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Salinity induces specific metabolic changes in sugarcane shoot explants in temporary immersion bioreactors

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Summary

There is a great demand of salt-tolerant sugarcane planting material in Cuba. Temporary immersion bioreactors (TIB) are effective to significantly increase sugarcane *in vitro* shoot proliferation rate from 1:4 in conventional containers to about 1:35. Sugarcane micropropagation in TIBs under NaCl stress may help screen mutants with salinity tolerance. We developed the experiment shown here to identify a NaCl concentration able to stress shoot in TIBs. At 30 days of culture initiation with different NaCl levels (0 - 200 mM), explant multiplication rate, shoot cluster fresh mass, and levels of aldehydes, chlorophylls, carotenoids and phenolics were determined in the plant material. Content of soluble phenolics in the culture medium was also evaluated. Addition of NaCl decreased shoot multiplication rate and fresh mass. Other statistically significant differences were recorded but the most important were noted in the increased contents of carotenoids, malondialdehyde, other aldehydes and soluble phenolics in the plants, and in the soluble phenolics in the culture medium. This research may be useful for future experiments of *in vitro* selection of new sugarcane genetic materials with NaCl tolerance. Fifty percent of multiplication rate was reduced with 89 mM NaCl which can be used to stress shoots during micropropagation in TIBs and eventually detect mutants with salt tolerance.

Keywords *In vitro* salt stress; *in vitro* selection; plant metabolites; *Saccharum sp.*; salinity

Introduction

There is a great demand of sugarcane planting material in Cuba, especially for salt-contaminated soils. Conventional methods of macropropagation, such as stem segments, do not satisfy the demand. Several researchers have developed protocols for micropropagation of different sugarcane varieties (Lee, 1987) and their results depended upon the genotype. Jiménez et al. (1995) recommended a micropropagation protocol for a Cuban variety (C-1051-73). However, the potential use of this micropropagation protocol is limited because of the relatively low multiplication rates (about 1:4). For wide spread application, it was necessary to develop more efficient methods.

Several systems for plant micropropagation have been established to increase proliferation and reduce production costs (Tisserat and Vandercook, 1985; Aitken-Christie and Davies, 1988; Simonton et al., 1991; Alvard et al., 1993). Our group developed a protocol for sugarcane (cv. C-1501-73) shoot formation in a temporary immersion bioreactor (TIB) (Lorenzo et al., 1998). This investigation was based on the protocol established by Jiménez et al. (1995), the shoot formation step was the only step modified and multiplication rate was increased from 1:4 to about 1:35 in 30 d of culture (Lorenzo et al. 1998; Lorenzo et al., 2001a; Lorenzo et al., 2001b).

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This positive result with TIBs might be caused by the combination of advantages of solid and liquid culture medium. Micropropagation on solid culture medium allows plant air exchange but nutrient uptake is limited to the explant basal surface. On the other hand, micropropagation in liquid culture medium increases nutrient uptake but hyperhydricity is frequently observed and is characterized by various degrees of morphological and physiological disorders including glassy, a waterlogged-tissue appearance and disordered growth in the shoot system, and more specifically in the leaves (Ziv, 1995). In a TIB, explants are covered with the culture medium only for a few minutes. Immersion allows nutrient uptake through the entire explant surface. However, the plant air exchange is restored after removing of culture medium. These two features are not usually combined in the classic liquid culture procedure (Alvard et al., 1993; Lorenzo et al., 1998; Escalona et al., 1999).

In vitro-induced mutagenesis (e.g. irradiation and chemical agents) combined with high plant multiplication rates may produce genetic variants (Larkin and Scowcroft, 1981; Brown et al., 1993; Cardoza and Stewart, 2004; Bhatia et al., 2005; Castillo et al., 2010) with improved agricultural performance in soils damaged by salt contamination. Many *in vitro*-induced mutants are agriculturally useless. Therefore, the development of efficient *in vitro* methodologies for early selection of salt-tolerant materials is essential (Ben-Hayyim and Kochba, 1982; Lutts et al., 2001; Gadakh et al., 2017; Hairuddin et al., 2017). Yet, it is important to note that the limitation of mutations is that no additional gene pool is brought into the plant material, for a better tolerance it might also be necessary to have such a germplasm (Acquaah, 2007).

