EFFECT OF TEMPERATURE AND CYTOKININ ON THE CAPACITY OF DIRECT SOMATIC EMBRYOGENESIS IN *Coffea arabica* L. GENOTYPES

Julieta Andréa Silva de Almeida[1], Rebeca Rocha Leal[2], Valéria Cristina Barbosa Carmazini[3], Marcus Vinicius Salomon[4], Oliveiro Guerreiro-Filho[5]

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ABSTRACT: The vegetative multiplication of *Coffea arabica* hybrids can be carried out by direct somatic embryogenesis. The objective of this study was to verify if temperature and cytokinins could influence the capacity for direct somatic embryogeneis in arabica genotypes. For this purpose foliar explants taken from adult plants of three *C. arabica* genotypes, hybrids 812 and 956 and cultivar Catuaí, were inoculated into a culture medium with ½ MS salts, 20.0 g l^{-1} sucrose and 30 µM 6-benzyladenine (6-BA) or 10 µM 2-isopenteniladenina (2-iP) and submitted to temperatures of 25 and 30 ºC in the absence of light. The treatments were evaluated with respect to the number of somatic embryos formed per foliar explant, at 270 days after the beginning of the experiment. A completely random experimental design was adopted with a 3 x 2 x 2 (genotype x temperature x plant growth regulator) factorial scheme, with ten replications per treatment and two explants in each. Temperature of 30 ºC favored significantly the formation of somatic embryos when compared to 25 ºC and this response pattern predominated amongst the three genotypes. On the other hand, 6-BA and 2-iso-pentenladenine caused similar answers for the majority of genotypes. The results of this study showed that temperature has a significant influence on the direct somatic embryogenesis capacity of *C. arabica* genotypes.

Index terms: Arabica hybrid, somatic embryo, 6-benzyladenine, 2-isopenteniladenina.

EFEITO DA TEMPERATURA E CITOCININA NA CAPACIDADE DE EMBRIOGÊNESE SOMÁTICA DIRETA EM GENÓTIPOS DE *Coffea arabica* L.

***RESUMO**: A multiplicação vegetativa dos híbridos de Coffea arabica pode ser obtida pela embriogênese somática direta. Objetivou-se, neste estudo, verificar se a temperatura e as citocininas 6-benziladenina (6-BA) and 2-isopentenladenina (2-iP) poderiam influenciar na capacidade de embriogênese somática direta de genótipos de arábica. Para tanto, explantes foliares provenientes de plantas adultas dos híbridos 812 e 956 e da cultivar Catuai Vermelho foram inoculados em meio de cultura com metade da concentração dos sais de MS, adição de 20 g l^{-1} de sacarose, 30 µM de 6-BA ou 10 µM de 2-iP, submetidos às temperaturas de 25 e 30 ºC, em ausência de luz. Foi adotado o delineamento inteiramente casualizado em esquema fatorial 3 x 2 x 2 (genótipo x temperatura x regulador de crescimento de planta), com dez repetições por tratamento e dois explantes em cada repetição. Os tratamentos foram avaliados quanto ao número de embriões somáticos formados por explante foliar, aos 270 dias do início do experimento. A temperatura de 30 ºC aumentou significativamente a formação de embriões somáticos quando comparado com 25 ºC. Por outro lado, as citocininas 6-BA e 2-iP causaram respostas similares para a maioria dos genótipos. Os resultados do presente estudo demonstraram que o fator temperatura tem influência significativa na capacidade de embriogênese somática direta de explantes foliares de genótipos de C. arabica.*

***Termos para indexação:** Híbrido de arábica, embrião somático, 6-benziladenina,* 2-isopenteniladenina.

1 INTRODUCTION

The vegetative multiplication of *Coffea* has been carried out by somatic embryogenesis (SE) especially for the clonal multiplication of arabica hybrids (BERTRAND et al., 2011; MORAIS; MELO, 2011).

