academicJournals

Vol. 16(24), pp. 1345-1353, 14 June, 2017
DOI: 10.5897/AJB2016.15733
Article Number: CFACE1664721
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Establishment, multiplication, rooting and acclimatization of *Cabralea canjerana* (Vell.) Martius

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Received 13 October, 2016; Accepted 28 April, 2017

Seedling production of canjerana (*Cabralea canjerana* (Vell.) Martius) has been limited by difficulty in germination, caused by recalcitrant behavior of their seeds. The objective of this study was to develop an efficient protocol for conservation and production of canjerana micropropagated plantlets. Seeds of canjerana were disinfected with 0, 2.5, 5.0, 7.5 and 10% of NaOCl solution, at immersion times of 10, 20 and 30 min to produce aseptic seedlings, which were cultivated on Murashige and Skoog (MS) and Woody Plant Medium (WPM) media. Nodal segments were cultivated in WPM medium supplemented with 0 and 2.5 µM of 6-benzylamine purine (BAP), kinetin (KIN) and thidiazuron (TDZ) or with 0, 1, 3, 6, 9 and 12 µM of BAP. Micro-cuttings were cultivated in MS and WPM media with 0 and 5.0 µM of indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA). The rooted micro-cuttings were acclimatized in a humid chamber in a greenhouse. The highest percentage of aseptic seedlings was produced with a solution of 7.5% of NaOCl and immersion times of 20 and 30 min. Adding BAP, KIN and TDZ or increasing concentrations of BAP to the WPM medium did not increase shoot number and length. Neither the base medium nor the auxin (IBA and NAA) had a significant effect on the survival of micro-cuttings after 60 days of cultivation, but the addition of 5.0 µM of NAA did increase the percentage of rooting and survival during the acclimatization. Both nodal segments and micro-stumps of canjerana have showed a low rate of multiplication. The addition of 5.0 µM of NAA to the basal medium increased the percentage of *in vitro* rooting and the percentage of survival during acclimatization of canjerana plantlets.

Key words: *In vitro* propagation, *in vitro* multiplication, *in vitro* rooting, acclimatization, microclonal hedge.

INTRODUCTION

To complement conventional tree breeding programs, three specific biotechnological techniques have been successfully used, namely, tissue culture, for *in vitro* propagation and conservation; molecular markers, to assess the genetic diversity within and among populations; and encapsulation and cryopreservation, for long-term conservation (Varshney and Anis, 2014). Among these techniques, tissue culture is an appealing approach for conservation, because it aids in the propagation of species with limited number of explants,

low seed yield due to inbreeding depression (Hendrix and Kyhl, 2000), complex and unresolved seed dormancy mechanisms (Merritt and Dixon, 2003; Merritt et al., 2007), and poor seed set and viability due to environmental stresses, such as drought, predation, and/or disease (Varshney and Anis, 2014). Tissue culture is an alternative for canjerana (*Cabralea canjerana* (Vell.) Martius), an important native tree species of many countries of South America. Canjerana seeds are difficult to store, having a drastic reduction in germination viability after 15 days of seed processing (Grunennvaldt et al., 2014). This species has an excellent quality of wood, being used in construction, recovery of degraded areas and by the timber industry (Carvalho, 2003; Pereira et al., 2011). Moreover, the fruit is used in popular medicine due to his biochemical properties against *Trypanosoma cruzi*, which causes chagasic disease (Fournet et al., 1996).

Micropropagation allows the *in vitro* conservation and plantlet production of selected genotypes at any time from a microclonal hedge, that is composed of the root system and the rest of the aerial part of excised plants (micro-stumps), from micropropagated plantlets produced by *in vitro* subcultures and the miniclonal hedge that consists of rooted mini-cuttings from conventional cutting method-derived sprouts (Sivarajan et al., 2014), and can be derived from seedlings, cuttings or micropropagation (micro-stumps) (Titon et al., 2006). Indeed, mini-cutting is one of the latest vegetative propagation techniques used for mass production of woody species plantlets (Hartmann et al., 2011).

Micropropagated plantlets are highly desirable as microclonal hedge material, because they have the advantages of high genetic and phytosanitary quality and physiological juvenility, which allows for the production of mini-cuttings with high competence for rooting (Varshney and Anis, 2014). Therefore, combining conventional breeding with biotechnological techniques enables the identification and multiplication of superior genotypes, necessary for rapid and uniform forest production (Haggman et al., 2014).