The present work shows effects of NaCl (0, 50, 100, 150, 200 mM) on sugarcane micropropagation in TIBs to induce conditions of salinity and osmotic shock with the plant tissues. NaCl have been used to simulate this stress in conventional tissue culture containers for many years (Ben-Hayyim and Kochba, 1982; Lutts et al., 2001; Fatima et al., 2015; Zaher-Ara et al., 2016; Carloni et al., 2017; Gadakh et al., 2017; Hairuddin et al., 2017). Sugarcane shoot multiplication rate, shoot cluster fresh weight, and levels of aldehydes, chlorophylls, carotenoids and phenolics were determined in the plant material. The content of soluble phenolics in the culture medium was also evaluated. This is a contribution to develop of a TIB-based selection method of sugarcane salt tolerance.

Materials and methods

Sugarcane meristems (cv. C-1051-73) were collected from field-grown plants and cultured following the protocol of Jiménez et al. (1995). Sugarcane *in vitro*-cultured plants after four subcultures were used as explants. Routine subcultures were at 30-d intervals. Bioreactors described in Fig. 1 (A, B) were implemented. Jiménez et al. (1995) culture medium, modified by Lorenzo et al. (1998), was

used: MURASHIGE and SKOOG (1962) inorganic salts and vitamins; 100 mg l^{-1} inositol; 30 g l^{-1} sucrose, 0.3 mg l^{-1} 6-benzyladenine and 1.0 mg l^{-1} paclobutrazol. Different levels of NaCl were supplemented to the culture medium at the beginning of the 30-d-long subculture: 0, 50, 100, 150, 200 mM. Each treatment involved three bioreactors (5 explants/bioreactor). Cultures were maintained at 25±1 ºC; 80 µmol m^{-2} s^{-1} (fluorescent light) and an 8 h photoperiod. At 30 d, shoot multiplication rate, shoot cluster fresh weight, and levels of malondialdehyde, other aldehydes, chlorophyll a and b, carotenoids and phenolics (soluble, cell wall-linked) were determined in the plant material. Content of soluble phenolics in the culture medium was also evaluated. Plant tissues were sampled from three independent replicates of 100 mg each (one from each bioreactor).

Chlorophylls were quantified following PORRA (2002), phenolics by the method of GURR et al. (1992), and malondialdehyde and other aldehydes as described in HEATH and PACKER (1968). To determine the levels of chlorophyll pigments, extraction was carried out with 5.0 ml acetone (80%, v:v). The samples were centrifuged (14086.8 × g, 4 °C, 15 min) and supernatants collected and absorbances at 646.6 and 663.6 nm recorded (RAYLEIGH, VIS-723G). Levels of carotenoids were also determined according to LICHTENTHALER (1987) and absorbance at 470 nm was measured. Phenolic compounds were extracted and quantified using a spectrophotometer by a colorimetric method based on reaction with Folin Ciocalteu reagent (mg gallic acid equivalents per g fresh weight). Malondialdehyde and other aldehydes were quantified by a colorimetric method based on reaction with thiobarbituric acid. Excretion of phenolics was determined using a modification of the Hoagland (1990) procedure. Culture medium (0.5 ml) was mixed with 4.5 ml distilled water and 0.5 ml Folin Ciocalteau reagent (50% v/v) added. The mixture was shaken, left for 5 min and one ml of saturated sodium carbonate solution added. The mixture was shaken again, left for 60 min, and the optical density was measured at 725 nm. Phenolic concentration was determined by a calibration curve using gallic acid as the standard.

