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Vegetative propagation of adult *Ilex paraguariensis* trees through epicormic shoots

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ABSTRACT. The difficulty and length of time required for seed germination of mate (*Ilex paraguariensis*), as well as the pressing need for clonal multiplication of improved genetic material, has resulted in several studies related to vegetative propagation in an effort to obtain rooted cuttings more quickly and with better genetic quality. Currently, the biggest challenge is propagating and rooting adult plants selected in the field without requiring clear cutting to generate conditions for the basal induction of juvenile sprouts. Therefore, the objective of this study was to develop a method to rescue adult mate plants through the generation of epicormic sprouts. To accomplish this, tree branches of mate that were at least 19 years of age were collected and packed in trays with sand for sprouting. Different solutions containing a mixture of sucrose and indole-3-butyric acid (IBA) were sprayed in branches at 29, 22, 15, 8 and 1 day(s) before collection. We conclude that the vegetative propagation of adult mate trees is technically efficient and requires no treatment with sucrose or IBA and results in the formation of plants suitable for planting or serving as mother plants for continuous multiplication via cloning.

Keywords: mate, shoot induction, rhizogenesis, cutting technique, cloning, vegetative propagation.

Propagação vegetativa de plantas adultas de *Ilex paraguariensis* por meio de brotações epicórmicas

RESUMO. A dificuldade e o longo tempo exigido para a germinação de sementes de erva-mate (*Ilex paraguariensis*), bem como a necessidade da multiplicação clonal de materiais genéticos melhorados resultaram em vários estudos relacionados a propagação vegetativa visando obter estacas enraizadas em curto espaço de tempo e com melhor qualidade genética. Atualmente, o maior desafio refere-se a propagação e enraizamento plantas adultas de erva-mate selecionadas a campo sem a necessidade de corte raso das matrizes para a indução de brotos basais juvenis. Objetivou-se desenvolver um método para resgatar plantas adultas de erva-mate por meio da indução de brotações epicórmicas em ramos destacados. Para tanto, galhos de árvores de erva-mate com 19 anos de idade foram coletados e acondicionados em bandejas com areia para emissão de brotações epicórmicas. Diferentes soluções contendo a mistura de sacarose e ácido indol-3-butírico (AIB) foram pulverizadas nos ramos aos 29, 22, 15, 8 e 1 dia(s) antes da coleta das brotações. A propagação vegetativa de árvores adultas de erva-mate foi tecnicamente eficiente e não há necessidade de tratamento com sacarose e/ou de AIB, resultando na formação de mudas adequadas para o plantio a campo ou também servindo como plantas-mãe para a multiplicação contínua por meio da clonagem.

Palavras-chave: erva-mate, indução de brotações, rizogênese, estaquia, clonagem, propagação vegetativa.

Introduction

Mate (*Ilex paraguariensis* St. Hil.), a species of the family Aquifoliaceae, is native to southern South America, more specifically found in Argentina, Uruguay, Brazil and Paraguay. Mate is used to make a stimulating drink that is consumed instead of coffee; the drink is greenish in color and contains caffeine, tannins and vitamins, such as B1, B2, B5, C, E, B-carotene, sucrose, fructose, folic acid, trigonelline, choline and many polyphenolic compounds (GUGLIUCCI; STAHL, 1995; PAGLIOSA et al., 2010; RACANICCI et al., 2008). It has been shown to be effective in the inhibition of colon cancer cell proliferation (MEJÍA et al., 2010).

Because of the difficulty of and length of time required for mate seed germination (CUQUEL et al., 1994), as well as the pressing need for the clonal multiplication (SANSBERRO et al., 1999) of improved genetic material (WENDLING et al., 2007), much research has been conducted in the field of *in vitro* and *ex vitro* propagation to obtain rooted cuttings more quickly and with better physiologic quality. Successful cloning is possible for juvenile material; however, similar to many other woody species, mature tissues show a low morphogenetic potential, which makes it difficult to clone mature trees by rooting cuttings or using *in vitro* techniques (TARRAGÓ et al., 2005). In the transition from juvenile to adult phases, plants undergo changes in their apical meristems, which occur in distinct periods of development and ontogenetic age, increasing the variability of rooting (HARTMANN et al., 2011; HUSEN, 2011; SCHWAMBACH et al., 2008; TARRAGÓ et al., 2005). Therefore, to clone forest trees, physiologically juvenile epicormic sprouts on the base of the tree are required or need to be inducted by rejuvenation with special techniques to rescue the rooting and growing capacity of the tree (HARTMANN et al., 2011).

