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COMPARISON OF THREE *in vitro* PROTOCOLS FOR DIRECT SOMATIC EMBRYOGENESIS AND PLANT REGENERATION OF *Coffea arabica* L. CVS. CATURRA AND CATUAÍ

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Palabras clave: *Coffea arabica*, Catuaí, Caturra, embriogénesis somática, medio de cultivo, capacidad embriogénica.

Keywords: *Coffea arabica*, Catuaí, Caturra, somatic embryogenesis, medium composition, embryogenic capacity.

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RESUMEN

Comparación de tres protocolos para la embriogénesis somática directa y la regeneración de plantas de *Coffea arabica* L. cvs. Caturra y Catuaí *in vitro*. La presente investigación tuvo como objetivo establecer una metodología para la inducción de la embriogénesis somática directa en las variedades de café Caturra y Catuaí. Se observó un efecto del genotipo en la inducción de la embriogénesis somática directa; con la variedad Caturra se obtuvo un mayor número de embriones somáticos ($2,71 \pm 0,45$). Los explantes de Caturra provenientes de plantas de 3 meses mostraron una mejor respuesta a la inducción de la embriogénesis somática directa que los explantes provenientes de plantas de 12 meses. De los explantes provenientes de plantas de 12 meses de Catuaí rojo no se obtuvo embriones somáticos en ninguno de los medios de cultivo evaluados. No hubo diferencias significativas en el número de embriones somáticos producidos a partir de la primera y segunda hoja de vitroplantas y los explantes provenientes de la parte distal, media y basal de las hojas de plantas de café. El medio de cultivo Yasuda resultó el más indicado para inducir la embriogénesis somática y el desarrollo de embriones somáticos en plántulas en Caturra. Mientras que para Catuaí rojo, fue el de Hatanaka.

ABSTRACT

A coffee (*Coffea arabica* L. cvs. Caturra and Catuaí) plant regeneration protocol via direct somatic embryogenesis was established. Vitroplant explants from first and second pair of leaves and explants from the distal, middle, and basal sections of 3 and 12 month-old plants were cultivated on the protocols described by Yasuda, Hatanaka and CATIE. Caturra somatic embryos were cultured on the same embryo induction media or DEV medium. The maximum number of somatic embryos (2.71 ± 0.45) was obtained from Caturra vitroplants. Explants from 3 month-old plants showed better response than 12 month-old Caturra explants. No somatic embryos were obtained in the *in vitro* protocols evaluated with 12 month-old Catuaí rojo plants. No differences were observed on the number of embryos produced from the first and second pair of vitroplant leaves and sections of the distal, middle, and basal coffee plant leaves. The Yasuda protocol was the most efficient to induce direct somatic embryogenesis and embryo-to-plant conversion for the Caturra variety, whereas Hatanaka's was the most suitable for the Catuaí variety.

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INTRODUCTION

Coffee is one of the most economic important crops for more than 50 countries in the world and it is highly valuable for beverage consumption. Out of more than 100 coffee species, only *Coffea arabica* L. (Arabica type coffee) and *C. canephora* Pierre (Robusta type coffee) are commercial species (Etienne 2006). In Latin America, Arabica type coffee is responsible for all the coffee production (Kumar 2006). In Costa Rica, Arabica type coffee is grown extensively with 80% of the area being cultivated with Caturra and Catuaí varieties (Zamora 1997).

In a genetic engineering program of coffee, it is necessary to develop an efficient *in vitro* culture system to obtain competent explants for plant transformation and regeneration (Fernández-Da Silva and Menéndez-Yuffá 2003). Among *in vitro* culture techniques, somatic embryogenesis has been used for micropropagation and genetic modification of higher plants (Ogita *et al.* 2002).

In coffee, this process can be achieved via direct somatic embryogenesis (DSE) from pro-embryogenic cells of the tissue in the absence of embryogenic callus or by indirect somatic embryogenesis (ISE) via callus formation (Jiménez 2001, Molina *et al.* 2002). This process in *Coffea arabica* has been histologically demonstrated by Sondahl *et al.* (1979), Michaux-Ferrière *et al.* (1989) and Quiroz-Figueroa *et al.* (2002). DSE has the advantage of reducing somaclonal variation and may be the sole procedure for obtaining somatic embryogenesis for those coffee genotypes recalcitrant to ISE (van Boxtel and Berthouly 1996). In addition, the time necessary for obtaining somatic embryos by DSE is shorter compared to ISE (Yasuda *et al.* 1985, Hatanaka *et al.* 1991, Bieysse *et al.* 1993, Loyola *et al.* 1999, Giridhar *et al.* 2004).

