Revista Ciência Agronômica, v. 41, n. 2, p. 272-278, abr-jun, 2010
Centro de Ciências Agrárias - Universidade Federal do Ceará, Fortaleza, CE
www.ccarevista.ufc.br

Scientific Article
ISSN 1806-6690

Standardized production of *Phyllanthus tenellus* Roxb. by plant tissue culture[1]

Produção padronizada de *Phyllanthus tenellus* Roxb. por cultura de tecidos vegetais

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Abstract - Exigencies as ethic plant raw material are part of the needs of modern phytotherapy. Micropropagation offers opportunities to obtain mass propagation of superior genotypes in short time. This study aimed to develop a protocol of direct and indirect organogenesis of *Phyllanthus tenellus* Roxb. Nodal segments from plantlets obtained by *in vitro* germination were subcultured in modified Murashige and Skoog medium added with different plant growth regulators: IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), GA_3 (3-giberelic acid) and KIN (kinetin). The highest proliferation rate was obtained using the combinations: IBA, KIN + GA_3 (3.5 mg L^{-1}) and IBA + KIN (2.4 mg L^{-1}). Rooting was intensified after 40 days, reaching 100% for all media with indole-3-butyric acid. Addition of 2,4 dichlorophenoxyacetic acid (2,4D) provided the best results for production of friable *calli*. Acclimatization was 100% effective for plantlets cultured in control medium, with decrease in survival rate in grown plantlets from media added with growth regulators.

Key words - Callus. Micropropagation. Medicinal plant.

Resumo - Exigências como matérias-primas vegetais fidedignas fazem parte das necessidades da fitoterapia moderna. A propagação *in vitro* permite a obtenção em massa de genótipos superiores, em curto espaço de tempo. Este estudo teve como objetivo desenvolver um protocolo de organogênese direta e indireta de *Phyllanthus tenellus* Roxb. Segmentos nodais de plântulas obtidas por germinação *in vitro* foram introduzidos em meio Murashige e Skoog adicionado de diferentes reguladores de crescimento vegetal: AIA (ácido indol-3-acético), AIB (ácido indol-3-butírico), AG_3 (ácido giberélico-3) e CIN (cinetina). A maior taxa de proliferação foi obtida utilizando as combinações: AIB, CIN + AG_3 (3,5 mg L^{-1}) e AIB + CIN (2,4 mg L^{-1}). A rizogênese foi intensificada após 40 dias de cultivo, atingindo 100% para todos os meios contendo AIB. A adição de ácido 2,4 diclorofenoxiacético (2,4D) proporcionou os melhores resultados para produção de calos friáveis. A aclimatização foi 100% eficaz para as plantas cultivadas em meio controle, com a diminuição da taxa de sobrevivência para as plantas cultivadas em meios adicionados de reguladores de crescimento.

Palavras-chave - Calogênese. Micropropagação. Planta medicinal.

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[1]Received 14/07/2009; accepted em 14/04/2010
Pesquisa financiada pela Capes/PROEX, FAPERJ. Parte da dissertação de mestrado do primeiro autor IBCCF/UFRJ
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Introduction

Nowadays, there is an increasing interest in the use of medicinal plant, but insufficient information, quality and control of some phytotherapeutical products offered is evident, and may not bring the results expected by professionals and users (VICTÓRIO; LAGE, 2008). Tissue culture techniques enable high efficiency and quick production of ethic and pathogens-free plants; besides provoke a considerable interest as a potential alternative to produce bioactive compounds. Plant growth regulators are fundamental to integrate and regulate plant development and their addition in culture media have been widely employed to improve morphogenesis and plant development (NAMDEO, 2007).

