Plant Cell Reports (1992) 11:204–206

Plant Cell Reports

Cryopreservation of embryonic axes of trifoliate orange (*Poncirus trifoliata* [L.] RAF.)

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Received August 30, 1991/Revised version received January 9, 1992 – Communicated by G. C. Phillips

ABSTRACT

Halved shoot bases of *Allium tuberosum* Rottl. ex Spreng. proliferated both axillary and adventitious shoots on B5 medium (1968) supplemented with either 6-benzylaminopurine (0.5 mg/l) or 1-naphthalene acetic acid (0.1 mg/l) and 2-isopentenyladenine (0.5 mg/l). *In vitro* shoots proliferated further numerous shoots upon subculture to fresh medium, and these shoots rooted spontaneously. Plantlets were transplanted successfully to soil and retained the diploid condition of the parents.

ABBREVIATIONS

B5, Gamborg *et al.*, (1968) medium; **BAP**, 6-benzylaminopurine; **2ip**, 2-isopentenyladenine; **NAA**, 1-naphthalene acetic acid

INTRODUCTION

Allium tuberosum Rottl. ex Spreng., commonly known as Chinese Chives, is widely distributed in China, Japan, South Asia and India from Kashmir to Kumaon in the northwest Himalayas extending to the northeastern region, at an altitude of 1500–2000 m. The leaves and young inflorescences of this non-bulbous species have a garlic-like flavour and are used by local inhabitants for culinary purposes. Due to over-exploitation and lack of organised cultivation, the wild populations have declined rapidly in their natural habitat. The loss of valuable genetic diversity may be mitigated by conservation of this species through *in vitro* methods.

Tissue culture techniques are being increasingly exploited for clonal multiplication and in vitro conservation of many *Allium* species which are sterile, possess considerable heterozygosity, or are to be freed of viruses and other pathogens (Novák *et al.*, 1986; El-Gizawy and Ford-Lloyd, 1987). The limited information on tissue culture of *A. tuberosum* includes regeneration of haploid plants from unpollinated ovaries (Hui-quiao and Hong-Yuan, 1989), the occurrence of apomictic plants from unpollinated ovule cultures (Kojima *et al.*, 1989), and plantlet regeneration from callus (Zee *et al.*, 1977). The present paper is the first report on clonal multiplication of *A. tuberosum* through shoot proliferation.

MATERIALS AND METHODS

Source of explants

Plants of *A. tuberosum* collected from wild stands in the natural habitat were maintained ex-situ (clonally propagated) at the NBPGR regional station at Srinagar. Cultures were initiated from shoot bases obtained from 200 field-grown plants. Following removal of the rhizome, roots and leaves (cut off 1 cm above the shoot base), the outer scales were removed from the shoot bases. Shoot bases were then washed in teepol detergent for 5 min followed by surface sterilization with 1% $HgCl_2$ (BDH) for 20 min. After rinsing with sterile distilled water five times, these bases (3–5 mm in diameter) were cut transversely at a height of 5 mm and a few mm of basal plate tissue were removed aseptically. These were explanted as such, or divided by a median vertical cut into longitudinal halves to destroy the main shoot apex. Following removal of the outer three layers of foliage leaf bases, these shoot halves with the inner 2–4 leaf bases and *ca* 1 mm of basal plate tissue attached, were implanted onto various media.

Subculture and rooting

For subculture and rooting, 6–8 week-old shoots excised from the proliferating mass were trimmed to 5 mm height and all existing roots cut off. They were explanted as such or separated into individual shoots before implanting onto various media.

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air flow of a laminar flow cabinet. At the end of each desiccation period, 10 seeds/20 axes were used for determining moisture content using a low constant temperature oven method (103 ± 2°C for 17 h) and the moisture content was calculated on a fresh weight basis. Fifteen of the remaining axes were cultured as controls for the respective desiccation period.

Freezing and thawing

The remaining 15 axes at each desiccation level were dispensed into a cryotube (NUNC, Roskilde, Denmark) of 1.8 ml capacity. Tubes wrapped in aluminum foil were then placed in cryocans and plunged directly into liquid nitrogen (LN) at -196°C, where samples were subjected to a freezing rate of -200°C/min. After 24 h of storage in LN, the cryotubes were thawed in a sterile water bath at 37 ± 2°C, and tested for germination. The experiment was repeated three times. A sample from the best treatments comprising 25 embryonic axes/cryotube, totalling 300 embryonic axes, were cryopreserved in LN for long-term storage (8 months).

Results and Discussion

The conservation efforts of Poncirus seeds have been hampered mainly by high moisture content and desiccation sensitivity. The viability of the seeds was not much affected when the whole seeds were desiccated from 36% moisture to 28% moisture, whereas a further reduction of 6% significantly affected the viability (Table 1). Seeds could not survive when desiccated below 20% moisture content, which was in agreement with the earlier report of Honjo and Nakagawa (1978). However, in the present study, cryopreserved embryonic axes of Poncirus showed a marked degree of tolerance to desiccation.

