DEVELOPMENT OF *Coffea arabica* L. SEEDLINGS OBTAINED FROM DIRECT SOMATIC EMBRYOGENESIS [1]

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ABSTRACT: This work aimed to develop embryos and acclimatize seedlings of *Coffea arabica* L. cv. Rubi produced by direct somatic embryogenesis. For the development of somatic embryos, were evaluated the effect of sucrose (0; 15; 30; 45 and 60 g.L^{-1}) and GA_3 (0; 2.5; 5 and 10 mg.L^{-1}). For the development of seedlings, were evaluated the influence of GA_3 (0; 2.5; 5; 10 mg.L^{-1}) and NAA (0; 0.25; 0.5; 1; 2 mg L^{-1}). The *in vitro* experiments were carried out in a growth chamber under light intensity of 32 µMol.m^{-2}.s^{-1}, at the temperature of ± 25 1ºC and photoperiod of 16 hours. The parameters evaluated were: number of leaves, length of the aerial part and fresh weight of the seedlings. For the seedling acclimatization process two types of substrates were tested (Plantmax® and Plantmax® plus carbonized rice peels (v/v 1:1) and fertilizer with slow-release (Osmocote®) (0, 0.5, 1 and 2 g of the fertilizer per cell). The results indicated that 5.81 mg L^{-1} GA_3 was efficient for the development of coffee somatic embryos and GA_3 (10 mg. L^{-1}) in combination with 1mg. L^{-1} NAA showed efficiency in the development of the seedlings obtained by direct somatic embryogenesis. The best substrate for acclimatization was Plantmax® mixed with carbonized rice peels and 1.68 g per cell of the Osmocote® fertilizer.

Key words: *Coffea arabica*, micropropagation, acclimatization, growth regulators, sucrose.

DESENVOLVIMENTO DE PLÂNTULA DE *Coffea arabica* L. ATRAVÉS DE EMBRIOGÊNESE SOMÁTICA DIRETA

RESUMO: *Objetivou-se com este trabalho, desenvolver embriões e aclimatar plântulas de* Coffea arabica *L. cv. Rubi, provenientes de embriogênese somática direta. No desenvolvimento de embriões somáticos, avaliou-se a influência de sacarose (0; 15; 30; 45 e 60 g.L^{-1}) e de GA_3 (0; 2,5; 5 e 10 mg.L^{-1}). No desenvolvimento de plântulas, avaliou-se a influência de GA_3 (0; 2,5; 5; 10 mg L^{-1}) e de ANA (0; 0,25; 0,5; 1; 2 mg L^{-1}). Os experimentos* in vitro *foram conduzidos em sala de crescimento com irradiância em torno de 32 µMol.m^{-2}.s^{-1} e fotoperíodo de 16 horas, com temperatura de 25 ± 1ºC. As variáveis avaliadas foram: número de folhas, comprimento da parte aérea e massa fresca da parte aérea. Na etapa de aclimatização de plântulas, foram testados dois tipos de substrato (Plantmax® e Plantmax® acrescido de casca de arroz carbonizada (v/v 1:1)) combinados com o fertilizante de liberação lenta Osmocote® (0, 0,5, 1 e 2 g do fertilizante por célula). Pelos resultados obtidos verificou-se que a utilização de 5,81 mg.L^{-1} de GA_3 apresenta eficiência no desenvolvimento de embriões somáticos de cafeeiro e as concentrações de 10 mg.L^{-1} de GA_3 e 1 mg.L^{-1} de ANA apresentam eficiência para o desenvolvimento de plântulas oriundas da embriogênese somática direta. O melhor substrato para aclimatização dessas plântulas foi o Plantmax® misturado à casca de arroz carbonizada, acrescido de 1,68 g por célula do fertilizante de liberação lenta Osmocote®.*

Palavras-chave: Coffea arabica, *micropropagação, aclimatação, regulador de crescimento, sacarose.*

1 INTRODUCTION

Somatic embryogenesis is one of the vegetative methods for propagation of *Coffea arabica*, which shows potential to be explored. Although the majority of the works in micropropagation of coffee through somatic embryogenesis uses the strategy of indirect embryogenesis (ALBARRAN et al., 2004; DUCOS et al., 2007), this system requires a long period to obtain the regenerated plants, being considered undesirable due to the highest probability of the occurrence of somaclonal variation in the regenerated plants.

