Rodriguésia 75: e01402023. 2024
http://rodriguesia.jbrj.gov.br
DOI: http://dx.doi.org/10.1590/2175-7860202475034

Plant anatomy: history and future directions

Histological study of adventitious rooting in *Acacia mearnsii* and *Ilex paraguariensis* mini-cuttings: insights into the so-called anatomical barrier

João Marcelo Santos de Oliveira[1,6], *Dilson Antônio Bisognin*[2], *Marlove Fátima Brião Muniz*[3], *Denise Gazzana*[4] & *Marjana Machado dos Santos*[5]

Abstract

Acacia mearnsii and *Ilex paraguariensis* are tree species of great social and economic importance in Brazil, demanding clonal cultivars. Their shoots possess a reservoir of totipotent cells with suitable potential for adventitious rooting, essential for mass production of high-quality seedlings. This study aimed to gain new insights into how anatomical barriers, *i.e.* sclerified tissues in the cortical region, may affect the adventitious rooting of cuttings from these species through histological examinations. For both species, histological analysis revealed significant diagnostic features. Tissue decay appears to be equivalent to an anatomical barrier in *A. mearnsii*. Starch abundance was notable in clones with higher rooting competence of *I. paraguariensis*, but they were not observed in the fundamental tissues of *A. mearnsii*, regardless of the rooting competence of the clone. The main differences in adventitious rooting were associated with the speed of response, initiated from cortical meristems, followed by differentiation of conductive tissue from newly formed tissue, connecting the periphery with the secondary vascular tissue. Thus, this newly formed tissue with parenchymatic structure provides the necessary structural basis for radial vascular connections. For both studied species, rhizogenesis presents distinct barriers to rooting, nevertheless these are not necessarily of anatomical nature.
Key words: *Cylindrocladium* sp., forest species, mini-cutting, rooting competence, starch.

Resumo

Acacia mearnsii e *Ilex paraguariensis* são espécies arbóreas de grande importância social e econômica no Brasil, que demandam cultivares clonais. Seus brotos possuem estoque de células totipotentes com potencial adequado para enraizamento adventício, essencial para a produção em massa de mudas de alta qualidade. Este estudo teve como objetivo obter novas perspectivas sobre como as barreiras anatômicas, ou seja, tecidos esclerificados na região cortical, podem afetar o enraizamento adventício de miniestacas dessas espécies por meio de estudos histológicos. Para ambas as espécies, a análise histológica revelou características diagnósticas significativas. O apodrecimento de tecidos parece ser equivalente a uma barreira anatômica em *A. mearnsii*. A abundância de amido foi notável em clones com maior competência de enraizamento de *I. paraguariensis*, porém não foram observados grãos de amido nos tecidos fundamentais de *A. mearnsii*, independentemente da competência de enraizamento do clone. As principais diferenças ao enraizamento adventício estavam associadas à velocidade de resposta, que se inicia a partir de meristemoides corticais, seguida pela diferenciação do tecido condutor a partir de um tecido neoformado com estrutura parenquimática, que conecta a periferia com o tecido vascular secundário. Portanto, esse tecido neoformado promove a base estrutural necessária para

[1] Federal University of Santa Maria, Department of Biology, Laboratory of Structural Botany, Santa Maria, RS, Brazil. ORCID: <https://orcid.org/0000-0002-6022-1767>.

[2] Federal University of Santa Maria, Department of Plant Science, Laboratory of Plant Breeding and Vegetative Propagation, Santa Maria, RS, Brazil. ORCID: <https://orcid.org/0000-0002-4754-9661>.

[3] Federal University of Santa Maria, Department of Plant Pathology, Laboratory of Plant Pathology, Santa Maria, RS, Brazil. ORCID: <https://orcid.org/0000-0001-7436-9589>.

[4] Federal University of Santa Maria, Graduate Program of Forest Engineering, Santa Maria, RS, Brazil. ORCID: <https://orcid.org/0000-0002-4245-4700>.

[5] Federal University of Santa Maria, Graduate Program of Agrobiology, Santa Maria, RS, Brazil. ORCID: <https://orcid.org/0000-0002-7682-5864>.

[6] Author for correspondence: joao.oliveira@ufsm.br

conexões vasculares radiais. Para ambas as espécies estudadas, a rizogênese apresenta barreiras distintas ao enraizamento, contudo estas não são necessariamente de natureza anatômica.

Palavras-chave: *Cylindrocladium* sp., espécies florestais, miniestacas, competência de enraizamento, amido.

Introduction

Most forest species are propagated by using seedlings. Sexual propagation results in highly variable populations for important traits, such as plant height, diameter, and yield potential, which hinders the use of adequate management practices. Asexual propagation is the best choice for species in which seedling production is dependent on either seed availability or quality. Asexual propagation results in uniform populations and ensures favorable combinations of traits, high levels of heterozygosity, and superior interactions of genetic variance (Pimentel *et al.* 2019). Among the asexual propagation techniques, cuttings are largely used for propagating horticultural and forest species because they are an efficient technique for maximizing genetic gain (Naidu & Jones 2016). Cuttings are prepared from stems, leaves, or roots, with stem cuttings being the most extensively used.

Mini-cutting is a variation of the cuttings technique. Mini-cuttings are the most important technique for cloning forest species, as demonstrated in the genus *Eucalyptus* L'Hér. (Naidu & Jones 2016), with a well-established protocol, and are widely used commercially by forestry companies (de Lima *et al.* 2022). It has been successfully used to improve the rooting competence of forest species, such as *Pinus pinaster* Endl. (Majada *et al.* 2011). Experimental protocols have been developed for other species, including *Ilex paraguariensis* A. St.-Hil., a species native to Brazil (Pimentel *et al.* 2019). The success of mini-cutting technology is directly associated with competence in the development of an adventitious root system.

Adventitious root formation is a highly controlled process that includes dedifferentiation and founder cell formation, cell division and primordia formation, and adventitious root growth (Lakehal & Bellini 2019). Adventitious root formation has a genetic component depending on the species and genotypes within the species, and an environmental component. Genetic differences among clones have been reported in the literature, such as rooting competence in *Eucalyptus* (Naidu & Jones 2016), *Cabralea canjerana* (Vell.) Mart. (Burin *et al.* 2020), and at the optimum time for mini-cutting rooting in hybrids of *E. benthamii* × *E. dunnii* (Brondani *et al.* 2012) and *Sequoia sempervirens* Endl. (Pereira *et al.* 2018).

Reserve accumulation in plant tissues, which is necessary for adventitious rooting, is affected by plant metabolism. Soluble sugar and protein consumption is associated with callus formation, whereas their accumulation is associated with adventitious rooting in *Ziziphus jujube* Mill. (Shao *et al.* 2018). Annual changes in soluble sugar and protein concentrations have been observed in the leaf and bark tissues of *Olea europaea* L. (Eris *et al.* 2007). The presence of leaves improves cutting rooting (Hartmann *et al.* 2011). Leaf reduction to half of the original area has been indicated for *I. paraguariensis* propagation by single-bud mini-cuttings (Pimentel *et al.* 2020). In this species, a significant inverse relationship was found between mini-cutting diameter and the percentages of survival and rooting (Gazzana *et al.* 2020a).

Adventitious rooting is a biological phenomenon that is commonly discussed in histological terms. Histology, anatomy, and structural studies in a broad way continue to be extremely important as, in terms of plant production, adventitious rooting resulting from experimentation remains a structural manifestation (Lovell & White 1986). Histological and anatomical studies have established a basis that has improved the understanding of adventitious rooting. In this context, the analysis of rooting potential as a function of the structure of the stems is remarkable, especially with respect to anatomical barriers. The study by Sachs *et al.* (1964), among others (Amissah *et al.* 2008; Monder *et al.* 2019), reinforced the need for histological studies, as the importance given to anatomical barriers, due to rooting failure was greatly reduced, in addition to the establishment of morphological markers of reference.