Table 1. Moisture content and viability of Poncirus trifoliata seeds during desiccation over silica gel.*

Time (h)	Moisture Content (%)	Viability (%)
0	36.3 ± 0.08	98.5 ± 2.51
4	34.3 ± 1.10	96.7 ± 5.2
10	30.5 ± 2.23	94.0 ± 2.44
18	28.5 ± 2.10	92.0 ± 1.41
24	26.0 ± 1.24	80.0 ± 2.87
28	22.0 ± 2.14	28.4 ± 3.57
50	20.0 ± 1.40	10.0 ± 4.42

*** Mean of three experiments with 50 seeds in each experiment**

Each embryonic axis of Poncirus trifoliata constitutes a small fraction of the entire dry mass of the seed, with the seed to embryonic axis ratio being 15:1. However, there is not much difference in axis moisture content as compared to the whole seed. The moisture content of fresh seeds was 36.3 ± 0.08, while that of freshly excised embryonic axes was 38.2 ± 1.0 on a fresh weight basis.

The freshly excised embryonic axes looked translucent with clearly distinguishable root and shoot apices (Fig. 1a), and showed 98.5% germination when cultured on MS medium supplemented with 0.2 mg/l each of NAA and BAP. Faster growth was recorded when cryopreserved axes were grown on medium supplemented with activated charcoal, which agrees with the earlier studies made by Withers (1979). Viable axes after cryopreservation when cultured over medium swelled and turned green within 49 h of culture (Fig. 1b). Root and shoot emergence was observed after one week of culture and well developed seedlings were recovered within 20 d of culture (Fig. 1c). Two to three month old plants (Fig. 1d and 1e) were vigorous and healthy, and later transferred to soil in pots with 60% (12 plants) establishment (Fig. 1f) and the rest died after 5 d of transfer.

The embryonic axes lost moisture rapidly in the sterile air flow cabinet (Table 2), showing reduction of about 18%, 20%, 22% and 24% on a fresh weight basis after 1, 2, 3, and 4 h of desiccation, respectively. A major proportion of the moisture loss occurred during the first hour of desiccation, and subsequent desiccation brought down the moisture at the rate of 2%/h for 3 consecutive hours, and lastly 3% in the fifth hour. Reduction in the moisture content of axes was, however, accompanied by a reduction in the survival rate. Embryonic axes desiccated for 4 h showed 76% germination, as compared to the undesiccated control which showed 98% germination.

Table 2. Moisture content and viability of excised embryonic axes of Poncirus during desiccation following cryopreservation in a laminar flow cabinet*

Time (h)	Moisture Content (%)	Viability (%) BC	Viability (%) AC
0	38.2 ± 1.0	98.0 ± 0.08	0
1	20.0 ± 0.18	96.0 ± 1.20	0
2	18.3 ± 0.84	90.0 ± 1.42	10 ± 1.0
3	16.0 ± 2.1	90.0 ± 2.24	50 ± 3.8
3.5	15.0 ± 0.67	80.0 ± 1.40	52 ± 3.0
4	14.0 ± 2.42	76.2 ± 1.48	68 ± 3.16
4.5	13.0 ± 1.2	50.8 ± 2.20	45 ± 2.1
5	11.0 ± 3.00	40.0 ± 3.42	40 ± 2.38

*** Mean of 3 experiments with 40 embryonic axes/treatment. BC: Germination before cryopreservation in 20 embryonic/treatment. AC: Recovery after 24 h and 8 months of cryopreservation at -196°C in 20 embryonic axes/treatment**

Cryopreservation was efficient only for the axes desiccated to 16% or lower moisture content. Freezing of embryonic axes at 14% moisture level in LN at -196°C showed a good recovery rate (68%), whereas embryonic axes possessing moisture contents at or above 20% lost viability completely when exposed to LN (Table 2). A set of embryonic axes with 14% moisture was cryopreserved at -196°C. When this sample was retrieved and cultured after 8 months of storage there was no evidence of any further loss in viability, showing the same results as the embryonic axes after

24 h of storage in LN. The seedlings obtained from cryopreserved embryonic axes were established in pots (Figs. 1c to 1f). The plants regenerated from cryopreserved embryonic axes were found to be morphologically uniform. The results of the present study indicate that the excised embryonic axes of Poncirus trifoliata are markedly tolerant to desiccation. Results showed that desiccation to a moisture level of 16% was found to be the critical moisture content, and 14% moisture content was optimal for the axes to successfully tolerate the low freezing temperature of -196°C, ranging from 24 h to 8 months storage.

