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de Rezende, Juliana Costa; Siqueira de Carvalho, Carlos Henrique; Ramia Santos, Ana Carolina;
Pasqual, Moacir; Batista Teixeira, João

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Multiplication of embryogenic calli in *Coffea arabica* L.

Juliana Costa de Rezende^{1*}, Carlos Henrique Siqueira de Carvalho², Ana Carolina Ramia Santos³, Moacir Pasqual⁴ and João Batista Teixeira⁵

¹Centro Tecnológico do Sul de Minas, Empresa de Pesquisa Agropecuária de Minas Gerais, Cx. Postal 176, 37200-00, Lavras, Minas Gerais, Brazil. ²Empresa Brasileira de Pesquisa Agropecuária-Embrapa Café, Brasília, Distrito Federal, Brazil. ³Fundação Procafé, Varginha, Minas Gerais, Brazil. ⁴Departamento de Agricultura, Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil. ⁵Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia, Empresa Brasileira de Pesquisa Agropecuária, Brasília, Distrito Federal, Brazil. *Author for correspondence. E-mail: julianacosta@epamig.br

ABSTRACT. The goal of this project was to evaluate the embryogenic callus induction of two *Coffea arabica* clones selected for their characteristics of rust resistance and high yield, as well as to compare their multiplication in two different media under both solid and liquid cultivation conditions. The protocol described by Teixeira et al. (2004) was used for callus induction in a randomized block design in which each clone was considered a treatment. Evaluation of callus induction was carried out 180 days after initiation by counting embryogenic calli. For callus multiplication, the treatments consisted of two different media [stage two of Albarran et al. (2004) and the multiplication medium described by Teixeira et al. (2004)] and two cultivation systems (solid and liquid). Evaluations were conducted by weighing calli 21, 42 and 63 days after initiation of the experiment. The two studied clones exhibited the same potential for embryogenic callus induction. The potential for embryogenic callus multiplication was influenced by the plant's genotype. When compared with the liquid system, the solid system displayed the highest level of embryogenic callus multiplication for the clones studied.

Keywords: culture media, cultivation systems, genotype.

Indução e multiplicação de calos embriogênicos de *Coffea arabica* L.

RESUMO. Este estudo foi realizado com o objetivo de avaliar a indução de calos embriogênicos de dois clones de *Coffea arabica*, selecionados para resistência à ferrugem e de alta produtividade e comparar a multiplicação desses calos em dois meios de cultura nos sistemas de cultivo gelatinoso e líquido. Para a indução de calos foi utilizado o protocolo descrito por Teixeira et al. (2004), em delineamento experimental inteiramente casualizado, sendo cada clone considerado um tratamento. A avaliação deste experimento foi realizada 180 dias após a instalação, por meio da contagem de calos embriogênicos formados. Para a multiplicação de calos, os tratamentos constituíram-se de dois meios de cultura (meio do estágio dois de Albarran et al. (2004) e meio de multiplicação de Teixeira et al. (2004)) e dois sistemas de cultivo (gelatinoso e líquido). As avaliações foram realizadas aos 21, 42 e 63 dias após a instalação do experimento, por meio da pesagem dos calos. Verificou-se que os clones avaliados apresentam o mesmo potencial de formação de calos embriogênicos. O potencial de multiplicação de calos embriogênicos é influenciado pelo genótipo. O sistema gelatinoso apresentou maior eficiência na multiplicação de calos embriogênicos dos clones estudados quando comparado ao sistema líquido.

Palavras-chave: meio de cultura, sistema de cultivo, genótipo.

Introduction

Somatic embryogenesis has been achieved in a great number of families and species of plants and has been used for basic studies of vegetable physiology and in more practical applications, such as micropropagation and genetic transformation. The potentially important application of embryogenesis for the accelerated multiplication of coffee plants was quickly noticed by the earliest studies, and numerous techniques that simplify this process have been developed that seek to establish

large-scale plant production and reduce seedling production costs.

Embryogenic calli can continue to originate somatic embryos for long time periods and after many subcultures. According to Pasqual et al. (1997), continuous somatic embryo production depends on the continuous proliferation of pro-embryogenic nodules from young somatic embryos during each subculture.