All data of this study were statistically evaluated using SPSS (Version 8.0 for Windows, SPSS Inc., New York, NY) to perform One-Way analysis of variance (ANOVA) and Tukey tests (p=0.05). The overall coefficients of variation (OCV) were calculated as follows: (standard deviation/average) * 100. In this formula, we considered the average values of the five NaCl levels compared (treatments) to calculate the standard deviation and average. Therefore, the higher the difference between the five treatments compared, the higher is the OCV (LORENZO et al., 2015). OCVs were classified as "Low" from 17.33 to 43.64%, "Medium" from 43.64 to 69.94% and "High" from 69.94 to 96.25%.

Results and discussion

NaCl decreased sugarcane shoot multiplication and fresh weight in a concentration-dependent way (Fig. 1C; Fig. 2A, 2B). With 200 mM NaCl, shoot multiplication rate reached 9.6% (3.3/33.9) of that obtained in the control treatment without NaCl, while fresh weight only reached 16.1% (1.4 g / 8.5 g). At biochemical level, several statistically significant differences among NaCl treatments were recorded (Fig. 2C-J) but "High" OCVs were only noted in the contents of carotenoids in the plants (Fig. 2G) and in the soluble phenolics excreted to the culture medium (Fig. 2J). Shoot multiplication rate (Fig. 2A) and the levels of carotenoids (Fig. 2G) and the excreted phenolics (Fig. 2J) were negatively correlated when NaCl was added. NaCl (0-200 mM) increased levels of carotenoids from 13.5 to 79.7 µg g^{-1} fresh weight (5.9 times, Fig. 2G); and excreted phenolics from 7.8 to 30.0 µg ml^{-1} culture medium (3.8 times, Fig. 2J).

Biochemical indicators with "Medium" OCVs also increased when salinity stress was imposed: 2.6 times the contents of malondialdehyde (0.10 / 0.04 nmol g^{-1} fresh weight; Fig. 2C); 3.7 times other aldehydes (1.4 / 0.4 nmol g^{-1} fresh weight, Fig. 2D); and 2.8 times soluble phenolics in the plants (4.4 / 1.5 µg g^{-1} fresh weight, Fig. 2H). Although statistically significant differences were recorded in the levels of chlorophyll a (Fig. 2E), b (Fig. 2F) and cell wall-linked phenolics (Fig. 2I), their OCVs were "Low" indicating a small effect of NaCl on them.

Plants have developed complex mechanisms for adaptation to the osmotic, ionic, and oxidative stresses that are induced by the salt stress (NAIK and AL-KHAYRI, 2016). Salinity has been associated with increases in abscisic acid (SHAFI et al., 2011), proline (BENHASSAINI et al., 2012), glycine-betaine (QUAN et al., 2004), poly-ols, sugar alcohols and soluble sugar concentrations (GURMANI

