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In vitro establishment of pepper-rosmarin nodal segments

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ABSTRACT

Pepper-rosmarin (*Lippia sidoides* Cham.) is a native medicinal plant from the Brazilian biome Caatinga. Its high economical importance comes from the antimicrobial properties of thymol and carvacrol, both present in the pepper-rosmarin essential oil. Nodal segments of pepper-rosmarin were established *in vitro* to evaluate different growth regulators. We tested four concentrations of IBA (0.0; 0.01; 0.05; and 0.1 mg L^{-1}) combined with six concentrations of BAP (0.0; 0.1; 0.5; 1.0; 2.0; and 4.0 mg L^{-1}) in assay 1; five concentrations of NAA (0.0; 0.1; 0.25; 0.5; and 1.0 mg L^{-1}) in assay 2; two concentrations of NAA (0.0 and 0.01 mg L^{-1}) combined with five concentrations of kinetin (0.0; 0.25; 0.5; 1.0; and 2.0 mg L^{-1}) in assay 3; five concentrations of IAA (0.0; 0.5; 1.0; 1.5; and 2.0 mg L^{-1}) in assay 4; and five concentrations of GA_3 (0.0; 0.1; 0.5; 1.0; and 1.5 mg L^{-1}) in assay 5. The use of BAP, IBA, NAA and KIN did not influence the number of shoots. The addition of 0.1 mg L^{-1} of NAA at the culture medium resulted in larger shoot length per explant, while the concentration of 1.0 mg L^{-1} of kinetin promoted larger shoot length in general. GA_3 did not affect the elongation of pepper-rosmarin shoots when cultivated *in vitro*.

Keywords: *Lippia sidoides*, medicinal plant, micropropagation, growth regulator.

RESUMO

Estabelecimento de segmentos nodais de alecrim-pimenta *in vitro*

O alecrim-pimenta (*Lippia sidoides* Cham.) é uma planta medicinal nativa da Caatinga, também conhecida como alecrim-pimenta, alecrim-bravo e alecrim-do-Nordeste. Possui propriedades antimicrobianas, graças à presença de timol e carvacrol em seu óleo essencial, o que lhe confere grande importância econômica. Este trabalho teve por objetivo estabelecer segmentos nodais do alecrim-pimenta *in vitro* testando diferentes reguladores de crescimento. No ensaio 1 foram testadas quatro concentrações de AIB (0,0; 0,01; 0,05 e 0,1 mg L^{-1}) combinadas com seis concentrações de BAP (0,0; 0,1; 0,5; 1,0; 2,0 e 4,0 mg L^{-1}); no ensaio 2, cinco concentrações de NAA (0,0; 0,1; 0,25; 0,5 e 1,0 mg L^{-1}); no ensaio 3, duas concentrações de NAA (0,0 e 0,01 mg L^{-1}) combinadas com cinco concentrações de cinetina (0,0; 0,25; 0,5; 1,0 e 2,0 mg L^{-1}); no ensaio 4, cinco concentrações de AIA (0,0; 0,5; 1,0; 1,5; e 2,0 mg L^{-1}) e; no ensaio 5, cinco concentrações de AG_3 (0,0; 0,1; 0,5; 1,0 e 1,5 mg L^{-1}). A utilização de BAP, AIB, NAA e CIN não influenciou o número de brotações. A adição de 0,1 mg L^{-1} de NAA no meio-de-cultura proporcionou maior comprimento dos brotos por explante, enquanto a concentração de 1,0 mg L^{-1} de CIN promoveu maior comprimento dos brotos. O uso de AG_3 não apresentou efeitos sobre o alongamento das brotações de alecrim-pimenta cultivadas *in vitro*.

Palavras-chave: *Lippia sidoides*, planta medicinal, micropropagação, reguladores de crescimento.

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Pepper-rosmarin (*Lippia sidoites* Chan.; Verbenaceae), a native bush from the Brazilian Northeastern region, where the biome *Caatinga* predominates, shows its major occurrence in the territory between Bahia and Ceará States. It is popularly known as *alecrim-pimenta*, *alecrim-do-Nordeste*, *alecrim-bravo* (Inneco *et al.*, 2000), and *estrepa-cavalo* (Innecco *et al.*, 2000; Matos, 2002). Pepper-rosmarin produces an essential oil abundant in thymol and carvacrol, which provides a very strong antimicrobial and antiseptic activity (Macambira *et al.*, 1988). Due to the great economical importance of such species, it may suffer a strong anthropic action and demographic reduction in its natural environment. The essential oil is used in the production of good quality shampoos, tooth pastes, and mouth washers, being also effective in the control of bacterial dental plaque (Nunes, 1999). Seeds from this species were not found. Its propagation is asexual, through the process of stem cutting, using its thinnest branches (Lorenzi & Matos, 2002) or herbaceous cuttings with leaves (Mendonça, 1997). Hence, the pepper-rosmarin micropopagation turns up as a technique of great application potential.

