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Micropropagation of *Pothomorphe umbellata* via direct organogenesis from leaf explants

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Abstract

The establishment of a micropropagation protocol for *Pothomorphe umbellata* was carried out using leaf segments cultured on 1/4 strength Murashige and Skoog medium supplemented with 0.5 mg l^{-1} 6-benzyladenine, 0.1 mg l^{-1} gibberelic acid added with 10 g l^{-1} sucrose. Rooting was achieved using MS medium devoid of growth regulators. An anatomical study confirmed shoot regeneration via direct organogenesis.

Abbreviations: BA – 6-benzyladenine; GA_3 – gibberelic acid; MS – Murashige and Skoog medium; MS/2 – 1/2 strength Murashige and Skoog medium; MS/4 – 1/4 strength Murashige and Skoog medium; NAA – naphtaleneacetic acid

Introduction

Pothomorphe umbellata (L.) Miq. is described in the Brazilian Pharmacopeia and is commonly known as pariparoba or caapeba. It belongs to the Piperaceae family and extracts of its root are used as colagogue and anti-hepatotoxic agents. Studies conducted by Felzenszwalb et al. (1987) with *P. umbellata* extracts revealed that it was not mutagenic.

Ethanolic extracts of *P. umbellata* have a high antioxidant activity comparable to tocoferol, which is attributed to its content of 4-nerolidylcatechol (Kijjoa et al., 1980; Barros et al., 1996). Also, 4-nerolidylcatechol dimers present in *P. peltata* extracts, show inhibitory activity against HIV (Gustafson et al., 1992).

Several Brazilian laboratories produce phytopharmaceuticals containing *P. umbellata*. However, there is no commercial production of this species in Brazil and its removal from the wild is leading to a rapid and progressive devastation of the species. Despite being an herbaceous plant, it grows very slowly and its seeds present a low germination index. The objective of this work was to establish a micropropagation protocol for *P. umbellata*, to describe the histology of the stages of culture regeneration and determine whether or not 4-nerolidylcatechol is present in micropropagated plantlets.

Material and methods

Sterilization

Leaves of one year-old *P. umbellata* plants grown at the University of Ribeirão Preto were cut into 1 cm^2 and washed in tap water for 12 hours, soaked in 1% (w/v) benomyl solution for 4 hours, then in a 0.25% calcium hypochlorite (w/v) solution for 30 minutes and finally immersed in gentamicin (100 mg l^{-1}) for 1 hour. Explants were placed directly onto the culture media without washing in water.

Inoculation

Disinfested explants were inoculated onto MS semi-solid medium (Murashige and Skoog, 1962), contain-

Table 1. Effect of salt concentrations and growth regulator on the development of *P. umbellata* explants

Culture medium	Growth regulators (mg l^{-1})	Mean of proliferating explants (%)	Mean number of shoots per explant	% of elongated plantlets	Mean height of shoots (cm)
1 MS	0.25 BA	–	–	–	–
2 MS	0.50 BA	–	–	–	–
3 MS	1.00 BA	–	–	–	–
4 MS	0.25 ANA	–	–	–	–
5 MS	0.50 ANA	–	–	–	–
6 MS	1.00 ANA	–	–	–	
7 MS	0.25 BA+0.25 NAA	77.3 A*	1.2 B		
8 MS	0.50 BA+0.25 NAA	90.0 A	2.7 A		
9 MS	1.00 BA+0.25 NAA	90.0 A	2.8 A		
10 MS	0.25 BA+0.50 NAA	80.0 A	1.7 B		
11 MS	0.50 BA+0.50 NAA	93.3 A	3.6 A		
12 MS	1.00 BA+0.50 NAA	100.0 A	3.1 A		
13 MS	0.25 BA+1.00 NAA	90.0 A	3.2 A		
14 MS	0.50 BA+1.00 NAA	93.3 A	2.9 A		
15 MS	1.00 BA+1.00 NAA	93.3 A	2.7 A		
16 MS	0.50BA+0.10 GA_3			6.6 C	1.0 B
17 MS/2	0.50 BA+0.10 GA_3			93.0 A	1.4 AB
18 MS/4	0.50 BA+0.10 GA_3			71.6 AB	1.2 B
19 MS/4	0.50 BA+0.50 GA_3			66.0 AB	1.6 AB
20 MS/4**	0.50 BA+0.10 GA_3			50.0 B	1.8 A