Although, DSE in coffee have been reported in *C. arabica* and *C. canephora* (Dublin 1981, Yasuda *et al.* 1985, Hatanaka *et al.* 1991, Bieysse *et al.* 1993, Molina *et al.* 2002, Quiroz-Figueroa *et al.* 2002, Giridhar *et al.* 2004), there are few reports concerning the optimization

of the factors influencing DSE in Caturra and Catuaí rojo varieties. This process depends on the genotype, physiological stage of the leaf, explant age, month of explant collection, plant growth regulator and gelling agent. DSE method can be adapted to different genotypes of coffee if the factors affecting this process are well controlled (Santana *et al.* 2004). Moreover, when choosing a culture protocol to induce and regenerate somatic embryos, it is essential to determine the yield of embryos produced by the explant, the time necessary to obtain them, and their capacity to germinate and develop into normal plants (Bieysse *et al.* 1993). For these reasons, the aim of this research was to validate a methodology to induce DSE for *C. arabica* varieties Caturra and Catuaí rojo by studying the influence of the genotype, explant source and *in vitro* protocol.

MATERIALS AND METHODS

Plant material

Vitroplants and 12 month-old coffee plants (*Coffea arabica* L.) were used as leaf donors from the Caturra and Catuaí rojo varieties. Also, leaves from 3 month-old plants of the Caturra variety were used. Seeds of Caturra and Catuaí rojo, collected from coffee plantations in Alajuela, Costa Rica, were washed and soaked for 24 h in distilled water with 2 drops of Tween 20, disinfected with 3.5% (v/v) sodium hypochlorite for 1 h and finally rinsed 3 times with sterile distilled water. The zygotic embryos were excised from the seeds and culture in baby food jars closed with polyethylene food wrap (Glad, Costa Rica) containing 20 ml of MS medium (Murashige and Skoog 1962) supplemented with Morel vitamins (1965), 100 mg l⁻¹ myo-inositol, 200 mg l⁻¹ casein hydrolysate, 400 mg l⁻¹ malt extract, 1 mg l⁻¹ 6-benzyladenine (BAP), 30 g l⁻¹ sucrose and 2 g l⁻¹ Phytigel; pH was adjusted to 5.6 before autoclaving for 21 min at 121°C and 1.07 kg cm⁻². *In vitro* plantlets developed from these embryos were cultured with 20 ml of the above medium under a 16 h light

photoperiod ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$) at $26 \pm 2^\circ\text{C}$ and transferred to fresh medium every 90 days. Three and 12 month-old coffee plants were grown under greenhouse conditions (80% relative humidity, temperature $18\text{--}35^\circ\text{C}$ and 12 h photoperiod).

Young leaves of greenhouse-grown plants were washed with distilled water and Tween 20 for 5 min and disinfected with a solution of Agri-mycin[®] 100 (Pfizer) and Benomyl[®] (DuPont) (1 g l^{-1} each) for 60 min. Then, in a laminar flow cabinet, they were disinfected with 1.6% (v/v) commercial sodium hypochlorite (NaOCl) for 30 min and with 1% (v/v) NaOCl for 5 min, followed by 3 rinses with sterile distilled water.

Induction of somatic embryogenesis

The first and second pair of vitroplant leaves and distal, middle and basal segments of the lamina from the first pair of leaves from three and twelve-month-old plants were used as explants. Leaf explants of 0.5 cm^2 , without the midvein and the margins, were cultured in 10 ml of Yasuda *et al.* (1985), Hatanaka *et al.* (1991), and CATIE (1988) medium contained in culture tubes (21x150 mm) closed with plastic closures. Yasuda *et al.* (1985) medium was gelled with Gelrite according to Bieysse *et al.* (1993). Thirty-nine treatments were established to evaluate DSE in a particular combination of explant source, the position of the explant in the leaf, and the culture media composition. Thirty explants were evaluated for each treatment. The percentage of explants with somatic embryos [(number of explants that produced embryos / the total number of explants) x 100] and the mean \pm SE of somatic embryos per explant was estimated after 12 weeks of culture. Also, the time required for the formation of globular somatic embryos was recorded.