Phyllanthus tenellus Roxb. is a member of Phyllanthaceae family, widely found in several places in Brazil, especially in Rio de Janeiro city (HOFFMANN et al., 2006). This species has been exploited as a source of herb medicines. Popularly known as "quebra-pedra" and "erva-pombinha", in folk medicine, its infusion is used as antibacterial and to treat kidney and urinary bladder disturbances, diabetes and intestinal infections (CALIXTO et al., 1997; LORENZI; MATOS, 2008). Morphological similarity among some *Phyllanthus* species hinders their identification and may explain in part the use of different species in popular medicine for the same purpose (GARCIA et al., 2004). In view of this, the current study aimed to establish a system of standardized production of raw material of *P. tenellus* using different tissue culture techniques to guarantee the supply of ethic and qualified plant material properly identified.

Material and methods

Plant material

Seeds were collected from plants of *Phyllanthus tenellus* Roxb. maintained in a greenhouse at Universidade Federal do Rio de Janeiro (Brazil) and were used as source of plant material to initiate tissue cultures. Voucher specimen was identified and is deposited at the Herbarium of National Museum of Rio de Janeiro (R 200872).

Tissue cultures

After *in vitro* seed germination (VICTÓRIO; LAGE, 2009), nodal segments (0.8-1.0 cm) were excised and inoculated in flasks (141 x 72 mm) containing MS (MURASHIGE; SKOOG, 1962) or MS reduced to half of NH_4NO_3 and KNO_3 solutions (MS½N). Media were supplemented with 3% sucrose, 1.48 μM thiamine-HCl, 2.43 μM pyridoxine, 4.1 μM nicotinic acid, 0.6 mM myo-inositol, pH adjusted to 5.8 ± 0.1, sterilized in autoclave at 120 °C and 1.1 kgf cm^{-2} were employed. To solidify media, 7.8 g L^{-1} of agar was used. Individual clones were selected and used in treatments. Cultures were maintained at 28 ± 2 °C, after previous evaluation of the adequate temperature for *P. tenellus* cultures, and maintained under white light (Sylvania F20 W T12), density of photon flux of 30 μmoles m^{-2} s^{-1}, photoperiod 16 h.

Different plant growth regulators IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), KIN (kinetin), BAP (6-benzylaminopurine) or GA_3 (3-giberelic acid) were tested in the following concentrations in mg L^{-1}: MS and MS½N (controls); MS + IAA 0.2 + KIN 0.5; MS + IAA 0.4 + KIN 0.8 + GA_3 0.8; MS½N + KIN 0.1 and 0.5; MS½N + IAA 0.2 + KIN 0.1; MS½N + IAA 0.2 + KIN 0.5; MS½N + IBA 0.2; MS½N + IBA 0.2 + KIN 0.1; MS½N + IBA 0.2 + KIN 0.5; MS½N + IAA 0.4 + KIN 0.8 + GA_3 0.3; MS½N + IBA 0.2 + KIN 0.8 + GA_3 0.3; MS½N + IBA 0.4 + KIN 0.8 + GA_3 0.3; MS½N + GA_3 0.3.

P. tenellus plantlets were subcultured each 60 days, in control medium for thirteen consecutive subcultures without reveal any significant differences in development. Each treatment consisted of three replicates with at least 25 nodal segments. Plant development was evaluated every 20 days, for two months according to the following parameters: number of shoots per explant; shoot height; number of lateral branches (perennial and deciduous) per shoot and presence of root and callus.

Callus and Root cultures

Leaves and shoot segments from 60-day-old plantlets were inoculated in flasks (72 x 59 mm) containing MS½N added with the following growth regulator, in mg L^{-1}: (0.8 and 1.0) KIN, (0.4) IAA, (0.3) GA_3 and (0.1; 0.5 and 1.0) 2,4D (2,4 dichlorophenoxyacetic acid). Explants were maintained under darkness condition. After 60 days, callogenesis percentage, the major length, color and type (friable or compact) of callus were evaluated. Color and type of callus were analyzed visually.

Root segments were excised from 60-day-old plantlets, and then inoculated in 250 mL erlenmeyer containing 50 mL of liquid MS½N. Treatments consisted of MS½N (control), MS½N + 0.4 mg L^{-1} IAA and MS½N + 1.0 mg L^{-1} 2,4D. Root cultures were maintained on a shaker at 100 rpm for 60 days under the same physical conditions above.

