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Full Length Research Paper

A rapid *in vitro* protocol for propagation of *Piper aduncum* and *Piper hispidinervum*, two species from Amazon region with multipurpose uses

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The morphogenic potential of nodal explants of *Piper aduncum* and *Piper hispidinervum* (Piperaceae) was investigated and a protocol for rapid micropropagation is described. An experiment based on the saline formulation of Murashige and Skoog (MS) and Wood Plant medium (WPM) combined with different N6-benzylaminopurine (BAP) and 1-naphthalene acetic acid (NAA) concentrations was evaluated. After determining the optimal concentration of growth regulators, the multiplication rates for the species, which were distributed in five subcultures, was assessed, and the number of plantlets produced in this period was recorded. After assessing the plants from all five subcultures, plantlets with well-developed root and shoot systems were transferred to pots containing substrate for acclimatization. The culture of nodal segments of *P. aduncum* and *P. hispidinervum* on hormone-free medium was shown to be a suitable method for micropropagation due to the high multiplication rate and good plant development. The use of BAP or BAP + NAA resulted to formation of vitrified multiple shoots and callus formation at the base of the microcuttings. Even at concentrations lower than 1 mg L^{-1}, the use of BAP resulted in vitrified multiple shoot and callus formation, without significantly improving the multiplication rates. For both species, the first subculture resulted in the greatest number of axillary buds, and mainly for *P. hispidinervum*, the MS medium was the most appropriate for *in vitro* multiplication of microcuttings. The species showed 100% root formation, and acclimatization of plants from the fifth subculture in a greenhouse resulted in 100% survival.

Key words: Spiked pepper, long pepper, micropropagation, dillapiol, safrole, morphogenesis, organogenesis.

INTRODUCTION

Piper aduncum commonly known as spiked pepper, and *Piper hispidinervum*, known as long pepper, are plant species found in the Amazon Region that have been generating economic interest due to their phytochemical properties.

Belonging to the Piperaceae family, these shrub-like species are a natural resource of great commercial value, due to their respective secondary compounds, dillapiol and safrole, which are the major compounds in the essential oils of these plants (Wadt et al., 2004; Estrela et

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Abbreviations: **BAP**, 6-Benzylaminopurine; **NAA**, α-naphthalene acetic acid; **IBA**, indole-3- butyric acid; **MS**, Murashige and Skoog's (1962) medium.

al., 2006).

The essential oil extracted from the branches and leaves of the spiked pepper consists of phenyl ether dillapiol, which has fungicidal, molluscicidal, larvicidal and insecticidal characteristics (Orjala et al., 1994; Rafael et al., 2008). Besides its use for pest control, the spiked pepper is also used by Amazon local populations, due its antiulcer, anti-haemorrhagic and anti-diarrhea characteristics, and to treat wounds (Fenner et al., 2006; Rodrigues and Carvalho, 2001). Long pepper, in turn, is noted for their constituent phenyl ether safrole (Rocha and Ming, 1999).

The main by-products obtained from safrole are heliotropin, which is widely used as a fragrance fixative, and piperonyl butoxide (PBO), which is one of the substances in the composition of piretrum-based biodegradable insecticides (Braga et al., 2005).

Despite their great commercial appeal, the spiked and long pepper species are still in the process of domestication, and are virtually unknown scientifically (Rocha and Ming, 1999). Researches on more efficient methods of propagation and cultivation that enable advances in plant breeding, or favor the rapid propagation of a large number of uniform plants of these species, are rare (Valle et al., 2006). Given that these species are still in the process of domestication, there is an urgent need for research to establish a mass production system that will enable their use in commercial crops, or for the studies of plant germplasm conservation, and genetic manipulations.

Micropropagation is a widely used technique, especially for the production of plantlets with economic potential. *In vitro* plant regeneration from the culture of axillary buds is important as it can generate clones, that is, plants that retain the characteristics of the mother plant (Ahmad et al., 2010).