Histological study

A histological analysis was carried out with segment of leaves with nodular callus, globular and torpedo stage somatic embryos. The explants were fixed for 24 h in a solution containing

10% formalin in a 0.2 M phosphate buffer. The explants were dehydrated in a graded series of ethanol and embedded in paraffin wax. Sections of $6 \mu\text{m}$ were stained with Haematoxylin-Eosin. Photographs were taken using a Nikon Eclipse E200 microscope and Kodak film at ASA 100.

Regeneration of somatic embryos

Caturra globular and torpedo stage somatic embryos obtained by the Hatanaka *et al.* (1991) and Yasuda *et al.* (1985) protocols were transferred either to the same medium or to the medium for development (DEV) described by van Boxtel and Berthouly (1996). In addition, somatic embryos obtained on the medium for embryo induction were transferred to the embryo development medium described by CATIE (1988). Ten replications with 10 somatic embryos were used for each germination medium. The somatic embryos were cultivated in baby food jars, closed with polyethylene food wrap (Glad, Costa Rica), at $26 \pm 2^\circ\text{C}$ under 16 h light photoperiod ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$). The germination percentage [(number of germinated somatic embryos / the total number of somatic embryos) x 100] was recorded after 4 weeks of culture. The composition of the culture media and growth conditions used for the induction of DSE and plant regeneration are given in Table 1.

Statistical analysis

Data were analyzed by one-way ANOVA and means values of data were separated with Tukey Unequal N HSD at $p < 0.05$ using STATISTICA 6.0 (StatSoft, Tulsa, OK, USA).

RESULTS

DSE started with the development of a creamy compact non-embryogenic callus (scar tissue) on the cut edge of the leaf explants after 2 weeks of culture on all the protocols evaluated for the Caturra and Catuaí rojo varieties (Figure 1A).

These calli did not show a continuous growth and somatic embryos developed on their surface (Figure 1B-C). Histological analysis showed a cluster of embryogenic cells which was composed of small isodiametric cells with a densely cytoplasm and a prominent nucleus (Figure 1D). These embryogenic cells continued a series of organized division and gave rise to globular somatic embryos (Figure 1E) and torpedo stage somatic embryos (Figure 1F). Identical histological development was observed in somatic embryos formed on explants from 2 of the genotypes studied. DSE in *Coffea arabica* cvs. Caturra and Catuaí rojo was demonstrated since globular somatic embryos had no apparent vascular connection with the mother tissue. These

somatic embryos had a suspensor-like structure (Figure 1E).

The embryo induction differed significantly between leaves derived from vitroplants of the Caturra and Catuaí rojo varieties ($p < 0.05$) regardless of the culture media composition and position of the leaf in the vitroplant. Explants from Caturra vitroplants produced more somatic embryos (2.7 ± 0.5) than Catuaí rojo vitroplants (1.4 ± 0.2) (Figure 2). Regardless the position of the leaf in the vitroplant, DSE was significantly higher when Caturra explants were cultured on the CATIE or Yasuda protocols (Table 2). For the Catuaí rojo variety, the higher percentage of explants with somatic embryos and number of embryos per explant was obtained on the Hatanaka

