

Microcutting propagation of *Eucalyptus grandis* x *E. urophylla* through clumps of axillary buds using different containers and substrates

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Abstract

This study aims to evaluate the microcuttings production in the micropropagation system via clumps of axillary buds in a clone of *Eucalyptus grandis* x *E. urophylla*, subjected to different types of containers and substrates. Clumps with six to eight differentiated buds of *Eucalyptus grandis* x *E. urophylla* established *in vitro* were used to test containers (polypropylene pot - 500 ml, glass flask - 250 ml; acrylic flask with gas exchange (AFGE) - 250 ml; and test-tube - 55 ml capacity) in a semisolid medium specific for *Eucalyptus* in a period of 35 days. For the substrates test, acrylic flask with gas exchange (AFGE) were used with different substrates (agar, average particle size vermiculite, and vermiculite: cellulose fiber in a 2:1 ratio) in a period of 35 days. The tests were installed in a completely randomized design (CRD). We evaluated the number of shoots larger than 0.5 cm per clump of bud, the number of microcuttings produced larger than 2 cm, the length of the longest microcutting (cm) and shoot vigor. Based on the obtained results, it was possible to observe that the best container to produce microcuttings larger than 2 cm was the polypropylene pot (500 ml). Glass flask (250 ml) was more advantageous to achieve greater production of microcuttings per square meter due to its capacity of better densification. The best substrates to produce microcutting larger than 2 cm using acrylic flask with gas exchange (AFGE) containers were agar or vermiculite.

Keywords: *In vitro* propagation; plant tissue culture; vegetative propagation.

Abbreviations: AFGE_acrylic flask with gas exchange, APDM_aerial part dry mass, APFM_aerial part fresh mass, BAP_6-benzylaminopurine, BIOAGRO_Institute of Biotechnology Applied to Agriculture, CO₂_carbon dioxide, CRD_completely randomized design, JADS_culture medium specific for *Eucalyptus* (Correia et al., 1995), HCl_chloridric acid, IBA_indole-3-butyric acid, LMC_length of the largest microcutting (cm), NAA_naphthaleneacetic, NaOH_sodium hydroxide, NMC > 2 cm_number of microcuttings larger than 2 cm, NMC > 2 cm m⁻²_number of microcuttings larger than 2 cm m⁻², NS > 0.5 cm_shoots larger than 0.5 cm, NS > 2 cm m⁻²_number of shoots larger than 0.5 cm m⁻², PVP-30_polyvinylpyrrolidone, RDM_root dry mass, RFM_root fresh mass, RT%_root percentage.

Introduction

Among the applications of micropropagation in the Brazilian Forest sector, the production of *Eucalyptus* by microcutting became one of the most important techniques. The microcutting has as its basic principle the clonal rejuvenation obtained *in vitro*. This technique allowed considerable gains in nursery productivity, mainly regarding to the increase of the percentages and quality of rooting as well as the time reduction for molt formation. Nonetheless, limiting factors make it difficult to use the technique on a commercial scale, among which the physical and operational structure dependence of a tissue culture laboratory can be highlighted, adjusted protocols for species and clones, and the high cost of production (Xavier et al., 2013). To solve the limiting factors of this technique, the need for basic studies to maximize the production of *Eucalyptus* microcuttings has been emphasized. Among such limitations, one of great importance that should be considered is the size of containers for plant development, given that the space for cultivation influences the composition of the gas phase on the recipient, and

consequently the culture growth and development (Xiao et al., 2011).

Several studies have already been carried out aiming at the best development of different cultures in different sizes and shapes of containers; for example, 438 ml glass flasks for *Vitis vinifera* cultivation (Monette, 1983); 125 ml glass flasks for *Actinidia chinensis* (Monette, 1986); 250 ml bowls for *Gossypium hirsutum* and *Gossypium arboreum* (Hazra et al., 2000); medium large sized test tubes for *Pfaffia glomerata* (Nicoloso and Erig, 2002); and 100 ml flasks for *Dendrobium nobile* culture (Moraes et al., 2010). Besides the different sizes and shapes, recipients with gas exchange can allow this exchange between the external and internal atmosphere of containers through natural ventilation, which also leads to the better development of the crops (Kozai, 2010), as observed in the following: glass jars with porous membranes in the increase of the number of shoots of *Azadirachta indica* (Rodrigues et al., 2012); in the highest growth and content of photosynthetic pigments of *Pfaffia glomerata* (Saldanha et

al., 2012); and porous membranes with increasing numbers of buds in *Olea europaea* (Pinheiro et al., 2013).

