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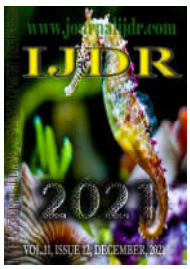

RESEARCH ARTICLE OPEN ACCESS

DIRECT ORGANOGENESIS AND *IN VITRO* REGENERATION OF PLANTS OF *PIPER MARGINATUM*

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ABSTRACT

Piper marginatum, popularly known in Brazil as Caapeba, is a native Amazonian plant of shrubby size, traditionally used for its active principles that act as therapeutic agents in wounds and pathologies. The objective of this work was to establish a protocol for the in vitro propagation of this species from leaf explants. For this, leaves were collected from two-year-old plants kept in a greenhouse at Embrapa Rondônia, in Porto Velho, Brazil. The leaves were subjected to disinfestation in a laminar flow chamber by immersion in 70% alcohol for one minute, followed by immersion in 2.0% sodium hypochlorite with 1.0 mL of Tween 20® for five minutes. Leaves were cut into 1.0 cm2 explants, which were inoculated into MS medium supplemented with 30.0 g L-1 sucrose, 6.0 g L-1 agar and variable concentrations of growth regulators benzylaminopurine (BA) (0.0, 1.13, 2.25 and 4.50 mg L-1) and 2,4-dichlorophenoxyacetic acid (2,4-D) (0.0, 1.11, 2.21 and 4.42 mg L-1) in factorial combination. The cultures were kept in a growth room at 25°C, under a 16-hour photoperiod. After 30 days, the number of shoots formed in the explants was evaluated. These shoots were subcultured on MS medium, under the conditions described, but without growth regulators. After 60 days, plantlet height, number of leaves, leaf area and root volume were evaluated. The treatment that contained 1.13 mg L-1 of BAP, in the absence of 2,4-D, resulted in the highest number of shoots and more vigorous plantlets after subculture, in relation to all evaluated variables. The plantlets were acclimatized in a greenhouse, with 50% shading and sprinkler irrigation three times a day, for their conversion into plants.

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INTRODUCTION

Piper marginatum Jacq.belongs to the Piperaceae family and is an aromatic species found in the Amazon region. Medicinal properties are attributed to this species, such as: antiseptic, astringent, anti-hemorrhagic and hemostatic. Its leaves contain a large amount of essential oil and tannic substances and alkaloid substances, from which its medicinal properties are fundamentally justified (Delgado-Paredes *et al*., 2012). The extract of its leaves has been used in folk medicine to treat liver and vesicle diseases and also as a tonic with carminative and antispasmodic action – previous chemical studies carried out on *P. marginatum* have described the occurrence of propiophenones, amides, flavonoids, phenylalkanoids and aristolactams (Reigada *et al*., 2007). Regarding the conventional ways of propagating this species, there are some limitations. The knowledge about *Piper* seeds is that their viability and germination sometimes present instability due to recalcitrance or even environmental conditions and ease of contamination by fungi (Abbasi *et al*., 2010; Ahmad *et al*., 2014; Padham, 2015).

Considering these limitations, *in vitro* techniques can be a biotechnological tool to multiply the species. This work aims to determine a protocol for *in vitro* regeneration of plants of the species *P.marginatum* Jacq. by inducing organogenesis in leaf explants.

MATERIAL AND METHODS

The experiments were carried out at the Plant Tissue Culture Laboratory at Embrapa (Brazilian Agricultural Research Corporation) in Porto Velho, Brazil. Leaves of *Piper marginatum* were collected at the experimental field from two years old plants and submitted to disinfestation procedures by washing with running tap water and a detergent agent for five minutes, immersion in 70% ethanol for one minute and in a 1.5% (v/v) sodium hypochlorite solution for 15 minutes, and then rinsed three times with sterile water. Under aseptic conditions, the leaves were cut into 1.0 cm^2 explants. These explants were inoculated into test tubes with 10.0 mL of an Murashige and Skoog (MS) basal culture medium supplemented with 30.0 g L^{-1} sucrose, 6.0 g L^{-1} agar and a factorial combination of the growth

Table 1. Average number of shoots per leaf explantof *P. marginatum* cultivated for 60 days in MS medium with factorial combinations of the growth regulators 2,4-D and BA

BA (mg L^{-1})	2,4-D (mg L^{-1})			
	0.00	1.11	2.21	4.42
0.00	-	-	-	-
1.13	5.9^{aA}	2.7^{bA}	1.8^{bA}	1.9^{bA}
2.25	3.9^{aB}	2.9^{aA}	2.1^{aA}	1.6^{aA}
4.50	3.9^{aB}	2.4^{aA}	2.2^{aA}	1.8^{aA}

*Averages followed by the same capital letter in the columns or small letter in the rows do not differ significantly at 5% probability by Tukey's test.