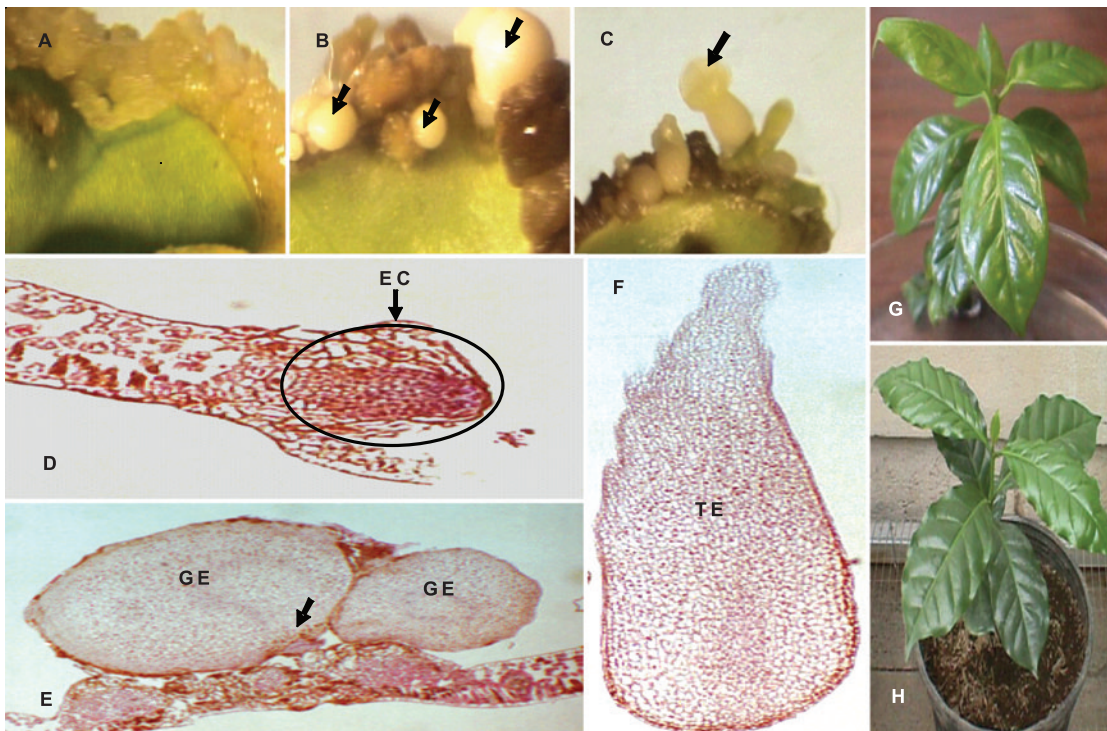


Fig. 1. Direct somatic embryogenesis in *Coffea arabica*. A. Compact nodular non-embryogenic callus (scar tissue) development on the cut edge of the leaf explants after 2 weeks of culture. B. Globular stage somatic embryo (arrows). C. Torpedo stage somatic embryo (arrow). D. Cluster of embryogenic cells (EC). E. Transversal section of globular stage somatic embryo (GE). F. Transversal section of torpedo stage somatic embryo (TE). G. Coffee plants regenerated from somatic embryos. H. Coffee plants acclimatized in the greenhouse.

Table 1. Composition of culture media and growth conditions used for the induction of somatic embryos by direct somatic embryogenesis from segments of coffee leaves (*Coffea arabica* L. cvs. Caturra and Catuaí rojo) and regeneration into plants.

Component ¹	Medium for embryo induction (CATIE 1988)	Medium for embryo development (CATIE 1988)	Hatanaka <i>et al.</i> (1991)	Yasuda <i>et al.</i> (1985)	Embryo development (DEV) (van Boxtel and Berthouly 1996)
Macroelements	MS/4	MS	MS/4	MS/4 + KH ₂ PO ₄ (42.5 mg)	MS
Microelements	MS/4	MS	MS/2	MS/2	MS
Fe EDTA	10.75	43	21.5	21.5	43
	-	Morel vitamins			
Piridoxine	1	-	1	1	1
Nicotinic acid	1	-	1	1	1
Thiamine	10		10	10	1
Calcium pantothenate	1	-	-	-	1
Biotin	0.01	-	-	-	0.01
BAP	4	0.3	-	1	0.3
2-iP	-	-	1	-	-
Sucrose	30	40	30	30	30
Myo- inositol	10	-	100	100	100
Gellin agent	Phytigel (3.2)	Phytigel (3.6)	Agar (9)	Gelrite (2.5) ²	Phytigel (2.2)
Growth conditions					
Light	Darkness	16 h light: 8 h dark photoperiod (30 μmol m ⁻² s ⁻¹)	12 h light: 12 h dark photoperiod (30 μmol m ⁻² s ⁻¹)	12 h light:12 h dark photoperiod (30 μmol m ⁻² s ⁻¹)	16 h light:8 h dark photoperiod (30 μmol m ⁻² s ⁻¹)

1 The concentration of mineral and organic components and plant growth regulators is expressed in mg l⁻¹; sucrose and gelling agent concentration is expressed in g l⁻¹.

2 Modified by Bieysse *et al.* (1993).

et al. (1991) protocol (Table 2). Nevertheless, on the Hatanaka *et al.* (1991) and Yasuda *et al.* (1985) protocols somatic embryos formed after 7 weeks of culture, whereas on the medium described by CATIE (1988) it did so after 12 weeks.

