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In vitro morphogenesis and cell suspension culture establishment in *Piper solmsianum* C. DC. (Piperaceae)

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RESUMO – (Morfogênese *in vitro* e estabelecimento de culturas de suspensão celular em *Piper solmsianum* C. DC. (Piperaceae)). *Piper solmsianum* é uma espécie herbácea do sudeste brasileiro onde vários compostos biologicamente ativos já foram identificados. O objetivo deste trabalho foi estabelecer suspensões celulares nesta espécie. Para tanto, foram utilizados explantes de pecíolos e folhas, retirados de plântulas cultivadas *in vitro*, os quais foram submetidos a diferentes combinações de reguladores de crescimento (AIA, ANA, 2,4-D e BAP). Foi obtida a neo-formação de raízes e brotos, estes últimos através do processo de organogênese indireta evidenciada por estudos histológicos. Para a indução e crescimento dos calos, foram avaliados, além das diferentes combinações de reguladores de crescimento, a suplementação ao meio de cultura de carvão ativado (1,5 $mg.l^{-1}$) e o regime de luz. Culturas mantidas na luz, em meio de cultura suplementado com 0,2 $mg.l^{-1}$ 2,4-D e 2 $mg.l^{-1}$ BAP e sem carvão ativado, resultaram em maior crescimento (massa fresca) dos calos. A partir destes calos, foram obtidas suspensões celulares, cuja dinâmica de crescimento e acúmulo de metabólitos foi estudado. Os resultados obtidos deverão ser utilizados para a caracterização de rotas biosintéticas em culturas *in vitro* em *P. solmsianum.*

Palavras-chave: calogênese, morfogênese, pariparoba, reguladores de crescimento, suspensões celulares

ABSTRACT – (*In vitro* morphogenesis and cell suspension culture establishment in *Piper solmsianum* C. DC. (Piperaceae)). *Piper solmsianum* is a shrub from Southeast Brazil in which many biologically active compounds were identified. The aim of this work was to establish a cell suspension culture system for this species. With this in mind, petiole and leaf explants obtained from *in vitro* plantlets were cultured in the presence of different plant growth regulator combinations (IAA, NAA, 2,4-D and BA). Root and indirect shoot adventitious formation, detected by histological analysis, was observed. Besides the different combinations of plant growth regulators, light regime and the supplement of activated charcoal (1.5 $mg.l^{-1}$) were tested for callus induction and growth. Cultures maintained in light, on a 0.2 $mg.l^{-1}$ 2,4-D and 2 $mg.l^{-1}$ BA supplemented medium, and in the absence of activated charcoal, showed the highest calli fresh matter increment. From a callus culture, cell suspension cultures were established and their growth and metabolite accumulation studied. The achieved results may be useful for further characterization of the activated secondary metabolites pathways in *in vitro* systems of *P. solmsianum*.

Key words: callogenesis, cell suspension culture, morphogenesis, pariparoba, plant growth regulators

Introduction

The Piperaceae family comprises 14 genera and ca. 1,950 species, of which the genus *Piper* is the largest, with more than 600 species described worldwide (Danelutte *et al.* 2003). Besides being of high commercial and economical importance, *Piper* species are medicinally used in different ways, such as in the Indian Ayurvedic system of medicine and in the folklore medicine of Latin America and the West Indies (Parmar *et al.* 1997). *Piper* species produce many biologically active compounds, including amides, flavanones, alkaloids, propenylphenols, lignans, neolignans, benzoic acids and chromenes (Parmar *et al.* 1997; 1998; Navickiene *et al.* 2003; Silva *et al.* 2002; Danelutte *et al.* 2003; Martins *et al.* 2003; Lago *et al.* 2004).

Piper solmsianum is a shrub measuring 1-3 meters tall, commonly found in Southern Brazil. Phytochemical studies revealed the presence of monoterpenes, sesquiterpenes, arylpropanoids, phenylpropanoids, lignans and neolignans, most of which showing biological activity against bacteria, fungus and the trypomastigote form of *Trypanossoma cruzi* (Moreira *et al.* 1995; Martins *et al.* 2000; 2003; Campos *et al.* 2005; 2007).

