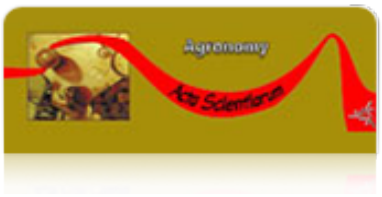

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Adventitious rooting and anatomical aspects of *Campomanesia phaea* stems

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ABSTRACT. Current literature is lacking regarding the vegetative propagation of the cambuci tree (*Campomanesia phaea* O. Berg Landrum). This study aimed to verify the efficiency of cutting techniques via the assessment of cuttings of varying types and sizes, sampling dates, and the influence of treatments containing plant growth regulator indolebutyric acid (IBA) combined with the antioxidant ascorbic acid across different immersion times. The species was found not to develop adventitious roots easily, and hence, the herbaceous and woody stem materials were subjected to histochemical studies to elucidate their anatomical and physiological processes. To this end, plant material was polymerized in glycol methacrylate resin, cut into sections, and stained using toluidine blue (0.05%) or via histochemical staining with PAS/naphthol blue black. Anatomical analysis of plant structures revealed the presence of phenolic compounds and sclerenchyma tissue, which in turn are expected to negatively impact the development of adventitious roots.

Keywords: cambuci; vegetative propagation; cuttings; plant anatomy; indolebutyric acid.

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Introduction

The cambuci tree (*Campomanesia phaea* O. Berg Landrum) is native to the Atlantic Forest biome, endemic to southeastern Brazil, and belongs to the Myrtaceae family, which also includes major species such as guava, jaboticaba, Brazilian cherries, and other fruit-bearing trees (Lorenzi, 2014). Its fruits possess many functional compounds relevant to human health owing to their antioxidant properties, such as tannins, phenolics, ascorbic acid, and fibers (Tokairin, Silva, Spricigo, Alencar, & Jacomino, 2018a). Cambuci fruits can be consumed either *in natura* or in the form of processed products, such as juice, liquor, ice cream, mousse, and cake, among other options (Azevedo, Silva, Jacomino, & Genovese, 2017; Tokairin, Bremer Neto, & Jacomino, 2018b).

Even though the production process of cambuci tree seedlings is restricted to the use of seeds (Lorenzi, 2014; Santoro, Brogio, Forti, Novembre, & Silva, 2020), there is a pressing need to develop and adopt methods that take advantage of the vegetative propagation techniques that would allow for the multiplication of identical plants (Teleginski, Zuffellato-Ribas, Koehler, Degenhardt-Goldbach, & Teleginski, 2018). This would ensure uniformity and homogeneity in the orchards (Trueman, McMahon, & Bristow, 2013) and favor the fixation of superior genotypes.

The propagation method known as cutting is a viable alternative since it takes advantage of cell dedifferentiation and the development of adventitious roots to regenerate new plants. The success rate of the technique is largely dependent on endogenous and exogenous factors. These factors are related to the stock plant's nutritional, physiological, and health status, as well as to its ontogenetic age, air temperature, light, and substrates used (Hartmann, Kester, Davies, Geneve, & Wilson, 2018).

It is widely known that rooting potential may be related to stem anatomy (Wendling, Brooks, & Trueman, 2015). It has been found that the anatomical structure of primary phloem combined with the degree of tissue sclerification contribute to the lack of development of adventitious roots in cuttings sampled from woody species (Mendonça et al., 2019). Therefore, elucidating the anatomy of plant tissues is key to understanding the process of rhizogenesis (Bortolini et al., 2008) as well as overcoming any difficulties that might arise in the development of adventitious roots (Lima et al., 2011).

The present work aimed to evaluate the adventitious rooting of cambuci cuttings of different types, sizes, and IBA doses, as well as stem anatomical properties that might be related to successful rhizogenesis.

Material and methods

Cutting and plant propagation

Five experiments using the cutting method were conducted in an experimental area located at the University of São Paulo (USP/Esalq), Crop Science Department in Piracicaba, state of São Paulo, Brazil (latitude 23º 42' 29" S, longitude 47º 37' 45" W, and 546 m elevation). Cuttings were collected from stock plants located in two separate locations: (a) commercial fields located in the Natividade da Serra municipality in São Paulo State, Brazil (latitude 23º 31' 27" S, longitude 45º 27' 12" W, and 720 m elevation), and (b) an experimental area belonging to the 'Coordination of Sustainable Rural Development' (CDRS, its acronym in Portuguese) located in the São Bento do Sapucai municipality in São Paulo State, Brazil (latitude 22º 40' 48.8" S, longitude 45º 44' 47.6" W, and 880 m elevation). In both locations, mother plants were 13 years old on the collection dates.