The *in vitro* cultivation process of forest species has different phases, emphasizing the initial establishment of *in vitro* cultures and the acclimatization of plants after rooting, which may be the major obstacles of micropropagation (Grattapaglia and Machado, 1998). *In vitro* establishment of forest species has several difficulties in obtaining tissues free from contamination caused by fungi and bacteria (Thorpe et al., 1991), while the major problem of acclimatization is the high mortality due to many species do not have the ability to convert from heterotrophic to autotrophic growth (Paiva and Oliveira, 2006).

Micropropagation of native trees has been accomplished in Brazil with *Handroanthus heptaphyllus* (Vell.) Mattos (Duarte et al., 2016; Pimentel et al., 2016), *Apuleia leiocarpa* (Vogel J. F. Macbride) (Lencina et al., 2016), *Ilex paraguariensis* (Saint Hilaire) (Tronco et al., 2015) and *Handroanthus impetiginous* (Mart. ex DC Mattos) (Martins et al., 2011), while in the USA with *Liquidambar styraciflua* (L.) (Durkovic and Lux, 2010), *Sequoia sempervirens* (D Don Endl.) (Ozudogru et al., 2011), and *Quercus alba* (L.) (Vieitez et al., 2012), among other species. Besides this, more studies on micropropagation of native trees species, including those with great quality of timber as canjerana, are needed, since only one study has been developed with micropropagation of this species as well as, the authors reported that this protocol needs to be optimized (Rocha et al., 2007).

Thus, the objective of this study was to develop a protocol for the micropropagation of canjerana from seeds of selected individuals, as a tool for conserving and producing plantlets of superior genotypes for field evaluations and breeding new cultivars.

MATERIALS AND METHODS

The study was carried out in the laboratories and greenhouses of the Center for Plant Breeding and Asexual Propagation, Department of Plant Science, Federal University of Santa Maria, Brazil. Eight experiments were carried out, including seed disinfection and germination, *in vitro* multiplication and rooting, and acclimatization in the greenhouse. To start the process, fruits of canjerana were collected from selected stock plants (Figure 1A), packed in a sealed plastic bag and stored in a cool and dry environment of the laboratory until they dehisced (Rocha et al., 2007). Seeds were extracted when the fruits first opened, and immediately pre-disinfected with a solution of 2.5% of sodium hypochlorite (NaOCl) for 10 min and rinsed three times with distilled water (Figure 1B).

For *in vitro* establishment of canjerana seedlings (Experiment 1), seeds were first washed with distilled H_2O in a sterilized metallic sieve before removing the aril. To obtain aseptic canjerana seedling, seeds were immersed in a 0, 2.5, 5.0, 7.5, or 10% solution of NaOCl, with one drop of Tween® 20 per 100 ml of distilled water for 10, 20 and 30 min and rinsed three times with sterile-distilled water. The 0% concentration contained only one drop of Tween® 20 per 100 ml of distilled water. All these procedures were accomplished in a laminar flow chamber under aseptic conditions. After disinfection, one seed was cultivated in glass culture tubes (15 ml) containing 5 ml of culture medium with 30 g L^{-1} of sucrose and 6 g L^{-1} of agar, with pH adjusted to 5.8, capped with aluminum foil and autoclaved for 20 min at 1 atmosphere.

The experiment was set up as a factorial, 5 × 3 (NaOCl concentrations and immersion times, respectively) in a complete random design with eight replications of five seeds. Germinated seeds (seeds with visible radicles) (Figure 1C) were scored every three days; to calculate the mean germination time (MGT) and the

