CRYOPRESERVATION OF *IN VITRO*-GROWN SHOOT TIPS OF CASSAVA

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ABSTRACT

An *in vitro* collection of cassava germplasm has been established at the Scientific Equipment Center of Kasetsart University's Research and Development Institute since 1985. Nearly 130 cultivars are presently maintained under slow-growth conditions. Most *in vitro* conservation methods are for short- to medium-term storage; they encounter various problems such as high costs of maintaining the stock, space requirements, risks of contamination and somaclonal variation with increasing time. For long-term conservation of plant germplasm, cryopreservation is a theoretical choice. Cryopreservation is based on the non-injurious reduction and subsequent interruption of metabolic functions of biological materials by reducing the temperature to that of liquid N (-196°C). However, the availability or development of a simple, reliable and cost-effective protocol and the subsequent regeneration of the plants are basic requirements for germplasm conservation. Vitrification is achieved by using a sufficiently high concentration of solutes, and by the use of rapid cooling rates to prevent the cell solution from freezing into ice; this ensures its transition into the amorphous or glassy state.

We first succeeded in the cryopreservation of *in vitro*-grown shoot tips of cassava (*Manihot esculenta* Crantz cv. CM3281-4) by a simple vitrification method. In the protocol, excised shoot tips from *in vitro* plantlets were precultured on solidified culture medium, supplemented with 0.3 M sucrose for 16 h; these were then treated with a mixture of 0.4 M sucrose and 2 M glycerol for 20 min at 25°C. The osmo-protected shoot tips were sufficiently dehydrated with a highly concentrated vitrification solution (designated PVS2) for 45 min prior to plunging into liquid N. Successfully vitrified shoot tips were rewarmed rapidly in water at 45°C and then plated on culture medium. These vitrified shoot tips developed shoots within three weeks after being recultured. The average rate of normal shoot formation was about 75%. Recently, the vitrification protocol was successfully applied to ten other cultivars of cassava. The average recovery rate was about 70%.

Further studies are necessary to develop a method that can be routinely applied to a wide range of lines showing diverse traits in gene banks.

INTRODUCTION

An *in vitro* collection of cassava germplasm has been developed at the Scientific Equipment Center of Kasetsart University's Research and Development Institute since 1985. Nearly 130 cultivars are presently maintained under slow-growth conditions. *In vitro* conservation methods are for short- to medium-term storage; they encounter various problems such as high costs of maintaining the stock, space requirements, risks of contamination and somaclonal variation with increasing time. Cryopreservation in liquid nitrogen appears to be a logical choice for long-term storage of plant germplasm with minimum space and maintenance requirements. Cryopreservation is based on the non-injurious reduction and subsequent interruption of metabolic functions of biological materials by reducing the temperature to that of liquid nitrogen (LN) at -196°C. However,

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the availability and development of a simple, reliable and cost-effective protocol are basic requirements for the long-term conservation techniques.

Cryopreservation of shoot tips of cassava using a conventional slow freezing method was reported by Escobar *et al.* (1997). However, this procedure is very complicated and time consuming and gives generally low rates of growth recovery Almost ten years ago, some new cryogenic procedures (vitrification, and encapsulation-dehydration techniques) were reported by Engelmann (2000) and by Sakai (1997; 2000). These protocols dehydrate a major part of the freezable water of specimens at non-freezing temperatures and enable them to be cryopreserved by being directly plunged into LN. These new protocols simplified the cryogenic procedures and increased the applicability to a wide range of plant materials, especially non-hardy tropical plants. Vitrification refers to a phase transition from a liquid into amorphous glass, while avoiding crystallization. The vitrification protocol requires the use of a highly concentrated vitrification solution, which sufficiently dehydrates explants so that they turn into a stable glass when cooled into LN. We first succeeded in the cryopreservation of cassava (cv. CM3281-4) by vitrification (Charoensub *et al.,* 1999).

However, to be able to apply the vitrification protocol to a wide range of cassava germplasm, it is essential to develop a simple and effective micropropagation method, which can supply a large number of uniform and suitable meristems required for successful cryopreservation. We succeeded in cryopreservation of ten cultivars of cassava (average rate of recovery growth about 70%), using standardized ideal apices, which are produced by mono-nodal cutting culture (Hirai and Sakai, 1999).

