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Indirect Effect of Agar Concentration on the Embryogenic Response of *Coffea canephora*

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ABSTRACT

Coffea leaf explants can present curvature of their edges after inoculation on solid culture medium, decreasing contact with the medium and leading to a less efficient embryogenic process. In the present study greater contact was shown between cv. 'Robusta' explants and the culture medium surface when an agar concentration of 3 g L^{-1} was used instead of 6 g L^{-1}, a concentration widely used for the majority of species inoculated *in vitro*. In addition, the treatment with 3 g of agar also resulted in greater efficiency of direct somatic embryogenesis of this species, verified by an increase in the number of sides of the explants showing the formation of embryogenic structures, and also from their size and the number of embryos formed. Thus the reduction in agar concentration allowed for greater physical contact between the edge of the explants and the surface of the culture medium, and also favoured the efficiency of somatic embryogenesis in leaf explants of *C. canephora* cv. 'Robusta 2264 Mar'.

Keywords: agar concentration, culture medium, curvature of the explant, direct and indirect somatic embryogenesis

INTRODUCTION

Coffea canephora cv. 'Robusta 2264 Mar' plants are highly heterozygous and thus multiplication via seeds becomes unviable if the objective is to obtain good uniformity in the field. Micropropagation of this species represents a viable alternative from both research and commercial points of view, and can be obtained via somatic embryogenesis, which has been successfully employed in coffee cultures.

Somatic embryogenesis of *Coffea* can occur directly as verified in the F1 Arabusta hybrid (*C. arabica* X *C. canephora)* (Dublin 1981) and *C. canephora* (Ramos *et al*. 1993), with the embryos forming directly from pre-embryogenic cells on the edges of explants, or indirectly, with the initial formation of calluses as in *C. arabica* (Sondhal and Sharp 1977), in eight *C. arabica* genotypes (Almeida *et al.* 2001) and in three *C. arabica* genotypes with reduced caffeine seed content (Almeida *et al.* 2006), from which cell niches can subsequently differentiate into embryos (Evans *et al*. 1981).

In the somatic embryogenesis of *C. canephora* the leaf explants have been observed to show curvature after inoculation on solid culture media, causing a reduction in contact between them and the medium. However it was preliminarily observed that greater contact between the leaf explants with curvature and the culture medium occurred when the agar was used at a lower concentration, showing a more gelatinous consistency. Thus in the present study the hypothesis was raised that a more gelatinous culture medium could favour contact between the edges of the explants and the culture medium surface.

In the somatic embryogenesis of the majority of species, the agar has been used at a variety of concentrations varying from 6 to 10 g L^{-1} (Street 1973), with no fixed concentration for this purpose. However, the effect of the agar concentration on *in vitro* vegetable tissue has been little discussed, since the agar presents diverse origins depending on the source of the raw material, the algae and the manufacturer. Scholten and Pierik (1998) tested different types and brands of agar and found difficulty in establishing an ideal type for the species studied (*Rosa hybrida* cv. 'Motrea'; *Lilium* cv. 'Enchantement' and *Sulcorebutia alba* (Cactus)). These authors also suggested that each plant species and each developmental process required its own choice of agar. Beruto and Curir (2006) showed that mineral and phenolic agar impurity content of three commercial agars (Oxoid, Merck and Roth) influenced the outcome of *Ranunculus asiaticus* micropropagation.

For *Coffea* considerable variation also exists with respect to the agar concentration used, such as 8 g L^{-1} for *C. arabica* (Sondhal *et al*. 1984), 9 g L^{-1} for *C. arabica* L. var. Typica (Yasuda *et al.* 1985), 6 g L^{-1} for *C. arabica* cv. 'Catimor' (Simões-Costa *et al*. 1999) and 8 g L^{-1} for *C. arabica* cv. 'Caturra' (Molina *et al*. 2002). However, despite the variation in agar concentration used in the media to induce somatic embryogenesis, agar is not the only factor responsible for its induction. Induction and the start of somatic embryogenesis can be affected by various factors, such as the developmental state of the donator plant of the explant, the type of explant and the environmental conditions (culture medium composition, light and temperature) (Fehér *et al.* 2003; Gaj 2004; Jiménez 2005).

Thus the objective of the present study was to verify the effect of a reduced agar concentration on the contact between leaf explant edges of *Coffea canephora* cv. 'Robusta 2264 Mar' and the solid culture medium surface.

