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DEVELOPMENT OF A CRYOPRESERVATION PROCESS FOR EMBRYOGENIC CALLUSES OF A COMMERCIAL HYBRID OF SUGARCANE (*SACCHARUM* SP.) AND APPLICATION TO DIFFERENT VARIETIES.

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SUMMARY: An efficient cryopreservation technique was set up with embryogenic calluses of sugarcane. Up to 92% regrowth was obtained from cryopreserved calluses of one variety after pregrowth with 0.3 M sucrose and 15% DMSO, freezing at 0.5°C.min-1 to -45°C and rapid thawing in a water-bath at 40°C. Numerous plantlets, comparable to that coming from control calluses, could be regenerated and grown in the greenhouse. This technique was successfully applied to calluses of 10 varieties out of 11 tested during three successive experiments.

KEY WORDS: sugarcane, *Saccharum* sp., cryopreservation, embryogenic callus.

INTRODUCTION

The development of plant breeding and tissue culture methods during the past decade has led to the production and handling of an increasing number of genotypes. Their maintenance is a major consumer of time, manpower, space and material. Various slow growth techniques have been set up in order to reduce that problem but only medium-term conservation can thus be achieved (1). Only cryopreservation (liquid nitrogen (LN), -196°C) presently offers a long-term conservation option.

Cryopreservation has been presently applied to more than 70 plant species in various forms, such as cell suspensions, calluses, meristems, zygotic and somatic embryos (2).

In order to ensure the large scale multiplication of sugarcane, various authors have developed methods permitting high frequency plant regeneration through somatic embryogenesis from leaf-derived embryogenic calluses (3, 4, 5, 6, 7). However, various problems have to be faced: the regeneration capabilities of these calluses progressively decrease over the successive subcultures (6). Moreover, the risks for somaclonal variation increase with the number of subcultures (8), particularly when culture takes place in the presence of exogenous growth regulators such as 2,4 dichlorophenoxyacetic acid (2,4-D), which is included in the medium utilized for the maintenance of sugarcane calluses. Somaclonal variation has been extensively described in sugarcane (9). Recovery of variant somaclones from embryogenic calluses has been reported recently (10). Therefore, it is important to develop an efficient cryopreservation technique which could ensure the long-term preservation of embryogenic calluses with high regeneration potential.

Cryopreservation experiments have been conducted on sugarcane by various authors. Resistance to freezing to -23 and -40°C was obtained with cell suspensions and calluses (11, 12). A limited number of albino plantlets were produced from cryopreserved calluses in 1984 by Ulrich *et al.* (13). More recently, high survival rates and recovery of whole plants were obtained by Jian *et al.* (14) using embryogenic calluses and Gnanapragasam and Vasil (15) using embryogenic cell suspensions but only one clone was used by each of these authors.

In this paper, we report the development of a cryopreservation technique for sugarcane embryogenic calluses produced using the process developed by Guiderdoni and Demarly (6). The technique was set up with one variety (Co6415)which has very high embryogenic potentialities and applied afterwards to 14 different commercial hybrids.

MATERIALS AND METHODS

Plant material

The material consisted of embryogenic calluses obtained from 15 commercial varieties of sugarcane (*Saccharum* sp.) taken from the *in vitro* collection of IRAT/CIRAD, Montpellier. Three varieties originated from Barbados (B70462, B70532, B75519), one from Barbados-Jamaica (BJ7058), four from Coimbatore, India (Co419, Co740, Co1001, Co6415), one from Hawaii, USA (H371933), one from Mayari, Cuba (My53173), one from Natal-Coimbatore, South Africa-India (NCo376),

three from Queensland, Australia (Q90, Q96, Q126), one from Sao Paulo, Brasil (SP701284).

One variety (Co 6415) was used for setting up the cryopreservation technique. The efficiency of the process was tested afterwards on the 14 other varieties.