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In vitro propagation of medicinal plant species has the advantage of compound production under controlled conditions, which means that cells of any plant can be easily multiplied to yield their specific metabolites (Vanisree *et al.* 2004). Some *Piper* cultures have already been established and their morphogenetic potential investigated, such as for *Piper longum* (Bhat *et al.* 1992; 1995; Soniya & Das 2002), *Piper nigrum* (Mathews & Rao 1984; Phillip *et al.* 1992), *Piper colubrinum* (Kelkar & Krishnamurthy 1998) and *Piper methysticum* (Briskin *et al.* 2001; Smith *et al.* 2002). High-frequency regeneration through somatic embryogenesis was achieved for *P. nigrum* and *P. colubrinum* (Joseph *et al.* 1996; Yusuf *et al.* 2001; Nair & Gupta 2006).

Plant cell cultures have been actively studied as a potential source of high-value biological compounds (Edahiro & Seki 2006). The major advantages of a cell culture system over the conventional cultivation of whole plants are: useful compounds can be produced under controlled conditions independent of climatic changes or soil conditions; cultured cells would be free of microbes and insects; the cells of any plants could easily be multiplied to yield their specific metabolites; automated control of cell growth and rational regulation of metabolite processes would reduce of labor costs and improve productivity (Vanisree *et al.* 2004). Establishment of cell suspension cultures capable of producing medicinal compounds at a rate similar or higher than intact plants have accelerated over the last few years, mostly due to optimization of the cultural conditions, selection of high-producing strains and employment of precursor feeding, transformation methods and immobilization techniques (Vanisree *et al.* 2004).

Besides the use for large-scale metabolite production, cell suspension culture technique may be employed for studying important biosynthetic pathways. In order to obtain high yields suitable for commercial exploitation, efforts have focused on studying the biosynthetic pathways of biologically active compounds through cell suspension culture (Dicosmo & Misawa 1995). Recently, Danelutte *et al.* (2005) carried out the only study aimed at establishing cell suspension cultures in Piperaceae and an investigation of the associated secondary metabolites in *Piper cernuum* and *Piper crassinervium*.

Although the recent findings on the vast quantity of biologically active compounds accumulated in *P. solmsianum* plants, there are no reports on *in vitro* propagation of this species. Thus, the aim of this study was to establish cell suspension cultures for further investigation on the biosynthesis of secondary metabolites in batch systems for this species.

Materials and methods

Establishment of aseptic cultures – Seeds of *Piper solmsianum* C.DC. were collected from the Parque Estadual da Serra do Mar, Ubatuba, Brazil. Seeds were surface sterilized in a solution of 1% sodium hypochlorite plus one drop of detergent for 20 min, followed by 30 s immersion in 70% ethanol. The material was rinsed five times with sterile, distilled water in a clear air cabinet, three to five surface-sterilized seeds were placed into test tubes containing culture medium.

Media and culture conditions – All cultures were maintained in a culture growth-room at 26 ºC, under a photosynthetic photon flux of 35 $\mu mol.m^{-2}.s^{-1}$ provided by cool white fluorescent tubes and photoperiod of 16 h. The materials grown in the dark were cultured in the same growth-room at 26 ºC. A MS (Murashige & Skoog 1962) basal culture medium, supplemented with 30 $g.l^{-1}$ of sucrose and 2 $g.l^{-1}$ of Phytagel (Sigma®), was used in all the steps described herein. The pH was adjusted to 5.7 prior to autoclaving at 121 ºC for 20 min.

Plantlet mass propagation – Prior to the study of *P. solmsianum* morphogenesis, *in vitro* mass propagation was carried out through axillary bud propagation. First and second axillary buds emerging from germinated plantlets were excised, vertically placed onto the basal hormone-free media and sub-cultured after 45 days of growth. This strategy permitted both obtaining large quantities and achieving a synchronized growth of the plantlet culture for the study of morphogenetic responses in this species.

