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Cryopreservation of non-encapsulated embryogenic tissue of sweet potato (*Ipomoea batatas*)

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ABSTRACT

Embryogenic tissue of the sweet potato (*Ipomoea batatas* (L) LAM) genotype TIB 10 was established from *in vitro* axillary shoot tips on Murashige and Skoog (1962) medium supplemented with 5 μM 2,4-dichlorophenoxyacetic acid. Embryogenic aggregates of fresh mass 9.0 - 12 mg were subjected to a rapid freezing protocol in liquid nitrogen following sucrose preculture and varying degrees of dehydration. Up to 50% of embryogenic explants survived rapid freezing after preculture on 0.4 or 0.7M sucrose only. Dehydration with silica gel to moisture contents in the range 18-41% improved the survival after cryopreservation of embryogenic tissue. Tissue dehydrated for intermediate periods exhibited poor survival. Following freezing, embryogenic tissue appeared to develop normally, retaining its competence to produce mature embryos and plantlets.

Abbreviations: BA, 6-benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; MS, Murashige and Skoog (1962) medium

INTRODUCTION

Somatic embryogenic tissue has a key role to play in sweet potato biotechnology. In particular, it is the preferred system for genetic transformation programmes and it offers opportunities for micropropagation and the development of synthetic seed technology. Consequently, it is important to develop storage systems for the long-term maintenance of genetic stability and embryogenic competence in the cell lines.

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In an earlier report we described the development of a cryopreservation protocol for sweet potato embryogenic tissue based on the technique of alginate encapsulation and dehydration (Blakesley *et al.* 1995). It involved the initial preculture of encapsulated immature embryogenic tissue on a medium containing high sucrose, dehydration in the sterile airflow of a laminar flow hood followed by a two-step freezing protocol using a programmable freezer. In the present study we have developed an alternative procedure for the cryopreservation of embryogenic tissue based on a high sucrose preculture, with or without a subsequent dehydration step and without encapsulation.

MATERIALS AND METHODS

Tissue culture

Somatic embryogenic cultures were established from axillary shoot tips (0.5 - 1.0 mm in length) excised from actively growing shoot cultures of the sweet potato genotype TIB 10. The axillary shoot tips were placed on Murashige and Skoog (MS) (1962) medium supplemented with 0.06M sucrose and 5 μM 2,4-D and incubated for 8 weeks. Embryogenic tissue proliferated as hard, compact shiny globular structures, and it was routinely subcultured onto fresh MS medium containing the same concentration of auxin every 4-6 weeks. To induce embryo maturation and germination, clumps of embryogenic tissue were transferred to semi-solid MS medium supplemented with 0.06M sucrose, 50 nM BA, 5 nM 1-naphthaleneacetic acid and 15 μM gibberellic acid (GA_3) for 10 d prior to transfer to a hormone-free MS medium supplemented with 0.06M sucrose. All cultures were incubated at

25°C with a 16h photoperiod at 50 μmol m^{-2} s^{-1} photosynthetically active radiation.

Cryopreservation

Embryogenic aggregates (9 - 12 mg fresh mass) were excised from proliferating embryogenic cultures and transferred to semi-solid MS medium supplemented with 0.1M sucrose for 3 d. They were subsequently transferred to MS medium supplemented with either 0.4M sucrose for 3 d, 0.7M for 3 d or 0.4M for 3 d followed by 0.7M for 2 d (Tables 1-3). In each experimental treatment, 3 replicate batches of 6 embryogenic aggregates were incubated on 25ml of medium contained in a 9cm Petri dish. Each replicate batch of embryogenic aggregates was then transferred to a filter paper carrier (2.0 x 0.5 cm) and dried in a 9cm Petri dish containing 2.0g of silica gel for up to 4 h. After dehydration, the carriers were transferred to 2 ml polypropylene cryovials (Camlab, UK) prior to rapid freezing. Freezing was achieved by plunging the cryovials directly into liquid nitrogen contained in a 2l Dewar flask. Moisture contents were estimated on a percentage fresh mass basis from 3 replicate batches of tissue dehydrated in an identical manner and weighed at 30 - 60 min intervals prior to final oven drying at 85°C overnight. Precise measurements however were difficult owing to the small, and variable mass and surface area of the explants.