We explored the genetic component of the adventitious root formation by selecting competent genotypes for propagation by mini-cuttings to maximize the gain from selection. We also performed cloning and histological studies of few forest species during this process, as *A. mearnsii* De Wild. and *I. paraguariensis*. *Acacia mearnsii* is a fabaceous tree that is native to Australia. It has

high economic and social importance in Rio Grande do Sul state, Brazil, where it is cultivated for tannin extraction, and cellulose and energy production. *Ilex paraguariensis* is an aquifoliaceous tree that naturally occurs in southern Brazil. Its thin branches and leaves are the main raw material for the tea and energy drinks industry (Cardozo Junior & Morand 2016), and cosmetic and pharmaceutical industries (Dartora *et al.* 2013), which has specific quality requirements and demands a continuous supply (Gazzana *et al.* 2020b).

Therefore, it is evident that knowledge of the species-specific barriers is the key to commercial success of mini-cuttings propagules. The aim of this study was to carry out histological studies to provide new insights into how barriers may affect adventitious rooting of *A. mearnsii* and *I. paraguariensis* mini-cuttings.

Material and Methods

The experiments were performed with propagules collected in a clonal mini-garden established in an acclimatized greenhouse at the Plant Breeding and Vegetative Propagation Center (MPVP), Department of Plant Science, Federal University of Santa Maria (UFSM), Santa Maria (RS), Brazil. The clonal mini-gardens of *A. mearnsii* and *I. paraguariensis* were established on benches in a closed soilless cultivation system, with coarse sand as the substrate and nutrient solution supplied to the culture bed by flood fertigation. Nutrient solution composition, distribution, and management practices were maintained as described by Gazzana *et al.* (2020b). For analyzes on *A. mearnsii* clones AB6, B5 and IA45 were used. For the analyzes on *Ilex paraguariensis*, clones 10SM07 (difficult-to-root) and 06SM15 (easy-to-root) were used.

Mini-cutting preparation and cultivation

Mini-cuttings were prepared by sectioning the shoots into single buds, and the leaves were reduced to 50% of the original area. Mini-cuttings were treated with a hydroalcoholic solution (1:1 v/v) of indolebutyric acid (IBA) at a concentration of 2,000 mg L^{-1} for 10 s, as defined in preliminary studies. Few *I. paraguariensis* mini-cuttings were not treated with IBA. The mini-cuttings were grown in 100-well trays containing equal proportions of a commercial substrate with pine bark base, medium vermiculite, and coarse-grained sand. They were placed on a bench inside a humidity chamber, with the relative humidity maintained above 95% by automated nebulization. Mini-cuttings of *I. paraguariensis* were collected at 0, 30, and 60 d, and those of *A. mearnsii* were collected at 0, 1, 2, 5, 10, 15 and 30 d of cultivation in a humidity chamber for anatomical investigations.

Sample preparation

The basal region of the mini-cuttings was first sectioned into ~ 1 cm pieces. Freehand cuts were made using a razor blade. The samples were fixed in two chemical solutions. A solution containing 3% glutaraldehyde and 0.1 M of sodium phosphate buffer with pH 7.2 (McDowell & Trump 1976), or a 6% solution of glyoxal and the same buffer (Dapson 2007). The samples were washed for 15 min, first in the buffer (pH 7.2) and then in distilled water, respectively.

Samples were subjected to continuous rotation in a microcentrifuge for 5–7 d in tubes containing 2 mL L^{-1} of Tween 20 or 5% Extran solution to remove epicuticular waxes and any lipophilic substances to facilitate subsequent infiltration in 2-hydroxyethyl methacrylate (HEMA). The samples were dehydrated in an ethyl alcohol series (O'Brien & McCully 1981), pre-infiltrated in HEMA and absolute ethanol solution (1:1 v/v) for 12 h, followed by infiltration in HEMA for ~ 4 h, and embedded in a Teflon holder until polymerization was completed (Gerrits & Smid 1983).

The samples were sectioned to 3, 4, and 5 µm thickness using a Leica RM2245 rotary microtome. Histological slides were stained with toluidine blue in 0.05% sodium benzoate buffer of pH 4.4 (Sidman *et al.* 1961). Total phenolic compounds were detected with toluidine blue (O'Brien *et al.* 1964), starch with Lugol's solution (Johansen 1940), starch and total polysaccharides with Schiff´s reagent and periodic acid (O'Brien & McCully 1981), and lipophilic substances with Sudan red 7 B (Brundrett *et al.* 1991). Schiff's reagent and periodic acid and Sudan red 7B histochemical tests were performed individually and in combination with toluidine blue.

To prevent rot disease, symptomatic mini-cuttings of *A. mearnsii* were disinfected in a 70% ethanol solution, followed by 1% solution of sodium hypochlorite. The mini-cuttings were transferred to Petri dishes containing potato dextrose agar medium, sealed, and cultivated at 25 °C under a 12 h photoperiod for 48 h.

Photographic documentation and analyses were performed using a Leica® DM 2000 and M80 microscope with a DFC295 image capture system, Zeiss AxioImager A2 microscope with a Zeiss MCr digital capture system, and ZEN (Zeiss™) software. The mini-cuttings were observed using a stereoscopic microscope (Olympus SZ51). Slides were prepared with fungal structures and visualized using an optical microscope (Zeiss Primo Star). Photomicrographic registration was performed with a Canon Rebel T5i camera with a 105 mm macro lens.

Results

General aspects of the mini-cuttings at time zero

In cross sections, the base of *A. mearnsii* mini-cuttings showed a combined structure of primary and secondary tissues. The primary tissues were the epidermis, fundamental tissue in the cortical region and pericycle, phloem, xylem, and the internal pith (Fig. 1a-b). The secondary tissues were represented by the cambium, phloem, and xylem (Fig. 1b). Based on the results presented below, the cortical region, sclerified and lignified pericycle, and primary and secondary phloem are highlighted. Callus formation occurred in these regions and tissues, through which the vascular connection between the adventitious roots and the stem vascular tissue was established, as well as the recruitment of cells for the formation of adventitious roots. Thus, the outermost cortical layer was typically composed of alternating collenchyma and chlorenchyma (Fig. 1a-b). Internally, the parenchymatic tissue differentiated into one or a few layers (Fig. 1b). The endoderm was composed of a layer of cells with a parenchymatic structure, with several cells being crystalliferous idioblasts (Fig. 1b). The pericycle was deep inside and composed of septate and sclereid fibers, both of which were usually alive (Fig. 1c). Sclerification and lignification of fibers was centripetal and gradual, cells were alive in most mini-cuttings, and higher number of cells was observed in young internodes. Even with relative lignification, the fibers divided transversally, becoming septate, in which, it was possible to distinguish basic aspects such as cytoplasm, vacuoles, and nucleus, in addition to pecto-cellulosic transverse walls (Fig. 1c). Although the pericycle was continuous, sclerification and lignification did not occur in all cells, as observed by the parenchymatic structure in some pericycle cells (Fig. 1b).

The stem structure of *I. paraguariensis* among the mini-cuttings investigated always presented with secondary xylem and phloem, although with a limited formation of tissues. Therefore, it still retained all the primary tissues (Fig. 2a), and in some cases, even presented general aspects of the primary organ. In the context of the present study, it was important to highlight the structure of the epidermis, pericyclic sclerenchyma, cortical region, and primary and secondary phloem (Fig. 2a-e).

