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FIELD PERFORMANCE OF SUGARCANE (*Saccharum* sp.) PLANTS DERIVED FROM CRYOPRESERVED CALLUSES

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Abstract

This study compared the field performance of sugarcane plants originating from three different sources: control, non-cryopreserved embryogenic calluses, cryopreserved embryogenic calluses and macropropagated material of the same commercial hybrid. Several agronomic traits were evaluated on 100 plants per treatment over a 27-month period covering the growth of the stool and of the first ratoon. Significant differences between treatments were observed only during the first six months of field growth of sugarcane stools. Stems produced from *in vitro* cultured material, irrespective of their cryopreservation status, had a smaller diameter and a shorter height than those produced from macropropagated material. These differences disappeared by12 months of stool field growth.

Keywords: sugarcane; *Saccharum* sp.; cryopreservation; embryogenic callus; macropropagated material; field performance.

INTRODUCTION

The sugarcane industry is among the most important businesses in Cuba. This industry is in dramatic need of planting material, which cannot be produced in sufficient quantities to meet the demand using classical macropropagation techniques. Biotechnological tools have been employed to increase the production of planting material. Castillo (1) has developed a protocol for sugarcane somatic embryogenesis, which allows mass production of plants from elite varieties. However, despite its success, this protocol has an important limiting factor, which lies with the progressive loss over time of the embryogenic potential of calluses. A simplified freezing protocol has thus been established for sugarcane embryogenic calluses which allows their conservation for the long-term with their embryogenic potential intact (15). It was successfully applied to three commercial hybrids (*Saccharum* sp. cv. CP52-43, C91-301 and C1051-73). The survival percentages of cryopreserved calluses ranged between 20 and 94% depending on the variety, and fully developed plantlets could be obtained from regenerating calluses. Embryogenic calluses of variety CP52-43 were stored for 14 months in liquid nitrogen without any effect on survival and plantlet production.

Before using cryopreservation as an additional tool in the overall conservation strategy for any plant material, it is essential to verify that the cryopreservation protocol developed does not have any destabilizing effect and that the plants produced from cryopreserved

explants are true to type. There exist an increasing number of reports indicating that no changes are observed in the material regenerated from cryopreservation (5). However, most of these experiments have been performed very soon after cryopreservation on a small number of individuals, often using material still cultured *in vitro* or after a very short period of growth *in vivo* and they concern mainly *in vitro* growth characteristics, or a limited number of biochemical or molecular markers. Only in a limited number of cases (e.g. 3, 5, 22) have plants been grown in the field for a long period allowing the assessment of agronomic characteristics.

In the case of sugarcane, numerous experiments have been conducted to study the field behaviour of micropropagated plants (7, 8, 11, 12, 13, 17, 19, 20), uncovering the occurrence of rejuvenation phenomena and of epigenetic changes. By contrast, only limited information is available concerning the stability of plants regenerated from cryopreserved material. RFLP analysis did not reveal any difference that could be attributed to cryopreservation between plants of one sugarcane variety produced from control and cryopreserved calluses (4) or cell suspensions (2). Plants produced from control and cryopreserved shoot tips of one variety were similar as regards pattern of two isoenzymatic systems (18) and no differences were observed for six agronomic traits observed during early growth *in vivo* of plants originating from control and frozen apices (9). However, to our knowledge, there are no published data on the field performance of sugarcane plants originating from cryopreserved material.

In this paper, the field performance of plants produced from embryogenic calluses of one sugarcane commercial variety cryopreserved using the protocol developed by Martinez-Montero *et al.* (15) was evaluated over a period of 27 months by observing several agronomic parameters. Similar observations were carried out simultaneously, for comparison, on plants produced from the same callus cultures, but which were not cryopreserved and on plants of the same variety originating from classical macropropagation.

MATERIALS AND METHODS

Plant material and in vitro culture

Embryogenic calluses were produced from immature inflorescence segments (3-5 mm) of the sugarcane commercial hybrid cv. CP52-43 (CP43-64 x CP38-34, Canal Point, USA) according to Castillo (1). Calluses were maintained in the dark at 25±2°C and subcultured every 20 d on basal medium consisting in MS (16) medium supplemented with 1.0 mg L^{-1} (2,4-D), 50 mg L^{-1} arginine and 500 mg L^{-1} proline. Calluses were subjected to the cryopreservation protocol after 120 d in culture.

