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Origin of adventitious roots in black walnut (*Juglans nigra*) softwood cuttings rooted under optimized conditions in a fog chamber

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Abstract High-quality black walnut (*Juglans nigra* L.) logs are of great economic value and are used in the manufacture of high-end products. Indigenous to the central hardwood region, black walnut has been commercially cultivated for many years, and genetic improvement and selections have resulted in superior timber genotypes. The recalcitrance of black walnut cuttings to form adventitious roots is the greatest hurdle for mass propagation of improved material. The goal of this research was to improve the frequency of adventitious root formation in black walnut cuttings, and investigate anatomical changes during root development. Softwood cuttings (15–20 cm) were collected from juvenile and mature sources of elite genotypes, dipped for 60 s in 31.1, 62.2, or 93.2 mM indole-3-butyric acid-potassium salt (K-IBA), or 36.9, 73.8, or 110.7 mM indole-3-butyric acid (IBA), and then inserted into a moist medium consisting of 3 perlite: 1 coarse vermiculite (v/v). Cuttings were placed in bench-top fog chambers or a mist bench for 5 weeks. To visualize anatomical changes during root formation, stems were fixed in formaldehyde, embedded in paraffin, serially sectioned, and stained on sequential days throughout root development. Rooting was greatest (72%) for cuttings exposed to 93.2 mM K-IBA and placed in the fog chamber, while cuttings treated with IBA rooted at lower frequencies (16–22.2%). Cuttings in the mist bench often deteriorated and rooted at lower frequencies independent of the auxin type. Anatomical analysis revealed adventitious root initials by day 16 and root primordia formation by day 18. Rooted cuttings survived acclimatization to the greenhouse.

Keywords Adventitious roots · Auxin · Cuttings · *Juglans* · Propagation

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Introduction

Eastern black walnut (*Juglans nigra* L.) is an integral species of the central and Eastern hardwood forests of the United States. Black walnut is rarely found in pure stands, but is highly prized for its timber (Williams 1990; Geyser and Rink 1998). Unique wood qualities such as figure, machinability, and color have made black walnut one of the most sought after hardwood trees for many high-end wood products including furniture, cabinetry, and veneer (Cassens 2004; Michler et al. 2007). As such, black walnut is an extremely valuable timber species. Extensive research has been conducted developing production systems to grow black walnut (Funk et al. 1979; Ponder Jr 1983; Ponder Jr and Baines 1988; Goodman et al. 2013, 2014), as well as generating superior timber genotypes (Beineke 1983; Victory et al. 2004). Black walnut unfortunately is recalcitrant to in vitro and vegetative clonal propagation methods on a commercial scale.

Established woody plant propagation techniques such as grafting, although routine, are not ideal for black walnut. Graft union death as a result of disease (Chen et al. 2013), scion-rootstock compatibility (Thomas et al. 2008), and the labor-intensive grafting techniques (Lowe and Beineke 1969) can influence success and survival. Coggeshall and Beineke (1997) found black walnut grafting success to be highly variable and inconsistent. Field trials have also shown that self-rooted *Juglans regia* (English walnut) outperformed grafted individuals of the same genotype (Hasey et al. 2001; Lopez 2001). Propagation via softwood cuttings, however, would be an ideal alternative to grafting to quickly replicate desired clones for timber or nut production, conservation efforts, and to complement breeding. Despite its potential value to commercial black walnut production, rooted cutting propagation of walnuts in unreliable. Farmer and Hall (1973) achieved a rooted frequency as high as 62% using a 49.2 mM indole-3-butyric acid (IBA) with seedling cuttings, although only on a small scale. Other reports of rooting black walnut cuttings required significant pre-treatment of stock plants and had a range of success (Shreve 1972; Shreve and Miles 1972; Farmer and Hall 1973; Carpenter 1975).

Our anatomical and physiological understanding of what drives adventitious root initiation in black walnut remains limited. One factor known to influence rooting is the ontogenetic age of the source material. A defining, and often limiting, characteristic of woody plant maturation was a sharp decline in rootability (Hackett 1988). This is of particular importance for clonal forestry as traits of interest are often not detectable until after the switch to a mature state. This study aimed to improve black walnut softwood cutting propagation, and to better understand factors influencing adventitious root development. The objective of this research was to determine how auxin type, indole-3-butyric acid-potassium salt (K-IBA) or IBA, and concentration in combination with rooting environment, fog or mist bench, influenced adventitious root formation survival. We also sought to document the histological origin of adventitious roots in easy-to-root juvenile cuttings and hard-to-root mature cuttings in order to develop a spatially explicit timeline of adventitious root formation.