*Means followed by the same letter do not differ statistically at p=0.05 according to the Tukey test.
**Medium had a reduced concentration of sucrose (10 g l^{-1}).

ing 30 g l^{-1} sucrose, supplemented with the concentrations and combinations of growth regulators as showed on Table 1. Medium (20) had a reduced concentration of sucrose (10 g l^{-1}). Experiments were carried out in glass flasks (8.5 cm height×5.0 cm i.d.) containing 30 ml of culture medium with 30 g l^{-1} of sucrose and 2.0 g l^{-1} Phytagel (Sigma), pH adjusted to 6.0 and autoclaved at 105 kPa for 15 minutes. The culture flasks were closed with polypropylene closures (Bellco) and sealed with plastic film (Parafilm 'M'), then maintained at 25±2 °C, 55–60% relative humidity under 16-h photoperiod with 40 μmol m^{-2} s^{-1} provided by cool white GE fluorescent lamps. Explants were kept on each medium for 2 subcultures, being transferred after 30 days of culture.

Plantlets with two pairs of leaves were placed on MS medium without growth regulators for rooting. After rooting, plantlets were transplanted into a styrofoam box (60×40 cm), containing substratum (Plantimax®). The boxes were kept inside a greenhouse at 70% relative humidity and watered daily. After 60 days plants were transplanted to the field.

The experimental design was fully randomized with three replicates of 10 explants per treatment. Data were analysed statistically by analysis of variance followed by the Tukey test, with the level of significance set at 5%.

Extraction of 4-nerolidylcatechol

Plantlets of 7 cm cultured *in vitro* (MS medium supplemented with 0.5 mg l^{-1} NAA and 0.5 mg l^{-1} BA) and plantlets from seeds of 7 cm were dried separately in an oven at 50 °C overnight. After grinding, the powder was extracted in 10 ml of chloroform using

ultrasound for 20 min. The extract was filtered and an aliquot applied to a silica gel plate concurrently with a standard of 4-nerolidylcatechol. TLC on silica gel 60 F254 (0.2-mm-thick plates) was developed with hexane-acetone solution (7:3) and detection was carried out by spraying with vanillin sulfuric as the color reagent, followed by heating at 100 °C.

HPLC analysis

Crude chloroform extracts were dissolved in methanol, filtered through a Sep-Pack C-18 cartridge and chromatographed on HPLC. Analysis of 4-nerolidylcatechol content was carried out using Shimadzu LC-10AD chromatograph, photodiode array detector, a CLC-CN-Shim-pack column (25 cm×4.6 mm), eluent system linear gradient 50–100% MeOH (20 min), 100% MeOH (5 min), flow rate 0.8 ml/min, detection at 284 nm. Quantitative analysis of 4-nerolidylcatechol was performed using a 3-point external calibration within a range of 0.13–2.06 μg μl^{-1}.

Histological analysis

Explants maintained in MS culture medium supplemented with 0.5 mg l^{-1} BA and 0.5 mg l^{-1} NAA were periodically removed (every 3 days during 60 days of culture) for histological analysis. Samples were fixed in Karnovsky solution (Karnovsky, 1965), dehydrated in alcoholic-ethyl series and infiltrated in glycol methacrilate (Reichert-Jung) resin. Sections (5 μm) were colored with Toluidine Blue (Sakay, 1973) and set up in synthetic resin (Permount).