Some authors have described protocols seeking to optimize the multiplication of embryogenic coffee calli (BOXTEL; BERTHOULY, 1996;

DUCOS et al., 2007a). Studies to establish better protocols were conducted using gelatinous media; however, the multiplication rates of these media were too low to be used in the large-scale production of coffee plants (SÖNDHAL; SHARP, 1977). In view of that result, there have been several attempts, with varying degrees of success, to establish somatic embryogenesis protocols in liquid media (AFREEN et al., 2002; DUCOS et al., 2007a and b; KUMAR et al., 2006).

Based on the published results mentioned above, this work was performed with the objective of evaluating the induction of embryogenic calli from two clones of *C. arabica* that were selected for high productivity and resistance to rust and comparing the multiplication of those calli in two culture media under gelatinous- and liquid-cultivation systems.

Material and methods

The experiments were performed in the Laboratório de Cultura de Tecidos da Fundação Procafé in Varginha, Minas Gerais State, using clones 3 and 28, which were selected for their resistance to rust and high productivity. The mother plant 3 is from the Siriema (*C. racemosa* x *C. arabica*) population and also exhibits resistance to the leaf-miner. The mother plant 28 was generated by crossing Red Catuaí IAC 44 (UFV Control: VER 209-2 = UFV 2144-260 EL 7) x Timor Hybrid CIFC 2750 (UFV Control: VER 209-2 = UFV 439-2).

The formation potential of embryogenic calli was first evaluated with leaf explants from clones 3 and 28. Well-developed leaves corresponding to the third pair were collected from the plagiotropic branches of the medial third of the adult mother plants and were taken to the laboratory.

The asepsis protocol consisted of immersion in 70% alcohol for 1 min. and disinfestation with sodium hypochlorite at a concentration of 2.4% active chlorine for 15 min. Soon afterwards, the leaves were rinsed three times with distilled, autoclaved water. After autoclaving at 121°C and 1 atm for 20 min., the culture medium was poured into 0.9 x 10 cm Petri dishes.

The callus induction protocol described by Teixeira et al. (2004) was used in this study. Initially, the explants were cultivated in primary medium (PM) for 30 days and were then transferred to secondary medium (SM), where they remained for 180 days. The experimental design was entirely random, using 33 replicates with nine explants per dish with each dish considered a replicate.

The analysis was conducted 180 days after the start of the experiment through the quantification of

embryogenic callus formation. Statistical analyses were undertaken using two models: the first with no factor (null) and the second considering the main effect of the hybrid. The Deviance Analysis was used. The residue analysis showed that the best adjustment was obtained from the quasibinomial family and the probit connection function. The standard deviation was determined, and being satisfactory, the Scott and Knott test for the comparison of averages was used. The analyses were performed using the GLM (Generalized Linear Models*) protocol due to the fact that the percentage data do not follow a normal distribution.

The embryogenic calli in this study were multiplied in a previously described multiplication medium (TEIXEIRA et al., 2004), with the medium being refreshed every 21 days over six months until the calli reached the necessary size to be used for a multiplication experiment. The treatments used were two culture media (ALBARRAN et al., 2004) stage two medium and Teixeira et al. (2004) multiplication medium), two cultivation systems (gelatinous medium and liquid medium) and evaluation time (21, 42 and 63 days) with five repetitions.

The Teixeira et al. (2004) multiplication medium is composed of half of the MS salts (MURASHIGE; SKOOG, 1962), thiamine (10 mg L⁻¹), pyridoxine (1 mg L⁻¹), glycine (1 mg L⁻¹), niacin (1 mg L⁻¹), myo-inositol (100 mg L⁻¹), L-cysteine (10 mg L⁻¹), hydrolyzed casein (100 mg L⁻¹), malt extract (200 mg L⁻¹), citric acid (250 mg L⁻¹), 2,4-D (5 µM), IBA (4.92 µM), 2iP (9.84 µM) and sucrose (20 g L⁻¹). Albarran et al. (2004) medium is composed of half of the MS salts, thiamine (10 mg L⁻¹), pyridoxine (1 mg L⁻¹), glycine (1 mg L⁻¹), niacin (1 mg L⁻¹), myo-inositol (100 mg L⁻¹), 2,4-D (4.5 µM) and kinetin (4.6 µM). Casein (100 mg L⁻¹) and sucrose (20 g L⁻¹) were added to this medium. Phytigel® at 3.4 g L⁻¹ was added to all of the gelatinous treatments.

