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Cryopreservation of Cassava Micropropagules using Simple Slow Freezing and Vitrification Techniques

[1]K.E. Danso and [2]B.V. Ford-Lloyd
[1]Department of Plant and Soil Sciences, Ghana Atomic Energy Commission,
Biotechnology and Nuclear Agriculture Research Institute, P.O. Box 80, Legon, Ghana
[2]School of Biosciences, The University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

Abstract: The objective of this study was to develop a simplified protocol for cryopreservation of shoot tips, nodal cuttings and embryogenic calli of cassava. Shoot tips, nodal cuttings or embryogenic calli of cassava (*Manihot esculenta* Crantz) were cryopreserved by slow freezing or vitrification. When nodal cuttings and embryogenic calli of cassava were cryopreserved using simplified slow freezing or vitrification, the slow cooling curve showed a faster cooling rate for embryogenic calli than shoot tips and nodal cutting explants. On subsequent immersion in liquid nitrogen, shoot tip explants showed only post-thaw callus formation while nodal cutting developed vestigial shoots which could not, developed into full plants. In contrast, all embryogenic calli survived cryopreservation by slow freezing with post-thaw viability depending on the sucrose concentration in the preculture medium. Post-thaw viability of callus clumps pretreated with 0.3 M sucrose was comparatively higher than 0.5 M sucrose but the mean number of embryos produced by both treatments was the same. However, cryopreservation of embryogenic callus clumps by vitrification did not result in post-thaw viability and subsequent embryogenic competence. This lost of post-thaw viability could not be attributed to phytotoxicity of Plant Vitrification Solution (PVS2) used to achieve cellular dehydration as the non cryopreserved controls treated with vitrification solution produced somatic embryos. This study has shown that the simplified slow freezing technique is a suitable cryogenic procedure which could be used for long term conservation of embryogenic cultures of cassava genetic resources particularly in laboratories where there are no programmable freezers.

Key words: Cryopreservation, embryogenic calli, slow freezing, shoot tips, somatic embryos, vitrification

INTRODUCTION

The slow cooling (or two-step) technique is the first classical cryogenic protocol developed for long-term storage of plant genetic resources. The technique requires the culture of explants on a medium with penetrating cryoprotective mixtures containing DMSO followed by two-step freeze-dehydration and is often preceded by cold acclimation. Cryoinjury by lethal ice crystal formation in tissue is avoided by freeze-dehydration involving slow, controlled cooling to a defined prefreezing temperature using a programmable freezer followed by rapid immersion of tissues in liquid nitrogen at -196°C. During the initial slow freezing (at about -40°C), the intracellular solution becomes highly concentrated and subsequent immersion in liquid nitrogen results in vitrification of the cytosol thereby avoiding cryoinjury (Gonzalez-Arnao *et al*., 2008).

Classical slow freezing is mainly used for cryopreservation of cell suspensions and calli (Panis and Lombardi, 2005). According to Engelmann (1997), the technique is unsuitable for long term storage of shoot apices, zygotic embryos and somatic embryos. However, Escobar *et al.* (1997) has reported of successful cryopreservation of cassava shoot tips by the slow freezing technique. Inherent limitation of the slow freezing technique is the need for a programmable freezer which makes the process expensive. Thus, a simplified slow cooling protocol that does not require the use complex freezing equipment is needed.

During the last decade several new cryopreservation protocols have been developed for storage of plant tissues (Engelmann, 1997). Among them are simplified slow freezing using domestic or laboratory deep-freezers together with Nalgene Cryo 1°C freezing container and vitrification (Panis and Thinh, 2001). In simplified slow freezing, plant tissues are precultured on a cryoprotective medium mostly sucrose followed by initial freeze-dehydration using an isopropanol-filled Nalgene Cryo

Corresponding Author: K.E. Danso, Department of Plant and Soil Sciences, Ghana Atomic Energy Commission, Biotechnology and Nuclear Agriculture, Research Institute, Legon, Ghana

1°C freezing container in a laboratory deep-freezer (-80°C) followed by a rapid immersion in liquid nitrogen. The isopropanol in the freezing container acts as template for ice nucleation around the cryovials resulting in movement of tissue water to the ice. It also ensures adequate tissue dehydration before storage in liquid nitrogen. Panis and Thinh (2001) used this simplified slow freezing procedure to cryopreserve cell suspension cultures of banana.

