

***In vitro* propagation and acclimatization of *Lippia rotundifolia*, an endemic species of Brazilian Campos Rupestres¹**

Propagação *in vitro* e aclimatização de *Lippia rotundifolia*, uma espécie endêmica dos Campos Rupestres Brasileiros

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ABSTRACT - The importance in folk medicine, combined to threats in their environment, becomes necessary to carry out studies involving large-scale propagation of *Lippia* genus. Although the tissue culture propagation is widely disseminated for medicinal plants, for *L. rotundifolia* any article was published yet. The present study aimed to establish an efficient protocol for micropropagation of *L. rotundifolia*. Nodal segments, taken from plants collected in the Espinhaço Range, were disinfected, and cultures were initiated on MS medium with PVPP (1 g L⁻¹), sucrose (3%) and agar (0.7%). The culture were maintained in a growth room at controlled conditions. Disinfestation procedures and the supply of PVPP on culture media resulted in both reduced contamination and phenol oxidation rates, with more than 90% of viable cultures. In the multiplication phase were tried different BAP and NAA combinations supplied to the MS medium. The treatment that resulted in highest multiplication rates was 0.33 µM BAP. The effects of NAA were evaluated for *in vitro* rooting. At 0.44 µM, rooting was 70% higher than that observed in the control. The acclimatization was held in trays with substrate, coated with translucent plastic and kept under shade. The plantlets were transferred to the greenhouse after 15 days and transplanted to plant beds after 30 days. The acclimatized plantlets bloomed one year after the transference to field conditions, showing that the *in vitro* culture did not affect the vegetative and reproductive development, which confirms the potential of micropropagation to reduce the extinction risk of *L. rotundifolia*.

Key words: Micropropagation. *In vitro* multiplication. *In vitro* rooting. *Ex vitro* acclimatization. Biodiversity conservation.

RESUMO - A importância na medicina popular, assim como as ameaças em seu ambiente, tornam necessária a realização de estudos envolvendo a propagação em larga escala de plantas do gênero *Lippia*. Embora a propagação *in vitro* seja amplamente disseminada para plantas medicinais, nenhum artigo foi publicado ainda com *L. rotundifolia*. O presente estudo teve como objetivo estabelecer um protocolo eficiente para a micropropagação dessa espécie. Segmentos nodais, retirados de plantas coletadas na Cadeia do Espinhaço, foram desinfetados antes da inoculação em meio MS suplementado com PVPP (1 g L⁻¹), sacarose (3%) e ágar (0,7%). Os tubos de ensaio foram mantidos em sala de crescimento sob condições controladas. O tratamento de desinfestação e a adição de PVPP aos meios de cultura resultaram na redução da infecção microbiológica assim como nas taxas de oxidação fenólica, proporcionando mais de 90% de culturas assépticas e viáveis. Na fase de multiplicação foram testadas diferentes combinações de BAP e ANA adicionadas ao meio MS. O tratamento que resultou em maiores taxas de multiplicação foi de 0,33 µM de BAP. Os efeitos do ANA foram avaliados no enraizamento *in vitro*. A 0,44 µM de ANA, o enraizamento foi 70% superior ao observado no controle. A aclimatização foi realizada em bandejas com substrato, revestidas com plástico translúcido e mantidas à sombra. As plantas foram transferidas para casa de vegetação após 15 dias e transplantadas para canteiros após 30 dias. As plantas aclimatizadas floresceram após um ano da transferência para condições de campo, demonstrando que os procedimentos de cultivo *in vitro* não afetaram o desenvolvimento vegetativo e reprodutivo das plantas, o que confirma o potencial da micropropagação para redução dos riscos de extinção de *L. rotundifolia*.

Palavras-chave: Micropropagação. Multiplicação *in vitro*. Enraizamento *in vitro*. Aclimatização *ex vitro*. Conservação da biodiversidade.