The media were adjusted to pH 5.8 ± 0.1 using 0.1 N NaOH or 0.1 N HCl before the autoclaving process, which was performed at 121°C and 1 atm for 20 minutes. In a laminar-flow chamber, 20 mL of the liquid medium was poured into 125 mL Erlenmeyer flasks, and soon afterwards, 160 mg of embryogenic calli were inoculated into the flasks. These flasks were sealed with laminated paper and were maintained in an orbital agitator at 100 rpm under the absence of light and at a temperature of 25 ± 2°C. The gelatinous media were poured into a 0.9 x 10 cm Petri dish and inoculated with nine embryogenic sections of 60 mg each. The dishes

*Computational program R® (R DEVELOPMENT CORE TEAM, 2008).

were sealed with PVC film and stored in wooden boxes under the absence of light and at a temperature of $25 \pm 2^\circ\text{C}$.

Measurements were conducted at 21, 42 and 63 days and involved weighing the calli using a precision scale placed in the laminar-flow chamber. At each evaluation, the culture medium was refreshed and the initial quantity of calli was adjusted, i.e., 160 mg of embryogenic calli in the case of the flasks and nine embryogenic sections of 60 mg each in the case of the Petri dishes.

The callus-increase percentage [I (%)] was calculated with the following formula: $I (\%) = [(FW \times 100)/(FW_{\text{initial}})] - 100$, where FW is the fresh weight (g) measured every 21 days and FW_{initial} is the fresh weight of the embryogenic callus initially inoculated in the flask or Petri dish. The I (%) was calculated with the intention of enabling a comparison between growth in liquid medium and in gelatinous medium, as the two media had different initial concentrations.

The callus-increase percentage was analyzed by the GLM procedure. The significance of the comparison was determined to a 5% probability by Student's t-test. As significant differences between the treatments and interactions were detected, further testing was undertaken. The regression analysis was used to study increase percentage values of calli in function of time analyzed. The clones' genotypes were analyzed separately in independent experiments, as it was not the objective of this experiment to compare them.

Results and discussion

Callus Induction

There was no significant difference between the clones studied (X_1) in the chi-squared test, demonstrating that their genotypes had no effect on the formation of embryogenic calli (Table 1). The two studied clones presented similar average percentages of embryogenic call formation (22% for

clone 3 and 20.31% for clone 28) despite their different genetic origins.

Table 1. Deviance analysis for presence of induced embryogenic calli in leaf explants of clones 3 and 28.

| Model | Model DF | Residual Deviance | Difference deviance | Difference DF. | p value |
|-----------|----------|-------------------|---------------------|----------------|---------------------|
| (1) Null | 57 | 40,835 | | | |
| (2) X_1 | 1 | 0,507 | 40,327 | 56 | 0,514 ^{ns} |

*ns - do not differ, by the chi-square test, to 5% significance. X_1 = genotype studied. X_1 , 03 e 28 clones.

This average percentage of callus formation is in line with results observed in prior studies. In the first work on the somatic embryogenesis of *C. arabica*, Söndhal and Sharp (1977) obtained 20% embryogenic callus formation using 5.4 μM ANA and 4.6 μM kinetin. Berthouly and Michaux-Ferriere (1996) obtained embryogenic callus formation starting at 20.8% from various genotypes of *C. canephora* when a primary medium was used containing 2.2 μM 2,4-D, 4.9 μM IBA and 9.8 μM 2-iP. Teixeira et al. (2004) obtained embryogenic calli formation in different *C. arabica* cultivars which values were 8% for cv. Mundo Novo and 100% for cv. Catuai.

Callus Multiplication

We observed that, for the two studied clones, the treatments with the gelatinous medium yielded a higher production of embryogenic calli at the end of 63 days than did the treatments with the liquid medium (Table 2). Due to the initial amount difference of inoculate used in both systems no statistical analyses aiming at comparing the cultivation system calli fresh mass was performed.