In the strategy of direct embryogenesis, the somatic embryos only appear from cicatricial callus, without passing through the phase of undifferentiated

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callus. This method involves the cultivation of explants under culture media, supplemented only with cytokinin (DUBLIN, 1981; YASUDA et al., 1985) or with the combination of auxin and cytokinin (PIERSON et al., 1983). Dublin (1981), suggested that propagation of coffee through direct somatic embryogenesis is suitable to keep the stability of the donator genotype.

The auxins and cytokinins have basic roles in somatic embryogenesis for some species of plants (MACIEL et al., 2003). In addition, according to Kochba et al. (1974), the presence of gibberellic acid in the culture media allows the initiation of a radicle meristematic zone and/or stimulates the development of an existing radicle zone.

Gibberellins have a notable effect in primary shoot elongation and its effect in tissues and in the growth center (meristems) is characterized by the increasing in cell size and/or cell division (NICKELL, 1982). In some cases, gibberellic acid was used to make somatic embryos in plants (GUERRA et al., 1998).

Due to the low photosynthetic capacity of cultivated plants *in vitro* there is a need to add carbohydrates to the culture media in order to supply metabolites, which are required for energy generation or as carbon skeletons for various biosynthetic processes involved in differentiation and in cellular growth.

The acclimatization is a crucial process in tissue culture which gives seedlings with high quality. The optimization of the acclimatization process involves adequate addition of nutrients, the use of right substratum, control of the growing environment, among others precautions (CATUNDA, 2004). Thus, there is a need in finding out the substrate which is uniform in its composition, rich in nutrients, with higher capacity for water retention and cationic exchange and absent in plagues, pathogens and weed seeds, and still, viable economically (MELO et al., 2001). However due to its composition, the substrates not always contains all the required nutrients for seedlings growth. In this case, addition of fertilizer to reach the level of nutrients is required for the development of the plants (MINAMI, 2000).

Protocols for acclimatization of somatic embryogenesis in Arabic coffee are well established. However, the results presented in the literature up to the present moment do not allow the application of the vegetative propagation at the commercial scale. Therefore, there is a need for research that aims to solve this question. Thus, this work had the objectives of evaluating the diverse factors that affect the development of embryos and seedlings of *Coffea arabica* L. cv. Rubi obtained from direct somatic embryogenesis and its posterior acclimatization.

2 MATERIAL AND METHODS

The experiments were performed in the laboratory of vegetable tissue cultures at the Department of Agriculture from the Federal University of Lavras, State of Minas Gerais. Murashige & Skoog (1962), media was solidified with 5 $g.L^{-1}$ of agar. Fifteen ml of the medium had the pH adjusted to 5.8 ± 0.1 and was distributed in tubes.

The following variables were evaluated: number of leaves, length of the aerial part and in the case of the experiments one and two, was measured the fresh weight of the seedlings. The unbalanced data were submitted to ANOVA by using the procedure GLM available at the computational application SAS® (SAS INSTITUTE, 1990). For the experiment number three, the data were subjected to ANOVA and the averages were compared with the Tukey test at the level of 5% probability, using the Sisvar® statistical program (FERREIRA, 2000).

