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Influence of Silver Nitrate on Somatic Embryogenesis Induction in Arabica Coffee (*Coffea arabica* L.)

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HIGHLIGHTS

- LFSE offers a simple and rapid way for somatic embryo and plant conversion.
- Somatic embryos is favored by the addition of $AgNO_3$.
- Somatic embryos were induced using leaf sections of in vitro plants within 8 weeks.

Abstract: The influence of silver nitrate (AgNO3), benzyladenine (BAP), and indole-3-acetic acid (IAA) on low frequency somatic embryogenesis (LFSE) induction in Caturra and Catuaí arabica coffee was evaluated. For the Caturra cultivar, the production of somatic embryos was significantly increased by adding AgNO3 to the semisolid culture medium. The highest average number of somatic embryos for this cultivar was obtained using 6.6 µM BAP, 2.85 µM IAA, and 40 µM AgNO3. In contrast, for the Catuaí cultivar, the highest average number

of somatic embryos was obtained using semisolid medium supplemented with 8.8 µM BAP, and 2.85 µM IAA. Using these protocols, somatic embryos were directly induced using leaf sections of in vitro plants of both coffee cultivars within 8 weeks. The somatic embryos developed into rooted plants with a 100% survival rate upon transfer to the greenhouse.

Keywords: coffee; silver nitrate; somatic embryos; plant growth regulators; tissue culture.

INTRODUCTION

Plant somatic embryogenesis (SE) has been defined as the formation of embryos from a single or group of haploid or somatic cells [1, 2]. Low frequency (LFSE) and high frequency somatic embryogenesis (HFSE) have been described. In the first type, somatic embryos are induced directly from pro-embryogenic cells of explants, while in the second, they originate from embryogenic callus [1]. It has been suggested that in LFSE the origin of somatic embryos is unicellular, whereas in HFSE has been described as unicellular or multicellular [3]. SE is a powerful biotechnological tool used to propagate elite plants or to conserve important genotypes [4]. Moreover, SE offers an efficient *in vitro* regeneration approach as a fundamental step in plant genetic improvement for studying basic aspects of ontogenesis of somatic embryos [5]. In *Coffea* spp., the first studies of SE have been reported at the beginning of 1970 [6]. Since then, a large quantity of LFSE and HFSE protocols have been optimized demonstrating that coffee is not a recalcitrant species for SE [4]. In the LFSE the somatic embryos are obtained faster (approximately 70 days) using only one medium meanwhile in HFSE several media are used and somatic embryo formation takes 9-10 months [4]. Although, in LFSE small number of somatic embryos are obtained (around 10 per explant) compared to hundreds of somatic embryos obtained per gram of embryogenic calli [4], the unicellular origin of somatic embryos in LFSE represents an advantage for the chemical and physical mutagenesis, genetic transformation and genetic editing, since prevents or reduces the appearance of chimeras [7]. In *C. arabica* and *C. canephora* many factors (such as genotype, explant type, the physiological state, age and growth conditions of the donor plants, the season of collection, nutrient composition of the medium, the volume of dissolved CO_2 or O_2 in the culture flask, and plant growth regulators) that affect LFSE induction have been studied [3, 8, 9, 10, 11, 12, 13]. However, few studies reported the effect of silver nitrate on LFSE using leaf explants of *C. arabica* L. and to the best of our knowledge it has not been analyzed using Caturra and Catuaí, which are two economic important producer cultivars in Costa Rica. Since SE is genotype dependent, the culture medium need to be modified for the different genotypes [7].Therefore, the objective of this study was to determine the influence of the benzyladenine (BAP), indole-3-acetic acid (IAA), and silver nitrate ($AgNO_3$) on low frequency somatic embryogenesis using leaf explants of *Coffea arabica* L. cultivars Caturra and Catuaí.

MATERIAL AND METHODS

In vitro coffee (*Coffea arabica* L. cvs. Caturra and Catuaí.) plants were used as explant donors. Leaf sections (0.5 cm^2) without midveins or margins were cultured in disposable petri dishes (94×16 mm) with the adaxial side in contact with 20 ml of MS medium [14] (Phytotechnology Laboratories) supplemented with 30 gL^{-1} sucrose, BAP, IAA, and $AgNO_3$ at the concentrations listed in Table 1 [15]. Cultures were maintained at 26±2 °C with a 16 h photoperiod (30 $\mu mol.m^{-2}.s^{-1}$). One hundred leaf disks were evaluated per treatment. The number of somatic embryos per explant was recorded after 8 weeks of culture. Statistical analysis was performed using one-way ANOVA and means were compared with Duncan's Test at p=0.05 using the program InfoStat [16]. Morphological features of the somatic embryos were determined using a histological analysis [17]. Globular embryos were transferred to baby food jars containing 20 mL of MS medium (Phytotechnology

Laboratories) supplemented with 6.6 µM BAP, 2.85 µM IAA, 40 µM $AgNO_3$, and 30 gL^{-1} sucrose. Jars were sealed with plastic wrap. Cultures were maintained at 26±2°C with a 16 h light photoperiod (30 $\mu mol.m^{-2}.s^{-1}$). The growth regulators and $AgNO_3$ used in the study were obtained from Sigma, St. Louis, MO, USA. The media pH was adjusted to 5.6 and the media was gelled with 8 gL^{-1} agar (Phytotechnology Laboratories) before autoclaving at 121 °C for 21 min. Regenerated plantlets were washed with water and transferred to pots containing autoclaved peat substrate (Plug Mix Perlite, VJ Centroamericana, San José, Costa Rica) and grown in the greenhouse at 18-35°C with a 12 h photoperiod and 80% relative humidity.