In the gelatinous-cultivation system, clone 3 produced, after 63 days, 5.06 g of calli in the Albarran et al. (2004) medium and 3.99 g of calli in the Teixeira et al. (2004) gelatinous medium (Table 1).

Table 2. Fresh mass of embryogenic calli (g) during the cultivation period and weight increase after 63 days for each culture medium and cultivation system under study, as determined for clones 3 and 28.

| Clone 3 | Fresh mass (g) | | | | Increase Weight (times) |
|-----------------------------------|---------------------------|------|------|------|-------------------------|
| | Cultivation period (days) | | | | |
| Culture medium / system | 0 | 21 | 42 | 63 | |
| Albarran et al. (2004) gelatinous | 0.54 | 1.07 | 2.55 | 5.06 | 9.37 |
| Albarran et al. (2004) liquid | 0.16 | 0.22 | 0.26 | 0.27 | 1.69 |
| Teixeira et al. (2004) gelatinous | 0.54 | 1.06 | 2.37 | 3.99 | 7.39 |
| Teixeira et al. (2004) liquid | 0.16 | 0.38 | 0.56 | 1.12 | 7.00 |
| Clone 28 | Fresh mass (g) | | | | Weight increase (times) |
| | Cultivation period (days) | | | | |
| Culture medium / system | 0 | 21 | 42 | 63 | |
| Albarran et al. (2004) gelatinous | 0.54 | 1.18 | 3.35 | 7.11 | 13.16 |
| Albarran et al. (2004) liquid | 0.16 | 0.36 | 0.74 | 0.99 | 6.19 |
| Teixeira et al. (2004) gelatinous | 0.54 | 1.02 | 2.60 | 6.40 | 11.85 |
| Teixeira et al. (2004) liquid | 0.16 | 0.42 | 0.60 | 1.58 | 9.87 |

For the liquid system, the media described by Albarran et al. (2004) and Teixeira et al. (2004) presented, after 63 days, 0.27 g and 1.12 g of calli, respectively. Clone 28, after 63 days, yielded a fresh mass of 1.58 g of calli from the Teixeira et al. (2004) medium and 0.99 g from the Albarran et al. (2004) medium. For the gelatinous cultivation system, the fresh mass of calli at the end of this period was 7.11 g with the Albarran et al. (2004) medium and 6.40 g with the Teixeira et al. (2004) medium.

The clones studied here presented varying average weight increases. The growth variation of clone 3 was from 1.69-fold to 9.37-fold, whereas clone 28 presented an average weight increase varying from 6.19-fold to 13.16-fold. In the Albarran et al. (2004) medium in the gelatinous-cultivation system (which mediated stronger growth), we observed that, at 63 days, clone 28 increased its weight 40.45% more than did clone 3. These results, which indicate that the multiplication potential of embryogenic calli is influenced by the clone's genotype, are in line with the results of Fuentes et al. (2000), which suggested that the somatic embryogenesis process is sensitive to culture conditions such as the composition of the medium, the physical environment of the culture, and the source and genotype of the explants.

Callus percentage increase

These results demonstrated the significance of the percentage increase of callus as functions of cultivation system and duration of cultivation, as well as the significance of the interactions between cultivation system and culture medium, cultivation system and duration, cultivation system and culture and medium and time factors.

The significant cultivation system and culture medium interaction suggests differentiated behavior between the levels of the cultivation system factor and each level of the culture medium factor (Table 3). For the Albarran et al. (2004) culture medium, there was a significant difference between the liquid- and gelatinous-cultivation systems, with the gelatinous system being statistically superior in the percentage increase of calli when compared with the liquid system. For the Teixeira et al. (2004) culture medium, a significant difference was not observed in the behavior of the cultivation systems.

Table 3. Average percentage increase of calli as a function of the cultivation system and the culture medium for clone 3.

| Cultivation System | Culture medium ¹ | |
|--------------------|-----------------------------|------------------------|
| | Albarran et al. (2004) | Teixeira et al. (2004) |
| Liquid | 67.07 b | 304.62 a |
| Gelatinous | 436.15 a | 359.64 a |

¹Averages followed by the same lower-case letter within a column do not differ significantly at 5% significance as measured by the Student's t test.