Morphogenetic potential study – The two youngest leaves (10 mm of diameter) and petioles (10 mm) were excised from 45 days old *in vitro* grown plantlets. Petiole and leaf explants were horizontally placed onto the culture medium. Leaf discs were placed with the abaxial side in contact with the culture medium. In order to investigate *P. solmsianum* morphogenetic potential, explants were cultured in a basal medium containing different combinations (0, 0.2 and 2 $mg.l^{-1}$) of the auxins 3-indolyl-acetic acid (IAA), 1-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and the cytokinin 6-benzylaminopurine (BA). In each treatment 10 repetitions with 5 explants for each experimental unit were tested. Explants were cultivated in the 16 h photoperiod condition for 30 days, and then analyzed by observing achieved morphogenetic responses.

Histological analysis – Explants from treatments that resulted in shoot differentiation from the morphogenetic potential study, were fixed in 0.2 M phosphate buffer: 2.5% paraformaldehyde solution (1:1) for 24 h. Material

was washed in phosphate buffer for 30 min and dehydrated by passing through a graded series of ethanol. Infiltration was carried out with ethanol: historesin (Leica®) (1:1) for 24 h, followed by 100% historesin for 2-3 h. Serial sections of 6µm were cut and stained with 0.5% toluidine blue O in 50% ethanol for 1 min, and then examined under a light microscope.

Establishment of callus and cell suspension cultures – For the establishment and optimization of callus culture conditions, petioles were inoculated into test tubes containing the MS basal medium and treatments that induced callogenesis. In each treatment 10 repetitions with 5 explants for each experimental unit were tested. The effect of light/dark regime, activated charcoal (1.5 mg.l^{-1}) supplement and growth regulator (2,4-D and BA) were analyzed through fresh matter (FM) increment after 30 days of culture. Data were analyzed by ANOVA and means were submitted to a 5% significance Student-Newman-Keuls (SNK) test. Cell suspension cultures were initiated by culturing callus in Erlenmeyer flasks containing the callus-optimized culture medium without Phytagel (Sigma®). Sedimented cell volume (SCV) and dry matter were determined during a culture cycle (42 days), in order to analyze both cell suspension and callus growth, respectively. Mean and standard error were applied to analyze the data.

Metabolite extraction in suspension cultures – Cells were isolated from culture medium (15 flasks), using 0.2 µm cellulose acetate filters (Sartorius®), after 15 and 40 days of culture and stored at -80 ºC prior to extraction. The lyophilized cells (200 mg) were ground to a fine powder under liquid nitrogen and then metabolites were extracted using a dichloromethane: methanol (2:1) solution for 30 min. Extract was concentrated on rotoevaporator. Metabolites in the culture medium (750 ml) were three-times extracted in ethyl acetate and then solvent was removed under vacuum using a rotoevaporator. Crude extracts were passed through reversed-phase Sep-Pak C18 cartridges prior to HPLC analysis.

HPLC analysis – Aliquots (20 µl) of stored samples were analyzed by HPLC using a 5 µm reverse phase column (Supelcosil C18, Supelco®). Mobile phase was constituted of acetonitrile in water and the gradient of acetonitrile was programmed to 30% over the first 8 min, from 30% to 100% between 8 and 35 min and 100% between 35 and 45 min, with 1 ml min^{-1} flow. The content was determined using a ultra-violet/visible detector at 254 nm.

Results and discussion

Germination of *Piper solmsianum* achieved approximately 35 and 80% after 4 and 8 weeks of culture, respectively. The establishment of *Piper* cultures is frequently slow and difficult. Fungal and bacteria contamination usually hampers culture establishment (Phillip *et al*. 1992; Bhat *et al*. 1995; Kelkar & Krishnamurthy 1998). However, in *P. solmsianum* only 10% of the inoculated seeds showed pathogen contamination. Axillary bud-mass propagation in a hormone-free MS basal medium resulted in plantlet formation, one plantlet for each axillary bud inoculum, and allowed for obtaining high quantities of plantlets to be used in morphogenesis investigation.

The morphogenetic potential of *P. solmsianum* in an MS basal medium supplemented with different concentrations of IAA, NAA, 2,4-D and BA, is summarized in Tab. 1. Petiole explants were the most responsive and besides callus formation, shoot organogenesis and rhizogenesis were also observed (Fig. 1).

