

MICROPROPAGATION AND CALLOGENESIS OF *Curcuma zedoaria* ROSCOE

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ABSTRACT: *Curcuma zedoaria* Roscoe (zedoary) is a medicinal properties-bearing Zingiberaceae from which rhizomes are commercially exploited. The objective of this work was to establish an *in vitro* protocol for micropropagation and callogenesis of *Curcuma zedoaria* Roscoe as alternative to improve plant production, turning economically feasible the exploitation of its secondary metabolites which present medicinal properties. Micropropagation by using shoot apexes produced by rhizome and from *in vitro* plants were carried out on Murashige & Skoog medium supplemented with 2.0 mg L⁻¹ benzyl amino purine and 30 g L⁻¹ sucrose. Plantlets were satisfactorily acclimated to greenhouse conditions by using plastic cover for at least 10 days. Treatment with endomycorrhiza at the *ex vitro* transferring time was beneficial to acclimatization, improving plant growth and development. Callus induction and growth were obtained by inoculating root segments on Murashige & Skoog medium supplemented with 1.0 mg L⁻¹ naphthalene acetic acid and incubation in the dark at 25 ± 2°C. Cell suspension cultures were established on liquid medium of same chemical composition and same culture conditions and a growth curve was obtained.

Key words: Zingiberaceae, *in vitro* culture, plant production, cells suspension, callus

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RESUMO: *Curcuma zedoaria* Roscoe (zedoaria) é uma Zingiberaceae com propriedades medicinais, da qual o rizoma é explorado comercialmente. O objetivo deste trabalho foi estabelecer um protocolo *in vitro* para a micropropagação e calogênese de *Curcuma zedoaria* Roscoe, como alternativa para melhorar a obtenção de plantas, tornando economicamente possível a exploração de seus compostos secundários que apresentam propriedades medicinais. A micropropagação usando ápices caulinares produzidos por rizoma ou por plantas cultivadas *in vitro* foi obtida em meio de cultura contendo os sais básicos de Murashige & Skoog suplementado com 2.0 mg L⁻¹ de benzil amino purina e 30 g L⁻¹ de sacarose. As plântulas obtidas foram aclimatadas em casa de vegetação utilizando-se cobertura plástica por no mínimo 10 dias. O tratamento das plântulas com endomicorrizas, no momento de transferência das plantas para casa de vegetação, foi benéfico para o processo de aclimatização, melhorando o crescimento e desenvolvimento das plantas. Calos foram obtidos pela inoculação de segmentos de raízes em meio de cultura contendo os sais básicos de Murashige & Skoog suplementado com 1.0 mg L⁻¹ de ácido naftaleno acético e mantidos no escuro. Culturas de células em suspensão foram estabelecidas em meio líquido de mesma composição e sob as mesmas condições de cultivo e uma curva de crescimento foi estabelecida para a espécie.

Palavras-chave: Zingiberaceae, cultura *in vitro*, produção, suspensão celular, calo

INTRODUCTION

Curcuma zedoaria Roscoe (zedoary) is a medicinal properties-bearing Zingiberaceae from which rhizomes are commercially exploited. Natural products from this species are widely used in perfumery, in food industry as condiment and dye, and medicine as well. In addition to the well-known effect of zedoary as a stomachic, it has been recently studied by its anti tumor (Kim et al., 2000), hepatoprotective (Matsuda et al., 2001), anti inflammatory (Jang et al., 2001) and analgesic (Navarro et al., 2002) effects. In spite of the great interest in ex-

ploiting more intensively this natural resource, several factors make its commercial exploitation unfeasibility. The propagation of this plant species has been only performed through rhizomes. Since *Curcuma* species are plants from tropical and subtropical regions, it is difficult for the rhizomes to elicit plant formation in the winter season. Furthermore, this vegetative propagation character confers low genetic variability to the species, reducing the chances of selecting a naturally-occurring clone, overproducing a desirable metabolite. A well established protocol including plant regeneration from cell would help plant population genetic variability.