In Figure 1, the average percentage increase in callus is presented for clone 3 as a function of time as determined for each culture medium and cultivation system. In callus multiplication with the gelatinous cultivation system, an increase of approximately 17.61% per day was observed using the Albarran et al. (2004) medium and 12.92% was observed using the Teixeira et al. (2004) medium. For the liquid-cultivation system, the increases were 1.25 and 12.72% for the Albarran et al. (2004) and Teixeira et al. (2004) media, respectively.

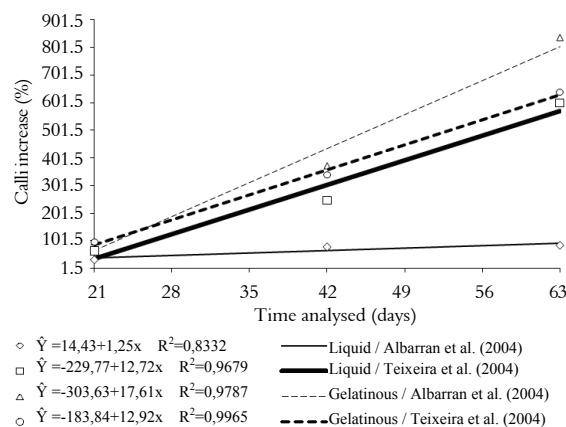


Figure 1. Average percentage increase of calli as a function of time as determined for each culture medium and cultivation system for clone 3.

These values are in line with those reported by Ducos et al. (2007a), who quantified the multiplication of embryogenic cells from *C. canephora* in liquid medium and observed a growth rate of 6.6 to 13.3% a day, starting with 1 g of calli and exhibiting a biomass growth of two to three times the initial amount, every 15 days.

Teixeira et al. (2004) observed that the fresh-matter weight at the end of various cultivation periods varied according to the initial cell density, reaching a maximum value of callus growth when the initial density was 8 g L⁻¹, which is the same density used in the present work in which the average fresh-weight increase at 63 days was 9.8-fold for clone 28 and 7.0-fold for clone 3 when the Teixeira et al. (2004) medium was used. The present work, therefore, corroborated the results obtained by those authors, who achieved a cell-density increase of nearly 10-fold after 56 days of cultivation (Table 1).

Clone 28

These results demonstrated the significance of the primary effects of cultivation system and time, as well as the significance of the interaction between cultivation system and time and culture medium and time. The increase in the percentage of calli for the

gelatinous-cultivation system was superior to that of the liquid-cultivation system (Figure 2). We found an increase in calli of approximately 23.6% per day when the gelatinous system was used and of about 10%, when the liquid cultivation system was used. It is likely that the low growth rate observed in the liquid medium is due to the low level of oxygenation to which the cellular suspensions were exposed.

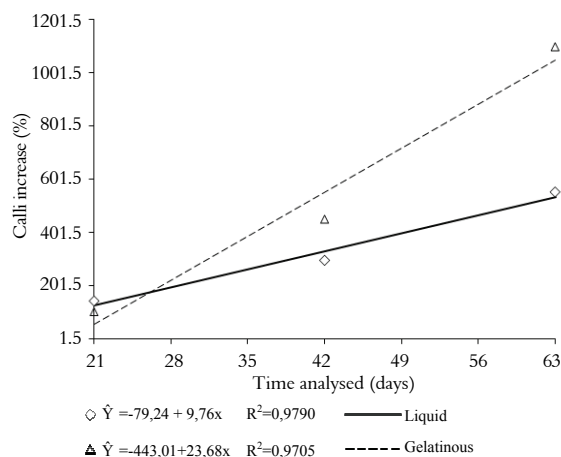


Figure 2. Average percentage increase of calli from clone 28 as a function of time as determined for each cultivation system.