Samples remained in liquid nitrogen for 1 h before they were thawed by placing the vials directly into a water bath at 35°C for 2 min. The carriers were then removed from the cryovials and transferred to semi-solid MS medium supplemented with 0.1M sucrose, where they rehydrated. Immediately after thawing the embryogenic tissue was incubated at 25°C in darkness for 48 h before the tissue was transferred to the light. Assessments of survival of embryogenic tissue were made regularly over subsequent weeks and the statistical significance between the total survival percentage in each treatment was tested using Chi-square analysis. The capacity of this tissue to produce mature embryos capable of germination was assessed using the regeneration media described above.

RESULTS

Incubation of embryogenic tissue on the high sucrose media resulted in a reduction in the moisture contents to 77-78% following incubation on 0.4M sucrose and 68-71% following incubation on 0.7M sucrose. Virtually all embryogenic aggregates survived a combination of high sucrose and evaporative dehydration, although the embryogenic competence was affected by the duration of dehydration (Tables 1-3) with a considerable reduction at very low moisture contents.

Table 1. Effect of dehydration only, and dehydration followed by rapid freezing on survival of embryogenic aggregates (9 - 12 mg) of TIB 10 precultured on MS medium supplemented with 0.1M sucrose for 3 d, followed by 0.4M for 3 d. Assessment made 4 weeks after treatment. n=18.

Dehydration time (h)	Moisture content (% FM)	Survival (%)			
		Dehydration only		Rapid freezing	
		Emb.	Non emb.	Emb.	Non emb.
0	77-78	100	0	50.0	50.0
1.0	66-68	100	0	11.1	55.6
2.0	46-54	100	0	22.2	33.3
3.0	18-28	66.7	33.3	33.3	38.9
4.0	10-18	16.7	83.3	0	72.2

Emb. = embryogenic tissue
Non emb. = reversion to non embryogenic tissue

Some of the embryogenic tissue was frozen immediately after the sucrose preculture without further evaporative dehydration. The survival of this tissue after rapid freezing was high, between 94.4 and 100%. Of this tissue, 44.4 - 50.0% of the explants retained their embryogenic competence, whilst the others proliferated entirely as a friable or mucilaginous callus (Tables 1-3). Explants dehydrated for 1 or 1.5 h exhibited a very low tolerance of freezing; the overall survival was considerably lower, and most of this tissue had lost its embryogenic competence (Tables 1-3).

Survival of embryogenic tissue following freezing improved with further dehydration to moisture contents in the range 18 - 41%, in explants precultured on MS medium supplemented with 0.7M sucrose (Tables 2 and 3). There was no significant difference ($p<0.01$) between the maximum survival of embryogenic tissue recorded with these treatments although the best survival, 88.9%, was obtained from embryogenic aggregates exposed to a more gradual increase in sucrose during the preculture phase (Table 3). Following cryopreservation, aggregates which retained their embryogenic competence produced new globular embryogenic structures from the

surface of the previous embryogenic tissue. The development of embryogenic structures was accompanied by the proliferation of a soft, friable mucilaginous callus and areas of red pigment were associated with the development of new embryogenic structures.

Table 2. Effect of dehydration only, and dehydration followed by rapid freezing on survival of embryogenic aggregates (9 - 12 mg) of TIB 10 precultured on MS medium supplemented with 0.1M sucrose for 3 d, followed by 0.7M for 3 d. Assessment made 4 weeks after treatment. n=18.

Dehydration time (h)	Moisture content (% FM)	Survival (%)			
		Dehydration only		Rapid freezing	
		Emb.	Non emb.	Emb.	Non emb.
0	68-70	100	0	44.4	50.0
1.0	54-59	94.4	5.6	5.6	5.6
1.5	42-52	94.4	5.6	16.7	5.6
2.0	25-41	100	0	55.6	44.4
2.5	18-31	88.9	11.1	61.1	38.9
3.0	14-21	61.1	38.9	27.8	50.0
4.0	7-16	16.7	50.0	11.1	72.2

Emb. = embryogenic tissue
Non emb. = reversion to non embryogenic tissue

Table 3. Effect of dehydration only, and dehydration followed by rapid freezing on survival of embryogenic aggregates (10 - 11 mg) of TIB 10 precultured on MS medium supplemented with 0.1M sucrose for 3 d, 0.4M sucrose for 3 d and 0.7M sucrose for 2 d. Assessment made 4 weeks after treatment. n=18.