On the other hand, independently of the culture media composition, when the first and second pairs of vitroplants leaves were used, no significant differences were observed in the number of somatic embryos produced for both varieties.

No somatic embryos were observed in explants of 12 month-old Caturra plants after 12 weeks of culture on the CATIE (1988) and Hatanaka *et al.* (1991) protocols. On the other hand, after 10 weeks of culture on the Yasuda *et al.* (1985) protocol, 40%, 20% and 13% of the explants from the distal, middle and basal sections of leaf laminas, respectively, showed embryogenic structures, but low averages of embryos per explant were obtained. For the Catauí rojo variety, none of the explants from 12 month-old plants produced embryos in any of the *in vitro* protocols used.

Somatic embryos were observed at the ninth week of culture using the different protocols and explants from 3 month-old Caturra plants. Regardless the position of the explant, the percentage of explants with somatic embryos and number of embryos per explant were higher when explants were cultured on the Yasuda *et*

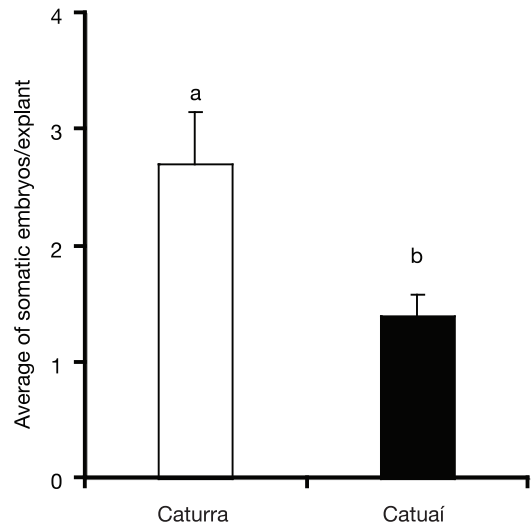


Fig. 2. Effect of genotype on direct somatic embryogenesis induction on explant from vitroplants of *Coffea arabica* cvs. Caturra and Catauí rojo regardless the *in vitro* culture protocol and position of the leaf in the vitroplant. Mean±SE. Values with the same letter are not significantly different with the Tukey Unequal N HSD test ($p < 0.05$).

al. (1985) protocol (Table 2). On the other hand, independently of the culture media composition, no significant differences were observed among distal (3.3 ± 0.6), middle (3.1 ± 0.7), and basal (2.0 ± 0.4) segments of the leaf lamina.

Table 2. Effect of the *in vitro* protocol on direct somatic embryogenesis induction in Caturra and Catauí rojo vitroplants and Caturra plants of three months-old after 12 weeks of culture.

In vitro protocol	In vitro plants				Plants of 3 months-old	
	Caturra		Catauí		Caturra	
	% ¹	Number SE ²	%	Number SE	%	Number SE
CATIE	50	3.3±0.8 a ³	21	0.6±0.2 b	41	1.5±0.3 b
Hatanaka	39	1.3±0.4 b	79	2.6±0.3 a	45	2.1±0.4 b
Yasuda	60	3.1±0.9 a	25	0.5±0.2 b	69	5.0±0.9 a

- 1 Percentage of explants producing somatic embryos were calculated using this formula [(number of explants that produced embryos / the total number of cultured explants) x 100].
- 2 Average number of somatic embryos per explant (mean ± SE).
- 3 Means within the column having the same letter were not significantly different by the Tukey (HSD) test for unequal N (Spjotvoll/Stoline) ($p < 0.05$).

In relation to the germination and the conversion of embryos into plants, differences in the regeneration protocols were observed. The highest percentage of regeneration was obtained with the Yasuda *et al.* (1985) protocol which differed significantly from the other treatments ($p < 0.05$) (Figure 3). Caturra somatic embryos developed into plants with 2 to 4 pairs of leaves (Figure 1G) and 100% of the regenerated plants were acclimatized and developed into adult plants (Figure 1H).