Materials and methods

Stock plants

Scion wood was originally collected from the canopy of mature elite black walnut genotypes at Martell Forest (West Lafayette, IN, USA) in March of 2012 and 2014, and grafted

onto wild-type seedling rootstocks following a modification of the protocol of Beineke (1984). Successfully grafted plants were overwintered 1 year in cold storage (3–4 °C) prior to being used. Mature softwood cuttings were collected from these 1- to 3-year-old grafted black walnut stock plants grown in the greenhouse. Seeds were collected at Martell Forest (West Lafayette, IN, USA) from mature elite black walnuts. Mature seeds were de-husked, cleaned, and stratified in moist peat moss at 5 °C in the dark for 120 days. Nuts were then germinated and grown in seedling trays (Polyflat 40 cm × 40 cm × 12.7 cm; Anderson Die and Mfg. Co., Portland, OR) in potting mix (1:1:1 (v/v/v), peat moss:perlite:vermiculite). Juvenile softwood cuttings were taken from 5- to 8-week-old seedlings of elite genotypes. Grafted and seedling stock plants were watered as needed, and fertilized with 5- to 6-month slow-release fertilizer 15-9-12 Osmocote® (The Scotts Miracle-Gro Co., Marysville, OH, USA) under ambient greenhouse conditions (22 ± 2 °C) with light that peaked between 1500 and 2000 μmol m^{-2} s^{-1} during the course of the study.

Softwood cuttings

Mature and juvenile softwood cuttings with four to six nodes (15–20 cm in length) were collected from only the apical portion of new growth of the same genotypes. Juvenile cuttings came from single stemmed seedlings, while mature cuttings were collected from multi-stemmed stock plants. Cuttings were similar morphologically with regard to stem diameter, internode length, and leaf number. Leaflets were trimmed to reduce total leaf area by 25–50%. The basal 3 cm of the cuttings were dipped for 60 s in 31.1, 62.2, or 93.2 mM K-IBA dissolved in deionized water, 36.9, 73.8, or 110.7 mM IBA dissolved in deionized water plus a few drops of 5 N NaOH, or deionized water (control), and then inserted into a moist medium of 3 perlite: 1 coarse vermiculite (v/v). Cuttings were maintained under ambient greenhouse conditions (May–November) under intermittent mist (15 s every 30 min) or in a bench-top fog chamber. The fog chamber consisted of three 53 L plastic containers (Sterilite, Townsend, MA). One container with lid attached acted as the water reservoir, where fog was generated by an Alpine 1 Jet Fogger (Alpine, Tokyo, Japan) and actively blown (30 min every 90 min) via polyvinyl chloride pipe by fan (80 mm × 80 mm × 12 mm; Sunon, Taiwan) into the fog chamber composed of the other two containers secured opening to opening (one on top of the other) without lids. For the duration of root formation, cuttings were kept under ambient light conditions (1000–1200 μmol m^2 s^{-1}). After 5 weeks, data were collected for number of rooted cuttings, number of roots per cutting, individual root length (cm), and number of lateral roots per cutting.

Anatomical analysis

To develop a spatially explicit timeline of morphological changes during root primordia development, the basal 3 cm of the shoots treated with 93.2 mM K-IBA (best rooting percentage) or deionized water (control) were used. Stem segments (1–5 cm) were collected on day 0, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20; and day 26 for mature only; and day 31, 33, and 35 for control only, and partitioned into 0.5 cm segments. Three biological replicates were taken for each day. Segments were immediately fixed in a modified Karnovsky's fixative (1.5% glutaraldehyde, 2% paraformaldehyde, 1 M phosphate buffer pH 7.2; Cat. No. 15732-10 Custom, Electron Microscopy Sciences, Hatfield, PA), rinsed for 10–15 min four times with phosphate buffer (0.5 M pH 6.8; Electron Microscopy Sciences, Hatfield, PA), serially dehydrated in ethanol to 100%, and then embedded in

paraffin wax (TissueTek Vip Cat. No. 62580-08, Electron Microscopy Sciences, Hatfield, PA). The embedded tissue was sectioned into 10 μm sections using a rotary microtome (Jung BioCut mod. 2035), and stained with 1% Safranin O and Fast Green (Jensen 1962). Histological sections were visualized with a light microscope (Zeiss Axioskop) using Spot™ imaging software (Idea camera model 27.2-3.1 MP color).