Several studies related to mate root cuttings have been conducted since 1930 (PRAT KRIKUN, 1995) in an attempt to develop suitable protocols for commercial use, but the results are not viable at the moment (BITENCOURT et al., 2009; BRONDANI et al., 2009), particularly if the original sprouts are from adult trees and are not from its base. Because of this, the biggest immediate challenge is obtaining rooting cuttings from selected adult plants without the need for clear cutting to induce the induction of basal juvenile shoots. The use of epicormic sprouts induced in cut branches is a potential alternative that may solve this problem. In this process, basal branches are collected and put into optimal environmental conditions for sprout induction (ROCHA, 2002; WENDLING et al., 2009). However, it is necessary to assess the rooting rate of these sprouts and, therefore, evaluate its efficacy as source material for the rooting cuttings technique used for mate.

Growth regulators are among the main factors that affect the rooting cuttings. The main plant regulator used for the vegetative propagation of adult plants is indole-3-butyric acid (IBA) (HARTMANN et al., 2011). In mate, studies have been carried out to evaluate the effect of growth regulators on the induction of *ex vitro* (BRONDANI et al., 2007, 2009; TARRAGÓ et al., 2005; WENDLING et al., 2007) and *in vitro* vegetative propagation (SANSBERRO et al., 1998, 1999, 2001), but none of these studies used induced epicormic sprouts from cut branches.

Sucrose applied in combination with IBA or alone can influence the induction of shoots or even encourage sprout formation (OVONO et al., 2009) with greater viability in rooting (BHARDWAJ; MISHRA, 2005; STENVALL et al., 2009), which highlights the importance of this substance in plant metabolism (PAVLINOVA et al., 2002; YANG et al., 2004). Nevertheless, little is known about the mechanism of these effects. The carbohydrate contents in plant tissues vary with the seasons (STENVALL et al., 2009), and the endogenous content is associated with the induction of new sprouts, especially in meristematic regions (KUMAR et al., 1999; PAVLINOVA et al., 2002). In addition, there are indications that the accumulation of carbohydrates in plant tissues is correlated with the capacity for adventitious rooting (STENVALL et al., 2009), emphasizing the importance of endogenous carbohydrate content in the successful cloning of selected genotypes.

Thus, this study aimed to develop a method of vegetative rescue of adult mate trees through the induction of epicormic shoots on cut branches and their subsequent rooting after treating the shoots with sucrose and IBA.

Material and methods

Shoot production

In November 2006, branches of 19-year-old mate trees were collected in the city of Colombo, Paraná State, Brazil. The branches were standardized to 60 cm long and 5-10 cm in diameter, and all sprouts were removed. Subsequently, the branches were placed vertically in trays (40 x 20 x 18.7 cm) containing 15 liters of medium sand and kept for 73 days in a greenhouse with an intermittent misting system, under relative humidity above 80% and temperature between 20 and 30°C.

Thirty days prior to sprout collection (43 days after installing the experiment), the spraying of Tween 20 began to break the surface tension of the water and increase treatment absorption by vegetative tissues in combination with the treatments (Figure 1). The treatments were comprised of a solution of sucrose (0, 2 and 4%) and IBA (0, 200 and 400 mg L^{-1}, dissolved in a solution of alcohol and water, 1:1, v/v) at 29, 22, 15, 8 and 1 day(s) before shoot collection. The S2 treatment (0% sucrose + 0 mg L^{-1} IBA) consisted of the application of a hydroalcoholic solution (water:alcohol, 1:1, v/v). An additional treatment (S1 – water) was installed, consisting of spraying the branches with distilled water.