Vitrification is also a simplified cryogenic procedure that has been employed for storage of both plant and animal tissues (Sakai *et al*., 1990; Brockbank *et al*., 2000). In vitrification, tissue dehydration is achieved by treatment with cryoprotective compounds followed by immersion in highly concentrated vitrification solutions (Sakai *et al*., 1990; Engelmann, 1997; Besnson, 1999) prior to storage in liquid nitrogen. Although, the use of vitrification solutions for tissue dehydration poses a toxic risk to plant tissue, the ability of cryoprotective compounds to vitrify at ultra low temperatures offers effective cryoprotection resulting in post-thaw viability. Cryogenic storage by vitrification has been reported for shoot tips of many plant species including cassava with variable post thaw recovery (Engelmann, 1997; Tagaki, 2000; Charoensub *et al*., 1999). There are, however, few reports on cryopreservation of embryogenic tissues of cassava by vitrification. In this study, we report on a simplified slow freezing protocol for cryopreservation of embryogenic tissues, nodal cuttings and shoot tips of cassava. Also, vitrification of embryogenic calli of cassava is discussed.

MATERIALS AND METHODS

Plant materials: The experiment was conducted at the School of Biosciences, the University of Birmingham laboratory in 2003. Five cassava accessions TME 2, TME 9, TME 2019, M. Col 1505 and Santom used for this study were obtained *in vitro*. Shoot tips and nodal cuttings were aseptically dissected from 8 week old plantlets. Somatic embryos were obtained from cyclic embryogenesis system as described by Raemakers *et al*. (1993) and Danso and Ford-Lloyd (2002). All cultures were incubated at 23-25°C under 16/8 h (light/dark) photoperiod with light supplied at an intensity of 40 μmol m^{-2} sec^{-1}.

Cryopreservation of shoot tips and nodal cuttings by slow freezing: Shoot tips and nodal cuttings of 8 to 12 week old plants of TME 2, TME 9, TME 2019 and M.Col 1505 were precultured on MS medium supplemented with 1 M sorbitol, 0.1 M DMSO and 4% sucrose as described by Escobar *et al*. (1997) or precultured on 0.3 M sucrose. After 3 days, explants were transferred to 30 mL sterile Universal Sterilin tubes and cryoprotected with 2 mL of cryoprotective mixture consisting of liquid (Murashige and Skoog, 1962) basal salts (MS) medium, I M sorbitol, 10% DMSO and 4% sucrose on ice for 2 h (Escobar *et al*., 1997). Samples were then dehydrated on sterile Whatman filter paper (90 mm) under laminar airflow hood for 1, 2 or 4 h and then put in 1.8 mL cryovials. The vials containing the samples and one with a sensor of a digital thermometer were put in a Nalgene Cryo 1°C freezing container filled with 250 mL of isopropanol (Fisher Chemical Company, UK). The freezing container with the samples was finally put in a -80°C laboratory deep-freezer (Sanyo Ultra Low Temperatures Freezer, Japan). The drop in temperatures was recorded every 5 min by the thermometer. At -33°C, the samples were allowed to remain in the deep freezer for a further 10 min to ensure effective dehydration before rapid immersion in liquid nitrogen in a Dewar flask for at least 1 h. Samples were then rapidly thawed in a warm water bath at 45°C for 90 sec, allowed to assume a room temperature and then cultured for regrowth on MS medium supplemented with 0.05 mg L^{-1} BAP and 0.01 mg^{-1} NAA (normal cassava multiplication medium). The percentage number of shoot tips and nodal cuttings which formed callus, roots or shoots, was recorded after 4 weeks of culture for regrowth.

Cryopreservation of embryogenic calli by slow freezing: Embryogenic calli of accession TME 9 were induced on MS medium supplemented with 8 mg L^{-1} 2, 4-D as described by Danso and Ford-Lloyd (2002). After 12 days, callus clumps were precultured on 25 mL solid MS medium supplemented with 2 μM $CuSO_4$, 8 mg L^{-1} 2,4-D and 0.3 M or 0.5 M sucrose, for 2 days and then dehydrated for 6 h under the laminar airflow hood. After dehydration, calli clumps were transferred to cryovials, placed in an isopropanol filled Nalgene Cryo 1°C freezing container and cryopreserved as described above. Cryopreserved calli clumps were cultured on MS supplemented with 0.1 mg L^{-1} BAP (MM, maturation medium) and the viability recorded 4 weeks after culture.

