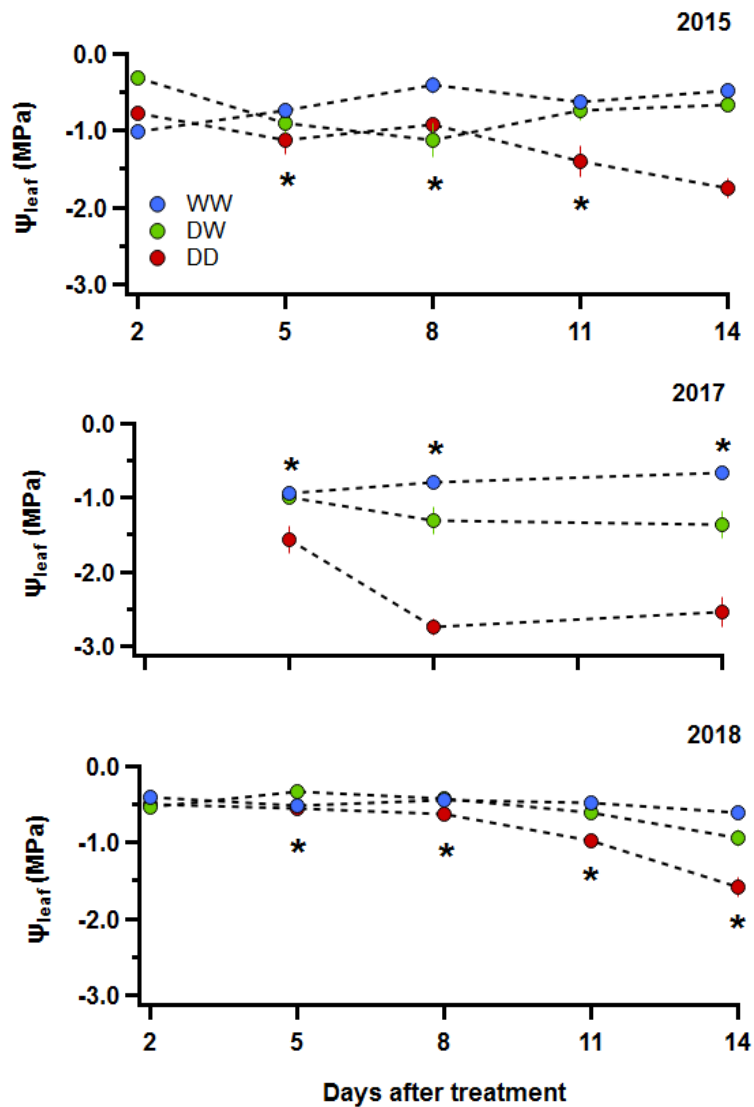


Fig. 4 Leaf water potential (Ψ_{leaf}) performance during a 15-days experiment repeated three different years (2015: $n=5$ trees per treatment; 2017 and 2018: $n=11$). Points represent the means \pm SE. Plants treatments were well-watered (WW), water withheld from the soil (DD) and water restricted at pot-level but provided via canopy soil (DW). Stars represent the level of significant difference between DD and DW each day: $P < 0.05$.

3.2 Photosynthetic performance parameters

3.2.1 Leaf net CO₂ Assimilation (A)

In general, light saturated net CO₂ assimilation rates (A) remained higher in leaves of the WW treatment, with average values between 7 and 12 $\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$ during the entire experimental period (Fig. 5). DW plants started with values similar to DD for the first 7 DAT; but by day 8 the effects of drought stress began to appear with leaves in the DW treatment showing a significant decrease of their assimilation rates to 3 to 6 $\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$ remaining low until the end of the experiment. Leaves in the DD treatment showed the greatest decrease after 8 DAT from 8 $\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$ to below 2 $\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$ and remained the lowest the entire experiment. Photosynthetic assimilation rate depends on stomatal conductance for CO₂ uptake. As we could see previously for stomatal conductance, the presence of canopy soil in the DW treatment maintained stomatal opening for a longer time despite drought conditions in the soil. Hence CO₂ assimilation continues to occur despite the water deficit conditions in the pot.