Leaf anatomy analysis

P. tenellus leaves from plantlets cultured in MS½N medium were used in histological analysis. Leaf samples were diaphanized and stained with safranin to verify the leaf blade venation and the stomata types at the

epidermal surfaces. The leaf clearing technique initially involved placing leaves in 50% sodium hypochloride. Leaves were then washed in distillated water for three times and after, they were run through 5% acetic acid for 1 min and 30% ethanol. Lastly, leaves were dyed with safranin (5 min), placed rapidly in 1% chloridric acid and washed in distillated water. The material was prepared on slides with 50% glycerin. Venation description was done according to Hickey terminology (1973).

For observation of leaf structure, plants were fixed in ethanol (70%) during 48 h. Three replicates of leaves and petioles samples from the third node were dehydrated in an ethanol series (80; 90 and 95% each hour), infiltrated and embedded in basic Historesin (Leica Microsystems, Germany) in eppendorf tubes (1 mL) and sectioned with rotary microtome (820 Spencer Microtome, American Optical Corporation, USA). Leaf sections of 8 μm thickness were stained in toluidine blue (0.05%) and prepared on permanent slides. The stained sections were examined and photographed by a Carl Zeiss optical microscope (model 4746.20-990).

Acclimatization

Sixty-day-old plantlets were transferred to commercial soil in seed plots of isopor with 9 cm^2 area, and kept in a greenhouse. Plants were protected from direct water with a transparent plastic film for 1 month. Survival percentage and plant height were evaluated after 30 days. After growth regulator treatment, some plantlets were subcultured in MS½N for eliminating any growth regulator residues and then be acclimatized.

Statistical analysis

Each experiment was done in a completely randomized design. Data were subjected to analysis of variance (ANOVA) and statistical average comparisons were made by Tukey's test at the 5% significance level. To infer if exist difference between two percentages; the Student's test ($P < 0.05$) was applied.

Results and discussion

The effects of plant growth regulators in plantlets development, in callogenesis, in root cultures; and, leaf anatomy description of *P. tenellus* leaves were the main results achieved in this research.

A simple reduction of salt solutions NH_4NO_3 and KNO_3 of MS improved rooting and plant development, besides to avoid glassy aspect of *P. tenellus* cultures (Table 1). Hyperhydricity is related to nitrate concentration (HAZARIKA, 2006).Comparing MS and

Table 1 - Morphogenic responses of *Phyllanthus tenellus* Roxb. plantlets under different growth regulators, after 60 days of culture

Media	Plant growth regulators (mg L^{-1})				Rooting	Number	Shoot height	Number of	Callus
	IAA	IBA	KIN	GA_3	(%)	of shoots	(cm)	branches /shoot	(%)
MS	-	-	-	-	31 a	1.5 ± 0.9 a	2.0 ± 1.2 a	6.2 ± 2.7 a	-
	0.2	-	0.5	-	15 a	1.6 ± 0.7 a	2.1 ± 1.3 a	5.3 ± 2,4 a	97 a
	0.4	-	0.8	0.3	22 a	1.7 ± 1.1 a	1.1 ± 0.5 a	2.0 ± 1.3 b	83 ab
MS½N	-	-	-	-	91 b	1.6 ± 0.8 a	2.2 ± 1.6 a	5.1 ± 2,4 a	-
	-	-	0.1	-	77 cd	1.2 ± 0.5 a	3.1 ± 1.9 a	5.8 ± 1.9 ac	4.1 c
	-	-	0.5	-	91 bd	1.6 ± 0.9 a	2.5 ± 1.4 a	5.9 ± 2.0 ac	-
	0.2	-	0.1		70 c	1.1 ± 0.3 a	2.6 ± 1.6 a	5.8 ± 1.8 ac	20 a
	0.2	-	0.5	-	81 bd	1.5 ± 0.6 a	4.4 ± 2.3 b	5.8 ± 2.5 ac	73 a
	-	0.2	0.1		100 b	1.3 ± 0.5 a	2.7 ± 1.2 a	5.8 ± 2.5 ac	88 a
	-	0.2	0.5	-	100 b	3.2 ± 1.6 bc	6.9 ± 4.5 c	4.8 ± 2.3 a	89 a
	-	0.2	-	-	100 b	1.2 ± 0.4 a	3.9 ± 2.0 a	7.4 ± 3.5 c	-
	0.4	-	0.8	0.3	28 a	2.4 ± 2,4 a	2.3 ± 1.9 a	3.2 ± 1.9 b	97 a
	-	0.2	0.8	0.3	100 b	3.5 ± 1.8 b	4.6 ± 3.2 b	4.8 ± 2.0 a	100 a
	-	0.4	0.8	0.3	100 b	2.7 ± 1.5 c	4.2 ± 2.2 b	4.9 ± 2.4 a	98 a
	-	-	-	0.3	53c	1.7 ± 0.9 a	2.3 ± 0.8 a	5.2 ± 1.5 a	9.4 c