Cryopreservation of embryogenic calli by vitrification: Embryogenic calli of cassava accession TME 9 and SM1-2075-1 Line 1 were induced on MS medium supplemented with 0.1 or 0.3 M sucrose and then transferred to a preculture MS medium supplemented with 0.3 M sucrose. After 4 days, callus clumps were transferred to 30 mL Universal Sterilin tubes and loaded with 2 mL of loading solution (2 M glycerol and 0.4 M sucrose in MS medium) for 20 min. Embryogenic clumps were vitrified with PVS 2 (30% glycerol, 15% ethylene

glycol, 15% DMSO all (v/v in 0.4 M sucrose) solution for 45 min. Half of the vitrified callus clumps were cryopreserved by immersion in liquid nitrogen while the remaining half served as controls. Cryopreserved callus clumps were rapidly thawed in a warm water bath for 90 sec. Both the cryopreserved clumps and the controls were cultured on MS medium supplemented with 0.3 M sucrose plus 8 mg L^{-1} 2,4-D for 1 day in the dark and then finally transferred to embryo maturation medium consisting of MS medium with 0.1 mg L^{-1} BAP. Cultures were initially incubated in the dark for 6 days prior to transfer to growth room incubation conditions. The percentage number of callus clumps with somatic embryos was recorded 4 to 6 weeks after culture on maturation medium.

Statistical analysis: Completely randomized design was used and data collected were subjected to Analysis of Variance (ANOVA) to separate means between treatments using the Minitab Statistical Software (version 12) where appropriate. Means which differed significantly, were separated using the Tukey's pair wise comparison. Chi square analysis was used to test for significant differences in post-thaw survival experiments.

RESULTS

Response of shoot tips and nodal cuttings to cryopreservation by slow freezing: Shoot tips and nodal cuttings of TME 2, TME 9, TME 2019 and M. Col 1505 precultured on 1 M sorbitol plus 0.1 M DMSO or 0.3 M sucrose remained green after 3 days. After cryoprotection on ice for 2 h with sorbitol-DMSO mixture and dehydration for 1 to 3 h, samples were subjected to slow cooling in isopropanol-filled freezing container in a laboratory -80°C deep freezer. The cooling curve for nodal cuttings and shoot tips of accession TME 2 is shown in Fig. 1. The cooling curve of all the remaining accessions followed the same pattern since the same procedure was used. There was an initial rapid cooling (decrease in temperature) of shoot tips at a rate of 1°C min^{-1} followed by a slow decrease in temperature to -35°C after which the samples were allowed to remain in the freezer for further 35 to 40 min before rapid immersion in liquid nitrogen. There was ice crystal formation on the surface of the cryovials before immersion in liquid nitrogen indicating initiation of ice by the isopropanol prior to immersion in liquid nitrogen. Upon subsequent immersion in liquid nitrogen and culture for regrowth, only shoot tip explants of accession TME 2 showed post thaw survival. However, post thaw viability depended on the preculture medium and duration of dehydration prior to slow cooling in the deep freezer.

Table 1: Response of nodal cuttings and shoot tips of cassava accession TME 9 to cryopreservation by slow cooling

Explant	Callus (%)	Plant recovery (%)
Shoot tip	35	0
Nodal cuttings	0	15

Explants were precultured on 1 M sorbitol plus 0.1 M DMSO for 3 days followed by immersion in liquid nitrogen. Twenty explants were cultured for each treatment

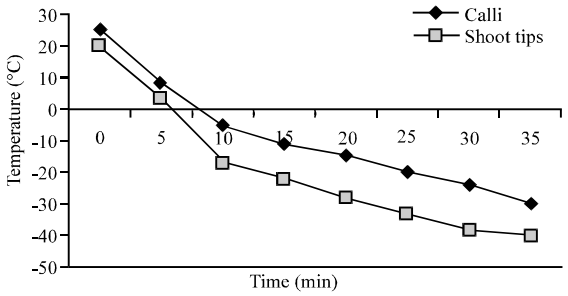

Fig. 1: Slow cooling curve for shoot tips of (TME 2) and embryogenic calli of TME 9

All explants precultured on 0.3 M sucrose followed by cryoprotection did not survive cryopreservation independent of the duration of dehydration. In contrast, response of shoot tips and nodal cutting explants precultured on DMSO depended on the duration of dehydration under the laminar airflow and the type of explants used. Explants dehydrated for 1 or 3 h prior to slow cooling and cryopreservation turned white and died within one week after cryopreservation while only those dehydrated for 2 h survived cryopreservation (Table 1). Of the viable explants, plant recovery depended on the type of explants; while 35% of shoot tips developed only calli, 15% of nodal cuttings developed directly into vestigial shoots which did not developed into complete plants even after prolonged culture for eight weeks.

