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Induction of Somatic Embryos of *Coffea arabica* Genotypes by 6-Benzyladenine

Julieta Andrea Silva de Almeida* • **Maria Bernadete Silvarolla**

Centro de Café 'Alcides Carvalho', Instituto Agronômico de Campinas, Avenida Barão de Itapura, nº. 1481, CEP 13.012-970, Campinas, São Paulo, Brazil

Corresponding author: * **julietasa@iac.sp.gov.br**

ABSTRACT

The objective of the present study was to verify the effect of adding 6-benzyladenine (6-BA) on somatic embryogenesis of *Coffea arabica* genotypes AC1, AC2, AC3 and Mundo Novo cv. 'IAC 376-4'. Rectangular foliar explants of these genotypes were inoculated into a single semi-solid culture medium consisting of ½ MS salts supplemented with 10, 15, 20, 30, 40 and 50 μM 6-BA, respectively and kept in the dark at 25°C. The treatments were evaluated with respect to the number of sides of the explant showing formation of structures, an estimate of the size of the structure formed by the explant and the total number of somatic embryos (SEs) produced. The formation of small structures (3 mm) on the borders of the explants of AC and 'Mundo Novo' was observed and these subsequently developed SEs when cultured in medium supplemented with lower concentrations of 6-BA tested (10, 15, 20 or 30 μM). In addition, SEs were also formed directly on the borders of the explants. Another aspect of the present study is the formation of SEs as a response to 6-BA as the sole growth regulator and their development in a single culture medium, in a single phase. This culture method results in a reduction in time, handling and consumables, thus being more advantageous, in addition to opening perspectives for its use with other *C. arabica* genotypes.

Keywords: 6-BA, coffee shrub, direct somatic embryogenesis

INTRODUCTION

The majority of vegetable species can be multiplied by means of somatic embryogenesis, the asexual reproduction model based on the cellular totipotencial concept, in which the somatic cell possesses all the genetic information necessary to induce the formation of a new plant (Vasil 2008). The somatic cell goes through alterations that lead to the embryogenic state, which could induce the initiation and development of a somatic embryo (SE) without the need for exogenous stimulation (Komamine *et al*. 1992; De Jong *et al*. 1993). The new SE starts as a bipolar structure with no vascular connection to the original tissue (Williams and Maheswaran 1986; Carman 1990; Dodeman *et al*. 1997; von Arnold *et al.* 2002; Quiroz-Figueroa *et al*. 2006) and then passes through histodifferentiation, composed of the globular, heart and torpedo developmental stages (Menéndez-Yuffá and Garcia 1997).

In *Coffea* somatic embryogenesis can occur by indirect (ISE) or direct (DSE) means and different genotypes have successfully responded to its application, although only some have the capacity for either ISE or DSE, whereas others have the capacity for both. In ISE there is an initial re-determination of the differentiated cells from the explant, followed by cell proliferation leading to the formation of a cell mass, the callus. The SEs are then formed from determined cells from sectors of this callus (Teixeira *et al* 2004; Almeida *et al.* 2008a). On the other hand, in DSE, the SEs are formed directly on cells at the edge of the leaf explant (Dublin 1981; Emons 1994; Yeung 1995; Almeida *et al.* 2007), which are pre-determined and competent for embryogenic development (Sharp *et al*. 1982; Gaj 2004).

But, normally, ISE is applied mainly to *C. arabica* genotypes whereas DSE is applied to *C. canephora* genotypes, and it has also been observed that ISE results in the formation of more SEs than DSE (Vieira and Kobayashi 2000). On the other hand it would be desirable to apply DSE to *C. arabica* more frequently, since when one compares ISE with DSE, it can be seen that the former presents both callogenesis and embryogenesis phases, whereas the latter occurs in a single culture medium in a single phase, resulting in a reduction in handling, the use of consumables and in the formation time of the SEs, thus being more advantageous (Altmann and Loberant 1998; Kumar *et al*. 2006).