DOI: 10.5935/1806-6690.20150041

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¹Recebido para publicação em 22/11/2013; aprovado em 25/03/2015

Parte do Trabalho de Iniciação Científica do segundo autor desenvolvido com bolsa do Programa BIC-UFJF

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INTRODUCTION

The Verbenaceae, one of the five most important eudicotyledonous families of the *Campos Rupestres* (dry, rocky grasslands), is distributed in almost all terrestrial ecosystems (GIULIETTI *et al.*, 2000). In the family, the genera *Lippia*, *Lantana* and *Stachytarpheta* are considered the most important (SALIMENA-PIRES, 1991). The genus *Lippia* belongs about 200 species that occur in South and Central America and tropical Africa. The most number of species, about 150, is found in Brazil, with a higher incidence in the Espinhaço Range, in Minas Gerais, and in Chapada Diamantina, in the state of Bahia (SALIMENA-PIRES, 1991). Species of genus *Lippia* present economic importance due to the wide use of its essential oils and its medicinal properties (PASCUAL *et al.*, 2001; SALIMENA-PIRES, 1991). In the Tropical America, Verbenaceae is quite used for its gastrointestinal properties and against respiratory diseases. However, in countries such Brazil and Guatemala, the Verbenaceae are used against skin diseases, burns and ulcers. In most cases, their leaves and flowers are used as a source of herbal drugs (PASCUAL *et al.*, 2001).

Lippia rotundifolia Cham. is a Verbenaceae shrub with underground xylopodium root system and oval leathery leaves. The plants present corymbs inflorescence with corolla lilacs strongly aromatic, containing many glandular trichomes. Leaves of *L. rotundifolia* are rich in monoterpenes. Limonene is also found in inflorescences and myrtenol, your derivative, is accumulated over in the leaves (LEITÃO *et al.*, 2008). Limonene is known for its medicinal properties, including action against tumors in mammals, and important drug in cancer therapies. To the myrtenol is also attributed the anticancer activity due to the inhibitory action of hepatocellular carcinoma and role in protecting membranes from damage caused by free radicals (BABU; PERUMAL; BALASUBRAMANIAN, 2012).

Considering that the anthropogenic perturbations in *Campos Rupestres* are increasing strongly in recent decades, that little is known about the impact of these actions on the plant populations, and that the risk of extinction of some endemic species are high, studies aiming at *Lippia* species conservation become necessary (VITTA, 2002). The tissue culture technique has been widely used in recent years for the propagation of medicinal plants (PARVEEN; SHAHAZAD, 2011). The *in vitro* clonal propagation through the axillary bud may to produce lots of plants in a limited space, regardless of the season and under controlled environment conditions (VARSHNEY; ANIS, 2012). For the success of *in vitro* procedures, it is essential to establish protocols for large-scale propagation, rooting and acclimatization of these plants. However, it is surprising that few *in vitro* studies

have been published with plants of the *Lippia* genus. In the literature, studies they were found only for *Lippia junelliana* (JULIANI *et al.*, 1999), *Lippia alba* (GUPTA; KHANUJA; SUSHIL, 2001), *Lippia filifolia* (PEIXOTO *et al.*, 2006) and *Lippia sidoides* (COSTA *et al.*, 2007).

Due to the endemism and the pharmacological activities of the Verbenaceae, this study aimed to develop an efficient protocol for *in vitro* propagation and *ex vitro* acclimatization of *L. rotundifolia*, from the perspective of reducing the risk of extinction of this species in its natural environment and its future use in pharmacological studies.

MATERIAL AND METHODS

In vitro Establishment:

Plants of *Lippia rotundifolia* Cham. were collected in the Espinhaço Range (Minas Gerais, Brazil) and established by vegetative propagation in the Experimental Station of Plants Propagation at the Federal University of Juiz de Fora (Minas Gerais, Brazil). Fertile specimens were deposited in the Herbarium of the Federal University of Juiz de Fora (CESJ) under number 31,376. Aiming to establish *in vitro* cultures, plants maintained under field conditions were subjected to surface sterilization with 1.72 mM Benomyl (DuPont®, USA), sprayed once a week for 30 days. Thirty nodal segments were collected from the plants and washed in running tap water added drops of commercial detergent for 1 h. Then, the explants were immersed in 70% ethanol (v/v) for 30 minutes and after in a solution of 1.72 mM Benomyl for 10 minutes. Finally, the nodal segments were immersed in commercial bleach solution (2% of active chlorine) at 30% (v/v) dilution, added of Tween 20 (20 µL) for 15 minutes. Subsequently, the explants were washed five times in distilled and autoclaved water and then inoculated on MS medium (Murashige; Skoog, 1962) at half strength salts and vitamins, without growth regulators. In order to control the phenolic oxidation, polyvinylpolypyrrolidone (PVPP, 1 g L⁻¹) was also supplied to the culture media. The occurrence of bacterial and fungal contamination as well as phenolic oxidation in tissues and culture media were evaluated daily.