Table 2. Averages of plantlet height, number of leaves, leaf area and root volumeof *P. marginatum*plantlets cultivated for 60 days in MS medium with factorial combinations of the growth regulators 2,4-D and BA

Growthregulators	Plantletheight (cm)	Numberofleaves	Leafarea (cm^2)	Root volume (cm^3)
1.13 mg L^{-1} BA	3.50 a	5.5 a	2.51 a	3,23 a
1.13 mg L^{-1} BA + 1.11 mg L^{-1} 2,4-D	3.15 ab	3.5 bc	1.30 b	2,07 b
1.13 mg L^{-1} BA + 2.21 mg L^{-1} 2,4-D	1.75 b	2.0 c	0.74 b	0,46 c
2.25 mg L^{-1} BA	1.75 b	4.0 b	0.84 b	1,61 b
2.25 mg L^{-1} BA + 1.11 mg L^{-1} 2,4-D	1.50 b	4.0 b	1.51 b	0,26 c
2.25 mg L^{-1} BA + 2.21 mg L^{-1} 2,4-D	2.00 b	0.0 d	0.71 b	0,20 c
4.50 mg L^{-1} BA + 2.21 mg L^{-1} 2,4-D	2.25 b	0.0 d	1.54 b	0,00 c

*Averages followed by the same letter in the columns do not differ significantly at 5% probability by Tukey's test.

regulators 2,4-Dichlorophenoxyacetic acid (2,4-D)(0.0, 1.11, 2.21 and 4.42 mg L^{-1}) and 6-Benzylaminopurine (BA)(0.0, 1.13, 2.25 and 4.50 mg L^{-1}), totalizing 16 treatments. The pH was adjusted to 5.8 and the medium autoclaved at 121°C for 20 minutes. After 60 days, the number of shoots formed in the explants was evaluated. These shoots were subcultured on MS mediumwithout growth regulators, under the conditions described, where the shoots were kept for 60 days for conversion into plantlets. After that, plantlet height, number of leaves, leaf area and root volume were evaluated.These data were submitted to analyses of variance and Tukey test at 5% of probability. The plantlets were successfully acclimatized in a greenhouse, with 50% shading and sprinkler irrigation three times a day, for their conversion into plants.

RESULTS AND DISCUSSION

There was no induction of shoots in the media without BA (Table 1). All the combinations of BA and 2,4-D induced shoots, but the media containing only BA showed higher average numbers of shoots per explant. The concentration of 1.13 mg L^{-1} BA, without 2,4-D, resulted in the highest average, 5.9 shoots per explant. In spite of the formation of shoots, some combinations of 2,4-D and BA gave rise to shoots that were not healthy and did not survive after their subculture in an MS medium without growth regulators. The combinations that allowed regeneration of healthy plantlets were: 1.13 mg L^{-1} BA, 1.13 mg L^{-1} BA + 1.11 mg L^{-1} 2,4-D, 1.13 mg L^{-1} BA + 2.21 mg L^{-1} 2,4-D, 2.25 mg L^{-1} BA, 2.25 mg L^{-1} BA + 1.11 mg L^{-1} 2,4-D, 2.25 mg L^{-1} BA + 2.21 mg L^{-1} 2,4-D, and 4.50 mg L^{-1} BA + 2.21 mg L^{-1} 2,4-D. The utilization of 1.13 mg L^{-1} BA, without 2,4-D, resulted in more vigorous plantlets after subculture, in relation to all evaluated variables, withthe highest averages of plantlet height, number of leaves, leaf area and root volume (Table 2). Specific supplementations of exogenous growth regulators are necessary for shoot induction in different *Piper* species. There are several studies describing the use of cytokinins and auxins, alone or in combination, for shoot formation in leaf explants. The successful of direct organogenesis was reported by Schwertner *et al*. (2008) in leaf explants of *P. umbellatum* with 0.5 mg L^{-1} BA. Delgado-Paredes *et al*. (2012) induced shoots in leaf explants of *P. aduncun*and *P. crassinervum* with 0.5 mg L^{-1} TDZ. Ahmad *et al*. (2010) observed organogenesis in leaf explants of *P. nigrum* with 1.5 mg L^{-1} BA + 1.0 mg L^{-1} GA_3. Malthi *et al*. (2016) induced callus and subsequently shoots in leaf explants of *P. longum* by using 1.5 mg L^{-1} BA + 1.0 mg L^{-1} 2,4-D and 3.0 mg L^{-1} BA, respectively. The plantlets were successfully acclimatized in a greenhouse, with 50% shading and sprinkler irrigation three times a day, for their conversion into plants.

CONCLUSION

Induction of shoots in *P. marginatum*leaf explants can be achieved in MS medium supplemented with 1.13 mg L^{-1} BA. The shoots develop into plantlets, which can be successfully acclimatized to give rise to plants.

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