Through micropropagation, it is possible to secure genetic gains in clonal populations, obtaining a large number of healthy and high quality plants in a small space within a short period of time, regardless of climatic factors and season. To obtain a large number of plantlets via micropropagation, the most important factors are the use of an appropriate culture medium, and the adjustment of plant growth regulators to optimal concentrations within the culture medium, as cytokinins for the induction and proliferation of *in vitro* axillary buds (Wu et al., 2009).

We therefore investigated the most suitable micropropagation protocol for the rapid production of *P. aduncum* and *P. hispidinervum* plantlets. Here, we report a protocol which can be used for the clonal multiplication of this plant species using nodal segments as explants and followed by the optimized conditions for *in vitro* rooting and further transfer into the greenhouse. To our knowledge, there are no reports on the micropropagation of these plant species.

MATERIALS AND METHODS

Seeds of *P. aduncum* and *P. hispidinervum*, obtained from the plants growing in the field gene bank of Embrapa Acre, Rio Branco, Acre, Brazil (9°58'29'' S, 67°48'36'' W), were used in this study. The seeds were surface-sterilized with 70% (v/v) ethanol for 2 min followed by treatment with 1.0 to 1.25% sodium hypochlorite for 15 min and rinsed three times with sterile distilled water. The surface-sterilized seeds were then carefully placed on MS (Murashige and Skoog, 1962) basal medium. The pH of medium was adjusted to 5.6 to 5.8 prior to autoclaving at 121°C for 15 min. The cultures for all the experiments were incubated in a culture room at 25 ± 2°C under a 16 h photoperiod at approximately 38 µmol s^{-1} m^{-2} photon flux.

After about 45 days, microcuttings of spiked pepper (*P. aduncum*) and long pepper (*P. hispidinervum*) with one to two axillary buds and approximately 0.8 cm in height were used as the source of plant material for the experiments. In all the experiments, 30 mL of culture medium were added to 250 mL flasks. 20 gL^{-1} sucrose was added to the culture media, and the pH was adjusted to 5.8 ± 0.1 before adding the Agar gelling agent (6 gL^{-1}). The culture media were autoclaved for 15 min at 121°C and 1.5 kgf cm^{-2} for sterilization.

First, an experiment based on the saline formulation used was carried out: MS (Murashige and Skoog, 1962) and WPM (Lloyd and McCown, 1980) combined with different BAP concentrations (0. 1. 2 and 3 mg L^{-1}) and NAA (0 and 0.05 mg L^{-1}).

Based on the results obtained in the previous experiment, a new test was performed to adjust the BAP concentration to lower concentrations than in the previous study: 0; 0.25; 0.50; 0.75 and 1.0 mg L^{-1}. At these BAP concentrations, only the MS saline formulation was used, according to the first experiment. In both experiments to assess the use of BAP, the assessment was performed after 30 days of cultivation. The variables recorded were: height of the regenerated shoots (measured from the base of the plant to the last bud, in cm), number of axillary buds, percentage of explants with multiple shoots, and percentage of explants with callus formation at the base.

After determining the optimal concentration of growth regulators, the multiplication rates for the species, which were distributed in five subcultures, was assessed, and the number of plantlets produced in this period was recorded. The spiked and long pepper microcuttings were then cultivated in MS and WPM medium, divided into five subcultures of 30-day intervals. At the end of the 30-day period, the microcuttings were assessed for shoot height (cm), number of buds, and percentage of explants with root formation, then transferred to new culture media as specified above, maintaining the same treatments.

After assessing the plants from all five subcultures, plantlets with well-developed root and shoot systems were gently removed from the flasks, washed in running tap water and transferred to pots containing Plantmax® substrate for acclimatization. The average temperature in the greenhouse was kept at 23 to 28°C, and the plantlets were maintained in a 50% shade and irrigated every eight hours by automatically-activated spray irrigation. Percentage of plant survival was recorded 4-weeks after transplant to *ex vitro* conditions.

The experiments were arranged in a completely randomized design with five replicates and six explants per replicate. Effects of treatments were tested by analyses of variance (one-way ANOVA) at the 5% level of significance using the statistical analysis system for microcomputers (SANEST) (Zonta and Machado, 1984); means were compared with Tukey's multiple range test at 95% significance. Prior to analysis, percentage data were arcsinsquare root-transformed, and data on number of buds were transformed according to the square root of (x + 0.5). Data on plant height were

not transformed.