Dehydration time (h)	Moisture content (% FM)	Survival (%)			
		Dehydration only		Rapid freezing	
		Emb.	Non emb.	Emb.	Non emb.
0	69-71	100	0	44.4	55.6
1.0	57-59	100	0	5.6	33.3
1.5	44-49	100	0	11.1	22.2
2.0	31-37	94.4	5.6	83.3	5.6
2.5	27-30	66.7	33.3	88.9	11.1
3.0	23-26	55.6	44.4	38.9	61.1
4.0	18-20	16.7	50.0	0	72.2

Emb. = embryogenic tissue
Non emb. = reversion to non embryogenic tissue

All embryogenic tissue which proliferated following cryopreservation appeared to be normal, and when transferred to the regeneration medium it had clearly retained its competence for maturation and germination, and plantlets were produced.

DISCUSSION

In the present paper we have demonstrated that it is possible to freeze small pieces of embryogenic tissue without using the technique, reported for a number of species (Dereuddre *et al* 1991; de Boucaud *et al* 1994) in which the embryogenic tissue is protected in an alginate bead. Furthermore, good survival following rapid freezing was obtained after preculture on a high sucrose medium without evaporative dehydration. Most published protocols using sucrose as the main cryoprotectant require extensive evaporative dehydration before good survival following freezing is obtained, and many authors do not report survival rates without such a treatment. Dumet *et al* (1993) however reported the cryopreservation of large heterogeneous clumps of oil palm somatic embryos (250 - 300 mg fresh mass) following sucrose preculture and evaporative dehydration. They also found that sucrose alone was sufficient to offer some protection to certain clones. In the present study, the moisture content of this non-dehydrated tissue immediately after preculture on sucrose was 68 - 78%, depending on the sucrose molarity. It is not clear how tissue with this high moisture content survived freezing, although it may be significant that the tissue was frozen rapidly. Other non-dehydrated tissues such as potato meristems have been shown to survive rapid freezing (Grout and Henshaw 1978).

In the present study, survival was severely reduced at the intermediate levels of dehydration. This may be a result of the inter- and intracellular water in the tissue being in a state of flux as the tissue quickly dried. Further dehydration to moisture contents of 18-41% however enhanced survival levels of embryogenically competent cells, and up to 88.9% survival was obtained. At these moisture contents it is likely that a glass transition, rather than ice crystallisation was obtained (Dereuddre and Kaminski 1992). This hypothesis could be verified using differential scanning calorimetry.

This technique may offer an alternative approach to that of 'encapsulation-dehydration' previously reported for the cryopreservation of small

embryogenic aggregates of sweet potato (Blakesley *et al.* 1995). It is technically simpler, as it does not necessitate the encapsulation of the tissue or the use of a sophisticated programmable freezer. However, without alginate protection, the tissue is more vulnerable, and the data shows that the period of evaporative dehydration is important. If the period of evaporative dehydration is too extensive following preculture on 0.7M sucrose, tissue can lose its embryogenic competence following freezing. This new technique will now be used to investigate the cryopreservation of a large range of sweet potato genotypes with, and without the evaporative dehydration step.

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REFERENCES

Blakesley D, Al Mazrooei S, Henshaw GG (1995) Plant Cell Reports 15, 259-263

Dereuddre J, Kaminski M (1992) J. Thermal Analysis 38: 1965-1978

Dereuddre J, Blandin S, Hassen H (1991) Cryo-letters 12: 125-134

de Boucaud MT, Brison M, Negrier P (1994) Cryo-letters 15: 151-160

Dumet D, Engelmann F, Chabrillange N, Duval Y (1993) Plant Cell Reports 12: 352-355

Grout BWW, Henshaw GG (1978) Ann. Bot. 42: 1227-1229

Murashige T, Skoog F (1962) Physiol. Plant. 15: 473-497