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Article

Comparison of Volatile Profile and Antioxidant Activity of *Piper divaricatum* G. Meyer (Piperaceae) Using Cuttings and Cell Tissue

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In this study, *P. divaricatum* (Piperaceae) plants were subcultured by cuttings and cell tissue in order to carry out a comparison of the volatile profile, phenolic compounds, and antioxidant activity. Propagation by cuttings used vermiculite substrate and Murashige-Skoog (MS) medium in the absence of growth regulators and rooting started at 15 days of growth providing a higher number of plants after 90 days. *In vitro* propagation was performed using shoot apices as explants in MS media supplemented with 0.5 mg mL^{-1} BA (6-benzyladenine). Volatile profiles analyzed by gas chromatography-mass spectrometry (GC-MS) showed as main compounds methyl eugenol, *E*-β-ocimene, and β-elemene in the *in vivo* and *in vitro* cultures. Phenolic contents determined by the Folin-Ciocalteu method had no significant difference at the end of 90 days of growth displaying a good linear correlation with antioxidant activity and phenylpropanoids amounts ($r > 0.7$). However, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging was higher for *in vitro* plants grown in comparison to *in vivo* plants cultured.

Keywords: vegetative propagation, micropropagation, essential oil, phenylpropanoids, phenolic compounds

Introduction

Plants are renewable resources providing raw material (like biomass: lignocellulosic) and phytochemicals (notably secondary metabolites) for different industrial applications, namely in the textile, construction, pharmaceutical, nutraceutical and cosmetic sectors.[1,2] Plant secondary compounds have high chemical and structural diversity and appear as non-volatile or volatile compounds. These compounds can be classified into four major classes: terpenoids, phenolic compounds, alkaloids and sulfur-containing compounds which can be antimicrobial, act as attractants/repellents, or as deterrents against herbivores.[2]

Due to the vast diversity of secondary metabolites present in the extracts and essential oils (EOs), the *Piper* species are considered an important source of bioactive compounds.[3,4] Among the metabolites of *Piper* EOs, we can highlight the phenylpropanoids as dillapiole, safrole, apiole, myristicin, eugenol, and methyl eugenol, which are

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identified in high amounts in the species of *P. aduncum*, *P. callosum*, *P. krukoffii* and *P. divaricatum*.[5,6] In the Amazon region, *P. divaricatum* G. Mey. EOs are characterized in two chemotypes: methyl eugenol (19.3-82.5%) and eugenol (7.3-39.7%), and chemotype elemicin (29.5-68.7%).[7] However, a higher safrole content in the leaves (98%), fruits (87%) and stems (83%) was reported for a specimen collected in Bahia State (Brazil).[8]

P. divaricatum EO chemotype methyl eugenol/eugenol has presented several biologic properties. The oil showed antioxidant capacity in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and the inhibition of lipid peroxidation in the system β-carotene/linoleic acid.[9] The oil displayed insecticidal potential against workers of the fire ant *Solenopsis saevissima* (Smith) (Hymenoptera: Formicidae), and the values of median lethal concentration (LC_{50}) in 48 h obtained was 301.7 mg L^{-1}.[10] Also, its antifungal activity was observed *in vitro* against the phytopathogenic fungi *Cladosporium cladosporioides*, *Cladosporium sphaerospermum*, and *Fusarium solani* f. sp. *piperis*.[7,10] For this reason, clones were cultivated in a greenhouse and inoculated by spores of *F. solani* f. sp. *piperis*, the causal agent of fusariosis in *Piper nigrum* (black pepper) and the plants displayed a total tolerance.[11]

Secondary metabolite production by plants can be affected by seasonality, circadian rhythm, plant development, luminosity, water availability, plant cultivation regime and temperature.[12] Plant cell culture can accelerate its synthesis of higher amounts in the short period favoring its accumulation in annuals and perennial plants.[13,14] Many strategies can be applied to induce or increase the secondary metabolite production, one of the most useful is the addition of compounds precursors or intermediates in the culture medium.[15,16] The *in vitro* culture techniques used to produce artemisinin was influenced by sugar concentrations, chilling treatment and UV-B radiation[17,18] and various elicitors including methyl jasmonate, gibberellic acid, salicylic acid and chitosan, which increased the production of artemisinin in different tissue cultures.[19] *Coffea arabica* suspension cells showed a significant increase of caffeine levels under aluminum treatment.[20]