RESULTS AND DISCUSSION

In general, the best results for shoot height and number of buds for the spiked pepper (*P. aduncum*) were observed in treatments T-1 (control) and T-5 (0 mg L^{-1} BAP + 0.05 mg L^{-1} NAA). On average, the culture media did not affect significantly these results. However, when the influence of culture media on shoot height was assessed, significant differences were observed in treatment T-1 only, where the WPM medium showed significantly superior results than the MS medium. With regard to the number of buds, there were no significant differences with regards to the culture media tested (Table 1).

When the percentage of explants with multiple shoots and callus was assessed, it was observed that the addition of BAP or BAP + NAA to the culture medium caused the formation of multiple shoots and calli in the explants, regardless of the saline formulation used (Figure 1-B). Spiked pepper microcuttings cultivated in MS culture medium with 1.0 mg L^{-1} BAP (T-2) resulted in 81.6% of explants with multiple shoot formation. However, in the WPM culture medium, the treatment which had the highest percentage of explants with multiple shoots was T-4, which consisted of 3.0 mg L^{-1} BAP, resulting in 73.1% of explants with multiple shoots formation (Table 1). For the callus formation variable, it was found that 67.0% of the microcuttings cultivated in MS culture medium consisted of 1.0 mg L^{-1} BAP (T-2) and showed callus formation, while in the WPM culture medium, the treatment containing 3.0 mg L^{-1} BAP (T-4) resulted in the highest percentage of callus formation in the cultivated explants (80.0%) (Table 1).

For the long pepper, the different treatments in MS culture medium presented no statistical difference for shoot height. But in general, the highest values for shoot height in MS and WPM culture medium were observed when the microcuttings were cultivated in treatments T-1 and T-5; both devoid of BAP (Table 2).

In relation to the height, the greatest number of formed axillary buds was observed in the treatments devoid of BAP (T-1 and T-5), regardless of the saline formulation used (Figure 1-A). In MS medium, treatments T-1 and T-5 generated 3.4 and 3.0 buds per explant, respectively, and in WPM medium, the values were 3.7 and 3.0 axillary buds per explants, respectively (Table 2). With respect to the percentage of explants with multiple shoot and/or callus formation, besides the treatment, the culture medium was also significant. The highest percentages of explants with multiple shoot and/or callus formation were also observed in the treatments con-taining BAP or BAP + NAA, regardless of the concen-tration tested (Table 2). For the long pepper, treatment T-5 (0 mg L^{-1} BAP + 0.05 mg L^{-1} NAA) resulted in a high percentage of explants with multiple shoots and callus formation when cultivated in WPM medium. Similar result was not observed for the spiked peppers. Regarding the saline formulations tested, it was found that microcuttings of long pepper, when cultivated in MS medium, showed a lower percentage of multiple shoot and callus formation than those cultivated in WPM medium (Table 2).

The interaction between cytokinin and auxin at lower concentrations may favor longitudinal growth, but this factor was not assessed in this study. Pescador et al. (2000), who also investigated species belonging to the Piperaceae family, found similar results to those found in this work when they cultivated nodes in a culture medium without growth regulator or with NAA and BAP. The authors observed that plants cultivated in BAP- or NAA-free culture medium had the highest values for shoot length, while the microcuttings cultivated in BAP or NAA medium showed multiple shoot formation at the base of the stem segment.

Due to the problems generated by the large number of multiple shoots, which in the vast majority were vitrified with calli, a second experiment was carried out with BAP at lower concentrations. However, this second study found that the use of BAP concentrations caused the formation of microcuttings containing both vitreous multiple shoots and calli (Table 3). For the spiked pepper, the use of BAP was statistically significant for the studied variables. For height and number of buds, the BAP-free treatment resulted in higher values, differing from the treatments with BAP. The average height and number of buds for the microcuttings cultivated in a BAP-free medium were 1.6 cm and 2.7 buds per microcutting, respectively (Table 3). For the treatments containing 0.25; 0.5; 0.75 and 1.0 mg L^{-1} BAP, height and number of formed buds showed no difference, and had lower values than the BAP-free treatment (control).

