Journal of Global Biosciences
ISSN 2320-1355
Volume 9, Number 5, 2020, pp. 7364-7376
Website: www.mutagens.co.in
DOI: www.mutagens.co.in/jgb/vol.09/05/090507.pdf

SCAN ME

Research Paper

PEG 6000 AND SUCROSE IN THE CONTROL OF THE DIRECT SOMATIC EMBRYOGENESIS CAPACITY IN *Coffea arabica* L.

Wilielle Cristina Freitas Neofiti[1], Priscila Fratin Medina[2], Gerson Silva Giomo[3] and Julieta Andrea Silva de Almeida[4]

[1] Coffee Center, Campinas Agronomic Institute, 13020-902, Campinas, São Paulo, Brazil

[2] Grain and Fiber Center, Campinas Agronomic Institute, 13020-902, Campinas, São Paulo, Brazil

[3] Coffee Center, Campinas Agronomic Institute, 13020-902, Campinas, São Paulo, Brazil

[4] Coffee Center, Campinas Agronomic Institute, 13020-902, Campinas, São Paulo, Brazil.

Abstract

Coffea arabica genotypes submitted to direct somatic embryogenesis produce small embryogenic structures and somatic embryos. In this species, somatic embryos are formed at the edges of leaf explants and on the surface of embryogenic structures, however both forms produce few embryos over a long period of time. Osmotic agents have the ability to promote the response of somatic embryogenesis in different species. The objective of this study it was to evaluate the effect of Polyethyleneglycol 6000 (PEG 6000) and sucrose at different concentrations in the direct somatic embryogenesis of *C. arabica*. Foliar explants of plants of the hybrids H8089, H8105, H8427 and of the cultivar Mundo Novo were cultured in a direct somatic embryogenesis induction culture medium with the addition of a combination of PEG 6000 (0, 2 %) and sucrose (0, 10, 20, 30 g/L). Explants from the four genotypes respond to all treatments with the exception of the those without sucrose. Treatments with 20 and 30 g/L of sucrose combined with 2 % PEG 6000 caused greater production of somatic embryos than the others. In addition, in general in these treatments the embryogenic structures were larger, which contributes to the promotion of the direct pathway, since they also form somatic embryos. The obtained results indicate that osmotic stress induced by PEG 6000 and sucrose can promote the direct route in leaf explants of *C. arabica*.

Key words: *Mundo Novo, hybrids, sucrose, PEG 6000, embryogenic structure*.

INTRODUCTION

Somatic embryogenesis is a vegetative multiplication process applied to the majority of species. Its occurrence is based on the cellular totipotency concept, in which the vegetable cell has the required genetic information to give origin to a complete and functional plant [1]. In this process, somatic embryos form from tissue cells of the explant [2]. Subsequently these embryos reach the plant state with preservation of the genetic characteristics of the explant donating plant [3]. However, despite scientific advances, the mechanisms that control the induction of somatic embryogenesis are still unknown [4].

Somatic embryogenesis can occur by indirect or direct routes [5, 6]. Genotypes of *Coffea arabica* can form somatic embryos by the indirect route, which occurs in two phases with the callogenesis followed by the formation of somatic embryos [7, 8] or direct route that occurs in a phase without the callogenesis [9, 10, 11, 12] or both.

In the direct path *C. arabica* explants first develop small embryogenic structures (up to 3 mm) with a hyaline color on their edges, as from the 14th day of cultivation, and somatic embryos. Somatic embryos are formed at the edges of leaf explants and too on the surface of embryogenic structures after 90 days [10]. But explants of this species submitted to direct somatic embryogenesis produce few somatic embryos and the process occurs over a long period of time [9, 10].

The efficiency of somatic embryogenesis can be affected by the genotype, the physiological conditions of the explant-donating plant, endogenous and exogenous hormones, type of culture medium, type of sugar and stress factors [13, 14, 15, 2, 16]. Stress factors have been related to promoting the acquisition of embryogenic competence in different species [17, 18, 19]. The stress-induction system tends to favor a greater formation of embryogenic calluses and somatic embryos in the indirect somatic embryogenesis in different species [20, 21]. But there is a lack of reports of the effect of osmotic stress on the direct somatic embryogenesis in *C. arabica*.