Experimental design and statistical analysis

Three biological replicates of 12 cuttings each were taken for each treatment and environment from May to September. The same five genotypes were used for juvenile and mature cuttings, and data were pooled for each maturation state. Data were analyzed with an analysis of variance (ANOVA) performed with R statistical software (Team 2006). Post-hoc Tukey's Honestly Significant Difference (HSD) comparison tests at the 5% level of probability were run on means shown to be significant by ANOVA.

Results

Softwood cuttings

While rooting success depended on all variables tested, maturation was the largest single factor that contributed to the frequency of adventitious root formation in black walnut cuttings. Despite having similar morphological features to juvenile cuttings, all cuttings from grafted mature genotypes failed to root regardless of auxin type, concentration, or environment (data not shown), and in the absence of exogenous auxin application (water control). Cutting mortality was greater in mature cuttings, as a result of stem rotting or leaf abscission. All juvenile cuttings also failed to root in the water control. A Tukey's HSD test ($P \leq 0.05$) found auxin type and concentration significantly influenced the number and length of adventitious roots as well as the number of lateral roots that formed with juvenile cuttings (Table 1). Overall, 93.2 mM K-IBA resulted in the greatest frequency of root formation for both fog and mist environments, 72.2 and 27.8%, respectively (Table 1).

Within each environment, auxin type had a positive effect on adventitious root formation. Cuttings exposed to K-IBA rooted at a higher frequency compared to IBA, independent of environment, and cuttings rooted in the fog chamber treated with K-IBA had significantly more roots (Table 1). Rooting environment also influenced rooting frequency, number and length of roots, as well as number of lateral roots. Cuttings placed in the fog chamber remained healthier throughout the rooting process, than those placed in the mist bench, and survived longer after rooting. Cuttings in the mist bench would often quickly turn necrotic and did not survive until data collection.

Fog chamber rooted cuttings formed roots at a higher frequency and had a greater number of roots overall (Table 1), although the only significant difference ($P \leq 0.05$) between the rooting environments occurred for the two best auxin treatments (62.2 mM K-IBA and 93.2 K-IBA) (Table 1). Among K-IBA concentrations, however, only the 31.1 mM treatment had significantly fewer and shorter roots (Fig. 1a; Table 1) as compared to the other treatments (Fig. 1b, c). Adventitious roots appeared healthy and actively growing in acropetally arranged files (Fig. 2a). Rooted cuttings were successfully acclimatized to ambient conditions where they resumed active shoot growth (Fig. 2b).

Table 1 Rooting environment and auxin application effects on the rooting of juvenile *Juglans nigra* L. cuttings

Auxin (mM)	Fog chamber				Mist bench			
	Root formation (%)	No. roots	Length (cm)	No. lateral roots	Root formation (%)	No. roots	Length (cm)	No. lateral roots
0	0	0a	0a	0a	0	0a	0a	0a
31.1 K-IBA	50.0	1.3 ± 0.2a	0.9 ± 0.2bc	2.0 ± 0.7ab	22.2	1.3 ± 0.5a	0.7 ± 0.3ab	2.7 ± 1.7a
62.2 K-IBA	63.9	3.2 ± 0.6b	1.6 ± 0.3 cd	6.8 ± 1.7b	22.2	1.1 ± 0.3a	0.8 ± 0.2ab	1.5 ± 0.7a
93.2 K-IBA	72.2	3.3 ± 0.4b	1.8 ± 0.2d	6.3 ± 2.0b	27.8	1.2 ± 0.3a	0.9 ± 0.3b	2.4 ± 0.9a
36.9 IBA	19.4	0.6 ± 0.2a	0.1 ± 0.03ab	0a	0	0a	0a	0a
73.8 IBA	22.2	1.1 ± 0.3a	0.8 ± 0.3abc	3.2 ± 1.3ab	19.4	0.7 ± 0.2a	0.7 ± 0.2ab	2.7 ± 1.1a
110.7 IBA	16.6	0.7 ± 0.3a	0.6 ± 0.2ab	2.5 ± 1.1ab	16.6	0.7 ± 0.2a	0.6 ± 0.2ab	2.4 ± 1.1a