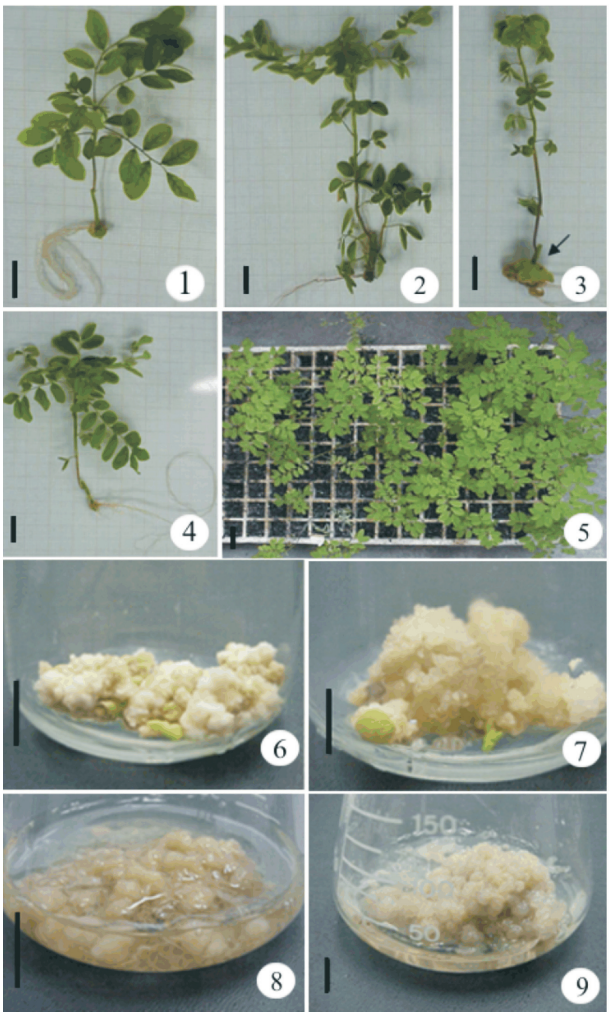
Different letters denote statistical differences ($p<0.05$, Tukey`s test). The results are the average ± SD. IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; KIN, kinetin; BAP, 6-benzylaminopurine; GA_3, 3-giberelic acid; MS, Murashige & Skoog

MS½N, the same combination of IAA 0.2 mg L^{-1} and KIN 0.5 mg L^{-1} induced significant differences in shoot height and rooting. Previous researches with other medicinal species so as *Calendula officinalis* also showed that reduced concentration of NH_4NO_3 and KNO_3 of MS salts resulted in better lateral shoot production and rooting (VICTÓRIO et al., 2008).