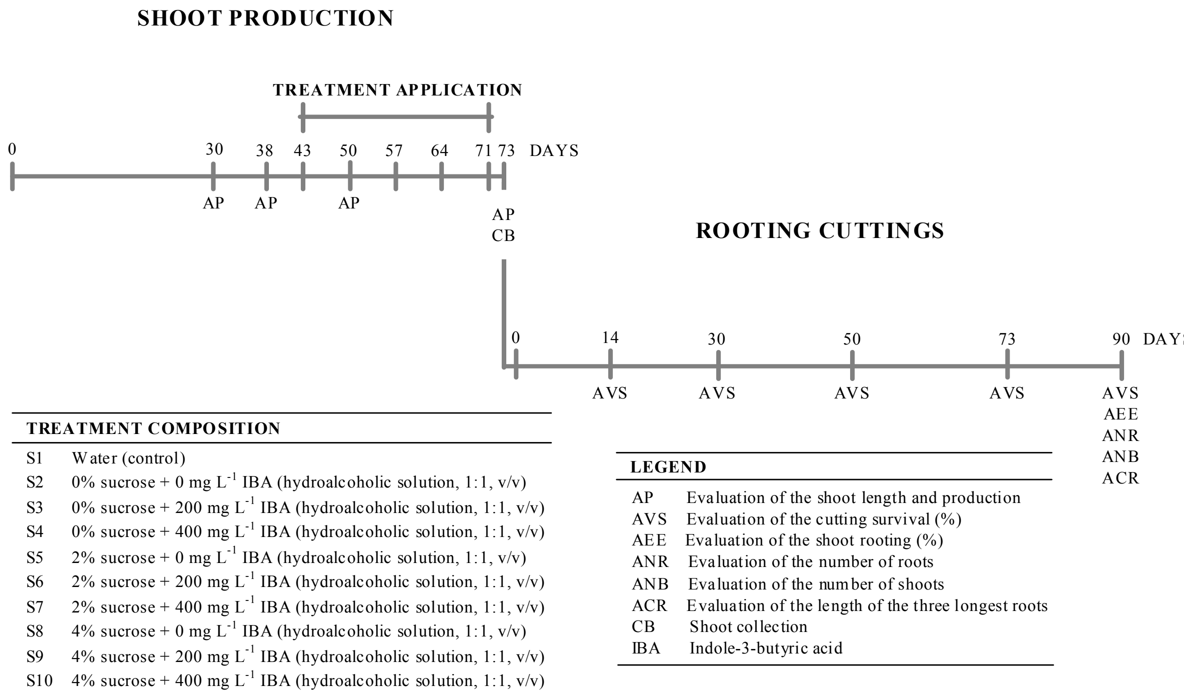

Figure 1. Summary of the experimental design of the study.

After the first shoots appeared on the branches (30 days), the number and length of sprouts were evaluated weekly. The sprouts on the branches were classified into two size classes, with shoots ≤ 4.0 cm assigned to class 1 and shoots > 4.0 cm to class 2. The experiment was conducted in a completely randomized factorial arrangement (2 x 10 x 2) with a split-plot in time. The factors consisted of two evaluation times (50 and 73 days), 10 solutions (S1: water, S2: 0% sucrose, S3: 200 mg L^{-1} IBA, S4: 400 mg L^{-1} IBA, S5: 2% sucrose, S6: 2% sucrose + 200 mg L^{-1} IBA, S7: 2% sucrose + 400 mg L^{-1} IBA, S8: 4% sucrose, S9: 4% sucrose + 200 mg L^{-1} IBA and S10: 4% sucrose + 400 mg L^{-1} IBA) and two classes of sprouts (C1 ≤ 4.0 cm and C2 > 4 cm), with 16 replications. One branch was used for each replication.

Rooting cuttings

The rooting potential of the shoots was evaluated. To do this, 73 days after placing the cut branches in sand, epicormic sprouts were collected and converted into semihardwood cuttings 4-8 cm long, all containing a terminal bud and leaf area reduced to 50%. The bases of the cuttings were immersed in IBA solution at 6,000 mg L^{-1} (alcohol:water, 1:1, v/v) for 10 seconds. They were then placed in plastic trays (40 x 20 x 18.75 cm) containing a substrate of carbonized rice hulls and placed in a greenhouse with an intermittent misting system for 90 days under relative humidity above 80% and temperature between 20°C and 30°C (Figure 2).

The survival of the cuttings was evaluated 90 days after the rooting of the cuttings. At the end of the experiment (90 days), the survival rate, percentage of rooting and sprouting, presence of callus and number and length of the three biggest roots per rooted cutting were determined.