Explant cells tend to die under extreme stress but can develop suitable adaptation mechanisms under moderate stress [22]. Osmotic stress normally influences the plant metabolism by altering the solute concentrations in the internal and external cell environments [23, 24, 25), compromising the events of growth and development [26].

Different types of osmotic agent have been used to change the osmotic potential of *in vitro* cultivation systems. Polyethyleneglycol 6000 (PEG 6000) influenced the somatic embryogenesis response of different species [27, 28, 29]. Sucrose, in addition to acting as an energy source for the *in vitro* vegetable tissues, can also act as an osmotic agent, and as such, is known to affect somatic embryogenesis in various species [30, 31, 32]. Thus, the objective of the present study was to verify the effect of PEG 6000 and sucrose in controlling the occurrence of direct somatic embryogenesis in *C. arabica.*

MATERIALS AND METHODS

Leaves collected up to the third pair of plagiotropic branches of adult *C. arabica* plants of the hybrids H8089, H8105, H8427 and of the cultivar Mundo Novo during the morning, located in an experimental area of the Campinas Agronomic Institute, São Paulo State, Brazil. Soon after collecting the leaves were washed in a detergent solution and rinsed in running water. They were then immersed in a 2 % sodium hypochlorite solution for twenty-five minutes, rinsed three times in distilled water and maintained in a moist chamber with ± 80 % humidity. The following day the leaves were disinfested again as described above. Rectangular explants (1.5 x 2.0 cm) were removed from the leaves.

In order to induce direct somatic embryogenesis, a culture medium with half the normal concentration of MS salts [33] was used with the addition of 30 g/L of sucrose and 30 µM of 6-BA (6-Benzyladenine) [9]. In addition, 0, 10, 20 and 30 g/L of sucrose 0 and 2 % PEG 6000 were also added to the culture medium. The pH value of the medium was then adjusted to pH 5.8 before autoclaving at 121 ºC and 1.5 atm for twenty minutes. For the germination of somatic embryos and the development of plants *in vitro* culture medium with half the concentration of MS salts without the addition of plant growth regulator was used.

The explants were inoculated in transparent glass pot (150 mL volume) containing 30 mL of culture medium. These explants were placed in the culture medium with the adaxial face in contact with the glass surface and incubated in the dark at 25 ºC. The somatic embryos obtained were transferred to the germination culture medium. The flasks with the embryos were kept in the presence of 16 hours of light and 8 hours of continuous darkness, at 25 ºC. Following the germination phase the embryonic axes

were transferred to growth and plant development medium. When the embryos reached the plant stage with four leaf pairs and root presence they were transferred to the acclimatization phase (Figure 3C).

For experiment was carried out using a completely random experimental design. In relation to the applied experiment each treatment with twenty repetitions and two explants each. The treatments were evaluated with respect to the capacity for somatic embryogenesis, as follows: the number of somatic embryos formed at 280 days throughout the experiments and estimation of the size of the embryogenic structures at 240 days. The data obtained from number of somatic embryos were analyzed statistically using the F test, and the means compared using Tukey's test, both at a level of 5 %. Data from estimation of the size of the embryogenic structures were expressed as mean standard error of the media.

RESULTS

Somatic embryos were formed in all treatments with sucrose with or without the addition of 2 % PEG 6000, except in those without sucrose who did not respond (Figure 1). In the treatments without PEG 6000, the greatest production of somatic embryos occurred mainly with 30 g/L sucrose, followed by 20 g/L and finally 10 g/L, with smaller numbers (Figure 1). At 280 days, with 30 g/L, without PEG, the genotype H8089 (Figure 1C) and the cultivar Mundo Novo (Figure 1A) produced the greatest average number of embryos by explant, followed by H8105 (Figure 1B) and H8427 (Figure 1D) with, respectively, 46.3, 26.4, 16.6 and 15.5 embryos. With 20 g/L, the cultivar Mundo Novo and the genotypes H8089 and H8105 formed the most embryos (22, 21.4 and 20.7) and the genotype H8427 formed the least embryos (13.7). On the other hand, numbers of embryos were formed with 10 g/L, obtaining 18.9, 17.5, 17.3 and 13.7 embryos, respectively from the genotype H8089, the cultivar Mundo Novo, H8105 and H8427 (Figure 1).