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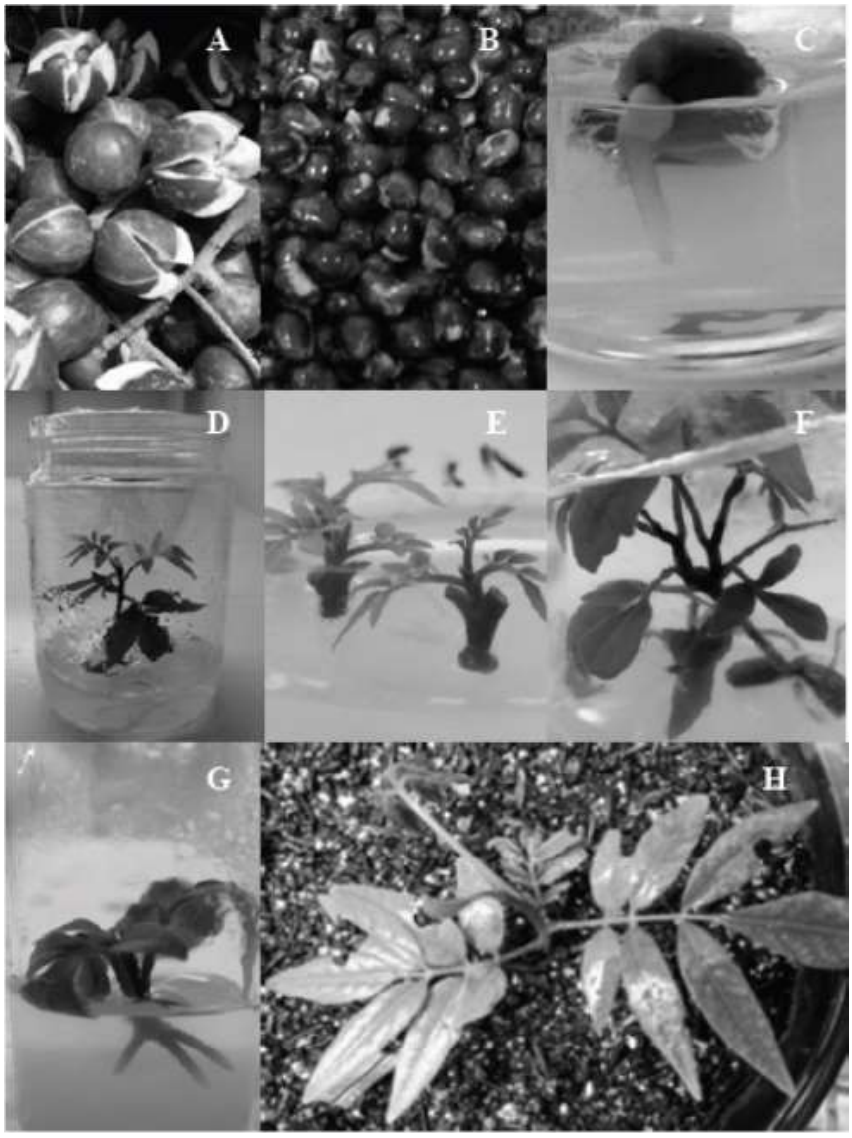

Figure 1. Process of plantlet production from selected canjerana stock plants: dehiscence of fruits of canjerana (A) disinfected seeds (B), *in vitro* germination (C). Aseptic seedlings of canjerana obtained from in vitro establishment and cultivated in different culture media for in vitro growth (D), which provided the explants used for in vitro multiplication (E). The microstump with new shoots (F), micro-cutting rooted in culture medium and ready for acclimatization (G) and micro-cutting acclimatizated in greenhouse (H).

index of germination rate (IGR).

MGT = (N1T1+N2T2+...+NnTn) / (N1+N2+...+Nn), and IGR = (N1/T1+N1/T2+...+Nn/Tn),

where N is the number of germinated seeds on a particular day and T is the time in days (Fick et al., 2007).

Thirty days after inoculation, the percentage of seeds that were free of fungi and/or bacteria, the germination rate and the proportion of normal seedlings were also evaluated.

To evaluate the effect of culture medium on *in vitro* growth (Experiment 2), aseptic seedlings of canjerana obtained from *in vitro* establishment were cultivated in three different culture media, MS (Murashige and Skoog, 1962), ½ MS (MS with half concentration of minerals and vitamins), and Woody Plant Medium (WPM) (Lloyd and McCow, 1981), with 30 g L^{-1} of sucrose and 6 g L^{-1} of agar (Figure 1D), with pH adjusted to 5.8, capped with aluminium foil and autoclaved for 20 min at 1 atmosphere.

The experiment was set up as a complete random design with four replications of four seedlings. The plant height, number of leaves and internodes and total length of roots were evaluated after 60 days of seedling cultivation.