Cryopreservation

Cryopreservation was performed according to the protocol developed by Martínez-Montero *et al.* (15). Calluses were transferred in 2 ml cryotubes before freezing and subjected to a 1 h pretreatment at 0°C with a cryoprotective solution consisting of 10% (v/v) dimethylsulfoxide and 0.5 M sucrose in standard liquid medium. Samples were frozen in a homemade ethanol bath consisting of a polystyrene box filled with 700 ml ethanol placed in a -40°C freezer, thus allowing an average cooling rate of 0.4-0.6°C min^{-1} between 0°C and -40°C. Ice crystallization was induced manually in the cryoprotective medium by briefly immersing the base of the cryotubes in liquid nitrogen. Once the temperature reached -40°C, the cryotubes were held for 2 h at this temperature before immersion in liquid nitrogen for storage. Samples were kept for 12 h at -196°C. Thawing took place by plunging the cryotubes in a +40°C water-bath for 1 min. Calluses were then transferred on standard culture medium for recovery and left for 40 days. For plant regeneration, cryopreserved and non-cryopreserved calluses were placed for 80 days on standard medium without 2,4-D and cultured at 27 ± 2°C, with a 16 h light/8 h dark photoperiod and a 40 µmol m^{-2} s^{-1} photon dose.

Acclimatization and field cultivation

After *in vitro* culture, sugarcane plants (2-4 cm high) were hardened in a greenhouse for 60 d (21). Once acclimatized, plants (20-30 cm high) originating from control and cryopreserved calluses were tested simultaneously to study their field performances. A third treatment consisting of macropropagated plants (6) was included in the experiment. These macropropagated plants were derived from stem axillary buds isolated from field-grown plants. Isolated buds were placed in a greenhouse for 60 d to allow bud sprouting before the establishment of the field experiment in a red ferralitic soil.

Experimental design and statistical analysis

Treatments were distributed following a randomised block experimental design including four repetitions per treatment. Experimental plots were 7.5 m long with 5 rows each. Intra-row spacing was 0.5 m. The commercial hybrid C266-70 (Co281 x POJ2878, INICA, Cuba) was planted as the experiment border. Fertilization at the time of planting included 75 kg ha^{-1} urea, 50 kg ha^{-1} P_2O_5 and 50 kg ha^{-1} K_2O. Additionally, 75 kg ha^{-1} urea were supplied 3 months after planting. The following measurements were performed on 100 plants originating from cryopreserved calluses, control calluses and macropropagated plants:

- After 6 months of stool field growth: number of stems per clump (*i.e.* number of suckers produced from the original plant); stem diameter (cm); stem length (m).
- After 12 months of stool field growth: number of stems per clump; stem diameter (cm); stem length (m); fibre percentage (w/w); juice brix (mass of sugar (g dry matter) per 100 g of juice, expressed in Brix degree); pol percentage in cane (total sugar content, expressed in % by mass); tons of pol ha^{-1}.
- After cutting of stools and 15 months of field growth of the first ratoon: number of stems per clump; stem diameter (cm); stem length (m); single stem fresh mass (kg); fibre percentage (w/w); juice brix; pol percentage in juice; pol percentage in cane; tons of pol ha^{-1}.

Each variable was tested for normal distribution (Kolmogorov-Smirnov, $p<0.05$) and unequal variances (Bartlet, $p<0.05$). Discrete variables were transformed (only for statistical analysis, not for data presentation) in accordance with $x = (0.5+x)^{0.5}$ and percentage variables according to $x = 2$ arcsine $((x/100)^{0.5})$. Analysis of variance (ANOVA) and Duncan's tests were carried out as well.

RESULTS

The results of the evaluation of field grown sugarcane plants after different periods are presented in Tables 1 (6 months of stool field growth), 2 (12 months of stool field growth) and 3 (15 months of field growth of the first ratoon). Significant differences between treatments were observed only during the first six months of field growth of sugarcane stools (Table 1). Stems produced from *in vitro* cultured materials, irrespective of their cryopreservation status, had a smaller diameter and a shorter height than those produced from macropropagated buds.

These differences disappeared during the course of the experiment as they were not observed anymore after 12 months of stool field growth (Table 2). No significant difference between treatments was observed for any of the parameters studied after 12 months of stool field growth (Table 2) and 15 months of field growth of the first ratoon (Table 3).

Table 1. Evaluation of several agronomic parameters after six months of field growth of sugarcane stools originating from cryopreserved calluses, control (non-cryopreserved) calluses and buds isolated from macropropagated plants. Data in rows followed by the same letter are not statistically different (ANOVA, Duncan test, $P<0.05$).