Cuttings were dipped in auxin for 60 s prior to insertion into moist rooting medium and placed in fog chamber or intermittent mist. Data were collected after 5 weeks. Values represent mean ± S.E. for 36 explants per treatment. Means in each column followed by the same letter were not significantly different according to a Tukey's HSD test ($P \leq 0.05$)

K-IBA, Indole-3-butyric acid-potassium salt; IBA, Indole-3-butyric acid

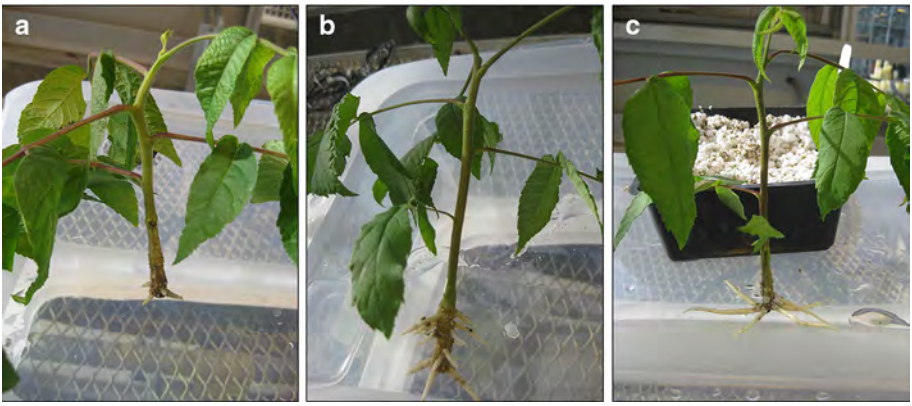

Fig. 1 Fog chamber rooted softwood cuttings of *Juglans nigra* 5 weeks after a 60 s dip in respective auxin treatment, **a** 31.1 mM K-IBA, **b** 62.2 mM K-IBA, **c** 93.2 mM K-IBA. For scale rooted cuttings were 15–20 cm in length

Anatomical analysis

Histological investigation revealed the origin of adventitious roots in black walnut softwood stem cuttings. Juvenile and mature cuttings displayed similar stem anatomy with the exception of the presence and abundance of sclerenchymatous phloem fiber tissue. In juvenile cuttings day 0, phloem fiber cells were found between the phloem and cortex and they grew in bundles forming a discontinuous ring along the transverse axis leaving gaps of parenchyma cells (Fig. 3a, b). Mature cuttings on day 0, however, had a darkly staining continuous ring of phloem fiber cells (Fig. 4a).

Juvenile cuttings by day 16 after auxin treatment displayed darkly stained nucleoli and nucleuses of dividing cells, forming root initials originating from the parenchyma cells in the phloem fiber gaps (Fig. 3c). By day 18 post-auxin treatment, root initials had developed into root primordia with organized cell files forming behind the root cap still within the phloem fiber gaps (Fig. 3d, e). Maturing adventitious roots were connected to the stem vasculature and elongating by day 20 (Fig. 3f), and appeared anatomically normal with mature root cap, quiescent center, and stele (Fig. 4f).

Cuttings from grafted mature genotypes, which failed to root, followed a different anatomical progression after auxin treatment. Minimal cell division was observed as far out as 18 days after treatment, and the continuous ring of phloem fiber cells remained intact (Fig. 4b). By day 20 in mature cuttings there was limited cell proliferation in the cortex with no visible organized cell division (Fig. 4c). Juvenile control (water) cuttings, which also failed to root, displayed indications of undirected cell division at day 20 (Fig. 4d), and by day 33 there were masses of undifferentiated callus tissue originating from the cortical region (Fig. 4e).