With respect to the combined treatments, with the addition of 2 % PEG, embryo production was greater principally with 20 and 30 g/L of sucrose and lower with 10 g/L for most of the genotypes (Figure 1). It should be highlighted too that most of the treatments combined with 2 % PEG produced more embryos than the equivalent treatment without PEG. For example, explants of the cultivar Mundo Novo in the presence of 10, 20 and 30 g/L of sucrose without PEG formed, respectively, after 280

days, 17.5, 21 and 26.4. However, in the presence of 2 % PEG these treatments had, respectively 20, 32.4 and 40.2 embryos and, in general, this response pattern was followed by the other treatments (Figure 1A). Somatic embryos of the four *C. arabica* genotypes formed in all treatments were transferred to the germination and plant growth media, respectively. When the plants had about six pairs of leaves and roots (Figure 3C) they were transplanted to the acclimatization phase. Part of these plants is being tested in a field condition to be evaluated for their agronomic characteristics.

In addition to somatic embryos, the response of formation of embryogenic structures at the edges of leaf explants was verified from the 15th day of cultivation (Figure 2). All the treatments showed an elevated percentage of these structures of above 70 %, with the exception of the explants without sucrose, which did not respond (data not shown). However, the size of the embryogenic structures was affected in a differentiated way by the sucrose combined, or otherwise, with PEG 6000.

In the absence of PEG, at 240 days the explants of all the genotypes formed larger embryogenic structures mainly with a dose of 30 g/L of sucrose (Figures 2). However, the size of the structures varied between the four genotypes for each sucrose dose without PEG. With the 30 g/L dose, the Mundo Novo cultivar (Figure 2A) and genotype H8427 (Figure 2D) formed larger structures with a mean size of 17 mm in both cases, whereas the sizes were smaller for H8089 (Figure 2C) and H8105 (Figure 2B), respectively 5.7 and 4 mm. In the treatment with 20 g/L, the Mundo Novo cultivar (Figure 2A) produced structures of 5.2 mm, and H8089, H8105 and H8427 below 4.3 mm (Figures 2C, B, D). With 10 g/L the largest size was 3.7 mm for H8089 (Figures 2C) and between 1.8 and 2.1 mm for the other genotypes (Figures 2A, B, D).

For the treatments combined with 2 % PEG, the explants formed larger embryogenic structures, principally with 30 g/L (Figure 3C) followed by 20 g/L (Figure 3D) of sucrose. The size of the structures also varied between the four genotypes. With 30 g/L of sucrose, the cultivar Mundo Novo (Figure 2A) formed 18 mm embryogenic structures, followed by the genotypes H8427, H8089 and H8105, respectively, with 9.1, 7.5 and 4 mm (Figures 2D, C, B). With 20 g/L the cultivar Mundo Novo and genotype H8089 produced the largest structures, followed by H8427 and H8105 with sizes of, respectively, 11, 10, 8.2 and 4.6 mm (Figures 2A, C, D, BF). With 10 g/L the sizes of the structures were smaller, between 2 and 6.6 mm (Figures 2A, B, C, D).