***In vitro* culture**

Establishment and culture of calluses

0.5 cm long sections of young rolled leaves excised just above the shoot apex of *in vitro* plantlets, as described by Guiderdoni and Demarly (6), were used to produce embryogenic calluses. The explants were inoculated in 90 mm diameter Petri dishes onto a solid callus induction medium containing macro and microelements and iron source according to Murashige and Skoog (16), vitamins according to Fuji (17), 34 $g.l^{-1}$ sucrose, 3 $mg.l^{-1}$ 2,4-D and solidified with 6 $g.l^{-1}$ agarose, adjusted to pH 5.6. The cultures were incubated for four weeks at 27± 1°C in the dark. The developing embryogenic calluses were then transferred and subcultured every four weeks on the same basal medium with 1 $mg.l^{-1}$ 2,4-D in the same environmental conditions.

Regeneration of plantlets

Six to seven weeks after thawing, regrowing calluses were transferred onto the standard medium without 2,4-D under a photoperiod of 12hrs/24 (50 $\mu E.m^{-2}.s^{-1}$) for shoot regeneration. After 12 to 15 weeks of culture in these conditions, rooted plantlets were transferred and grown in the greenhouse in Jiffy peat pellets on a synthetic medium.

Cryopreservation

Pregrowth/cryoprotection

Pieces of 9 to 10 week old calluses, about 3 to 4 mm in diameter (± 30 mg) were used. They were precultured for 0, 24 or 48 hrs in liquid medium with various sucrose concentrations (0.1 to 1.5 M).

Cryoprotective treatment consisted of a 1-hour culture of the calluses at 0°C in sterile 1 ml cryotubes, in liquid medium with various sucrose concentrations (0.1 to 1.5 M), to which dimethylsulfoxide (DMSO) could be progressively added over a period of 30 min until the final concentration (5 to 25%, v/v) was reached. In the experiments concerning the effects of freezing and thawing rates and the application to the other varieties, the cryoprotective solution used comprised 0.3 M sucrose and 15% DMSO.

Freezing

Controlled freezing was carried out using a programmable freezing apparatus (Minicool LC 40, from L'Air Liquide). A range of -20 to -80°C was used to test the

effect of the prefreezing temperature. A prefreezing temperature of -40 or -45°C was used to test other variables. A range of 0.1 to 5°C.min^{-1} was used to test the effect of the freezing rate. A freezing rate of 0.5°C.min^{-1} was used in experiments to test other variables. Crystallization in the cryoprotective medium was induced manually at a temperature intermediate between the crystallization and the nucleation temperature of the medium, by briefly pinching the ampoules with forceps previously cooled in liquid nitrogen. Once the prefreezing temperature was reached, the samples were thawed directly or immersed in liquid nitrogen, where they were kept for at least one hour.

Thawing

The cryotubes were immersed in a water-bath thermostated at 40°C (20, 40 or 60°C in the experiments concerning the effect of the thawing temperature), until melting of ice was complete.

Recovery of pretreated and frozen calluses

Each sample was poured into an empty Petri dish and the calluses were transferred into a 90 mm Petri dish containing 25 ml of standard solid medium, covered with a filter paper. Transfers were performed every 15 days.

When high sucrose levels had been used during pretreatment, the sucrose concentration was progressively lowered by daily subcultures on media with reduced sucrose content until the standard concentration was reached. The calluses were cultured in the same environmental conditions (27 ± 1°C, in the dark).

Viability and recovery assessment

Ten to twenty pieces of embryogenic calluses were used in each condition. The survival rate, measured 21 to 35 days after thawing, corresponded to the number of calluses which showed measurable regrowth during the recovery period. The regrowth rate, measured 35 to 40 days after thawing, corresponded to the fresh weight increase of the calluses during the recovery period. It was expressed in % of the untreated control. Plantlet production was estimated after 90 days of culture on the regeneration medium. It corresponded to the total number of plantlets equal or higher than 10 cm produced from 6 calluses randomly chosen in each condition.

Application to other varieties

Three independent experiments were performed with 14 additional clones, using the cryopreservation protocol set up with clone Co 6415. For each of these trials, 10 calluses were sampled from the same initial cultures. The only differences between experiments were the prefreezing temperature (-45°C in the first and second trial ; -50°C in the third) and the age of the calluses (10 week-old calluses in the first experiment ; 15-week old calluses in the second and the third). Results were expressed as previously described. Survival was recorded 42 days after thawing and recovery 52 days after thawing.