In micropropagation, culture media are supplemented with growth regulators, whose role is to supply the possible deficiencies of the hormonal endogenous levels in explants (Castro *et al.*, 2002). For *Lippia integrifolia* (Gris.) Hier, the culture medium Murashige and Skoog (MS) complete or with a reduction to half of the salt, supplemented with 1 mg L^{-1} of indole butyric acid (IBA) provided a good multiplication rate (Passera & Ambrosetti, 1999). For *Lippia micromera* Shau. in DC. var. *helleri* (Britt.), it was observed that the best multiplication results occurred using the MS medium supplemented with 0,1 mg L^{-1} of naphthalene acetic acid (NAA) + 2 mg of L^{-1} of 6-benzylaminopurine (BAP) (Capote *et al.*, 1999). In *Lippia alba*, cultivar Kavach, there were excellent results for apical buds placed in MS medium containing 2,0 mg L^{-1} BAP (Gupta *et al.*, 2001).

The microprogation has been used in the multiplication of several species with medicinal properties. Passera & Ambrosetti (1999) have developed a fast method for *Lippia integrifolia* propagation by the use of nodal segments in full or half MS medium (Murashige & Skoog, 1962). In *Lippia junelliana*, (Juliani Junior *et al.*, 1999), after studying different propagation methods, the best results were obtained with apical buds and nodal segments in MS medium supplemented with 4.4 µM of 6-benzylaminopurine (BAP) or 0.04 ìM of indolebutyric acid (IBA) + 4.4 µM of BAP. In *Lippia alba* cultivar Kavach, plant regeneration was obtained by using apical buds in MS medium, containing 2.0 mg L^{-1} of BAP (Gupta *et al*., 2001). For *Lippia micromera* var. *helleri*, it was noticed that the best multiplication results occurred when using the MS medium, adding 0.1 mg L^{-1} of naphthalene acetic acid (NAA) + 2,0 mg L^{-1} of BAP (Capote *et al.*, 1999). Gibberellins, in the form of gibberellic acid (GA_3), when used in *in vitro* multiplication, induce shoot elongation as their main effect, such as observed in *Rollinia mucosa* (Figueiredo *et al.*, 2001) and macela (*Egletes viscose* (L.) Less) (Diniz *et al*., 2003).

The aim of this work was to evaluate the use of different growth regulators in the *in vitro* multiplication of pepper-rosmarin.

MATERIAL AND METHODS

The assays were held at the Tissue Culture and Plant Breeding Laboratory, at the Department of Agricultural Engineering of the Federal University of Sergipe (UFS). Two-bud nodal segments were used as primary explants. Mother plants grew in greenhouse and were sprayed with 4 g L^{-1} of benomyl, two days before inoculation. The nodal segments, once collected, were washed in current water for 30 minutes, emerged in ethylic alcohol 70% for 30 seconds, and then in a sodium hypochlorite solution 0,8% for 16 minutes. After that, they were washed three times in distilled and autoclaved water in a laminar flow camera (Costa, 2006). The explants were inoculated in 250 ml flasks with 25 ml in MS medium (Murashige & Shoog, 1962), containing 7 g L^{-1} of agar, and with pH adjusted to 5.7 ± 0,1 before autoclaving (121 ± 1° C and 1,05 atm for 15 minutes). For the multiplication of pepper-rosmarin the following assays were held:

Assay 1: the influence of BAP and IBA - The experimental design was completely randomized in a factorial scheme 4 x 6, where four IBA concentrations (0.0; 0.01; 0.05; and 0.1 mg L^{-1}) were combined with six BAP concentrations (0.0; 0.1; 0.5; 1.0; 2.0; and 4.0 mg L^{-1}). The assay was carried out with five replications, each replication with four two-explant flasks.

Assay 2: the influence of NAA - The experimental design was completely randomized with five treatments (0.0; 0.1; 0.25; 0.5, and 1.0 mg L^{-1} of NAA) and five replications, each replication with four two-explant flasks. We added 3 g L^{-1} of active charcoal to the MS medium (Costa, 2006).