RESULTS

In this work, the effect of BAP, IAA, and $AgNO_3$ on LFSE induction in Caturra and Catuaí cultivars was evaluated (Table 1). For the Caturra cultivar, the formation of somatic embryos was significantly superior on culture medium with added $AgNO_3$ than on medium devoid of this compound (Figure 1). Somatic embryo production was lower at BAP concentrations of 8.8 µM and 11.1 µM than 6.6 µM in treatments with added $AgNO_3$ (Table 1). On the other hand, the complementation of the culture medium with $AgNO_3$ did not stimulate the production of somatic embryos in the Catuaí cultivar (treatment E) (Figure 2D, 2E, and 2F), indicating that the response to $AgNO_3$ was dependent on genotype. The highest average of somatic embryos for this cultivar was obtained on semisolid medium supplemented with 8.8 µM BAP and 2.85 µM IAA (Figure 2B) (Table 1). Somatic embryos did not form in either cultivar on culture medium devoid of BAP, IAA, and $AgNO_3$ (treatment A) (Table 1).

Table 1. Effect of BAP, IAA, and $AgNO_3$ on direct somatic embryogenesis in leaf explants of Caturra and Catuaí vitroplants after 8 weeks of culture.

	Treatments (µM)			Number of somatic embryos per explant (mean±SE)	
	BAP	**IAA**	**$AgNO_3$**	**Caturra**	**Catuaí**
A	-	-	-	0±0 c[1]	0±0 d
B	6.6	2.85	-	0.3±0.3 c	0.4±0.2 c d
C	6.6	2.85	40	3.3±0.3 a	1.2±0.2 b
D	8.8	2.85	-	0.4±0.3 c	2.4±0.2 a
E	8.8	2.85	40	2.3±0.3 b	1.0±0.2 b c
F	11.1	2.85	-	0±0 c	0.8±0.2 b c
G	11.1	2.85	40	2.4±0.2 b	0.7±0.2 b c

[1] Means followed by the same letter are not significantly different at p=0.05 using Duncan's test.

During the LFSE process, four developmental stages of Caturra somatic embryos were observed (Figure 3). Globular-shaped somatic embryos were bright (Figure 3A) and their cells showed a prominent nucleus and a dense cytoplasm. A suspensor-like structure connected the globular embryos with parent explants (Figure 3B). Heart-shaped embryos showed a well-defined procambium and a notch on the tip (Figure 3C and 3D). Torpedo stage somatic embryos (Fig. 3E) showed a distinct procambium (Figure 3F). Finally, the torpedo stage somatic embryos formed small plantlets with distinct cotyledon leaves, apical shoots and root meristems (Figure 3G). The cotyledon primordia formation began with the division of the procambium in the shoot axis (Figure 3H).

Using the protocol described here, it was possible to induce somatic embryos directly from leaf sections of in vitro plants of both coffee cultivars within 8 weeks. The somatic embryos developed into rooted plants with a survival rate of 100% upon transfer to the

greenhouse. In this study, the induction of somatic embryos, development of plants and greenhouse acclimatization of in vitro culture plantlets required 14 months. A protocol for LFSE induction from leaf segments of *C. arabica* L. cv Caturra to acclimatization of in vitro culture plantlets in the greenhouse is shown in Figure 4.

DISCUSSION

Several factors influence SE in coffee, including genotype, explant type, the physiological state, age and growth conditions of the donor plants, the season of collection, nutrient composition of the medium, the volume of dissolved CO_2 or O_2 in the culture flask, and plant growth regulators [4,5].

Similarly to our results, previous studies have demonstrated that silver nitrate (30–60 µM) stimulates organogenesis and SE in *C. canephora* [18, 19], *C. dewevrei* [12], *C. arabica* [19]. In this sense, a possible explanation of the positive effect of $AgNO_3$ could be that this compound promotes in vitro regeneration by inhibiting ethylene action and limiting explant oxidation and necrosis. Moreover, the inhibition of ethylene production has positive effects by enhancing the biosynthesis of polyamines, which participate in growth and development of plants as well as in basic biological process [20]. Likewise to the results of the present work, previous studies have demonstrated that coffee somatic embryogenesis is promoted by the combined use of auxins and cytokines [4, 5]. Similarly to our results, the formation of somatic embryos was favored by the combination of BAP, IAA, and $AgNO_3$ in *C. dewevrei* [16] and *C. arabica* and *C. canephora* [19].

Similar histological studies in coffee indicated that low frequency somatic embryo formation from leaf segments began with the rapid division and proliferation of the most mitotically active subepidermal cell layers. In this sense, it has been reported that small, isodiametric, densely cytoplasmic cells suffered a series of organized divisions originating somatic embryos [3, 17].

As previously reported in coffee, somatic embryos are obtained quicker using LFSE (about 7-12 weeks) than HFSE [4, 17, 21].

CONCLUSION

A protocol for LFSE was presented for simple and rapid formation of somatic embryo and plant conversion from leaf explants of Caturra and Catuaí using BAP, IAA and $AgNO_3$. This protocol does not involve the formation of embryogenic callus. The faster response provides a new scenario for coffee genetic modification.

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