The control of the occurrence of somatic embryogenesis in *Coffea* genotypes is strongly associated with plant growth regulators. *C. arabica* ISE is established through the use of the auxin 2,4-D (2,4-dichlorophenoxyacetic acid) and of kinetin for callogenesis, and of NAA (α-naphthaleneacetic acid) and kinetin for the induction of embryogenesis (Sondhal and Sharp 1977; Almeida *et al.* 2005; Samson *et al.* 2006; Almeida *et al.* 2008a). In *C. canephora,* the control of DSE is associated mainly with only the use of a cytokinin, like 2-iP (N6-(2-isopentyl)adenine), zeatin or kinetin (Hatanaka *et al*. 1991; Ramos *et al.* 1993; Hatanaka *et al* 1995; Almeida *et al.* 2007). The cytokinin 6-benzyladenine (6-BA) promotes bud sprouting in nodal explants of coffee trees (Garcia and Rafael 1989; Sondhal *et al* 1985; Ramos and Almeida 2005), but the literature also reports few studies relative its use to induce DSE in *C. arabica* such as the varieties 'Típica' (Yasuda *et al*. 1985), cv. 'Caturra' (Molina *et al*. 2002); cv, 'IAPAR 59' (Ayub and Gebieluca 2003) and the cultivars 'Rubi', 'Catuaí Vermelho' and 'IAPAR 59' (Barrueto-Cid and Cruz 2004). However, these results are reduced and little broadcast, but they open the possibility for the application of DSE with different *C. arabica* genotypes, since these would form SEs just with the use of the 6-BA and in a single culture medium. Thus the present study intended to characterize the formation of SEs of the *C. arabica* genotypes AC1, AC2 and AC3 and of the 'Mundo Novo' IAC 376-4 cultivar using 6-BA in a single culture medium.

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MATERIALS AND METHODS

In this study, leaves collected up to the third pair of plagiotropic branches of the *C. arabica* genotypes AC1, AC2 and AC3 and the 'Mundo Novo' IAC 376-4 cultivar (MN cv.) and established in a container (10 L) with clay soil, under greenhouse conditions, with a 40% sunlight reduction screen at Campinas Agronomic Institute. Plants were fertilized every six months and the pots were irrigated daily. The leaves were collected in February and August 2006, with an average temperature of 35 and 28°C, respectively, inside the greenhouse. Soon after collection, the leaves were washed with a detergent solution and then with running water. The leaves were then immersed in a 2% sodium hypochlorite solution for 25 min, rinsed in distilled water three times, maintained in a moist chamber (approx. 80% relative humidity) for 24 hrs, and then immersed in a 2% sodium hypochlorite solution again (Ramos *et al*. 1993). Rectangular (2 cm^2) explants were obtained from these leaves and inoculated into culture medium in transparent glass flasks (200 mL), with the adaxial face in contact with the surface of the medium. The flasks contained 30 mL of culture medium and were maintained in the dark at 25°C. The embryos formed from these explants were transferred to flasks containing 30 mL of embryo germination medium and maintained in the light for 16 hrs at 25°C. Subsequently the germinated SEs were transferred to seedling growth medium and maintained in the light for 16 hrs.

For the induction of embryogenesis, a medium containing half the salt concentrations found in MS (Murashige and Skoog 1962) was used, with the addition of sucrose (20 g/L) and different concentrations of 6-BA. The pH of the medium was adjusted to 5.8 and it was then solidified with agar (5 g/L) and autoclaved at 121°C and 1.5 atm. for 20 min. To germinate the SEs, a medium containing half the salt concentration found in MS but without the addition of the plant growth regulators was used, and the same medium was also used for the growth of the seedlings.

Two experiments were carried out, the first using 10, 15 and 20 µM 6-BA and the second 30, 40 and 50 µM. Each treatment consisted of eight repetitions, with one explant in each. The treatments were evaluated with respect to the number of sides of the explant showing structure formation, an estimate of the size of the structure formed by the explant and the total number of SEs produced.

A completely random experimental design was adopted, and the data obtained transformed ($\sqrt{x} + 1$), statistically analyzed using the F-test and the means compared using Tukey's test, both at the 5% level (Gomez and Gomez 1984).

RESULTS

In a preliminary study, the formation of small structures on the borders of the explants of the AC genotypes and 'Mundo Novo' cultivar of *C. arabica* were first verified, and these subsequently developed SEs on their surface when cultivated in a single culture medium with just the addition of 5 µM of 6-BA. It was observed that these structures oxidized before forming SEs. SEs were also formed directly on the borders of the explants. However the number of SEs was reduced (data not shown) and therefore other concentrations of 6-BA were tested aiming to optimize their production. Thus a further two experiments were carried out, the first using 10, 15 and 20 µM 6-BA and the second 30, 40 and 50 µM.