Somatic embryos may be induced via indirect (ISE) or direct (DSE) pathway (QUIROZ-FIGUEROA et al., 2006; YANG et al., 2010). Indirect somatic embryogenesis consists of two phases, the first corresponding to calogenesis giving rise to the callus and the differentiation of the somatic embryo occurs in the second phase, as from determined callus cells (GAJ, 2004). On the other hand, in DSE, the embryos are formed in a single phase, without calogenesis (GATICA-ARIAS; ARRIETA-ESPINOZA; ESPINOZA-ESQUIVEL, 2007). But, usually the ISE is used more often than the direct pathway to obtaining somatic embryos of Arabic genotypes (VIEIRA; KOBAYASHI, 2000).

The capacity of SE can be influenced by various factors, such as the physiological characteristics of the plant that donated the explants (KLCOVA; HAVRENTOVA; FARAGO, 2004) and the in vitro cultivation conditions with respect to: type of culture medium (LÓPEZ-GÓMEZ

[1,2,3,5]Centro de Café 'Alcides Carvalho' - Instituto Agronômico de Campinas - 13001-970 - Campinas - SP - julietasa@iac.sp.gov.br, rebeca.rochaleal@gmail.com, valeria_carmazina@yahoo.com.br, oliveiro@iac.sp.gov.br
[4]Coordenadoria de Assistência Técnica Integral/CATI - Av. Brasil, 2340 - Bairro Vila Itapura - 13070-178 - Campinas - SP mvinicius@cati.sp.gov.br

et al., 2010), carbon dioxide in the environment of the culture vessel (BARBÓN; JIMÉNEZ; PREIL, 2008); type of plant growth regulator (GIRIDHAR et al., 2004a, 2004b; KUMAR; RAMAKRISHNA; RAVISHANKAR, 2007; PEREIRA et al., 2007); light regime (ALMEIDA et al., 2008; DE-LA-PENA; GALAZ-AVALOS; LOYOLA-VARGAS, 2008), agar concentration (ALMEIDA; CARMAZINI; RAMOS, 2007), osmotic potential of the culture medium (AHMAD; JAVED; ASHRAF, 2007; FERRIE; KELLER, 2007) and temperature (ORTOLAN et al., 2007; TORRES-VIÑALS et al., 2004).

Plant growth regulators play a significant role in the control of somatic embryogenesis (JIMÉNEZ, 2005). The induction of DSE in *Coffea* is strongly associated with the addition of cytokinins to the culture medium, in the absence of auxins (ALMEIDA; SILVAROLLA, 2009; PAPANASTASIOU et al., 2008).

Temperature acts in a significant way in the occurrence of the events of plant growth and development in nature. However, a lack of studies on the effect of temperature on the capacity of SE is noticed in *Coffea*. The influence of the temperature on the different events of the embryo regeneration route in SE has been little studied. The majority of the studies found in the literature was related to the application of low temperatures as a heat shock treatment in ears, shoots, flowers and anthers to obtain haploid plants (CALIC-DRAGOSAVAC; STEVOVIC, ZDRAVKOVIC-KORAC, 2010; JAVED et al., 2007; MORAES et al., 2004; WANG; CAMPBELL, 2006). There are few references to the influence of this factor in the SE of other species, not related to the cultivation of anthers, as like *Cydonia oblonga* (MORINI et al., 2004) and *Picea abies* (KVAALEN; JOHNSEN, 2008). According to George and Davies (2008), there is no single, previously defined temperature for the in vitro cultivation of all species, and in general, 25 ºC is most used. Although different species are successfully multiplied under this condition, each species may have its own optimum temperature. Thus it is possible that temperatures other than 25 ºC could also influence DSE in *C. arabica*. The objective of the present study was to verify the influence of temperature associated with two cytokinins, 6 benzylaminopurine and 2-isopenteniladenina, on the direct somatic embryogenesis of three *Coffea arabica* genotypes.