The use of BAP resulted in an increase in the percentage of explants with multiple shoots and calli. There was no multiple shoot and callus formation when the explants were cultivated in a BAP-free culture medium. On the other hand, multiple shoot and callus formation was observed in all other treatments containing BAP (Table 3).

For the long pepper, BAP concentrations showed no difference in number of buds. For all other variables, the use of BAP showed statistically different results for the different concentrations tested. Maximum values for shoot height were obtained in the BAP-free treatment, with shoots measuring, on average, 1.3 cm. Similar to the results for spiked pepper, the treatments containing BAP led to multiple shoot and callus formation, but similar results were not observed in the BAP-free treatment (Table 3).

Studies on micropropagation with species belonging to the Piperaceae family have reported that the use of plant

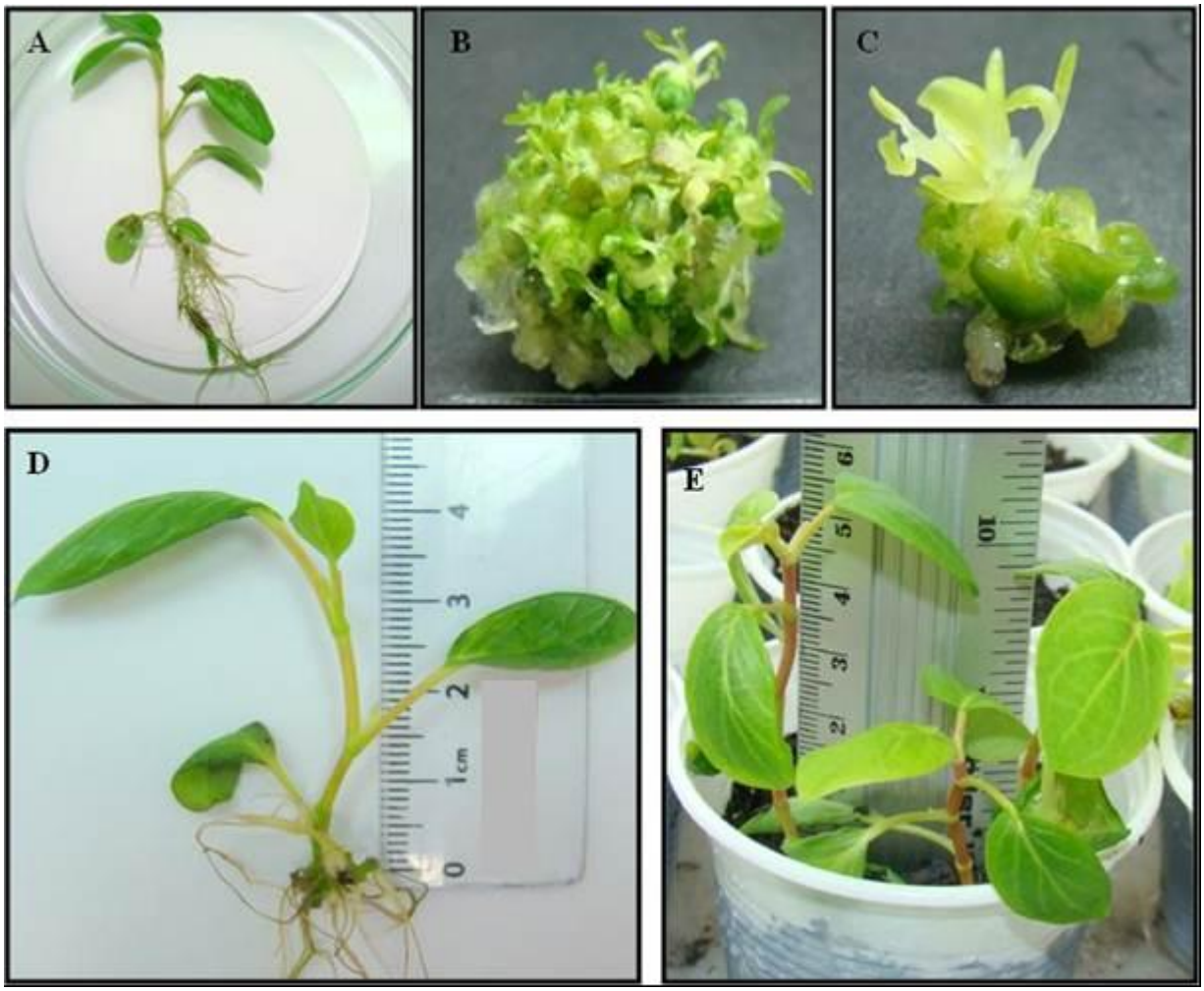

Figure 1. Long pepper and spiked pepper shoots obtained via micropropagation. (A) Microcuttings cultivated in BAP-free medium. (B) Vitreous appearance of an explant cultivated in MS medium containing 1.0 mg L^{-1} BAP. (C) Vitreous appearance of microcutting with abnormal shoots cultivated in MS medium containing BAP. (D) Microcutting cultivated in MS medium after fifth subculture, with root formation. (E) Acclimatized plants with normal growth in the greenhouse, 30 days after planting.