As a source of fine chemicals, plants have traditionally been grown in large plantations. Recently, new technologies have been developed which may replace plantation systems as a source of plant chemical products. The key to technical and economic feasibility rests on the ability to induce and select genetically stable whole plants or cell cultures that overproduce specific chemicals and the development of scale-up technology that exploits the biological capabilities of plant cells and promotes efficient production (Whitaker & Evans, 1987). To reach these goals, several reports of *in vitro* culture of species from the Zingiberaceae family have been published (Illg & Faria, 1995; Sharma & Singh, 1997; Borthakur et al., 1998; Shirin et al., 2000; Mello et al., 2001; Salvi et al., 2002). This work reports the feasibility of the utilization of tissue culture techniques to establish a protocol for micropropagation and cell suspension culture of *Curcuma zedoaria* Roscoe, which can be a source for secondary metabolites production.

MATERIAL AND METHODS

Plant material

After cleaned with brush and tap water, randomly collected fresh rhizomes from areas of the greater São Paulo (23°32'51"S; 46°38'10"W) were planted on plastic trays filled with sterilized vermiculite and allowed to sprout in greenhouse for 10 days. Intact buds and young shoots were excised from rhizomes and washed with tap water and with 2.0% sodium hypochloride solution for five minutes. Under aseptically conditions, shoots had their external leaves removed and together with buds were washed a second time in 2.0% sodium hypochloride solution and left under agitation for 10 minutes, following by washing the plant materials three times with sterile distilled water. Finally the meristem and shoot apices were excised and inoculated on culture medium.

Micropropagation

Test of different growth regulators - Rhizome bud meristems and shoot apices from greenhouse or *in vitro* growing plants were inoculated on a modified Murashige & Skoog (1962) medium (MS) containing 0.5 mg L⁻¹ nicotinic acid, thiamine and pyridoxine, 20 mg L⁻¹ glycine, 100 mg L⁻¹ myo-inositol, 30 g L⁻¹ sucrose and 8.0 g L⁻¹ agar as solidifying agent. Several concentrations of the growth regulators benzyl amino purine (BAP) (0; 0.5; 1.0; 1.5; 2.0; and 3.0 mg L⁻¹) and naphthalene acetic acid (NAA) (0; 0.2; 0.5 and 1.0 mg L⁻¹), either alone or in combination, were also added to the culture medium. The inoculated materials (rhizome bud meristems and shoot apices) were cultured in growth room at 25 ± 2°C, 31 μmol m⁻² s⁻¹, and a photoperiod of 16/8hours (light/dark) conditions. Transfer to fresh medium and subculturing were carried out every 30 days.

Test of different phenological age of shoots -

To test the effect of the phenological age of shoots on micropropagation, shoot apices were excised from shoots at different development stages and classified as bud, young shoot, developed shoot and adult plant. They were cultured on MS medium containing 2.0 mg L⁻¹ of BAP and development was evaluated as fresh matter accumulation and number of new shoots/explant at 0, 10, 30, 60 and 90 days.

Acclimatization

The *in vitro* micropropagated plants were grouped in three different sizes (Table 1) and individually transferred to 200-mL plastic cups filled with a mixture of vermiculite and sand (3:1 v/v) as substrate for acclimatization. Plants were then covered with plastic bags and moistened with Hoagland & Arnon (1950) nutrient solution. The plastic cover was removed at 0, 5, 10, 15 and 20 days.

Fifty days after acclimatization started, plants were tested to the effect of mycorrhiza treatment on the acclimatization and development of the *in vitro* obtained zedoary plants; acclimatization was also carried out on substrate inoculated with vesicle arbuscular endomycorrhizal fungus *Glomus etunicatum* (400-spore per 5 mL for inoculum). The effect was monitored by comparing the size and dry matter of the aerial part and of the root system of the treated and non-treated plants.

Callus induction

The induction of callus was pursued by inoculating leaf tissue (blade discs, sheath base, sheath top), root tissue (apex and medium region) and shoot apex, on a modified MS medium containing 0.5 mg L⁻¹ nicotinic acid, thiamine and pyridoxine, 20 mg L⁻¹ glycine, 100 mg L⁻¹ myo-inositol, 30 g L⁻¹ sucrose, and 8.0 g L⁻¹ agar as solidifying agent. Concentrations (0; 1.0; 5.0; 10.0 and 20.0 mg L⁻¹) of the growth regulators 2,4 dichloro phenoxyacetic acid (2,4-D) or NAA were supplemented to the culture medium. The inoculated materials were cultured in growth room at 25 ± 2°C, 31 ¼mol m⁻² s⁻¹, in the dark or under a photoperiod of 16/8hours (light/dark) conditions. The evaluation of callus formation was performed 50 days after inoculation.