Table 1. Responses of *Piper solmsianum* C. DC. explants to combinations of BA with IAA, NAA or 2,4-D after 30 days of culture. N = no response; R = root formation, S = shoot formation, C = callus formation; +++ more than 15 buds per explant; ++ 6-14 buds per explant; + 1-5 buds per explant.

	BA (mg.l^{-1})	IAA (mg.l^{-1})			NAA (mg.l^{-1})		2,4-D (mg.l^{-1})	
		0	0.2	2	0.2	2	0.2	2
Petiole	0	N	R (++)	R (++)	N	R (++)	C	C
	0.2	N	S (+)	S (+)	S (++)	S (+)	C	C
	2	N	N	S (++)	S (+++)	S (+)	C	C
Leaf	0	N	R (+)	R (++)	N	R (++)	R (+)	N
	0.2	N	S (+)	S (++)	N	N	S (+++)	C
	2	N	S (++)	S (++)	N	S (++)	S (+++)	S(+++)

Root formation was observed only in the absence of BA. IAA treatments resulted in rhizogenesis in both 0.2 and 2 mg.l^{-1} concentrations, whereas in NAA treatments, this morphogenetic response was only observed in the 2 mg.l^{-1} concentration (Tab. 1). Media supplemented with the synthetic auxin 2,4-D were inefficient for root formation. Only leaf explants treated with 0.2 mg.l^{-1} 2,4-D resulted in rhizogenesis.

Shoot organogenesis was the most frequently observed morphogenetic response (Tab. 1). Bhat *et al.* (1995) observed high regeneration rates using different explants in *P. longum*, *P. betle* and *P. nigrum*. After thirty days of culture, shoot organogenesis was observed in thirty-eight percent of the treatments. The application of both auxin and cytokinin regulators was essential for this response. In *P. solmsianum*, BA was essential for shoot induction, although this regulator is not essential for other *Piper* species (Mathews & Rao 1984; Philip *et al.* 1992; Bhat *et al.* 1992; 1995; Aminuddin-Johri

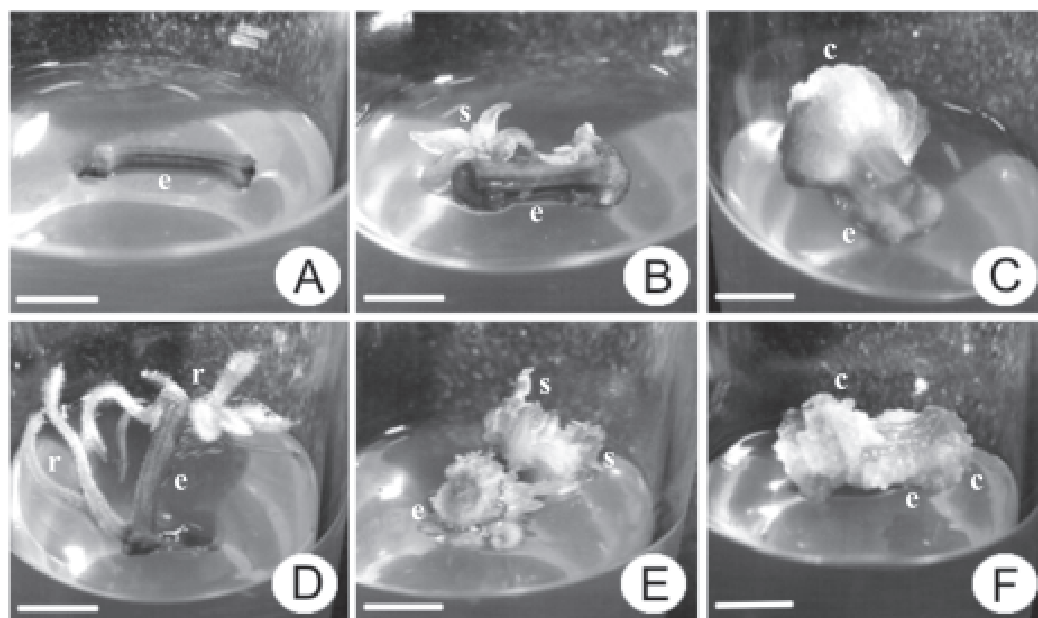

Figure 1. Responses of *in vitro Piper solmsianum* C. DC. petiole explants after 30 days of culture. A. Control. B. 2 mg.l^{-1} BA and 2 mg.l^{-1} IAA. C. 2 mg.l^{-1} BA and 0.2 mg.l^{-1} 2,4-D. D. 2 mg.l^{-1} IAA. E. 2 mg.l^{-1} BA and 0.2 mg.l^{-1} NAA. F. 0.2 mg.l^{-1} BA and 0.2 mg.l^{-1} 2,4-D. C, R, S - callus, root and shoot formation, respectively; E - petiole explant. Bars = 3.4 cm.