Additions of KIN and IBA or KIN, IBA and GA_3 in medium improved the best proliferation rate and shoot elongation in *P. tenellus* (Table 1), that are important parameters to consider in large-scale production. Media without growth regulators produced plantlets with satisfactory height and rooting to acclimatization, however the regenerative incidence of shoots per explant was low. The use of growth regulators may induce about 2.5 times more number of shoots than control medium (MS½N). KIN plus IBA and GA_3 improved shoot number production of *P. tenellus* in about 50%. The maximum production of shoots per explant was 3.5 and 3.2 in the media containing the following growth regulators 0.2 mg L^{-1} IBA, 0.8 mg L^{-1} KIN and 0.3 mg L^{-1} GA_3 or 0.2 mg L^{-1} IBA and 0.5 mg L^{-1} KIN, respectively. Better results were observed when growth regulators were used in combinations. A higher level of KIN enhanced adventitious root formation. Still, for KIN and IBA combination, the increasing in KIN concentration up to 0.5 mg L^{-1} improved shoot number from 1.32 to 3.21 (Table 1 and Figure 1 and 2). Bhattacharyya e Bhattacharya (2001) verified that KIN and IAA in combination were more efficient than BAP to induce new shoots per explant in *P. amarus* cultures. In tissue cultures of *P. caroliniensis*, KIN induced higher number of shoots than BAP at same concentrations (CATAPAN et al., 2001).

Combination of KIN, IAA and GA_3 resulted in reduced rooting and shooting, probably due to auxin type and its concentration since replace IAA to IBA (0.2 and 0.4 mg L^{-1}) improved rooting, number of shoots and the growing in height of *P. tenellus* plantlets. The smallest concentration of IBA was enough to improve rooting of *P. tenellus* in 100% (TAB. 1 and FIG. 3 and 4), despite of 98% incidence of basal callus. Rooting was intensified from 40 days of culture. *P. tenellus* plantlets showed fragile aspect when compared with *ex vitro* plants probably due to anatomical and physiological epigenetic alterations resulted of the *in vitro* conditions. The fragile aspect was characterized by thick stems and small leaves that easily fall. Fragility has been reported as an inherent feature of plantlets obtained by tissue culture techniques as result of low light intensity and changes of abiotic factors under *in vitro* condition.

The addition of any growth regulators in media induced basal callus in *P. tenellus* plantlets. In the same way, IAA (1.0 and 3.0 mg L^{-1}) or KIN (3.0 mg L^{-1}) inhibited the shoot regeneration and increased callogenesis process in tissue cultures of *P. amaro* (BHATTACHARYYA; BHATTACHARYA, 2001). Studies with *P. stipulatus* have resulted in 100% of callogenesis using from 1.25 to 5.0 µM of KIN, while the same concentrations of IAA or IBA did not induce callogenesis in *P. caroliniensis* (CATAPAN et al., 2001). *In vitro* flowering was also observed under growth regulator effects (VICTÓRIO; LAGE, 2009).



Figures 1-9 - *Phyllanthus tenellus* Roxb. raw material. 1. KIN 0.2 mg L^{-1}. 2. KIN 0.5 mg L^{-1}. 3. KIN 0.2 mg L^{-1} + IBA 0.1 mg L^{-1} (detail to basal callus). 4. IBA 0.2 mg L^{-1}. 5. Thirty-day-old acclimatized plants. 6. Callus obtained from leave culture in MS with addition of 2,4D. 7. Callus obtained from nodal segment, treated with 2,4D. 8. Root culture in MS (control) medium. 9. Root culture in 2,4D. Bar = 1 cm

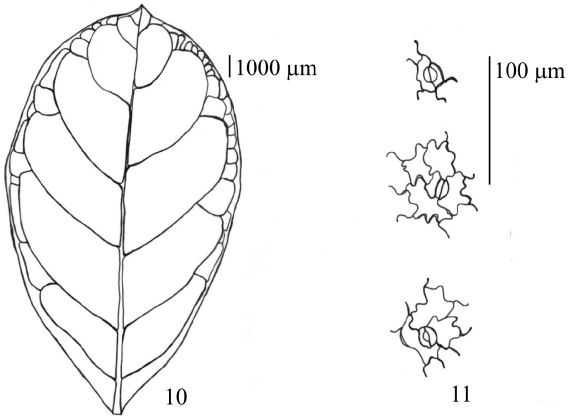
Acclimatized plantlets from IBA, KIN and GA_3 or KIN and IBA media showed 81.9% and 13.6% survival

