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INDUCTION OF SOMATIC EMBRYOGENESIS AND PLANT REGENERATION OF SUGARCANE cv. RB925345 FROM SOMATIC EMBRYOS

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ABSTRACT - The aim of this study was to establish a protocol for induction of somatic embryogenesis and regeneration of somatic embryos of sugarcane (cultivar RB925345). Best results was obtained using concentration of 13.5 µM of 2,4-dichlorophenoxyacetic acid, 45 days of incubation on induction medium, one subculture and 15 days of incubation on regeneration medium, providing 47.5% of embryogenic *calli* and 38.9% of plants regenerated. Adjustments are necessary to this protocol to improve the embryogenic calli production rate and regeneration of somatic embryos.
Key words: *Saccharum* spp., 2,4-dichlorophenoxyacetic acid, plant tissue culture, somatic embryogenesis.

INDUÇÃO DE EMBRIOGÊNESE SOMÁTICA E REGENERAÇÃO DE PLANTAS DE CANA-DE-AÇÚCAR cv. RB925345 DE EMBRIÕES SOMÁTICOS

RESUMO - O objetivo deste estudo foi estabelecer um protocolo de indução de embriogênese somática e regeneração de embriões somáticos de cana-de-açúcar (cultivar RB925345). Melhores resultados foram obtidos utilizando a concentração de 13.5 µM do 2,4-ácido diclorofenoxiacético, incubando por 45 dias em meio de indução, realizando um subcultivo e incubando por 15 dias em meio de regeneração, alcançando uma taxa de produção de 47,5% de calos embriogênicos e 38,8% de plantas regeneradas. Ajustes são necessários neste protocolo para melhorar a taxa de produção de calos embriogênicos e regeneração dos embriões somáticos.
Palavras-chave: *Saccharum* spp., 2,4-ácido diclorofenoxiacético, cultura de tecido vegetal, embriogênese somática.

INTRODUCTION

Sugarcane crop stands out in the world mainly because it is a renewable energy source for producing ethanol and contributing to produce 65% of world sugar (DIBAX et al., 2011). Sugarcane is cultured in subtropical and tropical regions of more than 70 countries and Brazil is the world leader in use of sugarcane as a source of renewable energy and sugar exports (DIBAX et al., 2011). Currently, sugarcane cultivars are results of interspecific crosses between *Saccharum officinarum*, *S. sinense*, *S. barbieri*, *S. spontaneum* and *S. robustum*. Important for Brazilian economy, genetic breeding programs increased the works with this species. Tissue culture is a powerful tool that can accelerate genetic breeding (ALVES et al., 2011). Thus, efficient protocols for *in vitro* regeneration should be established. Due to influence of genotype on morphogenic response, it is essential to adapt protocols performed for each cultivar. *In vitro* propagation of sugarcane was performed for several cultivars through callus culture (RASHID et al.; ATHER et al., 2009), meristems (BURNER; GRISHAM, 1995), organogenesis and somatic embryogenesis (LAKSHMANAM et al., 2006) and immature leaves (KHAN et al., 2009; RAMGAREEB et al., 2010).

Ho and Vasil (1983) obtained somatic embryogenesis from explants of sugarcane inoculated on medium supplemented with auxin, and plant regeneration from embryogenic calli obtained from immature leaves was observed by Nieves et al. (2008) using MS medium (MURASHIGE; SKOOG, 1962) supplemented with 2.4-D (2,4-dichlorophenoxyacetic acid). Sugarcane genetic breeding program RIDESA (REDE INTERINSTITUCIONAL DE DESENVOLVIMENTO DO SETOR SUCROALCOOLEIRO) have been developing several cultivars. Cultivar RB925345 was developed by Universidade Federal de São Carlos (UFSCar). Main characteristics of this cultivar are a fast development, early maturation, resistance against many diseases, high productivity, and high content of fiber and sucrose (RIDESA, 2010). The aim of this research was to obtaining a specific protocol to induction of somatic embryogenesis and regeneration of somatic embryos of sugarcane (cultivar RB925345).