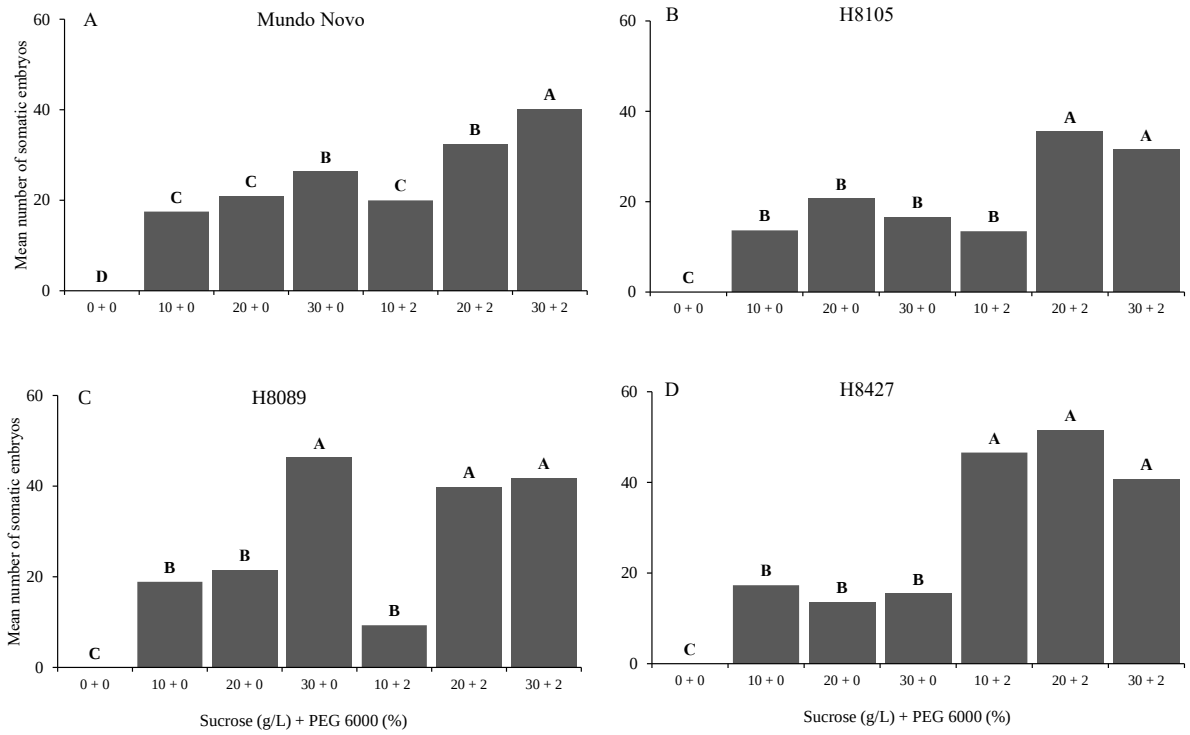

Figure 1: Number of somatic embryos formed in *C. arabica* leaf explants inoculated in a direct somatic embryogenesis culture medium with addition of sucrose and PEG 6000 addition, both in different concentrations, kept in continuous dark and at 25 ºC, after 280 days from the beginning of the experiment.