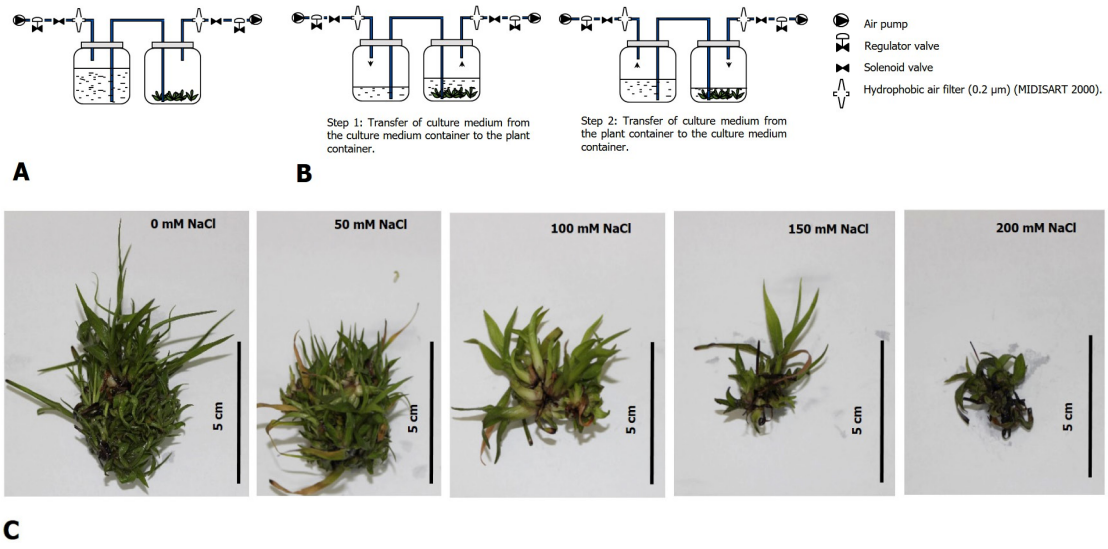

Fig. 1: Design of the temporary immersion bioreactor used and typical shoots produced. **A** Temporary immersion bioreactor. **B** More of operation of a temporary immersion bioreactor. Immersions (2 min each) were performed every 3 h during 30 d. **C** Typical phenotypes of sugarcane shoots produced in temporary immersion bioreactors with different levels of NaCl. Glass containers with 300 ml of total capacity, 200 ml of liquid culture medium, and silicone tubes were used. Five explants per container were cultured (40 ml medium / explant). Shoots were free in the bottom of containers. The design of temporary immersion bioreactor was previously described by LORENZO et al. (1998) and ESCALONA et al. (1999).