RESULTS

Effect of pregrowth conditions

Increasing the preculture duration and the sucrose concentration in the preculture medium had detrimental effects on the survival and regrowth of pretreated calluses (Table 1). Without preculture, survival was optimal with 0.1 and 0.5 M sucrose, and regrowth maximal for 0.5 M sucrose. No survival nor recovery were observed after prefreezing to -40°C or freezing in LN.

Effect of sucrose and DMSO concentration during the cryoprotective treatment

Survival of pretreated calluses was generally high (up to 100%) whatever the sucrose and DMSO concentrations (Table 2). After prefreezing to -40°C, no survival was obtained without DMSO. High survival rates were observed with 0.1 M sucrose and 5 to 20% DMSO, and with 0.3 and 0.5 M sucrose added with 5 to 25% DMSO. However, in these latter cases, DMSO was toxic when employed at high levels (20 to 25%). After freezing in LN, survival was considerably lowered with 0.1 M sucrose and all DMSO concentrations,except with 20%. With 0.3 and 0.5 M sucrose, survival was generally comparable to that obtained after prefreezing to -40°C.

Regrowth of pretreated calluses was satisfactory. After prefreezing to -40°C, regrowth was generally lowered and decreased with increasing concentrations of DMSO. After freezing in LN, regrowth was reduced for calluses pretreated with 0.1 M sucrose and low DMSO concentrations (5 and 10%) but was comparable to that observed with prefrozen samples pretreated with higher DMSO concentrations (15 to 25%). With higher sucrose levels, no decrease was observed when DMSO concentration was between 5 and 15% (for 0.3 M) and 5 to 20% (for 0.5 M). Higher DMSO concentrations were toxic and regrowth was suppressed. In comparison with untreated controls (Fig. 1a), regrowth was progressively delayed according to the successive phases of the treatment: pretreated (Fig. 1b), prefrozen (Fig. 1c) and cryopreserved (Fig. 1d) calluses.

Effect of prefreezing temperature

With prefrozen controls, survival decreased only for prefreezing temperatures lower than -50°C (Table 3). After freezing in LN, survival was optimal and comparable to that obtained with prefrozen controls for temperatures between -40 and -50°C. The same observations could be made as concerns the regrowth of prefrozen and cryopreserved calluses.

Effect of freezing rate

In the case of prefrozen controls, survival was high (80 to 100%) whatever the freezing regime (Table 4). Growth recovery was maximal with the higher freezing rates (2.5 and 5°C.min^{-1}). After freezing in LN, survival was optimal for freezing rates of 0.1 and 0.5°C.min^{-1} (Table 4).It decreased with higher cooling rates and no survival was

obtained for a freezing regime of 5°C.min^{-1}. Regrowth was maximal for a freezing rate of 0.1°C.min^{-1} and satisfactory for 0.5°C.min^{-1}. No regrowth was obtained for cooling rates higher than 2.5°C.min^{-1}.

Effect of thawing temperature

Survival was observed in all thawing conditions (Table 5). It was optimal for a temperature of 40°C, and decreased progressively with increasing thawing temperatures. The same observations were performed as concerns the effect of thawing temperature on the regrowth of frozen calluses.

Production of plantlets from cryopreserved calluses

The production of plantlets was measured with calluses prefrozen to various temperatures and cryopreserved (Table 6). With prefrozen controls, the number of plantlets regenerated decreased for prefreezing temperatures lower than -55°C. With cryopreserved calluses, the number of plantlets obtained was maximal for prefreezing to -45 and -50°C. Fully developed plantlets, similar to that obtained from untreated controls, were produced from all regenerating calluses. All were successfully weaned and grown in the greenhouse (Fig. 2).