Parameter measured	Origin of stools		
	Cryopreserved calluses	Control calluses	Buds from macropropagated plants
Number of stems per clump	5.14 ab	5.76 a	4.92 b
Stem diameter (cm)	1.53 b	1.40 b	1.81 a
Stem length (m)	0.56 b	0.45 b	0.91 a

Table 2. Evaluation of several agronomic parameters after 12 months of field growth of sugarcane stools originating from cryopreserved calluses, control (non-cryopreserved) calluses and buds isolated from macropropagated plants. No statistical differences were found (ANOVA) between treatments.

Parameter measured	Origin of stools		
	Cryopreserved calluses	Control calluses	Buds from macropropagated plants
Number of stems per clump	9.80	11.55	9.32
Stem diameter (cm)	2.58	2.51	2.66
Stem length (m)	1.65	1.59	1.70
Juice brix (Brix °)	22.07	21.22	24.69
Fibre percentage (w/w)	11.55	11.23	11.52
Pol percentage in juice (% by mass)	19.17	18.37	21.35
Pol percentage in cane (% by mass)	17.14	16.21	18.94
Tons of pol ha^{-1}	9.96	8.28	10.71

Table 3. Evaluation of several agronomic parameters after 15 months of field growth of the first sugarcane ratoon originating from cryopreserved calluses, control (non-cryopreserved) calluses and buds isolated from macropropagated plants. No statistical differences were found (ANOVA) between treatments.

Parameter measured	Origin of ratoon		
	Cryopreserved calluses	Control calluses	Buds from macropropagated plants
Number of stems per clump	8.57	9.65	10.29
Stem diameter (cm)	2.52	2.57	2.65
Stem length (m)	1.93	1.88	2.34
Single stem mass (kg)	1.20	1.15	1.24
Juice brix (Brix °)	12.9	12.17	11.50
Fibre percentage (w/w)	23.22	23.02	22.68
Pol percentage in juice (% by mass)	20.45	20.07	19.55
Pol percentage in cane (% by mass)	17.86	17.63	17.89
Tons of pol ha^{-1}	8.74	9.75	10.97

DISCUSSION

This study has demonstrated that the differences observed for several agronomic characters between stools originating from cryopreserved and control calluses, and macropropagated material after 6 months of field growth disappeared progressively with time, as no differences could be uncovered in stools after 12 months nor after 15 months of field growth of the first ratoon.

It confirms all the already published evidence that indicates the stability of plant material regenerated after cryopreservation (5, 10). The originality and importance of this study lie with the large numbers of samples studied (100 plants per treatment), the extended duration of the experiment (27 months in the field, with two culture cycles) and the type of characters measured (agronomic traits). Indeed, most stability studies have been performed very soon after cryopreservation on a small number of individuals, often using material still cultured *in vitro* or after a short period of growth *in vivo* and they concern mainly *in vitro* growth characteristics, or a limited number of biochemical or molecular markers. Only very few published reports on this topic have dealt with a comparable number and duration of components. No differences have been noted in the vegetative and floral development of several hundreds of palms regenerated from control and cryopreserved oil palm embryogenic cultures, but no detailed account of the observations made has been published (5). The most comprehensive study is the comparison of the field behaviour of banana plants regenerated from control and cryopreserved cell suspensions (3), which showed that two out of the eleven descriptors analyzed differed between control and cryopreserved material during the first culture cycle but that, similarly to our observations, these differences disappeared during the second culture cycle.

An interesting result in our study is that plants regenerated from control and cryopreserved calluses displayed the same differences in comparison with those originating from macropropagated material. These differences are therefore not induced by cryopreservation but are due to the fact that both groups of plants originated from *in vitro* cultured material. It is indeed a well known phenomenon that tissue culture induces temporary changes in the behaviour of *in vitro* cultured plants during their early *in vivo* growth phase (23). Such changes can be induced by *in vitro* culture conditions including, notably, low light intensity, high humidity, limited gas exchanges, presence of high sucrose concentrations and growth regulators in the medium.

In the case of sugarcane, changes in the field behaviour of plants have been frequently observed after *in vitro* culture. Several authors have reported an increase in the number of new stems per clump (8, 11, 20), which generally induces a reduction in the stem diameter and mass. Peña and Stay (19) and Palmera *et al.* (17) stated that, with sugarcane *in vitro* culture stimulated growth and vigour, induced rejuvenation and generally improved agricultural performance. Many authors (1, 12, 13, 14, 24) indicate that such differences disappear during the course of field growth and the first clonal multiplication, as observed in our experiments.

In conclusion, the results obtained in this study validate the cryopreservation protocol developed by Martinez-Montero *et al.* (15) for embryogenic calluses. Cryopreservation of embryogenic calluses will thus be incorporated in the scheme established by the Centro de Bioplantas for mass production of *in vitro* sugarcane plants by means of somatic embryogenesis.

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