Plant tissue culture techniques are the most frequently used as biotechnological tools for basic and applied purposes. Its applications include investigation on plant developmental processes, functional gene studies, commercial plant micropropagation, generation of transgenic plants with specific industrial and agronomical characteristics, plant breeding and crop improvement, virus elimination from infected materials to render high-quality healthy plant material, preservation and conservation of germplasm of vegetatively-propagated plant crops, and rescue of threatened or endangered plant species. Additionally, plant cell and organ cultures are of interest for the production of secondary metabolites of industrial and pharmaceutical interest.[21] In this sense, the propagation of *P. divaricatum* is an important strategy for its application to secondary metabolite production or even in genetic enhancement of *P. nigrum* through cloning and multiplication of plants in large scale in a short time interval.

Experimental

Cutting propagation

P. divaricatum was propagated by cuttings of branches containing a single node with 6 cm of length. All cuttings were planted individually into plastic trays with twenty-four cells containing vermiculite. Plants were irrigated twice *per* day during bioassay and every three days it was applied 10 mL of Murashige and Skoog (MS) medium without growth regulator. The cuttings were kept in a greenhouse. Every 30 days shoot apices were excised from cuttings with roots and leaves, and used as explants for micropropagation. Seedlings regenerated from micro-cutting were sterilized with Derosal 0.2% fungicide solution daily for seven days. Leaves were harvested at 30, 60 and 90 days of growth to analyze essential oils, phenolic compounds and antioxidant activity.

Cell tissues

Shoot apical explants were sterilized in sodium hypochlorite 0.2% (v/v) (20 min), followed by rifampicin solution 3.0% (30 min), and then treated with 0.5% fungicide solution (Derosal) for 2 h. The explants were rinsed in sterile distilled water and cultured on MS or $^{1}/_{2}$MS medium[22] in the presence and absence of antibiotics streptomycin, amoxicillin and rifampicin (100 or 200 mg L^{-1}). The supplementation of media was composed by addition of growth regulators 6-benzyladenine (BA) or α-naphthalene acetic acid (NAA) at the concentration of 5 mg L^{-1}. The pH was adjusted to 5.8 priorly and solidified with 2.0 g L^{-1} Phytagel, and then it was sterilized at 121 °C for 20 min. Explants were kept at 25 ± 3 °C under 16 h light *per* daily photoperiod with irradiance intensity during the light period of 30 m^{-2} s^{-1}. Cultures were checked regularly for contaminations, and those that presented infection symptoms were immediately discarded. Treatments were composed of three replicates.

Essential oil extraction and preparation of *P. divaricatum* extracts

The leaves were collected from plants *in vivo* and *in vitro* cultured at 30, 60 and 90 days of growth and then the essential oils and extracts were obtained. The volatile compounds were extracted from 3 g of leaves of each treatment by simultaneous distillation extraction by Likens-Nickerson apparatus, using *n*-pentane (3 mL) as solvent.[23] After 2 h of extraction, the organic fraction was collected. Fresh leaves (2.0 g) was extracted by percolation with ethyl acetate (72 h) and residual solvent was removed under reduced pressure.

Chemical composition analysis of essential oils

The oils were analyzed on a GCMS-QP2010 Ultra system (Shimadzu Corporation, Tokyo, Japan), equipped with an AOC-20i auto-injector and the GCMS-Solution software containing the NIST[24] and Adams[25] libraries. An Rxi-5ms (30 m × 0.25 mm; 0.25 µm film thickness) silica capillary column (Restek Corporation, Bellefonte, PA, USA) was used. The conditions of analysis were: injector temperature of 250 °C; oven temperature programming of 60-240 °C (3 °C min^{-1}); helium as carrier gas, adjusted to a linear velocity of 36.5 cm s^{-1} (1.0 mL min^{-1}); split mode injection for 1 µL of sample (oil 3 µL:hexane 500 µL); split ratio 1:20; ionization by electronic impact at 70 eV; ionization source and transfer line temperatures of 200 and 250 °C, respectively. The mass spectra were obtained by automatic scanning every 0.3 s, with mass fragments in the range of *m/z* 35-400. The retention index was calculated for all volatile components using a homologous series of C8-C40 *n*-alkanes (Sigma-Aldrich, St. Louis, USA), according to the linear equation of Van den Dool and Kratz.[26] The quantitative data regarding the volatile constituents were obtained by peak-area normalization using a GC 6890 Plus Series, coupled to flame ionization detector (FID), operated under similar conditions of the gas chromatography-mass spectrometry (GC-MS) system. The components of oils were identified by comparing their retention indices and mass spectra (molecular mass and fragmentation pattern) with data stored in the GCMS-Solution system libraries.