Application to other varieties

In the first experiment (Table 7), 10 out of the 14 varieties tested showed survival (ranging from 10 to 80% of the controls), but regrowth was very low, compared with the results obtained previously with Co6415. Regeneration was obtained from 6 varieties only. In the second experiment, survival was observed with the 11 varieties tested. Regrowth was still low, but higher than in the first experiment with varieties that had shown high survival rates. Regeneration was impossible with 4 varieties that had recovered in the first trial (B70532, BJ7058, Co1001, H371933). However, the contrary was observed with 4 other varieties (Co740, NCo376, Q90, Q96). In the last experiment, results were generally low (survival with 8/11 varieties, regeneration with 5/11 varieties only). When observing the mean results of these 3 successive experiments, regeneration was observed with 10 out of the 11 varieties used in every trial. Only one variety was responsive in every trial, five in two trials and four in one trial. Regeneration of numerous plantlets could be obtained from almost all regrowing calluses.

DISCUSSION/CONCLUSION

The technique set up with clone Co6415 gave good results since with the optimal conditions, up to 92.1% recovery could be obtained from cryopreserved calluses and the plantlets produced from these calluses were similar to that originating from control samples. The conditions defined in this study were different from that used by other authors. For cryoprotective treatment, a mixture of polyethyleneglycol,

glucose and DMSO was used by Ulrich et al. (13) ; Jian *et al.* (14) and Gnanapragasam and Vasil (15) employed a mixture of sorbitol and DMSO. Freezing conditions were also different: 1°C.min^{-1} down to -30°C (13) or -40°C (14) followed by a plateau at the prefreezing temperature. Thawing was performed using a similar technique. High survival and recovery rates could also be obtained in the two more recent papers (14, 15).

This study also allowed to emphasize the importance of various aspects of a cryopreservation process. Sucrose is a widely employed cryoprotective substance. High concentrations and long pregrowth durations can be easily withstood by plant tissues when sucrose is employed in solid medium as in the case of oil palm somatic embryos (0.75 M for 7 days) (18).This is the same in liquid medium when samples are embedded (and thus protected) in alginate beads, as with potato meristems (19) or carrot somatic embryos (20). In other cases, the optimal pregrowth durations are shorter and the optimal concentrations lower (21). Higher levels and/or longer pregrowth durations have rapid toxic effects, as observed with sugarcane embryogenic calluses. In our study, survival after freezing in LN was obtained only when using a binary cryoprotective mixture of sucrose and DMSO, which is the cryoprotectant most commonly employed. Mixtures of cryoprotectants are recognized as generally more efficient and less toxic than one substance only at the same total concentration (21). It is interesting to note that if regrowth was maximal with the highest sucrose levels tested (0.3 and 0.5 M) added with 5 to 15% DMSO, it occurred also with the lowest sucrose content (0.1 M) and a high level of DMSO. This underlines the fact that the total concentration of the cryoprotectants has also to be taken into account.

The necessity of precisely defining the two main parameters of freezing, i.e. the freezing rate and prefreezing temperature was illustrated during this work. The range of freezing rates ensuring cell survival greatly varies with the species. With oil palm somatic embryos, survival could be obtained with cooling rates ranging from 0.5 to 200°C.min^{-1} (22). However, with *Pucciniella distans* cell suspensions, 50% of the cells withstood freezing at 1°C.min^{-1} but only 15% at 0.5°C.min^{-1} (23). Sugarcane embryogenic calluses showed an intermediate behaviour since satisfactory regrowth was obtained with 0.1 and 0.5°C.min^{-1}. For any species, the optimal prefreezing temperature lies between -30 and -50°C, which was observed in this study. No plateau at the end of the controlled freezing sequence was performed, as used by Jian *et al.* (14) and Gnanapragasam andVasil (15). From a practical point of view, the use of a 0.5°C.min^{-1} freezing regime may be preferable to 0.1°C.min^{-1}, due to the extended duration of freezing in this latter case (7.5 hours to reach -45°C).