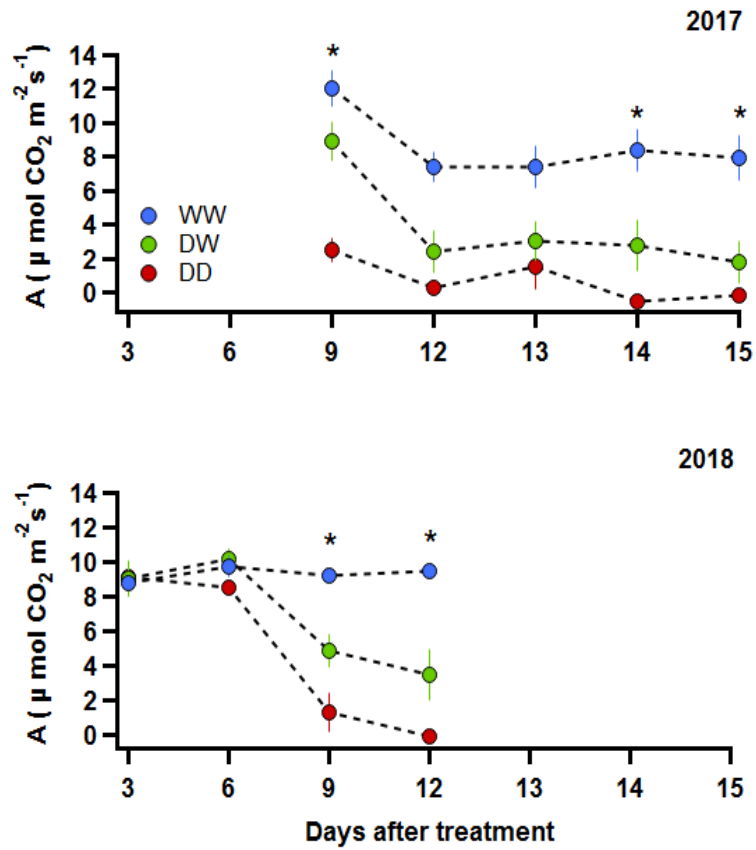


Fig. 5 CO₂ assimilation rates during a 15-days experiment repeated twice (2017 and 2018: n=11). Points represent the means \pm SE. Plants treatments were well-watered (WW), water withheld from the soil (DD) and water restricted at pot-level but provided via canopy soil (DW). Stars represent the level of significant difference between DD and DW each day: $P \leq 0.05$.

3.2.2 Chlorophyll fluorescence (Fv/Fm) performance

Corresponding to the results for CO₂ assimilation rates (*A*) and leaf water potential (ψ_{leaf}), chlorophyll fluorescence reflected a similar pattern for all the treatments during the three years (Fig. 6). Trees in the different treatments began to show differences by day-6 to day-8, with DD having the lowest values (0.6 ± 0.05 to 0.5 ± 0.05) as initial indicators of mild stress. The trajectory of leaves in the DW treatment decreased at a slower rate compared to DD, and started showing stress symptoms around 11 DAT. At the final stage, all DD plants reached the lowest values of fluorescence (0.0 ± 0.05 to 0.1 ± 0.05) In contrast, leaves in the DW treatment maintained values of 0.65 ± 0.05 and 0.45 ± 0.05 for the last 4 days, for 2015 and for the last two years (2017 & 2018) respectively (Fig. 6). This suggests that the additional water from the canopy soil mitigated the effects of drought at the whole-plant level evidenced by the sustained Fv/Fm indicating that the activity of the light harvesting machinery is maintained, contributing directly to photosynthesis.

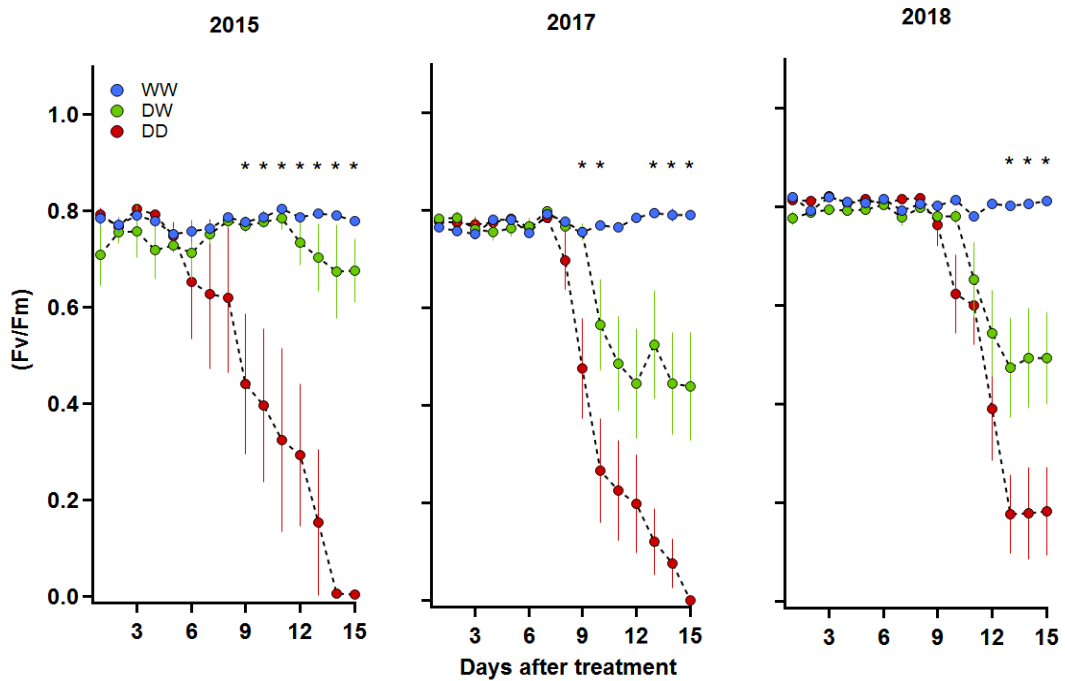


Fig. 6 Chlorophyll fluorescence (Fv/Fm) performance during a 15-days experiment repeated three different years (2015: n=5 trees per treatment; 2017 and 2018: n=11). Points represent the means \pm SE. Optimal values are in the range of 0.79 to 0.84, with lower values indicating impaired light-harvesting machinery. Plants treatments were well-watered (WW), water withheld from the soil (DD) and water restricted at pot-level but provided via canopy soil (DW). Stars represent the level of significant difference between DD and DW each day: $P \leq 0.05$.