***In vitro* culture conditions:** All cultures were kept in test tubes 2.5 x 15 cm. The culture media was the MS medium supplemented with 3% sucrose (w/v) and 0.7% agar (w/v). The pH of the culture media was adjusted to 5.7 ± 0.1 before autoclaving, performed for 20 min at 120 °C and 1 atm pressure. The tubes were capped with polyethylene autoclavable closures and sealed with 15 µM PVC film. Cultures were maintained in a growth chamber with controlled temperature (26 ± 1 °C), photoperiod (16-8 h) and light (40 µmoles m⁻² s⁻¹).

***In vitro* multiplication:** Thirty days after the establishment of aseptic cultures, studies concerning *in vitro* multiplication were performed. Nodal segments, measuring 2-3 cm were inoculated vertically on culture media in full strength of MS salts and vitamins, supplied with 6-benzylaminopurine (BAP: 0; 0.33; 0.66 or 1 μ M) and α -naphthalene acetic acid (NAA: 0; 0.001; 0.01 or 0.1 μ M) in all combinations, for a total of 16 different treatments, with 10 replicates each. After 40 days of inoculation, we assessed the shoot and the root numbers and the height of the largest shoot.

***In vitro* rooting:** The *in vitro* plantlets rooting was evaluated using nodal segments, measuring 2-3 cm, previously stabilized and maintained on MS medium, without growth regulators. The nodal segments were inoculated in MS medium at the total strength of the salts and vitamins and supplied of NAA (0; 0.11; 0.22; 0.33 or 0.44 μ M), for a total of 5 different treatments, with 10 replicates each. After 45 days, the cultures were evaluated considering of the number and size of shoots and roots.

***Ex vitro* acclimatization:** Fifty rooted plantlets were removed from the test tubes and their roots were washed in tap water. Then the material was transplanted into polystyrene trays filled with substrate Plantmax HT®, covered with clear plastic. The plants were kept under shade and watered for 15 days until complete wetting of the substrate. Subsequently, the trays were transferred to a greenhouse with automated micro-sprinkler irrigation. After 45 days, the seedlings were transferred to beds filled with soil/sand/cattle manure, in the proportion 3:2:1 (v/v/v) and irrigated weekly.

Statistical Analyses: In all trials, the count data were normalized using $\sqrt{x + 0.5}$, and the measurement data employing $\log(x + 1)$. The experiments were performed in a completely randomized design (CRD) and the data analyzed by ANOVA. Means were compared with Tukey's test at 5% probability using the software SAEG (version 9.1).

RESULTS AND DISCUSSION

***In vitro* establishment:** The pharmacological potential (PASCUAL *et al.*, 2001) and the anthropogenic threats in their natural environment (GIULIETTI *et al.*, 2000; VITTA, 2002), makes it necessary to perform studies concerning alternative techniques of culture and propagation of *Lippia rotundifolia*. Tissue culture is a propagation procedure that enables rapid achievement of clones, regardless of adverse environmental influences. The success of micropropagation depends primarily on *in vitro* establishment of aseptic cultures free of phenolic oxidation. The PVPP showed high efficiency in the control of phenolic oxidation avoiding

tissue and culture medium browning. Less than 2% of explants showed browning caused by the action of polyphenol oxidases. The efficiency of PVPP in the control of phenolic oxidation is documented in the literature. The PVPP is an amorphous polymer without physiological activity (BASHA, 2011), used for *in vitro* establishment of genotypes that is prone to phenolic oxidation (BUSSELEZ *et al.*, 2012). The PVPP acts by complexing phenolic and alkaloids compounds released in response to tissue injury, reducing the toxic effects on the cells (FOLCH-CANO; OLEA-AZAR; SPEISKY, 2013; MALIK *et al.*, 2010; AMIN; JAISWAL, 1988).