Response of embryogenic calli to cryopreservation by slow freezing: Embryogenic callus clumps of accession TME 9 precultured on 0.3 or 0.5 M sucrose plus 8 mg L^{-1} 2, 4-D were dehydrated under the laminar flow hood to achieve approximately 75% moisture loss prior to slow cooling. Upon transfer of callus clumps to isopropanol bath in the deep freezer, the cooling curve was similar to that of shoot tips and nodal cuttings as the same device and isopropanol was used as template to initiate extracellular ice for dehydration (Fig. 1). However, the slow cooling curve indicated that embryogenic calli had a faster cooling rate than the shoot tips.

Upon subsequent cryopreservation, callus clumps showed post thaw viability and embryogenic competence (Fig. 2a, b). However, post thaw viability and subsequent embryogenic competence were dependent on the sucrose

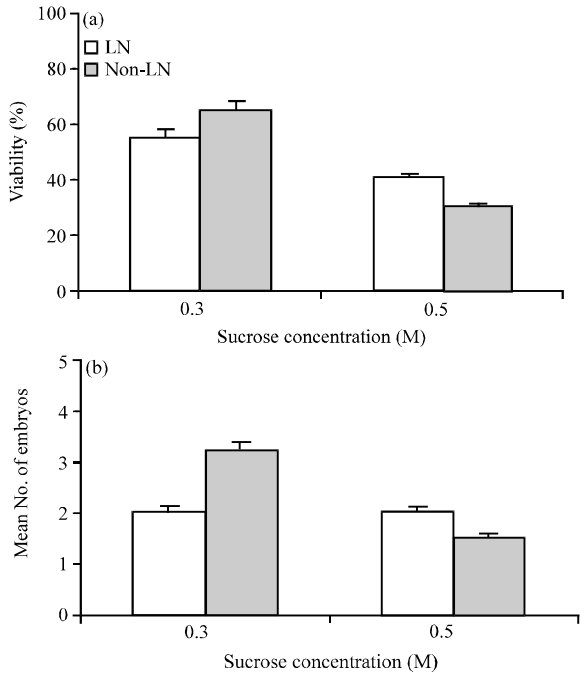

Fig. 2(a-b): Post-thaw (a) viability and (b) mean number of embryos by embryogenic clump of TME 9 cryopreserved by slow freezing. Twenty embryogenic calli were cultured per treatment

Table 2: Response of callus clumps of TME 9 to cryopreservation by vitrification

Sucrose concentration (M)	Post-thaw response	Non-LN
0.0	Viability (%)	100.00
	Mean No. of embryos	9.6±1.2
0.1	Viability	100.00
	Mean No. of embryos	9.9±0.6

The total number of embryos cultured varied from 10 to 20. LN indicates cryopreserved and Non-LN indicates non cryopreserved controls

concentration in the cryoprotective medium. Callus clumps precultured on 0.3 M sucrose developed somatic embryos mostly via callus formation whereas with 0.5 M sucrose, embryo developed directly without callus phase intervention. Fifty five percent and 40% of callus clumps cryoprotected on 0.3 and 0.5 M, respectively were viable and almost all the viable clumps were embryogenically competent independent of the sucrose concentration. However, there were no significant differences ($p \leq 0.05$) between the cryopreserved clumps and non-cryopreserved controls when Chi square analysis was performed. Also post-thaw viability of callus clumps precultured on 0.3 and 0.5 M sucrose did not show significant differences ($p \leq 0.05$).

The mean number of embryos produced by clumps was the same for clumps cryoprotected with 0.3 and 0.5 M sucrose. However, the percentage viability and mean number of embryos produced from clumps cryoprotected with 0.5 M sucrose followed by slow freezing was comparatively higher than non cryopreserved controls but the difference is not significant ($p \leq 0.05$).

Response of embryogenic calli to cryopreservation by vitrification: All embryogenic callus clumps of TME 9 induced on MS without sucrose or 0.1 M sucrose followed by cryopreservation by vitrification lost post-thaw viability and subsequent embryogenic competence. Non-cryopreserved control callus which were only treated with vitrification solutions without cryopreservation were however embryogenically competent indicating that vitrification solutions were not toxic to the calli (Table 2). The viability of callus clumps of TME 9 were independent of the sucrose concentration in the induction medium as there were no significant differences between post-thaw viability and mean number of embryos produced.