For *in vitro* multiplication (Experiment 3), aseptic seedlings of canjerana were also used as source of explants. The explants were separated into basal, intermediate and apical segments that were 0.5 to 1.0 cm in length and contained one axillary bud. The explants were planted in flask (150 ml) containing 40 ml of WPM medium with 0 or 2.5 µM of 6-benzylamine purine (BAP), 30 g L^{-1} of sucrose

and 6 g L^{-1} of agar. The pH was adjusted to 5.8, capped with aluminium foil and autoclaved for 20 min at 1 atmosphere.

The experiment was set up as a factorial 2 × 3 (BAP concentration and type of segments) in a complete random design with five replications of four segments. Shoot number and length (cm) were evaluated after 60 days of cultivation (Figure 1E).

Basal and intermediate segments of canjerana aseptic seedlings were used to study the effect of different cytokines and BAP concentrations on *in vitro* multiplication. For experiment 4, segments of canjerana were planted in WPM medium containing 2.5 µM of BAP, kinetin (KIN), or thidiazuron (TDZ) added with 30 g L^{-1} of sucrose and 6 g L^{-1} of agar. For experiment 5, segments of canjerana were planted in WPM medium containing 0, 1.0, 3.0, 6.0, 9.0, or 12 µM of BAP added with 30 g L^{-1} of sucrose and 6 g L^{-1} of agar. The pH adjusted to 5.8, capped with aluminum foil and autoclaved for 20 min at 1 atmosphere. Both experiments were carried out in complete random design, with eight replications of six segments. Shoot number and length (cm) were evaluated after 60 days of cultivation.

After excising the nodal segments used as explants for the previous experiments, the root system and the rest of the aerial part of the aseptic seedlings (micro-stumps) were transferred to MS or WPM medium added with 30 g L^{-1} of sucrose and 6 g L^{-1} of agar (Figure 1F) (Experiment 6). The pH adjusted to 5.8, capped with aluminum foil and autoclaved for 20 min at 1 atmosphere. The experiment was set up in a complete random design, with eight replications of six micro-stumps. The percentage of micro-stump survival and the number of micro-cuttings produced per micro-stump were evaluated after 60 days of cultivation.

For *in vitro* rooting (Experiment 7), micro-cuttings were grown in MS or WPM basal media, with 0 and 5.0 µM of indolebutyric acid (IBA) or naphthalene acetic acid (NAA), added with 30 g L^{-1} of sucrose and 6 g L^{-1} of agar. The pH adjusted to 5.8, capped with aluminum foil and autoclaved for 20 min at 1 atmosphere. The micro-cuttings were 1 to 2 cm in length and had two leaves.

The experiment was a 2 × 3 factorial (base media and auxin concentrations) in a complete random design, with five replications of four micro-cuttings. The percentage of rooting was evaluated after 60 days of cultivation (Figure 1G).

For the acclimatization of canjerana plantlets (Experiment 8), the rooted micro-cuttings from the three culture media were planted in plastic pots containing 150 cm^3 of a commercial substrate (organic pine bark base, H.Decker Company, Santa Catarina, Brazil) for acclimatization. The pots were placed in the shade under a bench for a week and irrigated twice a day. The pots were then placed on the top of the bench and irrigated once a day, until the substrate exceeds the field capacity, and kept in a greenhouse with a mean temperature of 22°C and 50% of shade checked automatically through a control board with a thermohygrometer. The experimental was a complete random design, with five replications of four rooted micro-cuttings. The survival percentage was evaluated after 30 days of acclimatization (Figure 1H).

Data were subjected to analysis of variance and for those variables with significant differences ($p \leq 0.05$), treatment means were compared by Tukey test or polynomial regression, as appropriate. The percentage data were transformed to arcsin $\sqrt{x/100}$ and the counting data to $\sqrt{x+0.5}$ to meet the statistical presuppositions, especially variance homogeneity. All analysis were done with software Estat (UNESP, Jaboticabal) program (Estat., 1994).

RESULTS AND DISCUSSION

The analysis of variance in experiment 1 showed a significant interaction ($p \leq 0.05$) between concentrations of NaOCl and immersion times for the percentages of disinfection and germination. Seeds treated with 7.5% of NaOCl for 30 min had more than 80% of disinfection and more than 90% of these seeds germinated (Figure 2A and B). The percentage of disinfection and vigor (time to germination) were more strongly associated with the concentration of NaOCl than the immersion time (Figure 2A). The highest percentages of disinfection and germination were observed with the concentration of 7.5% of NaOCl for the three immersion times (Figure 2B).