et al. 1993; Kelkar *et al.* 1996; Kelkar & Krishnamurthy 1998). In leaf explants, all IAA and BA combinations resulted in shoot organogenesis, whereas only 2 mg.l^{-1} NAA plus 2 mg.l^{-1} BA treatments resulted in this morphogenetic response. In 2,4-D treatments, adventitious shoot formation was observed in 2 mg.l^{-1} BA concentration treatments and in 0.2 mg.l^{-1} 2,4-D plus 0.2 mg.l^{-1} BA combinations. In petiole explants, shoot organogenesis was observed in all auxin and cytokinin combinations. Nevertheless, the synthetic auxin 2,4-D did not induce root or shoot regeneration in these explants.

Preferential regions of tissue differentiation were observed during *P. solmsianum* morphogenetic potential investigation (Fig. 1). In petioles, root adventitious formation at the cut end of the explant was very evident and is probably associated with the presence and distinct distribution of endogenous regulators. In *P. longum* this process was detected throughout vessels and in the proximal region of leaf explants (Soniya & Das 2002; Kelkar & Krishnamurthy 1998).

Histological analysis revealed that adventitious shoot formation was achieved through indirect organogenesis (Fig. 2A). The 30-day cultured leaf and petiole explants showed the formation of elongated, vacuolated and loosely arranged tissue, characteristic of friable calluses, at the end of the cut section (Fig. 2A-B). Cylindrical meristemoids were observed at the inner part of this tissue (Fig. 2A-B), while the outer cells of calluses underwent division, leading to the formation of densely cytoplasmatic isodiametric cells with pro-eminent and enlarged nuclei (Fig. 2B-C). Notch-like structures were formed in these meristematic regions, as has been described for *P. longum* indirect shoot-formation (Bhat *et al.* 1995), which subsequently resulted in the formation of shoot buds (Fig. 2C). Leaf primordia were also seen around the developing shoot- apex (Fig. 2D).

In plants, both auxins and cytokinins are necessary for cell transition from G1 to S cell cycle phase and from G2 to M cell cycle phase (Stals & Inzé 2001). In *P. solmsianum*, callus formation was observed in all petiole explants treated with 2,4-D. However, in leaf-explants a small brown callus was only observed in the 0.2 mg.l^{-1} BA plus 2 mg.l^{-1} 2,4-D treatments, suggesting differences in sensitivity between the explants used. In order to obtain white, friable, fast growing calluses, the light/dark regime and supplementation with activated charcoal were studied for callus formation from petiole explants, together with the different combinations of growth regulators that induced callogenesis. Light-effect is one of the most important factors in plant morphogenesis (Morini *et al.* 2000). In *P. solmsianum*, light-grown materials revealed FM increment than did that from the dark condition (Fig. 4). Rapid medium and explant browning is common during the establishment of *Piper* cultures, and is mainly due to the presence of phenolics that under oxidation produce growth inhibitor polyphenols and quinones