The first experiment (Table 1), hereinafter referred to as E1, was carried out using herbaceous cuttings ranging from 10-15 cm in length and displaying two pairs of leaves (Figure 1) collected from mother plants in January 2018 (sprouting season). A bevel cut was applied to the lower end of each cutting (next to a vegetative bud). The cuttings were subjected to a 15 s immersion in ascorbic acid (5 g L^{-1}), followed by a 10 s immersion in a hydroalcoholic solution of indole butyric acid (IBA; LS Chemicals, 99% purity) with the following concentrations: 0, 1,500, 3,000, 4,500, or 6,000 mg L^{-1}. Upon preparation, cuttings were placed in 72-cell Styrofoam trays filled with medium-grade vermiculite and kept in an intermittent nebulization chamber to prevent desiccation.

Trials were carried out following a complete randomized design, with five treatments replicated four times. Experimental units consisted of five cuttings, and hence, a total of 100 cuttings were employed in this study. Evaluations were performed at 30, 60, and 90 days after trial start (DAS) when mortality and leaf retention (expressed as the average number of leaves per cutting) were evaluated. At 90 DAS, callogenesis (expressed as the percentage of cuttings that had developed calluses) and rooting were also determined.

In addition to the trial described above, four other experiments were carried out. Three trials employed herbaceous cuttings, whereas a separate trial utilized woody cuttings (Table 1; Figure 1). Trials followed similar experimental designs and assessments as previously stated (E1). Trial number two (E2) tested for the effect of a longer immersion period in IBA that lasted for 12 h rather than 10 s as in E1. Therefore, IBA concentrations decreased 10-fold to 0, 150, 300, 450, and 600 mg L^{-1}.

Cutting sizes were decreased for the third trial (E3) to a maximum of 8 cm in length with a single pair of developed leaves per cutting (Table 1). Besides the aforementioned bevel cut performed on the lower end of each cutting (Figure 1), lesions were artificially made using a scalpel immediately before immersion in ascorbic acid and quick immersion in IBA (similar rates as in E1). Experiments 1, 2, and 3 (E1, E2, and E3) were performed in the summer season, during which the mother plants were in full sprouting season.

A fourth experiment (E4) was performed by maintaining similar IBA and ascorbic acid concentrations and immersion times to those in E1 (Table 1). However, the herbaceous cuttings used in this trial were limited to a maximum length of 8 cm, all displaying a single pair of developed leaves whose blades were split in half (Figure 1). Such cuttings were collected from mother plants in spring 2018 when plants had started to sprout. These displayed basal lesions due to the process of removal and detachment from mother plants, unlike the cuttings from previous trials cut using pruning shears.

Table 1. List of experiments carried out, depicting information regarding the type of cutting, ascorbic acid, and indole butyric acid (IBA) rates and immersion times, season during which the trial took place, and peculiarities regarding the method for cutting preparation.

Trial ID	Type of Cutting	Ascorbic acid		IBA		Season	Cutting length and additional information
		Rates (g L^{-1})	Immersion time	Rates (mg L^{-1})	Immersion time		
E1	Herbaceous	5	15 s	1,500 to 6,000	10 s	Summer	Longer cuttings (10-15 cm) with two pairs of leaves
E2	Herbaceous	5	15 s	150 to 600	12 hours	Summer	Longer cuttings (10-15 cm) with two pairs of leaves
E3	Herbaceous	5	15 s	1,500 to 6,000	10 s	Summer	Lesions (scalpel); smaller cuttings (8 cm) with one pair of leaves
E4	Herbaceous	5	15 s	1,500 to 6,000	10 s	Spring	Smaller cuttings (8 cm) with one pair of leaves
E5	Woody	5	15 s	1,500 to 6,000	10 s	Autumn	Lesions (scalpel); longer cuttings (10-15 cm) with one pair of leaves

Figure 1. Different types of cambuci (*Campomanesia phaea* O. Berg Landrum) cuttings employed. (A) Herbaceous cutting displaying two pairs of leaves (trials ID number: E1 and E2); (B) herbaceous cutting with a single pair of leaves (trial E3); (C) cuttings with a single pair of leaves and leaf blades cut in half (trial E4); and (D) woody cutting with a single pair of leaves (trial E5). Bar = 5 cm.