2 MATERIAL AND METHODS

Leaves were collected from the third pair of branches of adult *Coffea arabica* plants of the cultivar Catuai Vermelho IAC 99 and the hybrids H20049C812 (812) and H20032C956 (956), developed by the genetic improvement program of the Instituto Agronômico de Campinas. The leaves were washed in a detergent solution and rinsed three times in running water, disinfested in a 2.5 % commercial sodium hypochlorite solution for 25 minute. This disinfested treatment was repeated twice, per two consecutive days. Rectangular 1.5 x 2.0 cm explants were obtained from these leaves, excluding the main vein, edges and apical and basal portions. The explants were inoculated with the abaxial side in contact with the culture medium.

The culture medium contained half strength MS salts (MURASHIGE; SKOOG, 1962), 20.0 g l-1 sucrose and 30 µM 6-BA (ALMEIDA; SILVAROLLA, 2009) or 10 µM 2-iP (ALMEIDA; CARMAZINI; RAMOS, 2007). The pH of the medium was adjusted to 5.8, solidified with 5 g l-1 agar and autoclaved at 121 ºC and 1.5 atm for twenty minutes. Volumes of 30 mL of the culture medium were added to transparent glass flasks (250 mL) and kept at 25 or 30 ºC ± 2 in the absence of light.

The treatments were evaluated at 270 days for their direct somatic embryogenesis capacity, to the number of somatic embryos produced per foliar explant. A completely random experimental design was adopted with a 3 x 2 x 2 (genotypes x temperature x plant growth regulator) factorial scheme, with ten replications per treatment and two explants in each. The data obtained from NE were analyzed statistically using the F test, and the means compared using Tukey's test, both at a level of 5 %.

3 RESULTS AND DISCUSSION

The explants of all the treatments presented somatic embryo forming capacity (NE) (Figure 1). It was not found any significant interaction between temperature and plant growth regulator.

The temperature of 30 ºC significantly increased the number of somatic embryos formed per foliar explant of the three genotypes when compared to 25 ºC (Figure 1). In this case, at 30 ºC, the Catuaí, 812 and 956 formed respectively 68.8, 71.9 and 20.0 embryos per foliar explant. Moreover, at 25 ºC, all of these genotypes had reduced number of embryos per explant.

On the other hand, 6-BA and 2-iP caused the same effect on the number of somatic embryos for the genotypes Catuaí (Figure 1D) and 812 (Figure 1E), but 6-BA increased the production of somatic embryos for the genotype 956 (Figure 1F).

Foliar explants of the hybrids 812, 956 and the cv Catuaí, submitted to DSE, formed two morphological types, small structures similar to callus (Figure 2A) and somatic embryos (Figure 2B), both occurring in an isolated mode or simultaneously on the edges of rectangular explants, in a single cultivation phase. The somatic embryos formed directly on the edges of the explants (Figure 2B) or on the surface of the structures similar to callus (Figure 2A), mainly when both were oxidized (data not shown).

Contact between the explants and, principally, the plant growth regulator present in the culture medium, is one of the factors that unleashes the events of induction, initiation and development of the somatic embryos (JIMÉNEZ, 2005). In previous studies it was shown that 30 µM 6-BA (ALMEIDA; SILVAROLLA, 2009) and 10 µM 2-iP (ALMEIDA; CARMAZINI; RAMOS, 2007) induced good somatic embryo formation in foliar explants of the genotypes of *C. arabica*, both cultivated at 25 ºC. In the present study, it was observed that 6-BA and 2-iP, in general, have similar response for the total of somatic embryos, except for the genotype 956 which showed greater quantity in the presence of 6-BA (Figure 2). These differences could be attributed to genetic response.

It was observed that although the treatments at 30 ºC in presence of 30 µM 6-BA or 10 µM of 2-iP promoted good embryo formation, the time for this to occur was very long, since it started at about 90 days in reduced amounts (data not shown), and only reached elevated production after 240 days (data not shown).