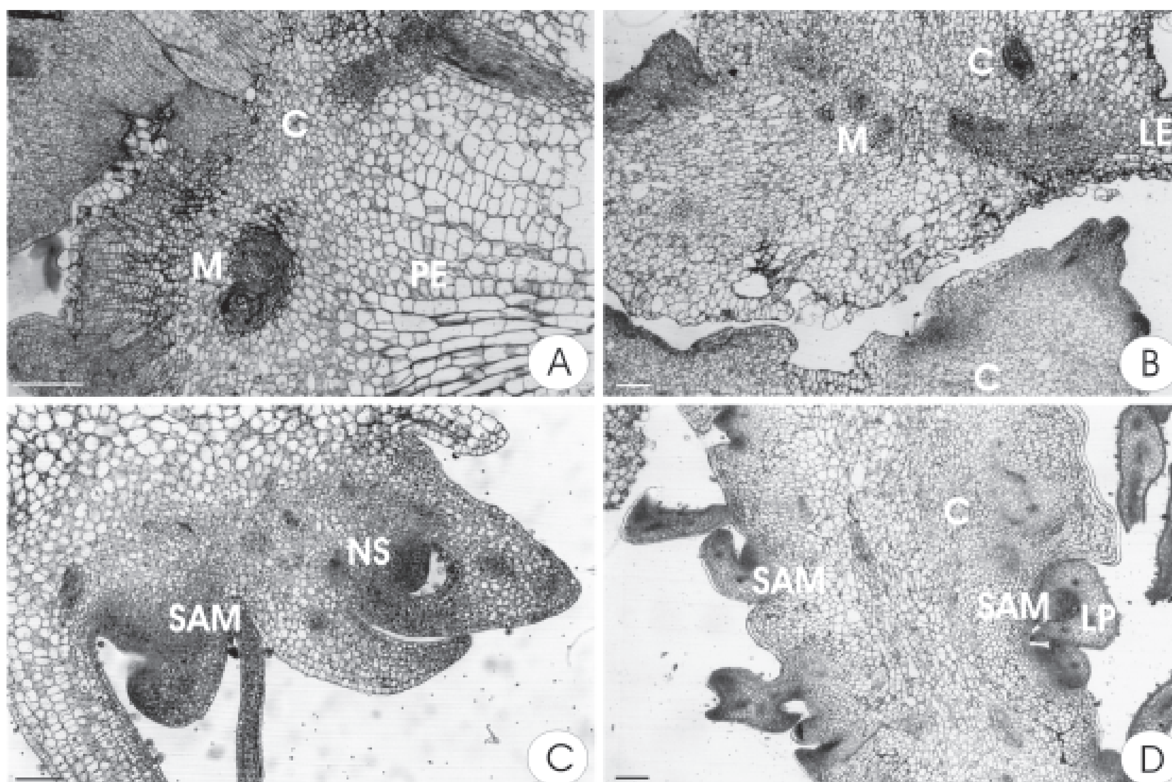

Figure 2. Histological analysis of *Piper solmsianum* C. DC. *in vitro* shoot regeneration. A-B. Callus formation from the cut end of petiole and leaf explants (M - cylindrical meristemoids, C - callus, PE - petiole explant, LE - leaf explant). C-D. Shoot regeneration by differentiation from the outer layer of callus cells (SAM - shoot apical meristem, NS - notch-like structure which will differentiate into shoot buds, LP - leaf primordia). Bars = 50 µm.

(Madhusudhanan & Rahiman 2000). Activated charcoal can adsorb inhibitory compounds secreted from plant tissues, and thus reduce their effect (Teixeira *et al.* 1994). In order to control polyphenol leaching, activated charcoal was supplemented into the culture medium. In general, although reducing the browning effect (data not shown), the supplementation of activated charcoal inhibited callus growth (Fig. 4). It is known that activated charcoal can absorb growth regulators, limiting their use in the culture, and consequently affecting both tissue growth and morphogenesis (Ebert & Taylor 1990). Among growth-regulator treatments, 0.2 $mg.l^{-1}$ 2,4-D plus 2 $mg.l^{-1}$ BA combination resulted in the highest MF increment (Fig. 4).

Optimized culture conditions (16 h photoperiod and supplementation with 0.2 $mg.l^{-1}$ 2,4-D plus 2 $mg.l^{-1}$ BA) were used for callus growth and investigation of cell suspension growth dynamics during a culture cycle. The *P. solmsianum* callus lag-phase was extensive during approximately 20 days. The exponential phase could not be well delimited. However, the stationary phase was reached at day 36 of culture (Fig. 3A). Cell suspension cultures, induced by callus cells at day 24 of culture, showed an increase in SCV from 0.6 ml to 2 ml after 42 days of culture, without subculture (Fig. 3B). The lag-