Another needed element to maximize shoots production is the nature of the substrate in which *in vitro* rooting occurs, since it considerably influences plant quality (Afreen-Zobayed et al., 2000). The conventional use of agar as a gelling agent has some drawbacks, such as the reduction of the oxygen coefficient, which affects the differentiation and development of the aerial and radicular plant part. In addition, the use of agar can cause a great difficulty on the circulation and nutrients absorption in the culture medium, limiting the development of some plant species to specific agars (Kirdmanee et al., 1995; Ichimura and Oda, 1998; Scholten and Pierik, 1998). Thus, alternative materials such as vermiculite and fibrous materials such as cellulose have been presented as options to increase productivity and decrease production costs (Afreen-Zobayed et al., 2000).

In this context, this work aimed to evaluate the microcuttings production in a micropropagation system through the proliferation of axillary buds of the *Eucalyptus grandis* x *E. urophylla* clone, subjected to different types of containers and substrates.

Results

Test with different containers

Based on the results, containers had a significant effect ($p \leq 0.05$) on the number of shoots larger than 0.5 cm ($NS > 0.5$ cm), number of shoots larger than 0.5 cm m^{-2} ($NS > 2$ cm m^{-2}), number of microcuttings larger than 2 cm ($NMC > 2$ cm), number of microcuttings larger than 2 cm m^{-2} ($NMC > 2$ cm m^{-2}), and length of the largest microcutting (cm) (LMC). However, the vigor feature showed no significant difference ($p > 0.05$) for the tested containers.

The production of $NS > 0.5$ cm showed statistical difference between the containers (Fig 1A). Polypropylene pot (500 mL) presented the average of 52 shoots, which was successively higher to the glass flasks (250 mL), AFGE (250 mL), and the test tube (55 mL).

However, when the production of $NS > 0.5$ cm m^{-2} (Fig 1B) is compared, the container that presented the highest production was the glass flask, with a mean of 6331 shoots m^{-2} , higher than the polypropylene pot (2311 shoots m^{-2}). The test tube produced on average 5277 m^{-2} shoots, not differing from glass flask. The AFGE showed 3775 m^{-2} shoots, so did not differ from the test tube production as well as from the polypropylene pot.

In relation to $NMC > 2$ cm, there were significant differences between containers, Fig 1C. The highest mean of microcuttings was observed on polypropylene pot (22 microcuttings per pot), the AFGE and the test tube presented the lowest production (9.5 and 1.5 microcuttings, respectively), whereas the glass flask did not show any difference in relation to the other containers.

When compared the $NMC > 2$ cm with the $NMC > 2$ cm m^{-2} , Fig 1D, there was a change in the production scale, the polypropylene pot showed to be the container with the lowest production (9770 microcuttings m^{-2}), differing only from the glass flask (2781 microcuttings m^{-2}). The other containers had no difference in production compared to the glass flask and the polypropylene pot.

The LMC showed statistical difference only between the glass flask, AFGE containers, and test tube (Fig 1E). The glass flask provided better growth of the microcuttings when compared to the AFGE and the test tube. However, for

polypropylene pot, the length of microcuttings did not show any difference between the tested containers.

Test with different substrates

The substrates tested showed significant differences ($p \leq 0.05$) in the number of microcuttings larger than 2 cm ($NMC > 2$ cm), length of the largest microcutting (LMC), shoots vigor, aerial part fresh mass (APFM), aerial part dry mass (APDM), root fresh mass (RFM), root dry mass (RDM), and root percentage (RT%). Only the number of shoots larger than 0.5 cm ($NB > 0.5$ cm) had no effect on the substrates tested (Fig 2).