Table 1 - Classification and morphological characteristics of the *in vitro* regenerated plants used for the acclimatization test.

Plant classification	Aerial Part		Root System			
	Height	Leaves	Roots	Diameter	Length	
	-----	cm	-----	cm	mm	cm
Type I	3-4	1-2	< 5	0.50	3	
Type II	5-8	3-4	7	0.75	4	
Type III	> 8	> 5	> 9	1.00	5	

Cell suspension culture

Callus formed on MS solid medium supplemented with 1.0 mg L⁻¹ NAA (the best callus inducing concentration; see Results and Discussion) were transferred into 250-mL Erlenmeyer flasks containing 75 mL of basic MS liquid medium and kept under agitation (100 rpm), in the dark at 25 ± 2°C for cell suspension culture establishment. Cell multiplication was monitored in experiments containing 1.0 g of fresh weight of cells per 75 mL of medium at 0, 4, 7, 11, 14, 18, 21, 25, 29, and 32 days after transfer, by measuring dry weight accumulation. The cell viability in the cell suspension culture was followed by the tetrazolium test (Dixon, 1985). This test consists of adding 8 mL of 0.5% 2,3,5 triphenyl-tetrazolium chloride solution (pH 7.5) to variable amounts of cells. After incubation and centrifugation, the pellet of cells is resuspended on 3 mL ethanol (95%). Cells are incubated and centrifuged and the supernatant used to read the absorbance at 485 nm against ethanol (95%) as a control.

Statistical Parameters

Trials were performed in totally randomized design. The test of growth regulators consisted of 24 treatments (six BAP concentrations and four NAA concentrations) with 20 replicates, and for different phenological age of shoots, four treatments with ten replicates. Acclimatization consisted of 30 treatments (three plant sizes, five time intervals of cover and the presence or absence of mycorrhiza) with ten replicates each. The experiments for callus induction consisted of 120 treatments (six explants, five concentrations of two growth regulators – 2,4-D and NAA – and two light conditions – light and dark) with 18 replicates each. The establishment of cell suspension cultures and their growth were evaluated at ten time intervals, with three replicates. All graphics were based on treatment means.

RESULTS AND DISCUSSION

Zedoaria has quickly micropropagation response *in vitro* by the diverse type of explants (Figures 1 and 2) producing plants that can be easily acclimated (Figure 3). Callus is also promptly induced by using root segments as explant, and when transferred to liquid medium, cell suspension culture is readily established (Figure 4).

Micropropagation

Test of growth regulators - The best results were obtained using rhizome bud meristems for the micropropagation of *C. zedoaria* Roscoe. The explants response to BAP and NAA, individually and in combination, were evaluated 60 days after inoculation (Figure 1). The occurrence of diverse morphogenetic types was observed, and could basically be classified into three

classes: a) explants presenting different levels of development with high micropropagation rate (> 2.0) and variable number of leaves, occurring in the presence of high BAP concentrations and absence of NAA; b) explants with no development or low level of development and low micropropagation rate (< 0.5), amorphous and oxidized, occurring in the presence of high concentration of both BAP and NAA; c) explants presenting root formation and low or no aerial part development and also low micropropagation rate (< 0.5), occurring in the presence of high NAA concentration and absence of BAP. The two last morphogenic types did not show complete developmental stages and were discarded. Culture media containing high BAP and NAA concentration or absence of BAP are not recommended for *C. zedoaria* micropropagation.

Micropropagation quantified by the number of shoots/explant was higher in the absence of NAA but was BAP-dependent when the NAA concentration was less than 1.0 mg L⁻¹, remaining itself steady until BAP concentration of 2.0 mg L⁻¹ and decreasing thereafter (Fig-