DISCUSSION

Induction of somatic embryos by direct somatic embryogenesis from leaves of *C. arabica* cv. Typica, Caturra rojo, Catuaí amarillo and Catimor (Yasuda *et al.* 1985, Bieysse *et al.* 1993, Calheiros *et al.* 1994, Molina *et al.* 2002, Quiroz-Figueroa *et al.* 2002, Fernandez-Da Silva *et al.* 2005) and *C. canephora* Pierre ex Frohner (Hatanaka *et al.* 1991) has been described. The results obtained

in this research using *C. arabica* corroborated a significant influence of the genotype, source of explant, and *in vitro* protocol on DSE for the Caturra and Catuaí rojo varieties.

An influence of the genotype was clearly observed since the Caturra variety produced more somatic embryos per explant than Catuaí rojo. In this sense, van Boxtel and Berthouly (1996) indicated that the variability in obtaining embryogenic calli from *C. arabica* is variety and hybrid dependant. Moreover, Molina *et al.* (2002) reported that DSE response of the genotypes of Catimor was between 12 to 72.7%. The embryogenic potential of coffee species is genetically determined (Bieysse *et al.* 1993, Molina *et al.* 2002, Santana *et al.* 2004).

In addition, our results corroborated that the explant source influenced DSE. In the present study, the number of somatic embryos was greater using explants from vitroplants followed by explants from 3 months and 1 year-old plants. A similar result was obtained by Bieysse *et al.* (1993) who reported a superior induction of

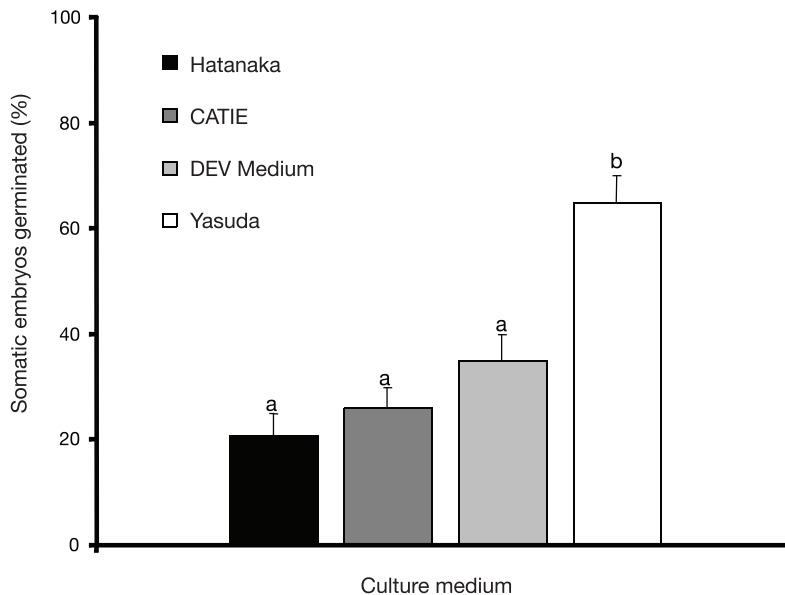


Fig. 3. Effect of the *in vitro* protocol on the germination of somatic embryos of *Coffea arabica* cv. Caturra. The percentage of germinated embryos after 4 weeks of culture on Hatanaka *et al.* (1991), the medium for embryo development (CATIE 1988), DEV medium (van Boxtel and Berthouly 1996) and Yasuda *et al.* (1985) protocol are showed. Values with the same letter are not significantly different with the Tukey Unequal N HSD test ($p < 0.05$).

somatic embryogenesis in explants coming from vitroplants (50%) of *Coffea arabica* cv. Caturra rojo than those produced on explants from young greenhouse plants (30%). Barry-Etienne *et al.* (2002) pointed out that in leaf tissue of vitroplants, the palisade parenchyma is composed of weakly comprised cells of different size and shape when compared to leaf tissue from seedlings. Also, leaves from vitroplants have less epicuticular wax. Therefore, this anatomic character could favour a better uptake of media components and growth regulators, which at the same time could improve the response of this tissue. In addition, the differential response of explants derived from vitroplants, 3 and 12 month-old plants could be related to the physiological stage of the donor plant which determines nutritional and plant growth regulator levels in the tissues (Villalobos and Thorpe 1991). In this context, vitroplants of coffee have higher contents of potassium, nitrogen and phosphorus, elements that play an important role in somatic embryogenesis induction (Barry-Etienne *et al.* 2002).