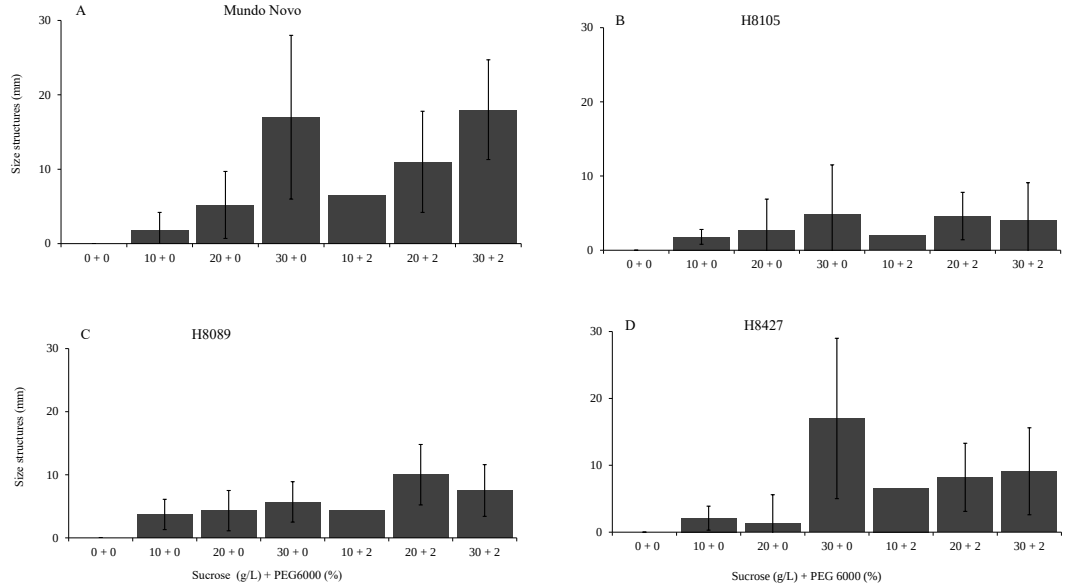

Figure 2: Estimation of the size of these structures formed in leaf explants of *C. arabica* grown in direct somatic embryogenesis culture medium with sucrose and PEG 6000 addition, both in different concentrations, kept in continuous dark and at 25 ºC, after 240 days of the beginning of the experiment.

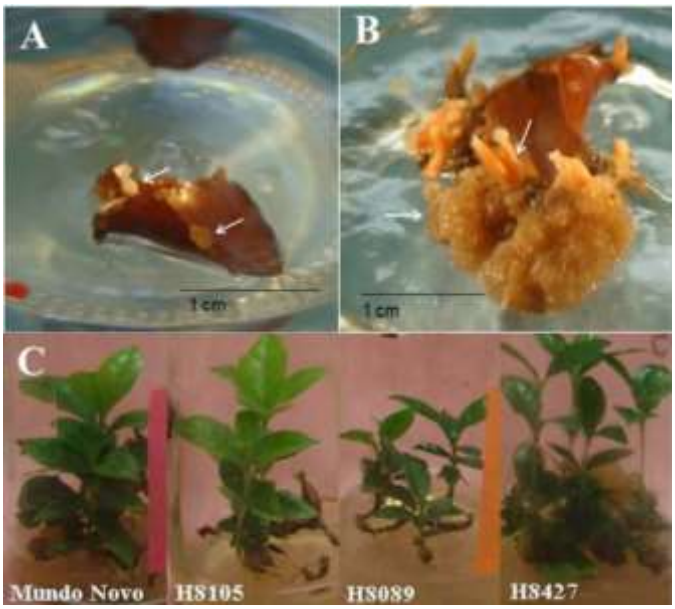

Figure 3: Morphological responses of *C. arabica* leaf explants inoculated in direct somatic embryogenesis induction medium in the absence of light at 25 ºC. A. Explant with 20 g/L of sucrose, without PEG 6000. Upper arrow: somatic embryo at the edge of the explant. Bottom arrow: small embryogenic structure. B. Explant with 30 g/L of sucrose and 2% PEG. Upper arrow: somatic embryo. Bottom arrow: larger embryogenic structure. (A and B: after 280 days of the beginning of the cultive). C. *In vitro* plants of the studied genotypes.

DISCUSSION

The results obtained in this study indicate that sucrose treatments combined with PEG 6000 promoted the capacity of direct somatic embryogenesis of explants from four *C. arabica* genotypes for increasing the production of somatic embryos and forming larger embryogenic structures, except in those without sucrose that did not respond. The treatments showing no response corroborate the fact that the explants depended on the energy from the sucrose in order to unleash somatic embryogenesis [34, 35, 36].

The treatments with 20 and 30 g/L of sucrose with 2 % PEG generally promoted the greater number of somatic embryos in the explants of the four genotypes submitted to the direct route compared to other treatments (Figure 2) as well as 30 g/L of sucrose without PEG. Possibly, these treatments induced a certain degree of intensity of osmotic stress that favored the acquisition of embryogenic competence and led to the formation of a higher production of somatic embryos. Authors argue that the stress not only promote dedifferentiation in tissues *in vitro* but it could also induce the production of somatic embryos [18]. Others found that vegetable tissue cells of

Arabidopsis interrupt their cell cycle soon after exposure to osmotic stress, remain latent for some time, and then proceed with cell proliferation, as their adaptation response to the stress [37]. In this study, perhaps the greater production of somatic embryos of *C. arabica* can be associated as an adaptation response of explant cells to applied osmotic stress.