Results and discussion

The regeneration of adventitious shoots of *Pothomorphe umbellata* from leaf explants was dependent on both auxin and cytokinin being present in the medium. Explants inoculated on culture medium supplemented with only BA or NAA did not show organogenesis.

The combinations of NAA and BA tested induced shoot proliferation and some adventitious roots (Table 1). However, the roots formed independently of the shoots. The height of the shoots was less than 0.5 cm in all the experiments and shoots were vitrified. The combination of 0.5 mg l^{-1} of NAA and 0.5 mg l^{-1} BA was considered more suitable for the organogenesis of *P. umbellata* as it induced the least vitrification (it was not possible to quantify vitrification due to the small size of the shoots). Adventitious shoots were arranged in rosettes.

The combination of GA_3 and BA was tested for shoot initiation and elongation. Although MS medium supplemented with 0.5 mg l^{-1} of BA and 0.1 mg l^{-1} of GA_3 promoted some shoot elongation, the number of elongated plantlets was low (6.6%). Plantlets cultured with 0.5 mg l^{-1} BA and 0.1 mg l^{-1} GA_3 but only half-strength MS medium, or even 1/4 strength, exhibited a high percentage of plantlets taller than 1 cm.

Besides promoting shoot elongation the MS/4 medium supplemented with 0.5 mg l^{-1} of BA and 0.1 mg l^{-1} GA_3 with 10 g l^{-1} sucrose suppressed vitrification. The induction of plantlet elongation using a reduced concentration of salts and sucrose has been shown for several species (Jelaska, 1987; Bonga and Aderkas, 1992) as well as GA_3 (Depommier, 1981). The established protocol for *P. umbellata* organogenesis indicates MS medium supplemented with 0.5 mg l^{-1} NAA and 0.5 mg l^{-1} BA and 1/4 strength MS containing 0.1 mg l^{-1} GA_3 and 0.5 mg l^{-1} BA with 10 g sucrose for shoot initiation and elongation respectively.

Morphogenetic potential of root, leaf, node and internode explants of species belonging to the Piperaceae family was evaluated by Bhat et al. (1995). The number of shoot buds induced varied according to the species, type of explant and treatment with growth regulators. *Piper longum* leaf explants formed shoot buds while *P. betle* and *P. nigrum* did not. Response of internodal and nodal explants was more effective than that of leaves but varied greatly (1–30) depending on the cytokinin level. Even at the best concentration, only 40% of *P. nigrum* nodal explants regenerated adventitious shoot buds.

Results presented here showed that leaves of *P. umbellata* have great organogenic potential not only for shoot formation but also for the production of roots, and that this ability is directly related to the presence of exogenous growth regulators in the culture medium. Plantlets maintained for 30 days on MS medium devoid of growth regulators showed 100% rooting. Rooted plantlets were transplanted to soil/sand and acclimatized to ambient (greenhouse) conditions after two months, with 100% survival.

Histological analysis

Organogenesis of plants of the genus *Piper* with and without callus has been recorded from stem, root or