Table 1. Statistical results for each response variable of poplar trees in three trials (each one of 15-days experimental period) under three different treatments: WW, DW and DD. Each trial consisted of 11 trees per treatment (2017 & 2018) and 5 trees per treatment in 2015; trial was included as a random factor and interaction for Treatment X Time was tested using a linear mixed model (LMM) and ANOVA. All results are presented as F-value (P-value). Results represent the significance of all data points, from 1-DAT to 15-DAT for all the three years. P values were considered significant if: $P \leq 0.05$.

See figures for specific comparisons and significance between DD and DW treatments on each experimental day of each trial.

Source	Response Variable	F value	Numerator df	Denominator df	P value
<i>Treatment</i>	SMT	41.47	2	77	<.0001
	Gs	59.59	2	74	<.001
	Ψ_{leaf}	34.75	4	75	<.0001
	A	82.14	2	47	<.0001
	Fv/Fm	43	2	77	<.0001
<i>Treatment X Time</i>	SMT	396.82	2	1143	<.001
	Gs	147.92	2	1103	<0.001
	Ψ_{leaf}	14.28	4	323	<0.0001
	A	5.78	2	252	0.0035
	Fv/Fm	269.48	2	1145	<0.0001

4. Discussion

In this study, we have demonstrated, in three different trials, that canopy water uptake from canopy soil occurs, and that is a significant water uptake mechanism, that positively affects *Populus sp* performance mitigating the effects of water deficit on water and carbon balance and extending plant survival (Fig. 4,5, S1). Several studies have confirmed water uptake from different tissues at the canopy level -either by foliage (Carter Berry et al. 2018; Eller et al. 2013, 2016; Limm et al. 2009) or directly through the bark (Cuneo, et al. 2018; Mason Earles et al. 2016). However, canopy roots, despite their nature, have not been considered of great importance in the canopy water uptake. Most of the aerial root research focused on root anatomy and indirect nutrient absorption (Hertel, 2011; Hertel & Köhler, 2010), but there is little experimental evidence of their functioning for water uptake. This is the first study that demonstrates the effect of canopy soil in physiological performance and water relations of whole-trees with induced canopy roots under drought in a novel experimental system.

Multiple studies highlighted the positive roles of canopy soil by itself (Gotsch et al. 2014; Gotsch et al. 2018) suggesting it as an important additional water source for the host tree. The high water-storage capacity of canopy soils (Hölscher et al. 2004; Köhler et al. 2007), and the fact that humidity, darkness and inorganic material are critical for the development of canopy roots (Nadkarni, 1994), suggest that some species of trees may have developed aerial roots as an adaptation to gain access to additional water sources at the canopy level. In the current study, we demonstrated in a series of controlled experiments that the onset of drought stress was delayed in trees with canopy soil and roots measured by multiple physiological parameters including leaf water potential, net CO₂ assimilation rates, chlorophyll fluorescence, and stomatal conductance.

4.1 Canopy water uptake: Two mechanisms

There are two possible mechanisms by which water can be transported from canopy soil to the host tree: bark absorption and through canopy roots. Previous studies have recognized the significant role of bark in rainfall interception and storage, noting that some water may be

absorbed by the xylem and could potentially recover hydraulic conductance from embolism (Earles et al., 2016; Mayr et al., 2014b). The cellular mechanism on how water is absorbed through a woody surface is still unknown, but there are some hypotheses; Earles (2018) suggests that water entry is partially facilitated by a symplastic route. When water enters in contact with bark surfaces, it quickly spread, refilling all those hydrophilic capillary spaces of the rhytoderma (the outermost layer composed of cortical cells and dead phloem). Therefore, rain or fog events could wet the bark surface in hours or minutes, increasing its water potential close to zero. Based on dye tracing experiments beneath the rhytoderma, water encounters the phellogen cell wall, where it moves by osmotic gradient through the hydrophilic and non-suberized regions of it and continues to the parenchyma via aquaporins channels (Laur & Hacke, 2014; Mayr et al., 2014a). Water movement across the cell membrane occurs by simple diffusion and lastly it is released directly to the xylem vessels, which are interconnected and embedded in parenchymatous tissue (Borchert & Pockman, 2005; F. C. Meinzer et al., 2003). Knowing that atmospheric water can be absorbed through the bark, it is also conceivable that water stored at canopy soil maintains bark surfaces covered and soaked for an extended period. As we noticed on our results, this canopy soil water ultimately can be taken up by the host tree and contribute significantly to its water potential (at least at the canopy level) (Fig. 4).