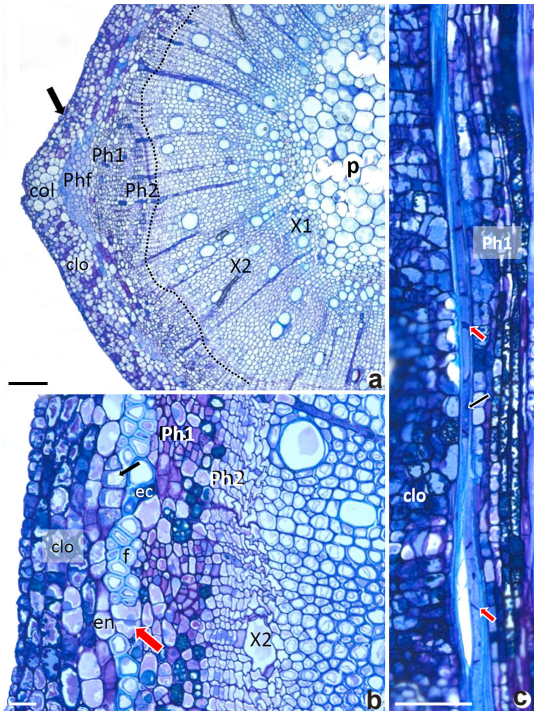

Figure 1 – a-c. Cross-sectional (a, b) and longitudinal (c) sections of the stem of *Acacia mearnsii* clone B5 at time zero – a. general appearance with indications of different primary and secondary tissues (arrow, epidermis; dotted line, vascular cambium); b. detail of the discontinuous pericyclic sclerenchyma region containing fibers (f), sclereids (ec) and cells with parenchyma structure (red arrow) [black arrow indicates crystalliferous idioblasts in endoderm (en)]; c. detail of a portion of the cortical and vascular region separated by septate fibers with emphasis on the transverse walls (red arrows) and nuclei (black arrows). Short forms: col = collenchyma; clo = chlorenchyma; Phf = phloem fiber bundle; Ph1 = primary phloem; Ph2 = secondary phloem; X2 = secondary xylem; X1 = primary xylem; p = pith. Scale bars = 50 µm.

The epidermis contained a single layer of cells when mini-cuttings were produced. In the cortical region, a layer of chlorenchyma was observed, and more internally, parenchymatic tissue (Fig. 2a-b), with an internal limit in the endoderm (Fig. 2a,b). The pericyclic sclerenchyma was generally not continuous (Fig. 2c,d) and was composed of fibers and sclereids (Fig. 2b-d). The thickness of this sclerenchyma varied from one to six layers, alternating with cells with a parenchymatic structure or crystalliferous idioblasts (Fig. 2c-d). The phloem region, comprising primary and secondary tissues, contained many parenchyma and companion cells (Fig. 2c-d). Small amounts of starch grains were identified in the chlorenchyma, endodermis, xylem rays, and medullary regions.

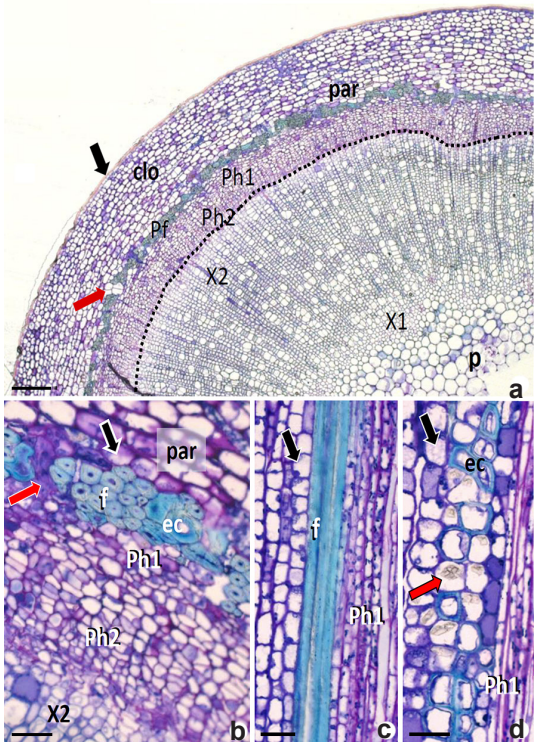

Figure 2 – a-d. Transverse (a-b) and longitudinal (c-d) sections of *Ilex paraguariensis* clones 10SM07 and 06SM15 at time zero (a,c,d. clone 10SM07; b. clone 06SM15) – a. general anatomy of the stem showing primary and secondary tissues (arrow = epidermis; red arrow = sclerenchyma discontinuity; dotted line = vascular cambium); b. detail of a portion of the pericyclic sclerenchyma with emphasis on the external parenchyma (pair) and internal primary phloem (Ph1) and secondary phloem (Ph2) and the discontinuity of sclerification (red arrow) and endoderm (black arrow); c. detail of fibers (f) of the pericyclic region and endoderm (black arrow); d. detail of sclereids (ec) of the pericyclic region, endoderme (black arrow) and crystals (red arrow). Short forms: clo = chlorenchyma; par = parenchyma; Pf = primary phloem fibers; f = pericyclic fibers; ec = sclereids; Ph1 = primary phloem; Ph2 = secondary phloem; X2 = secondary xylem; X1 = primary xylem; p = pith. Scale bars = 50 µm.

Adventitious rooting process of the mini-cuttings

Among the evaluated clones of *A. mearnsii*, a clear reaction to cultivation was observed in the first 20–26 h. The endoderm is the tissue that responds initially with dedifferentiation and cell proliferation, although primary phloem cells may also respond (Fig. 3a) or proliferate (Fig. 3b) in both the primary and secondary phloem regions. Such proliferation occurred in several samples without the due accompaniment of the external portion of the cortex and epidermis, and these tissues were therefore compressed, with extensive accumulation of phenolic compounds in the remaining tissue in these regions (Fig. 3b). In locations where reactions were observed in the primary phloem, discontinuity of the sclerenchyma was common (Fig. 3a).

In general, after the initial process of endodermal cell proliferation in *A. mearnsii*, the phloem region actively contributes to new cells through primary and secondary tissues. Thus, at 30 d of cultivation, the phloem proliferation process, together with cortical tissue, generated important structural consequences for the process with projection of new tissue between sclerenchyma groups and the formation of conductive tissues, which were already connected to the stem vascular tissue (Fig. 3c). Endoderm and phloem cells around the sclerenchymatic clusters remained divisible (Fig. 3d). The tissues derived from the phloem and endoderm differentiated into groups of vascular elements (Fig. 3c) and roots were formed (Fig. 3d). This process took place within 30 d, although samples were not collected and the exact period is still unknown. In samples of clone AB6 of *A. mearnsii*, only the reaction of the cortical region to the culture was common (Fig. 4a), without impeding root formation (Fig. 4b). Although the volumes of fundamental tissue between sclerenchyma clusters were smaller, there was differentiation of conductive elements between adventitious roots and stem vascular tissue (Fig. 4b). Furthermore, in the region of the