MATERIAL AND METHODS

Leaves from the 1st and 2nd pairs of each branch from adult plants of the genotype *C. canephora* cv. 'Robusta 2264 Mar', maintained in a container (50 L) under field conditions at the Campinas Agronomic Institute, were collected during the spring and summer seasons of 2001 and 2002, respectively. The leaves were first disinfested in a detergent solution to remove dust, and then dipped in a 2% sodium hypochlorite solution for 20 minutes and finally rinsed three times in sterile distilled water. After disinfestation, square

Received: 3 October, 2007. Accepted: 8 November, 2007.

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explants were extracted (2.0 cm^2), excluding the areas of the main nervure, the edge and the apical and basal parts of these leaves, and inoculating the explants in a pre-culture medium, aimed at the selection of non-contaminated explants. The pre-culture medium consisted of half-strength MS salts (Murashige and Skoog 1962) with the addition of 0.3 g L^{-1} peptone and 0.1 g L^{-1} yeast extract (Sondahl *et al*. 1984), without the addition of growth regulators. For each treatment, ten Petri dishes were used, each containing six explants, which were maintained in the dark at 25ºC.

Healthy explants were transferred individually to flasks (290 mL) containing 50 mL of callus induction medium, MS salts, 2.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 5 µM Kinetin (Sondahl *et al*. 1979); or were treated by direct embryogenesis with half the MS salts and the addition of 10 µM 2iP (N6-(2-isopentyl)adenine) (Ramos *et al*. 1993).

Two experiments were carried out with square explants (2.0 cm^2 of size) inoculated with their adaxial surface in contact with the medium superficie. In the first experiment the explants were inoculated into the callus induction medium with the addition of different agar concentrations: 2, 3, 4 and 6 g L^{-1}. Twenty repetitions of each treatment were carried out and they were evaluated with respect to the contact between the edges of the explant and the culture medium surface, scoring as follows: ***1***. lack of contact between the explant edges and the culture medium surface; ***2***. explant with all its edges in contact with the culture medium.

In the second experiment, the explants were submitted to direct and indirect somatic embryogenesis induction media, both with agar concentrations of 3 and 6 g L^{-1}, with ten repetitions for each treatment. Subsequently, 120 days after the start of the experiment, the explants with embryogenic structures from the direct embryogenesis induction medium were transferred to the germination medium, consisting of half the MS salts without the addition of growth regulators, each treatment consisting of ten repetitions. The calluses resulting from indirect embryogenesis were transferred to the embryogenesis induction medium, consisting of half the MS salts with the addition of 0.5 µM 1-naphthalene acetic acid (NAA) and 2.5 µM kinetin, the treatments being carried out with ten repetitions.

The treatments were evaluated with respect to the number of sides of the explants showing the formation of embryogenic structures (direct somatic embryogenesis) or of calluses (indirect somatic embryogenesis), an estimation of the size of the callus and/or of the mass of embryogenic structure and the number of embryos formed.

In the present study, the term embryogenic structure referred to the globular structures developing from the cells on the edges of the explants when in the direct embryogenesis induction medium. All the treatments were maintained at 25ºC in the dark, except for the direct somatic embryogenesis, whose explants remained in the light as from 120 days from the start of the experiment. For the two experiments a random block statistical design was chosen, and the data obtained were analysed statistically (Pimentel-Gomes 2000) using the "F" test at 5%, the means being compared by Tukey's test at the 5% level.

RESULTS AND DISCUSSION

Coffea canephora cv. 'Robusta 2264 Mar' is a species with the capacity for both direct and indirect somatic embryogenesis. Leaf explants of this species frequently show curvature at their edges soon after inoculation into the culture medium, decreasing the efficiency of the process.

In the first experiment, the effect of agar concentrations of 2, 3, 4 and 6 g L^{-1} on contact between the explant edges and the culture medium surface was tested, and it was shown that the explants showed better contact with the medium surface when the agar concentration was 3 g L^{-1} (**Fig. 1**). At 2 g L^{-1}, the medium was too gelatinous and fragile, becoming incapable of physically supporting the explants.