Although some water can be absorbed through the bark, the hydraulic resistance generated by the cambial zone and woody tissue in the path from bark to xylem, is considerably higher compared to bare stem surfaces or unsuberized regions, where water has direct contact with parenchyma and vascular tissue. The presence of canopy roots on branches provides an additional and more efficient water entry, facilitating and increasing the conductance and water uptake from canopy soils. In this study, canopy roots were induced by air-layering (Hartmann et al., 2011); most of the roots were at early stages of development (i.e., 2nd and 3rd order) during the experiment and were constituted essentially of unsuberized fine roots with fully developed structures, thus, we can assume that nearly 80% of the root biomass present was functional and performed as primary exchange surface with the canopy soil and could be responsible for most of the water absorption (Cantillo et al., 2020; Gambetta et al., 2013; Kennedy et al., 2010; Nadkarni et al., 2004).

4.2 Canopy soil effect in plant water budget

Poplars are sensitive to soil water deficit and known to exhibit various drought responses such as decreased leaf area, leaf abscission, enhanced root growth, increase water use efficiency (WUE), stomatal closure, and osmotic adjustment among others (Larcheveque et al. 2011). The fact that in most of the trials (2015 & 2017), trees with canopy soil (CS) ended up with a higher soil water content compared to plants without it under drought conditions (Fig. 2), suggests three different explanations: (1) A reduction of plant's total water use by restricting or diminishing transpiration rate (i.e. decreasing stomatal conductance), which maintained soil water budget greater and relatively stable for a longer time than trees without CS; (2) a redistribution of water from canopy soil towards other internal tissues -most probably to branches and leaves at the distal ends of the canopy, rehydrating and providing enough water to be used for transpiration, reducing the uptake of underground soil water; (3) a redistribution of water from the canopy soil towards trunk and other parts at the proximal ends of the tree by reverse flow, and eventually to the underground roots. This third mechanism of water redistribution could have been eventually released directly into the soil and contributed into keeping soil moisture tension values higher than trees without CS.

The presence of canopy soil resulted in significant improvements in plant water status (Fig. 3 & 4). Although plants were under three different treatments, we observed that drought stress symptoms started to be more significant after 8 to 9 DAT. During this first period, the main water source was likely from the pot regardless of the presence of canopy soil as an extra water source in DW trees. By day 10, stomatal conductance and water potential decreased drastically for DD plants as a response to the low soil water content, which was insufficient to recover the daily and night water lost through transpiration (Fig. 3 & 4). On the other hand, DW trees with canopy soil as an additional water source were able to prolong their stomatal activity and maintain a greater leaf water potential likely on branches with canopy soil and their neighboring branches. Although the amount of water absorbed from the CS may not have been sufficient to maintain an optimal stomatal conductance and leaf water potential as shown in WW plants ($\Psi_{\text{leaf}} < -1$ MPa), the results strongly suggest that canopy water uptake alleviated and contribute to preserve mildly transpiration rates until the end of the experiment (Fig. 3).

Despite the fact that in the last trial (2018), the results have been more variable (Fig. 2 & 3), the 2018 results on water potential and other physiological parameters (i.e., photosynthesis and fluorescence) reinforced the findings from 2015 and 2017 experiments (Fig. 5 & 6). Initially, plants were mainly using water from soil underground to replenish water lost from transpiration. As a response of water depletion on soil underground, plants reduced their stomata opening neglecting the presence of an extra-water source aboveground. We suggest that water from canopy soil was available and used to sustain a minimum water potential, yet not enough to fully replenish transpiration water lost, but sufficient to maintain a stable carbon assimilation rate until the end of the experiment.

4.3 Canopy soil effect on photosynthetic performance

An additional water source aboveground that contributed to increase leaf water potential, at least at the canopy level, ultimately showed an indirect effect on plant performance. At the end of the experiment, there was evidence that plants holding CS look greener and healthier than plants without them (Fig. 7, Supplemental data). The CO₂ assimilation rates were maintained more steadily in DW plants ($\sim 3\text{-}6\mu\text{mol CO}_2\text{ m}^{-2}\text{s}^{-1}$), despite the absence of underground soil water (Fig. 2), demonstrating a solid evidence of the significance and contribution by the canopy soil. It is important to highlight that in nature, canopy soil is mostly extended and occupies larger areas, covering $\sim 80\%$ of the surface of the stems and branches; In this experiment, there were only three discrete canopy soils systems of $\sim 15\text{-}20$ cm in length by $\sim 8\text{-}10$ cm thick added per tree, covering around $\sim 10\%$ of each branch, even so, the effect on a smaller scale of the significant contribution of the canopy soil in tree performance was evident. Additionally to the sustained photosynthetic activity in DW, chlorophyll fluorescence did not show stress symptoms immediately in DW plants, despite of being under the same conditions of water deficiency as DD plants; which supports the aforementioned positive effect of CS water, as an extra hydraulic aid that influences the overall activity of the plant (Fig. 6).