The $NMC > 2$ cm for the substrates agar or vermiculite was statistically higher ($p < 0.05$) to the substrate vermiculite: cellulose fiber. The production in AFGE presented 9.6, 5.6, and 1.2 microcuttings larger than 2 cm, using as substrate agar, vermiculite and vermiculite: cellulose fiber, respectively (Fig 2A).

For LMC, the agar culture was statistically higher in relation to the others (Fig 2B), plants in this medium presented a microcuttings length of 8.5 cm on average, followed by vermiculite with 4.6 cm and finally the substrate with vermiculite: cellulose fiber, with 2.2 cm per microcutting, which was statistically lower than the others.

Fig 2C shows that the percentage of root emission in the substrate for treatments with agar or vermiculite did not present statistical differences among them, they presented 100% and 83% of root emission, respectively. The use of vermiculite: cellulose fiber presented on average 50% of rooting, it was then statistically lower, compared to the other substrates.

The adopted pattern for vigor presented a statistical difference between the use of vermiculite: cellulose fiber and the other treatments, Fig 2D. Vermiculite: cellulose fiber had the lowest grade (1.4) for vigor feature. Whereas, the treatments that used agar or vermiculite as substrate showed vigor statistically similar, with grades of 3.0 and 2.3, respectively.

The treatments with agar or vermiculite, Figs 3A and 3B, showed more vigorous microcuttings, greater quantity and length as well as greater presence of roots. The opposite occurred in the vermiculite: cellulose fiber treatment, Fig 3C, in which the microcuttings showed smaller sizes and production, with presence of leaf callus.

The aerial part fresh mass (APFM) of plants grown on the substrate vermiculite: cellulose fiber (0.489 g) was statistically lower than those grown on agar (2.183 g) and vermiculite (1.695 g) (Fig 4). Though, when compared agar and vermiculite, there was no significant difference in MFPA. For aerial part dry mass production (APDM), the agar substrate (0.426 g) was higher to the other substrates tested, the vermiculite substrate presented intermediate weight (0.292 g), while the vermiculite: cellulose fiber system produced lower values when compared to the others.

The Fig 4 shows the mean values of the root fresh mass (RFM) and root dry mass (RDM) of the plants in the tested substrates. In this way, plants cultivated in the substrate containing vermiculite had a higher mean (0.666 g), while the lowest value was observed for vermiculite: cellulose fiber (0.077 g). The agar system showed intermediate values (0.506 g). However, for root dry mass (RDM), the agar system was significantly higher to the others (0.105 g), while vermiculite and vermiculite: cellulose fiber did not present statistical differences, with a mean of 0.038 and 0.011 g, respectively.