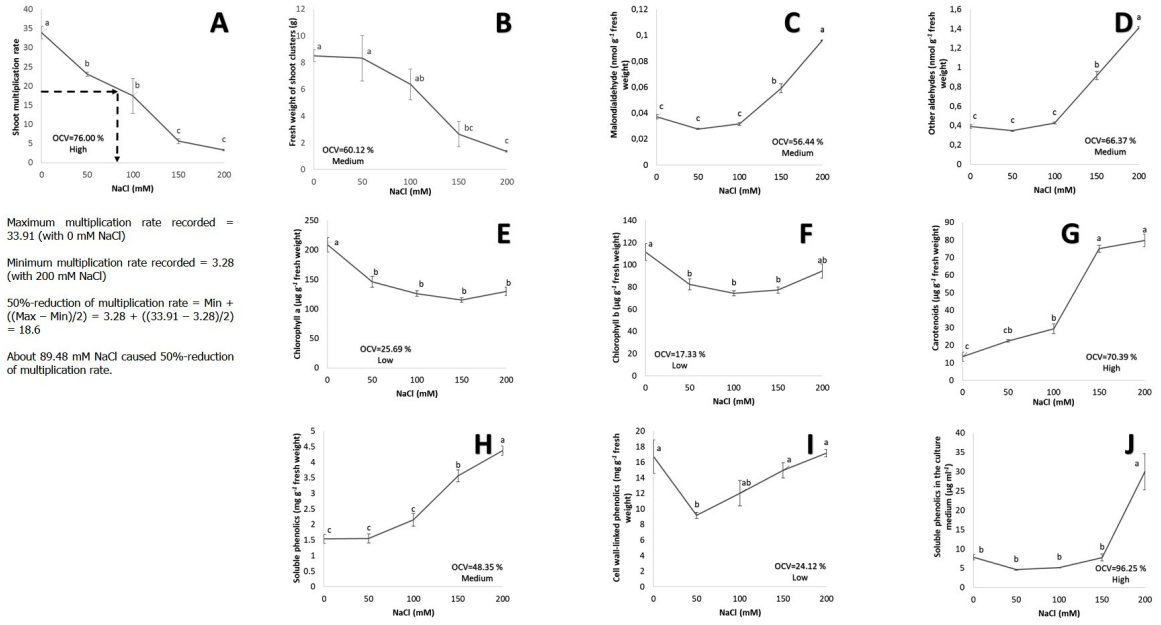

Fig. 2: The effect of sodium chlorine on sugarcane during microprogagation in temporary immersion bioreactors. Shoot dusters were formed around each explant. Vertical bars represent SE. Results with the same letter are not statistically different (One-way ANOVA, Tukey, p>0.05). Overall coefficient of variation (OCV) = (Standard deviation/Average) * 100. To calculate this coefficient, the five average values were considered. The higher the difference among results, the higher the overall coefficient of variation. Classification of OCVs: "Low" from 17.33 to 43.64%, "Medium" from 43.64 to 69.94% and "High" from 69,94 to 96.25%.

et al., 2007). Salinity stress also decreases plant growth (West et al., 2004; Munns, 2005), nutrient uptake (Abdelgadir et al., 2005), K^+:Na^+ ratio (Díaz-López et al., 2012), stomatal aperture and density (Huang et al., 2009), hexoses, sucrose and starch (Arbona et al., 2005) and chlorophyll contents (Rivelli et al., 2012).

Exposure to salinity is known to induce or stimulate the production of secondary plant products, such as phenols, terpenes, and alkaloids (Winkel-Shirley, 2002; Haghighi et al., 2012; Selmar and Kleinwächter, 2013; Boestfleisch et al., 2014; Boestfleisch and Papenbrock, 2017). *Catharanthus roseus* grown under salt stress showed increased levels of the alkaloid vincristine (Misra and Gupta, 2006; Fatima et al., 2015). In *Grevillea*, a significant increase in anthocyanin concentration was reported under salinity exposure in both the salt-tolerant *Grevillea ilicifolia* and the salt-sensitive *Grevillea arenaria* (Kennedy and De Filippis, 1999). In *Datura innoxia*, salt treatment increased the total alkaloid content in young leaves, and the results indicated that at the organ level, tropane alkaloid accumulation was related to plant growth (Brachet and Cosson, 1986). Salinity also increased the diamine and polyamine content in *Oryza sativa* (Krishnamurthy and Bhagwat, 1989).

In our experiment with sugarcane micropropagated in TIBs with NaCl; levels of soluble phenolics in the culture medium and shoots, carotenoids, malondialdehyde and other aldehydes were the clearest indicators of stress. These results agree with previous experiments conducted in different culture systems and plant species submitted to abiotic stressors (Winkel-Shirley, 2002; Haghighi et al., 2012; Rivelli et al., 2012; Selmar and Kleinwächter, 2013; Boestfleisch et al., 2014; Hernández et al., 2015; Boestfleisch and Papenbrock, 2017).

Despite the extensive studies on the effects of salinity stress on plants, the influence of NaCl on sugarcane shoots as micropropagated in TIBs, provide a new perspective. Multiplication rates shown in Fig. 2A indicate that 50% reduction was obtained with 89 mM NaCl. This concentration can be used to stress shoots during micropropagation in TIBs and eventually identify mutants with salinity or osmotic tolerance.

Several *in vitro* selection methodologies to produce mutants able to grow under salinity have been described. For instance, callus cultures have been stressed *in vitro* with NaCl in rice (Lutts et al., 2001), citrus (Ben-Hayyim and Kochba, 1982), sugarcane (Gadakh et al., 2017) and onion (Hairuddin et al., 2017). Although the new genotype tolerance requires additional confirmation under a field environment, *in vitro* selection allows some research cost reductions because there is no inclusion of a large number of susceptible materials in expensive field trials.

Author contribution

D.G., L.H., B.V., J.M., M.C., M.E., M.H., L.Y., G.T.S.B., C.C.T., J.P. and J.C.L. designed the research; D.G., L.H., B.V., J.M. and M.C. conducted the experiments; D.G., M.E., M.H., L.Y., G.B., C.C.T., J.P. and J.C.L analyzed the data and wrote the paper; and J.C.L. had primary responsibility for the final content. All authors have read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest Authors do not have any conflict of interests.