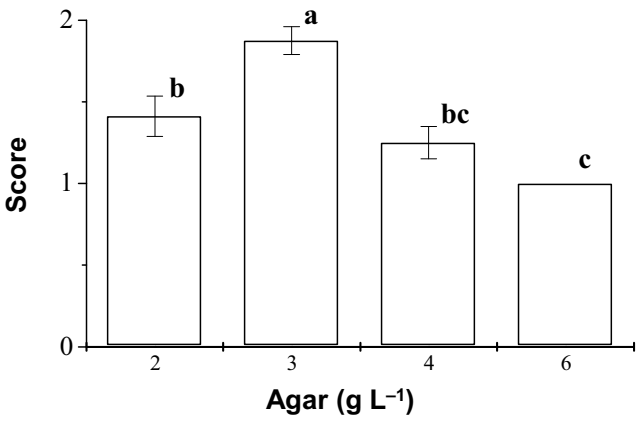

Fig. 1 Effect of different concentrations of agar in the contact between the *C. canephora* cv. 'Robusta 2264 Mar' leaf explants and the surface of the culture medium. Scoring as follows: **1**. lack of contact between the explant edges and the culture medium surface; **2**. explant with all its edges in contact with the culture medium.

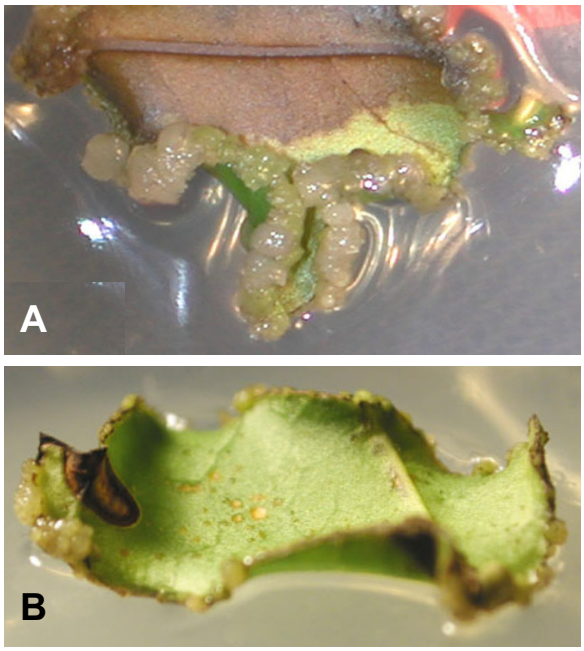

Fig. 2 Characterization of the contact between the *C. canephora* cv. 'Robusta 2264 Mar' leaf explants and the surface of the medium with addition of different concentrations of agar. (**A**) 3 g L^{-1} of agar: the medium is in contact with the border of the explant; (**B**) 6 g L^{-1} of agar: explants with curvature without contact with the medium.

Three g L^{-1} favoured the best physical accommodation of the explants on the culture medium surface, providing a greater surface contact between the two (**Fig. 2A**), than those at 6 g L^{-1} (**Fig. 2B**). Preliminary observations also showed that 22.0% of the explants of this genotype presented curvature when inoculated onto the culture medium with 3 g L^{-1} of agar as compared to 47.3% on medium with 6 g L^{-1}. Thus it was shown that a reduction in agar concentration favoured contact between the explant and the culture medium. The size and orientation of the explant can also affect the *in vitro* response (Nhut *et al.* 2001). However, in the present study only square explants (2 cm^2) were used, with their adaxial surfaces in contact with the upper face of the culture medium. For somatic embryogenesis of *Coffea* explants this form and disposition has been used as the pattern since the pioneer studies were carried out with this species (Sondhal and Sharp 1977; Dublin 1981; Michaux-Ferrière *et al.* 1989) except for the size which can vary.

However, another fact to be considered is that the reduction in gelling agent concentration can cause hyperhydricity