The last trial (E5; Table 1) for evaluation of this technique employed woody cuttings collected from mother plants in autumn 2018 when cutting collection and preparation is not recommended because of the predominant environmental conditions of low temperatures and scarce precipitation (Hartmann et al., 2018). Cuttings were 10-15 cm in length and displayed a single pair of leaves and a bevel cut at the lower end (Figure 1). A scalpel was used to artificially create lesions on each cutting, followed by immersion in ascorbic acid and quick immersion in IBA, as in trial 1 (E1).

Data from trials E1-E5 were subjected to analysis of variance (ANOVA) and treatment means compared using Tukey's HSD test ($\alpha = 0.05$) when appropriate. Analyses were performed using SAS® v.9.4. software (SAS Institute Inc., Cary, NC, USA).

Analyses of cambuci tree anatomical features

Based on the results obtained for rooting and callus formation on cambuci cuttings, basal portions of woody cuttings employed in trial number 5 (E5) and herbaceous shoots of one-year-old cambuci trees were collected and subjected to further studies focused on anatomical features.

Plant samples originating from cuttings that did not develop roots or any visual calluses were fixed using Karnovsky's solution (2.5% glutaraldehyde, 4% paraformaldehyde, 0.1 M sodium cacodylate) and a series of 60 s vacuum treatments to improve solution absorption into plant tissues. After a period of 24 hours in Karnovsky's solution, plant samples were subjected to dehydration through a sequence of increasing ethyl alcohol concentrations (30, 50, 70, 90, and 100%). Dehydrated samples were then pre-infiltrated with 1:1 (v v^{-1}) ethanol-resin solution for 48 hours, pure historesin for 72 hours, followed by polymerization to allow the formation of blocks for further analyses.

Histology slides were prepared by subjecting polymerized blocks to sectioning using a rotary microtome (820 Spencer Microtome; American Optical Corporation), and 5 µm thick sections were obtained. These were then placed on slides for staining using two different techniques. The first was a periodic acid-Schiff

(PAS)/naphthol blue black staining method (Fisher, 1968) to allow for the observation of glycogen, basal membranes, cell walls, neutral and acidic polysaccharides, glycosidic radicals in glycoproteins, starch, and phenols (PAS) as proteins (naphthol blue black). The second was differential staining using toluidine blue (0.05% w v^{-1}; pH 3.2; O'Brien, Feder, & McCully, 1964) for the observation and characterization of tissue anatomy, mostly pertaining to the differences between cells displaying lignified or non-lignified walls.

Following staining procedures, histology slides were mounted between the microscope slides and coverslips using the Entellan® mounting medium. Upon drying, slides were viewed using light microscopy. Images were taken and recorded using a Zeiss Axio Imager microscope (Carl Zeiss Microscopy, LLC, White Plains, NY) mounted with a digital camera and Image-Pro Plus 6.3 software (Media Cybernetics, Inc., Rockville, MD) was used for image processing.

Results and discussion

Cambuci tree cuttings

High cambuci cutting mortality and low foliar retention were observed across all trials (E1-E5). Leaf loss occurred more intensively between 30 and 60 days after the start of the trial (DAS) incurring cutting death and a generalized lack of foliar retention at 90 DAS (data not shown). Of all the studies, only E3-related procedures allowed for a significant level of foliar retention at 90 DAS (p-value = 0.3135; data not shown). However, despite this fact, no root or callus formations at the base of the cuttings were observed in all trials (E1-E5). Premature leaf loss in cambuci cuttings is expected to have hindered the process of adventitious root formation since leaf retention is key to the maintenance of photosynthetic activity and carbohydrate synthesis as well as being a source of auxins and cofactors related to rhizogenesis (Souza, Xavier, Leite, Santana, & Leite, 2013; Hartmann et al., 2018).