MATERIAL AND METHODS

Culture conditions

Cultures were maintained in growth chamber, temperature of 25 ± 2°C, without light (to induction of embryogenic *calli*) and under cold white fluorescent light (to regeneration of somatic embryos), with photon flux density of 50 µmol m^{-2} s^{-1}, 16/8 light/dark photoperiod. Cultures were performed in glass flasks (10 cm diameter and 20 cm height), containing 30 mL of culture medium and sealed with rigid polypropylene caps. All media had

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pH adjusted to 5.8 and were autoclaved for 20 min at 120°C and 1.1 atm.

Induction of embryogenic *calli*

All study was developed in Plant Tissue Culture Laboratory - Embrapa Clima Temperado. Immature meristematic leaf-rolls of sugarcane (cultivars RB925345) were used as explants. Explants were collected from 6 month-old plants originated through vegetative propagation and cultured in experimental field. Explants were surface-sterilized by immersion in 70% alcohol (v/v) for 1 min followed by 2.5% sodium hypochlorite (v/v) for 15 min. They were subsequently rinsed three times in sterile distilled water and cut into sections about 3 mm. Explants were incubated in glass flasks containing callus induction medium composed of MS medium supplemented with 20 g sucrose, 7.0 g agar, 100 mg PVP, 100 mg myo-inositol, 0.5 mg nicotinic acid, 0.15 mg citric acid, 1 mg thiamine, 50 mg cysteine, 2 mg glycine, 50 mg arginine, 0.5 mg pyridoxine and 50 mL coconut water, and supplemented with three different concentrations (4.5, 13.5 and 31.5 µM) of 2,4-dichlorophenoxyacetic acid (2,4-D). Explants were incubated for three different times (30, 45 and 60 days) in absence of ligth. After each period of incubation, samples were evaluated according to following variable: percentage of explants producing embryogenic *calli*.

Regeneration of somatic embryos

Embryogenic *calli* masses obtained after 45 days of incubation were subcultured three times (21 days for each subculture interval) in induction medium. After each subculture, somatic embryos of sugarcane were subdivided in samples of 4.0 mm^2 and transferred to MS medium supplemented with 20 g sucrose, 7.0 g agar, 100 mg PVP, 100 mg myo-inositol, 0.5 mg nicotinic acid, 0.15 mg citric acid, 1 mg thiamine, 50 mg cysteine, 2 mg glycine, 50 mg arginine, 0.5 mg pyridoxine and 100 mL coconut water. These materials were maintained in growth chamber. After three different times (15, 30 and 45 days) of incubation on regeneration medium, samples were evaluated according to the following variable: percentage of embryogenic samples producing shoots.

Statistical analysis

Experimental design to induction of embriogenic *calli* was completely randomized in 3 x 3 factorial scheme (time of incubation in induction medium and concentration of 2,4-D in induction medium) with 12 replicates and 5 explants per experimental unit. For regeneration of somatic embryos, experimental design was completely randomized in 2 x 3 x 3 (time of incubation in regeneration medium, concentration of 2,4-D in induction medium and number of subcultures) factorial scheme with four replicates and four explants per experimental unit. Treatments effect were analysed by ANOVA and means were compared by Tukey's multiple range test using the package ExpDes (FERREIRA et al., 2013) in software R (R CORE TEAM, 2014).

RESULTS AND DISCUSSION

Embryogenic *calli* induction

Morphogenetic response in explants began after 21 days after isolation in all treatment, but only embriogenic *calli* from induction medium supplemented with 13.5 and 31.5 µM of 2,4-D was regenerated (Figure 1). Analysis of variance showed no interaction between time of incubation in induction medium and concentration of 2,4-D (Table 1). Better results of induction of embryogenic *calli* were obtained using medium supplemented with 31.5 µM of 2,4-D (86.8%) and time of incubation of 45 or 60 days (58.0 and 56.6%) (Table 2).