respectively, after 30 days of transplantation to field. The low survival may be due to basal callus, short shoots and ineffective rooting. Plantlets cultured in medium added of auxin, cytokinin and gibberellin achieved 8.1 ± 3.7 cm height, while plantlets from medium contained cytokinin and auxin reached 4.0 ± 1.0 cm. Sixty-day-old plantlets cultured in control medium (MS½N) showed 100% of adaptation to the *ex vitro* environment (Figure 5). These plantlets achieved the height of 11.0 ± 5.6 cm. Flowers and fruits were verified in all plants after one month of acclimatization. Within 50 days of acclimatization, plants should be transferred to soil.

Callus production was achieved from 20 day of culture and it was intensified during 60 days from nodal segment and leaf explants. IAA and GA_3 induced the formation of small and compact callus of *P. tenellus* in white color (Table 2). Callogenesis reached 100% in medium with addition of 1.0 mg L^{-1} 2,4D in both types of explants (Figure 6 and 7). 2,4D is generally related to the callogenesis induction in culture of different species by promoting high cell division rate with consequent differentiation of the plant tissues. The addition of 2,4D at 0.1 or 1.0 mg L^{-1} concentration stimulated shoot development and rooting in darkness. The use of 2,4D resulted in friable callus in green, brown, gray or yellow colors (Table 2). At 1.0 mg L^{-1} callogenesis was not effective, while 0.8 mg L^{-1} KIN concentration induced white and compact *calli*. Color changes may be related to secondary metabolites production. The culture condition established in the current work induced the formation of friable fast-growing *calli* from nodal segment explants resulting in a suitable system for *in vitro* biomass and metabolite production and a resource to initiate suspension cell cultures.

With respect to root cultures, it was observed the production of friable callus as cited by Victório and Lage (2007). In addition, several small nodules were observed in roots (Figure 8 and 9).

Morphological and anatomical descriptions are the first parameters of botanic authentication for guarantee quality control of herbal medicines. Such characteristics are considerable aspects to confirm *Phyllanthus* species and to contribute to raw material supplementation for phytotherapics. In order to certificate *P. tenellus* species, the leaves were analyzed in their morphology and anatomy. Leaf venation of *P. tenellus* is pinnate type, camptodromous pattern, characterized by gradual decreasing in secondary venations in the leaf apical section (Figure 10). The leaf is amphistomatic and the greatest concentration of stomata occurred on the abaxial surface. The following stomata types were observed, paracytic, anisocytic and anomocytic (Figure 11). According to Metcalfe (1963) the occurrence and distribution of the stomata types present high value in taxonomic identification. Transverse sections from leaf blade showed uniestratified epidermis (Figure 12 and 13). The pattern of leaf epidermis and parenchymas were similar of those revealed to *ex vitro* plants (Garcia et al., 2004). Palisade parenchyma is unistratified and the spongy parenchyma



Figures 10-11 - Sixty-day-old diaphanized leaf of *Phyllanthus tenellus* Roxb. plantlets. 10. Leaf venation: pinnate type with camptodromous pattern. 11. Stomata types in both sides (up to down): paracytic, anisocytic and anomocytic

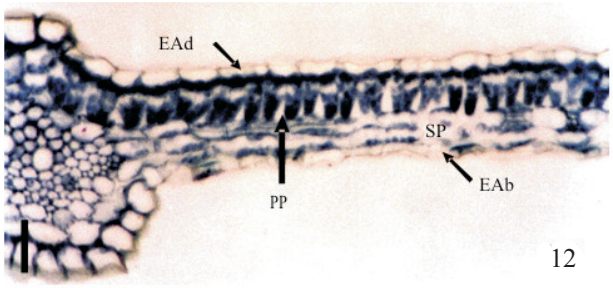
Table 2 - Callogenesis obtained from nodal segments of *Phyllanthus tenellus* Roxb. after 60 days in culture on MS medium supplemented with 2,4D