In addition, the highest percentage of disinfection was found when seeds were treated with 7.5% of NaOCl for 20 or 30 min (Figure 2A), which was also associated with the highest vigor, quantified by the lowest mean germination time (Figure 2D), the highest index of germination rate (IGR) (Figure 2E) and the highest percentage of normal seedlings (Figure 2C). Therefore, the immersion times of 20 and 30 min where the most effective seed disinfection treatments without affecting germination and vigor. Seeds treated with 7.5 and 10% of NaOCl may have increased the degree of seed moisture (Sofiatti et al., 2008) and the permeability of seed tissues, reducing their germination time (Figure 2D) and increasing their germination rate. It probably occurred, because sodium hypochlorite is a strong oxidant and its action may change the properties of cell membranes of the integument or provide additional oxygen to the seed (Rocha et al., 2007).

In this study, a higher concentration of NaOCl and longer time of immersion than Rocha et al. (2007) was used without significantly affecting seed germination (Figure 2A) and vigor (Figure 2D). In canjerana seeds, concentrations of up to 10% of NaOCl for 30 min could be used, since it had a similar effect on seed germination and vigor as the 7.5% solution. After this work, this strategy is being used for *in vitro* establishment of aseptic seedlings of canjerana.

In the experiment 2, the base culture medium affected significantly seedling growth (Table 1). The WPM and MS medium resulted in taller seedlings, with more leaves and internodes and total length of roots than the ½ MS medium. Similar results were observed in explants of seedling from *Hagenia abyssinica* (Bruce J.F. Gmel), which presented either a similar *in vitro* growth, when cultivated in MS or WPM media (Feyissa et al., 2005). In contrast, plantlets of *Punica granatum* (L.) cultivated in WPM medium were apparently more vigorous and with longer shoots when compared with MS culture medium, which is an important feature in micropropagation (Valizadeh et al., 2013). Although, the most appropriate culture medium depends upon the species and type of explant; in the present study, aseptic seedlings of canjerana had a similar growth in both MS and WPM media. For this reason, WPM medium was hereinafter used on *in vitro* multiplication of canjerana, due to the fact that this culture medium developed by Lloyd and McCow (1981) has been the most widely used in studies