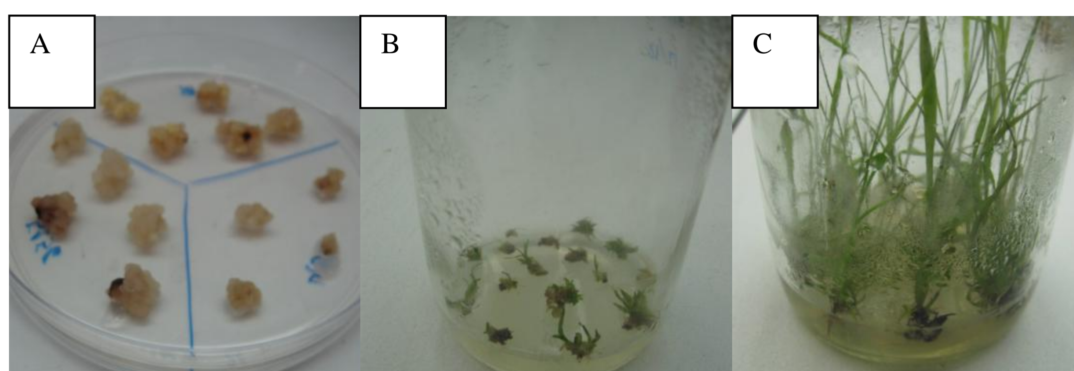

FIGURE 1 - Regeneration of sugarcane from somatic embryos (cultivar RB925345). A = subculture of embriogenic *calli* after 45 days of incubation in induction medium. B = regeneration of somatic embryos after subcultures. C = plants regenerated from somatic embryos after 45 days in regeneration medium.

TABLE 1 - Analysis of variance of variables time of incubation in induction medium (time) and concentration of 2,4-D adding to induction medium (concentration).

Source	GL	SQ	QM	Fc	p-value
Time	2	8840	4420	11.516	0.00003**
Concentration	2	84540	42270	110.133	0.00000**
Time x concentration	4	485	121	0.316	0.86670
Residual	99	37997	384		
Total	107	131863			

** = significant at 1% by F test.

TABLE 2 - Production of embryogenic *calli* of sugarcane (cultivar RB925744).

Percentage of explants producing embryogenic *calli*			
Time (days)	Mean	Concentration (µM)	Mean
30	38.2 B*	4.5	18.5 C
45	56.6 A	13.5	47.5 B
60	58.0 A	31.5	86.8 A

*Means followed by same letter do not differ by Tukey's test at 5%.

Using growth regulation 2,4-D, all treatments produced embryogenic *calli*, differently of the negative control (MS medium without 2,4-D). These results are similar to data found by Ho and Vasil (1983), where these authors studied the of embryogenic *calli* induction in sugarcane (clone 68-1067). According to studies of Raemakers et al. (1995), induction of embryogenesis *calli* in sugarcane occur just when the culture medium is supplemented with auxin. Growth regulator 2,4-D, a auxin, is essential to induction of embryogenic *calli* and is widely used to produce somatic embryos (GRATTAPAGLIA; MACHADO, 1990). Nevertheless, higher concentrations of 2,4-D in culture medium produce *calli* with mucilaginous appearance (HO; VASIL, 1983).

The results of this study show clearly that embryogenic *calli* production is directly influenced by concentration of the growth regulator 2,4-D and by time of incubation of the explants in induction medium (Table 2). These results are similar to studies of Ho and Vasil (1983) and Desai et al. (2004), where these authors studied this process in sugarcane genotype CoC-671.

Somatic embryos regeneration

Analysis of variance showed interaction only between amount of subculture performed and induction medium (concentration) provider of embryogenic *calli* (Table 3). Better results of morphogenetic response of regeneration were obtained using somatic embryos subcultured on induction medium supplemented with 13.5 µM of 2,4-D and performing one subculture on this induction medium (38.9%) (Table 4). According these results, induction medium supplemented with 31.5 µM of 2,4-D show higher somatic embryos production rate (86.8%), but these materials obtained showed low regeneration rate (5.6%). Nevertheless, induction medium supplemented with 13.5 µM of 2,4-D showed 47.5% of production of somatic embryos and 38.9% of regeneration. Embryogenic *calli* from induction medium supplemented with 4.5 µM of 2,4-D not survived after first subculture and consequently removed of the analyzes.

Similar results was showed by Ho and Vasil (1983), where the authors verified that sucrose can act in cellular morphogenetic response and root development during shoots regeneration. Likewise important is the use of organics complex and essential amino acids the regeneration medium (LITZ; GRAY, 1995). Immature somatic embryos provide low shoots production rates (RAEMAKERS et al., 1995). This problem is called early germinations and is the main problem in development of somatic embryos (LITZ; GRAY, 1995).