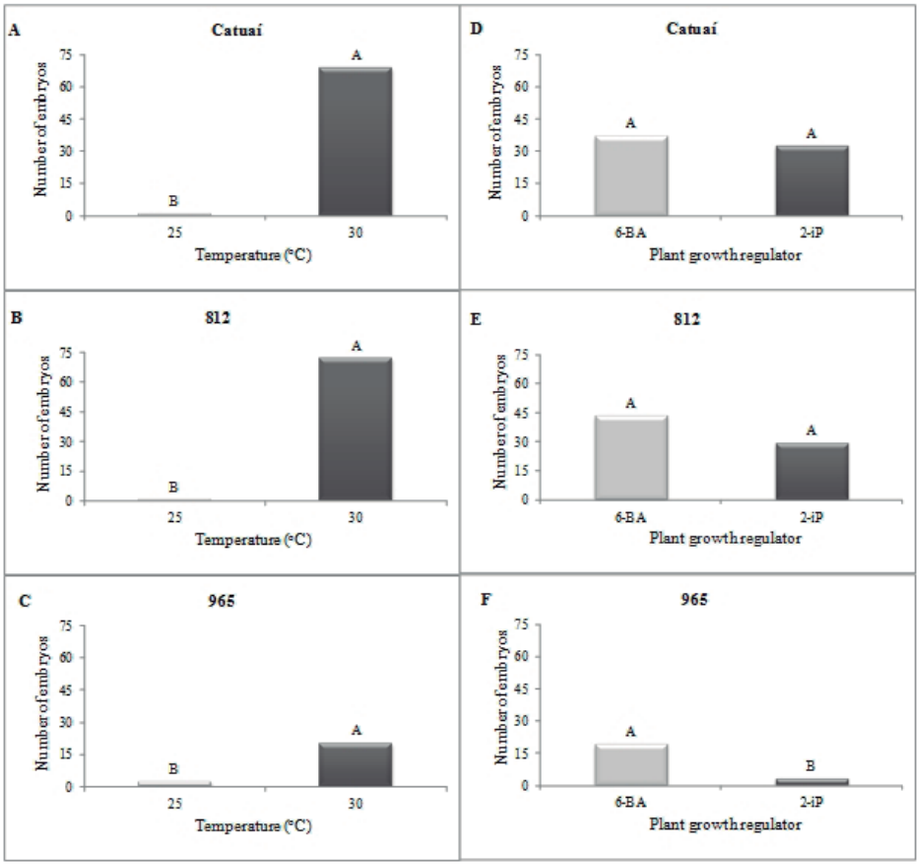

FIGURE 1 - Effect of temperature (***A,B,C***) and plant growth regulator (***D,E,F***) in the number of somatic embryos formed per foliar explant of three genotypes of *Coffea arabica* submitted to direct somatic embryogenesis, maintained in the dark, at 270 days after the beginning of the experiment.