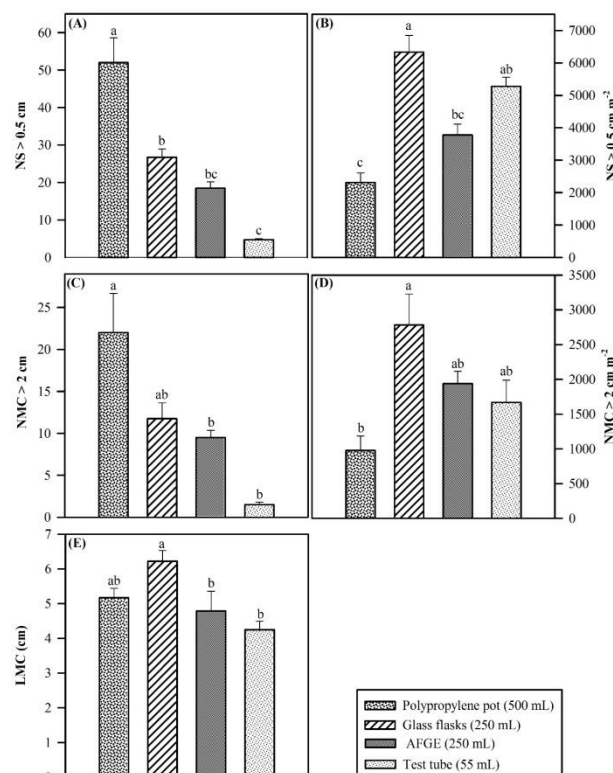


Fig 1. Features observed in micropropagation of *Eucalyptus grandis* x *E. urophylla* clone in different containers. (A) Number of shoots > 0.5 cm (NS > 0.5 cm); (B) Number of shoots > 0.5 cm m⁻² (NS > 0.5 cm m⁻²); (C) Number of microcuttings > 2 cm (NMC > 2 cm); (D) Number of microcuttings > 2 cm m⁻² (NMC > 2 cm m⁻²); and (E) Length of the largest microcutting (LMC, cm). *Means followed by same letter do not differ by Tukey test at 5% probability. Bars represent standart error (\pm SE).

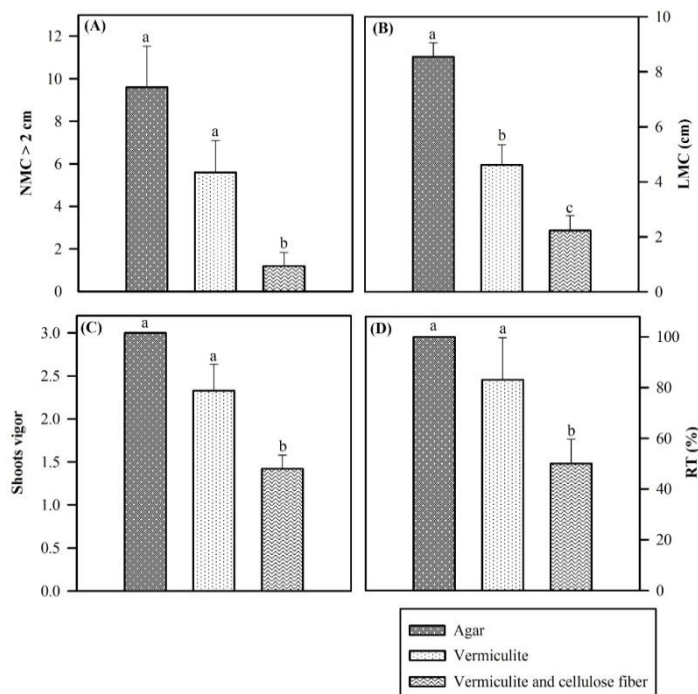


Fig 2. Features observed in micropropagation of *Eucalyptus grandis* x *E. urophylla* clone in different substrates. (A) Number of microcuttings > 2 cm (NMC > 2 cm); (B) Length of the largest microcutting (LMC, cm); (C) Shoots vigor; and (D) Root percentage (RT%). *Means followed by same letter do not differ by Tukey test at 5% probability. Bars represent standart error (\pm SE).

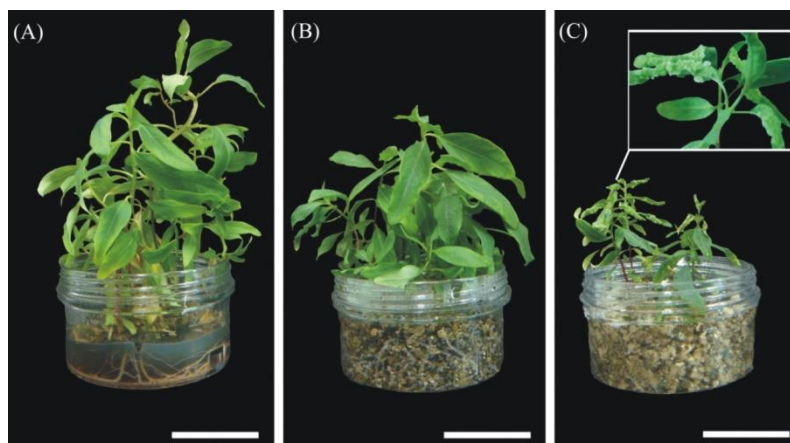


Fig 3. *Eucalyptus grandis* x *E. urophylla* clone at 30 days *in vitro* cultivation AFGE system during the elongation phase on different substrates. (A) Agar + semisolid medium JADS; (B) Vermiculite + liquid medium JADS; and (C) Vermiculite: cellulose fiber + liquid medium JADS. In detail, the presence of leaf callus. Bars: 30 mm.