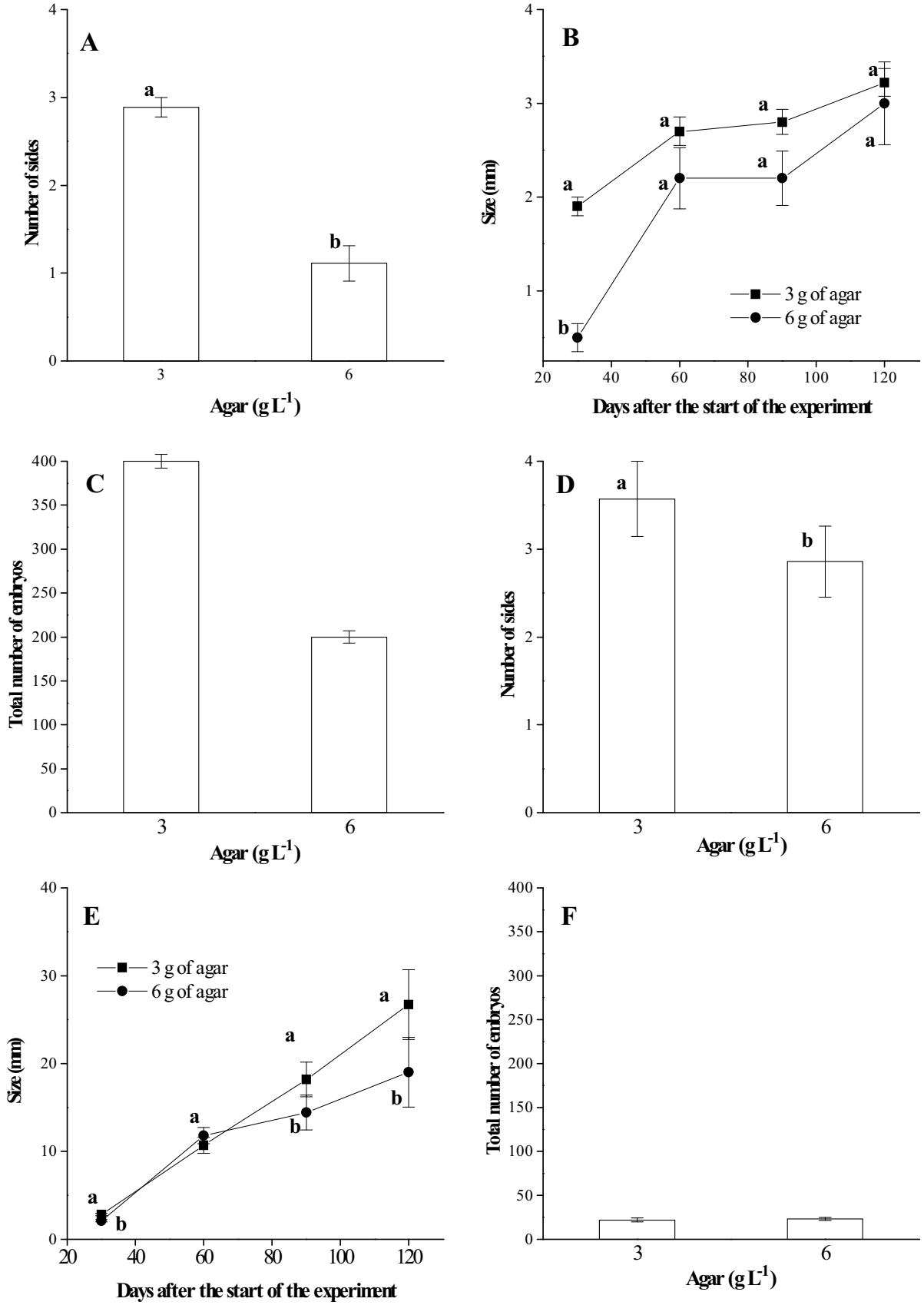

Fig. 3 Effect of 3 and 6 g L^{-1} agar on the somatic embryogenesis in explants of the *C. canephora* cv. 'Robusta 2264 Mar', at 25°C in the dark. (**A**) Number of sides of the explants with the formation of embryogenic structures. (**B**) Estimation of the size of the embryogenic structures mass. (**C**) Total number of embryos. (**D**) Number of sides of the explants with the formation of calluses. (**E**) Size of calluses. (**F**) Total number of embryos. Scoring (in **A** and **D**) of the number of sides of the explants showing the formation of embryogenic structures (direct somatic embryogenesis) or calluses (indirect somatic embryogenesis): **1.** One side of the explant showing formation of embryogenic structures or calluses. **4.** Four sides of the explant showing formation of embryogenic structures or calluses. The means were compared by Tukey's test at P = 5%, except for **C** and **F**. Each treatment had ten repetitions.

in the *in vitro* vegetable tissue (Ebrahim and Ibrahim 2000; Ivanova *et al.* 2006). Hyperhydricity (previously vitrification) is a physiological disorder causing morphological and biochemical alterations in the vegetable *in vitro* (Debergh *et al.* 1992) and this response too, can be associated with the genotype (Pérez-Tornero *et al.* 2001). However, no hyper-

hydricity was found in this work. In addition, the reduction in the agar concentration could reduce the maintenance costs of the tissue culture procedures.