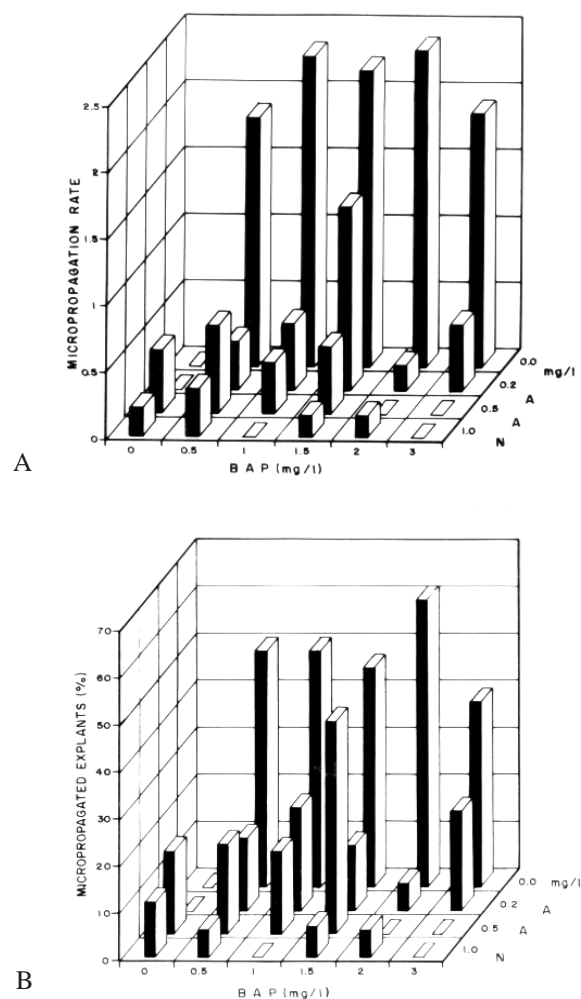


Figure 1 - Micropropagation of *C. zedoaria* plants in different BAP X NAA concentration treatments. (A) micropropagation rate (shoots/explant); (B) occurrence of the morphological type a shoot.

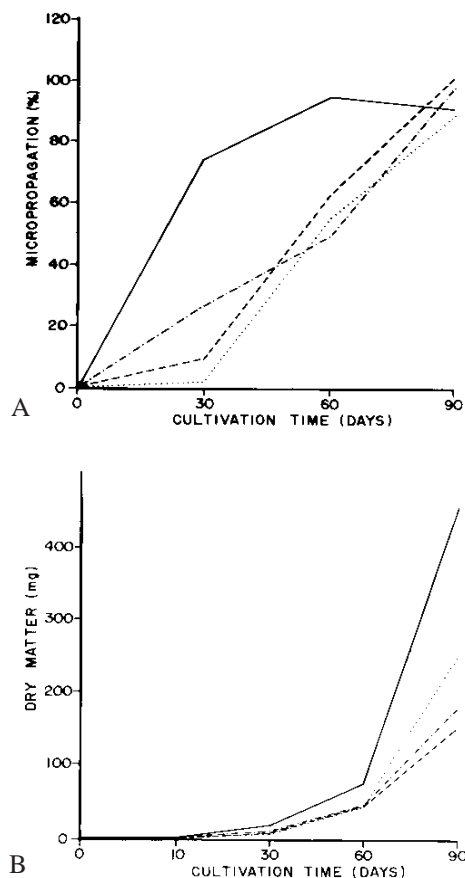


Figure 2 - Results obtained per explant (rhizome bud or shoot apex) isolated from different phenological ages *C. zedoaria* plants (___ rhizome bud; --- young shoot; —.—, developed shoot; adult plant). (A) micropropagation rate (shoots/explant), (B) dry matter accumulation per explant.

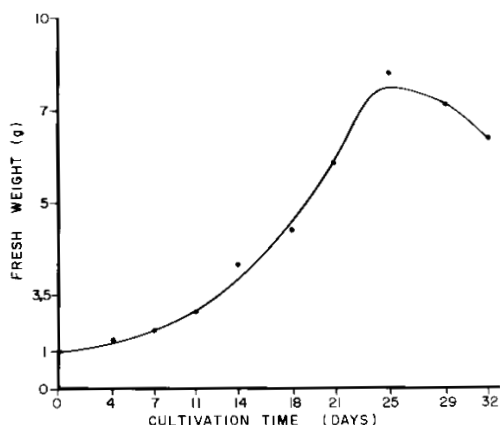


Figure 4 - Growth curve of *C. zedoaria* cell suspension cultures as fresh matter accumulation.

ure 1A). The frequency of plants with well-formed shoot (hereafter called morphogenetic type a) is shown in Figure 1B. Micropropagation of *Curcuma zedoaria* Roscoe is BAP-dependent and is inhibited by NAA.