Total phenolic content (TPC)

The Folin-Ciocalteu method was employed to determine the total phenolic (TPC) content of ethyl acetate extract. The extracts were dissolved in methanol at an initial concentration of 40 mg mL^{-1} and then diluted in water. Aliquots of 500 µL of the aqueous solution, 250 µL of Folin-Ciocalteu reagent (1.0 M) and 1250 µL of sodium carbonate (75 g L^{-1}) composed the reaction mixture. After 30 min, the absorbance was measured at 760 nm and 25 °C (UV-Vis spectrophotometer, Biosystems RA2708, Costa Brava, Spain). Gallic acid at concentrations of 0, 0.5, 1, 2, 4, 6, 8 and 10 mg L^{-1} was used to prepare the calibration curve. The TPC was expressed as gallic acid equivalents (GAE) in milligrams *per* gram of extract (mg GAE g^{-1}).[27]

Antioxidant activity

The extracts were dissolved in methanol, and aliquots (50 µL) were mixed with DPPH radical solution (60 µM, in methanol). After 60 min, the absorbance of reaction mixture was measured at 517 nm, and then the percentage of DPPH radical scavenging (I) was calculated using the equation:

$$I(\%) = \left[1 - \frac{A_{sample}}{A_{control}}\right] \times 100 \quad (1)$$

where A_{sample} and $A_{control}$ are the absorbance of test samples and of the control, respectively.

The total antioxidant capacity was expressed as Trolox equivalents (TE), which was calculated from a Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) curve at concentrations range of 1 to 10 µg mL^{-1}.

Statistical analysis

All data were expressed as means ± standard deviation. Analysis of variance was conducted by Bonferroni test using GraphPad 5.0.[28] Differences at $p < 0.05$ were considered statistically significant.

Results and Discussion

Propagation of *P. divaricatum*

The establishment of protocol propagation of *P. divaricatum* by cuttings was successful with a higher survival rate (95%) using vermiculite substrate and MS medium in the absence of growth regulators. Rooting were started at 15 days of growth, and a higher number of plants after 90 days were obtained (Figure 1).

In the scientific literature, there are few studies on *Piper* species propagation by cuttings. *P. aduncum* cutting propagation was performed using applications of NAA at concentrations of 300 and 600 mg L^{-1} and sand as the substrate.[29] *P. hispidum* propagation was developed from two types of cuttings (apical and basal) using washed sand and a commercial substrate. The rooting was higher in the

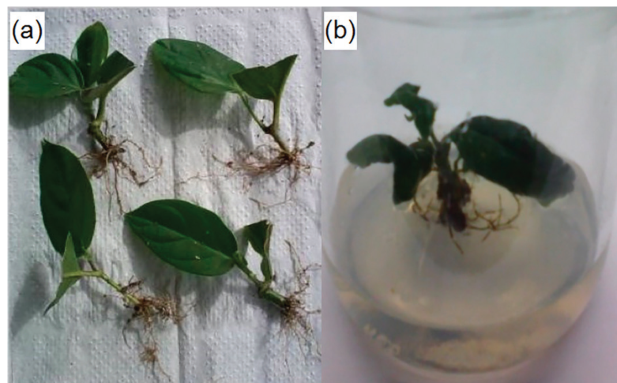

Figure 1. Propagation vegetative of *P. divaricatum*. (a) Cutting propagation using vermiculite substrate and MS medium in the absence of growth regulators; (b) plantlets regenerated in MS medium supplemented with 0.5 mg mL^{-1} BA (6-benzyladenine).

apical cuttings (85.67%) than the basal cuttings (74.47%). However, for this species, the substrates soil + poultry manure and/or soil + guarana shell were shown to produce high-quality cuttings.[30] Cuttings of *P. arboreum* were treated with different concentrations of indole-3-butyric acid (IBA) (0, 2000, 4000, 6000 and 8000 mg L^{-1}) and maintained in a greenhouse with intermittent irrigation, temperature, and humidity control and displayed greater results for aerial part development independent of the IBA concentration.[31]