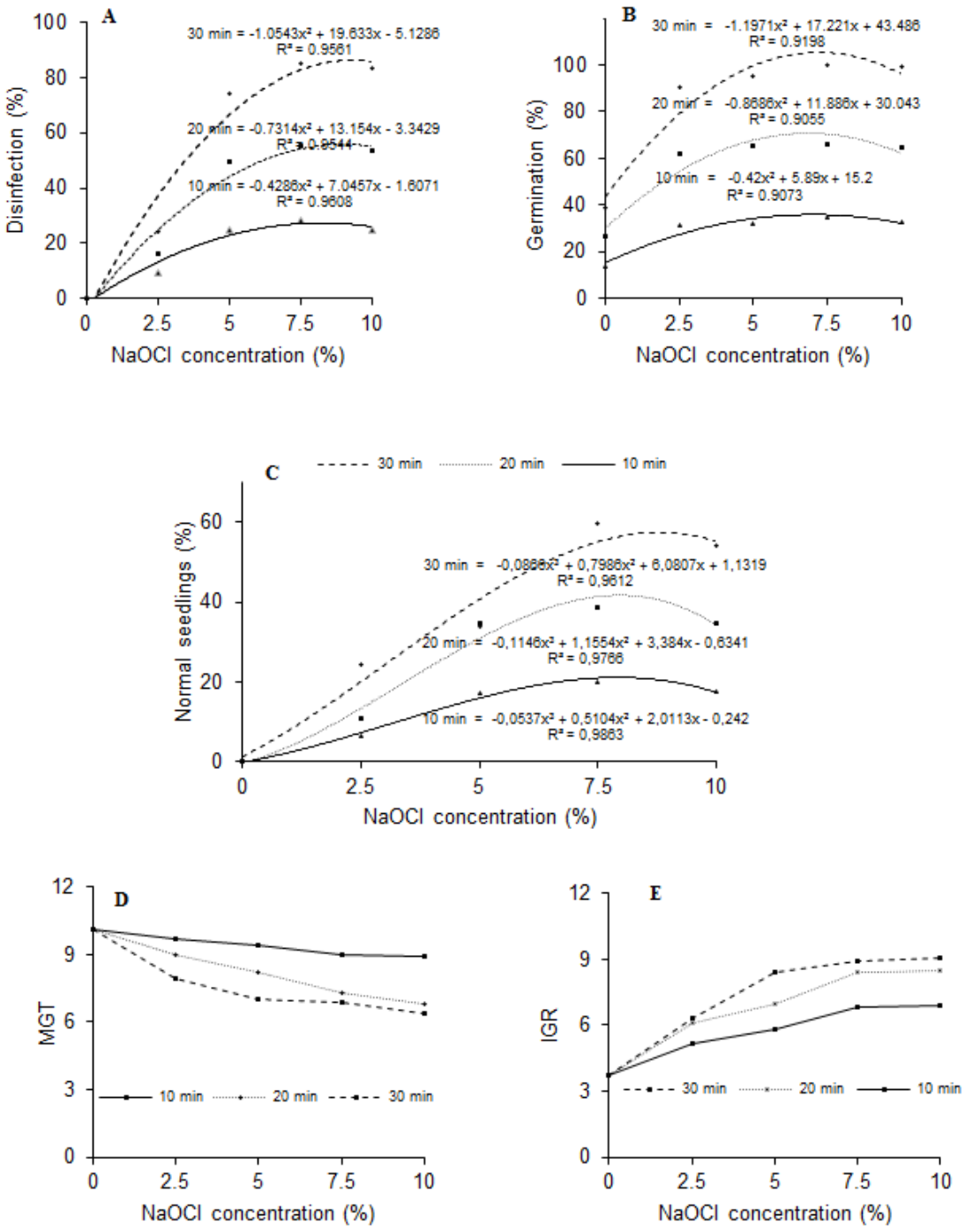

Figure 2. Percentages of not infected seeds (A), rates of germination (B), percentages of normal seedlings (C), mean germination time (MGT) (D), and the index of germination rate (IGR) (E) of canjerana seeds immersed in five different concentrations of NaOCl at different times.

involving propagation of woody species, as is the case of canjerana.

With regards to cytokines (Experiment 3), there were no significant interactions ($p \geq 0.05$) between BAP concentrations and the source of explants for the number and length of shoots after 60 days (Table 2). The largest number and length of shoots were verified in WPM medium supplemented with 6 µM of BAP, but with no statistically significant differences between the treatments (Supplemental Figure 1). It is likely that the greatest

Table 1. Length of aerial parts, number of leaves and internodes, and total length of roots of canjerana seedlings cultivated in three culture media for 60 days.

Treatment	Length of aerial parts (cm)	Number of leaves	Number of internodes	Total length of roots (cm)
WPM	3.7[a]*	5.1[a]	2.5[a]	10.3[a]
MS	3.6[a]	4.5[a]	2.5[a]	10.2[a]
½ MS	2.4[b]	2.5[b]	2.1[b]	6.6[b]

*Means values followed by the same letter are not significantly different by Tukey's test at 5% of probability.

Table 2. Shoot number and length of apical, intermediate and basal segments of canjerana cultivated in two concentrations of 6-benzylamine purine (BAP) for 60 days.

Treatments	Shoot number	Shoot length (cm)
Concentration		
BAP (0 µM)	0.3[a]*	0.2[a]
BAP (2.5 µM)	0.5[a]	0.5[a]
Type of segment		
Basal	0.9[a]	0.6[a]
Intermediate	0.4[ab]	0.4[ab]
Apical	0.0[b]	0.0[b]

*Means values followed by the same letter are not significantly different by Tukey's test at 5% of probability.

growth of shoots was observed in the basal and intermediate segments, independent of BAP concentration in the media. Thus, the lowest multiplication rate observed on apical explants of canjerana, might have occurred probably due to the adventitious shoots regenerated from the apical portion, which presented less vigor and therefore is being more sensitive to ethylene accumulation (Sá et al., 2012). Similar results were reported by Anis and Faisal (2005), who observed a higher rate multiplication in nodal segments of *Psoralea corylifolia*.