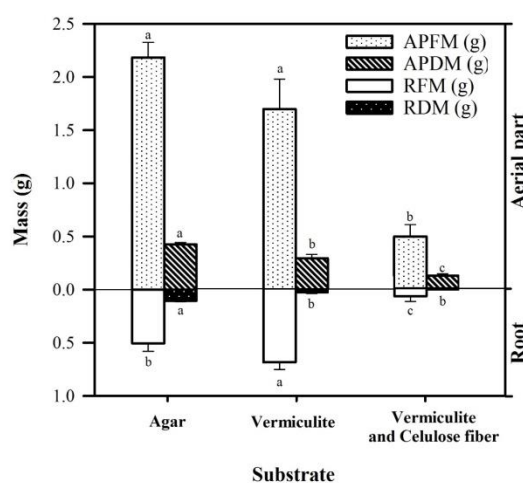


Fig 4. Features observed in micropropagation of *Eucalyptus grandis* x *E. urophylla* clone in different substrates. APFM: aerial part fresh mass (g); APDM: aerial part dry mass (g); RFM: root fresh mass; and RDM: root dry mass. *Means followed by same letter do not differ by Tukey test at 5% probability. Bars represent standard error (\pm SE).

Discussion

When compared, the shoot productivity > 0.5 cm and microcuttings productivity > 2 cm in the containers, without considering the space occupied by the container in the culture rack; the large volume containers produced higher values in relation to the features evaluated. McClelland and Smith (1990) in their work with five forest species have found results that corroborate the values found in this study. They observed a better microcutting production in large volume containers such as 350 mL magentas and 200 mL glass flasks, which were higher to the 60 mL test tube.

Some studies also had showed the largest *in vitro* production in large-volume containers (Monette, 1983; Hazra et al., 2000). These results may be related to internal environmental factors that affect the development of explants, where smaller flasks tend to have low concentrations of carbon dioxide and high concentrations of ethylene, as well as may also be affected by light irradiation, air temperature, and relative humidity (Zimmerman, 1995; Xiao et al., 2011).

The abovementioned factors may limit the flow of photosynthetically active photons, decrease the rate of transpiration and photosynthesis, and can also cause lower absorption of water and nutrients, which leads to a reduction

of the growth rate and development of explants (Nguyen and Kozai, 2005; Zobayed, 2006; Xiao et al., 2011). The AFGE container did not perform well compared to the glass flask and the polypropylene pot. Zobayed et al. (2000) in their work with *Eucalyptus camaldulensis* observed gains in mass and photosynthetic rate in plants raised in containers with gas exchange, when compared to those raised in a closed system using rigid lids MagentasTM. On the other hand, when the production of buds > 0.5 cm m^{-2} and microcuttings > 2 cm m^{-2} were estimated, the largest flask (500 mL pot) was the one that produces the least, when compared to the other containers. This fact was related to the space occupied by the container in the tissue culture rack. Since the smaller containers were easier to organize and placed in a denser way, they presented a greater number of clumps m^{-2} , thus, higher productivity per area. The best results for the number of microcuttings > 2 cm, root emission percentage and vigor were observed on the substrates of agar and vermiculite system. The length of the largest microcutting was an exception, in which presented better results only for the agar system. Kirdmanee et al. (1995), in the photoautotrophic cultivation of *Eucalyptus camaldulensis* with alternative substrates (agar system, GelriteTM, plastic net, and