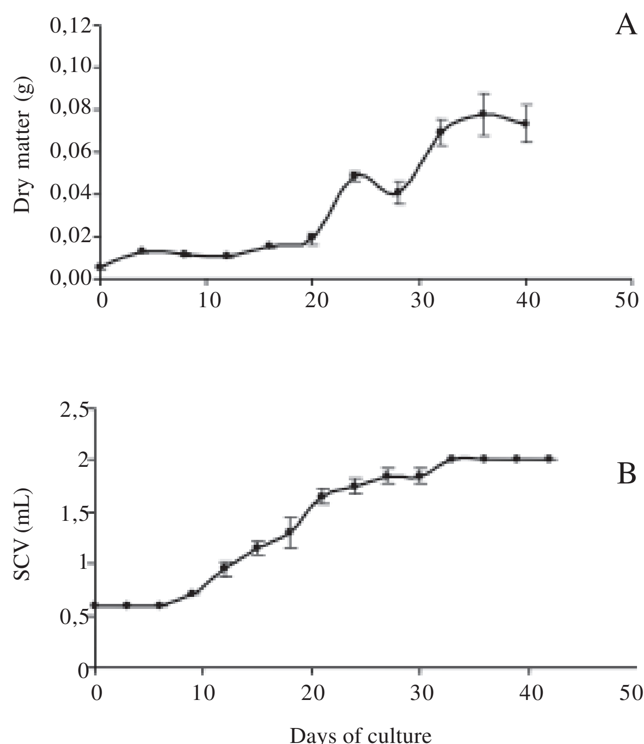

Figure 3. *Piper solmsianum* C. DC. callus (A) and cell suspension growth (B) during 40 and 42 days of culture, respectively. SVC - sedimented cell volume.

phase lasted only 6 days, followed by a 12-day exponential phase, when the growth-rate increased 10% per day. The stationary phase was reached at day 30 of culture. In a suspension culture with a finite medium-volume, an S-shaped growth-curve is generally observed as the start inoculum passes through a series of characteristic growth phases initiated by a lag phase, a period of cell adaptation into the new culture medium, followed by a high rate of cell division, the exponential phase, and then a linear growth phase. The rate of cell growth is reduced during the stationary growth-phase (Szabados *et al.* 1993)

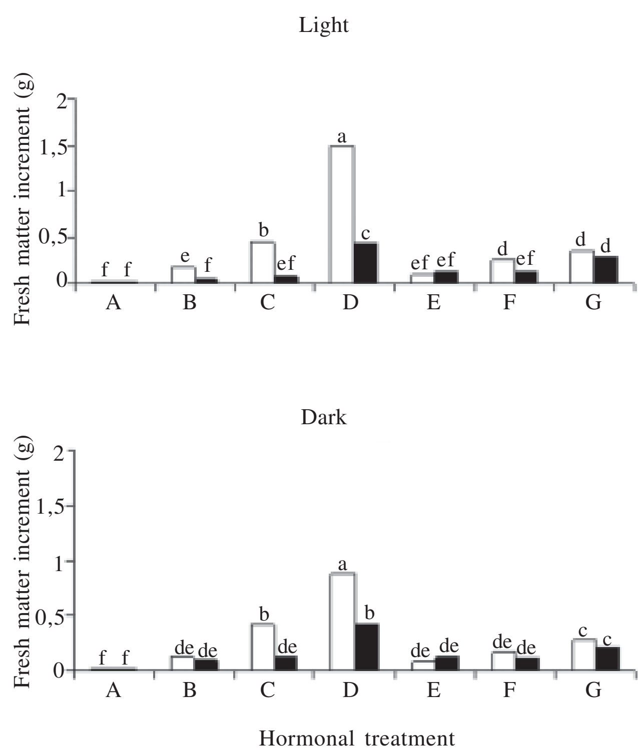

Figure 4. *Piper solmsianum* C. DC. callus fresh matter increment (g) after 30 days of culture in different conditions. Hormonal treatments: A - control; B - 0.2 mg.l^{-1} 2,4-D; C - 0.2 mg.l^{-1} 2,4-D+0.2 mg.l^{-1} BA; D - 0.2 mg.l^{-1} 2,4-D+2 mg.l^{-1} BA; E - 2 mg.l^{-1} 2,4-D; F - 2 mg.l^{-1} 2,4-D+0.2 mg.l^{-1} BA; G - 2 mg.l^{-1} 2,4-D+2 mg.l^{-1} BA. (□ = no activated charcoal; ■ = activated charcoal).