In the experiment 4, the addition of different cytokines in the media did not increase significantly the shoot number and length, and in fact TDZ significantly reduced the number of shoots (Table 3). In addition, in experiment 5, increasing the concentration of BAP in the media did not significantly affect shoot number and length (Supplemental Figure 1). Regardless of treatment, the number of shoots of canjerana was low. A low number of shoots were also observed with *Balanites aegyptiaca* Del. Varshney and Anis (2014) and it is in agreement with previous studies of canjerana (Rocha et al., 2007). According to these authors, the low number of shoots is because these species have erect stems and a small tree crown, which may affect the rate of *in vitro* multiplication and even the sprouting of vegetative material from the microclonal hedge. Another possibility would be that the physiology of canjerana segments results in a monopodial development. According to Nicoloso and Erig (2002), in *Pfaffia glomerata* (Spreng Pedersen), the physiological condition of the apical segment induced the expression of monopodial development, which is an expression of the relationship between the indoleacetic acid and cytokines (Hartmann et al., 2011).

Table 3. Shoot number and length of nodal segments of canjerana cultivated in different cytokinins for 60 days.

Cytokinins	Shoot number	Shoot length (cm)
BAP (2.5 µM)	0.8[a]*	0.5[a]
KIN (2.5 µM)	0.6[a]	0.5[a]
Control	0.6[a]	0.4[a]
TDZ (2.5 µM)	0.2[b]	0.2[a]

*Means values followed by the same letter are not significantly different by Tukey's test at 5% of probability.

In this study, there was no difference in shoot number and length even with the application of 12 µM of BAP. This inability to establish stabilized shoot cultures as well as a slow growth rate in microculture indicated recalcitrance of this species. This might be due to the exposure of explants to higher cytokine concentrations during the induction phase may have led to the accumulation of cytokines, which inhibited further shoot multiplication and growth (Malik et al., 2005).

Table 4. Percentage of survival of micro-stumps and number of micro-cutting per micro-stump of canjerana cultivated in two culture media for 60 days.

Culture media	Survival (%)	Number of micro-cuttings
WPM	70.0[a]*	1.40[a]
MS	40.0[b]	0.95[a]

*Means values followed by the same letter are not significantly different by Tukey's test at 5% of probability.

Table 5. Percentages of survival and rooting of canjerana micro-cuttings cultivated on two base medium, with or without two auxins after 60 days and the percentage of survival of rooted micro-cuttings after 30 days of acclimatization in a humid chamber in the greenhouse.

Treatments	Survival (%)	Rooting (%)	Survival (%)
Base medium			
WPM	75.0[a]*	20.0[a]	-
MS	65.0[a]	31.6[a]	-
Auxins			
NAA (5.0 µM)**	70.0[a]	57.5[a]	62.5[a]
IBA (5.0 µM)***	67.5[a]	20.0[b]	37.5[b]
Control (0 µM)	72.5[a]	0.0[c]	0.0[c]

*Means followed by the same letter are not significantly different by Tukey's test at 5% of probability. **NAA: Naphtalene acetic acid. ***IBA: indolebutyric acid.

In the experiment 6, the culture media did not affect significantly the production of micro-cuttings per micro-stump, but the WPM medium resulted in a higher percentage of micro-stump survival than the MS medium (Table 4). As observed with nodal segments, micro-stumps also showed a small number of shoots resulting in a low yield of micro-cuttings, with an average of 1.1 micro-cutting per micro-stump, compared to *Eucalyptus* species hybrids with a production between 1.5 and 2.3 micro-cuttings per micro-stump (Wendling at al., 2000). According to Titon et al. (2003), few shoots emitted by micro-stump is a normal response after decapitation of the apex, possibly due to the persistence of apical dominance. However, it is expected that through a proper management of micro-stumps by performing successive collections of micro-cuttings, causes a loss of apical dominance and increase the number of vegetative propagules of canjerana.