Previous studies showed that water absorbed through the leaves during foliar water uptake (FWU) influences positively leaf and stem water potentials, improving hydraulic

conductivity and increasing leaf water content (Johnson et al., 2012; Meinzer et al., 2006). However, long-term effects of these improvements on photosynthetic activity and water fluxes has been difficult to quantify. Indirectly, Berry and Smith (2013) assumed an enhancement in net carbon gain based on a greater stomatal conductance response to FWU, although to what extent these daily gains occur, and context is still unknown (Berry et al., 2014). On the other hand, Steppe et al. (2018) and Nguyen et al (2017) demonstrated the important role of FWU in turgor maintenance, growth and hydraulic conductivity (Nguyen et al., 2017; Steppe et al., 2018), but with little evidence of net effect on cumulative growth or carbon storage. Regardless the absorption strategies between FWU and CS tested in this study, some tractable analogies are found since both mechanisms imply a water subsidy at the canopy level. Our results supports Eller et al (2013), where *Drimys brasiliensis* saplings were able to survive for a long period of time only under fog water conditions and without any additional water input, allowing plants to keep a positive carbon gain and growth at higher levels than the completely drought treatment, similar to what we found in our experiment, where plants with canopy soil (DW) maintained its ecophysiological performance relatively stable and greater compared to DD plants.

4.4 Ecophysiological importance

According to our results, plant water relations and physiological performance between treatments were markedly influenced by the presence of canopy soil, suggesting that under specific environmental conditions, particularly drought, the existence of CS can function as a biologically meaningful source of water to compensate the water deficit in ground soil.

Like FWU and its significant role in plant water balance, canopy soil, by its own composition and ability to accumulate large amounts of water, becomes an additional hydraulic subsidy for the host tree but through a distinct pathway. In the present study, we argue that this canopy water, not only is being used to alleviate drought conditions, helping to maintain tree water potential, stomata conductance and consequently CO₂ assimilation rate, but also could potentially function as localized reservoir for hydraulic recovery to cope with temporal variations in water demand and availability, at least at the canopy level.

The fact that the canopy soil is located aboveground, offers physical advantages in terms of water transport. Since canopy soils are positioned near the same height of leaves and branches, water transport from a canopy source is easier and more efficient, due to the reduced distance, gravimetric potential, and resistance compared to water transport from the underground soil. Thus, canopy soil might provide an ecophysiological benefit for the tree, especially in drought conditions, when there is a greater vulnerability to embolism given the high tensions that must be exerted to move water upwards to leaves and branches.

Since our experiments were performed on young isolated trees under greenhouse conditions, our results on the effects of canopy water uptake on plant performance need to be interpreted with caution to extrapolate to individuals in field conditions. However, if water can reach leaves and other branches of the tree, all the effects associated to hydraulic conductivity recovery by improving and changing water potential gradient could serve to repair embolism (Berry et al. 2018). Moreover, since branch surface is completely wet or soaked, facilitates water absorption through the bark and provides more energetically favorable pathways for water movement to nearby xylem conduits (Earles et al., 2016). Thus, bark covered by wet canopy soil could work as an absorption surface, that could be enhanced by canopy roots and help localized embolism removal.

4.5 Conclusions and future directions

In this study we demonstrated that the presence of canopy soil is an advantage for coping with conditions of water stress, since additional water aboveground can be used by the host tree mitigating the effects on water potential and plant performance. However, there are still many key knowledge gaps that limit a more complete understanding of water movement. We demonstrated that the effect of water stress is mitigated by the canopy soil. Future studies may focus on quantification of the amount water uptake by the canopy soil in a tree, and to what extent this gain of water have an effect in a longer-term. The answer to these questions will allow us to comprehend how much of the soil-plant-atmosphere-continuum (SPAC) can be decoupled and to what extent plants are able to survive with subsidiary water sources.

There are mounting evidence that water can be absorbed through canopy roots or bark (Cantillo et al., 2020; Earles et al., 2016; Kennedy et al., 2010; Mayr et al., 2014b; Nadkarni & Primack, 1989) More detailed studies on root and bark hydraulic conductance, supported by plant performance experiments with trees with CS with and without roots are required to better understand the mechanisms and extent of water absorbed by either pathway. As we observed in our results, the effect of CS in one of the trials (2018 experiment) was inconsistent from the others, this raises the question- when the canopy soil is functional and what environmental factors determine its functionality. More explorations of the linkages between canopy water uptake and hydraulic functioning, plant growth and risk of mortality will enhance our ability to predict plant responses to novel climates.