Using coconut water in regeneration medium provide benefic effects to morphogenetic and development of shoots (GEORGE et al., 2008). Nevertheless, compounds concentrations in coconut water are not regular in fruits or industrialized products. These irregular concentrations can be responsible for variations in the regeneration rate of somatic embryos.

Regenerated shoots from somatic embryos were produced in this experiment using regeneration medium without auxin, as reported by Falco et al. (2000) and Snyman et al. (2006). After regeneration process, shoots were subcultured in regeneration medium for 21 days, transferred to polypropylene pots containing autoclaved vermiculite and acclimated in glasshouse.

TABLE 3 - Analysis of variance of the variables time of incubation on regeneration medium (time), concentration of 2,4-D adding to induction medium of subcultures (concentration) and number of subcultures performed (subculture).

Source	GL	SQ	QM	Fc	p-value
Concentration	1	2604.16667	2604.1667	8.0357	0.0075**
Time	2	902.77778	451.38889	1.3929	0.2614
Subculture	2	2569.44444	1284.7222	3.9643	0.0278**
Time x concentration	2	69.44444	34.72222	0.1071	0.8987
Concentration x subculture	2	2569.44444	1284.7222	3.9643	0.0278*
Time x subculture	4	277.77778	69.44444	0.2143	0.9288
Time x concentration x subculture	4	277.77778	69.44444	0.2143	0.9288
Residual	36	11666.6666	324.07407		
Total	53	20937.5000	395.04717		

*, ** = significant at 1% and 5% by F test, respectively.

TABLE 4 - Regeneration of sugarcane somatic embryos (cultivar RB925744). Percentage of embryogenic samples producing shoots.

	Concentration (µM)	
Subcultures	13.5	31.5
1	38.9 aA*	5.6 aB
2	8.3 bA	5.6 aA
3	11.1 bA	5.6 aA

*Means followed by same lower case letter in the column and capital letter on the line do not differ by Tukey’s test at 5%.

CONCLUSION

We concluded in this research that induction of embryogenic *calli* and regeneration of somatic embryos cultured under conditions used were responsive.

Better results were obtained using 13.5 µM de 2,4-D in induction medium, cultured for 45 days (47.5%), one subculture in the induction medium and incubating for 15 to 45 days in regeneration medium (38.9%).

The protocol used for sugarcane (cultivar RB925345) in this research need more adjustments to improve efficiency in the production of somatic embryos and consequently plant regeneration.

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BIBLIOGRAPHIC REFERENCES

ALVES, S.A.O.; LEMOS, O.F.; SANTOS FILHO, B.G.; SILVA, A.L.L. *In vitro* embryo rescue of interspecific hybrids of oil palm (*Elaeis oleifera* x *Elaeis guineensis*). **Journal of Biotechnology and Biodiversity**, Palmas, v.2, n. 2, p.1-6, 2011.

ATHER, A.; KHAN, S.; REHMAN, A.; NAZIR, M. Optimization of the protocols for *callus* induction, regeneration and acclimatization of sugarcane cv. Thatta-10. **Pakistan Journal of Botany**, Karachi, v. 41, n.2, p. 815-820, 2009.

BURNER, D.M.; GRISHAM, M.P. Induction and stability of phenotypic variation in sugarcane as affected by propagation procedure. **Crop Science**, Madison, v.35, n.3, p.875-880, 1995.

DESAI, N.S.; SUPRASANNA, P.; BAPAT, V.A. Simple and reproducible protocol for direct somatic embryogenesis from cultured immature inflorescence segments of sugarcane (*Saccharum* spp.). **Current Science**, Bangalore, v.87, n.6, p.764-768, 2004.

DIBAX, R., ALCÂNTARA, G.B., BESPALHOK FILHO, J.C.; MACHADO, M.P.; OLIVEIRA, Y.; SILVA, A.L. L. Plant regeneration of sugarcane cv. RB931003 and RB98710 from somatic embryos and acclimatization. **Journal of Biotechnology and Biodiversity**, Palmas, v.2, n.3, p.32-37, 2011.

FERREIRA, E.B.; CAVALCANTI, P.P.; NOGUEIRA, D.A. **Experimental designs package. R package version 1.1.2.** 2013. Available from: <http://CRAN.R-project.org/package=ExpDes>. Acess: 30 jun. 2018.