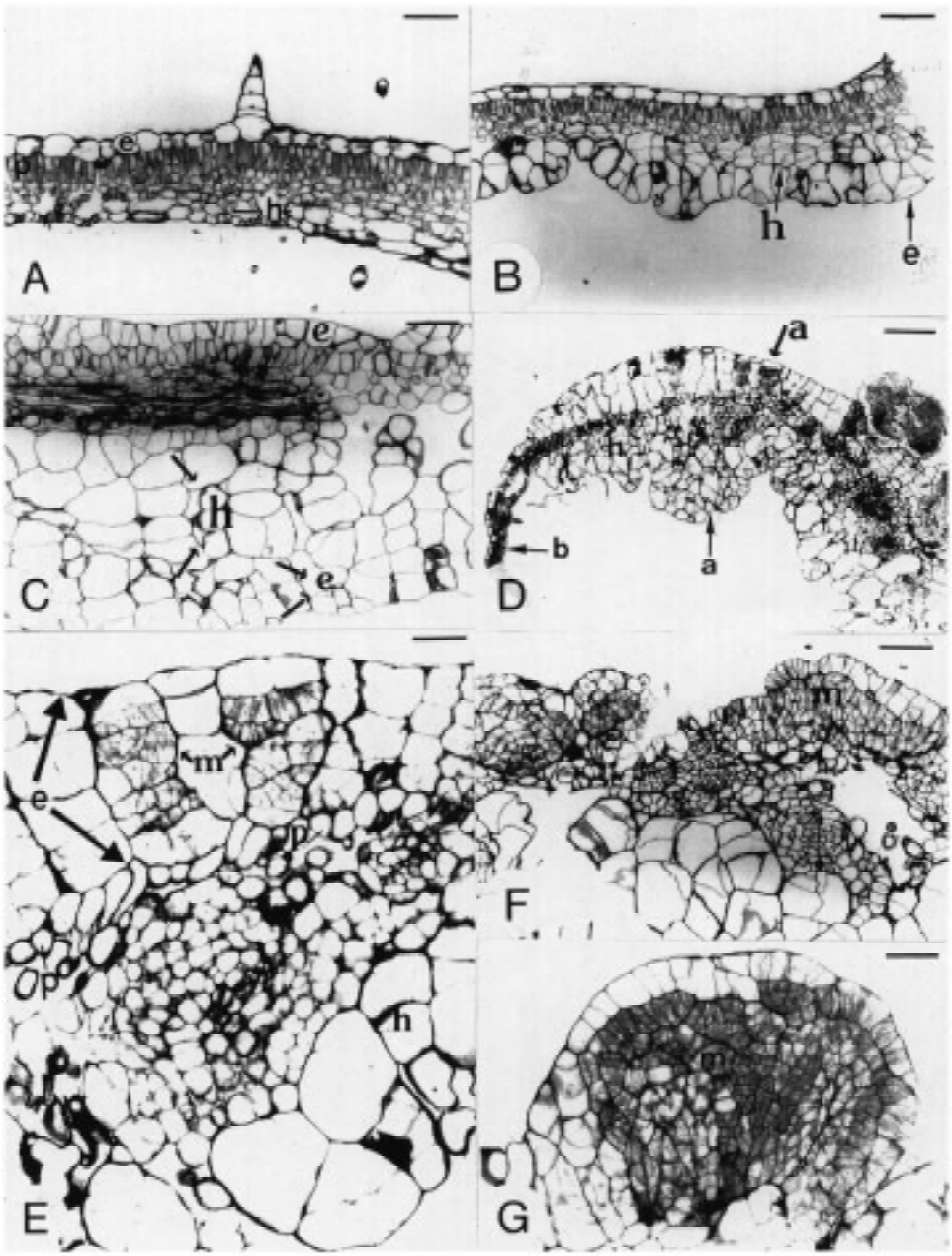

Figure 1. Transverse sections of *Pothomorphe umbelata* (L.) Miq. leaf used as explant. (*A*) At the inoculation time (bar=102.3 μm). (*B*) After 6 days of culture, some epidermal (e) and hypodermal (h) cells dedifferentiated and underwent division (arrows) (bar=204.6 μm). (*C*) After 30 days of culture, the mesophyll became thicker as the number of cell layers increased due to the divisions of the hypodermal (h) and epidermal (e) cells (arrows) (bar=204.6 μm). (*D*) Leaf blade exhibiting two different sectors (a, b) after 35 days of culture (bar=552.4 μm). (*E*) Detail of the sector a; the adaxial epidermis became thicker (e) and the meristemoids (m) originated from some epidemal cells that underwent unequal divisions giving rise to daughter cells with different sizes which exhibited dense cytoplasm, conspicuous nucleus and nucleolus. The palisade tissue (p) consisted of short rounded cells (bar=102.3 μm). (*F-G*) Meristemoid (m) development (bars=204.6 μm).