In the *in vitro P. divaricatum* propagation, the shoot apical explants were sterilized with a fungicide solution composed by benzimidazole and the antibiotics amoxicillin or streptomycin at a concentration of 200 mg L^{-1}. The explants treated with both antibiotics reduced the bacterial contamination to 50% with no difference between their effects. The culture media MS and ½ MS displayed differences in explant regeneration in the presence of growth regulator. MS supplemented with 0.5 mg mL^{-1} BA induced callus formation that differentiated into stem and leaves and promoted the multiplication of new explants at 90 days, after the third subculture. In this stage, explants exhibited shoot with leaves, and its shoot tips were excised and transferred to fresh medium containing the same hormonal combinations for multiplication.

In vitro Piper cultures have been established and its morphogenetic potential investigated. *P. nigrum* was cultured by callus induction and shoot regeneration from leaf and petiole explants. Leaf had a better callogenic response on explants cultured in MS supplemented with 0.5 mg mL^{-1} of BA pure or in combinations of 1.5 mg L^{-1} BA + 1.0 mg L^{-1} NAA. Shoot organogenesis were achieved onto MS medium supplemented with 1.0 mg L^{-1} BA pure or 1.5 mg L^{-1} BA + 1.0 mg L^{-1} gibberellic acid (GA3), or with MS medium supplemented with 1.5 mg L^{-1} thiodiazoran or 1.5 mg L^{-1} IBA. The elongated shoots were rooted on MS medium supplemented with different concentrations of IBA pure or in various combinations with NAA and indole-3-acetic acid (IAA).[32,33] Petiole callogenesis was induced from MS medium supplemented with 0.5 mg L^{-1} BA and shoot formation was recorded for 0.5 mg L^{-1} BA and roots were recorded for 2.0 mg L^{-1} of IBA.[34]

MS medium supplemented with 1 mg L^{-1} IAA and 0.5 mg L^{-1} BA was suitable for induction of multiple shoots in shoot tip and leaf base explants in *P. betle*. Callus, multiple shoots, and roots were formed on medium containing 0.5 mg L^{-1} 6-benzyaminopurine (BAP) + 1 mg L^{-1} IAA.[35] The better regeneration of *P. crocatum* was obtained from internodes in MS medium. The supplementation of MS media with 5.0 mg L^{-1} BAP, 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and charcoal was the most effective in shoot initiation, proliferation and showed less browning.[36]

P. longum showed multiple shoots induced from nodal segments in MS supplemented with BA (0.5 to 5 mg L^{-1}), IAA (0.2 to 1 mg L^{-1}) and NAA (0.5 to 1 mg L^{-1}) and the best medium for rooting was MS with 1 mg L^{-1} BA and 0.2 mg L^{-1} IAA.[37] *P. auritum* showed organogenesis derived from leaf tissue in MS medium supplemented with different combinations of 2,4-D (0.5 to 2.0 mg L^{-1}) and 1.5 mg L^{-1} kinetin for the regeneration of callus and shoot, respectively. All plants elongated when using a medium consisting of 0.1 mg L^{-1} 2,4-D and 1.0 mg L^{-1} kinetin, and the rooting was successful on one-half MS basal medium supplemented with 2.0 mg L^{-1} IAA.[38]

Essential oils chemical composition

GC and GC-MS techniques were used in the analysis of the EO chemical composition obtained from *P. divaricatum* leaves and resulted in an average above than 90.0% of total identification. Phenylpropanoids were the predominant compound class identified in the leaf oils with methyl eugenol as the main component in all samples. The plants cultured by cuttings showed a higher phenylpropanoid production at 30 days of growth in comparison to plants cultivated by cell tissue culture. However, the phenylpropanoid concentrations between these treatments did not display differences at 60 and 90 days of growth.