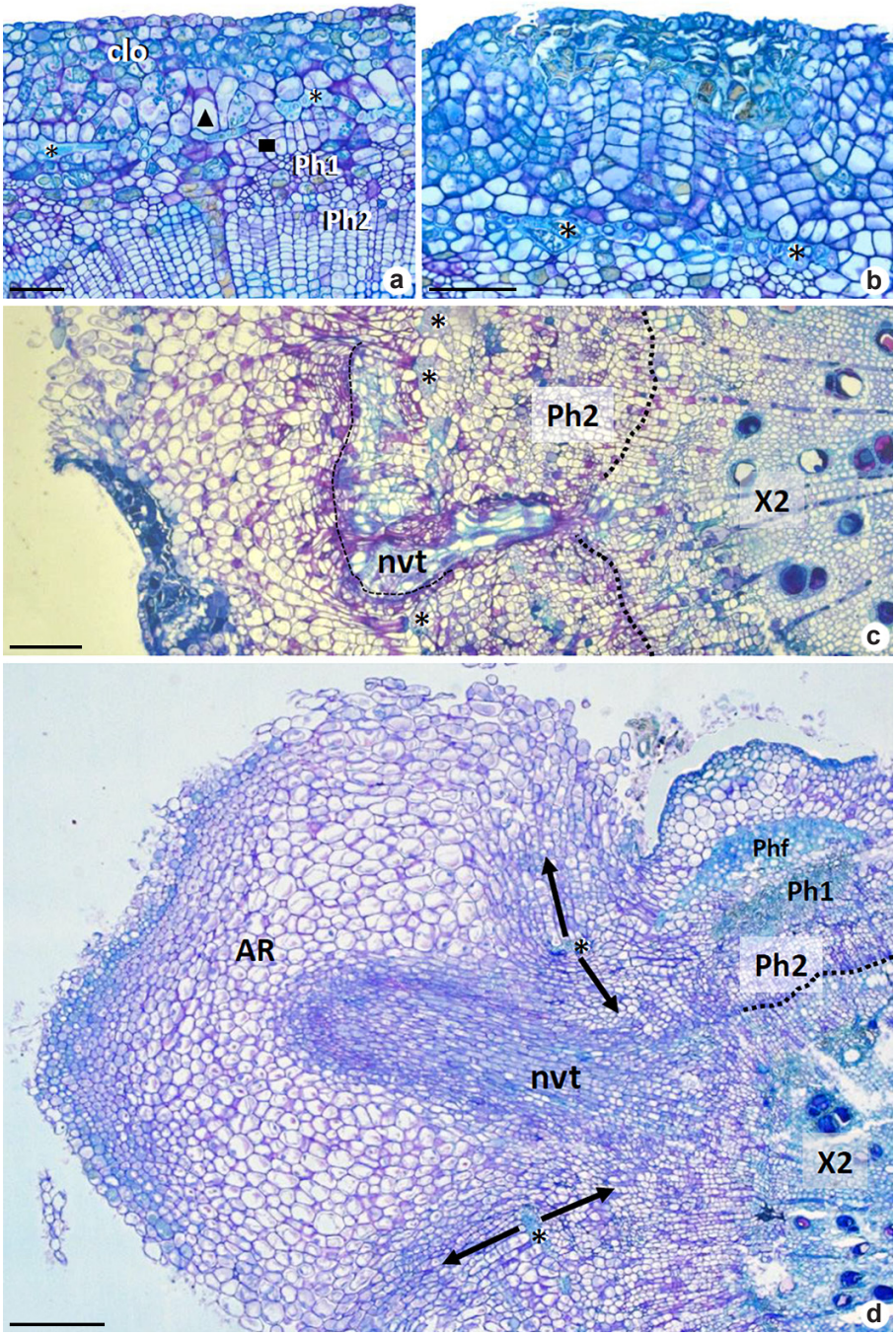

Figure 3 – a-d. Cross sections in the base region of the of mini-cuttings of *Acacia mearnsii* clones – a. clone IA45 after 24 h of cultivation. Black triangle = endoderm reacting to cultivation; black square = primary phloem cells reacting to cultivation. Fiber groups (*); b. clone AB6 after 24 h of cultivation. Fiber groups (*); c. clone B5 after 30 d of cultivation. Neoformed vascular tissue (nvt) has a cambium line through the vascular and cortical regions (dashed line). Dotted line = vascular cambium; fiber groups (*); d. clone B5 after 30 d of culture showing a region with adventitious root (AR) and established vascular connection (nvt). Fiber groups (*) far apart with space filled with newly formed tissue. Arrows indicate the direction of proliferation from fiber clusters. Dotted line = vascular cambium. Short forms: Short forms: clo = chlorenchyma; Phf = primary phloem fibers; Ph1 = primary phloem; Ph2 = secondary phloem; X2 = secondary xylem; X1 = primary xylem. Scale bars: a-c = 50 µm; d = 200 µm.

vascular connection between adventitious roots and stems, cambial activity was lost (Fig. 3d). The understanding of how the vascular groups in the callus are spatially differentiated is limited due to the loss of orientation within the axial and radial planes of symmetry of the cutting.

The rooting process of *Acacia mearnsii* mini-cuttings (Fig. 5a) was accompanied by hyphal proliferation of *Cylindrocladium* sp. (Fig. 5b-c) with extensive tissue dissolution, mainly of the vascular cambium (Fig. 5d,f) that was observed at 48 h of cultivation (Fig. 5d). Hyphae were observed within the dissolved tissues and inside cells (Fig. 5e). Consequently, a discontinuity occurred between the live tissues that surrounded the cambium and through which vascular connections to the adventitious roots would be built (Fig. 5f). This tissue dissolution is acropetal (Fig. 5a), and ended up being colonized in all or almost the entire circumference. In affected mini-cuttings, it is common for the roots to emerge far above the basal region where the infection occurred (Fig. 5a), in addition to the emergence of a few roots facing the same direction, or even the emergence of a single root (Fig. 5a).

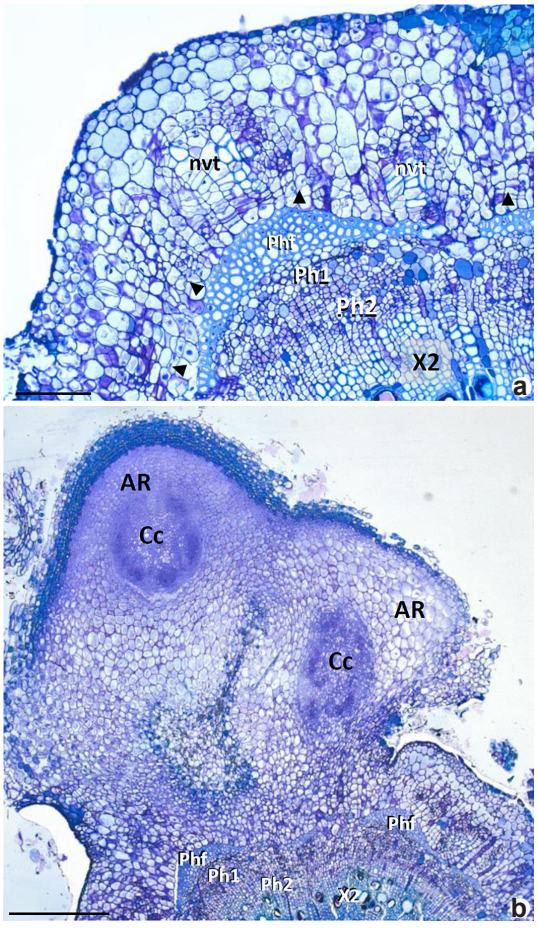

Figure 4 – a-b. Cross sections in the region of the base of the mini-cuttings of clone AB6 of *Acacia mearnsii* – a. 30 d of cultivation. Neo-formed vascular tissue (nvt) in the cortical region; b. general aspects showing region with emergence of two adventitious roots (AR). Short forms: Black triangle = endoderm reacting to culture; Phf = primary phloem fibers; Ph1 = primary phloem; Ph2 = secondary phloem; X2 = secondary xylem; Cc = central cylinder. Scale bars: a = 100 µm; b = 200 µm.

In *Ilex paraguariensis*, both the evaluated clones (06SM15 and 10SM07) exhibited three common characteristics: (a) both produced roots, (b) exhibited identical processes of callogenesis and differentiation of adventitious roots, and (c) the roots always formed at the base of the mini-cuttings, that is, through the regions exposed by the cut at the time of the mini-cutting preparation (Fig. 6a-b). In rare situations, there was a combined emergence of adventitious roots above the region of the cut, and therefore, through the cortical region and epidermis (Fig. 6b), presenting aspects of direct rhizogenesis.

The initial process of mini-cutting differentiation in *I. paraguariensis* was marked by cell proliferation on both sides of the pericyclic sclerenchyma, mainly cells of the primary and secondary phloem, and cells of the inner portion of the cortical region, including the endoderm (Fig. 6c). Rapid proliferation produced two effects. One of these is macromorphological, which makes the base of the mini-cuttings bulky (Figs 6b). The second effect, histologically, is the discontinuity of the pericyclic sclerenchyma, that is interrupted synchronously by the cortical and phloem tissues (Fig. 6c). Thus, the previously isolated cortical and vascular regions (Fig. 2a) became continuous. In general, cortical tissues react within the first millimeter of the wound together with phloem tissues. Above the base, the main contribution to callus is the phloem. Cells from these new tissues were recruited and differentiated into conductive elements that connected the adventitious roots to the stem conductive tissues (Fig. 6c-d), and the process was completed before 30 d of culture.