Regarding explant type, the results obtained in this research demonstrate that it is possible to induce DSE using the first and second pair of vitroplant leaves. In contrast, Quiroz-Figueroa *et al.* (2002) found that this type of explant did not show embryogenic response using Caturra rojo and Yasuda *et al.* (1985) culture medium. In addition, Quiroz-Figueroa *et al.* (2002) showed that explants from the distal part of the leaf were less responsive than those from the basal part of the leaves. Instead, we did not observe differences among sections coming from the distal, middle and basal sections of the leaves of 3 month-old greenhouse plants.

The culture medium described by CATIE (1988) and Yasuda *et al.* (1985) were the best for DSE induction in Caturra (Table 2). Yasuda *et al.* (1985) in *C. arabica* cv. Typica mentioned that 186 ± 90 explants formed somatic embryos. Moreover, Quiroz-Figueroa *et al.* (2002) reported and average of 5 somatic embryos per explant and 70% of the leaf explants of *C. arabica* cv. Caturra rojo showed somatic embryos. In

contrast to the results obtained in this study, Bieysse *et al.* (1993) did not obtain somatic embryos using Caturra rojo and Yasuda *et al.* (1985) culture medium. Probably differences in physiological state of the leaf, explant age and month of explant collection may account for the results obtained in both studies.

Moreover, induction of embryos in the Yasuda protocol was completed in 7 weeks whereas using CATIE (1988) protocol it was accomplished in 12 weeks. DSE induction and embryo formation required 8 to 15 weeks in *C. arabica* and *C. canephora* (Yasuda *et al.* 1985, Hatanaka *et al.* 1991, Bieysse *et al.* 1993, Molina *et al.* 2002, Quiroz-Figueroa *et al.* 2002). Thus, compared to previous studies, the response time was reduced by at least 1 week using the conditions validated in the present work.

Furthermore, the Yasuda *et al.* (1985) protocol is simpler than that of CATIE (1988) since it uses only 1 medium to induce both DSE and the regeneration of plantlets. Besides, Yasuda *et al.* (1985) was the most efficient in stimulating the DSE in explants from different sources. These results could be related to differences in the mineral salts and the gelling agent compared to the other media. García and Menéndez (1987) observed that production of somatic embryos of coffee was greater in media solidified with gelrite than with agar. The Yasuda culture medium contained gelrite, which might allowed water and nutrients to be more easily absorbed by the explant and consequently favoring somatic embryogenesis (Bieysse *et al.* 1993). Moreover, Yasuda culture medium has a higher concentration of KH_2PO_4 . This fact is important as it has been demonstrated that a high absorption of phosphorus, nitrate and ammonium is required to trigger somatic embryo formation in coffee (Albarrán 1999). Also, phosphorus is one of the mineral elements most absorbed by the embryogenic cells of coffee (Albarrán 1999, Solano 2001).

On the other hand, the culture medium described by Hatanaka *et al.* (1991) was the best for DSE induction in Catuaí rojo (Table 2). A similar result was obtained by Calheiros *et al.*

(1994) who reported an average of 17 somatic embryos per explant of Catuaí amarillo using Hatanaka *et al.* (1991) medium culture. Moreover, Hatanaka *et al.* (1991) indicated that 100% of the leaf explants of *C. canephora* cultured on 5 μ M 2-iP showed somatic embryos and each explant formed more than 100 somatic embryos.

The conversion of somatic embryos into plantlets occurred predominantly with the Yasuda *et al.* (1985) protocol. A similar result was obtained by Albarrán (1999) who reported that the mineral salts and vitamins of the Yasuda culture medium with concentrations of BAP between 4 and 6 mg l^{-1} were more efficient to stimulate the formation of somatic embryos from a cellular suspension of a hybrid F1 of *Coffea arabica* (Caturra x ET531), as well as for the conversion of the embryos into plants.

A simple and rapid protocol was validated for direct embryo formation and plant regeneration from leaf explants of Caturra and Catuaí rojo. The protocol of Yasuda *et al.* (1985) is the most suitable for Caturra whereas that of Hatanaka *et al.* (1991) is the most effective for Catuaí rojo. These protocols do not require formation of embryogenic callus and reduce response time, providing a new panorama for mass propagation of coffee. Furthermore, since genetic engineering of coffee is the subject of intensive research, *i.e.* to confer resistance to *H. hampei*, the *in vitro* protocols reported in this study could be used to obtain competent target tissue (somatic embryos and plants) for genetic modification.

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