Normally, in the direct route, explants of this species usually form small embryogenic structures up to 3 mm in size that can form somatic embryos [10] (Figure 3A). But in this study the treatments with by 30 g/L of sucrose combined or not with 2 % PEG also caused the formation of larger embryogenic structures in *C. arabica* explants (Figures 2, 3B). Then, larger embryogenic structures are important because they also form somatic embryos on their surface [10] which contributes to increase promoting the somatic embryogenesis response. It is also noted in this study that the larger embryogenic structures (Figures 2, 3C) were generally similar in size and texture to the calluses formed on the *C. arabica* explants when submitted to the indirect somatic embryogenesis. Normally, for callus induction in the indirect route, 30 g/L of sucrose and 2.4 D are used [8]. But, in this study for induction of the direct pathway, 30 g/L of sucrose and cytokinin were used, without any auxin, which caused the formation of larger embryogenic structures, similar to the calluses developed by the indirect route of *C. arabica* [7, 8]. It is possible that this response is due to osmotic stress induced by PEG and sucrose. Endogenous vegetable hormones interact with applied stress leading to the acquisition of embryogenic competence [19]. It was verified in *Oryza sativa* that osmotic stress altered the regulation of the endogenous levels of auxins and other hormones, favoring the occurrence of callogenesis and regeneration [34]. The mechanism underlying auxins and stress-induced somatic embryogenesis are not known, but both conditions induce biosynthesis of endogenous auxins [19]. The synthesis of endogenous auxins is considered a crucial step in the change towards totipotent growth. But for a more adequate conclusion about the development of larger embryogenic structures in the direct pathway, explants of *C. arabica* should be further studied in relation to the effect of osmotic stress in this pathway.

Throughout this whole study too it was noted that the three H8105, H8089 and H8427 hybrids responded in differentiated ways to the factors evaluated, these genotypes are being improvement phase and still have genetic instability while the Mundo Novo

cultivar is stable because it is a cultivated material. But, although the hybrids are genetically unstable, they in general showed similar responses to treatments with PEG and sucrose.

The osmotic stress induced by sucrose combined with 2 % of PEG 6000 positively influenced the control of direct somatic embryogenesis in leaf explants of four *C. arabica* genotypes, showing an outstanding formation of larger embryogenic structures and an increase in the production of somatic embryos, these being relevant contributions to the somatic embryogenesis of this species.

ACKNOWLEDGEMENT

The authors thank to the Process 11/10090-1 supported by Fundação de Apoio À Pesquisa do Estado de São Paulo (FAPESP).

REFERENCES

[1] Horstman, A.; Bemer, M. and Boutilier, K., 2017, A transcriptional view on somatic embryogenesis. Regeneration. 4(4): 201-216.

[2] Elmer, K.E.S., 2013, Factors regulating somatic embryogenesis in plants. In: Aslam J, Srivastava PS, Sharma MP, editors. Somatic embryogenesis and gene expression. New Delhi: Narosa Publishing House, p.56-81.

[3] Mendez-Hernandez, H.A.; Ledezma-Rodriguez, M.; Avilez-Montalvo, R.N.; Juárez-Gómez, Y.L. and Skeete, A., 2019, Signaling overview of plant somatic embryogenesis. Front. Plant Sci. 10: 1-15, Article 77.

[4] Pais, M.S., 2019, Somatic embryogenesis induction in woody species: The future after OMICs data assessment. Front. Plant Sci. 10: Article 240. doi.org/10.3389/fpls.2019.00240.

[5] Williams, E.G. and Maheswaran, G., 1986, Somatic embryogenesis: factors influencing coordinated behavior of cells as an embryogenic group. Ann. Bot. 57: 443-462.

[6] Fehér, A., 2019, Callus, dedifferentiation, totipotency, somatic embryogenesis: what these terms mean in the era of molecular plant biology? Front. Plant Sci. 10: 1-11.