In the first experiment, the genotype AC1 showed an average of 3 to 4 sides for the parameter of number of sides of the rectangular explant showing the formation of structures, and the others showed 1 to 2 sides (**Fig. 1A**). It was also observed that the size of these structures was small for all the genotypes, with an average of 3 mm (**Figs. 1B, 2A**). SE formation occurred both on the structures (**Fig. 2B**) and directly on the explant border (**Fig. 2C**) and started about 120 days after starting the experiment, but in reduced numbers (data not shown). After 230 days, AC3 showed the greatest number of SEs for all the 6-BA concentrations, followed by AC2 just for 15 µM (**Fig. 3A**), whereas 'Mundo Novo' maintained a reduced number throughout the whole experiment. But, after 390 days, all genotypes

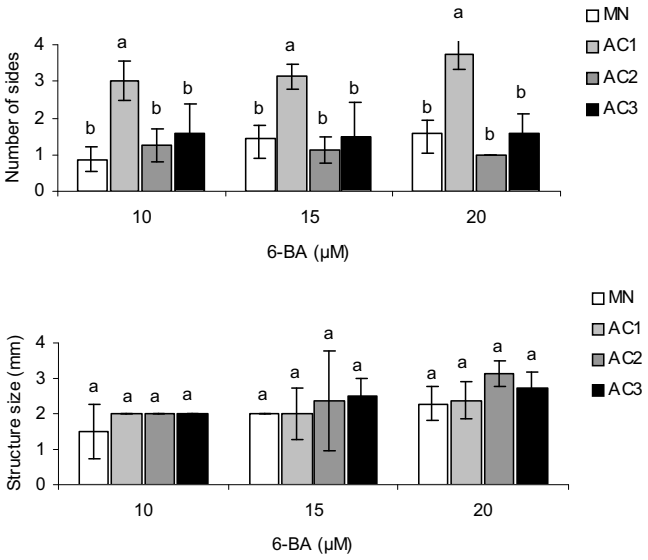

Fig. 1 Effect of different concentrations of 6-BA on the somatic embryogenesis in leaf explants of the AC genotypes and MN cultivar of *C. arabica* when cultivated in a single culture medium and were maintained in the dark at 25°C. (**A**) Number of sides of the rectangular explant showing the formation of structures. (**B**) Estimation of the size of structures. The means were compared by Tukey's test at P = 0.05, only within each dose of 6-BA. Each treatment had eight repetitions.

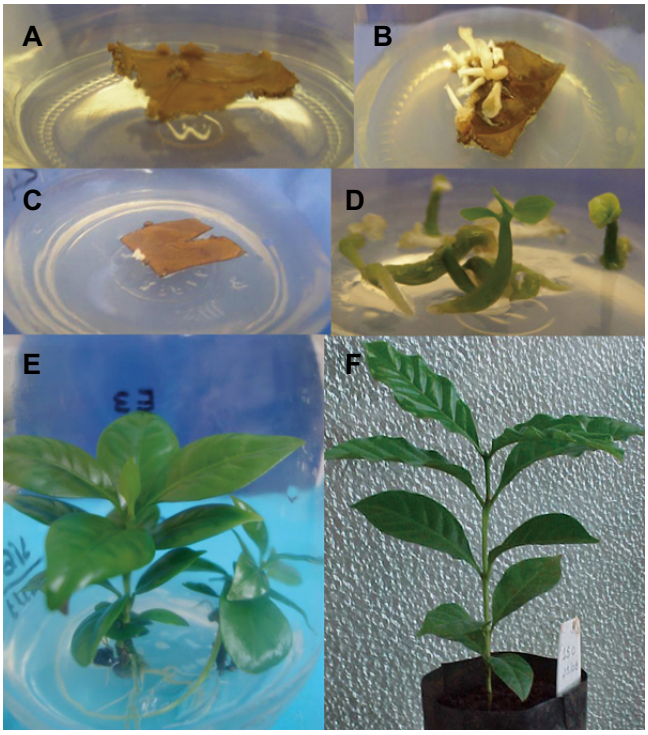

Fig. 2 Somatic embryogenesis on leaf explants of the AC1 genotype of *C. arabica* when cultivated in a single culture medium with addition of 6-BA. (**A**) Formation of small structures on the borders of the explant. (**B**) Embryos developed on the surface of the structures. (**C**) Embryos formed directly on the borders of the explant. (**D**) Germinated embryos. (**E**) Seedlings. (**F**) Plant in nursery condition.