Human and animal rights This research did not involve experiments with human or animal participants.

Informed consent: Informed consent was obtained from all individual participants included in the study. Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

References

Abdelgadir, E., Oka, M., Fujiyama, H., 2005: Characteristics of nitrate uptake by plants under salinity. J. Plant Nutr. 28, 33-46.

Acquaah, G., 2007: Principles of Plant Genetics and Breeding Blackwell, Oxford, UK.

Aitken-Christie, J., Davies, H., 1988: Development of a semiautomated micropropagation system. Acta Hort. 230, 81-87

Alvard, D., Cote, F., Teisson, C., 1993: Comparison of methods of liquid medium culture for banana micropropagation. Plant Cell Tiss. Org. Cult. 32, 55-60.

Arbona, V., Marco, A.J., Iglesias, D.J., López-Climent, M.F., Talon, M., Gómez-Cadenas, A., 2005: Carbohydrate depletion in roots and leaves of salt-stressed potted *Citrus clementina* L. Plant Growth Reg. 46, 153-160.

Ben-Hayyim, G., Kochba, J., 1982: Growth characteristics and stability of tolerance of citrus callus cells subjected to NaCl stress. Plant Sci. Lett. 27, 87-94.

Benhassaini, H., Fetati, A., Hocine, A.K., Belkhodja, M., 2012: Effect of salt stress on growth and accumulation of proline and soluble sugars on plantlets of *Pistacia atlantica* Desf. subsp. atlantica used as rootstocks. Biotechnol. Agron. Soc. Environ. 16, 159-165.

Bhatia, P., Ashwath, N., Senaratna, T., Krauss, S.L., 2005: Genetic analysis of cotyledon derived regenerants of tomato using AFLP markers. Curr. Sci. 88, 280-284.

Boestfleisch, C., Wagenseil, N.B., Buhmann, A.K., Seal, C.E., Wade, E.M., Muscolo, A., Papenbrock, J., 2014: Manipulating the antioxidant capacity of halophytes to increase their cultural and economic value through saline cultivation. AoB PLANTS 6:plu046. DOI: 10.1093/aobpla/plu046

Boestfleisch, C., Papenbrock, J., 2017: Changes in secondary metabolites in the halophytic putative crop species *Crithmum maritimum*, *Triglochin maritima* and *Halimione portulacoides* as reaction to mild salt stress. PLoS ONE 12:e0176303. DOI: 10.1371/journal.pone.0176303

Brachet, J., Cosson, L., 1986: Changes in the total alkaloid content of *Datura innoxia* Mill. subjected to salt stress. J. Exp. Bot. 37, 650-656.

Brown, P., Lange, F.D., Kranz, E., Lörz, H., 1993: Analysis of single protoplasts and regenerated plants by PCR and RAPD technology. Mol. Gen. Genet. 237, 311-317.

Cardoza, V., Stewart, C.N., 2004: Brassica biotechnology: progress in cellular and molecular biology. *In Vitro* Cell Dev. Biol.-Plant 40, 542-551.

Carloni, E., Tommasino, E., Colomba, E.L., Ribotta, A., Quiroga, M., Griffa, S., Grunberg, K., 2017: *In vitro* selection and characterization of buffelgrass somaclones with different responses to water stress. Plant Cell Tiss. Org. Cult. DOI:10.1007/s11240-017-1220-9:1-13

Castillo, N.R.F., Bassil, N.V., Wada, S., Reed, B.M., 2010: Genetic stability of cryopreserved shoot tips of *Rubus* germplasm. *In Vitro* Cell Dev. Biol.-Plant 46, 246-256.

Díaz-López, L., Gimeno, V., Lidón, V., Simón, I., Martínez, V., García-Sánchez, F., 2012: The tolerance of *Jatropha curcas* seedlings to NaCl: an ecophysiological analysis. Plant Phys. Bioch. 54, 34-42.