In the experiment 7, the base media and type of auxin had no significant effect on micro-cutting survival after 60 days of cultivation, but the addition of 5.0 µM of NAA increased the percentage of rooting and also the percentage of survival during acclimatization (Table 5). The addition of NAA to the base medium resulted in a higher percentage of rooting (57.5%) than the addition of IBA (20%) (Table 5). This percentage of rooting is not as high as the 87.5% found with segments of canjerana cultivated in ½ MS medium added with 5.0 µM of IBA reported by Rocha et al. (2007). This similar result reported by Rocha et al. (2007), was achieved with a tropical medicinal tree species, *Garcinia indica* (Thouars) Choisy in 66.6 to 91.66% of shoots cultivated on half-strength MS medium supplemented with 10 µM of IBA, while 52.77 to 77.77% shoots developed roots on NAA supplemented media (Malik et al., 2005).

In the experiment 8, the addition of 5.0 µM NAA to the base medium also increased significantly the percentage of survival during acclimatization from 37.5 to 62.5% (Table 5), although these values were not very high. Based on preliminary studies with different compositions of substrates, it is likely that higher percentages of survival during acclimatization can be achieved by selecting appropriate combination of substrate composition and keeping high air humidity in the wet chamber, with a relative humidity of approximately 85%, supplied automatically by a climate control.

Conclusion

To obtain vigorous and aseptic seedlings, seeds of canjerana can be treated with a 7.5% of sodium hypochlorite solution for 30 min and inoculated in a 30 g L^{-1} of sucrose and 6 g L^{-1} of agar, with pH adjusted to 5.8.

These seedlings can be grown in either WPM or MS media to increase the number of propagules produced per genotype. Nodal segments will not root in MS and WPM media; however, shoots produced from micro-stumps do root when 5.0 µM of NAA is added to the basal media. Nodal segments of canjerana and micro-stumps have a low rate of multiplication; therefore, tissue culture will be most valuable in maintaining superior genotypes of canjerana *in vitro* and as a source of stock plants for the microclonal hedge. The micropropagation of canjerana, as other native tree species, has a high potential for maintaining germoplasm *in vitro*, accelerating the breeding scheme for the identification of desirable genotypes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are grateful to Coordination for the Improvement of Higher Level Personnel (Capes) and the National Council for Scientific and Technological Development (CNPq) for the research fellowships.

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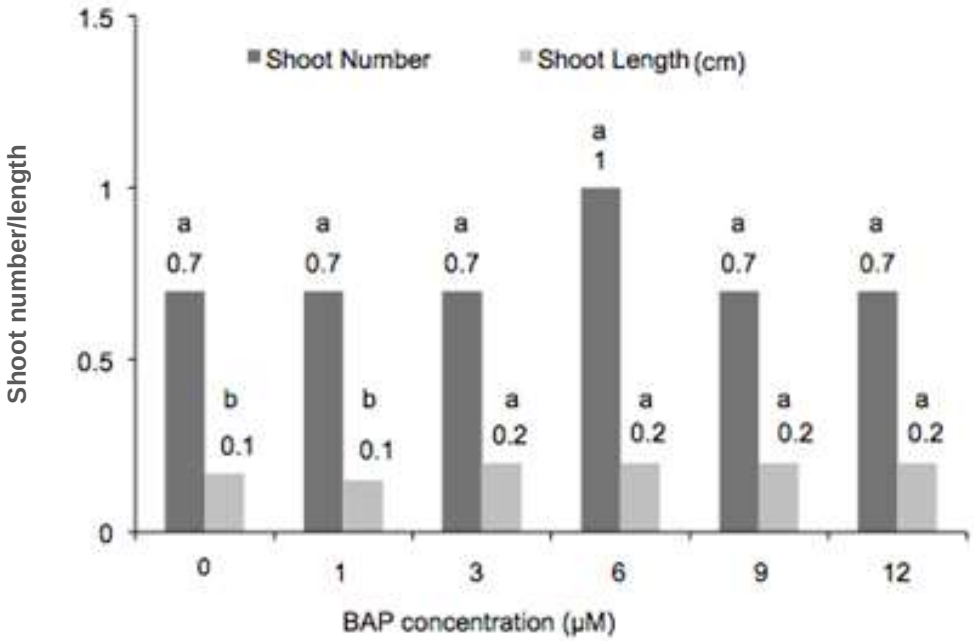

Supplemental Figure 1. Shoot number and length of nodal segments of canjerana cultivated in different concentrations of 6-benzylamine purine (BAP) for 60 days.