DISCUSSION

Seeds are ideal propagules for long term storage of plant genetic resources. However, in cassava sexual propagation results in genetic segregation, thus seed banking is not suitable for conservation of the crop. Cryopreservation is therefore the only option for long-term conservation of the diverse cassava genetic resources. Thus, simplified slow freezing and vitrification were used to cryopreserve shoot tips, nodal cuttings and embryogenic callus clumps of cassava.

Shoot tips and nodal cutting explants cryopreserved using slow freezing resulted in post-thaw viability expressed as callus formation and vestigial shoots recovered from shoot tips and nodal cuttings, respectively. According to Engelmann (1997), complex tissues such as shoot tips and well organized somatic embryos do not often respond to cryopreservation by slow freezing, an observation similar to the findings in this report. However, Reed *et al.* (2001) reported that 0-10% and 30-40% of *Ribes* species survived cryopreservation by slow freezing in different laboratories using different programmable freezers. Similarly, Escobar *et al.* (1997) reported 70% plant recovery rate in cassava shoot tips after cryopreservation by slow freezing. The differences between the report presented in this study and that of Escobar *et al.* (1997) can be attributed to the differences in cryogenic equipment used. Escobar *et al.* (1997) used a programmable freezer for slow freezing to achieve a controlled critical temperature for tissue dehydration while in this report isopropanol-filled Nalgene Cryo 1°C freezing container (isopropanol bath) was used, thus making it difficult to control or maintain the critical temperature at

-35°C. According to Reed *et al*. (2001), the slow cooling method is critically dependent on precise control of cooling rates and ice nucleation and is achieved by using a programmable freezer. With programmable freezers, ice nucleation is automatically initiated externally and samples can be cooled at a slower rate while an isopropanol bath has no such facilities. Thus, it was difficult to control the cooling rate thereby leading to excessive tissue dehydration.

Callus clumps of accession TME 9 were viable and embryogenically competent after cryopreservation by slow freezing. Successful cryopreservation of somatic embryos by slow freezing has been reported in horse-chestnut (*Aesculus hippocastanum*) with 43% embryo recovery after pretreatment with 0.75 M ABA (Jekkel *et al*., 1998). Using a similar Nalgene Cryo 1°C freezing container, Panis and Thinh (2001) obtained post-thaw viability of cell suspension cultures of banana using fluorescein diacetate test (FDA) but indicated that there was no regrowth. They argued that lack of regrowth could be due either to excessive tissue dehydration which continues after -35°C, or insufficient dehydration since in the Nalgene box no ice crystallization phase could be applied. In this report 40 to 55% of callus clumps precultured on 0.5 or 0.3 M sucrose, respectively survived cryopreservation by slow cooling and this might be due to effective cryoprotection by sucrose. Sucrose is known to stabilise cellular membranes and proteins during dehydration and freezing thus maintaining the liquid crystalline state of membrane bilayers (Panis *et al*., 1996; Thomashow, 1999). Also, sucrose cryoprotection increase endogenous sugar levels in the cytosol and accumulate specific hydrophilic proteins such as dehydrins which stabilize cell membranes (Jitsuyama *et al*., 2002), thus enhancing acquisition of freezing tolerance in embryogenic tissues. Besides, the culture of the cryopreserved clumps on MS medium with 2,4-D supplement immediately after cryopreservation may have reactivated embryogenic cell proliferation before transfer to maturation medium.

Cryopreservation of embryogenic callus clumps by vitrification neither resulted in post-thaw viability nor embryogenic competence. Escobar *et al*. (2001) have also made a similar observation that cryopreservation of friable embryogenic callus of cassava after treatment with PVS2 or PVS3 did not result in post-thaw viability. Although, the exact reason for lack of post-thaw viability could not be elucidated from this study, it may be attributed to the sequential two step dehydration caused by osmotic dehydration of vitrification solution followed by freeze-dehydration which may have caused disorganization of cells of cryopreserved callus clumps resulting in loss of viability.

CONCLUSION

The study has shown that simple laboratory (or domestic) freezer could be used to achieve post-thaw viability of embryogenic callus clumps in a simplified slow freezing procedure. The technique is cost effective and can be used to conserve embryogenic cultures of cassava for genetic transformation particularly, in laboratories where there is no sophisticated programmable freezer in the developing countries. In contrast, vitrification of shoot tips, nodal cuttings and embryogenic calli did not result in post thaw survival. Further investigations on the use of vitrification should be done to improve the technique for long term conservation of cassava.

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