Somatic Embryogenesis from Three Coffee Cultivars: ‘Rubi’, ‘Catuaí Vermelho 81’, and ‘IAPAR 59’

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Abstract. Leaf explants of *Coffea arabica* cv. Rubi, ‘Catuaí Vermelho 81’, and ‘IAPAR 59’ were placed onto MS basal medium with 4 μM of picloram for 8–12 weeks in the dark at $27 \pm 2^\circ\text{C}$ (standard temperature). Explants (‘Rubi’ and ‘Catuaí Vermelho 81’) with callus were transferred to a SP basal medium plus 0, 12, 24, and 48 μM BAP, whereas for ‘IAPAR 59’, only 48 μM BAP was added. After 8 weeks for ‘Rubi’ and ‘Catuaí Vermelho 81’ and after 12 weeks for ‘IAPAR 59’, all cultivars showed indirect embryogenesis. Embryogenic material was transferred to a SP basal medium without BAP, in light (16-hour photoperiod) and standard temperature. In a second experiment, leaf explants belonging to all three cultivars were inoculated in petri dishes with SP basal medium plus 0, 2.5 and 5 μM BAP, in the dark at standard temperature. After 8 weeks, only ‘IAPAR 59’ produced direct embryos. These were transferred to SP basal medium. About 30% of embryos developed abnormalities. Normal embryos were successfully transferred to soil.

Coffee is cultivated in several Brazilian states. In the last 6 years, two million hectares of commercial coffee plantation produced ≈ 25 million sacks (60 kg each) per year (Brazil, 2000). The Brazilian or international markets, except Africa, prefer *Coffea arabica* L. cultivars to *C. canephora* Pierre cultivars (Gonçalves et al., 1998). *Coffea arabica* cultivars are still undergoing a breeding and improvement program to achieve better agronomic performance. Breeding programs using the classic methods are long and expensive. It is also necessary to shorten the time to obtain hybrids and to establish the new cultivars. Tissue culture offers a means for asexual propagation of hybrids with important agronomic traits, such as short size, high productivity, and precocious fruit ripening.

Significant progress in somatic embryogenesis is reported for several cultivars of *C. arabica* or *C. canephora* (Sondahl and Sharp, 1977; Staritsky, 1970), but there are no reports for *C. arabica* ‘Rubi’, ‘Catuaí Vermelho 81’, and ‘IAPAR 59’, three important cultivars used by farmers in Brazil. In this study we demonstrate the potential for somatic embryogenesis of these cultivars. This work provides a technological platform for classical genetics

and genetic transformation for improving crop production in the field.

Materials and Methods

Two experiments were carried out using leaf segments from the upper half of 6-month-old seedlings (20–30 mm long), cultivated in SP basal medium modified from Barrueto Cid et al. (1999). The SP basal medium contained half-strength MS (Murashige and Skoog, 1962) macrosalts, full strength MS microsalts, without KI, and half-strength MS Fe-EDTA, 1 $\text{mg}\cdot\text{L}^{-1}$ each of: calcium pantothenate, pyridoxine HCl, and nicotinic acid; thiamine HCl 10 $\text{mg}\cdot\text{L}^{-1}$, inositol 50 $\text{mg}\cdot\text{L}^{-1}$, sucrose 20 $\text{g}\cdot\text{L}^{-1}$, Phytigel 2.5 $\text{g}\cdot\text{L}^{-1}$. All explants from three *C. arabica* cultivars, ‘Rubi’, ‘Catuaí Vermelho 81’, and ‘IAPAR 59’, were cultured with the adaxial side in contact with the medium, and their approximate size was 10 mm $\times$ 5 mm.

Experiment 1. Leaf explants of the cultivars were cultured in darkness at $27 \pm 2^\circ\text{C}$ on MS basal medium, with 0 or 4 μM picloram (4-amino-3,5,6-trichloropicolinic acid). All treatments and control (0 μM picloram) contained 10 explants per petri dish (90 mm $\times$ 15 mm) and all were replicated four times. Each treatment had 10 petri dishes, for a total of 40 dishes per cultivar.

After 8 weeks for ‘Rubi’ and ‘Catuaí Vermelho 81’, and after 12 weeks for ‘IAPAR 59’, the callused explants were transferred to SP basal medium plus BAP (*N*⁶-benzylaminopurine) at : 0, 12, 24, 48 μM for the cultivars Rubi and Catuaí Vermelho 81, but IAPAR 59 only on 48 μM . Ten petri dishes were used per treatment per cultivar, with five explants in

each. The material was cultured in the dark at $27 \pm 2^\circ\text{C}$ for 4 weeks.

Experiment 2. Leaf explants of the cultivars were inoculated in petri dishes with SP medium with 0, 2.5, or 5 μM BAP, in the dark at $27 \pm 2^\circ\text{C}$ for 8 weeks. For each cultivar 10 dishes were used per treatment, with five explants per dish.

After 4 weeks in the first experiment and 8 weeks in the second, individual embryos (50 per cultivar), were transferred to SP basal medium for germination and growth. Cultivars were grown with a 16-h photoperiod, at 30 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at $27 \pm 2^\circ\text{C}$ (laboratory conditions).

After 45 d, plantlets from both experiments, with an average of height of 20 mm and root length of 15 mm, were transferred to plastic pots (90 mm $\times$ 70 mm) with damp vermiculite, covered with transparent plastic bags and kept under laboratory conditions for 2 weeks. After 15 d in vermiculite, the plantlets were transferred to soil and kept in a greenhouse with uncontrolled environmental temperature [$\approx 25^\circ\text{C}$ and 70% relative humidity (RH)] and were irrigated periodically as needed.