In the second experiment the effect of agar concentrations of 3 and 6 g L^{-1} on the response of direct and indirect somatic embryogenesis was tested for leaf explants (**Fig. 3**), with the objective of verifying if the greater contact between the explant and the culture medium could influence the response.

For direct somatic embryogenesis, it was shown that in the medium with 3 g L^{-1} of agar, the explants presented a greater number of sides showing the formation of a mass of embryogenic structures than with 6 g L^{-1} of agar (**Fig. 3A**). Initially the size of the mass of these structures was greater in the medium with 3 g L^{-1} than in that with 6 g L^{-1}, but by the end of the experiment this difference between the experiments had disappeared (**Fig. 3B**). For indirect somatic embryogenesis, it was also shown that 3 g L^{-1} of agar favoured the response, since the explants showed a greater number of sides forming callus (**Fig. 3D**) and the calluses were larger (**Fig. 3E**). Possibly the substances present in the medium were more available to the explants in the more gelatinous medium, favouring the embryogenics response. On the other hand, commercial agar is a source of a wide variety of organic and inorganic contaminants (Dodds and Roberts 1982; Beruto and Curir 2006), which can interfere with the culture medium, such as, for example, altering its hydric potential (Ghashghaie *et al.* 1991; Williams 1995; Beruto *et al.* 1999), and this would probably influence the *in vitro* tissue differentiation model. The hydric potential of the environment can significantly influence the development of vegetable tissue, and thus this aspect also deserves to be studied in more detail for *in vitro* cultures.

The cv. 'Robusta' explants were also submitted to the induction of direct and indirect somatic embryogenesis in the light. However, in indirect embryogenesis, the explants only formed small (3 mm) oxidized calluses and in direct embryogenesis, the explants showed a reduced formation of embryos (data not shown).

The somatic embryogenic capacity of *Coffea* depends on several conditions such as the genotype (Molina *et al.* 1999), the season during which the plant material was collected (Santana *et al.* 2004; Almeida *et al.* 2005), the temperature and illumination (Simões-Costa *et al.* 1999), the exogenous growth regulator concentration (Yasuda *et al.* 1985) and medium composition (Fuentes *et al.* 2000). In addition, although the induction of this process is not affected by the agar, it can have an indirect influence. Thus, in this study it was shown that a reduction in the agar concentration indirectly favoured the somatic embryogenesis of cv. 'Robusta' since it permitted greater physical contact between the edge of the explant and the surface of the culture medium. Hence, when choosing the gelling agent it is important to consider the type, brand and its concentration, since this can indirectly influence the efficiency of the development of the *in vitro* vegetable tissue.

The embryogenic structures obtained in the direct embryogenesis medium and the calluses formed on the explants in the indirect embryogenesis medium were transferred to the embryogenesis germination and induction media, respectively, also with the addition of 3 and 6 g L^{-1} of agar, 120 days after the start of the experiment. For direct embryogenesis, more embryos formed from the explants maintained in the medium with 3 g L^{-1} of agar than in those with 6 g L^{-1} of agar (**Figs. 3C, 4**). For indirect embryogenesis, the number of embryos formed was reduced, less than 30, in both agar concentrations (**Fig. 3F**), indicating that the reduction in the agar concentration only favoured the start of the development of callus (**Fig. 3E**), but did not promote the formation of embryos. Although cv. 'Robusta 2264 Mar' is capable of direct and indirect somatic embryogenesis, the direct route is preferentially more efficient than indirect embryogenesis (Julieta AS Almeida, pers. obs.). It has also been verified for other species *in vitro*, that a reduction in the agar concentration can alter the pattern of development. This was shown in *Ceratozamia hildae* proembryos (Moon *et al*. 2004), and in *Artemisia dracunculus* nodal explants (Mackay and Kitto 1988), presenting a decrease in the proliferation rate and growth, and in *Chrysanthemum morifolium*, showing an increase in budding (Karim *et al*. 2003). Romberger and Tabor (1971) also verified an increase in dry mass of *Picea abies* plantlets cultivated in a low agar concentration medium, and attributed this to the increase in diffusion of enzymes and other molecules through the more dilute agar to the vegetable tissue.