regulators promote the development of multiple shoots (Ahmad et al., 2010; Anand and Rao, 2000). Soniya and Das (2002) reported that the use of BAP in association with kinetin promoted the development of multiple shoots in the apical buds of Piper longum. In this study, the use of BAP also promoted the highest percentage of multiple shoots formed in microcuttings of spiked pepper and long pepper, but their use has not been feasible due to vitrification of the multiple shoots (Figure 1B and C). Vitrification or hyperhydricity is a morphological and physiological disorder found in micropropagated plants. Morphologically, it is characterized by plants presenting a vitreous consistency and stiff, brittle leaves. Physiologically, plants with this aspect also show decreased chlorophyll content and proteins associated to photosynthesis, increased intracellular water content and decreased ion composition found in plant cells (Hazarica, 2006).

With regards to the species that do not require plant growth regulators for *in vitro* development, Blank et al. (2008) tested different BAP concentrations to determine the optimal concentration of this cytokinin for the generation of axillary buds in *Lippia sidoides*. The authors claim that the control treatment resulted in the formation of a higher number of shoots and, thus, the addition of BAP was unnecessary for the *in vitro* propagation of this species. For the micropropagation of aloe (*Aloe vera*), Debiasi et al. (2007) studied different BAP concentrations, to determine a protocol that would allow the formation of a higher number of shoots. According to the authors, the BAP-free treatment promoted the highest multiplication rate, with formation of 2.5 shoots per

Table 1. Effect of MS and WPM media and different BAP and NAA combinations on shoot length (cm), multiplication rate, and percentage of explants with multiple shoots and callus formation at the base during the micropropagation of *Piper aduncum.*