Detection of starch grains

In *Acacia mearnsii*, only a few starch grains were observed, and other polysaccharides were not observed in the fundamental tissues of either pre-

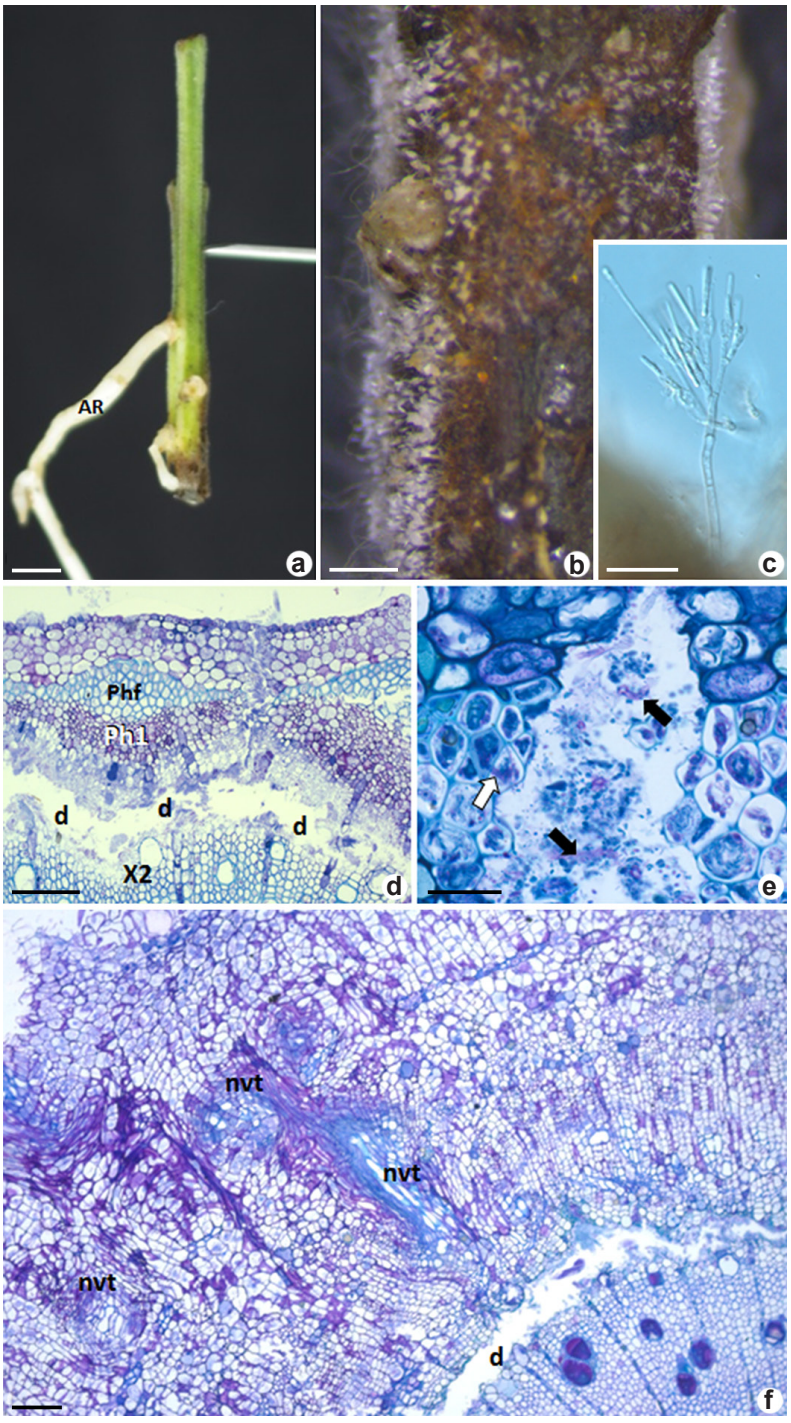

Figure 5 – a-f. Side view (a-b), cross-sections of mini-cuttings (d-f) of *Acacia mearnsii* clones, and (c) side view of conidiophores and conidia of *Cylindrocladium* spp – a. clone B5 at 30 d of cultivation; b. general appearance of the mini-cutting showing presence of *Cylindrocladium* spp. *sporulation*; c. conidiophores and conidia of *Cylindrocladium* spp. under differential and interferential contrast; d. clone B5, 48 h of culture showing dissolution of the cambial region (d); e. clone AB6, detail of infected region with the presence of extracellular hyphae (black arrows) and intracellular hyphae (white arrow); f. clone B5 at 30 d of culture with dissolution of the cambial region (d). Short forms: AR = adventitious root; Phf = primary phloem fibers; Ph1 = primary phloem; X2 = secondary xylem. Scale bars: a = 200 µm; b = 500 µm; c = 50 µm; d = 50 µm, e = 25 µm; f = 50 µm.

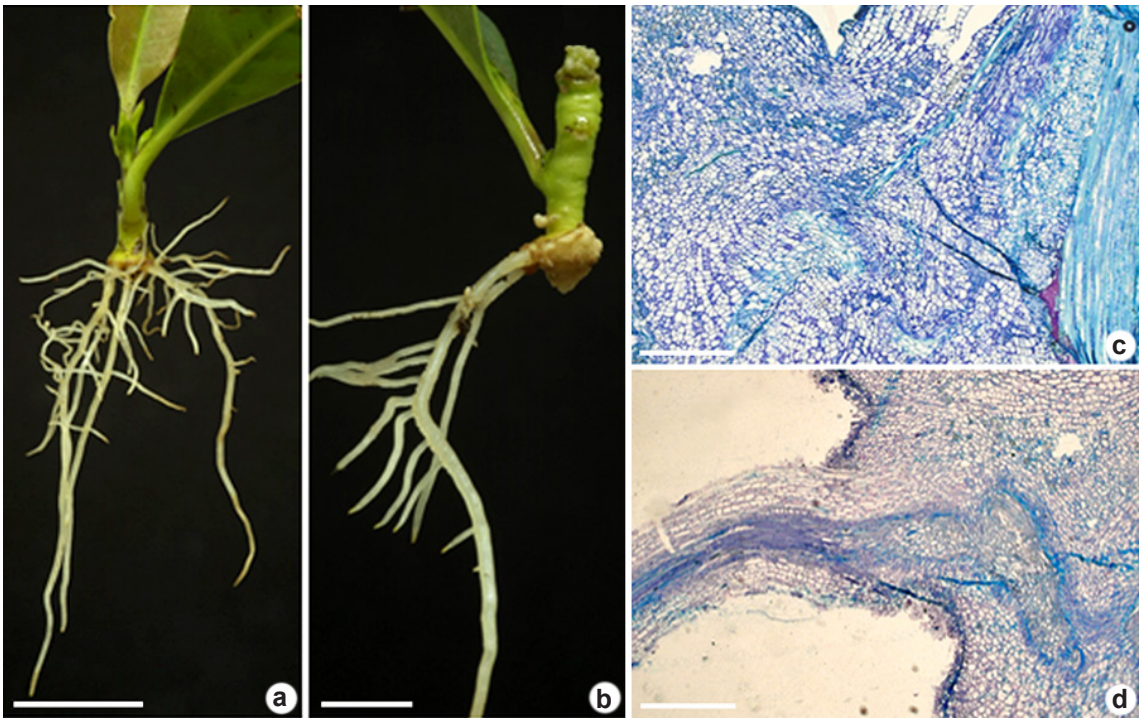

Figure 6 – a-d. Side views of stems from clone 13SM05 (a)(b) Longitudinal sections of stems from clone 10SM07 (c) (d) of *Ilex paraguariensis* at 30 days of cultivation – a. adventitious roots emerged from the base in the mini-cutting; b. adventitious roots emerged at the mini-cutting base and adventitious root emerged above the base (arrow); c. black dotted lines indicate continuity of newly formed vascular tissues through sclerenchyma clusters connecting the callus to the stem vascular tissues, red dotted lines indicate pericyclic fibers; d. black dotted lines indicate continuity of newly formed vascular tissues through the callus with a central cylinder (Cc). Short forms: nvt = neo-formed vascular tissue; X2 = secondary xylem; cls = callus. Scale bars: a-b. 10 mm; c-d. 400 μm.

(shoot) or neo-formed (callus) tissues, regardless of the rooting competence of the studied clone (Fig. 7a-b). In the 06SM15 clone of *I. paraguariensis* with greater rooting competence, the presence of large amounts of starch grains in cells was generalized to pre- and neo-formed tissues (Fig. 7c), unlike clones with lower rooting competence (Fig. 7d). In both the species studied, there was no accumulation of polysaccharides in vacuoles.