The spraying of Benomyl in the plants maintained *ex situ* and also their inclusion in the culture medium coupled with the others aseptic procedures performed in the laboratory were quite effective, resulting in more than 90% cultures free of fungi and bacteria colonies. The methodology used in disinfection method of *L. rotundifolia* showed high efficacy, which was similar to that used successfully with other Verbenaceae such as *L. filifolia* (PEIXOTO *et al.*, 2006), *Verbena litoralis* (BRAGA *et al.*, 2012) and *Bouchea fluminensis* (RESENDE *et al.*, 2014).

***In vitro* multiplication:** In the multiplication phase, the explants showed highest propagation rates when inoculated on MS medium supplied with 0.33 μ M BAP, with average of 2.6 shoots/explant (Figure 1). The higher concentrations of this growth regulator resulted in reduction in the shoots multiplication, suggesting sensitivity of this species to cytokinins. The supply of NAA does not significantly stimulated the proliferation of shoots, beyond that auxin had not significantly interacted with BAP.

In spite of BAP is widely reported in the literature for Verbenaceae micropropagation (BRAGA *et al.*, 2012; PEIXOTO *et al.*, 2006; VIDYA *et al.*, 2012; RESENDE *et al.*, 2014), for *L. rotundifolia* a tendency of phytotoxicity was observed in response to increasing in BAP concentration, which resulted in reduction in the number and in the size of shoots, besides to the occurrence of hyperhydricity. Typically, BAP is more efficient than cytokinins derived from adenine on *in vitro* propagation phase (SHIVA PRAKASH; PENTAL; BHALLA-SARIN, 1994; SHUKLA *et al.*, 2012; THIYAGARAJAN; VENKATACHALAM, 2012). In *in vitro* cultures of *L. alba*, elongated shoots were observed when BAP was added to the culture medium (GUPTA; KHANUJA; SUSHIL., 2001).

Although cytokinins are usually supplied in culture media aimed at breaking apical dominance and stimulate the initial shoots formation (THIYAGARAJAN; VENKATACHALAM, 2012), its effectiveness in the process depends not just on the absolute concentration,

but also on its auxin interaction. In this phase of micropropagation, the highest multiplication rate was observed in response to 0.33 μM BAP + 0.01 μM NAA (Figure 1), however, without significant differences in relation to other auxin concentrations. Even so, the multiplication rate found for *L. rotundifolia* (3.1 shoots/explant) contrasts with the results for other Verbenaceae. For *Lippia filifolia*, about nine fold more shoots/explant were found (PEIXOTO *et al.*, 2006). For *Lippia juneliana*, the maximum shoot number obtained was also higher than in the present study (JULIANI *et al.*, 1999). These results demonstrate specificity in response to *Lippia* genotypes, which suggest the necessity for adjustment of protocols to improve the efficiency of *in vitro* propagation for each species.

Besides presenting a relatively reduced propagation rate, callus formation and hyperhydricity were also observed in cultures of *L. rotundifolia* in the treatments where BAP was supplied. Although the hyperhydricity and calli occurrence were generally observed *in vitro* only at higher cytokinins concentrations (SHUKLA *et al.*, 2012), *in vitro* cultures of *L. rotundifolia* showed that physiological abnormality in every one of BAP concentration (Figure 2a).

Among other adverse effects, the hyperhydricity may result in weakly rooting seedlings and in abnormal plantlets, with low stomatal efficiency and reduced amount of chlorophyll, which sometimes results in reduced seedling survival on acclimatization

(KEVERS *et al.*, 2004). To avoid the effects caused by hyperhydricity, BAP at 0.33 μM seems to be the most suitable concentration for *in vitro* multiplication of *L. rotundifolia*. According Shiva Prakash; Pental; Bhalla-Sarin (1994), the relative efficiency of cytokinins on shoot proliferation follows the order BAP > kinetin > zeatin > adenine sulfate. Recent studies demonstrating the existence of more powerful sources of this class of plant growth regulators such as N-(2-Chloro-4-pyridyl)-N-phenylurea (4-CPPU) and, specially, Thidiazuron (TDZ) (ROLLI *et al.*, 2012). The TDZ, besides acting as a synthetic cytokinin, promotes the overexpression of natural cytokinins, being successfully used for some species to stimulate shoots multiplication at concentrations below than 1 μM (VARSHNEY; ANIS, 2012). Although the *in vitro* propagation observed for *L. rotundifolia* have been relatively small, any other method of sexual or vegetative reproduction overcomes the micropropagation when comparing the multiplication rate/explant, which emphasizes the importance of tissue culture as an efficient method for large-scale propagation of this species. In addition, the *in vitro* germplasm conservation is possible regardless of biotic and edaphoclimatic conditions. Although not assessed in this study, the TDZ might be the only cytokinin which stimulates a higher shoots proliferation in *L. rotundifolia*. However, in the literature, there are several studies that found a direct correlation between TDZ and hyperhydricity (HUETTEMAN; PREECE, 1993; MALIK *et al.*, 2010).