BAP + NAA	Shoot length (cm)			Multiplication rate			Explants with multiple shoots (%)			Explants with callus formation (%)		
(mg L^{-1})	MS	WPM	Average	MS	WPM	Average	MS	WPM	Average	MS	WPM	Average
Control	1.7^{aB}	4.0^{aA}	2.8^{a}	3.2	3.7	3.4^{a}	0.0	0.0	0.0^{d}	0.0	0.0	0.0^{b}
1.0 + 0.0	0.5^{bA}	0.5^{cA}	0.5^{c}	1.7	1.1	1.4^{b}	81.6	44.5	63.0^{a}	67.0	28.5	47.7^{a}
2.0 + 0.0	0.5^{bA}	0.5^{cA}	0.5^{c}	1.5	1.5	1.5^{b}	55.5	50.0	52.7^{ab}	56.5	34.2	45.3^{a}
3.0 + 0.0	0.5^{bA}	0.5^{cA}	0.5^{c}	1.0	0.4	0.7^{b}	14.6	73.1	44.0^{abc}	28.5	80.0	54.2^{a}
0.0 + 0.05	1.0^{abA}	1.2^{bA}	1.1^{b}	2.4	1.4	1.9^{ab}	0.0	0.0	0.0^{d}	0.0	0.0	0.0^{b}
1.0 + 0.05	0.5^{bA}	0.5^{cA}	0.5^{c}	1.5	1.0	1.2^{b}	4.8	20.0	12.4^{bcd}	23.7	8.0	16.0^{ab}
2.0 + 0.05	0.5^{bA}	0.5^{cA}	0.5^{c}	1.3	0.4	0.8^{b}	15.0	27.0	21.0^{abcd}	61.5	27.0	44.2^{a}
3.0 + 0.05	0.5^{bA}	0.5^{cA}	0.5^{c}	0.9	1.0	0.9^{b}	11.0	1.3	2.0^{cd}	56.0	11.0	32.0^{ab}
Average	0.7^{A}	1.0^{A}		1.8^{A}	1.3^{A}		23.0^{A}	27.0^{A}		36.6^{A}	23.6^{A}	

Mean values followed by the same lowercase letter within columns and uppercase letter within line are not significantly different according to Tukey's test ($P < 0.05$).

Table 2. Effect of MS and WPM media and different BAP and NAA combinations on shoot length (cm), multiplication rate, and percentage of explants with multiple shoots and callus formation at the base during the micropropagation of *Piper hispidinervum.*

BAP + NAA	Shoot length (cm)			Multiplication rate			Explants with multiple shoots (%)			Explants with callus formation (%)		
(mg L^{-1})	MS	WPM	Average	MS	WPM	Average	MS	WPM	Average	MS	WPM	Average
Control	1.4^{a}	1.0^{a}	1.2^{a}	3.4	3.7	3.5^{a}	0.0^{bA}	0.0^{bA}	0.0^{b}	0.0^{bA}	0.0^{bA}	0.0^{d}
1.0 + 0.0	1.0^{a}	0.0^{b}	0.5^{ab}	1.2	0.0	0.6^{b}	44.0^{abB}	96.7^{aA}	70.3^{a}	2.9^{bB}	100.0^{aA}	51.5^{bc}
2.0 + 0.0	0.1^{a}	0.3^{ab}	0.2^{bc}	0.2	0.3	0.2^{b}	82.0^{aA}	75.4^{aA}	78.7^{a}	76.3^{aA}	90.0^{aA}	83.2^{abc}
3.0 + 0.0	0.3^{a}	1.6^{a}	0.9^{ab}	1.2	0.7	0.9^{b}	85.0^{aA}	55.5^{abA}	70.2^{a}	85.3^{aA}	76.3^{aA}	81.0^{abc}
0.0 + 0.05	1.2^{a}	1.7^{a}	1.4^{ab}	3.0	3.0	3.0^{a}	0.0^{bB}	85.3^{aA}	43.0^{ab}	0.0^{bB}	85.3^{aA}	43.0^{c}
1.0 + 0.05	0.0^{a}	0.3^{ab}	0.1^{c}	0.0	0.4	0.2^{b}	19.0^{abB}	80.0^{aA}	49.0^{a}	100.0^{aA}	98.7^{aA}	99.5^{a}
2.0 + 0.05	0.7^{a}	0.3^{ab}	0.5^{abc}	0.7	0.4	0.5^{b}	29.0^{abA}	44.5^{abA}	38.0^{ab}	95.2^{aA}	92.0^{aA}	93.6^{ab}
3.0 + 0.05	0.0^{a}	0.1^{b}	0.0^{c}	0.0	0.2	0.1^{b}	28.0^{abA}	28.0^{abA}	28.4^{ab}	100.0^{aA}	97.1^{aA}	98.5^{a}
Average	0.6^{A}	0.7^{A}		1.2 A	1.0 A		36.0^{B}	58.2^{A}		57.4^{B}	80.0^{A}	

Mean values followed by the same lowercases letter within columns and uppercases letter within line are not significantly different according to Tukey's test ($P < 0.05$).

explant.