Experiment 1: Development of the somatic embryos

There were used somatic embryos obtained by direct embryogenesis from coffee seedling leaves *Coffea arabica* L., cv. Rubi MG 1192 originated from *in vitro* culture of zygotic embryos. The zygotic embryos were induced in MS media (MURASHIGE & SKOOG, 1962), with 50% of salts, with 20.0 mg L^{-1} of GA_3, 6.0 mg L^{-1} of kinetin, 8.0 $mg.L^{-1}$ of NAA, 100 $mg.L^{-1}$ of hydrolyzed casein and 400 $mg.L^{-1}$ of malt extracts. The tubes were kept during 150 days with light intensity of 32 $\mu Mol.m^{-2}.s^{-1}$ at the temperature of 25 ± 1°C. The somatic embryos at the torpedo stage were individualized in tubes (one embryo per tube) and kept during 30 days in MS media, aiming homogenization of the materials for the beginning of the first experiment. The treatments consisted of four combinations of GA_3 at the concentrations of (0; 2.5; 5.0 and 10.0 $mg.L^{-1}$) and five sucrose concentrations (0; 15; 30; 45 and 60 g L^{-1}) added to MS media.

The experimental design used was entirely randomly in factorial scheme 4 x 5, with four repetitions and three tubes per parcel. Each tube contained one explant. The evaluation of the experiment was performed after 150 days of the beginning of the experiments.

Experiment 2: Development of the seedlings

Seedlings of *Coffea arabica* cv. Rubi around 1 cm length from the first experiment which were kept during 30 days in MS media, aiming homogenization of the material was used as explants. The treatments consisted of a combination of four GA_3 concentrations (0; 2.5; 5 and 10 $mg.L^{-1}$) and five concentrations of NAA (0; 0.25; 0.5 and 1 $mg.L^{-1}$) added to the MS media. The tubes were kept during 60 days with light intensity of 32 $\mu Mol.m^{-2}.s^{-1}$ at the temperature of 25 ± 1ºC. The experimental design used was entirely randomly in factorial scheme 4 x 4, with four repetitions and three tubes per parcel. Each tube contained one explant. The evaluation of the experiment was performed after 60 days of the beginning of the experiments.

Experiment 3: Acclimatization of the seedlings

Seedlings from *Coffea arabica* cv. Rubi, originated from the second experiment, were transplanted into polystyrene trays with 72 cells with a volume of 120 mL. The trays were kept in a bench with metallic mesh. Following the trays contained the seedlings, were placed inside a green house equipped with intermittent nebulization system. The bench was protected at its superior part, above the nebulization pipe and in the border, with meshes that allowed 50% of shade.

In the beginning of the experiments, the seedlings were with three couple of true leaves and were divided in three blocks according to its height. There were used seedlings of about 2.0 cm of length and with the presence of root system. The treatments consisted of two types of substrate (Plantmax hortaliças ® and Plantmax hortaliças ® plus carbonized rice peels) (v/v at the proportion of 1:1), and four concentrations of fertilizer of slow-release for each 55 liters of substrate (0, 0.5, 1 and 2 g of the fertilizer per cell). The commercial Plantmax® substrate is made of vermiculite and ground pines peels composed and enriched with nutrients and also with carbonized rice peels. The nutrients of this substrate were complemented with fertilizers of slow-release, named Osmocote®, at the formulation of 15-10-10 + micronutrients.

The experimental design used was random blocks, with three blocks and five plants per parcel, containing in each cell tray one unique seedling. After 150 days, the experiment was evaluated.

3 RESULTS AND DISCUSSION

Experiment 1: Development of the somatic embryos

There was significant effect for the GA_3 concentrations tested. The significant effect was observed for length of the aerial part and number of leaves. There was no significant difference regarding interactions among the factors by the F test. The best result for length of the aerial part (Figure 1), was obtained with the use of 5.81 $mg.L^{-1}$ of GA_3 (1.45 cm). This growth regulator is involved in several important physiological activities, being effective in growth, specially, in shoot elongation (CROCOMO & CABRAL, 1988). These results are in accordance with the results found by Rezende et al. (2001), which showed better results with 5 $mg.L^{-1}$ of GA_3 for immature embryos of *C. canephora* Pierre ex Froehn.