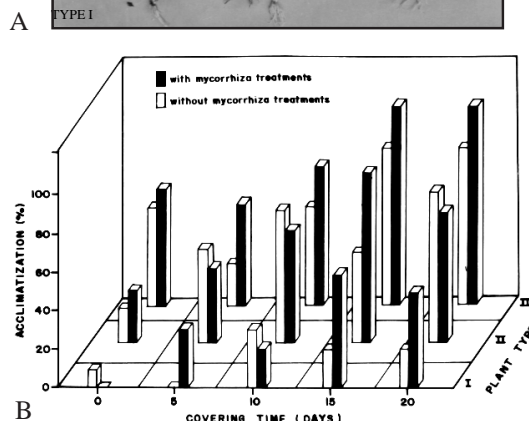
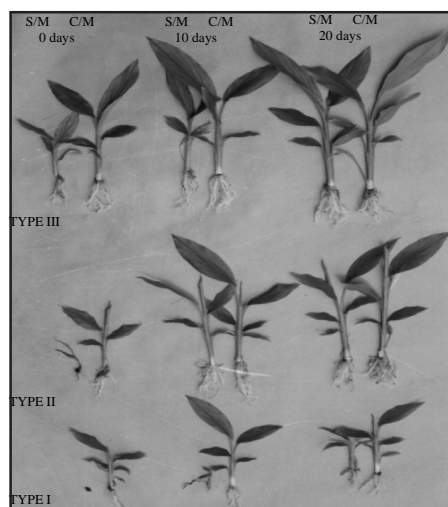


Figure 3 - Effect of plantula classification types (I, II and III), mycorrhization (C/M - with mycorrhiza treatment, S/M - without mycorrhiza treatment) and covering time (days) on the acclimatization process of the *in vitro* regenerated *C. zedoaria* plants. (A) photo, (B) histogram.

Being a monocotyledonous crop, *Curcuma zedoaria* does not allow great explant diversity for micropropagation. Shoot apices have been the responsive explant often used. Rhizome bud meristems were the best explant source for micropropagation (Figure 2). However, rhizome explants may not be the ideal ones, hence they add to the protocol septic problems resulting from the presence of soil residues. Additionally, zedoary rhizomes are dormant during winter. Therefore, micropropagated plant shoot apices are preferred since sterile explants would be available all year-round.

Test of phenological age of shoots - The effect of the phenological development stage of the explant on the micropropagation rate (number of shoots per explants) and dry matter produced per explant of zedoary are shown in Figures 2A and 2B, respectively. Rhizome buds are more efficient for both parameters evaluated. At 30 days of culture, rhizome buds showed 72.3% micropropagation while young shoot, developed shoot and shoot from adult plants presented 10.3%, 25.3% and 1.6%, respectively.

However, at 90 days after inoculation, similar micropropagation rate was observed for all explants (Figure 2A). As far as micropropagation potential is concerned, rhizome bud present a greater advantage since it responds rapidly producing plantlets in a shorter period of time in comparison to other explant types. The dry matter accumulation was similar for all four types of explants until 30 days after inoculation. Then, shoots originated from buds started to accumulate dry matter and, at 90 days, it presented three times more dry matter than the explants from young and developed shoot and twice more than explants from adult plants.

Acclimatization

Questions always arise concerning the appropriate size or age of the plants leaving the *in vitro* condition to overcome stress conditions occurring during acclimatization. Also, how long should they stay in greenhouse before they can be transferred to the field? These questions were satisfactorily answered herein. Furthermore, the possible beneficial effect of plant treatment with mycorrhiza on the acclimatization capacity was also tested with good results.

The results of the acclimatization of the micropropagated plants carried out with plants of three different sizes (Table 1) in the presence or absence of the endomycorrhizal fungus *Glomus etunicatum* are shown in Figure 3 (A and B). A direct relationship was found among plant size, length of the period under plastic cover, mycorrhiza treatment and the acclimatization ability of the plants. Plants of type III size were the most successful (97% acclimatization after 15 days under plastic cover) and of type I the least (20% acclimatization after 10 days covering) on overcoming the *ex vitro* stress conditions. Micorrhiza treatment consistently improved performance of types I and II plants, while micorrhiza treatment of type II plants did not show consistent response. The accumulation of dry matter by the aerial part and by the root system showed that mycorrhiza-treated plants were able to accumulate twice as much dry matter than untreated plants, in both parts of the plants, and for all three types. This difference was more pronounced around the 5th and the 10th day after inoculation.