For rooting analyses, the experimental design was completely randomized. The factors consisted of 10 solutions (S1: water, S2: 0% sucrose, S3: 200 mg L^{-1} IBA, S4: 400 mg L^{-1} IBA, S5: 2% sucrose, S6: 2% sucrose + 200 mg L^{-1} IBA, S7: 2% sucrose + 400 mg L^{-1} IBA, S8: 4% sucrose, S9: 4% sucrose + 200 mg L^{-1} IBA and S10: 4% sucrose + 400 mg L^{-1} IBA) with four replications. The number of cuttings per plot ranged from 2 to 12 depending on the production of epicormic sprouts.

Statistical analysis

The data from both experiments were submitted to the Hartley test ($p < 0.05$) to check the homogeneity of variance among the treatments. The data were analyzed by ANOVA ($p < 0.05$ and $p < 0.01$). Based on the results of the ANOVA, the data were compared by Duncan's test ($p < 0.05$).

Results and discussion

The generation of epicormic shoots (Figure 2A) capable of rooting (> 4 cm) from cut branches of mate occurred regardless of the application of sucrose or IBA (Figure 2B-E). However, the application of sucrose and IBA, depending on the concentrations used, caused different behaviors in terms of the number and length of sprouts (Figure 3).

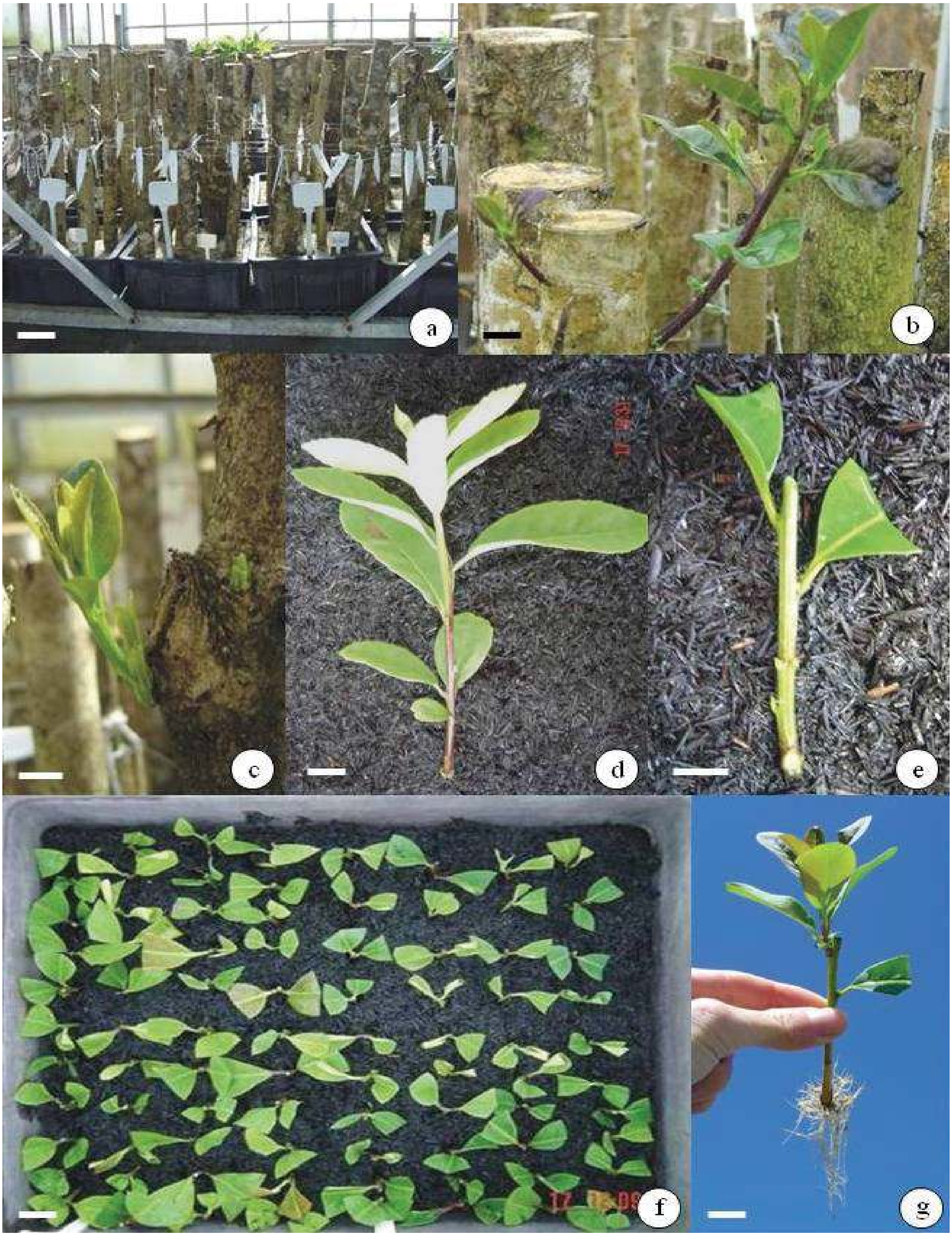

Figure 2. *Ilex paraguariensis* rooting cuttings from cut branches of mature trees. (a) arrangement of branches in the greenhouse, bar: 10.0 cm, (b) epicormic sprouting on cut branches, bar: 2.0 cm, (c) epicormic sprout detail, bar: 2.0 cm, (d) bud collected at 73 days, bar: 1.0 cm, (e) prepared cutting, bar: 1.0 cm, (f) design used for planting the cuttings in a box containing carbonized rice hull, bar: 2.5 cm (g) rooted cutting, bar: 1.5 cm.

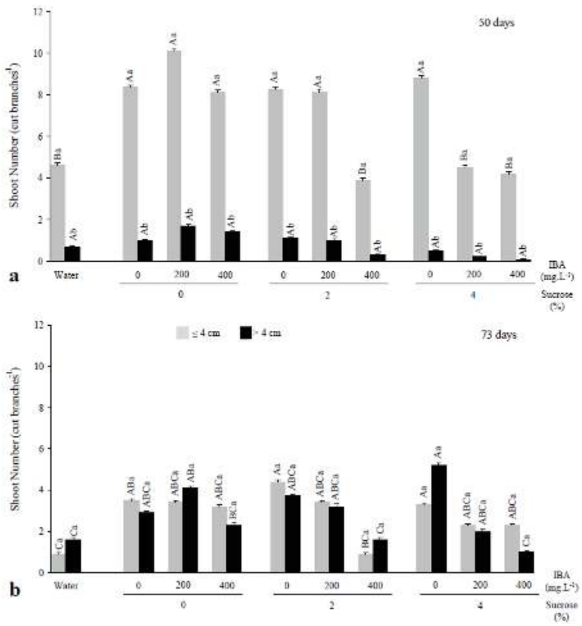

Figure 3. Effects of sucrose and IBA on the induction and size of the shoots of *Ilex paraguariensis* at 50 (a) and 73 (b) days. The mean data (average ± standard deviation) of 16 cut branches. The means followed by the same letter, where uppercase represents the treatments and lowercase represents the shoot sizes, do not differ statistically (ANOVA followed by Duncan's test, $p < 0.05$). Water = control, IBA = indole-3-butyric acid.