Figure 1 - Averages of shoot number in cultures of *L. rotundifolia* in response to BAP and NAA, 40 days after *in vitro* inoculation. Means followed by the same lower-case letters (comparing BAP doses) and upper-case letters (comparing NAA doses) are not different according to Tukey's test at 5% of probability

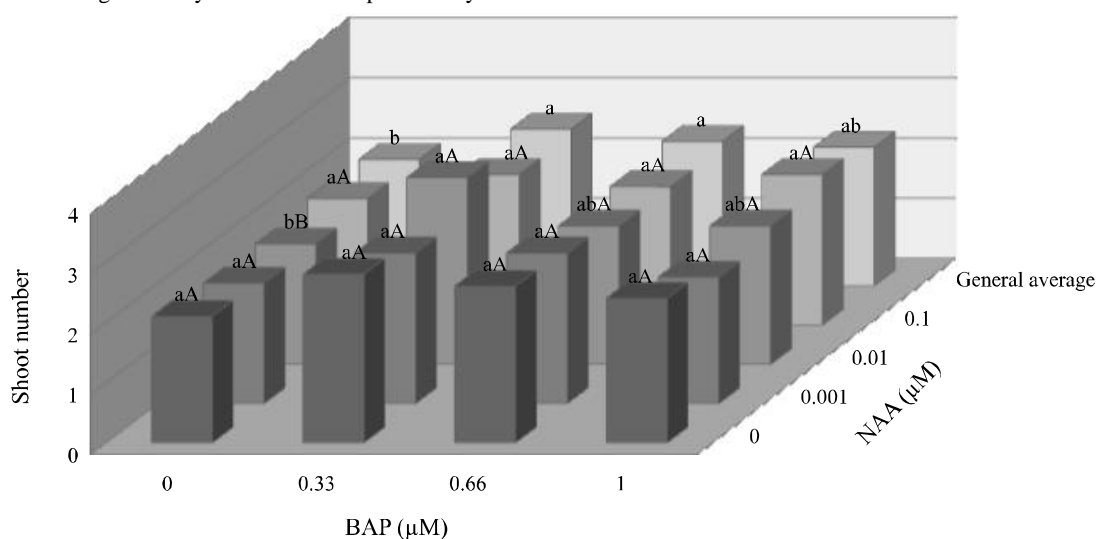
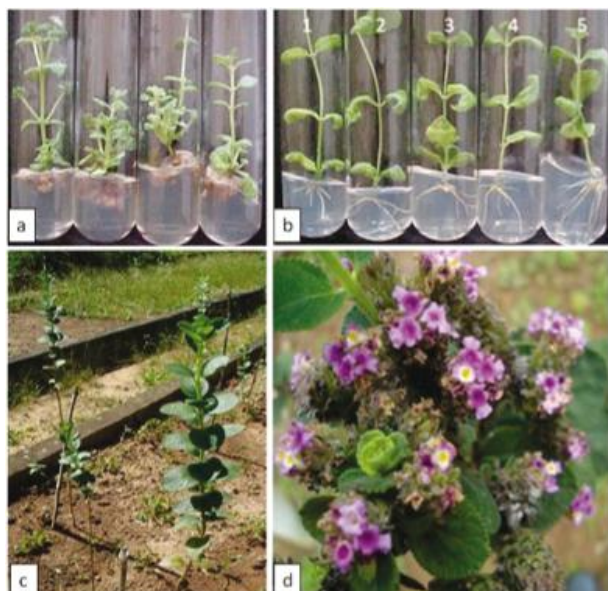