Thawing is only rarely taken into consideration. Rapid thawing is usually performed in a 40°C water-bath, in order to avoid recrystallization phenomena which occur between -80 and -30°C and can be lethal to the cells. Increasing the thawing

temperature can lead to dramatic improvements of the results : Reuff *et al.* (24) obtained a two-fold increase in survival of a *Coleus blumei* cell suspension by increasing the temperature of the water-bath to 60°C. On the other hand, with grape embryogenic cell suspensions (25), this temperature led to a sharp decrease in survival. In the case of sugarcane embryogenic calluses, such drastic differences were not observed, since the optimal temperature was 40°C but regrowth was obtained with all the other thawing conditions tested.

An original approach of this study was the application of the process to a large number of genotypes, which presently is still rarely performed. With oil palm somatic embryos, great variations were observed in the recovery rate of more than one hundred clones (26). This was also noted during our experiments with embryogenic calluses of sugarcane. It appears that a minimal survival rate has to be reached in most of the cases, in order to obtain plant regeneration from frozen material. Regeneration could be observed on 10 out of the 11 varieties tested in every successive experiment. Only one variety gave positive results in each case, five in two experiments and four in one trial only. Moreover, the results of these trials were generally lower than that obtained when setting up the cryopreservation technique with Co6415. This could be due to various factors. Even if the calluses were sampled on the same initial cultures, the second and third experiments were performed with material which had spent five additional weeks in culture. The calluses used at these different dates may not be at the same physiological and developmental stage. Observations also indicated that the type of calluses used were sligthly different between Co6415 and the other varieties, the former ones comprizing well individualised embryos, the latter ones less structured embryogenic nodules. Various parameters of the cryopreservation process may also be modified, particularly during the freezing procedure, which may improve the results with the other varieties.

From a practical point of view, this paper showed that cryopreservation of embryogenic calluses is possible for a large number of varieties, since only 4 out of 14 the varieties tested, i.e. 29%, did not withstand the protocol set up with Co6415. However, a part of the cryopreserved calluses has to be thawed immediately and checked for reproliferation and plant production for each experiment before long-term storage of the rest of the material may be foreseen. Additional work, both on the propagation and the cryopreservation technique should allow in the near future the routine use of cryopreservation for embryogenic calluses of sugarcane.

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Table 1: Effect of preculture duration and sucrose concentration on the survival and growth recovery of pretreated (0°C), prefrozen (-40°C) and cryopreserved (LN) bryogenic calluses.

Preculture duration (hrs)	Sucrose (M)	Survival (%)			Growth recovery (%)		
		0°C	-40°C	LN	0°C	-40°C	LN
0	0.1	100	0	0	65	0	0
	0.5	100	0	0	106	0	0
	1.0	85	0	0	57	0	0
	1.5	75	0	0	54	0	0
24	0.1	75	0	0	29	0	0
	0.5	85	0	0	37	0	0
	1.0	0	0	0	0	0	0
	1.5	0	0	0	0	0	0
48	0.1	50	0	0	23	0	0
	0.5	45	0	0	31	0	0
	1.0	0	0	0	0	0	0
	1.5	0	0	0	0	0	0

Table 2: Effect of sucrose and DMSO concentration during the cryoprotective treatment on the survival and growth recovery of pretreated (0°C), prefrozen (-40°C) and cryopreserved (LN) embryogenic calluses.

Sucrose (M)	DMSO (%, v/v)	Survival (%)			Growth recovery (%)		
		0°C	-40°C	LN	0°C	-40°C	LN
0.1	0	100	0	0	39	0	1
	5	100	90	27	48	63	24
	10	100	100	11	127	46	2
	15	85	50	30	39	20	16
	20	100	80	67	72	24	38
	25	79	0	17	60	7	13
0.3	0	100	0	0	98	0	0
	5	100	100	100	175	57	59
	10	100	100	100	93	41	44
	15	100	100	100	62	43	47
	20	100	50	14	64	13	0
	25	100	40	8	40	17	0
0.5	0	100	0	0	105	0	0
	5	100	100	100	68	42	85
	10	100	100	70	130	68	46
	15	100	100	100	68	34	33
	20	100	57	100	77	16	17
	25	100	50	10	74	5	0

Table 3: Effect of the prefreezing temperature on the survival and growth recovery of prefrozen and cryopreserved (LN) embryogenic calluses.