Positive effect of GA_3 was also found by others authors in *C. Arabica* for the cultivar Acaiá Cerrado (Pereira et al. 2007), and for the cultivar Catuaí Vermelho LCH 2077-2-5-44 (Cavalcante-Alves et al.

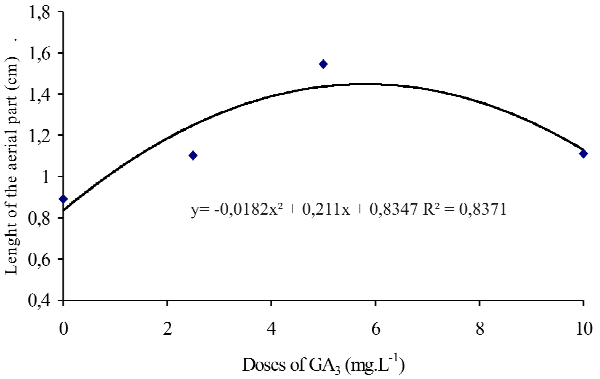

Figure 1 – Length of the aerial part of seedlings from somatic embryos of *C. arabica* cv. Rubi obtained *in vitro* and cultivated in different concentrations of GA_3.

1999). Custers (1982) and Norstog (1972) pointed out that gibberellins is a group of growth regulators that is capable of acting in the embryo by stimulating its growth.

However, concentrations above 5.81mg.L^{-1} promoted inhibition of the development of the embryo probably due to the phototoxic effect of this regulator. This result agreed with Válio (1976), which proposed that low concentrations of gibberellic acid promoted seed germination in coffee seed whereas high concentrations may cause dead of the embryos.

The low requirement of gibberellic acid verified in this variable is explained by the fact that the embryos may have certain endogenous quantities of this hormone (CHAGAS et al., 2005; JIMENEZ et al., 2001). Takaki et al. (1979), showed decrease in germination when coffee seeds were treated with gibberellic acid. For these authors, the decrease in seed germination was caused by the increase in enzymes (Cellulase and others), which were induced by the growth regulator causing degradation of cell wall materials.

Experiment 2: Development of the seedlings

There was significant effect for the concentration of GA_3 for length of the aerial part. The concentration of NAA showed significant in relation to the fresh weight of the seedlings. There was no significant effect for the interaction in none of the characteristics evaluated at the level of 5% of probability by the F test.

Since de dada relate to length of the aerial part did no show normality of the residues, they were transformed in $\left(\sqrt[5]{x+1}\right)/0,2$. The results showed crescent increase in length of the aerial part when was increased the GA_3 concentrations, obtaining seedlings with 1.23 cm (real dada) at the concentration of 10 mg.L^{-1}(Figure 2). Others authors also had good results when they used higher concentrations of the regulator. Pereira et al. (2007), which used 14.2 mg.L^{-1} of GA_3 associated to 0.5 mg.L^{-1} of NAA and Cavalcante-Alves et al. (1999), which observed in the presence of 9 mg.L^{-1} of BAP and 20.0 mg.L^{-1} of GA_3 higher number of sprout with length superior 1 cm. These results are different from the ones showed by Carvalho et al. (1999), which worked with orthotropic gemma of 'Catuaí Vermelho' and found lower number of sprouting, when they increased the GA_3 concentration.

The affect of GA_3 in plant growth is through the regulation of cell division and elongation (TAKAHASHI et al., 1988) and extensibility of the cell walls (METIVIER, 1986; RAVEN et al., 1992). The positive influence of the regulator in the size of various species is confirmed by: Ben-Gad et al. (1978) in *Citrus limettioides* Tanaka; Muller & Young (1982), with *Citrus aurantium* L.; Coelho et al. (1983), for hybrids seedlings of Cleópatra x *Poncirus trifoliata* (L.) Raf., and Modesto et al. (1996), for seedlings of lemon 'Cravo'.