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SUPPLEMENTARY MATERIAL

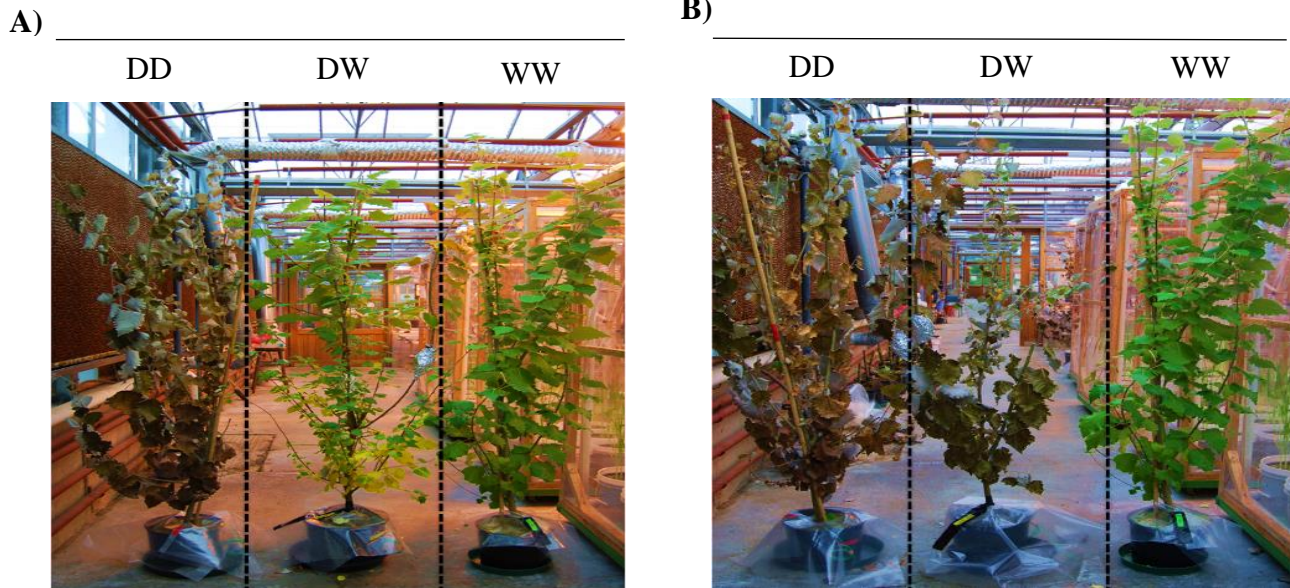


Fig. S1 Poplar trees (*Populus* spp.) under three different water treatments at the end of a 15-days experiment: DD (Dry Pot-Dry canopy), DW (Dry pot – Wet canopy soil), WW (Well-watered). More details about treatments in the Material & Method section. A) Significant qualitative differences among treatments, DW looks healthier and with still green leaves compared to DD at the end of the experimental period. B) Differences among DW and DD treatments are less evident, and both presented stress symptoms, even though DW have canopy soil water available.

CHAPTER 5

Conclusions & Future directions

1. Conclusions

This study shows for the first time that the presence of an additional water source in the form of canopy soil mitigates the effects of belowground soil water deficit. Furthermore, canopy water uptake is facilitated by canopy roots (adventitious aerial roots) developed by the host tree underneath and into the canopy soil. Canopy roots are directly connected to the vascular system of the host tree harboring the canopy soil which enables redistribution of water absorbed from the canopy throughout the tree, possibly by reverse and bi-directional flow. This hydraulic redistribution helps to maintain carbon and water balance at the canopy level (neighbor branches and leaves) under conditions of dry belowground soil. In conclusion, the current study suggests that the presence of canopy soil and roots, when hydrated, prolongs tree survival by decoupling the tree from the soil-plant-atmosphere continuum (SPAC), alleviating the tree's dependence on belowground soil water when water availability is low.

1.1 Canopy roots (CR) are functional and possibly hydraulically more efficient than terrestrial roots (TR) (Chapter 2).

In Chapter 2, similarities between canopy roots (CR) and terrestrial roots (TR) were seen in structure, morphology and anatomy (including root basic features) suggesting that aerial roots are also functional and can take up water regardless of where they have originated (stem or branches) or developed (canopy soil).

The main differences between primary root systems and adventitious roots are their origin from the radicle and from parts of the plant not originating from the embryonic roots, respectively. Because of their origin on unusual parts of the plant, adventitious roots of some species have developed a structure and function slightly different from those of the primary root system. Nevertheless, a principal physiological function of both root systems is to extract water

and nutrients from their surroundings. The fact that adventitious roots have an aerial habit enables these roots to exploit a part of the plant's environment inaccessible to the primary root system (Barlow, 1986). For instance, CR arising from branches into canopy soil can take up water from the canopy, as demonstrated in this study, additional to the water available in the belowground soil.

Water uptake by roots, and root hydraulic conductance depend not only on the type of soil, but also on environmental conditions influencing the roots and trees. The hypothesis that CR are more hydraulically efficient than TR, possibly because CR grew in permanently well-watered canopy soil while TR developed in dry soil, was partially supported. TR presented suberized endo- and exodermis (bark tissue) but also had larger diameter xylem vessels compared to CR. In contrast, CR presented more lateral roots, non-suberized cell walls and smaller diameter xylem vessels. Previous studies have demonstrated that the radial water pathway is more critical than axial water movement in determining flux of water from soil to the vascular system. Based on these findings, we can infer that CR have higher hydraulic conductance than TR, because anatomically they have less resistance for water flow in the radial direction.

1.2 Canopy soil water uptake is facilitated by canopy roots and redistributed throughout the tree by reverse or bi-directional flow (Chapter 3).

In Chapter 3, results demonstrated that the ability to grow CR into canopy soil enables water absorption and redistribution of water by the canopy. These findings challenge the unidirectional soil-plant-atmosphere continuum (SPAC), although follows within the conceptual framework that water movement should be considered as a continuum involving all potential water sources. In this case, the establishment of a water potential gradient between moist canopy soil and dry terrestrial soil ($\Psi_{\text{canopysoil}} > \Psi_{\text{soil}}$) was enough to induce reverse flow in *Populus* trees.

Hydraulic redistribution from branches to the rest of the plant, opposite in direction described by the unidirectional SPAC, reduces the energy required and resistance generated to move water from belowground water resources to aboveground branches and leaves, possibly decreasing cavitation risk and maintaining leaf water potential.