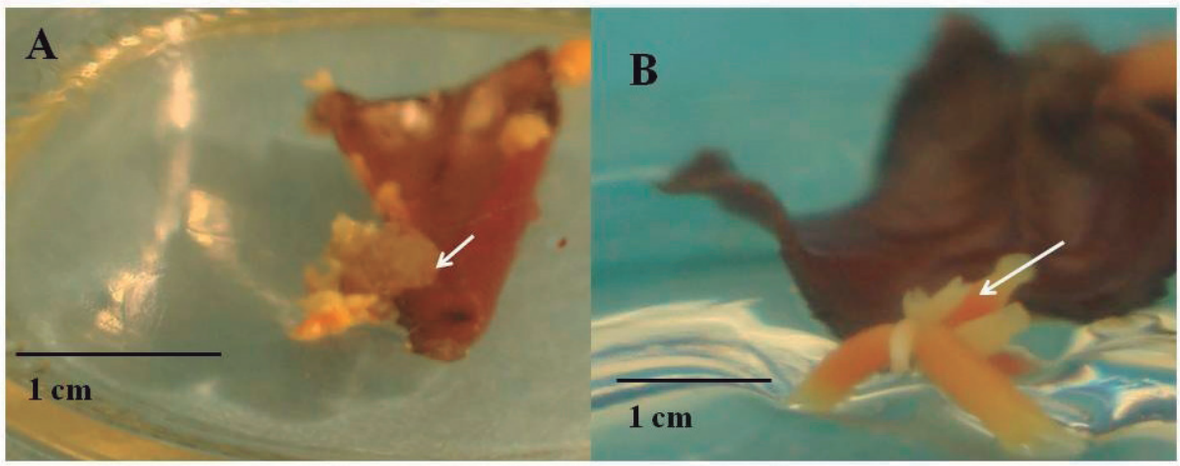

FIGURE 2 - Presence of structures similar to callus (**A**) and somatic embryos (**B**) on foliar explants of *Coffea arabica* submitted to direct somatic embryogenesis, at 30 ºC.

This long time was also observed in other studies with genotypes of arabica (ALMEIDA; SILVAROLLA, 2009). Perhaps, this long time to the beginning of the formation of somatic embryos by direct way is associated with the cellular plasticity. The vegetative cells show developmental plasticity (FEHÉR, 2008). According to Schlichting and Smith (2002) a phenotype plasticity is any change in an organism's characteristics in response to an environmental signal. The plasticity can be influenced by the given physiological state of the cell (NIKLAS, 2008) which is determined by its genetic and developmental conditions and by environmental cues (MAL; LOVETT-DOUST, 2005), as temperature (ATKIN et al., 2006) and plant growth regulation (FARNSWORTH, 2004).

Thus the results of the present study showed that the temperature influences the DSE capacity of *C. arabica* genotypes. Fehér (2008) discussed that the occurrence of somatic embryogenesis is not only due to genetic control, but it could also depend on the physical environmental conditions (GEORGE; DAVIES, 2008). Thus the exact biochemical expression of the cells in culture could be considerably modified by the conditions imposed by the medium.

4 CONCLUSIONS

The temperature of 30 ºC increased the number of somatic embryos produced in foliar explants of *C. arabica* when compared to 25º C. 6-BA and 2-iP had the same effect on the induction of somatic embryos in most genotypes.

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6 REFERENCES

AHMAD, M. S. A.; JAVED, F.; ASHRAF, M. Iso-osmotic effect of NaCl and PEG on growth, cations and free proline accumulation in callus tissue of two indica rice (*Oryza sativa* L.) genotypes. **Plant Growth Regulation**, Dordrecht, v. 53, p. 53-63, 2007.

ALMEIDA, J. A. S.; CARMAZINI, V. C. B.; RAMOS, L. C. S. Indirect effect of agar concentration on the embryogenesis responses of *Coffea canephora.* **Fruit, Vegetable and Cereal Science and Biotecnology**, Tokyo, v. 1, p. 121-125, 2007.

ALMEIDA, J. A. S. et al. Embriogênese somática em genótipos de *Coffea arabica* L. **Coffee Science**, Lavras, v. 3, n. 2, p. 143-151, 2008.

ALMEIDA, J. A. S.; SILVAROLLA, M. B. Induction of somatic embryos of *Coffea arabica* genotypes by 6-benzyladenine. **International Journal of Plant Developmental Biology**, Tokyo, v. 3, n. 1, p. 5-9, 2009.

ATKIN, O. K. et al. Phenotypic plasticity and growth temperature: understanding interspecific variability. **Journal Experimental Botany**, London, v. 57, n. 2, p. 267-281, 2006.

BARBÓN, R.; JIMÉNEZ, E.; PREIL, W. Influence of *in vitro* environment on somatic embryogenesis of *Coffea arabica* L cv Caturra rojo: the effects of carbon dioxide on embryogenic cell suspensions. **Plant Cell Tissue and Organ Culture**, Dordrecht, v. 95, n. 2, p. 155-161, 2008.

BERTRAND, B. et al. Performance of *Coffea arabica* F1 hybrids in agroforestry and full-sun cropping systems in comparison with American pure line cultivars. **Euphytica**, Dordrecht, v. 181, p. 147-158, 2011.