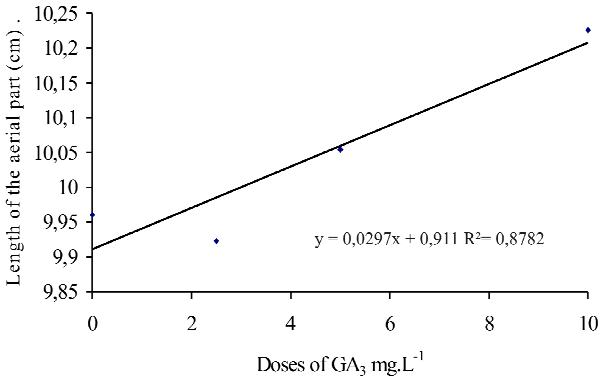

Figure 2 – Length of the aerial part of *C. arabica* cv. Rubi seedlings obtained *in vitro* and cultivated in different concentrations of GA_3.

For the fresh weight, there was increasing effect up to the maximum concentration of NAA in the media, reaching 0.22 g at the concentration of 1 mg.L^{-1} (Figure 3). Others authors had good results using inferiors concentrations of NAA. For example, Pereira et al. (2007), showed better results for fresh weight of the aerial part of seedlings when they were obtained through somatic embryogenesis of the coffee cultivar Acaiá Cerrado, with 0.75 mg.L^{-1} of NAA. Andrade et al. (2001), working with zygotic embryos of *C. arabica* cv. Catuaí Vermelho, showed superior results for fresh weight when they added 0.53 mg.L^{-1} of NAA in the media.

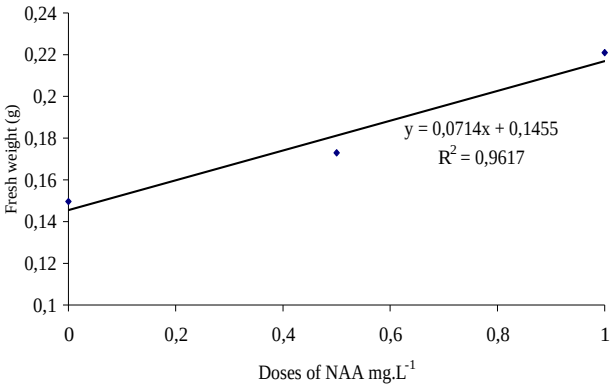

Figure 3 – Effect of the different concentrations of NAA in the seedling fresh weight of *C. arabica* cv. Rubi cultivated *in vitro*.

Experiment 3: Acclimatization of the seedlings

There was significant effect of the substrate under the number of leaves and of the concentrations of fertilizer under the height of the seedlings. The interactions among the factors did not show significant relation for none of the characteristics. The Plantmax® mixed with carbonized rice peels (v/v 1:1), showed higher number of leaves only when Plantmax® was used (Figure 4). Theses results are in accordance with Vallone (2004), which working with coffee seedlings, concluded that the replacement of commercial substrate by carbonized rice peels allows faster development of the seedlings.

The carbonized rice peels mixed together with the substrates are being studied for seedlings production and according to Minami (1995), its have flocculated format, dark color, soft, easy handling, with great capacity of drainage, slow alkaline pH, low capacity for water retention, rich in Ca and K, free of plagues and pathogens due to the carbonization process.

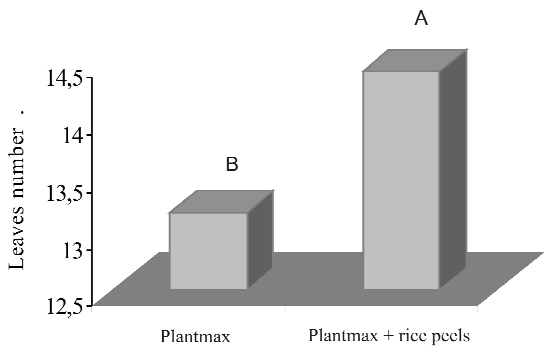

Figure 4 – Effect of different substrates in the number of leaves of *C. arabica* cv. Rubi seedlings. Averages followed by the same letter do not differ by the Tukey test at the level of 5% of probability.