1.3 Water from canopy soil mitigates the deleterious effects of low water availability in below-ground soil, extending plant survival and maintaining carbon and water balance (Chapter 4).

Chapter 4 illustrated that canopy soil (CS) can play an important role in plant survival during drought conditions. Specifically, CS provides a significant amount of water that compensates for the lack of water in the below-ground soil, extending the survival and activity of trees with canopy soil. The redistribution of water from aboveground to other tree organs alleviates drought stress by decoupling the SPAC, reducing reliance of the tree on its main water source underground. In this way, similar to the effects of foliar water uptake (FWU) (Steppe et al., 2018), water coming from CS could serve as an additional subsidy that buffers declines in turgor and hydraulic conductivity, raising the water potential locally at the canopy level, increasing the water potential gradient and resulting in reverse flow observed in the previous experiments. This allows plants to maintain transpiration, stomatal conductance, and photosynthesis over the experimental period.

2. Future directions

Considering that this was one of the first studies conducted to understand the physiological effects of canopy soil and roots on trees under conditions of water deficit, our results contribute to the understanding of tree hydraulics and water relations. To consider the role of canopy soil water uptake in whole-plant water budgets, we have correlated the presence of canopy soil and canopy roots to plant water and carbon balance. The implications for this are significant, as canopy soil water uptake could mitigate water deficits, maintaining leaf water potential as well as contributing to the hydration of mesophyll cells or vasculature. This study so far represents the beginning of canopy soil research and its physiological impacts. Still required are improvements in both methodological and technical aspects, and attention to the many key knowledge gaps that limit a more complete understanding of how this system works. This dissertation presents an introduction of a long pathway for research into canopy soil and roots to be considered within the framework of plant water relations.

In the next section, I address questions focused on technical and experimental design improvements and key questions surrounding future research including environmental scenarios in which canopy soil water uptake is likely to occur, the precise pathways of water flow, canopy nutrient uptake and the net effects of canopy soil on plant water balance and climate change.

2.1 Were isotopic or sap flow sensor data sufficient to track water movement? What other methods can be used to confirm water redistribution?

In Chapter 3, the combination of isotopic and sap flow results suggested that water absorbed through canopy roots or bark could move considerable distances within the plant and potentially is utilized in distal leaves and wood vasculature. However, the results presented here were variable and did not show statistically significant differences. More precise quantification and tracing of water movement will require new methods capable of higher resolutions and multiple dimensions. Imaging techniques such as micro CT (Brodersen et al., 2011) and MRI (Zwieniecki et al., 2013) combined with modeling of root hydraulics and field-based studies using dendrometers and stem psychrometry will expand our understanding of canopy soil water uptake. In order to understand better water movement in a reverse or bidirectional flow, and the driving forces that determine specific water fluxes, future research needs to contemplate not only plant water potential and soil moisture availability, but also hydraulic conductivity and capacitance across the tree body. This will contribute to a more integrated model of water movement pathways.

2.2 What would be the effect of CS during low rainfall periods under natural conditions?

Several studies have challenged the unidirectional SPAC model, most of them based on foliar water uptake (Burgess & Dawson, 2004; Nadezhdina et al., 2010; Oliveira et al., 2005; Simonin et al., 2009). Despite the fact that all the experiments in this study were done under controlled greenhouse conditions, and CS effects were limited by surface area and amount, this is the first report to confirm reverse flow from CS and assess its significance in plant survival and performance.

Further studies in natural scenarios and conditions are necessary to understand better if CS net effects allow for greater growth or carbon storage, and to determine their linkages with

hydraulic functioning and risk of mortality. Ultimately, parameterization of new ecosystems and global climate models should start considering other water sources used by vegetation for carbon fixation and evapotranspiration besides terrestrial soil water to better understand the long-term vegetation dynamics.

2.3 What other impacts CS would have in the host tree, if nutrients accumulated aboveground are also considered? Consideration of nutrient accumulation from canopy soil may increase the significance of canopy soils and roots in forest ecosystems.

The development of canopy roots underneath CS implies that this type of adventitious root is not simply a natural “accident” or meaningless adaptation, as has been seen for some tree species with aerial roots. The fact that canopy roots respond to similar factors that stimulate their growth and development as do terrestrial roots, and that they are anatomically indistinguishable, suggest that they are useful adaptations that could facilitate and exploit the use of resources at the forest upper level. Although nutrient absorption was not evaluated in this study, it is known that in addition to storing large quantities of water, CS is also able to accumulate large amounts of allochthonous nutrients and organic material from the decomposition of epiphytes and their associated biota. Therefore, CS besides being an extra-water source, could also become an “auxiliary” source of nutrients for the host tree (Haristoy et al., 2014; Nadkarni, 1981).

A few studies have reported mycorrhizae and nitrogen-fixing bacterial associations in canopy roots of multiple tree species (Kennedy et al., 2010; Nadkarni, 1981). These indicate that canopy roots are also taking up nutrients from canopy soil, and the absorption is supported by microbial symbioses. Although water absorption can occur either by roots or through bark, nutrients are most likely only be absorbed through canopy roots. Therefore, the presence of these root systems aboveground would be even more important, not only in conditions of drought but nutrient deficit in terrestrial soils. More research should be done to understand the significance of canopy soil nutrients in plant performance and nutrient cycling at the forest ecosystem level.