At 30 days of growth, the plants cultivated by cuttings showed as major compounds methyl eugenol (63.16%), *E*-β-ocimene (9.79%) and β-elemene (3.93%). The main difference observed was the lower concentrations of eugenol, β-elemene and eugenyl acetate identified at amounts of 4.89, 8.99 and 6.45% in the cuttings and 1.24,

3.93 and 0.0% in the cell tissue. At 60 days of growth, the production of monoterpene hydrocarbons such as α-pinene, β-pinene, myrcene, limonene, *Z*-β-ocimene, *E*-β-ocimene, *allo*-ocimene was higher in the plants cultured by cuttings (20.22%) than plants cultivated by cell tissue (8.52%). *E*-β-Ocimene amounts showed an increase from 7.22 to 9.69% in the cuttings and a remarkable decrease from 9.67 to 0.88% in the cell tissue. Additionally, there was in the cuttings the production of 2*E*-hexenal (1.70%), an aldehyde of low molecular weight. After 90 days of growth, the concentrations of oxygenated sesquiterpenoids were higher in the cuttings (10.86%) than plants cultured *in vitro*. Regarding the variation of the major compounds, the plants cultivated by cuttings displayed high amounts of *E*-β-ocimene (8.69%) and low concentrations of β-elemene (4.73%) in comparison to plants cultured by cell tissue. However, the average production of methyl eugenol has an increase from 63.35 to 80.31% in the interval of 30 to 90 days of growth not displaying quantitative differences in both cultivation methods (Table 1).

Table 1. Volatile compounds of *Piper divaricatum* leaves cultured by cuttings and cell tissue at 30, 60, and 90 days of growth

Compound	RI[a]	RI[b]	30 days		60 days		90 days		Literature[8] / %
			Cuttings / %	Cell tissue / %	Cuttings / %	Cell tissue / %	Cuttings / %	Cell tissue / %	
2*E*-Hexenal	846	847				1.70*	0.15		
α-Pinene	932	936			3.91	2.87	0.19	0.04–	0.2
β-Pinene	974	977			3.79	2.96	0.09		0.3
Myrcene	988	989		0.22*	0.89	0.62–	0.18	0.19	0.2
β-Phellandrene	1025	1027							0.5
Limonene	1024	1023	0.37	0.05+	1.37	0.66–	0.43	0.14–	
Z-β-Ocimene	1032	1036	0.29	0.27	0.46	0.39	0.23	0.26	
E-β-Ocimene	1044	1045	**7.22**	**9.79+**	**9.67**	0.88–	**6.11**	**8.69+**	1.7
allo-Ocimene	1128	1128		0.17*	0.13	0.14	0.07	0.12+	
Carvacrol	1298	1298				0.23*			
δ-Elemene	1335	1337	0.06	0.02		0.04*	0.03	0.03	
Eugenol	1356	1357	4.89	1.24–	0.13	0.06–	0.22	0.78+	**23.6**
α-Copaene	1373	1376							0.1
β-Elemene	1389	1392	**8.99**	3.93–	3.56	0.59–	**7.20**	4.73–	2.8
Methyl eugenol	1403	1413	**63.54**	**63.16**	**72.99**	**69.56**	**80.95**	**79.67**	**63.8**
β-Caryophyllene	1417	1419	2.33	1.00–	1.25	2.30+	2.09	1.24–	1.2
β-Copaene	1431	1426	0.17	0.06–	0.02	0.09+	0.13	0.07–	0.2
γ-Elemene	1434	1434				0.06*			
α-Humulene	1452	1453	0.30	0.05–	0.03	0.15+	0.14	0.07–	0.3
Germacrene D	1484	1484							0.7
β-Selinene	1489	1486							0.1
E-Methyl isoeugenol	1491	1488			0.05	0.35+	0.01	0.01	
trans-Muurola-4(14),5-diene	1493	1488	1.77	1.21	0.44	1.13+	1.27	1.28	
Eugenyl acetate	1521	1525	**6.45**						1.7
Elemicin	1550	1555	0.47	0.33–	0.07	0.40+	0.17	0.26+	0.4
Spathulenol	1577	1580				0.04*			0.1
Caryophyllene oxide	1582	1584							0.3
Viridiflorol	1592	1591							0.1
Selin-11-en-4α-ol	1658	1659				0.06*			0.2
Apiole	1677	1678							0.1
Monoterpenes hydrocarbons			7.88	10.5	20.22	8.52	7.30	9.44	3.3
Oxygenated monoterpenoids						0.23			
Sesquiterpenes hydrocarbons			13.62	6.27	5.30	4.36	10.86	7.42	5.6
Oxygenated sesquiterpenes						0.55			0.6
Phenylpropanoids			**73.35**	**64.73**	**73.24**	**70.37**	**81.35**	**80.72**	**89.6**
Others			0.07		0.07	1.93	0.18		
Total			96.87	81.50	98.83	85.51	99.69	97.58	99.1

[a]RI: retention index (Adams, 2007;[25] NIST 2011);[24] [b]RI calculated. *: compounds produced only *in vitro* cultured; +: compounds produced *in vitro* with increase above of 30%; –: compounds produced *in vitro* with decrease of 30%. Major compounds with percentage above than 5.0% are highlighted in bold.