produced an elevated number of SEs for all the 6-BA concentrations (**Fig. 3B**) except for MN, which continued with little SE production. Also, the explants oxidized 90 days after the start of the experiment, and the structures formed on the explants, 40 days after their formation (data not presented), suggesting that oxidation was necessary for SE induction, since the initiation of SE formation occurred after 120 days.

In the second experiment, the explants of all the geno-

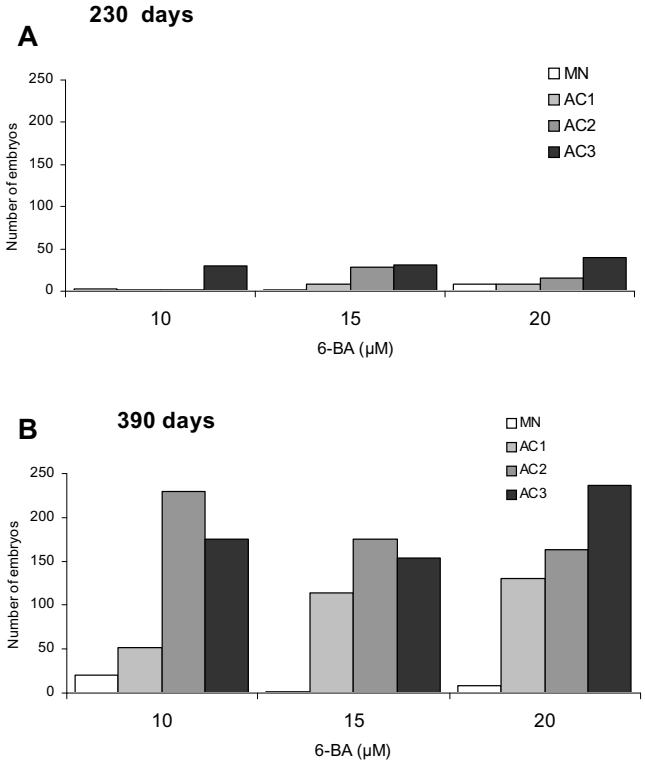

Fig. 3 Effect of different concentrations of 6-BA on the number of embryos developed from structures or formed directly on the borders of the explants of the AC genotypes and cv. 'MN' of *C. arabica* when cultivated in a single culture medium and were maintained in the dark at 25°C. Each treatment had eight repetitions. The data are cumulatives for each treatment.

types showed the formation of structures, AC3 showing the greatest number of sides with the formation of such structures at the concentrations of 30, 40 and 50 µM of 6-BA (**Fig. 4A**). These structures were also of reduced size (**Fig. 4B**) as in the previous experiment. On the other hand, the formation of SEs only occurred for explants maintained in 30 µM of 6-BA, nothing being formed in the other treatments (**Fig. 4C**). Considering the genotypes tested, AC3 formed the greatest number of genotypes followed by AC1, whereas AC2 and 'Mundo Novo' showed little response.

The SEs obtained in both experiments germinated (**Fig. 2D**), reached the seedling stage (**Fig. 2E**) and rooted seedlings reached approximately 70% survival under acclimatization conditions (**Fig. 2F**).