vermiculite), found better substrate growth results with vermiculite, followed by the plastic net, Gelrite™, and agar. The worst results in all evaluated features were observed on the vermiculite: cellulose fiber system, compared to vermiculite and agar. Similar results were found in the work of Khan et al. (2002), where the use of Florialite™ (substrate based on vermiculite and cellulose fiber) did not show promising results for *Eucalyptus tereticornis* compared to the conventional system (agar). These authors concluded that the shoot height, the number of new nodal segments generated per explant, and the multiplication coefficient were significantly higher under photomixotrophic conditions (30 g L⁻¹ sucrose and 400-450 µmol mol⁻¹ increase CO₂) than under a variety of photoautotrophic cultural conditions (400-450 and 1400-1500 µmol mol⁻¹ CO₂ and Florialite™ with 400-450 µmol mol⁻¹ CO₂ increase). Higher values of fresh and dry mass of the aerial part as well as of the radicular part were observed in the environment with agar and vermiculite, and smaller masses were observed in the system with vermiculite: cellulose fiber. Therefore, substrates with higher porosities can influence the formation of a root system, while in agar, evidence showed that there are structural abnormalities of the root tissues (Kataoka, 1994). There is also evidence that, depending on the physical and chemical nature of the substrate, the rooting and growth capacity may also be affected (Zobayed et al., 2000). The use of substrates, as cellulose fiber, may lead to greater difficulty in inoculating the explants due to its texture; it can often lead the explants present difficulties of remaining in the medium, resulting in lower growth rates (Afreen et al., 1999); these observations were corroborated by this study results.

In the light of the aforementioned results and studies, it was evident the great variation of responses according to the species, the objective of the work and substrates inserted, but the use of alternative substrates plays an important role in the micropropagation process (Afreen et al., 1999).

Materials and methods

Plant material and in vitro culture conditions

The experiments were conducted at the Vegetable Tissue Culture Laboratory II of the Institute of Biotechnology Applied to Agriculture (BIOAGRO) at the Federal University of Viçosa, located in Viçosa - MG. Clumps containing six to eight differentiated buds were obtained from the micropropagation of the *Eucalyptus grandis* x *E. urophylla* clone, established *in vitro* by 25 subcultures under heterotrophic conditions, in test tubes with 10 mL JADS culture medium (Correia et al., 1995), with the following composition: 30 g L⁻¹ sucrose (Vetec™), 100 mg L⁻¹ myo-inositol (Sigma™), 800 mg L⁻¹ PVP-30 (polyvinylpyrrolidone - Vetec™), 0.5 Mg L⁻¹ of BAP (6-benzylaminopurine - Sigma™), 0.01 mg L⁻¹ NAA (naphthaleneacetic acid - Sigma™) and 7 g L⁻¹ agar (Merck™). Then, the clumps were transferred to the same container type and culture medium, which the concentration of BAP had reduced from 0.5 mg L⁻¹ to 0.3 mg L⁻¹. In both steps, the pH was adjusted to 5.8 with 1 M HCl or 1 M NaOH prior to the addition of agar. The culture medium was sterilized in an autoclave at a pressure of 1.5 atm and a temperature of 121 °C for 20 minutes. The cultures remained in a tissue culture room to growth at 25 ± 2 °C for a 16-hour photoperiod with irradiance of 33 µmol m⁻² s⁻¹ (quantified by LI-COR™ radiometer, LI-250A Light Meter), provided by

two tubular fluorescent lamps (Special Daylight, 40W, Osram™, Brazil).