Discussion

Softwood cuttings of *J. nigra* were successfully clonally propagated and continued to grow normally after rooting and acclimatization. The highest rooting frequencies 63.9 and 72.2%, and highest number of roots per cutting 3.2 ± 0.6 and 3.3 ± 0.4, were achieved

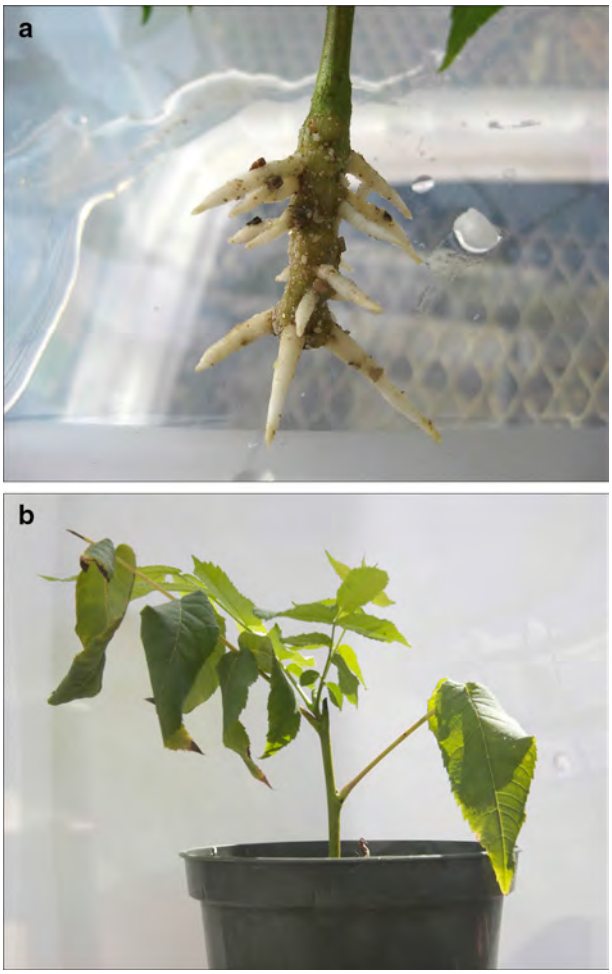

Fig. 2 Successfully rooted juvenile black walnut cutting, **a** healthy adventitious roots actively growing in vertical files, **b** successfully acclimatized rooted black walnut cutting resuming active shoot growth

when juvenile cuttings were exposed to 62.2 or 93.2 mM K-IBA, respectively. These rooting frequencies were similar or greatly exceeded cutting propagation systems in other *Juglans* species. *Juglans cinerea* (butternut) cuttings rooted as high as 87.5% when treated with 74 mM IBA (Pijut and Moore 2002; Pijut 2004), *J. hindsii* × *J. regia* cuttings rooted from 30 to 80% when treated with 24.9–33.1 mM K-IBA (Reil et al. 1998). Serr (1964) reported best results with hardwood cuttings of *Juglans* hybrids when soaking the basal end of the cuttings for 24 h in 1.1 mM IBA. *Juglans regia* rooting has been reported to range from 0 to 14.5% when IBA was used (Gautam and Chauhan 1990; Gunes 1999; Tajbakhsh et al. 2009), and *J. hindsii* (northern California black walnut) cuttings rooted at 100% with an acid pre-treatment prior to application of IBA (Lee et al. 1976).

Auxin type had a positive impact on black walnut root formation in both fog and mist environments. Cuttings achieved higher rooting percentages with significantly more roots ($P \leq 0.05$) when dipped for 60 s in K-IBA compared to IBA, and increasing K-IBA