DISCUSSION

In the first experiment is showed that the production of embryos gradually increased with time for the AC genotypes and remained low for 'Mundo Novo'. In another study, it was found that AC1 genotype formed a larger number of SEs than 'Mundo Novo' when their foliar explants were inoculated into a semi-solid medium added of 6-BA and polyethylene glycol 6000 (Almeida *et al*. 2008b). Besides, the application of ISE showed again this difference, the AC1, AC2 and AC3 genotypes produced more number of embryos than 'Mundo Novo' (Almeida *et al.* 2008a). Another way, treatments with 6-BA induced satisfactory multiple sprouting from the nodal explants of 'Mundo Novo' (Ramos and Almeida 2005). So, all these observations indicated that maybe the micropropagation of 'Mundo Novo' is more efficient by process of budding than somatic embryogenesis. But, the low efficiency of somatic embryogenesis capacity of 'Mundo Novo' can be explained in terms of cell competence. Jiménes (2001) stated that one can observe both competent and incompetent sections on a single callus, indicating that genetically identical cells respond differently to a particular stimulus, and that only a minority are responsive. Thus, in this study, this could infer that the explants of the 'Mundo Novo' may form few embryos due to the absence of competent cells. For the first experiment it could also be said that the elevated formation of embryos verified to the AC genotypes after 390 days was due to the explant cells that became competent after this period. This suggests that the acquisition of such competence could be attributed to this long period of time, which could have represented a stress condition that indirectly acted as an induction factor for formation. According to Merkle *et al*. (1995), various changes can occur in the reprogramming of a cell into the competent state. Firstly the current gene expression is terminated and substituted by an embryogenic program, which does not occur in all the cells at the same time. Thus the process of the acquisition of embryogenic competence by a somatic cell involves both the reprogramming of the gene expression model and changes in the morphology, physiology and metabolism, which can occur over a long period of time as observed in the present study. Thus these alterations reflect de-differentiation, activation of cell division and a change in cell destiny (Namasivayam 2007)**.**

Another aspect of the present study refers to the induction, initiation and differentiation of SEs as a response to 6-BA as the only growth regulator, which suggested that the control of the induction of these embryos on the *C. arabica* genotypes studied may be similar to that verified to the majority of *C. canephora* genotypes (Hatanaka *et al.* 1991; Ramos *et al*. 1993; Hatanaka *et al.* 1995; Almeida *et al*. 2007). The literature also presents some reports on the induction of somatic embryos of *C. arabica* as a response solely to the use of cytokinin, such as the 'IAPAR 59' and the Sarchimor hybrid to the 6-BA and/or thidiazuron (Ayub and Gebieluca 2003), or the cultivars 'Rubi', 'Catuai 81' and 'IAPAR 59' to 6-BA (Barrueto-Cid and Cruz 2004). Quiroz-Figueroa *et al*. (2002) also obtained somatic embryos from *C. arabica* explants cultivated in a single culture medium with just 6-BA, which initiated as small, densely cytoplasmatic, isodiametric cells. The influence of cytokinins on the induction of somatic embryos of *C. arabica* has also been observed in other systems, such as previously formed calluses, pulsed for 24 hours in a liquid medium containing 6-BA (Papanastasion *et al*. 2008).

In the first experiment, the number of embryos formed was greatest for the AC2 and AC3 genotypes for all 6-BA concentrations tested, whereas in the second experiment this response only occurred in the treatment with 30 µM and only AC3 produced a larger number of embryos. These results evidence the differentiated sensitivity of these genotypes to different concentrations of 6-BA. Another way, regardless of being trees of a same progeny, the results suggested genetic differences in the capacity of somatic embryogenesis among AC genotypes. This fact is corroborated by the application of ESI which results showed that AC1 genotype produced the highest number of embryos than AC2 and AC3 (Almeida *et al.* 2008a).

Another observation of this study is that, in both experiments, somatic embryos were formed either directly from the border of the explants or from the structures that had developed at the explant borders. Besides, the results concerning the structures developed by the explants of all the genotypes showed that these were all of very reduced size, 3 mm. In addition, it could also be suggested that these structures had a function similar to that of the callus in ISE while the formation of embryos directly on the border of the explants, classified as DSE. Thus, in the present study the formation of somatic embryos in genotypes AC and MN occurred both by DSE and ISE. Another way, Acuna (1993) studied somatic embryogenesis in foliar explants of 'Caturra' and the hybrid Timor inoculated into a single culture medium with the addition of 6-BA and/or 2iP, and showed too the formation of two types of structures he called microcalluses: small compact globular calluses constituted of rounded cells, and the larger non-amorphous compact calluses containing elongated parenchymatous cells. Da Silva *et al*. (2005) also inoculated *C. arabica* cv. 'Catimor' ex-