According to Puchalski & Kämpf (2000), the carbonized rice peels shows space for aeration, this means that the volume of the macro pores is superior to 42% and the total porosity is superior to 80%, theses are ideal characteristics for the use of this substrate in small volumes. Carlesso et al. (2002) and Silva et al. (2003), evaluated different substrate for production of *C. arabica* and *C. canephora* seedlings, they showed inferior results with the use of the Plantmax® substrate in the absence of others compound.

On the other hand, there was no significant difference for height of the plants. Although, the result showed no significant difference, the seedling showed superior height when the commercial Plantmax® substrate was used (Figure 5), in opposition to the number of leaves. Anyway, higher number of leaves may be more interesting to the acclimatization process, since it indicates presence of photosynthetic apparatus.

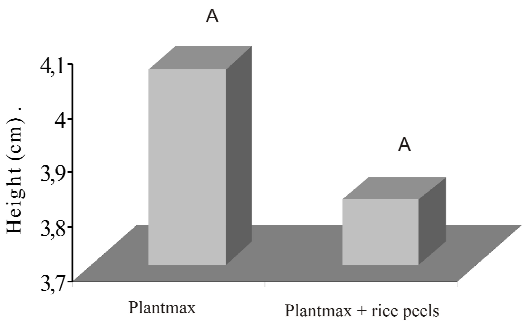

Figure 5 – Effect of the different substrates in the height of *C. arabica* cv. Rubi seedlings. Averages followed by the same letter do not differ by the Tukey test at the level of 5% of probability.

The concentration of 1.68 g of Osmocote® per cell was the concentration that showed plants with superior height, decreasing after that (Figure 6). This result is quite similar to the results obtained by Melo et al. (2001), which showed that the concentration of 1g of the fertilizer of slow-release per container allowed superior seedling development in commercial substrate.

Likewise, Rangel et al. (2002), observed among others substrate, that Plantmax combined with the concentration of 1.8 g of fertilizer per container showed precocity in the formation of the *C. canephora* seedlings in nursery. This last result is in accordance with the present work, mainly considering the volume of the container of 120 mL, which is exactly the same as the volume of cell used.

It is important to point out that, after one year and half from the beginning of the experiments, the seedling were ready to be sown with ideal size and number of leaves.

This is an advantage, since somatic embryogenesis allows multiplication of elite plants *in vitro*, guaranteeing uniformity and keeping the genetic improvement made by the selection.

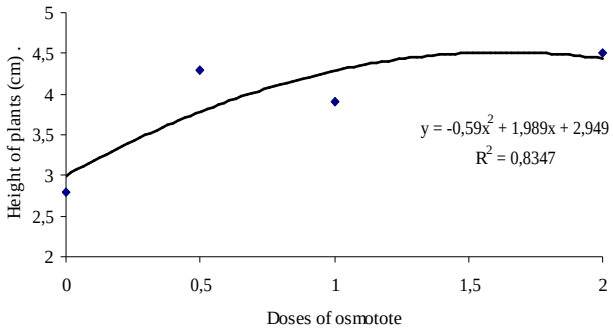

Figure 6 – Height of the *C. arabica* cv. Rubi seedlings grown at different concentrations of Osmocote®.

4 CONCLUSIONS

The use of 5.81 mg.L^{-1} of GA_3 increased the development of somatic embryos of coffee and the concentration of 10 mg.L^{-1} of GA_3 and 1 mg.L^{-1} of NAA showed to be effective for the development of seedlings originated from somatic embryogenesis.

The substrate Plantmax® (v/v 1:1) when mixed with carbonized rice peels plus 1.68 g per cell of fertilizer of slow-release (Osmocote®), was the best substrate for acclimatization of the seedlings of coffee cv. Rubi originated from somatic embryogenesis.

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