For the production of plantlets in successive subcultures, the number of spiked pepper subcultures was statistically significant for height (cm) and number of buds, and not significant for the percentage of rooted microcuttings. The factor culture medium was not significant for any of the variables studied, and the interaction subculture x culture medium showed a statistically significant difference in the number of buds only. For the long pepper, the number of subcultures and the type of saline formulation used resulted in statistically significant differences for the variables height and number of formed buds. For the variable percentage of rooted microcuttings, none

Table 3. Effect of BAP on shoot length, multiplication rate, and percentage of explants with multiple shoots and callus formation at the base during the micropropagation of *Piper aduncum* and *Piper hispidinervum* in MS medium.

BAP (mg L^{-1})	*Piper aduncum*				*Piper hispidinervum*			
	Shoot length (cm)	Multiplication rate	Explants with multiple shoots (%)	Explants with callus formation (%)	Shoot length (cm)	Multiplication rate	Explants with multiple shoots (%)	Explants with callus formation (%)
0.0	1.6^{a}	2.7^{a}	0.0^{c}	0.0^{b}	1.3^{a}	1.5^{a}	0.0^{b}	0.0^{b}
0.25	1.0^{b}	1.0^{b}	48.0^{a}	100.0^{a}	1.1^{b}	1.2^{a}	27.5^{a}	100.0^{a}
0.50	0.9^{b}	1.0^{b}	27.7^{b}	100.0^{a}	1.2^{ab}	1.2^{a}	39.6^{a}	100.0^{a}
0.75	0.9^{b}	1.0^{b}	23.8^{b}	100.0^{a}	1.1^{b}	1.5^{a}	48.0^{a}	100.0^{a}
1.0	0.8^{b}	1.0^{b}	52.0^{a}	100.0^{a}	1.0^{b}	1.0^{a}	37.2^{a}	100.0^{a}

Mean values followed by the same lowercase letters within columns are not significantly different according to Tukey's test ($P < 0.01$).

Table 4. Micropropagation rates from nodal segments of *Piper aduncum* and *P. hispidinervum* after 5 subcultures of multiplication.

	Shoot length (cm)			Multiplication rate			Shoot rooting (%)		N° shoots accumulated	
N^{0} Subcultures	MS	WPM	Average	MS	WPM	Average	MS	WPM	MS	WPM
Piper aduncum										
1	3.7	4.4	4.0^{a}	3.4^{aB}	4.2^{aA}	3.8^{a}	100	100	3.4	4.2
2	1.8	2.3	2.2^{b}	2.2^{bB}	2.9^{bA}	2.5^{bc}	100	100	7.48	12.2
3	2.6	2.1	2.3^{b}	2.7^{abA}	2.8^{bA}	2.7^{b}	100	100	20.2	34.1
4	1.5	1.2	1.3^{b}	2.6^{bA}	2.3^{bcA}	2.4^{bc}	100	100	52.5	78.4
5	2.4	2.2	2.3^{b}	2.3^{bA}	2.0^{cA}	2.1^{c}	100	100	121.0	157.0
Average	2.4^{A}	2.4^{A}		2.6^{A}	2.8^{A}		100^{A}	100^{A}		
Piper hispidinervum										
1	5.0^{aA}	2.2^{aB}	3.6^{a}	5.0^{aA}	4.2^{aB}	4.6^{a}	100	100	5.0	4.2
2	1.5^{bA}	1.0^{bA}	2.2^{b}	2.3^{bcA}	1.9^{bA}	2.1^{b}	100	100	11.5	8.0
3	1.7^{bA}	1.1^{bB}	1.4^{b}	2.7^{bA}	2.0^{bB}	2.3^{b}	100	100	31.0	16.0
4	1.3^{bA}	0.9^{bA}	1.1^{b}	2.3^{bcA}	1.9^{bA}	2.1^{b}	100	100	71.4	30.3
5	1.9^{bA}	1.0^{bB}	1.4^{b}	2.0^{cA}	1.1^{cB}	1.5^{c}	100	100	143.0	33.0
Average	2.3^{A}	1.2^{B}		2.8^{A}	2.2^{B}		100^{A}	100^{A}		

Mean values followed by the same lowercase letters within columns and uppercase letters within line are not significantly different according to Tukey's test ($P < 0.05$).

of the factors used were significant. For both species, the first subculture resulted in the highest values related to shoot height and number of buds (Table 4).