Discussion

General aspects of the mini-cuttings at time zero

Regarding anatomical aspects, it should be noted that the stems of *A. mearnsii* and *I. paraguariensis* were highly similar. Some tissue and cytological aspects varied, mainly in terms of tissue distribution in the cortical region, sclerification, and lignification in the pericycle and primary phloem fibers.

Adventitious rooting process of the mini-cuttings

In the present study, there was a discontinuous sclerenchymatic layer in the pericyclic region of the internodes, and this discontinuity was amplified as a function of callus formation. Sclerenchyma discontinuity is one of the variations, considering continuous (Bryant & Trueman 2015; Sá *et al.* 2022) or absence of sclerenchyma (Harbage *et al.* 1993; Ahkami *et al.* 2009) is also reported. Beakbane (1961) and Goodin (1965) considered the sclerified layer as a factor that reduces the rooting potential, including in *A. mearnsii* (Beakbane 1961), thus acting as a barrier to rooting. In the present study, there was no evidence of its effects on the rhizogenic potential of clones already tested in either species, especially in those considered to have low rooting potential. Altamura (1996), in general, considered that in species with greater rhizogenic potential, the sclerenchymatic barrier

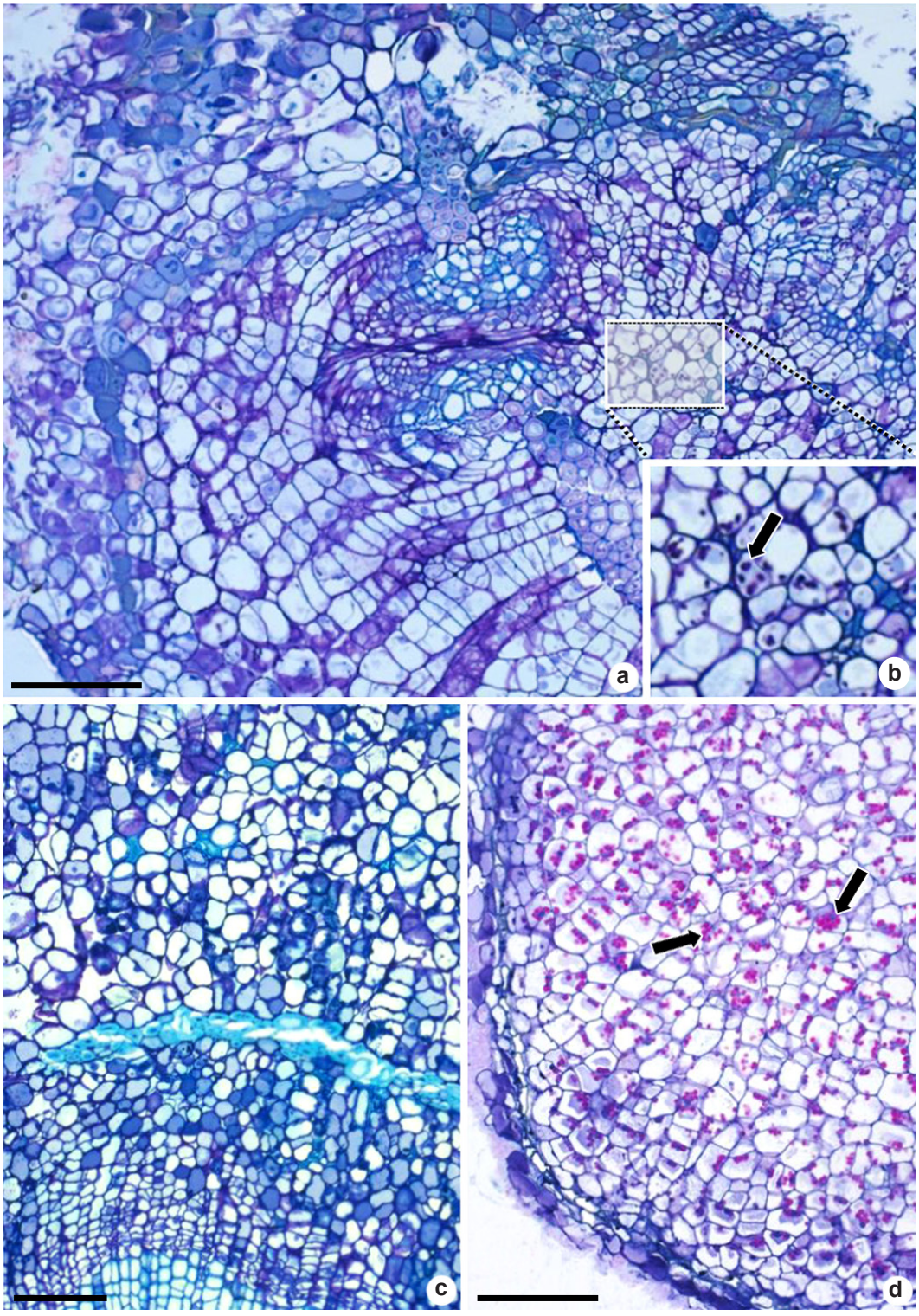

Figure 7 – a-d. Cross-sections of mini-cuttings. (a)(b) *Acacia mearnsii*. (c)(d) *Ilex paraguariensis* – a. clone B5. General aspect of a stem region with the presence of callus. Few cells with starch grains are seen. Rectangle indicates site of occurrence of starch cells derived from phloem callogenesis; b. detail of starch cells (arrow); c. detail of the shoot region after callogenesis in clone 10SM07 with starch-free tissues; d. detail of a portion of callus in clone 06SM15 with tissues containing a large numbers of amiliferous cells (arrows). Scale bars: a,c = 200 µm; d = 400 µm.

may be present, but discontinuously, when comparing such potentials in species whose sclerenchymatic layer is continuous. Loss of continuity of peripheral sclerified tissue can be achieved by culture conditions that induce cell proliferation and growth, such as exposure to mist and hormone treatment (Sachs *et al.* 1964), or hormone application alone (Altamura 1996), increasing potential sites of vascularization and/or root emergence. Sachs *et al.* (1964) present, as part of their argument against the sclerenchymatic layer being the cause of non-rooting or potential reduction, that the mini-cutting structure does not have any barrier in the cut region, which is the point of root emergence, having proven such a possibility in *Anacardium occidentale* L., according to Jásik & De Klerk (1997) in *Olea europaea* L. (Altamura 1996), *Coffea robusta* L.Linden (Reaño 1940) and *Dianthus caryophyllus* L. (Stangler 1956), where "root primordia are initiated, grow down inside the fiber band and emerge through the cut base" (Lovell & White 1986).

The proliferation of cortical and phloem tissues is relatively discrete in the species studied, mainly in *A. mearnsii*, once callus formation imposes a lower morphological alteration. Rhizogenesis is therefore indirect and necessarily passes through the stages of rapid stimulation, tissue reaction with proliferation and dedifferentiation, formation of meristemoids, initial differentiation of adventitious roots, and differentiation of conductive tissue, which is concomitant with the growth of adventitious roots. In addition, the process is acropetously active and vertical series of new tissue are formed, which are relatively longer in *A. mearnsii*. In this context, in genotypes with lower rooting competence, if rooting is indirect, the anatomical barrier becomes discontinuous with tissue filling, it is possible that the anatomical barrier or structural barrier does not exist.

For both species, the sclerified tissue in the cortical region seems to be important to the process, as intense cell proliferation occurred in the parenchymatic tissues surrounding them, a process that is considered essential for the formation of vascular tissue, which is similar to that described for *Malus domestica* (Suckow) Borkh. "Jork 9" (Jásik & De Klerk 1997), among others (Altamura 1996). Therefore, sclerified clusters appear to contribute to callus formation, vascularization and rooting.