Assay 3: the influence of kinetin and NAA - The experimental design was completely randomized, with treatments in a factorial scheme 2 x 5, corresponding to two NAA concentrations (0.0 and 0.01 mg L^{-1}) combined with five kinetin concentrations (0.0; 0.25; 0.5; 1.0, and 2.0 mg L^{-1}). The experiment was carried out with four replications, each replication with four two-explant flasks. We added 3 g L^{-1} of active charcoal and 200 mg L^{-1} of cefotaxime-sodium to the MS medium (Costa, 2006).

Assay 4: the influence of IAA - The experimental design was completely randomized with five treatments (0.0; 0.5; 1.0; 1.5; and 2.0 mg L^{-1} of IAA) and five replications of four two-explant flasks each. We added 3 g L^{-1} of active charcoal and 200 mg L^{-1} of cefotaxine-sodium to the MS medium (Costa, 2006).

Assay 5: the influence of GA_3 - Due to the lack of shoot elongation in the previous assays, five concentrations of GA_3 were tested (0.0; 0.1; 0.5; 1.0; and 1.5 mg L^{-1}) in a completely randomized experiment, with five replications, each with four two-explant bottles. We added 3 g L^{-1} of active charcoal to the MS medium. The GA_3 was cold sterilized, in a continuous laminar flow camera (Millipore, 2,2 µm) and added to the medium during the cooling process (40 to 50° C).

In all assays, cultures were kept in BOD in absence of light, for seven days after inoculation, and then transferred to a growing room with temperature of 25 ± 2° C, photon irradiance of 30 µmol m^{-2} s^{-1}, and photoperiod of 16 hours. In 30 days of inoculation, the following characteristics were evaluated: shoot number and length (cm), number of leaves, and dry mass of the aboveground part (DMAP). Number of leaves and shoots and DMAP were transformed into square root of (x+ 0,5) in assays 1 and 2. The same transformation was applied to shoot length in assay 1. Data were submitted to the variance analysis. When the F test was significant, means were compared by the Tukey test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Assay 1: the influence of BAP and IBA - There was no significant interaction between BAP and IBA in the micropropagation of pepper-rosmarin for none of the analyzed characteristics. Concerning isolated effects of both growth regulators, concentration 2 mg L^{-1} of BAP induced the lowest shoot number per explant, although not differing statistically from concentrations 0.1; 1.0; and 4.0 mg L^{-1} (Table 1). BAP has been frequently applied to the *in vitro* multiplication for several species, and there has been variation in the concentrations for the shoot induction, as in *Tagetes* sp. (0.5 mg L^{-1} of BAP) (Turchetto *et al*., 2005), chapéu-de-couro (*Echinodorus cf. scaber* Rataj.) (1.0 mg L^{-1} of BAP) (Pereira *et al.*, 2000a), yagrumo-macho (*Didymopanax morototoni*) (1.0 mg L^{-1} of BAP) (Mantovani *et al.*, 1999), and the Korean-grape (*Vitis thumbergii* Sieb. et Zucc.) (0.5 mg L^{-1} of BAP) (Lu, 2005).

Regarding shoot length by explant, the treatments did not differ statistically among themselves. The absence of increment in the shoot height due to the use of BAP was reported before in the

in vitro multiplication of the grapevine rootstock Paulsen 1103, causing an inhibitory effect in the shoot growth (Lucas *et al*, 2006). Considering the number of leaves, the use of 4 mg L^{-1} BAP produced the highest value, not differing statistically from the treatments with 0 and 1.0 mg L^{-1} (Table 1). Concentrations of 0 to 0.5 mg L^{-1} provided the highest values for the aboveground dry mass, although not differing statistically from 0.1 and 1.0 mg L^{-1}, while these ones did not differ either from concentrations 2.0 and 4.0 mg L^{-1} (Table 1).

Concerning the presence of IBA, there were no significant differences for none of the evaluated characteristics. Divergent results were obtained in the micropropagation of *Lippia juneliana*, in which 1.0 mg L^{-1} of BAP combined with 0.008 mg L^{-1} of IBA were efficient in shoot induction (Juliani Júnior *et al*., 1999).

Assay 2: the influence of NAA - The studied NAA concentrations did not influence significantly nor shoot and leaf number, neither aboveground dry mass (Table 1). In relation to shoot length, a significant difference among treatments was noticed. The concentration 0.1 mg L^{-1} induced the longest shoots, although not differing from the check treatment and concentrations 0.25 and 0.5 mg L^{-1}, but only from the concentration 1.0 mg L^{-1} (Table 1). These results show that higher concentrations of such auxin may inhibit shoot length.