The chemical profile of plants cultured using both methods was similar to previous reports[6,8] for *P. divaricatum* leaves with predominance of phenylpropanoids, particularly methyl eugenol. Secondary metabolite production can be drastically altered in response to different growth regulators in the culture medium.[39,40]

In this study, the levels of oxygenated sesquiterpenoids were higher in plants cultivated by cuttings than cell tissue during the entire period. Previous studies reported different effects promoted by plant growth in sesquiterpene amounts, for example in *Micromeria* species (Lamiaceae) cultured *in vitro*. In the case of *Micromeria pulegium*, the percentage of total sesquiterpenoids in micropropagated plants ranged from 5.62% plant growth regulator-free medium compared to 10.60% with BA-supplemented medium and both were considerably higher than in wild-growing plants (1.47%).[41] However, in *Micromeria croatica*, the percentage of total sesquiterpenoids in micropropagated plants was lower than in wild-growing plants.[42]

Other species as *Ocimum basilicum* and *O. sanctum*, characterized primarily by the presence of monoterpenes and phenylpropanoids, showed differences in their essential oil chemical compositions according to culture conditions. The main compounds produced in field plants, micropropagated plants *in vitro* from shoot apices, and acclimated plants (*ex vitro*) were α-muurolol (30.62%), methyl eugenol (27.38%) and linalool (23.31%), respectively.[43] Furthermore, MS concentration and the growth regulator types have qualitatively and quantitatively influenced the growth and volatile composition in *O. basilicum*, a higher amount of methyl eugenol with the media 2MS and ¼MS, and a higher content of linalool and 1,8-cineole with MS and woody plant media (WPM) were observed.[44] When the precursor phenylalanine was added to the medium, it enhanced the production of eugenol by *Ocimum sanctum* (holy basil) cultured by tissue culture.[45]

Phenolic content and antioxidant activity

Phenolic content in the samples was determined by the Folin-Ciocalteu method, and no statistical differences between plants cultivated by cuttings and cell tissue (Figure 2A) were observed, except at 60 days of growth. Secondary metabolite profiles of plants are influenced by the development stage when it was collected.[46] In *P. divaricatum* leaves, an increase of 60% in the accumulation of phenolic compounds was observed, depending on the time from 30 to 90 days of growth. The antioxidant activity was considerably higher in the cuttings at 30 (96.5 ± 4.9 / 14.1 ± 0.9 mg TE g^{-1}) and 90 days (53.3 ± 1.1 / 90.9 ± 1.9 mg TE g^{-1}) of growth in comparison to cell tissue (Figure 2B).

Phenolic compounds are commonly found in edible and non-edible plants and showed numerous biological effects including antioxidant activity.[47] The values of DPPH inhibition, TPC and phenylpropanoids amounts were submitted to linear regression analysis to find a correlation. TPC displayed a similar correlation with antioxidant activity in the cuttings (coefficient of determination (R^2) = 0.614, correlation coefficient (r) = 0.78) in comparison to plants cultured by cell tissue (R^2 = 0.491, r = 0.70). In addition, the TPC showed a good correlation with phenylpropanoids amounts in the plants cultivated *in vivo* (R^2 = 0.546, r = 0.74) as well as *in vitro* method (R^2 = 0.533, r = 0.73). However, we did not find a correlation between DPPH radical scavenging and phenylpropanoids amounts.