According to Curtis (2005), the liquid cultivation system can provide strongly anaerobic conditions due to the reduced level of oxygen inside the tissue, which is caused by the transfer limitations and physiological limitations of the tissue. De Faria et al. (2003) compared the multiplication of embryos in liquid medium containing 50 and 80% dissolved oxygen and obtained improved multiplication of calli and embryo regeneration at 80% dissolved oxygen, probably due to the enhanced metabolic activity of the cellular suspensions in the medium with increased oxygen content.

Although the liquid cultivation system employed in this experiment was studied under agitation conditions, which allow more penetration of oxygen within the culture medium and improve the distribution of recently-solubilized oxygen, the gelatinous-cultivation system exhibited a higher rate of callus growth. This fact can be explained by the high intensity of oxygenation that occurs on the surface of solid media, because the explants are in direct contact with the air, which results in oxygen being more available for metabolism (PINTO et al., 1995). Therefore, the use of gelling agents creates improved aeration conditions for the base of the explants. It is worth emphasizing that the culture medium used in the present study was a gelatinous medium in which low concentrations of the gelling agent were applied, thereby allowing the diffusion of nutrients in the medium.

In spite of the higher efficiency of the gelatinous-cultivation system observed in the present work, many authors affirm that the use of liquid culture is a prerequisite for the automation and cost-reduction required for commercial propagation systems (DE FERIA et al., 2003; DUCOS et al., 2008). However, the advantages of cultivation in liquid media are frequently counterbalanced by the disadvantages, such as cell asphyxia, hyperhydricity, the need for complex equipment (ETIENNE et al., 2006) and higher culture medium consumption.

In the present work, for the gelatinous cultivation system, 25 mL of culture medium was employed for the inoculation of 0.54 g of calli, which resulted in an average weight at the end of 63 days of 4.25 g for clone 3 and of 6.75 g for clone 28 (Table 2). To inoculate the same 0.54 g of calli in liquid medium, 67.5 mL of culture medium would be necessary, representing an increase of at least 227.5% in the volume of medium to be used, and the rate of growth under the liquid system remains lower.

In Figure 3, the average percentage increase of calli is presented as function of time as determined for each medium. The percentage increase of callus provided by the Albarran et al. (2004) and Teixeira et al. (2004) media were quite similar, with an increase of about 15.5% a day when Albarran et al. (2004) medium was used and of approximately 18% when the Teixeira et al. (2004) medium was used, indicating that the two culture media support similar rates of multiplication.

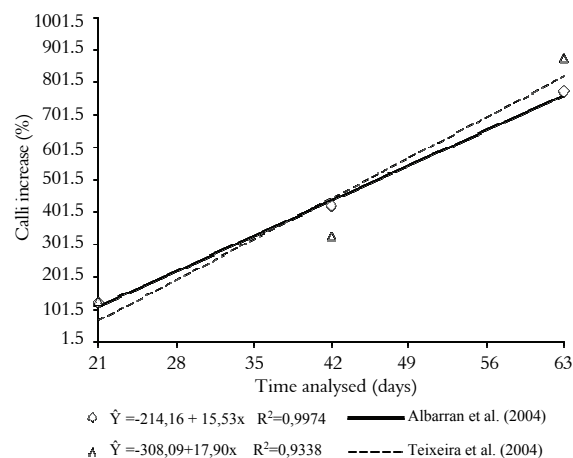


Figure 3. Average percentage increase of calli as a function of time as determined for each culture medium for clone 28.

In the medium developed by Teixeira et al. (2004), two auxins (5.0 μM of 2,4-D and 4.92 μM of IBA) and one cytokinin (9.84 μM of 2-iP) are used, whereas in the Albarran et al. (2004) medium, just one auxin

(4.5 μM of 2,4-D) and one cytokinin (4.6 μM of kinetin) are used. Although auxins and cytokinins of different types and concentrations were used, the average percentage increase of calli they provided were quite similar, indicating that embryogenic calli present high plasticity in their response to the culture medium.

Conclusion

The clones analyzed here exhibit similar potential for embryogenic callus formation. Additionally, the embryogenic callus multiplication potential was found to be influenced by the clone's genotype. The gelatinous cultivation system demonstrated greater efficiency for the multiplication of embryogenic calli from the clones studied here when compared with the liquid system.

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