For the long pepper, significant differences between the saline formulations tested were found. For the variables height and number of formed buds, the results for the MS culture medium were statistically higher than those of the

WPM medium. In the first subculture, microcuttings cultivated in MS medium showed an average height of 5.0 cm, with 5.0 buds per microcutting. For the microcuttings cultivated in WPM medium in the first subculture, the values found for height and number of buds per microcuttings were 2.2 and 4.2 cm, respectively (Table 4).

Despite the decrease in the number of buds from the first subculture, the multiplication rate was unchanged in the subsequent subcultures. The majority of studies that carry out successive subcultures present a gradual decrease in the number of buds over the subcultures. Flores et al. (2006) subcultivated nodes of Brazilian Ginseng (*Pfaffia tuberosa*) over five successive subcultures. According to the authors, the largest bud formation occurred in the first subculture and remained stable in the following subcultures. In other works, the successive subcultures did not cause a reduction in the multiplication rate; this was the case of *P. glomerata* subcultivated for six months, which showed no decrease in the average number of axillary buds when subcultivated during this time period (Maldaner et al., 2007).

Regardless of the number of subcultures or the type of saline formulation used, microcuttings of *P. aduncum* and *P. hispidinervum* showed 100% root formation (Table 4), indicating that these species have morphogenic potential for the development of roots, without the need for external stimuli (Figure 1D).

From the average values found in the five subcultures of spiked pepper and long pepper microcuttings, it was possible to estimate the potential number of plantlets to be produced based on time (TME). Thus, considering the average number of long pepper buds cultivated in MS or WPM medium and accumulated in each subculture, at the end of the five successive subculture periods, it would be possible to produce around 143 and 33 plantlets per microcutting, for each inoculated microcutting, respectively. For the spiked pepper, cultivation in MS or WPM medium would provide the formation of 121 and 157 plantlets per initially inoculated microcutting (Table 4).

Regarding the saline formulations tested, the use of MS salts in the *in vitro* cultivation of long pepper and spiked pepper microcuttings is suggested, given that on average, saline formulations showed no differences in height and number of buds for the spiked pepper, while for the long pepper, the MS medium was the one that presented the best results.

The acclimatization of plants of long pepper and spiked pepper obtained in the fifth subculture showed a 100% survival rate when kept in the greenhouse (Figure 1E). In relation to the percentage of root formation and survival rate during acclimatization, Pereira et al. (2000), attempting to develop a micropropagation protocol for *Pothomorphe umbellate*, also a species of the Piperaceae family, obtained similar percentages to those found in this study. According to the authors, *P. umbellata* plants kept for 30 days in growth regulator-free medium showed 100% root formation, and these same rooted plants, when acclimatized in a greenhouse, resulted in 100% survival. The plants developed normal leaves and no detectable morphological variations were scored.

This study highlights a complete micropropagation protocol for *P. aduncum* and *P. hispidinervum* through nodal segments as explants. In summary, the results achieved here suggest that for the spiked pepper and long pepper, the formation of the largest number of axillary buds, with the greatest length of aerial part, occurs in a BAP-free medium. The use of BAP in the micropropagation stage results in the formation of vitreous multiple shoots and callus formation at the base of the microcuttings. Even at concentrations lower than 1 mg L^{-1}, the use of BAP resulted in multiple shoot and callus formation, without significantly improving the multiplication rates of the species studied. For both species studied, the first subculture resulted in the greatest number of axillary buds, and, mainly for *P. hispidinervum*, the MS saline formulation is the most appropriate for *in vitro* multiplication of microcuttings. The species showed 100% root formation, and acclimatization of plants from the fifth subculture in a greenhouse resulted in 100% survival.

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