The results also indicated that immersion in the growth regulator IBA resulted in high cutting mortality and leaf loss (data not shown). The latter was more intensely observed in trial number 2 (E2) in which a longer immersion time was used (Table 1). In such trials, up to 80% leaf loss and a cutting survival rate of only 5% was observed at 30 DAS (< 0.0001; data not shown) when IBA was used at the highest rate (IBA at 600 mg L^{-1}). Accordingly, severe leaf abscission was also reported when red guava (*Psidium cattleianum* Sabine) and pineapple guava (*Acca sellowiana* Berg.) cuttings were exposed to 400 mg IBA L^{-1} for 16 or 24 hours, respectively; these also belong to the Myrtaceae family (Nachtigal, Hoffmann, Kluge, Fachinello, & Mazzini, 1994; Franzon, Antunes, & Raseira, 2004). The authors attributed such negative effects to the phytotoxic effects might follow after immersion in IBA for longer periods of time.

Santoro, Mikami, Souza, and Roberto (2010) reported that adventitious root formation in herbaceous guava (*Psidium guajava* L.; Myrtaceae) cuttings do not take place if cuttings lose all leaf area, despite being exposed to optimal environmental conditions (e.g., light, temperature, and relative humidity), further indicating the need for foliar retention in adventitious rhizogenesis. Furthermore, the actual effects of leaf retention might vary according to the type of cutting utilized. For instance, Sasso, Citadin, and Danner (2010) observed that root formation in herbaceous jabuticaba (*Plinia cauliflora* L.) cuttings was dependent on the presence and retention of leaves, whereas root formation in woody cuttings was not. Therefore, intense foliar abscission might have adversely affected rooting in herbaceous and woody cambuci cuttings.

For most trials employing herbaceous cambuci cuttings, a negative effect of increasing IBA concentrations on both cutting survival and leaf retention took place (data not shown). Interestingly, such an effect was not observed for woody cuttings.

Similarly, Sasso et al. (2010) observed that elevated IBA rates applied to the base of herbaceous jabuticaba cuttings were inhibitory to root formation but resulted in greater rooting when applied to woody cuttings.

In contrast, herbaceous *Campomanesia aurea* O. Berg (popularly known in Brazil as '*guabirobinha-do-campo*') cuttings collected in spring were more responsive to IBA application than woody cuttings collected in winter (Emer, Schafer, & Fior, 2018). Moreover, both positive and negative effects were verified in herbaceous guava cuttings exposed to IBA (Colombo et al., 2008; Yamamoto et al., 2010; Kareem et al., 2016; Singh, Krishan, & Singh, 2018).

These contradictory results regarding IBA applications and their effects on cutting rooting for other Myrtaceae plant species, as also reported here for cambuci, further emphasize that the use of this growth regulator is intimately connected to other key factors such as the type of material, IBA rates, and plant species.

The mother plant's ontogenetic age (Lattuada, Spier, & Souza, 2011) and its physiological and hormonal statuses also play a major role in this process in addition to the environmental conditions at their site of origin (Xavier, Wendling, & Silva, 2013), indicating the need for a better understanding of how these variables interact.

Anatomical analyses

The analysis of samples from herbaceous stems (Figure 2A) indicated the presence of a layer of unistratified epidermal cells containing trichomes. However, cortex tissues were shown to be multistratified with 6 to 8 cell layers and parenchyma cells of different sizes displaying schizogenous secretion cavities adjacent to the epidermis. The presence of sclerenchyma tissue forming a discontinuous sheath at the cortical region was also observed near the phloem, which is, in turn, presented as a bicollateral vascular bundle.

Despite their herbaceous morphological aspect, cutting samples displayed secondary growth as indicated by the conspicuous vascular cambium. Fibers and brachysclereids petrous cells are randomly distributed along the pith (or medulla) region (Figure 2A) as were crystal idioblasts containing druses that were also visible in the cortical and medullar parenchyma.

Low rooting potential in a given plant species, as reported here for the cambuci tree, is often associated with the occurrence of sclerenchyma tissues located externally to the root meristem development region (Hartmann et al., 2018; Mendonça et al., 2019).

Sclerenchyma tissue displays cells with thick, lignified secondary walls that function primarily in water conduction and mechanical support (Appezzato-da-Glória & Carmello-Guerreiro, 2013; Evert, 2013). However, such tissue might tend to act as a physical barrier for the development of root primordia. Nevertheless, a direct causal relationship cannot be established (Wendling et al., 2015). Despite not being the only factor that can impede the formation of root primordia (Hartmann et al., 2018), the occurrence and perivascular continuity of sclerenchyma cells has been correlated with poor rooting in cuttings prepared from woody species (Lima et al., 2011; Paiva & Gomes, 2011). In cambuci, there is no perivascular continuity of sclerenchyma tissue cells suggesting that it might have a low influence in this process.