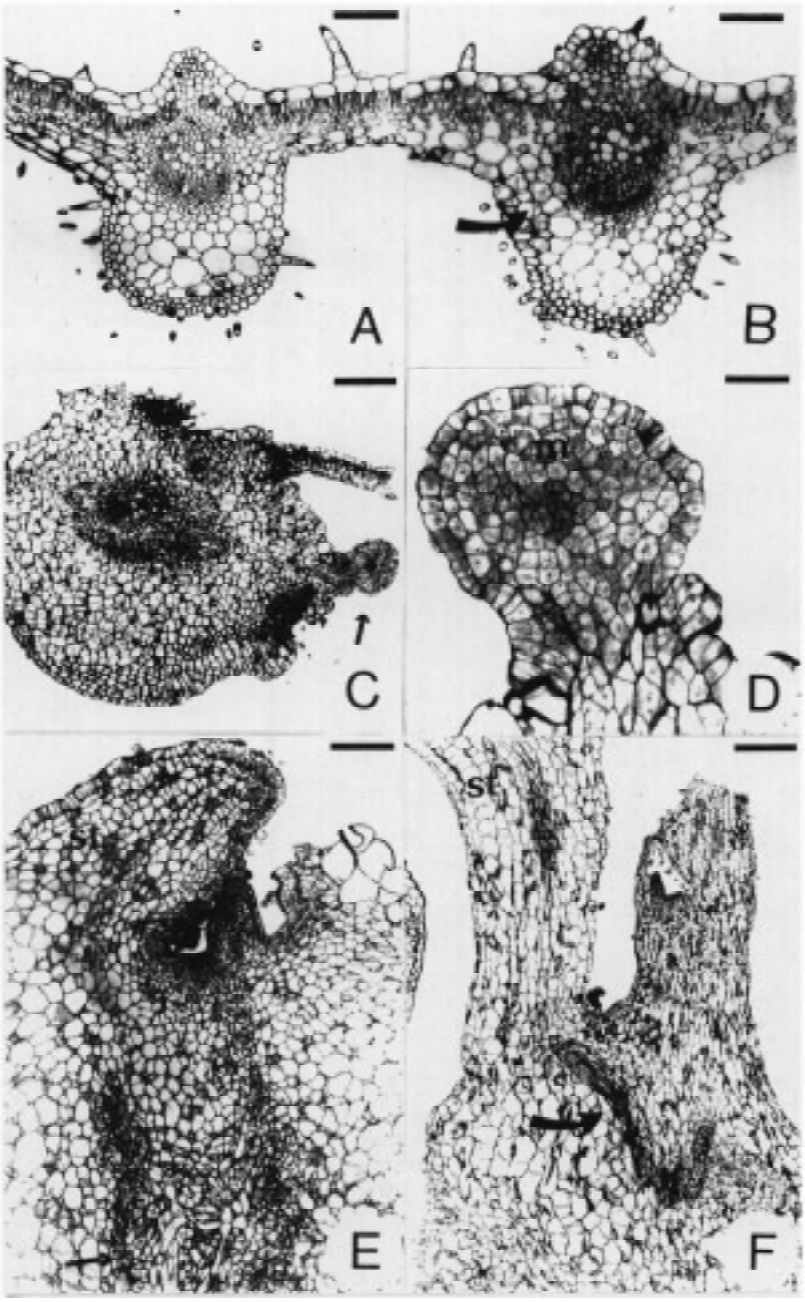

Figure 2. (*A-D*) Transverse sections from midrib of *Pothomorphe umbelata* (L.) Miq. leaf used as explant. (*A*) At the inoculation time (bar=204.6 μm). (*B*) After 6 days of culture, some epidermal and parenchyma cells dedifferentiated and underwent division (arrow) (bar=204.6 μm). (*C*) Meristemoid originated from epidermal and subepidermal cells of the midrib abaxial surface (arrow) after 25 days of culture (bar=552.4 μm). (*D*) Meristemoid (m) consisted of densely cytoplasmic cells (bar=102.3 μm). (*E-F*). Longitudinal sections of the shoot buds after 40 days of culture; vascular connection with the explant tissue (arrows). St=stipule-like structure (bar=204.6 μm and 552.4 μm, respectively).

leaf explants (Bhat et al., 1992, 1995; Kelkar et al., 1996). Johri and Aminuddin (1996) reported somatic embryogenesis in *Piper betle*.