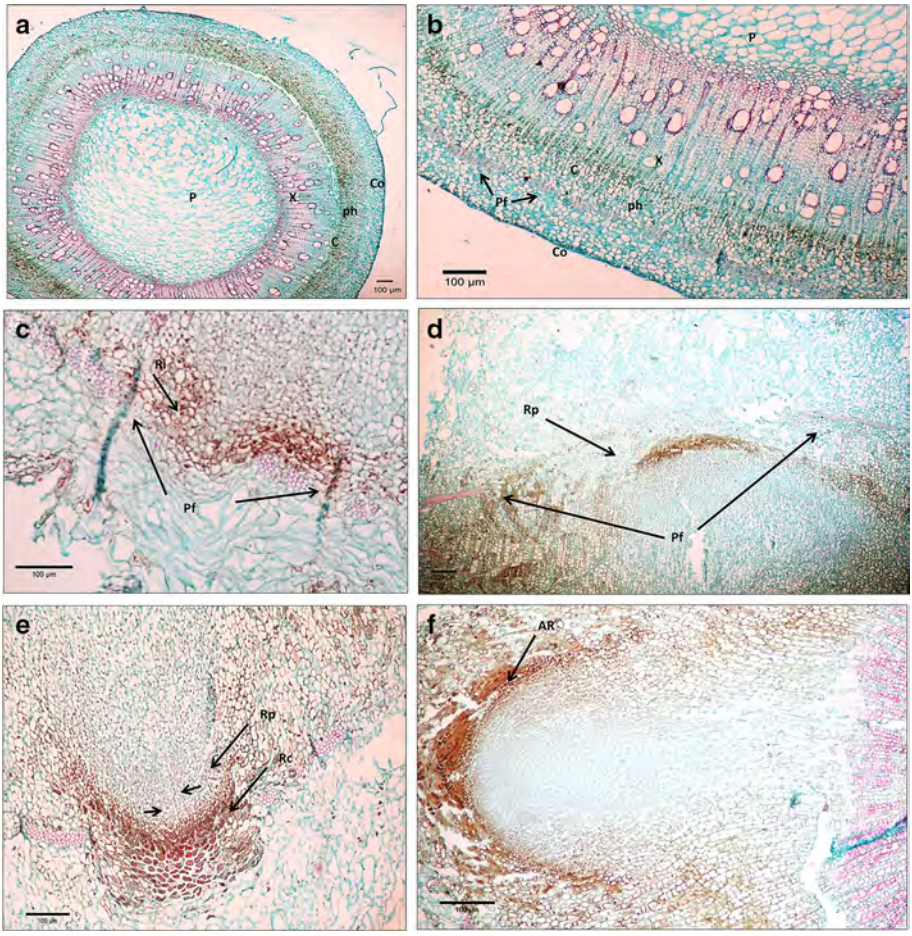

Fig. 3 Transverse sections of juvenile black walnut (*Juglans nigra*) cuttings 0–20 days after auxin treatment, **a**, **b** morphological structure of cutting base on day 0; close-up of vasculature **b** in which diffuse porous vessel elements were found within the xylem, flanked centrifugally by the cambium, phloem, a discontinuous ring of phloem fibers, and finally the cortex, **c** Day 16, darkly staining nuclei and nucleoli dividing to form a root initial in the gaps between phloem fiber tissue, **d** Day 18 longitudinal view of root primordia forming within phloem fiber gaps, **e** Day 18, maturing root primordia with cells organizing into darkly staining root cap and centripetally cells organized linear files (*arrows*) within the meristematic zone, **f** Day 20, fully developed adventitious root elongating and emerging from cutting. *AR* Adventitious root; *C* Cambium; *Co* Cortex; *ph* Phloem; *Pf* Phloem fibers; *P* Pith; *Rc* Root cap; *Ri* Root initial; *Rp* Root primordia; *X* Xylem. *Scale bar* 100 µm

improved the number and length of roots until an optimum was reached at 93.2 mM. The superiority of K-IBA could be a result of the increased solubility of the IBA salt (Hartmann et al. 2011). This increased solubility would allow for improved translocation to potential rooting origin sites of auxin within black walnut stems. This is the first report of using K-IBA to root *J. nigra* softwood cuttings, although K-IBA has been effective to root *J. cinerea* cuttings (Pijut and Moore 2002; Pijut 2004). Previous research with black walnut, however, had exclusively used IBA as the auxin type and cuttings were rooted under