Figure 2 - *In vitro* propagation and *ex vitro* acclimatized *Lippia rotundifolia*. a. Multiplication phase showing shoots proliferation, hyperhydricity and basal callus on microcuttings in response to MS + BAP (0.33, 0.66 or 1 μ M). b. Rooting phase: 1 - MS (control); 2 - MS + NAA 0.1 μ M; 3 - MS + NAA 0.2 μ M; 4 - MS + NAA 0.3 μ M; 5 - MS + NAA 0.4 mM. c. Plants after completed *ex vitro* acclimatization. d. Detail of inflorescence in one year acclimatized plant of *Lippia rotundifolia*

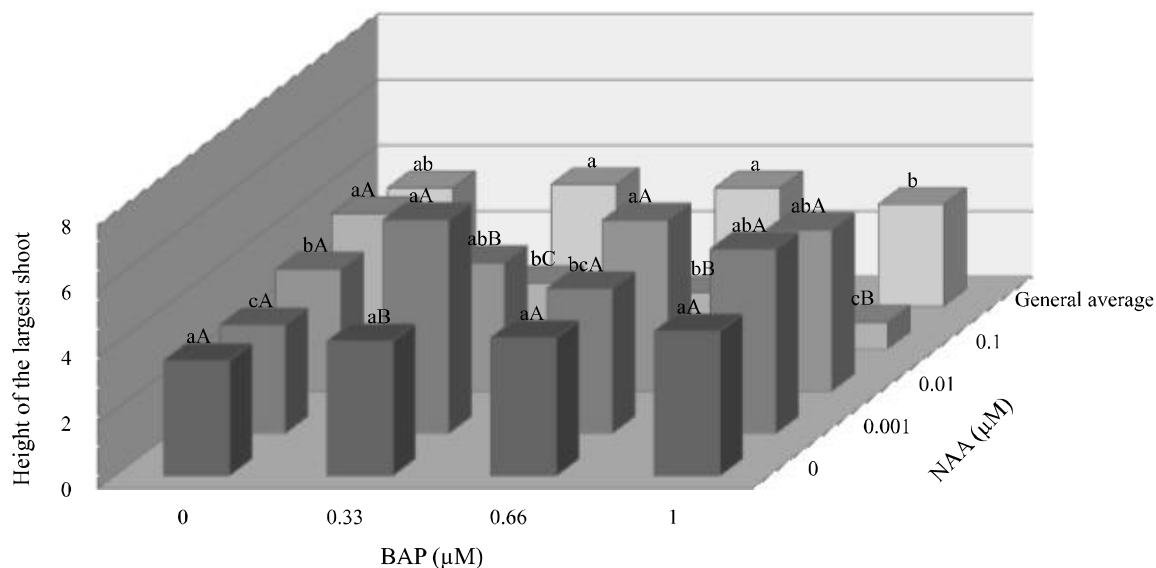


Significant interaction between BAP and NAA were observed in relation to the height of the largest shoot and also for the number of roots. In the multiplication phase, shoots more elongated were observed in response to 0.33 μ M BAP + 0.001 μ M NAA (Figure 3). In higher concentrations of both growth regulators, reduction in the size of shoots was observed, suggesting that, for *L. rotundifolia*, the optimal concentration range is below to the limits assessed. In this phase of micropropagation, *in vitro* root formation was observed only in the absence of BAP, with best results in response to 0.1 μ M NAA (Figure 4).

Besides to the rooting inhibiting, the addition of BAP stimulated the development of callus at the shoots base and also the occurrence of hyperhydricity (Figure 2a). This response is typical for the effects of cytokinins on *in vitro* adventitious root formation, which occurs in response to the imbalance of the endogenous ratio between natural auxins and cytokinins (SANTOS; ARRIGONI-BLANK; BLANK, 2012). These results show that *in vitro* rooting phase should not be suppressed from the *L. rotundifolia* micropropagation protocol.

***In vitro* rooting:** The supply of NAA increased the number of roots in the microcuttings. In the presence of 0.44 μ M NAA, nearly to eight adventitious roots have been produced, an increase of 70% compared to the control which, in turn, does not differ from the other NAA concentrations (Figure 5).