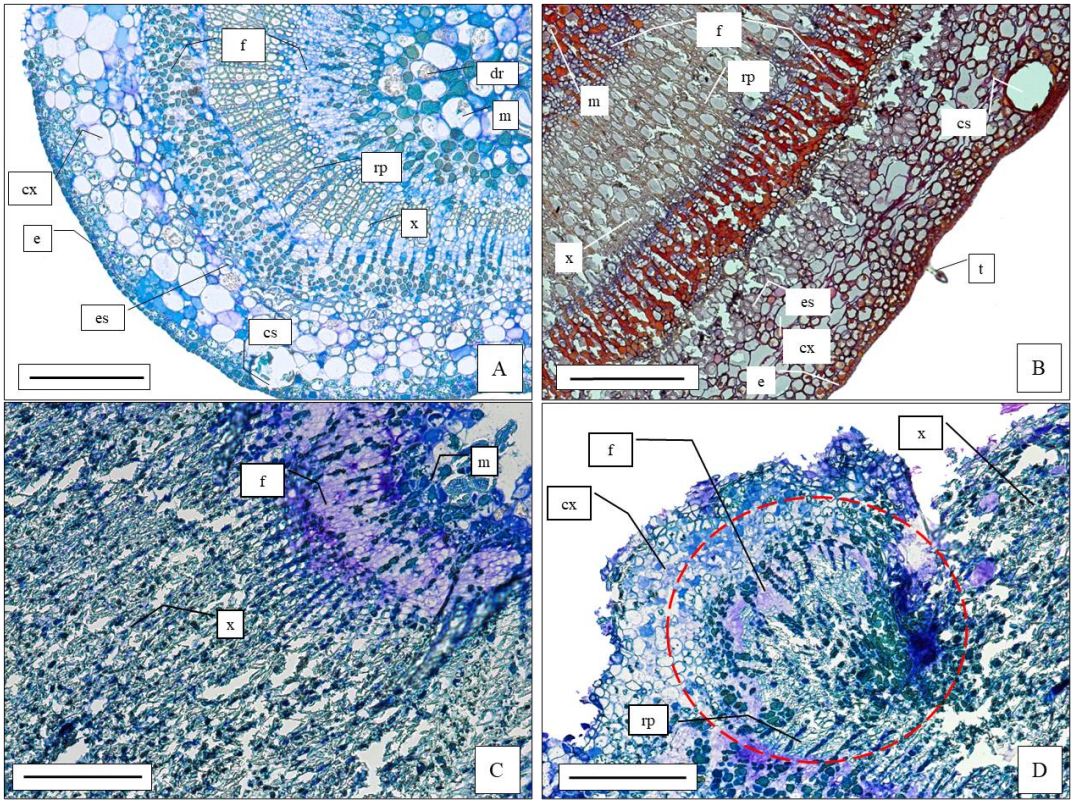

Figure 2. Cross-section of herbaceous cambuci shoots which were subjected to staining with toluidine blue (0.05%) (A). PAS (periodic acid/Schiff reagent) and Naphthol blue black reactions (B). Cross-section of basal portions in cambuci woody stems, depicting modified xylem tissues which were subjected to the propagation process (C). Cross-section of external regions at the basal portion of woody stems in which the development of differentiated tissue (red circle), as well as the development of new tissues displaying anatomical conformation analogous to the original stem are highlighted (D). Letter coding: cs = secreting pit; cx = cortex; dr = druses; e = epidermis; es = sclerenchyma; f = phloem; m = medulla (Pith); rp = parenchyma ray cells; t = trichome; x = xylem. Bar = 200 µm.