In the evaluations at 30 and 38 days after the placement of the branches in the greenhouse, prior to implementing any treatment of sucrose and IBA, only sprouting below 4 cm was observed (data not shown). Averages of 5.5 and 5.8 shoots per branch at the evaluation after 30 and 38 days, respectively, were observed (data not shown).

At 50 days after the placement of branches in the greenhouse (seven days after the first spraying of the treatments), no significant effect on the shoots was observed after the application of sucrose or IBA individually for shoots larger or smaller than 4 cm. However, the combined treatment of 2% sucrose and 400 mg L^{-1} of IBA or 4% sucrose and 200 or 400 mg L^{-1} IBA resulted in a reduction in the production of shoots longer than 4 cm (Figure 3). Likewise, at 73 days (30 days after the first spraying of the treatments), the application of sucrose alone did not affect the production of shoots (Figure 2B-C). However, when IBA was applied at a concentration of 400 mg L^{-1}, alone or in combination with sucrose, there was a reduction in the production of shoots (Figure 3).

In both evaluations at 50 and 73 days, there was clearly a reduced production of shoots in the control treatment (distilled water) as compared to almost all of the other treatments, and especially in relation to the treatment without sucrose or IBA (alcohol only) (Figure 3).

Just as was observed in the production of shoots, the survival (Figure 2F) and rooting (Figure 2G) of the specimens from cut branches were not positively affected by the application of sucrose or IBA, either individually or in combination. The application of sucrose alone, however, reduced the cutting survival and rooting, regardless of the concentration used. In contrast to the production of shoots, these characteristics were affected significantly in a negative manner, likely due to the presence of alcohol in the control treatment (0% sucrose and 0 mg L^{-1} IBA). It is noteworthy that in the survival evaluation in relation to rooting, there was a loss of 44%, indicating that not all live cuttings were rooted (Figure 4).