A comparison of the rooted mini-cuttings showed a marked difference in *A. mearnsii* due to the easy separation of the bark, the absence of rooting at the base of the mini-cuttings, and the formation of few roots or only one root. The adventitious root structure of *A. mearnsii* is predominantly different from that observed in other studies, including *I. paraguariensis*, both due to the smaller number of roots formed and due to its asymmetric distribution. This deficiency in root system formation is maintained during the growth and development of plants in the field and is associated with the high percentage of dislodging observed in some clones in the first years of cultivation. The inclusion of histological studies was also motivated by these characteristics, although the study hypothesis was that only intrinsic structural differences could be associated with both the type and rooting potential. Therefore, the context is very similar to the usual assessments of rooting potential associated with structural barriers.

In *A. mearnsii* the same conditions necessary for maximizing the rooting process favor disease development, in this case caused by the fungus *Cylindrocladium* sp. To our knowledge, this is not the first report of rot disease in *A. mearnsii* mini-cuttings. Twenty-nine endophytic fungi have been isolated from asymptomatic mini-cuttings (Duin *et al.* 2018), and the genus *Calonectria* De Not. is the most common cause of rot disease (Duin *et al.* 2017).

It should be noted that among the investigated clones of *A. mearnsii* differing in rooting competence, there was a single and robust radial vascular connection with an adventitious root. Notably, fungal infection is not present in these places, which may indicate that hyphal proliferation may be inhibited.

Detection of starch grains

In the studied species, among the different clones, starch in a reasonable amount was identified only in the mini-cuttings of the 06SM15 clone of *I. paraguariensis*, which has already been highlighted for presenting the best rooting rates (Pimentel *et al.* 2019). In general, results related to starch are usually considered controversial because of the lack of a pattern regarding rooting. Thus, in some studies, similar to the present one, there is a positive relationship between starch and adventitious rooting (Reuveni & Raviv 1980; Aslmoshtaghi & Shahsavar 2010; Pimentel *et al.* 2020), whereas other studies observed the opposite (Denaxa *et al.* 2012), or no correlation between the presence of starch and adventitious rooting (Ferriani *et al.*

2008; Lima *et al.* 2011), as also observed in the present study for *A. mearnsii*. Thus, starch would be an interesting marker only for *I. paraguariensis*. In the cases of *A. mearnsii* and *I. paraguariensis*, complementary studies integrating soluble sugars, among other factors, and adventitious rooting are necessary.

Although there is a positive relationship between starch and/or other sugars and rhizogenesis, this is not a cause-and-effect relationship (Del Rio *et al.* 1991; Rahman *et al.* 2002). However, excess starch may indicate an excess of carbohydrates and consequent availability in at least one clone of *I. paraguariensis*. There does exist a demand for structural sugars and energy sources, mainly for species with indirect rooting, as the calluses themselves are important drains, and their differentiation ends up overlapping the rooting itself. In addition to the fact that the species in the present study, even considering their different rooting potentials, present a relatively fast process, as they already had functional roots 30 d after the start of cultivation. Thus, there is a demand for several different morphogenetic processes over a short period of time.

The use of the periodic acid-Schiff (PAS) method in the present study was associated with the possibility of detecting sugars, mainly hexoses in the cytoplasm or vacuoles (Kiernan 1981), which has been successful in plant reproductive cells (Oliveira *et al.* 2015). However, for both species in the present study, the analyses did not allow for conclusions regarding the presence of other sugars. In general, there is a correlation between starch, its mobilization, and other sugars, which has been demonstrated in adventitious rooting using biochemical methods (Reuveni & Raviv 1980; Del Rio *et al.* 1991; Rahman *et al.* 2002; Ferriani *et al.* 2008; Aslmoshtaghi & Shahsavar 2010; Lima *et al.* 2011).

For both the species, histological analysis contributed substantially to diagnosis. In *Acacia mearnsii*, the absence of tissue appears to be an anatomical barrier. For *Ilex paraguariensis*, the abundance of starch was remarkable in clones with the greatest rooting competence. In *A. mearnsii*, starch grains were not observed in fundamental tissues regardless of the rooting competence of the clone.

Although there are some differences, the formation of adventitious roots in both species and the studied clones followed a similar pattern, from the recruitment of cells during callus formation, adventitious vascularization, and the indirect formation of functional roots. The main differences were associated with the response speed for adventitious rooting that starts from cortical meristemoids, followed by the differentiation of conductive tissue from a neo formed one, which connects the periphery with the secondary vascular tissue. Therefore, this neo-formed tissue promotes the structural basis necessary for radial vascular connections. Both the studied species have barriers to rhizogenesis, but these are not necessarily anatomical in nature.

Acknowledgements

The authors are grateful to the Coordination for the Improvement of Higher Education Personnel (CAPES - Finance Code 001); the National Council for Scientific and Technological Development (CNPq - Grant 302388/2019-2) of the Ministry of Science and Technology of Brazil; and Tanagro SA (Agreement AGTT 011/2019), for scholarships and partially supporting this research.

Data availability statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

References

Ahkami AH, Lischewski S, Haensch KT, Porfirova S, Hofmann J, Rolletschek H, Melzer M, Franken P, Hause B, Druege U & Hajirezaei MR (2009) Molecular physiology of adventitious root formation in *Petunia hybrida* cuttings: involvement of wound response and primary metabolism. New Phytologist 181: 613-625.

Altamura MM (1996) Root histogenesis in herbaceous and woody explants cultured *in vitro*. A critical review. Agronomie 16: 589-602.

Amissah JN, Paolillo DJ & Bassuk N (2008) Adventitious root formation in stem cuttings of *Quercus bicolor* and *Quercus macrocarpa* and its relationship to stem anatomy. Journal of the American Society for Horticultural Science 133: 479-486.

Aslmoshtaghi E & Shahsavar AR (2010) Endogenous soluble sugars, starch contents and phenolic compounds in easy- and difficult-to-root olive cuttings. Journal of Biological & Environmental Sciences 4: 83-86.

Beakbane AB (1961) Structure of the plant stem in relation to adventitious rooting. Nature 192: 954-955.

Bisognin DA (2011) Breeding vegetatively propagated horticultural crops. Crop breeding and applied biotechnology 11: 35-43. Available at <https://www.scielo.br/j/cbab/a/JLNSzyYxYCyzFhNqfKmtXCt/?format=pdf&lang=en>. Access on 18 July 2022

Brondani GE, Wendling I, Brondani AE, Araujo MA, Silva ALL & Gonçalves AN (2012) Dynamics of adventitious rooting in mini-cuttings of *Eucalyptus benthamii* x *Eucalyptus dunnii*. Acta Scientiarum-Agronomy 34: 169-178. DOI: 10.4025/actasciagron.v34i2.13059

Brundrett MC, Kendrick B & Peterson CA (1991) Efficient lipid staining in plant material with Sudan Red 7B or Fluoral Yellow 088 in polyethylene glycol-glycerol. Biotechnic & Histochemistry 66: 111-116.

Bryant PH & Trueman SJ (2015) Stem anatomy and adventitious root formation in cuttings of *Angophora*, *Corymbia* and *Eucalyptus*. Forests 6: 1227-1238.

Burin C, Batistel SC, Lohmann G & Bisognin DA (2020) Rooting competence of mini-cuttings of *Cabralea canjerana* clones in different seasons. Ciência Rural 50: e20190451. DOI: https://doi.org/10.1590/0103-8478cr20190451

Cardozo Junior ELC & Morand C (2016) Interest of mate (*Ilex paraguariensis* A. St.-Hil.) as a new natural functional food to preserve human cardiovascular health – a review. Journal of functional foods, 21: 440-454. DOI: 10.1016/j.jff.2015.12.010

Dapson RW (2007) Glyoxal fixation: how it works and why it only occasionally needs antigen retrieval. Biotechnic & Histochemistry 82: 161-166.

Dartora N, Souza LM, Paiva SMM, Scoparo CT, IacominiM, Gorin PAJ, Rattmann YD & Sassaki GL (2013) Ramnogalacturonano de *Ilex paraguariensis*: um potencial adjuvante em sepsia tratamento. Carbohydrate Polymers 92: 1776-1782.

de Lima MS, Araujo MM, Berghetti ALP, Aimi SC, Griebler AM, Somavilla LM, Santos OP & Valente BMRT (2022) Mini-cutting technique application in *Corymbia* and *Eucalyptus*: effects of mini-tunnel use across seasons of the year. New Forests 53: 161-179. DOI: https://doi.org/10.1007/s11056-021-09851-4

Del Rio C, Rallo L & Caballero JM (1991) Effects of carbohydrate content on the seasonal rooting of vegetative and reproductive cuttings of olive. Journal of Horticultural Science 66: 301-309.