The anatomy of *P. umbellata* leaf explants (Figures 1A and 2A) followed the pattern described by Moraes et al. (1987). The first histological alteration occurred after the sixth day of culture (Figures 1B and 2B). In the leaf blade, the abaxial epidermal cells increased in size and, simultaneously with hypodermal cells, divided in several planes (Figure 1B, arrows). In the midrib (Figure 2B), some cells from the adaxial and abaxial epidermis and from the fundamental parenchyma exhibited dense cytoplasm and conspicuous nucleus and nucleolus (Figure 2B, arrow). Some of these cells underwent division.

Between days 6 and 35 of culture the mesophyll became gradually thicker as the number of cell layers increased due to the divisions of the hypodermal and epidermal cells (Figure 1C, arrows). The palisade tissue that used to consist of narrow and elongated cells (Figure 1A) presented short rounded cells (Figure 1E). These changes were not observed over the entire leaf blade. On Figure 1D, a very differentiated region (section a) can be observed apart from one region that kept characteristics similar to the original explant (section b).

The midrib (Figure 2A) also increased in size due to the pronounced meristematic activity of the fundamental parenchyma and epidermal cells mainly in the abaxial face (Figure 2B-C). By the 25th day of culture, the periphery of the explant showed meristemoids consisting of densely cytoplasmic cells (Figure 2C-D) distributed on the midrib surface, indicating the early stages of shoot bud differentiation.

The meristemoid differentiation on the leaf blade was observed after 35 days of culture. This showed the same histological characteristics as those described for the midrib. All leaf blade meristemoids were from adaxial epidermal cells (Figures 1E-F). These meristemoids, by further cell division, gave rise to small protrusions of tissue (Figure 1G) which gradually became green and organized into a growing point as described in *Cichorium endivia* (Vasil and Hildebrandt, 1966) and in *Passiflora* (Appezzato-da-Glória et al., 1999). After 40 days of culture, the shoot bud development (Figure 2E-F) and its vascular connection with the explant tissue (arrows) was observed. The histological examination revealed that shoots developed *de novo* from superficial layers of tissue and the pattern of shoot bud origin and development was very similar to that previously described for other species in the literature (Mohamed et al., 1992; Malik and Saxena, 1992). According to Hicks (1980), shoot regeneration is found to be direct if there is no callus formation as verified in the present study.

Concurrently with the development of these initial shoot buds, the explant continued its cell division and by the 50th day of culture it was possible to see a mass of callus tissue. At this stage, some adventitious roots primordially originated internally in meristematic zones near the vascularization of the explant. Appezzato-da-Glória (1999) also observed in *Passiflora* that roots arose from meristematic tissue located in the deeper regions of the explant while shoots arose from marginal meristematic areas.

Histological analysis suggests that there is a high frequency of direct morphogenesis from leaf explants of *Pothomorphe umbellata*. Plantlets obtained from direct organogenesis were normal at maturity and showed no phenotypic variations. Overall results show that the established protocol is adequate for both mass propagation and *Agrobacterium*-mediated or direct genetic transformation of the species.

Chromatographic analysis of extracts of *P. umbellata* maintained *in vitro* revealed that micropropagated plantlets were able to produce 4-nerolidylcatechol. The yield of 4-nerolidylcatechol in *in vitro* cultured plantlets was 26.1 mg/g d.w. while plants from seeds accumulated 32.2 mg/g d.w.

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