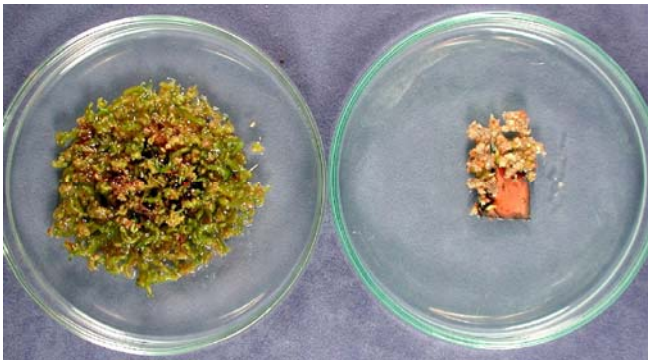

Fig. 4 Effect of 3 (left) and 6 g L^{-1} (right) agar on the direct somatic embryogenesis in explants of *C. canephora* cv. 'Robusta 2264 Mar' maintained at 25°C in the dark.

Table 1 Number of embryos formed via direct and indirect somatic embryogenesis from leaf explants of the *C. canephora* cv. 'Robusta 2264 Mar'. The number of embryos was evaluated after 120 days that the embryogenic structures and calluses were transfered to the germination and embryogenesis induction medium, respectively, maintained at 25°C in the dark.

Embryogenesis induction medium → **Germination medium**			
Direct embryogenesis			
Agar (g L^{-1})	**3**	**6**	**Total**
3	274	141	415
6	145	59	204
Total	**419**	**200**	**619**
Indirect embryogenesis			
Agar (g L^{-1})	**3**	**6**	**Total**
3	0	0	0
6	22	23	45
Total	**22**	**23**	**45**

Table 1 shows the data referring to the number of embryos formed, considering the agar concentrations of 3 and 6 g L^{-1}, when the explants were first introduced into the direct (forming embryogenic structures) and indirect (forming callus) embryogenesis induction media, and after their transfer to the embryogenic structure germination medium and embryogenesis induction medium, respectively. With direct embryogenesis, the explants previously maintained in 3 and 6 g L^{-1} of agar formed more embryos when transferred to the medium with 3 g L^{-1} of agar than to that with 6 g L^{-1} of agar. This greater formation of embryos in the former medium was possibly due to the greater contact attained between the explants and the more gelatinous medium, thus permitting that a greater number of cells on the edges of the explants remained exposed to the nutrients and growth regulators, consequently favouring the induction of somatic embryos.

From the results obtained it can be seen that the reduction in agar concentration favoured physical contact between the edges of the *C. canephora* cv. 'Robusta 2264 Mar' explants and the culture media surface, indirectly promoted the capacity for direct somatic embryogenesis and could also lead to a reduction in the costs of tissue culture procedures, due to the use of a smaller amount of agar. There are no available studies in the literature on this aspect. However, sometimes the reduction in the response of an *in vitro* soma-

tic tissue, although associated with physiological disorder, may in fact be due to a lack of contact between the tissue and the medium.

There is currently a widespread tendency for large-scale micropropagation of *Coffea* somatic embryos in bioreactor systems. According to a recent review by Ducos *et al*. (2007) different types of bioreactor systems can be applied to *Coffea.* These systems may produce hundreds of embryos from embryogenic cells, which will subsequently attain the seedling state. The two systems for producing the somatic embryos of *Coffea*, bioreactors and conventional tissue culture are different technologies, but the initial phase of the bioreactor system requires embryogenic cells. These cells are originated from a callus produced via indirect somatic embryogenesis which generally is formed from an explant cultivated in a medium with the addition of a gelling agent. Thus since bioreactor technology is a reality used to obtain somatic embryos on a large scale, studies such as the present one that are trying to improve the efficiency of somatic embryogenesis are important. Improvements in conventional somatic embryogenesis will also contribute indirectly to the efficiency of bioreactor technology.

ACKNOWLEDGEMENTS

The authors are grateful for the financial support given by the Consórcio Brasileiro de Pesquisa e Desenvolvimento do Café.

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