CALIC-DRAGOSAVAC, D.; STEVOVIC, S.; ZDRAVKOVIC-KORAC, S. Impact of genotype, age of tree and environmental temperature on androgenesis induction of *Aesculus hippocastanum* L. **African Journal of Biotechnology**, Nairobi, v. 9, n. 26, p. 4042-4049, 2010.

DE-LA-PENA, C.; GALAZ-AVALOS, R. M.; LOYOLA-VARGAS, V. M. Possible role of light and polyamines in the onset of somatic embryogenesis of *Coffea canephora.* **Molecular Biotechnology**, Totowa, v. 39, n. 3, p. 215-224, 2008.

FARNSWORTH, E. Hormones and shifting ecology throughout plant development. **Ecology**, Durham, v. 85, n. 1, p. 5-15, 2004.

FEHÉR, A. The initiation phase of somatic embryogenesis: what we know and what we don't. **Acta Biologica Szegediensis**, Hungary, v. 52, n. 1, p. 53-56, 2008.

FERRIE, A. M. R.; KELLER, W. A. Optimization of methods for using polyethylene glycol as a non-permeating osmoticum for the induction of microspore embryogenesis in the *Brassicacea.* ***In Vitro*** **Cellular & Developmental Biology Plant**, Berlin, v. 43, n. 4, p. 348-355, 2007.

GAJ, M. D. Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. **Plant Growth Regulation**, Dordrecht, v. 43, p. 27-47, 2004.

GATICA-ARIAS, A. M.; ARRIETA-ESPINOZA, G.; ESPINOZA-ESQUIVEL, A. M. Comparison of three *in vitro* protocols for direct somatic embryogenesis and plant regeneration of *Coffea arabica* L. cvs. Caturra and Catuaí. **Agronomia Costarricense**, San José, v. 31, n. 1, p. 85-94, 2007.

GEORGE, E. F.; DAVIES, W. Effects of the physiological environment. In: GEORGE, E. F.; HALL, M. A.; DE KLERK, G. (Ed.). **Plant propagation by tissue culture**. 3rd ed. Netherlands: Springer, 2008. p. 423-464.

GIRIDHAR, P. et al. Direct somatic embryogenesis from *Coffea arabica* L. and *Coffea canephora* P. ex Fr. under the influence of ethylene action inhibitor-silver nitrate. **Acta Physiologiae Plantarum**, New York, v. 26, n. 3, p. 299-305, 2004a.

GIRIDHAR, P. et al. Thidiazuron induced somatic embryogenesis in *Coffea arabica* L. and *Coffea canephora* P. ex Fr. **Acta Botanica Croatica**, Ottawa, v. 63, n. 1, p. 25-33, 2004b.

JAVED, M. A. et al. The role of alternating culture temperatures and maltose in enhancing the anther culture efficiency of salt tolerant indica rice (*Oryza sativa* L.) cultivars, Pokkali and Nona Bokra. **Plant Biotechnology**, Tokyo, v. 24, p. 283-287, 2007.

JIMÉNEZ, V. M. Involvement of plant hormones and plant growth regulators on *in vitro* somatic embryogenesis. **Plant Growth Regulation**, Dordrecht, v. 47, p. 91-110, 2005.

KLCOVA, L.; HAVRENTOVA, M.; FARAGO, J. Cultivar and environmental conditions affect the morphogenic ability of barley (*Hordeum* vulgare) scutellum-derived calli. **Biologia**, Btatislava, v. 59, n. 4, p. 501-504, 2004.

KUMAR, V.; RAMAKRISHNA, A.; RAVISHANKAR, G. A. Influence of different ethylene inhibitors on somatic embryogenesis and secondary embryogenesis from *Coffea canephora* P. ex Fr. ***In Vitro*** **Cellular Developmental Biology Plant**, New York, v. 43, n. 6, p. 602-607, 2007.

KVAALEN, H.; JOHNSEN, O. Timing of bud set in *Picea abies* is regulated by a memory of temperatura during zigotic and somatic embryogenesis. **New Phytologist**, Cambridge, v. 177, p. 49-59, 2008.