Denaxa NK, Vemmos SN & Roussos PA (2012) The role of endogenous carbohydrates and seasonal variation in rooting ability of cuttings of an easy and a hard to root olive cultivars (*Olea europaea* L.). Scientia Horticulturae 143: 19-28.

Duin IM, Higa AR, Santos AF, Coelho TAV, Rezende EH & Auer CG (2017) Etiologia e possíveis fontes de inóculo para a ocorrência da podridão de miniestacas de acácia-negra. Summa Phytopathologica 43: 297-302.

Duin IM, Coelho TADV, Gracia PC, Santos ÁFD, Pimentel IC & Auer CG (2018) Identificação de fungos endofíticos em miniestacas de acácia-negra. Summa Phytopathologica 44: 278-280.

Eris A, Gulen H, Barut E & Cansev A (2007) Annual patterns of total soluble sugars and proteins related to coldhardiness in olive (*Olea europaea* L. 'Gemlik'). The Journal of Horticultural Science and Biotechnology 82: 597-604. DOI: 10.1080/14620316.2007.11512279

Ferriani AP, Mayer JLS, Zuffellato-Ribas KC, Cleusa BONA, Koehler HS, Deschamps C & Oliveira MC (2008) Cutting and anatomy of vassourão-branco. Scientia Agraria 9: 159-166.

Gazzana D, Pimentel N, Lohmann GT, Maculan LG & Bisognin DA (2020a) Vegetative propagation of mate from shoot cuttings induced by coppicing of selected plants. Semina: Ciências Agrárias 41: 1849-1860.

Gazzana D, Pimentel N, Lohmann GT, Spanevello JdF & Bisognin DA (2020b) Selection between and within half-sibling progenies of *Ilex paraguariensis* for adventitious rooting of mini-cuttings. Crop Breeding and Applied Biotechnology 20: 4. DOI: 10.1590/1984-70332020v20n4a56

Gerrits PO & Smid L (1983) A new, less toxic polymerization system for the embedding of soft tissues in glycol methacrylate and subsequent preparing of serial sections. Journal of Microscopy 132: 81-85.

Goodin JR (1965) Anatomical changes associated with juvenile-to-mature growth phase transition in *Hedera*. Nature 208: 504-505.

Harbage JF, Stimart DP & Evert RF (1993) Anatomy of adventitious root formation in microcuttings of *Malus domestica* Borkh.Gala'. Journal of the American Society for Horticultural Science 118: 680-688.

Hartmann HT, Kester DE, Davies FT Jr. & Geneve RL (2011) Plant propagation: principles and practices. 8th ed. Prentice-Hall, Boston. 915p.

Jásik J & De Klerk GJ (1997) Anatomical and ultrastructural examination of adventitious root formation in stem slices of apple. Biologia Plantarum 39: 79-90.

Johansen DA (1940) Plant Microtechnique. McGraw-Hill, London. 530p.

Kiernan JA (1981) Histological & histochemical methods: theory & practice. Pergamon Press, Oxford. 344p.

Lakehal A & Bellini C (2019) Control of adventitious root formation: insights into synergistic and antagonistic hormonal interactions. Physiologia Plantarum 165: 90-100.

Lima DM, Biasi LA, Zanette F, Zuffellato-Ribas KC, Bona C & Mayer JLS (2011) Rooting capacity of *Maytenus muelleri* Schwacke cuttings with indolebutyric acid application related to anatomical

aspects. Revista Brasileira de Plantas Medicinais 13: 422-438.

Lovell PH & White J (1986) Anatomical changes during adventitious root formation. *In*: Jackson MB (ed.) New root formation in plants and cuttings. Developments in plant and soil sciences. Vol. 20. Springer, Dordrecht. Pp. 111-140.

Majada J, Martínez-Alonso C, Feito I, Kidelman A, Aranda I & Alía R (2011) Mini-cuttings: an effective technique for the propagation of *Pinus pinaster* Ait. New Forests 41: 399-412.

McDowell EM & Trump BF (1976) Histologic fixatives suitable for diagnostic light and electron microscopy. Archives of Pathology & Laboratory Medicine 100: 405-414.

Monder MJ, Kozakiewicz P & Jankowska A (2019) Anatomical structure changes in stem cuttings of rambler roses induced with plant origin preparations. Scientia Horticulturae 255: 242-254.

Naidu D & Jones N (2016) Evaluation of mini-cuttings technique as a propagation system for *Eucalyptus* hybrids©. Proceedings of the 2015 Annual Meeting of the International Plant Propagators' Society 1140: 1-6. DOI: https://doi.org/10.17660/ActaHortic.2016.1140.1

O'Brien T, Feder NMEM & McCully ME (1964) Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59: 368-373.

O'Brien TP & McCully ME (1981) The study of plant structure principles and selected methods. Termarcarphi Pty, Melbourne. 357p.

Oliveira JMS, Martins MS, Dorneles MP & Freitas CC (2015) Starch distribution in anthers, microspores and pollen grains in *Aechmea recurvata* (Klotzsch.) LB Sm. *Dyckia racinae* LB Sm. Acta Botanica Brasilica 29: 103-112.

Pereira MO, Ângelo AC, Navroski MC, Nicoletti MF, Nascimento B, Sá ACS, Gasparin E & Lovatel QC (2018) Roots of mini-cutting of clones of *Sequoia sempervirens* in different seasons of the year. CERNE 24: 452-460. DOI: https://www.researchgate.net/publication/332396621

Pimentel N, Lencina KH, Kielse P, Rodrigues MB, Somavilla TM & Bisognin DA (2019) Produtividade de minicepas e enraizamento de miniestacas de clones de erva-mate *Ilex paraguariensis* A. St. Hil. Ciência Florestal 29: 559-570.

Pimentel N, Pedroso MF, Lencina KH, Oliveira JMS & Bisognin DA (2020) Anatomical characterization of the adventitious roots of mate (*Ilex paraguariensis* A. St.-Hil.) mini-cuttings. Brazilian Archives of Biology and Technology 63. DOI: 10.1590/1678-4324-2020190359

Rahman N, Awan AA, Nabi G & Ali Z (2002) Root initiation in hard wood cuttings of olive cultivar coratina using different concentration of IBA. Asian Journal of Plant Sciences 1: 563-564.

Reaño PC (1940) Histological study and observations on the effects of some synthetic growth substances on the stem tip cuttings of coffee. Philippine Agriculturist 29: 87-99.

Reuveni O & Raviv M (1980) Importance of leaf retention to rooting of avocado cuttings. Journal of the American Society for Horticultural Science 106: 127-130.

Sá FP, Gomes EN, Maggioni RA, Wendling I, Helm CV, Sant'Anna-Santos BF & Zuffellato-Ribas KC (2021) Biochemical and anatomical features of adventitious rhizogenesis in apical and basal mini-cuttings of Ilex paraguariensis. New Forests 53: 1-20.

Sachs RM, Loreti F & De Bie J (1964) Plant rooting studies indicate sclerenchyma tissue is not a restricting factor. California Agriculture 18: 4-5.

Shao F, Wang S, Huang W & Liu Z (2018) Effects of IBA on the rooting of branch cutting of Chinese jujube (*Zizyphus jujuba* Mill.) and changes to nutrients and endogenous hormones. Journal of forestry research 29: 1557-1567. DOI. https://doi.org/10.100s11676-017-0557-67

Sidman RL, Mottla PA & Feder N (1961) Improved polyester wax embedding for histology. Stain Technology 36: 279-284.

Stangler BB (1956) Origin and development of adventitious roots in stem cuttings of *Chrysanthemum*, carnation, and rose. Cornell University Agricultural Experiment Station Memoir 342: 1-24.

Area Editor: Dr. João Paulo Basso-Alves
Received on August 28, 2023. Accepted on March 04, 2024.