Escalona, M., Lorenzo, J.C., González, B., Daquinta, M., Borroto, C., González, J.L., Desjardines, Y., 1999: Pineapple micropropagation in temporary immersion systems. Plant Cell Rep. 18, 743-748.

Fatima, S., Mujib, A., Tonk, D., 2015: NaCl amendment improves vinblastine and vincristine synthesis in *Catharanthus roseus:* a case of stress signalling as evidenced by antioxidant enzymes activities. Plant Cell Tiss. Org. Cult. 121, 445-458.

Gadakh, S., Patel, D., Singh, D., 2017: Use of RAPD markers to characterize salt and drought lines of sugarcane. Int. J. Adv. Res. Biol. Sci. 4, 50-57.

Gurmani, A.R., Bano, A., Saleem, M., 2007: Effect of ABA and BA on growth and ion accumulation of wheat under salinity stress. Pak. J. Bot. 39, 141-149.

Gurr, S., McPherson, J., Bowles, D., 1992: Lignin and associated phenolic acids in cell walls. In: Wilkinson, D.L. (ed.), Molecular Plant Pathology, 51-56. Oxford Press, Oxford.

Haghighi, Z., Modarresi, M., Mollayi, S., 2012: Enhancement of compatible solute and secondary metabolites production in *Plantago ovata* Forsk. by salinity stress. J. Med. Plants Res. 6, 3495-3500.

Hairuddin, R., Yamin, M., Hama, S., 2017: Application methods for conventional and modern development of onion (*Allium cepa* L.) in abiotic stresses. In: Junaid, R. (ed.), Proceeding International Conference on Natural and Social Science, ICONSS, B8-51 - B8-57. Palopo Cokroaminoto University, Makassar.

Heath, R., Packer, J., 1968: Photoperoxidation in isolated chloroplast: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem Biophys. 125, 189-198.

Hernández, L., Loyola-González, O., Valle, B., Martínez, J., Díaz-López, L., Aragón, C., Vicente, O., Papenbrock, J., Trethowan, R., Yabor, L., Lorenzo, J.C., 2015: Identification of discriminant factors after exposure of maize and common bean plantlets to abiotic stresses. Not. Bot. Horti. Agrobo. Cluj.-Nap. 43, 589-598.

Hoagland, R.E., 1990: *Alternaria cassiae* alters phenylpropanoid metabolism in Sicklepod (*Casia obstusifolia*). J. Phytopathol. 130, 177-187.

Huang, X.Y., Chao, D.Y., Gao, J.P., Zhu, M.Z., Shi, M., Lin, H.X., 2009: A previously unknown zinc finger protein, DST, regulates drought and salt tolerance in rice via stomatal aperture control. Gen. Dev. 23, 1805-1817.

Jiménez, E., Pérez, J., Gil, V., Herrera, J., García, Y., Alonso, E., 1995: Sistema para la propagación de la caña de azúcar. In: Estrade, M., Riego, E., Limonta, E., Tellez, P., Fuente, J. (eds.), Avances en Biotecnología Moderna. Elfos Scientiae, La Habana, Cuba.

Kennedy, B., De Filippis, L., 1999: Physiological and oxidative response to NaCl of the salt tolerant *Grevillea ilicifolia* and the salt sensitive *Grevillea arenaria*. J. Plant Physiol. 155, 746-754.

Krishnamurthy, R., Bhagwat, K., 1989: Polyamines as modulators of salt tolerance in rice cultivars. Plant Physiol. 91, 500-504.

Larkin, P.J., Scowcroft, W.R., 1981: Somaclonal variation – a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60, 197-214.

Lee, T., 1987: Micropropagation of sugarcane (*Saccharum* spp.). Plant Cell Tiss. Org. Cult. 10, 47-55.

Lichtenthaler, H.K., 1987: Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Meth. Enzymol. 148, 350-382.

Lorenzo, J., Ojeda, E., Espinosa, A., Borroto, C., 2001a: Field performance of temporary immersion bioreactor-derived sugarcane plants. *In Vitro* Cell Dev. Biol. - Plant. 37, 803-806.