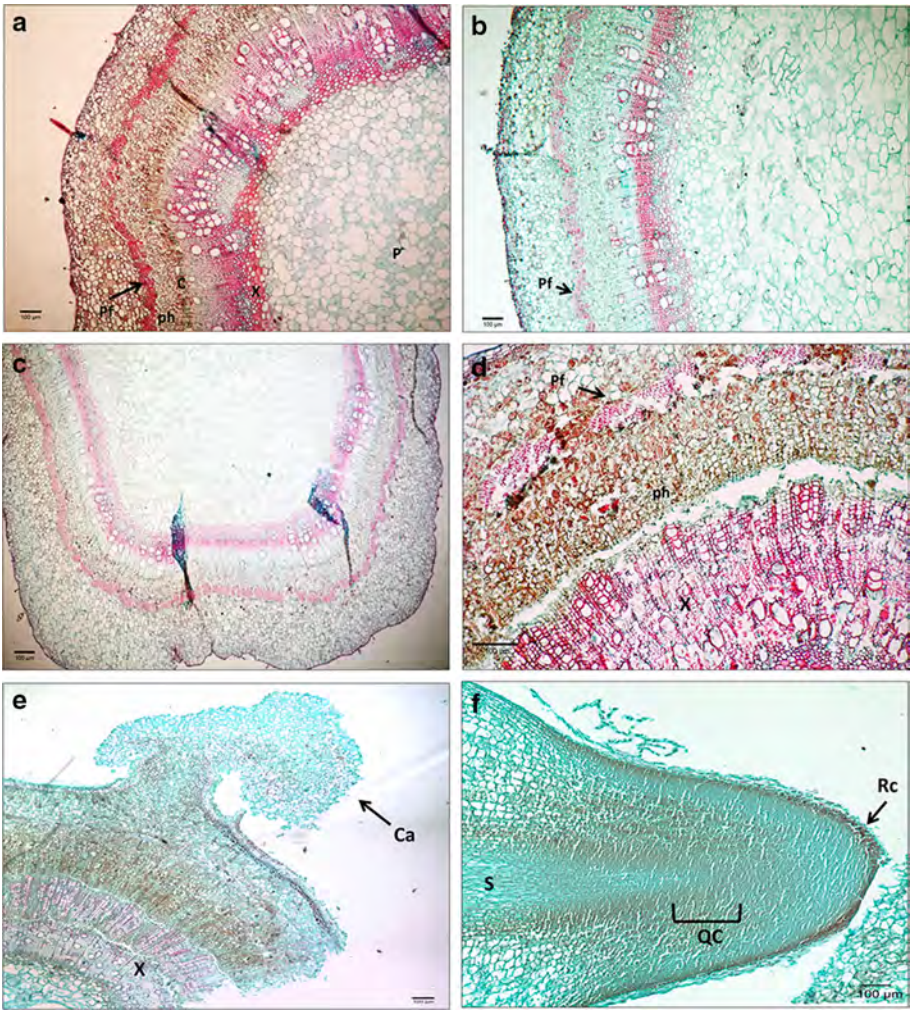

Fig. 4 Transverse sections of black walnut (*Juglans nigra*) cuttings after root induction, **a** Mature cutting day 0 displaying similar anatomical features to juvenile cuttings with the exception of a continuous phloem fiber ring centrifugally to phloem elements, **b** Mature cutting day 18 showing limited cell division and no root primordia development, **c** mature cutting day 20 with enlarged cortical region, **d** Juvenile control (water) cutting day 20, no organized cell division within phloem fibers gaps, **e** Juvenile control (water) cutting day 33, extensive callus formation arising from the cortex, **f** Adventitious root from juvenile cutting day 30 showing normal morphology. *Ca* Callus; *C* Cambium; *Co* Cortex; *ph* Phloem; *Pf* Phloem fibers; *P* Pith; *QC* Quiescent center; *Rc* Root cap; *S* Stele; *X* Xylem. *Scale bar* 100 µm

intermittent mist. These studies often had limited numbers of replicates or necessitated extensive pretreatments, which made commercial production impractical.

Farmer and Hall (1973), using 49.2 mM IBA, achieved an average of 55–62% rooting under intermittent mist, but cuttings required etiolation, girdling with a copper wire, and treatment with the fungicide benomyl. Soaking the basal ends of black walnut cuttings in ethephon for 6 h resulted in 60–70% rooting, but these did not survive long-term (Carpenter 1975). Again using 25–49 mM IBA, Shreve and Miles (1972) and Shreve (1972)

achieved rooting as high as 100%, but with as few as 10 cuttings per treatment, reproducibility was limited. We also found that when rooting occurred in a fog chamber compared to intermittent mist, cuttings remained healthier for longer and were significantly better for all of the variables recorded. The compound leaves of black walnut led to rapid desiccation in the mist bench, despite reduction in leaf area, and there was often mineral salt build-up on the foliage that also contributed to necrosis. Similar to other findings with black walnut, intermittent mist often led to necrotic cuttings that did not survive for the duration of the study (Farmer 1971). We determined fogging created more amenable conditions for maintaining healthy cuttings while adventitious roots were forming. Juvenile black walnut cuttings were repeatedly rooted at a high rate (72.2%) in a fog chamber with 93.2 mM K-IBA. K-IBA and fog were essential for success.