Assay 3: the influence of kinetin and NAA - There was nor significant interaction between kinetin and NAA, neither kinetin significant effects over shoot and leaf number (Table 1). Different results were obtained in *Cissus sicyoides* establishment and *in vitro* multiplication, in which the best rates for shoot numbers and length were reached in the presence of 0.99 mg L^{-1} of kinetin and 0.58 mg L^{-1} of NAA (Abreu *et al.*, 2003). Positive effects were also observed in the multiplication of *Salix humboldtiana* (Pereira *et al*., 2000b) and *Ficus carica* L. (Fraguas *et al.*, 2004).

Table 1. Shoot number and length, leaf number, and aboveground dry mass of *in vitro* explants of pepper-rosmarin as function of BAP, NAA, and kinetin concentration in MS culture medium (número e comprimento de brotos, número de folhas e massa seca da parte aérea de explantes *in vitro* de alecrim-pimenta, em função de concentrações de BAP, NAA e cinetina no meio de cultura MS). São Cristóvão, UFS, 2006.

BAP (mg L^{-1})	Shoot number[1]	Shoot length (cm)[2]	Leaf number[1]	Dry mass of the aerial part (g)[1]
0	2.4 a	0.34 a	9.4 ab	0.008 a
0.1	2.0 ab	0.35 a	9.0 ab	0.007 ab
0.5	2.3 a	0.34 a	9.1 ab	0.007 a
1	1.9 ab	0.26 a	8.8 ab	0.005 ab
2	1.6 b	0.27 a	6.8 b	0.004 b
4	2.2 ab	0.30 a	10.8 a	0.005 b
CV (%)	14.48	7.32	21.60	0.31
NAA (mg L^{-1})				
0.00	2.3 a	0.62 ab	24.0 a	0.021 a
0.10	2.9 a	0.83 a	23.7 a	0.037 a
0.25	2.6 a	0.70 ab	18.3 a	0.034 a
0.50	2.7 a	0.43 ab	18.0 a	0.027 a
1.00	2.2 a	0.34 b	15.0 a	0.015 a
CV (%)	18.82	9.98	23.59	1.77
kinetin (mg L^{-1})				
0.00	3.3 a	0.88 ab	18.5 a	0.024 a
0.25	3.0 a	0.72 b	16.7 a	0.018 ab
0.50	2.5 a	0.77 b	14.0 a	0.016 b
1.00	2.9 a	1.17 a	18.0 a	0.022 a
2.00	3.3 a	0.81 b	18.7 a	0.023 a
CV (%)	21.51	26.32	23.28	17.77

Means followed by the same letter in the columns , did not differ from each other, Tukey test, $p \leq 0,05$ (médias seguidas de mesma letra nas colunas não diferem estatisticamente entre si pelo teste de Tukey, $p \leq 0,5$). [1/] Data transformed to arc sen of the square root of (x+0.5), for assays 1 (BAP x IBA) and 2 (NAA) (dados transformados para arco seno de raiz de (x + 0,5), para o ensaio 1 (BAPxAIB) e 2 (ANA)); [2]/Data transformed to arc sen of the square root of (x+0.5) for assay (dados transformados para arco seno de raiz de (x + 0,5) para o ensaio 1).

Shoot length was significantly altered in response to differences in the kinetin concentration. The concentration 1.0 mg L^{-1} resulted in the longest shoots, although not differing from the check treatment (Table 1). Concerning aboveground dry mass, it was noticed that the absence of kinetin improved mass accumulation, although not differing from the concentrations 0.25, 1.0, and 2.0 mg L^{-1}.

Assay 4: the influence of IAA - Shoot number and length and number of leaves did not present significant differences as function of the use of IAA. This suggests that the auxin endogenous level in the explants was sufficient for their regeneration.

Assay 5: the influence of GA_3 - GA_3 did not induce the expected shoot elongation in pepper-rosmarin. In fact, there were no significant differences among treatments in any of studied characteristics. In this study, a high percentage of vitrified plants was observed (data not showed) as the concentration of GA_3 was increased. Similar results were obtained in *Tournifurtia paniculata* nodal segments (Bertolucci *et al.*, 2000) and in macela (Diniz *et al.*, 2003), in which the presence of GA_3 induced hyperhydration and alteration in leaf shape.

We concluded that the use of BAP, IBA, NAA, and kinetin did not influence shoot number. The addition of 0.1 mg L^{-1} of NAA or 1.0 mg L^{-1} of kinetin to the culture medium improved shoot length. GA_3 did not have any effect on pepper-rosmarin shoot length under *in vitro* culture conditions.

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