Lorenzo, J.C., González, B., Escalona, M., Teisson, C., Espinosa, P., Borroto, C., 1998: Sugarcane shoot formation in an improved temporary immersion system. Plant Cell Tiss. Org. Cult. 54, 197-200.

Lorenzo, J.C., Blanco, M.A., Peláez, O., González, A., Cid, M., Iglesias, A., González, B., Escalona, M., Espinosa, P., Borroto, C., 2001b: Sugarcane micropropagation and phenolic excretion. Plant Cell Tiss. Org. Cult. 65, 1-8.

Lorenzo, J.C., Yabor, L., Medina, N., Quintana, N., Wells, V., 2015: Coefficient of variation can identify the most important effects of experimental treatments. Not. Bot. Horti. Agrobo. Cluj.-Nap. 43, 287-291. DOI: 10.15835/nbha4319881.

Lutts, S., Kinet, J., Bouharmont, J., 2001: Somaclonal variation in rice after two successive cycles of mature embryo derived callus culture in the presence of NaCl. Biol. Plant 44, 489-495.

Misra, N., Gupta, A.K., 2006: Effect of salinity and different nitrogen sources on the activity of antioxidant enzymes and indole alkaloid content in *Catharanthus roseus* seedlings. J. Plant Physiol. 163, 11-18.

Munns, R., 2005: Genes and salt tolerance: bringing them together. New Phytol. 167, 645-663.

Murashige, T., Skoog, F., 1962: A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant 5, 473-497.

Naik, P.M., Al-Khayri, J.M., 2016: Abiotic and biotic elicitors – Role in secondary metabolites production through *in vitro* culture of medicinal plants. In: Shanker, A.K., Shanker, C. (eds.), Abiotic and Biotic Stress in Plants - Recent Advances and Future Perspectives, 247-277. InTech, Al-

Hassa. DOI: 10.5772/61442

Porra, R., 2002: The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. Photosynth. Res. 73, 149-156.

Quan, R., Shang, M., Zhang, H., Zhao, Y., Zhang, J., 2004: Engineering of enhanced glycine betaine synthesis improves drought tolerance in maize. Plant Biotechnol. J. 2, 477-486.

Rivelli, A., De Maria, S., Pizza, S., Gherbina, P., 2012: Growth and physiological response of hydroponically grown sunflower as affected by salinity and magnesium levels. J. Plant Nutr. 33, 1307-1323.

Selmar, D., Kleinwächter, M., 2013: Influencing the product quality by deliberately applying drought stress during the cultivation of medicinal plants. Ind. Crops Prod. 42, 558-566.

Shafi, M., Bakht, J., Khan, M., Khan, M., Raziuddin, M., 2011: Role of abscisic acid and proline in salinity tolerance of wheat genotypes. Pak. J. Bot. 43, 1111-1118.

Simonton, W., Robacker, C., Krueger, S., 1991: A programmable micropropagation apparatus using cycled liquid medium. Plant Cell Tiss. Org. Cult. 27, 211-218.

Tisserat, B., Vandercook, C.E., 1985: Development of an automated plant culture system. Plant Cell Tiss. Org. Cult. 5, 107-117.

West, G., Inze, D., Beemster, G.T.S., 2004: Cell cycle modulation in the response of the primary root of *Arabidopsis* to salt stress. Plant Physiol. 135, 1050-1058.

Winkel-Shirley, B., 2002: Biosynthesis of flavonoids and effects of stress. Curr. Op. Plant Biol. 5, 218-223.

Zaher-Ara, T., Boroomand, N., Sadat-Hosseini, M., 2016: Physiological and morphological response to drought stress in seedlings of ten citrus. Trees 30, 985-993.

Ziv, M., 1995: *In vitro* acclimatization. Automation and environmental control in plant tissue culture, 493-516. Springer.

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