[7] Söndahl, M.R. and Sharp, W.R., 1977, High frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. Z. Pflanzenphysiol. 81: 395-408.

[8] Almeida, J.A.S., Silvarolla, M.B., Fazuoli, L.C., Stancato, G.C., 2008, Embriogênese somática em genótipos de *Coffea arabica* L. Coffee Sci. 3: 143-151.

[9] Almeida, J.A.S., Silvarolla, M.B., 2009, Induction of somatic embryos of *Coffea arabica* genotypes by 6-Benzyladenine. Int. J. Plant Dev. Biol. 53: 5-8.

[10] Almeida J.A.S.; Leal, R.R.; Carmazini, V.C.B.; Salomon, M.V. and Guerreiro-Filho, O., 2016, Characterization of the morphological events in the somatic embryogenesis in the direct somatic of *Coffea arabica* L. genotypes. Plant Cell Biot. Mol. Bio. 17: 393-403.

[11] Chone, R.M.S.; Rocha, D.I.R.; Monte-Bello, C.C.; Pinheiro, H.P.; Dornelas, M.C.; Haddad, C.R.B. and Almeida, J.A.S., 2018, Brassinosteroid increases the cytokinin efficiency to induce direct somatic embryogenesis in leaf explants of *Coffea arabica* L. (Rubiaceae). Plant Cell Tissue Organ Cult. 135: 63-71.

[12] Alves, I.S.; Carmazini, V.C.B.; Santos, C.D. and Almeida, J.A.S., 2018, 2 - Isopentenyladenine in the induction of direct somatic embryogenesis capacity of *Coffea arabica* L. Cienc. Rural. 48: 1-5.

[13] Fehér A., Pasternak T.P. and Dudits D., 2003, Transition of somatic plant cells to an embryogenic state. Plant Cell Tiss. Org. Cult. 74: 201-228.

[14] Gaj, M.D., 2004, Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Regul. 43:27-47.

[15] Deo, P.C.; Tyagi, A.P.; Taylor, M.; Harding, R. and Becker, D., 2010, Factors affecting somatic embryogenesis and transformation in modern plant breeding. The South Pacific Journal of Natural and Applied Sciences. 28: 27-40.

[16] Isah, T., 2016, Induction of somatic embryogenesis in woody plants. Acta Physiol. Plant. 38(118): 1-22.

[17] De Jong, A.J.; Chmidt, E.D.L. and De Vries, S.C., 1993, Early events in higher-plant embryogenesis. Plant Mol. Biol. 22: 367-377.

[18] Zavattiere, M.A.; Frederico, A.M.; Lima, M.; Sabino, R. and Arnholdt-Shmitt, B., 2010, Induction of somatic embryogenesis as example of stress-related plant actions, Electron. J. Biotechn. 13: 1-9.

[19] Fehér, A., 2015, Somatic embryogenesis - Stress-induced remodeling of plant cell fate. Biochim. Biophys. Acta. 1849: 385-405.

[20] Cabrera-Ponce, J.L.; López, L.; León-Ramírez, C.G.; Jofre-Garfias, A.E. and Verver-y-Vargas, A., 2015, Stress induced acquisition of somatic embryogenesis in common bean *Phaseolus vulgaris* L. Protoplasma. 252: 559-570.

[21] Carloni, E.; Tommasino, E.; Colomba, E.L.; Ribotta, A.; Quiroga, M.; Griffa, S. and Grunberg, K., 2017, *In vitro* selection and characterization of buffelgrass somaclones with diferent responses to water stress. Plant Cell Tissue Organ Cult. 130: 265-277.

[22] Demeter, Z.; Kanalas, P.; Máthé, C.; Cseke, K.; Szöllösi, E.; M-Hamvas, M.; Jámbrik, K.; Kiss, Z. and Mészáros, I., 2014, Osmotic stress responses of individual white oak (*Quercus section, Quercus subgenus*) genotypes cultured in vitro. J. Plant Physiol. 171: 16–24.