Figure 3 - Averages of height of the largest shoot in cultures of *L. rotundifolia* in response to BAP and NAA, 40 days after *in vitro* inoculation. Means followed by the same lower-case letters (comparing BAP doses) and upper-case letters (comparing NAA doses) are not different according to Tukey's test at 5% of probability



For *Lippia filifolia*, the *in vitro* rooting was also promoted by NAA in the same concentration range (PEIXOTO *et al.*, 2006). The rhizogenesis occurs through induction, initiation and expression phases. In every one of these developmental stages, auxins are critically and fundamentally important (SAUER; ROBERT; KLEINE-VEHN, 2013). However, the auxins should be supplied in suitable concentration, since excessive levels of these growth regulators inhibit the adventitious rooting, which occurs in response to ethylene production (GÜREL; WREN, 1995).

In the presence of 0.44 μM NAA, the roots showed a higher elongation (Figure 5) and improved morphological development, with more ramifications and greater thickness. In response to NAA, the cultures also showed no hyperhydricity symptoms nor the callus formation observed in multiplication phase in response to BAP (Figure 2b). Morphological and physiological abnormalities are reported during *in vitro* rooting, especially necrosis in shoots and callus development at the base of the explants (PARVEEN; SHAHZAD, 2011). However, in the present study, microcuttings of *L. rotundifolia* showed no problems, which was also no found to *Lippia filifolia* (PEIXOTO *et al.*, 2006), *Verbena litoralis* (BRAGA *et al.*, 2012),

and *Bouchea fluminensis* (RESENDE *et al.*, 2014), another Verbenaceae. The supply of NAA, at all the concentrations evaluated, did not result in increasing in the shoots number and also was not effective in stimulating the shoots elongation (Figure 5).

Figure 4 - Averages of root number in cultures of *L. rotundifolia* in response to BAP and NAA, 40 days after *in vitro* inoculation. Means followed by the same letters do not differ by Tukey's test at 5% probability

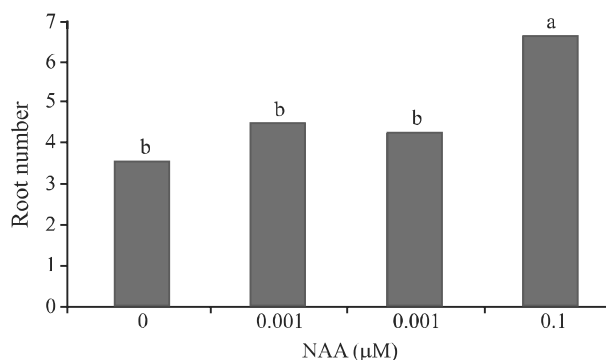
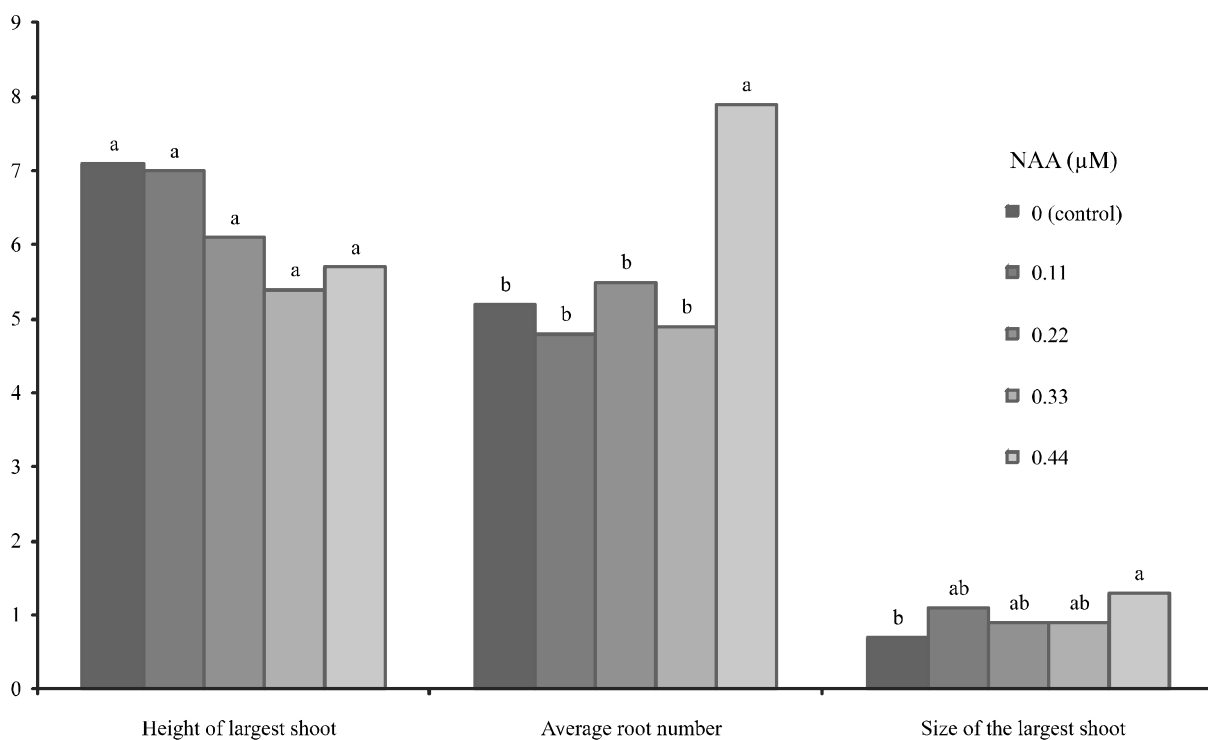