The ontogenetic age of the cuttings was one of the single greatest factors controlling root formation, as all cuttings of mature origin failed to root. The greater occurrence of mortality with mature cuttings during rooting, could be a possible explanation for reduced adventitious root formation. Morphological and physiological restraints have also been linked to rooting recalcitrance in *Prunus avium* (Dick and Leakey 2006). The importance of juvenility to rooting of cuttings from trees has been reported in walnut and other species (Shreve and Miles 1972; Ballester et al. 1999; Hartmann et al. 2011). The effects of maturation on adventitious root formation has been well documented (Hackett 1988; Pijut et al. 2011). Vegetative phase change and the resulting loss of rootability has been linked to a number of factors including molecular mechanisms and fluctuations in auxin homeostasis (Wang et al. 2011; Rasmussen et al. 2015; Xu et al. 2016). In the absence of exogenous auxin no roots formed in black walnut cuttings, however callus formation was present originating from cortex tissue. Similar findings have been previously reported for black walnut (Shreve and Miles 1972). This indicated that auxin treatment was not necessary for dedifferentiation, but was required for adventitious root initiation in black walnut cuttings.

Anatomical analysis elucidated the origin of adventitious roots in juvenile black walnut cuttings. Although adventitious roots may arise from different locations among genera (Jackson 1986), histological evidence showed that black walnut roots originated from the gaps between phloem fiber bundles. These gaps presumably maintain throughout the length of the longitudinal axis, as adventitious roots often formed in vertical files along the stem. Adventitious root initials were not visible within the gaps until day 16, and mature roots did not begin to elongate until day 20. This delay in the progression of root formation contrasts with easy-to-root cuttings from trees such as *Populus,* but could not be described as indirect root formation as there was no intervening callus stage (Hartmann et al. 2011; Zhao et al. 2014). Progenitor cells were most likely parenchyma cells because of their location, isodiametric shape, and ability to change their cell fate (Esau 1977). Although this is the first described origin of adventitious roots in black walnut, a report on *J. regia* 'Hartley' shoots described anomalous parenchyma rays centripetal to the cortex potentially giving rise to adventitious roots (Avanzato and Cappellini 1988). Similar origins of adventitious roots have also been noted in *Laurus nobilus* L. (bay laureal) (Parlak and Semizer-Cuming 2012).

In black walnut, one explanation for the failure of cuttings from a mature origin to root could be the absence of competent progenitor cells. Cross-sections of mature stems revealed a continuous ring of phloem fiber cells, thereby eliminating potential sites of adventitious root origin that were present in juvenile cuttings. Similar differences between juvenile and mature stem anatomy have been observed in *J. nigra* × *J. regia* hybrids (Claudot et al. 1992) with respect to fiber abundance and location. Auxin application alone in black walnut was not sufficient to initiate adventitious root development, as

parenchymatous cells within phloem fiber gaps must also be present. In addition to previously documented molecular and hormonal cues, these findings provide evidence for an anatomical mechanism as a possible explanation of adventitious root formation recalcitrance in black walnut. In mature black walnut stems increased fiber development reduced potential origins of root formation. Similarly, Maynard and Bassuk (1996) found in *Carpinus betulus* L. *fastifiata* a positive correlation between the percentage of sclereid-free gaps and percentage of adventitious root formation, highlighting the need for specific progenitor cells for adventitious root development to occur. The importance of anatomy and the abundance of sclerenchymatous tissue to rooting ability has been documented in other woody species as well (Beakbane 1961; Vieitez and Vieitez 1976). Our findings suggest that sclerenchymatous tissue does not act as a mechanical barrier, but its formation instead eliminates cells capable of perceiving signals to changes in cell fate. This first report of a spatially explicit timeline of adventitious root development in black walnut is integral for guiding future studies on the molecular controls of root formation. The optimization of a vegetative propagation system for a highly valuable hardwood tree, such as black walnut, would be of great interest for commercial production, to tree breeders, and for conservation efforts.

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