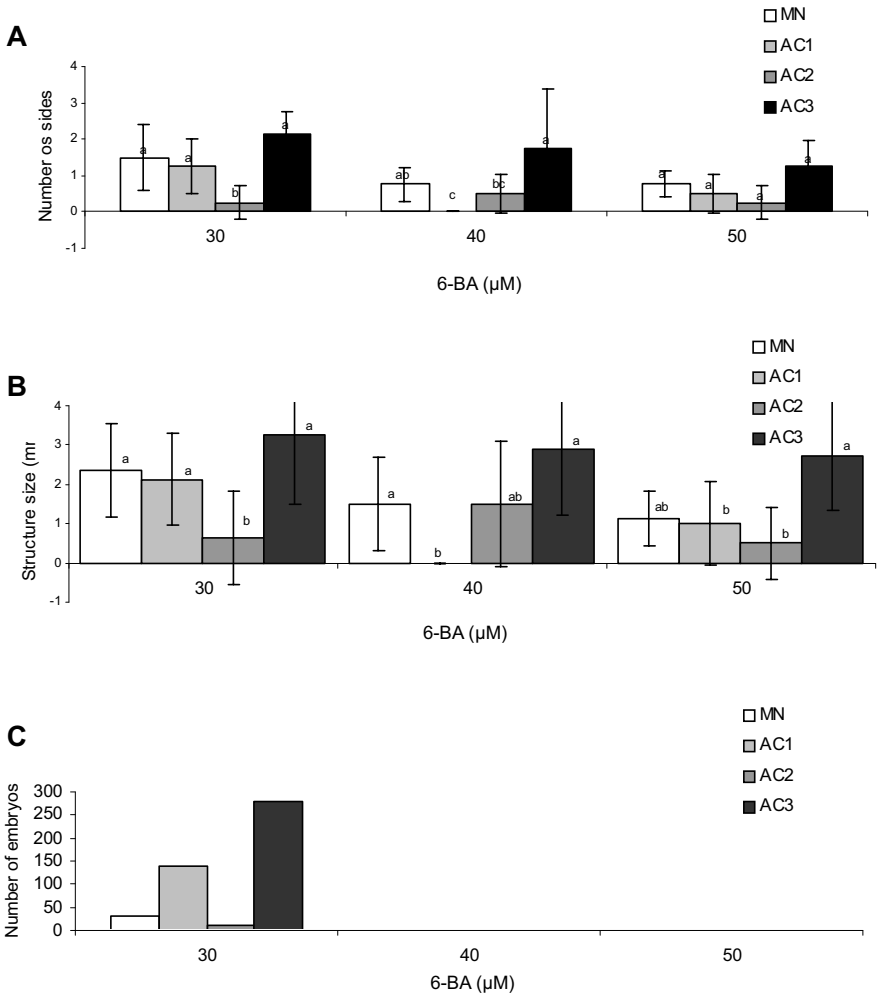

Fig. 4 Effect of different concentrations of 6-BA on the somatic embryogenesis in explants of the AC genotypes and MN cv of *C. arabica* maintained in the dark at 25°C. (**A**) Number of sides of the explants with the formation of structures. (**B**) Estimation of the size of the embryogenic structures. (**C**) Total number of embryos. The means were compared by Tukey's test at P = 0.05 only within each dose of 6-BA, except for **C** whose the data are relative for the total number of embryos. Each treatment had eight repetitions.

plants into a single medium with just 6-BA, and observed the formation of calluses with the initiation of embryos, although it was not clear to the authors if these were initiated by DSE or ISE. Another interesting aspect of this study is that both the structures formed from the borders of the explants and explant edges themselves maintained the same pattern in terms of size, color and morphology throughout the course of the experiment. However, both the structures and the explant edges showed continuous formation of new embryos, a feature that allows to characterize the two systems as embryo multiplication matrices. Wann (1988) suggested that the phenomenon of the direct formation should be considered as the cloning of pre-embryogenic determined cells (PEDC), constituting a large scale reproduction of pro-embryos, and the role of growth regulators can be considered as agents to stimulate division that result in more PEDCS. So, maybe, in this case the 6-BA controls the cloning of embryos.

Although ISE is the via most employed to obtain somatic *C. arabica* embryos, the results of the present study provided some evidence of the possibility of forming them via DSE with a reduction in time, handling and consumables, also opening perspectives for its application with other genotypes of this species.

CONCLUSIONS

Foliar explants of the *C. arabica* AC1, AC2 and AC3 and the cv. 'Mundo Novo' genotypes investigated formed somatic embryos in a single culture medium and in response to a single plant growth regulator, 6-BA.

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