Figure 5 - Effects of NAA on *in vitro* rooting of microcuttings of *L. rotundifolia*, 40 days after *in vitro* inoculation. Means followed by the same letters do not differ by Tukey's test at 5% probability



Ex vitro acclimatization: Rooted seedlings from culture media containing 0.44 μ M NAA were acclimatized *ex vitro*. More than 80% of the plantlets removed from the test tubes survived to acclimatization. In this process, the plantlets were initially maintained in greenhouse under shade. After an initial period of two weeks, the plantlets were transferred to the greenhouse and kept under a micro-sprinkler, twice a day, for five minutes. After 45 days, the acclimatized plants were transferred to beds exposed to direct sunlight and watered weekly. In this condition, the plants showed faster vegetative growth reaching, after 12 months, about 1-1.5 m in height and exhibited a typical morphology of the species (Figure 2c), with leathery leaves strongly aromatic. The acclimatized plants from *in vitro* rooting phase showed normal vegetative development and underground rooting stem system like xylopodium, characteristic of the species, which allow the plants regrowth after the wildfires (SALIMENA-PIRES, 1991). The plants under field conditions also showed typical reproductive development. The flowering occurred in spring (Figure 2d), showing that *in vitro* protocols and culture conditions did not affect the life cycle of the species. Additionally, the literature showed that the *in vitro* culture may increase the content of terpenoids, revealing that the medicinal properties might be improved in response to *in vitro* culture (ARRIGONI-BLANK *et al.*, 2011; SILVA *et al.*, 2013).

In this work, the large-scale micropropagation of *L. rotundifolia* protocols were accomplished with absolute success considering the high plant survival observed in acclimatization phase. After the restoration of plants under field conditions were observed no change in the phenotypic characteristics among *in vitro* propagated individuals and the donor plants, showing that the protocols used are efficient for *in vitro* propagation and conservation of *L. rotundifolia*. Nowadays, several plants of *L. rotundifolia* derived from *in vitro* cultures are completely adapted to field conditions, serving as sources of new propagules for different studies, allowing the maintenance of *ex vitro* germplasm of this important species in *Campos Rupestres*.

CONCLUSIONS

1. The field and laboratories disinfection procedures and the supply of PVPP on culture media resulted in both reduced microbiological infection and phenol oxidation rates, with more than 90% of viable aseptic cultures;
2. The BAP at 0.33 μ M provides the highest multiplication rates;

3. The NAA at 0.44 μ M provides a rooting 70% higher than in the control;
4. The acclimatization process was very successful, with more than 80% of plantlets survived;
5. The acclimatized plants bloomed one year after the transference to field conditions, showing that the *in vitro* culture did not affect the vegetative and reproductive development, which endorses the potential of micropropagation to reduce the extinction risk of *L. rotundifolia*.

ACKNOWLEDGEMENTS

We thanks the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Programa de Apoio à Publicação da Universidade Federal de Juiz de Fora (PROPESQ-UFJF) for the financial support.

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