Test with different containers

After 30 days, clumps in the multiplication culture medium containing six to eight differentiated buds were transferred to different containers, as polypropylene pots (500 ml capacity), glass flasks (250 ml capacity), Agripot™ (AFGE - acrylic flask with gas exchange and 250 mL capacity), and test tubes (55 ml capacity). The amount of culture medium in each container was variable per number of explants, with 100 mL for 10 clumps in polypropylene pot; 40 mL for 4 clumps in glass bottle; 30 mL for 3 clumps for AFGE (Agripot™); and 10 mL for 1 clump in test tube. The used culture medium was JADS, with 30 g L⁻¹ sucrose, 100 mg L⁻¹ myo-inositol, 800 mg L⁻¹ PVP-30, 0.05 mg L⁻¹ BAP, 25 mg L⁻¹ of IBA (indole-3-butyric acid - Sigma™), and 7 g L⁻¹ of agar. The pH was adjusted to 5.8 with 1 M HCl or 1 M NaOH prior to the addition of agar, and the medium was autoclaved at a pressure of 1.5 atm and temperature of 121 °C for 20 minutes. The containers remained in a tissue culture growth room at 25 ± 2 °C for a 16-hour photoperiod with irradiance of 33 µmol m⁻² s⁻¹ (quantified by LI-COR™ radiometer, LI-250A Light Meter) provided by two tubular fluorescent lamps (Special Daylight, 40W, Osram™, Brazil).

Test with different substrates

After 30 days, clumps in the multiplication culture medium, that contained six to eight differentiated buds were transferred to the AFGE recipient with different substrates: 1) traditional system in semi-solid medium with agar; 2) liquid medium with vermiculite of average granulometry (30%); and 3) liquid medium and a mix of vermiculite and cellulose fiber washed in a 2: 1 ratio. The used culture medium was JADS with 30 g L⁻¹ sucrose, 100 mg L⁻¹ myo-inositol, 800 mg L⁻¹ PVP-30, 0.05 mg L⁻¹ BAP, 0.25 Mg L⁻¹ IBA and 7 g L⁻¹ agar. Though, agar was added only to the substrate containing the semisolid medium, the pH was adjusted to 5.8 with 1M HCl or 1M NaOH, and the medium was autoclaved at a pressure of 1.5 atm and temperature of 121 °C for 20 minutes. The treatments remained in a growth room at 25 ± 2 °C for a 16-hour photoperiod with irradiance of 33 µmol m⁻² s⁻¹ provided by two tubular fluorescent lamps (Special Day Light, 40W, Osram™, Brazil).

Experimental evaluations

The evaluations were performed after 35 days of cultivation of the treatments. The assessments were made with regard to the number of shoots larger than 0.5 cm per clump, number of microcuttings produced larger than 2 cm, length of the largest microcutting (cm), and shoots vigor based on a grade scale varying from 1 (Low), 2 (Medium) and 3 (High). In addition to these evaluations, were estimated in the containers experiments the number of shoots larger than 0.5 cm⁻² and the number of microcuttings produced larger than 2 cm⁻², considering the arrangement of the respective containers in the space of 1 m⁻². In the substrate experiment the percentage of root emission, fresh and dry aerial part mass, and fresh and dry root mass were evaluated.

Experimental design and analysis

The tests were installed in a completely randomized design (CRD). For the experiment with container, four types were tested: polypropylene pot, glass flask, AFGE and test tube, with four replications. For each type of container a quantity of clumps of differentiated buds were inoculated. For the substrate experiment, three types - agar, vermiculite, and vermiculite: cellulose fiber - were tested, with five replications. In each plot (AFGE) were inoculated three clumps of differentiated buds. The data analysis was performed in the R statistical software Version 3.3.1 (R Core Team, 2016), using the ExpDes package, Version 1.1.2 (Ferreira et al., 2013). Normality assumption of the residue, by Shapiro-Wilk, and homogeneity of the variance, by Bartlett, were verified at the 5% level of significance. Once confirmed the non-significance of these tests, which indicates the normality of the residue and homogeneity of variances, the evaluated features were subjected to analysis of variance. When the p value of variance was less than 0.05 and the F was significant, the treatments means were compared by the Tukey test, at the 5% level of significance.

Conclusion

In conclusion, the best container for microcutting production larger than 2 cm and for number of shoots > 0.5 cm was the polypropylene pot (500 mL). When greater microcutting production larger than 2 cm and number of shoots > 0.5 cm per square meter was aimed, the glass flask (250 mL) was more advantageous due to its better possibility of densification. The best substrates to produce microcuttings larger than 2 cm with good shoots vigor and root percentage using the AFGE container were agar or vermiculite.

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