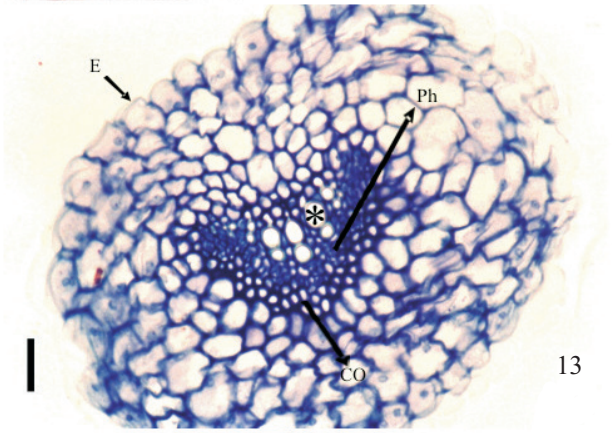
Media (2,4D mg L^{-1})	%	Length$^{\kappa}$ (cm)	Aerial part	Root	Color	Type	
						F	C
0.1	100 a	1.6 ± 0.5 a	x	x	Y	x	x
0.5	100 a	1.9 ± 0.6 a	-	-	B/G	x	-
1.0	100 a	1.8 ± 0.5 a	xγ	xγ	G/LY	x	-

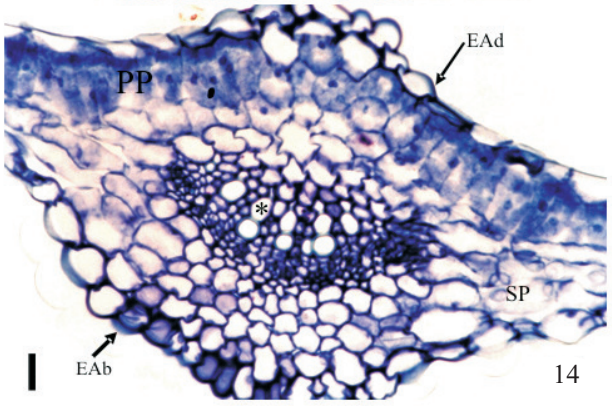
F, friable; C, compact; Y, yellow; B, brown; G, gray; LY, light yellow. Different letters denote statistical differences ($p<0.05$, Tukey`s test). $^{\gamma}$Only one callus. $^{\kappa}$Major length of callus. 2,4D, 2,4 dichlorophenoxyacetic

consists of two layers of cells. The main vein in transverse section is biconvex. The petiole showed cylindrical transversal section and parenchymatic tissue involves the central vascular system in arc.

Idioblasts containing monocrystals were observed in the palisade parenchyma in agreement with anatomy of *ex vitro* plants (ALQUINI; TAKEMORI, 2000). Even from triturated plant material the crystals and stomata are references to identify this species through leaf anatomy analyses.







Figures 12-14 - Transverse sections of 60-day-old *Phyllanthus tenellus* Roxb. leaves obtained from tissue culture. 12. Transverse section of foliar blade: epidermis adaxial (E Ad), epidermis abaxial (E Ab), palisade parenchyma (PP) and spongy parenchyma (SP). 13. Transverse section of petiole: epidermis (E), collenchyma (CO), xylem (*), phloem (Ph). 14. Transverse section of the main median nervure. Bar = 20 µm

Conclusion

P. tenellus is a widely medicinal herb used in Brazil. The large number of species of *Phyllanthus* genus and their similarity is a disturbed and a risk in folk medicine. As result of the present study, *in vitro* systems permit the standardized propagation of *P. tenellus* raw material under controlled and aseptic conditions showing the relevance of this study together with anatomical pattern of this species as a certificate of safe and quality. Furthermore, the establishment of different tissue culture techniques reveals other alternatives to acquire therapeutic metabolites from *P. tenellus* under aseptic and controlled conditions.

Acknowledgements

This research was supported by CAPES, FAPERJ and Laboratório Simões Ltda**.**

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