The highest acclimatization rate occurs after 15 days in greenhouse and apparently independent on the plant size tested for both treated and untreated mycorrhiza conditions. According to other investigations carried out in our laboratory (results not reported here) this seems to be true for plant species such as African violet, pineapple, and strawberry. There is a minimal size, in the case of zedoary type III plants, that will confer acclimatization higher than 90% after 15 days in greenhouse. It also became apparent that the higher the stress, the better the mycorrhiza treatment response, apparently to the type I plants (Figure 3). The enhanced development of zedoary

plants by mycorrhiza treatment probably results from the increase of the root absorption area, which results in high absorption of water and nutrients (Vidal et al., 1992; Estrada-Luna et al., 2000; Locatelli & Lovato, 2002). Untreated zedoary plants required a much longer time under plastic covers to show complete acclimatization, and their survival rate was always under 25%. On the other hand, the mycorrhiza-treated plants had survival rate two to three times higher than those untreated (Figure 3).

Longer time under plastic cover showed to be a requirement for the acclimatization of all three types of plants, mainly, the smaller type I.

Callus induction

Concentrations of 2,4-D did not show any positive response for callus induction in any of the tested types of explants when evaluation was carried out 50 days after inoculation. On the other hand, treatment of diverse tissues of *Curcuma zedoaria* with different NAA concentrations produced variable callus induction responses. Callogenesis occurred on the shoot apex, leaf sheath base, root apex and root medium region explants. Occurrence of relevant callogenesis was inversely proportional to NAA concentration. Best results were observed when explants from roots (root apexes and median portion of roots) were maintained at NAA concentrations of 1.0 mg L⁻¹ (Table 2). Complete absence of light was a requirement for best callus induction. The calli were yellowish and nodular with morphogenic aspects. Plant can be regenerated from these calli by indirect organogenesis (Mello et al., 2001). According to these authors, histological analysis revealed that callus was formed from hypertrophied cortical parenchyma cells of the explant. Some of these cells underwent division while the surrounding cells accumulated starch. Callus was capable of shoot bud regeneration after 70 days.

Table 2 - Callus induction from types of explants and in culture media with concentrations of NAA maintained in the light (L) or in the dark (D).

Explant	NAA									
	0.0		1.0		5.0		10.0		20.0	
	L	D	L	D	L	D	L	D	L	D
	----- mg L ⁻¹ -----									
shoot apex	-	-	-	+	-	++	+	++	-	+
sheath base	-	-	+	+++	+	++	+	++	-	+
sheath top	-	-	-	+	-	+	-	+	-	+
blade disc	-	-	-	-	-	-	-	-	-	-
root apex	-	-	+	++++	++	+++	+	+	+	+
root medium region	-	-	+++	++++	+	+	+	+	-	+

- no induction; + induction; ++ low production of callus; +++ medium production of callus; ++++ high production of callus

Cell suspension culture

Calli obtained on solid MS culture medium containing 1.0 mg L^{-1} NAA when transferred to a same liquid medium did not show any significant cell multiplication before four weeks after transfer. At the end of the 4th week, whitish granular cell aggregates started to be formed in the culture medium and maintained during subsequent subculturing. A typical cell growth curve constructed from any of subsequent subculturing is shown in Figure 4. This curve shows a lag phase of around five days, followed by an exponential phase of growth lasting approximately 15 days, ending up in the stationary phase. The whole growth curve took approximately 25-30 days to be completed and presented about 8 to 10 folds fresh matter accumulation. The cell viability determined by the tetrazolium test was, on average, above 70%.

A system for using plants of a particular species for production of phytochemicals should provide a choice to use either plant tissue or cell suspension. The crop biomass production depends on a very reliable plant propagation method not always already developed for every plant species. Some of the factors limiting the feasibility of this procedure are geographic location of the target species, absence of seed production, seed dormancy and length of the species cycle (Babu et al., 1992; Kackar et al., 1993; Illg & Faria, 1995). Plant tissue culture techniques could be a reliable strategy to supply any required amount of micropropagated plants at cost not exceeding the conventional way to propagate plant species (Mello et al., 2000). Cell suspension culture is a requirement for the production of chemicals from plants in a way quite similar to that used for microorganisms, where the utilization of bioreactor becomes feasible. The bottleneck of this biotechnological process lies on the ability of metabolites excretion by plant cells.

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