LÓPEZ-GÓMEZ, P. et al. Influence of explant and culture medium on somatic embryogenesis of coffee leaves. **Revista de Fitotecnia Mexicana**, Chapingo, v. 33, n. 3, p. 205-213, 2010.

MAL, T. K.; LOVETT-DOUST, J. Phenotypic plasticity in vegetative and reproductive traists in an invasive weed, *Lythrum salicaria* (Lythraceae), in response to soil moisture. **American Journal of Botany**, Columbus, v. 92, n. 5, p. 819-825, 2005.

MORAES, A. P. et al. Effect of temperature shock on soybean microspore embryogenesis. **Brazilian Archives of Biology and Technology an International Journal**, Curitiba, v. 47, n. 4, p. 537-544, 2004.

MORAIS, T. P.; MELO, T. Biotecnologia aplicada ao melhoramento genético do cafeeiro. **Ciência Rural**, Porto Alegre, v. 41, n. 5, p. 753-760, 2011.

MORINI, S. et al. Effect of high and low temperature on the leaf regenerating capacity of Quince BA29 rootstock. **Acta Horticulturae**,The Hague, v. 658, p. 591-597, 2004.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tabacco tissue cultures. **Physiologia Plantarum**, Copenhagen, v. 15, p. 473-497, 1962.

NIKLAS, K. J. Functional adaptation and phenotypic plasticity at the cellular and whole plant level. **Journal Bioscience**, Uberlândia, v. 33, n. 4, p. 1-8, 2008.

ORTOLAN, A. R. et al. Efeito da temperatura e luminosidade na regeneração *in vitro* de plantas de trigo. **Scientia Agraria**, Curitiba, v. 8, n. 1, p. 61-65, 2007.

PAPANASTASIOU, I. et al. Effect of liquid pulses with 6-benzyladenine on the induction of somatic embryogenesis from coffee (*Coffea arabica*) callus cultures. **Plant Cell Tissue Organ Culture**, Dordrecht, v. 92, n. 2, p. 215-225, 2008.

PEREIRA, A. R. et al. Embriogênese somática direta em explantes foliares de *Coffea arabica* L. cv. Acaiá cerrado: efeito de cinetina e ácido giberélico. **Ciência e Agrotecnologia**, Lavras, v. 31, n. 2, p. 332-336, mar./abr. 2007.

QUIROZ-FIGUEROA, F. R. et al. Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. **Plant Cell Tissue Organ Culture**, Dordrecht, v. 86, p. 285-301, 2006.

SCHLICHTING, C. D.; SMITH, H. Phenotypic plasticity: linking molecular mechanisms with evolutionary outcomes. **Evolutionary Ecology**, Netherlands, v. 16, p. 189-211, 2002.

TORRES-VIÑALS, M. et al. Large-scale production of somatic embryos as a source of hypocotyl explants for *Vitis vinifera* micrografting. **Vitis**, Siebeldingen, v. 43, n. 4, p. 163-168, 2004.

VIEIRA, L. G. E.; KOBAYASHI, A. K. Micropropagação do cafeeiro. In: SIMPÓSIO DE PESQUISAS DOS CAFÉS DO BRASIL, 1., 2000, Poços de Caldas. **Anais...** Poços de Caldas: Consórcio Brasileiro de Pesquisa e Desenvolvimento do Café, 2000. p. 147-167.

WANG, Y.; CAMPBELL, C. Effects of genotypes, pretreatments and media in anther culture of common (*Fagopyrum* esculentum) and self-pollinating buckwheat. **Fagopyrum**, Ljubljana, v. 23, p. 29-35, 2006.

YANG, J. L. et al. Direct somatic embryogenesis from pericycle cells of broccoli (*Brassica oleracea* L. var. italic) root explants. **Plant Cell, Tissue and Organ Culture**, Dordrecht, v. 100, n. 1, p. 49-58, 2010.