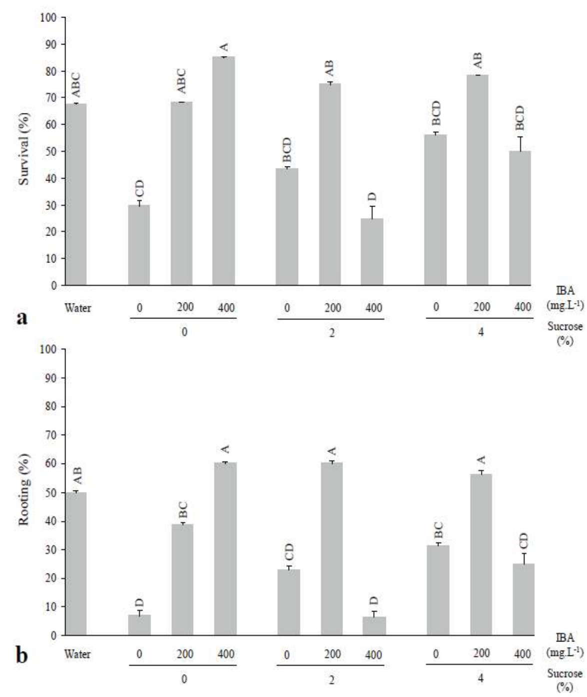

Figure 4. Effects of sucrose and IBA on survival (a) and rooting (b) of *Ilex paraguariensis* cuttings at 90 days. The mean data (average ± standard deviation). The means with at least one equal letter did not differ statistically (ANOVA followed by Duncan's test, $p < 0.05$). Data transformed by arcsine$[(n+0.5)/100]$. Water = control, IBA = indole-3-butyric acid, n = sampled data.

The number and strength of roots did not respond positively or negatively to the application of sucrose or IBA individually. The application of 400 mg L^{-1} IBA had a negative effect on these characteristics, regardless of the sucrose concentration (Figures 5 and 6).

Similarly, the number of shoots was not positively influenced by the use of sucrose or IBA. In this case, only the application of IBA at 200 mg L^{-1} and sucrose at 2 or 4% did not have a negative effect (Figure 7). The percentage of cuttings with shoots as assessed after 90 days in the greenhouse was not influenced positively by the application of sucrose or IBA, without, however, significantly reducing the number of cuttings with shoots (data not shown) (Figure 2F).

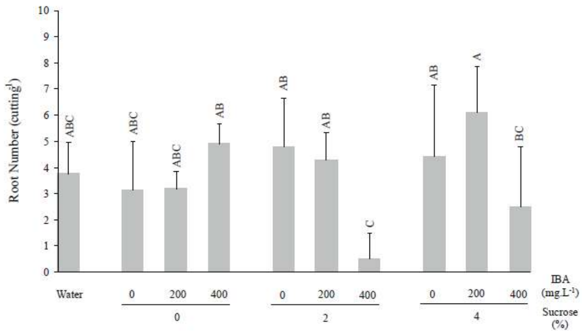

Figure 5. Number of roots on cuttings of *Ilex paraguariensis* in a greenhouse at 90 days. The mean data (average ± standard deviation). The means with at least one equal letter did not differ statistically (ANOVA followed by Duncan's test, $p < 0.05$). Data were transformed by $(n/10)^{0.5}$. Water = control, IBA = indole-3-butyric acid, n = sampled data.

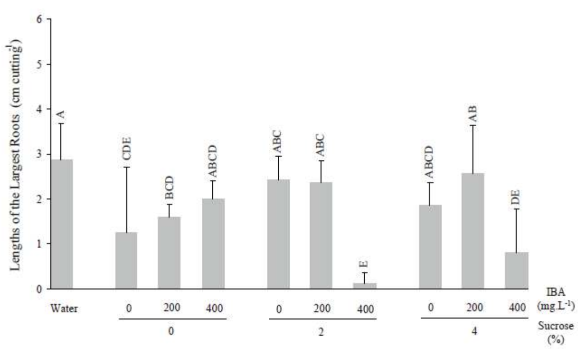

Figure 6. Lengths of the three largest roots on *Ilex paraguariensis* cuttings in a greenhouse at 90 days. The mean data (average ± standard deviation). The means with at least one equal letter did not differ statistically (ANOVA followed by Duncan's test, $p < 0.05$). Water = control, IBA = indole-3-butyric acid.