Although cells in the suspension culture are undifferentiated, some may be induced to start a differentiation process during the period of slowed and stationary growth (George 1993). Generally, only a small amount of secondary metabolites is being produced at the exponential phase of growth, as nutrients and common precursors are being allocated to primary metabolism, this resulting in biomass increment (Bourgaud *et al.* 2001). When there is a reduction in growth rates and cells start to differentiate, plant cell metabolism is shifted to the biosynthesis of secondary metabolites, thus indicating biochemical differentiation and consequent activation of those enzymes responsible for secondary metabolite production (Payne *et al.* 1991; Bourgaud *et al.* 2001). In *P. solmsianum* cell suspension culture a vast quantity of metabolites were detected in the culture medium, suggesting that cells are capable of excreting metabolites into the medium (Fig. 5). As predicted, extracts from the culture medium at the stationary phase (40 days of culture) showed the highest variety of accumulated compounds. This is an attractive feature for large scale production, as compounds can be easily recovered from

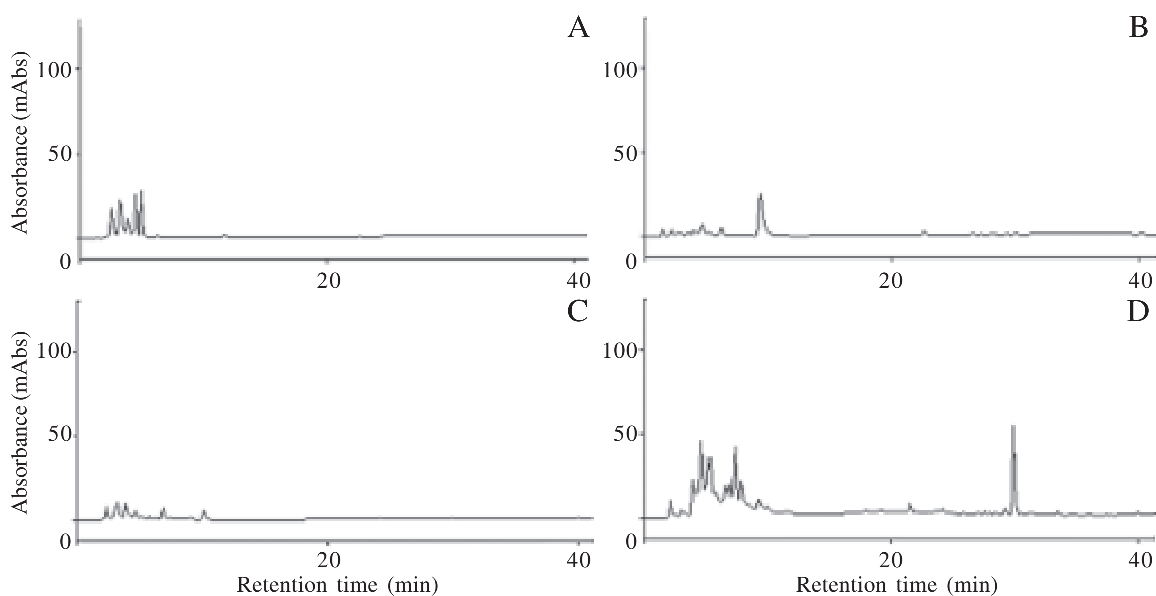

Figure 5. Chromatograms of *Piper solmsianum* C. DC. cell suspension cultures during 45 days of culture: A. Cells at the exponential phase (15 days). B. Culture medium at the exponential phase (15 days). C. Cells at stationary phase (40 days). D. Culture medium extract at the stationary phase (15 days).

the suspension culture without interfering with cell growth.

P. solmsianum proved to be highly amenable to cell-culture techniques. The identification of secondary metabolites accumulated in cell suspension culture may provide important information for characterization and studying phenolic metabolites biosynthesis, especially of those related to the phenylpropanoids and lignan pathway.

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