FALCO, M.C.; TULMANN NETO, A.T.; ULIAN, E.C. Transformation and expression of a gene for herbicide resistance in Brazilian sugarcane. **Plant Cell Reports**, Heidelberg, v.19, n.12, p.1188-1194, 2000.

GEORGE, E.F.; HALL, M.A.; KLERK, G.J. **Plant propagation by tissue culture.** 3[a] ed. Amsterdam: The Background Springer, 2008, v.1, p.501.

GRATTAPAGLIA, D.; MACHADO, M.A. **Micropropagação.** In: TORRES, A.C.; CALDAS, L.S. (Ed.). Técnicas e aplicações da cultura de tecidos de plantas. Brasília: ABCTP/EMBRAPA - CNPH, p.99-169, 1990.

HO, W.J.; VASIL, I.K. Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.). I. The morphology and physiology of callus formation and the ontogeny of somatic embryos. **Protoplasma**, Vienna, v.118, n.3, p.169-180, 1983.

KHAN, I.A.; DAHOT, M.U.; SEEMA, N.; YASMEEN, S.; BIBI, S.; RAZA, G.; KHATRI, A.; NAQVI, M.H. Direct regeneration of sugarcane plantlets: a tool to unravel genetic heterogeneity. **Pakistan Journal of Botany**, Karachi, v.41, n.2, p.797-814, 2009.

LAKSHMANAN, P.; GEIJSKES, R.J.; WANG, L.; ELLIOT, A.; GROF, C.P.L.; BERDING, N.; SMITH, G.R. Development and hormonal regulation of direct shoot organogenesis and somatic embryogenesis in sugarcane (*Saccharum* spp. interspecific hybrids) leaf culture. **Plant Cell Report**, Heidelberg, v.25, n.10, p.1007-1015, 2006.

LITZ, R.E.; GRAY, D.J. Somatic embryogenesis for agricultural improvement. **World journal of Microbiology e Biotechnology**, Netherlands, v.11, n.4, p.416-425, 1995.

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

NIEVES, F.; SAGARRA, R.; GONZALEZ, Y.; LEZCANO, M.; CID, M.; BLANCO, A.; CASTILLO, R. Effect of exogenous arginine on sugarcane (*Saccharum* sp.) somatic embryogenesis, free polyamines and the contents of the soluble proteins and proline. **Plant Cell, Tissue and Organ Culture**, Netherlands, v.95, n. 313, p.13-20, 2008.

R FOUNDATION FOR STATISTICAL COMPUTING. **R Development Core Team.** R: a language and environment for statistical computing. Vienna, Austria, 2009. Available from: <http://www.R-project.org>. Acess: 20 mai. 2018.

RAEMAKERS, C.J.J.M.; JACOBSEN, E.; VISSER, R.G.F. Secondary somatic embryogenesis and applications in plant breeding. **Euphytica**, Netherlands, v.81, n.1, p.93-107. 1995.

RAMGAREEB, S.; SNYMAN, S.J.; VAN ANTWERPEN, T.; RUTHERFORD, R.S. Elimination of virus and rapid propagation of disease-free sugarcane (*Saccharum* spp. cultivar NCo376) using apical meristem culture. **Plant Cell, Tissue and Organ Culture**, Netherlands, v.100, n.2, p.175-181, 2010.

RASHID, H.; ALI KHAN, S.; ZIA, M.; CHAUDHARY, M.F.; HANIF, Z.; CHAUDARY, Z. Callus induction and regeneration in elite sugarcane cultivar hsf-240. **Pakistan Journal of Botany**, Karachi, v.41, n.4, p.1645-1649, 2009.

RIDESA. REDE INTERUNIVERSITÁRIA PARA DESENVOLVIMENTO DO SETOR SUCROALCOOLEIRO. **Catálogo nacional de variedades "RB" de cana-de-açúcar.** Curitiba: Rede Interuniversitária para Desenvolvimento do Setor Sucroalcooleiro, 2010. 80p.

SNYMAN, S.J.; MEYER, G.M.; RICHARDS, J.M.; HARICHARAN, N.; RAMBAREEB, S.; HUCKETT, B.I. Refining the application of direct embryogenesis in sugarcane: effect of the developmental phase of leaf disk explants and the timing of DNA transfer on transformation efficiency. **Plant Cell Report**, Heidelberg, v.25, n.10, p.1016-1023, 2006.