Prefreezing temperature) (°C)	Survival (%)		Growth recovery (%)	
	prefrozen	LN	prefrozen	LN
-20	92	0	99	0
-25	100	0	98	1
-30	100	10	82	14
-35	90	30	75	28
-40	90	78	56	73
-45	100	100	90	92
-50	100	73	45	80
-55	60	9	43	7
-60	56	20	62	15
-80	0	0	1	0

Table 4: Effect of the freezing rate on the survival and growth recovery of cryopreserved embryogenic calluses.

Freezing rate (°C/min)	Survival (%)		Growth recovery (%)	
	-45°C	LN	-45°C	LN
0.1	100	73	82	50
0.5	87	67	66	30
1	80	13	49	6
2.5	80	27	110	0
5	100	0	156	0

Table 5: Effect of thawing temperature on the survival and growth recovery of cryopreserved embryogenic calluses.

Thawing temperature (°C)	Survival (%)	Growth recovery (%)
20	17	19
40	77	30
60	55	23
80	38	12

Table 6: Effect of the prefreezing temperature on the number of regenerating calluses and the total number of plantlets higher than 10 cm produced from 6 prefrozen and cryopreserved (LN) embryogenic calluses.

Prefreezing temperature (°C)	Prefrozen		LN	
	Regenerating calluses	Total nbr of plantlets	Regenerating calluses	Total nbr of plantlets
-20	4/6	128	0/6	0
-25	5/6	425	0/6	0
-30	6/6	330	1/6	33
-35	5/6	260	3/6	135
-40	3/6	159	2/6	42
-45	6/6	468	6/6	258
-50	5/6	460	6/6	372
-55	4/6	116	1/6	93
-60	4/6	344	1/6	37
-80	0/6	0	0/6	0
Non frozen control	4/6	220		

Table 7: Results of three successive cryopreservation experiments with embryogenic calluses of 14 different varieties of sugarcane.
Survival and regeneration are expressed as a function of the number of calluses cryopreserved. Regrowth is expressed in % of the control.
*: no data

Clone	Experiment 1			Experiment 2			Experiment 3			Mean value (%)		
	Survival	Regrowth	Regeneration	Survival	Regrowth	Regeneration	Survival	Regrowth	Regeneration	Survival	Regrowth	Regeneration
B70462	0/10	0	0/6	*	*	*	*	*	*	0	0	0
B70532	1/10	6	1/6	2/8	1	0/6	1/5	6	0/6	17	4	5
B75519	0/10	0	0/6	1/10	1	0/6	0/4	0	0/6	4	0	0
BJ7058	3/10	8	1/6	1/10	1	0/6	0/5	0	0/6	16	3	5
Co419	4/10	8	1/6	2/10	14	1/6	1/8	1	0/6	25	8	11
Co740	7/10	2	0/6	9/10	29	3/6	6/10	64	4/6	73	32	39
Co1001	3/10	16	1/6	1/10	1	0/6	1/9	6	1/6	17	8	11
H371933	7/10	13	2/6	3/10	1	0/6	0/10	0	0/6	33	5	11
My53173	0/10	0	0/6	*	*	*	*	*	*	0	0	0
NCo376	2/10	3	0/6	6/10	16	3/6	2/10	11	1/6	33	10	22
Q90	8/10	7	0/6	10/10	28	4/6	1/7	1	0/6	70	12	22
Q96	7/10	5	0/6	8/10	76	3/6	6/10	39	2/6	70	40	28
Q126	5/10	13	3/6	4/8	55	2/6	1/6	5	1/6	38	24	33
SP701284	0/10	0	0/6	*	*	*	*	*	*	0	0	0