[23] Bray, E.A., 2001, Plant Response to Water-deficit Stress. In: Encyclopedia of Life Sciences. Chichester: John Wiley & Sons, Ltd, p. 1-7.

[24] Mahajan, S. and Tuteja, N., 2005, Cold, salinity and drought stress: An overview. Arch. Biochem. Biophys. 444: 139-158.

[25] Osakabe, Y.; Osakabe, K.; Shinozaki, K. and Tran, L-S.l., 2014, Response of plants to water stress. Front. Plant Sci. 5: 1-8.

[26] Poolman, B.; Blount, P.; Folgering, J.H.A.; Friesen, R.H.E.; Moe, P.C. and Heide, T. der, 2002, How do membrane proteins sense water stress? Mol. Biol. 44: 889-902.

[27] Viji, M.; Maheswari, P.; Karuppanapandian, T. and Manoharan, K., 2012, Effect of polyethylene glycol and mannitol on somatic embryogenesis of pigeonpea, *Cajanus cajan* (L.) Millsp. Afr. J. Biotech. 11(45): 10340-10349.

28] Elmaghrabi, A.M.; Rogers, H.J.; Francis, D. and Ochatt, S.J., 2017, PEG induces high expression of the cell cycle checkpoint gene *wee* in embryogenic callus of *Medicago truncatula*: potential link between cell cycle checkpoint regulation and osmotic stress. Front. Plant Sci. 8: 1-12.

[29] Maheshwari, S. and Manoharan, K., 2019, Induction of somatic embryogenesis from embryonal axis, cotyledonary node and leaf explants of green gram [*Vigna radiata* (L.) Wilczek.]. Int. J. Sci. Basic Appl. Research. 9(5): 420-429.

[30] Shi, X.; Dai, X.; Liu, G. and Bao, M., 2009, Enhancement of somatic embryogenesis in camphor tree (*Cinnamomum camphora* L.): osmotic stress and other factors affecting somatic embryo formation on hormone-free medium. Trees. 23: 1033-1042.

[31] Lema-Ruminska, J.; Goncerzewicz, K. and Gabriel, M., 2014, Influence of abscisic acid and sucrose on somatic embryogenesis in cactus *Copiapoa tenuissima* Ritt. forma mostruosa. Sci. World J. Article ID 513985: 1- 7.

[32] Mishra, V.K. and Singh, R.M., 2016, Sorbitol and sucrose- induced osmotic stress on growth of wheat callus and plantlet regeneration. Curr. Trends Biotechnol. Pharm. 10(1): 5-12.

[33] Murashige, T.; and Skoog, F., 1962, A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

[34] Lee, S.T. and Huang, W.L., 2014, Osmotic stress stimulates organogesis in callus of rice (*Oryza sativa* L.) via auxin signaling and carbohydrate metabolism regulation. Plant Growth Regul. 73: 193-204.

[35] Navarro, B.V.; Elbl, P.; Souza, A.P.; Jardim, V.; Oliveira, L.F.; Macedo, A.F.; Santos, A.L.W.; Buckeridge, M.S. and Flo, E.I.S., 2017, Carbohydrate-mediated responses during zygotic and early somatic embryogenesis in the endangered conifer, *Araucaria angustifolia.* Plos One. 12: 1-20. e0180051 doi.org/:10.1371/journal.pone.0180051.

[36] Grzyb, M.; Kalandyk, A.; Waligórski, P. and Mikula, A., 2017, The content of endogenous hormones and sugars in the process of early somatic embryogenesis in the tree fern *Cyathea delgadii* Sternb. Plant Cell Tiss. Org. Cult. 129: 387-397.

[37] Skirycz, A.; Claeys, H.; De Bodt, S.; Oikawa, A.; Shinoda, S.; Andriankaja, M.; Maleux, K.; Eloy, N.B.; Coppens, F.; Yoo, S.-D.; Saito, K. and Inzé, D., 2011, Pause-and-stop: The effects of osmotic stress on cell proliferation during early leaf development in *Arabidopsis* and a role for ethylene signaling in cell cycle arrest. Plant Cell. 23: 1876-1888.