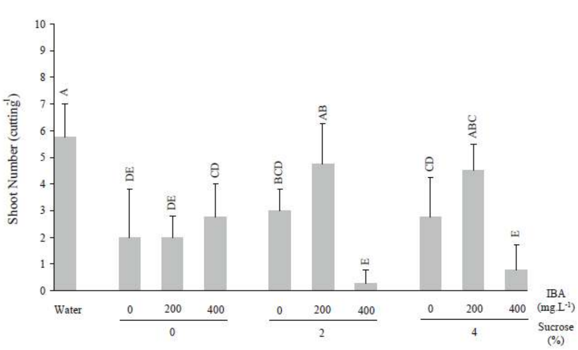

Figure 7. Number of shoots on *Ilex paraguariensis* cuttings in a greenhouse at 90 days. The mean data (average ± standard deviation). The means with at least one equal letter did not differ statistically (ANOVA followed by Duncan's test, $p < 0.05$). Water = control, IBA = indole-3-butyric acid.

Our results show that the use of sucrose and IBA on cut shoots does not result in greater strength or number of shoots suitable for rooting (> 4 cm) nor does it result in qualitatively better rooting (Figures 3, 4 and 5). Likewise, the strength of rooted cuttings, as evaluated by the number and length of the three largest roots and number of shoots, was not positively influenced by the application of IBA alone or in combination with sucrose (Figures 6 and 7).

The formation of shoots on the cut branches of mate that did not receive any treatment of sucrose or IBA demonstrates the feasibility of this method in the induction of shoots suitable to root, which agrees with results obtained by Rocha (2002) for other forestry species. Our data on rooting, on the other hand, demonstrate that the induction of buds on cut shoots can be used to rescue adult genotypes and the cuttings production of mate through the rooting process.

The increased production of shoots in the alcohol-containing treatment in relation to virtually all other treatments suggests a positive influence of alcohol on sprouting. This finding suggests that ethanol is a carbon source capable of supplying the demand for carbohydrates required by vegetative propagules. The potential of ethanol and methanol as carbon sources has been proposed in previous studies. An effect of ethanol and other aliphatic alcohols have been observed on plant growth, development and senescence (GUDJÓNSDÓTTIR; BURSTRÖM, 1962; SATLER; THIMANN, 1980) in germinating pea seedlings (COSSINS; TURNER, 1963), and they have been suggested to be a carbon source for growth in *Chorella vulgaris* (BACH; FELLIG, 1958; STREET et al., 1958) and oat seedlings (MER, 1958, 1961). The effect of ethanol on root formation in cuttings of *Phaseolus aureus* was associated with the production of ethyl-glucoside and carbohydrates in the presence of light (MIDDLETON et al., 1978).

On the other hand, alcohol was harmful to the survival and rooting of cuttings (Figure 4), suggesting a toxic effect in the absence of IBA. Yamamoto et al. (2010) attribute this effect to a possible toxicity of alcohol, which is able to inhibit the bud development processes, causing yellowing and leaf drop, inducing a higher rate of mortality in vegetative propagules. Furthermore, organic solvents, such as ethanol, methanol and acetone, may act as solubilizers of endogenous auxins in cuttings (BHATTACHARYA et al., 1985). However, Bhattacharya et al. (1985) found that ethanol, methanol and acetone stimulated the formation of adventitious roots in cuttings derived from etiolated hypocotyls of *Vigna radiata*. These authors suggested that ethanol, methanol and acetone could negate the requirement for other carbohydrates. In their results, they observed that sucrose and organic solvents used alone significantly increased the formation of roots; however, when combined, they suppressed rooting, probably because the level of carbon sources became supraoptimal and affected the balance.