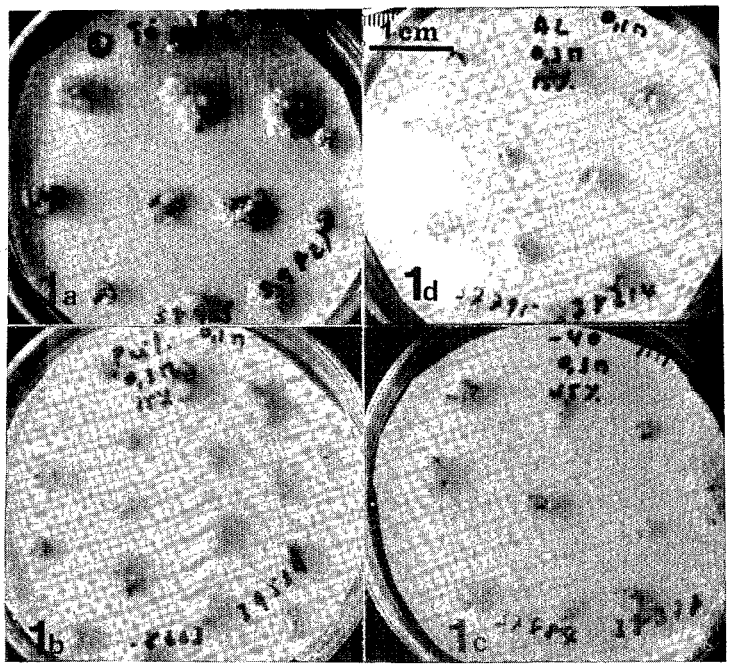

Figure 1: Effect of pretreatment (1b), prefreezing (1c) and cryopreservation (1d) on the regrowth of embryogenic calluses, in comparison with untreated controls (1a).

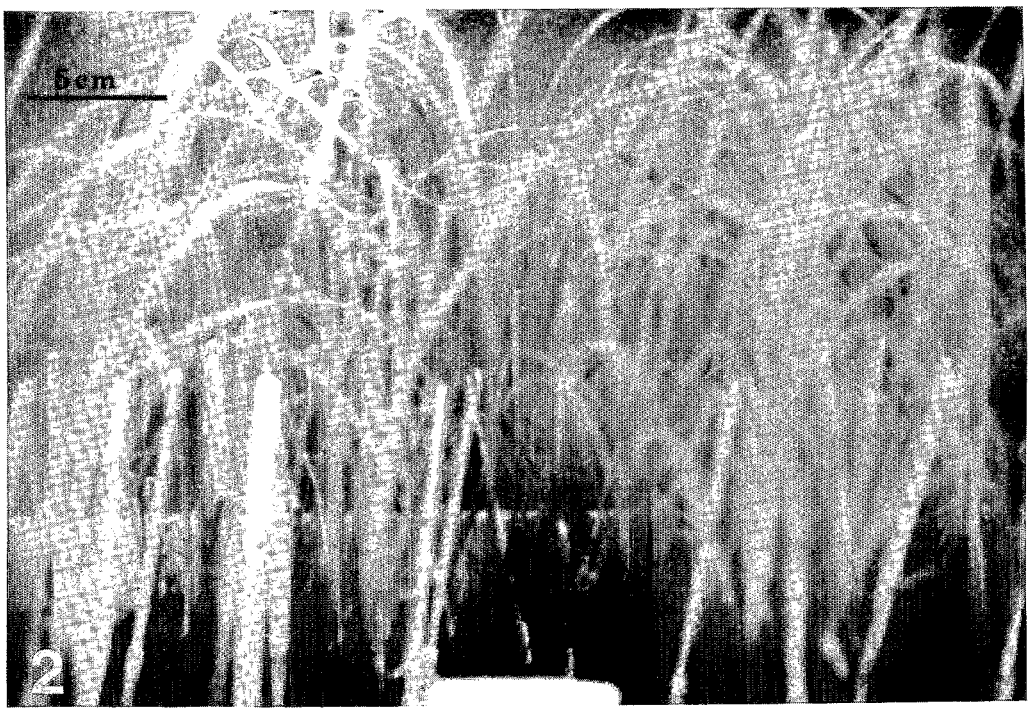

Figure 2: Plantlets regenerated from cryopreserved calluses after transfer in the greenhouse.