2.4 What are the specific environmental conditions and signals that induce the formation of canopy roots? Is the activity of canopy roots constrained to a specific season?

Knowledge about aboveground adventitious roots (canopy roots) anatomy, their absorptive capacities for water and/or nutrients under natural conditions, and the physiology of their initiation and growth is limited. Nadkarni (1994) reported that the stimuli that induce canopy roots involve more than the presence of wet epiphyte mats, suggesting that simply moisture or darkness do not trigger the growth of canopy roots. On the other hand, the variety of nutrients from the epiphytic communities accessible by the host trees via these root systems has a positive feedback between the growth of epiphytes and the nutrition of their host trees (Nadkarni & Primack, 1989). A possible explanation of this mechanism is that the presence of root systems appears to provide better substrate for epiphyte colonization than does bare bark by hardening the surface of the stem and providing more locations for colonization by epiphytes propagules and retention of water and nutrients. As the epiphyte community develops, the host tree may derive more leachates from the epiphytes and put forth more adventitious roots, which would in turn create better substrate for further epiphyte colonization (Nadkarni, 1994).

Rooting initiation involves the formation of new root meristems, which comprises complex changes in the metabolism, and the interaction of numerous endogenous factors in the developmental shift leading to adventitious root formation, both at the biochemical and molecular level. Cells are said to be competent for root generation when they can respond directly to an inducing stimulus (usually wounding and or/ auxin) by the direct formation of root primordia. It is well known that auxins play a fundamental role in the formation of adventitious roots, but they are not the only determinant; other compounds that interact with auxins and are involved in plant stress and defense responses are also related in the rooting process, such as phenols, polyamines, peroxidases and flavonoids (Haissig et al., 1994; Hand, 1994).

Less studied but equally important is the carbon allocation required for the development of adventitious roots. To understand better adventitious rooting, it is not enough to know what patterns of C allocation are in roots and shoots; it is necessary to know how they occur and their significance to plant function. Considering multiple factors that regulate C allocation, such as plant growth patterns, the species-specific factors that determine root morphology and the ability

to produce adventitious roots, including the environmental factors, all these integrated with the source-sink dynamic, will be necessary to understand the success or failure of rooting. Therefore, C allocation is of key importance to root adventitious plant biology because it may help explain the rooting availability of some species, as well as the successful field performance of adventitiously rooted species (Bloom et al., 1985; Friend et al., 1994)

Whole-plant source-sink relationships are affected strongly by seasonal changes and respond to defoliation, pruning and stress. Dry seasons will promote C allocation to develop new or larger roots that reach groundwater supplies or other water sources to overcome water deficit (Hill et al., 2013; Rosado et al., 2011; Vervoort & van der Zee, 2012). Therefore, canopy root formation can be very dynamic and may depend on whole plant patterns of carbon allocation. Increasing shoot or belowground root strength can result in decreased C allocation to canopy roots. Ultimately, the challenge will be integrating whole-plant C allocation dynamics and their seasonal effects to understand better canopy roots development.

2.5 Canopy soil water uptake, plant hydraulics and their linkage to climate change

Considering that many tree species in tropical and temperate rainforests develop canopy soil and roots on their branches, and are constantly exposed to rainfall and cloud dynamics, it is highly relevant to understand and measure plant-water relations accurately, especially when considering climate change effects in plant distribution patterns, vegetation response dynamics to climate, crop productivity, and food security. For instance, unifying plant hydraulic traits into dynamic global vegetation models will be useful to improve predictions of forest response to climate change (Berry et al., 2019).

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VITA

Johanna Cantillo was born in Bogotá, Colombia. She became interested in Plant Physiology during her undergraduate studies in Biology at the National University of Colombia (Universidad Nacional de Colombia). For her final project of her bachelor's in biology, she worked on Postharvest Biology of Fruits. After, she spent multiples years as a researcher assistant at different institutions: Laboratory of Plant Physiology and Biochemistry of the Department of Biology, National University of Colombia under the supervision of Dr. Luzmarina Melgarejo and Laboratory of Plant Physiology and Biochemistry, SINCHI, Amazon Institute of Scientific Research working on postharvest physiology and lastly at Laboratory of Mycology and Phytopathology at the Andes University (LAMFU) under the supervision of Dr. Silvia Restrepo, working on plant phytopathology. Johanna began her Ph.D. studies in 2012 under the supervision of Dr. Elizabeth Van Volkenburgh and co-advised by Dr. Soo-Hyung Kim at the University of Washington. During her time at UW, she held various teaching assistant positions in the Biology department. In addition, she spent a significant amount of time training and mentoring undergraduates in the lab. Johanna has participated to various outreach activities at the UW Biology Greenhouse and collaborated to an outreach program sponsored by the Nasa space grant consortium at UW. In addition to her dissertation work, Johanna coauthored a peer-reviewed article on endophytes and plant stress mitigation published on the journal *Microbial Ecology*.