Divergences between antiradical activity and phenolic compounds are reported in the literature, once these methods present different chemical and molecular mechanism.[48] DPPH assay based on the reduction of alcoholic DPPH solution by a substance or complex mixture through the donation of hydrogen atoms or electrons, while the Folin-Ciocalteu is a method which involves a single electron

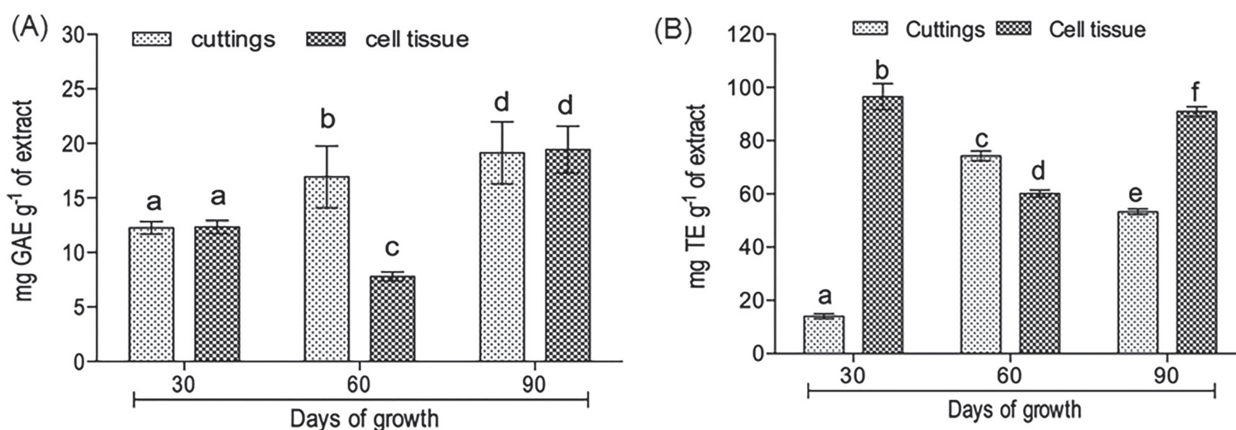

Figure 2. (A) Phenolic content and (B) antioxidant activity in the *P. divaricatum* leaf extracts cultured by cuttings and cell tissue at 30, 60 and 90 days of growth.

transfer from the substrate, presumably a phenol.[49-51] Also, it is essential to highlight that the Folin-Ciocalteu reagent has no differentiation among phenolic combinations as sugars, aromatic amines, ascorbic acid, and even inorganic substances can react with Folin-Ciocalteu providing an inexact result.[52]

Different levels of phenolics have been reported to the same species according to its propagation methods. *In vitro* culture of *Cucumis anguria* (Cucurbitaceae) showed a significant increase in phenolic content, flavonoid concentrations and antioxidant potential when compared to *in vivo* plants. For this, MS culture media was supplemented with saccharose 3.0% and a combination of 3.0 mM TDZ (thidiazuron), 1.0 mM IBA and 75 mM spermidin.[53] The same effects were observed for *in vitro* cultures of *Coleonema pulchellum* (Rutaceae). However, in addition to plant growth regulators, organic inducers (OES) such as casein hydrolyzate, hemoglobin, glutamine, and mebendazole were used.[54]

Callus induction of *Ephedra strobilacea* (Ephedraceae) was performed in a standard MS medium with the following hormonal addition of 1.5 mg L^{-1} NAA and 1 mg L^{-1} kinetin. The extracts were evaluated by FRAP (ferric reducing antioxidant power) and Folin-Ciocalteu methods. The samples displayed an antioxidant activity and phenolic content around five times higher in the wild plants than the callus.[55] The extracts of *Trifolium pratense* (red clover) *in vivo* and *in vitro* grown plants as well as callus tissue of red clover were tested for the evaluation of their antioxidant activities. The highest amounts of total phenolic and total flavonoids content were found in methanol extract of *in vivo* grown plants. The samples grown *in vivo* and callus tissue displayed a comparable antioxidant activity with high ferric reducing power, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and DPPH radical scavenging, hydrogen peroxide scavenging and chelating power.[56]

Conclusions

In this study, we have reported the establishment of vegetative propagation protocols for *P. divaricatum*, a native shrub from the Amazon region. The regeneration was successful by cuttings with no growth regulators added while in the cell tissue micropropagation it was necessary. After 90 days of growth, the plants cultured in both methods displayed the same volatile profile with the maintenance of its bioactive compounds as methyl eugenol. Furthermore, the phenolic contents and antioxidant activity had a low variation. Our results showed that the propagation of *P. divaricatum* plants in large scale is recommended by cutting, once this protocol is more economically viable for the production of secondary metabolites.

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