The PAS histochemical test highlights the presence of soluble phenolic compounds which are stained orange. Such compounds were observed in most tissues from herbaceous cambuci shoots (Figure 2B) ranging from epidermal cells and phloem parenchyma ray cells to the medullary parenchyma. Although not as intense, such compounds were also stained and visualized in cortical parenchyma cells, phloem, and xylem parenchyma ray cells. Soluble phenolic compounds are considered secondary metabolites of plant metabolism and display many chemical forms and functions, playing diverse roles such as in the defense against herbivores, attraction of pollinators, and UV light absorption (Taiz, Zeiger, Møller, & Murphy, 2018). A similar occurrence of phenolic compounds has been observed in other members of the Myrtaceae family, such as guava and uvaia (*Eugenia pyriformis* Cambess.), grumixama (*E. brasiliensis*), and eucalyptus (*Eucalyptus urophylla*; Duarte & Paula, 2005; Donato & Morretes, 2007; Gomes et al., 2009; Mendonça et al., 2019).

According to Mendonça et al. (2019), the accumulation of phenols in plant tissues might stimulate or inhibit the process of adventitious root formation. The negative influence resulting from the synthesis or oxidation of phenolic substances is well known in other Myrtaceae family members (Fachinello, Hoffmann, & Nachtigal, 2005). While studying the essential oil and ethanolic extract from *C. phaea* trees, Lorençoni et al. (2020) identified 41 different compounds, including some flavonoids. However, literature is still lacking regarding the identification and classification of compounds found in its stems and their influence over adventitious root development.

The region at which phenolic compounds occur in cambuci stems coincides with the main regions of root primordia development, that is, xylem and phloem parenchyma ray cells (Mayer, Biasi, & Bona, 2006; Hartmann et al., 2018), and thus corroborate our findings pertaining to the generalized lack of root or callus formation in cambuci cuttings. This might have contributed to the observed lack of rooting in cambuci cuttings due to their negative impact on adventitious root formation.

Observations of basal stem portions in woody cambuci cuttings revealed a similar anatomical organization found in herbaceous shoots (Figure 2C and D). Even though differentiation of root tissues had not taken place, cells within the xylem parenchyma ray cells showed levels of activity, as indicated by the synthesis of new tissues, identical to those in the shoots. The new tissues were found to overlap the original tissues (Figure 2D) displaying characteristics commonly found in calluses.

Adventitious rooting might occur following two distinct anatomical patterns, which are commonly referred to as direct and indirect (Goulart, Xavier, Iarema, & Otoni, 2014). In the direct pattern, competent cells initiate cell division upon induction in a polar pattern. However, in the indirect pattern, a state of non-responsiveness is generally observed until callus formation, which is followed by the formation of adventitious roots (Hartmann et al., 2018).

Our findings regarding the absence of root tissue differentiation strongly suggest low totipotency capacity in cells located at the base of cambuci cuttings, a contrasting result when compared to corresponding tissues in plant species in which root formation takes place more easily. According to Goulart et al. (2014), in hard-to-root plant species, root formation commonly originates from scar tissues; however, roots can still be expected to originate from cells able to maintain their dedifferentiation ability.

Root primordia may originate from different regions and plant tissues which become meristems (Evert, 2013). Tissues displaying greater dedifferentiation capabilities are young tissues located in the secondary phloem and parenchyma ray cells surrounding the phloem, cambium, or calluses (Hartmann et al., 2018).

At the basal portion of woody cambuci cuttings, deteriorated plant tissue was observed nearby intact tissues (Figure 2C and D), along with low amounts of newly formed secondary tissue which further indicates low vascular cambium activity and slow growth in this species (Mayer et al., 2006).

According to Fachinello et al. (2005), the application of exogenous auxin such as IBA should promote the activation of cells located in the vascular cambium which promotes cell multiplication and rhizogenesis. However, in plant species that do not root easily from cuttings, the absence of rooting cofactors or high amounts of inhibitory compounds (such as phenolics) might prevent adventitious root formation, especially when it occurs in regions of root primordia development (Lima et al., 2011; Mendonça et al., 2019).

Cambuci tree domestication is still in its initial steps, and thus, technical and scientific understanding regarding this species is still scarce. This study sheds light on factors limiting adventitious root formation in both woody and herbaceous cambuci cuttings. It should be mentioned, however, that the occurrence of phenolic compounds as well as sclerenchyma tissues observed in cuttings might not be the only factors related to the lack of root development.

Conclusion

Cambuci propagation using both herbaceous and woody cuttings do not constitute a viable option for vegetative propagation of this species. In cambuci cuttings, the presence of phenolic compounds might have a greater negative influence on adventitious root formation than the physical barrier imposed by the sclerenchyma tissue.

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