Sucrose is the primary source of carbohydrates in plants and as such is responsible for their metabolic reactions (PAVLINOVA et al., 2002), which can positively influence the induction of shoots and roots in cuttings (STENVALL et al., 2009). Classical studies have already reported that root induction decreases proportionally with the decrease of carbohydrates in the culture medium (LANE, 1978), and there is a strong interaction of the carbohydrate level and endogenous hormonal levels, which affects morphogenic and/or organogenic processes (CORUZZI; ZHOU, 2001; KUMAR et al., 1999). Our results regarding the effects of sucrose corroborate those of Romano et al. (1995) in their study of adventitious root formation in *Quercus suber*. The importance of preconditioning at high carbohydrate concentrations, after the increased concentration of carbohydrates in plant tissues promoted by the use of sucrose in combination with phytohormones, has been demonstrated (BHARDWAJ; MISHRA, 2005; STENVALL et al., 2009). In under controlled experimental conditions, Ovono et al. (2009) also reported that an increased carbohydrate concentration in plant tissues promoted the formation of larger tissues and organs, such as leaves, stems and adventitious roots, and many carbohydrates can be accumulated as reserves in different phases of the plant cycle.

Bigger shoots responded satisfactorily to the treatments tested; however, it was observed that higher concentrations of IBA resulted in the mortality of shoots of smaller size, especially below 2 cm, which necrosed. The S2 treatment (i.e., water:alcohol, 1:1, v/v), representing only the solvent used to prepare the IBA solution, also showed symptoms of necrosis on the leaves, probably due to the presence of alcohol, considering its surfactant and dehydration properties (HARTMANN et al., 2011).

Data from our study indicate that the induction of epicormic shoots on cuttings of mate is a technically efficient method for vegetative rescue of mature trees of this species. Rocha (2002), working in a greenhouse with branches of approximately one meter placed in 20 L pots containing sand, were able to produce 3.3 cuttings per meter at 40 days for *Cariniana legalis*. At 20 days, they obtained an average production of 2.0 per meter of branch for *Swietenia macrophylla*, *Anadenanthera macrocarpa* and *Cedrela fissilis*. The number of shoots per branch after evaluation in the greenhouse is in agreement with the observations of Brondani et al. (2008) when using the automated greenhouse, but the genotype factor (HARTMANN et al., 2011) and seasonality effect (STENVALL et al., 2009) must be considered, which may influence this trait.

On the other hand, the tissue competence failure may reflect on the lack of receptors of that phytohormonal class that can induce the organogenic process (CARY et al., 2001; MACARTHUR et al., 2009; SMET et al., 2009). Phytohormone inactivation can occur through the combination of sugars derived from hydrolysis of sucrose, glucose and fructose, thereby altering the balance of active molecules of auxin and cytokinin (CORUZZI; ZHOU, 2001), directly affecting adventitious root formation in vegetative propagules (BHARDWAJ; MISHRA, 2005).

The rooting percentages reported here are consistent with those observed in mate by Brondani et al. (2007, 2008) and Wendling et al. (2007), demonstrating the feasibility of the propagation method. The work of Almeida et al. (2007), using *Eucalyptus cloeziana*, underscored the feasibility of generating epicormic shoots induction on cuttings without, however, obtaining adventitious rooting of cuttings from shoots. This lack of rooting, according to the authors, was ascribed to the position of the tissue in the tree, i.e., the low capacity for adventitious rooting of physiologically mature tissue in relation to more juvenile ones. Other causes for a lack of rooting have been linked to factors such as the age and/or juvenility of the propagules (ALMEIDA et al., 2007; BHARDWAJ; MISHRA, 2005; BRONDANI et al., 2010, 2012; HARTMANN et al., 2011; SCHWAMBACH et al., 2008; STAPE et al., 2001; WENDLING et al., 2010).

In the case of growing mate, the cuttings technique is only indicated for the rescue of selected adult genotypes, given that the overall rates of rooting are not satisfactory (BRONDANI et al., 2009; TARRAGÓ et al., 2005), in addition to the fact that a minicuttings technique has already been developed for the species, with good results (BRONDANI et al., 2007, 2008; WENDLING et al., 2007). Thus, we suggest using the present developed cloning procedure on a commercial scale through the propagation of cuttings and subsequently mass multiplication by the minicuttings technique, which, according to published data (WENDLING et al., 2010), results in higher rooting rates and root strength when compared to the traditional cuttings technique.

Conclusion

The application of IBA and sucrose did not influence the induction of epicormic sprouts or shoot rooting.

The rescue method, inducing epicormic shoots on branches of mate, was efficient and presents a strategy that can be used for the production of rooted cuttings.

This propagation method can be applied to other species that sprout from cut branches, which may serve as an alternative method to clone superior genotypes without cutting the selected mother tree.

Acknowledgements

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