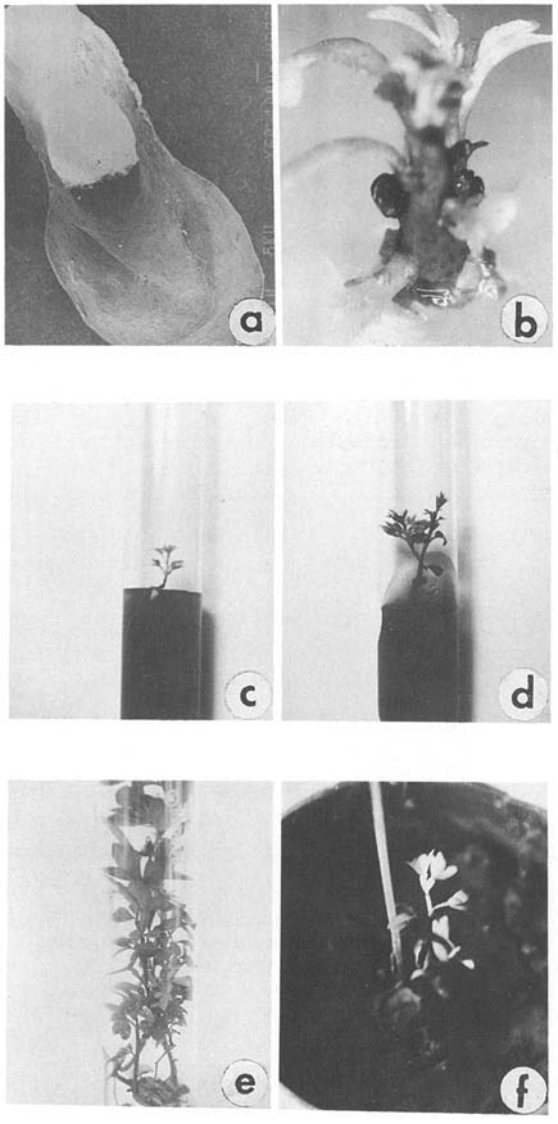

Fig. 1. Plant regeneration from cryopreserved (24 h in LN) embryonic axes of Poncirus trifoliata. (a) Photograph showing root and shoot apices of isolated embryonic axis, (b) Initiation of leaf primordia from shoot apex after 1 week of culture, (c) 20 d old seedling, (d) One month old seeding, (e) Three month old seedling, (f) Cryopreserved seedlings in pot.

In most of the reports of successful cryopreservation, excised embryos or embryonic axes have been used for desiccation sensitive species, i.e., zygotic embryos of Citrus (Mumford and Grout, 1979); oil palm (Grout et al., 1983), Veitchia and Howea (Chin et al., 1989), coconut (Chin et al., 1989); Hevea (Normah et al., 1986), and tea (Chaudhury et al., 1991), where these systems withstood freezing after being subjected to partial desiccation. Cryopreservation of nucellar cells of Citrus sinensis (naval orange) has been reported by Kobayashi et al., (1990), providing additional evidence for the feasibility for long-term genetic conservation.

The desiccated embryonic axes do not lose viability after rapid cooling, and storage at the temperature of LN (-196°C). At such temperatures, there should be no change in the tissue, either genetic or developmental, over a period of decades (Ashwood-Smith and Grant, 1977; King and Roberts, 1979). This situation, together with an ease to develop independent plants in vitro from embryonic axes, may provide an effective technique for the long-term conservation of desiccation-sensitive species. Studies are underway to assess the genetic integrity of cryopreserved plants by cytological, biochemical and morphological analyses.

Acknowledgments

Authors thank Dr. R.S. Rana, Director, NBPGR for providing the facilities and Department of Biotechnology, Government of India, for financial support.

References

Ashwood-Smith MJ, Grant E (1977) In: Elliot K, Whelan J (eds) The freezing of mammalian embryos, CIBA Symposium 52. Elsevier, Holland, pp 251-271

Bajaj YPS (1984) Curr Sci 53:1215-1216

Chaudhury R, Radhamani J, Chandel KPS (1991) Cryoletters 12:31-36

Chin, HF, Krishnapillay B, Alang ZC (1988) Cryoletters 9:372-379

Chin, HF, Krishnapillay B, Hor YL (1989) Pertanika 12:183-186

Grout BWW, Shelton K, Prichard HW (1983) Ann Bot 52:381-384

Honjo H, Nakagawa K (1978) In: Akihama T and Nakajima K (eds) Long-term preservation of favorable germplasm in arboreal crops. Fruit Tree Res Stn, Tsukuba, Japan, pp 31-35

King MW, Roberts EH (1979) The storage of recalcitrant seeds achievements and possible approaches. International Board for Plant Genetic Resources, Rome

Kobayashi S, Ikeda, Nakatani M (1978) In: Akihama T and Nakajima K (eds) Long-term preservation of favorable germplasm in arboreal crops. Fruit Tree Res Stn, Tsukuba, Japan, pp 8-12

Kobayashi S, Sakai A, Ohyama I (1990) Plant Cell Tissue Organ Culture 23:18-20

Marin ML, Duran-Vila N (1988) Plant Cell Tissue Organ Culture 14:51-57

Mumford PM, Grout BWW (1979) Seed Sci Tech 7:407-410

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

Normah MN, Chin HF, Hor YL (1986) Pertanika 9:299-303

Radhamani J, Malik SK, Chandel KPS (1991) Seed Sci Tech 19 (in press)

Reitz HJ (1984) Outlook on Agriculture 13:140-146

Withers LA (1979) Plant Physiol 63:460-467