Experimental design and statistical analysis were performed using arc sine transformation, analysis of variance (ANOVA), and Duncan’s multiple range tests for embryogenic callus percentage and somatic embryos number ($P = 0.05$).

Results and Discussion

All explants of ‘Rubi’, ‘Catuaí Vermelho 81’, and ‘IAPAR 59’ produced callus on the first basal medium with picloram. No callus was observed on the control explants. This is the first time that picloram was used to induce callus in coffee. It was not necessary to add another plant growth regulator. By contrast, Molina et al. (2002) used 2,4-dichlorophenoxyacetic acid (4.5 μM) and kinetin (37 μM). The percentage and type of callus was not reported. In our case, the callus tissues were beige or white, humid, compact, and emerged from the adaxial side of the midvein. The cut edges of the leaves produced less callus (Fig. 1a).

In the SP basal medium with BAP, embryos were produced by indirect somatic embryogenesis (ISE) from the brown/beige callus. The high-frequency embryogenesis described in other studies (Sondahl and Sharp, 1977) was not observed. Embryos were initiated only at the peripheral base of the callus. Somatic embryos were white (Fig. 1b), or reddish for ‘IAPAR 59’. A comparison of BAP concentrations showed that 48 μM significantly promoted embryogenic callus and somatic embryo formation. In ‘Rubi’ and ‘Catuaí Vermelho 81’, this performance was superior to that of ‘IAPAR 59’ (Table 1). Table 1 shows that the percentage of embryogenic callus varied between 40% to 26% at 48 μM BAP. This concentration was the most effective for embryo production. These percentages are lower than Berthouly and Michaux-Ferrière’s (1996) results. They used *C. canephora* cultivars and different ratios of plant growth regulator for the ISE process. Broadly speaking, it seems that *C.*

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Fig. 1. (1a) *Coffea arabica* 'Rubi' leaf explant showing callus induction on MS medium with 4 μ M picloram at 8 weeks (1.5 $\times$); (1b) 'Rubi' indirect somatic embryogenesis induction on SP medium without BAP (2.5 $\times$); (1c) plantlet from 1b after 30 d (20 mm); (1d) typical 'Rubi' plant from 1c transferred to soil in greenhouse, 50-d-old (100 mm).

Table 1. Indirect somatic embryogenesis in coffee from leaf callus showing percentage of embryogenic callus and the number of somatic embryos recovered after 8–12 weeks on 4 μ M picloram and 4 weeks on BAP.

Cultivar	BAP (μ M)				No. of embryos			
	Embryogenic callus (%)							
	0	12	24	48	0	12	24	48
Rubi	---	8 c	10 c	40 a	---	12 c	15 c	80 a
C.V. 81	---	4 c	8 c	36 a	---	3 c	3 c	90 a
IAPAR59	---	---	---	26 b	---	---	---	44 b

Data shown are original and not arc sine transformed. Means with different letters are significantly different ($P = 0.05$) according Duncan's multiple range comparison procedure.

canephora genotypes have a greater tendency for embryogenesis than *C. arabica*.

The number of embryogenic clusters from 'Rubi' and 'Catuaí Vermelho 81' varied from one to three per callus. In the case of 'IAPAR 59', only one cluster per callus was common. In all clusters, two types of embryos (torpedo stage) were observed: large tubular and compact (primary), and small translucent white (secondary). The latter were generally observed emerging from the surface of the hypocotyl base of the primary embryos.

We observed that 'Rubi' and 'Catuaí Vermelho 81' did not show any embryogenic or callus response on medium with 0–5 μ M BAP after 8 weeks. However, 'IAPAR 59' showed direct somatic embryogenesis (DSE) without passing through the callus stage. At 2.5 μ M BAP, a total of 31 embryos was counted from 12% of leaf explants. At 5 μ M, 68 embryos were recovered from 30% of leaf explants.

DSE was initially reported in *C. arabusta*, *C. canephora* and more recently in *C. arabica*. For *C. arabusta*, a high BAP/2,4-D ratio and high levels of adenine and malt extract were used (Dublin, 1981). For *C. canephora*, a high percentage of DSE was obtained with isopentenyladenine (2-ip), plus inhibitors of action and ethylene synthesis (Fuentes et al., 2000; Hatanaka et al., 1995). For *C. arabica*, BAP level (13 μ M) yielded 12% to 73% DSE (Molina et al., 2002). Taken together, the embryogenic capacities for these species remain difficult to evaluate.

About one-third of the embryos showed abnormalities such as the lack of root or shoot development. Plantlets were not established. Similar problems were also reported by Herman and Haas (1975), indicating that more research is needed to solve this problem. The remaining two-thirds produced somatic plantlets that were phenotypically similar to

seedlings (Fig. 1d). Figure 1c presents the typical 'Rubi' embryogenic pattern. For the other cultivars, we also observed a similar ISE. Cultivar IAPAR 59 showed both DSE and ISE (data not shown). Our experience shows that when the coffee plantlets were vigorous, with a stem and root (e.g., Fig. 1c), they could be transferred successfully to soil in greenhouse under $\approx 25^\circ\text{C}$ and 70% RH.

Our results confirm that, in coffee, somatic embryogenesis is cytokinin-dependent. At zero or low BAP concentration, either no embryos or only a few embryos were formed. In summary, we offer a simple and an useful alternative protocol for ISE or DSE from leaf explants in *C. arabica* cultivars Rubi, Catuaí Vermelho 81, and IAPAR 59. The protocol for ISE requires a MS medium with 4 μ M picloram for 8–12 weeks followed by SP medium with 48 μ M BAP. For DSE with 'IAPAR 59', the protocol requires SP medium plus 5 μ M BAP.

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