MATERIALS AND METHODS

In vitro-grown plantlets of cassava (*Manihot esculenta,* cv. CM3281-4) were maintained on MS inorganic medium (Murashige and Skoog, 1962) containing 100 mg/l inositol, 1 mg/l thiamine HCl, 0.02 mg/l BA, 0.1 mg/l GA, 0.01 mg/l NAA, 3% sucrose and 7.5 g/l agar, pH 5.6 at 25°C under a 12 h photoperiod (50 μmol s^{-1} m^{-2}). They were subcultured every two months. The nodal segments, consisting of one lateral bud and a 0.5 cm long stem, were taken from 2-month-old stock cultures and densely cultured (30 nodal segments) on the same medium as mentioned above in Petri dishes (10 cm diameter, 2 cm height). These segments developed young axillary shoots and leaves after culture under the light conditions **(Photo 1).** The apices (about 1 mm long with two leaf primordia) were excised from the 2-day old axillary shoots (about 1 cm height) for cryopreservation.

The excised apices were precultured on MS inorganic medium supplemented with 0.3 M sucrose for 16 h. They were placed in a 1.8 ml cryo-tube and then osmo-protected with a mixture of 2 M glycerol and 0.4 M sucrose (LS solution) for 20 min at 25°C. After removing the LS solution, a highly concentrated vitrification solution (PVS2, 7.8 M) (Sakai *et al.,* 1990) was added and gently mixed. After being replaced once with fresh PVS2, apices were dehydrated with PVS2 for 45 min at 25°C. The cryo-tubes in which apices were finally suspended in 0.5 ml of PVS2, were plunged into LN (-196°C) and held for at least 2 h. After being rapidly warmed in water at 45°C by vigorously shaking for about 1 min, PVS2 was drained from the cryo-tubes and replaced with MS inorganic medium containing 1.2 M sucrose for 20 min. Then cryopreserved apices were transferred onto

sterilized filter paper discs over a solidified culture medium in Petri dishes. After one day, they were transferred again to fresh medium.

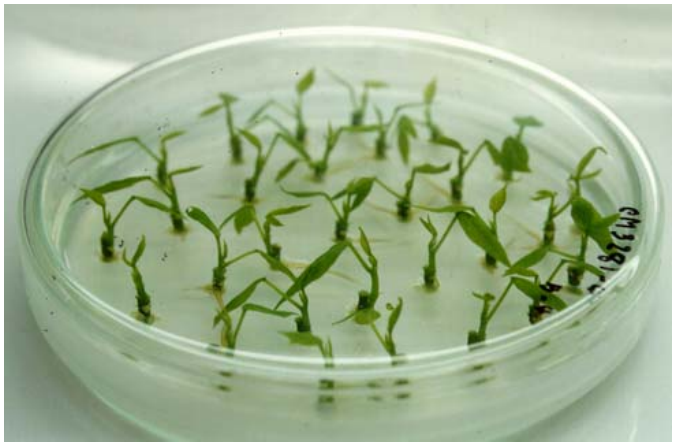

Photo 1. The mono-nodal segments developed young axillary shoots after being cultured[1)] *for 12 days.*

[1)] The nodal segments, consisting of one lateral bud were densely cultured in a Petri dish containing MS inorganic medium supplemented with 100 mg/l inositol, 1 mg/l thiamine HCl, 0.02 mg/l BA, 0.1 mg/l GA, 0.01 mg/l NAA, 3% sucrose and 7.5 g/l agar.

In this study, ten cassava cultivars (CM3281-4, CM323-52, CM3401-2, Hanatee, MInd 9, AMM22, MKUC, KU50, Rayong 1 and Rayong 90) were tested for their cryopreservation by vitrification.

RESULTS AND DISCUSSION

The key factors for the successful cryopreservation by vitrification are the acquisition of osmo-tolerance to PVS2 solution and the mitigation of injurious effects during the dehydration process. To enhance the osmo-tolerance of excised apices, they were precultured with 0.3 M sucrose for 16 h, followed by the treatment with 2 M glycerol and 0.4 M sucrose for 20 min before being dehydrated with PVS2 solution. To determine the optimal time of exposure to PVS2 solution, osmo-protected apices were treated with PVS2 at 25°C for different lengths of time before being plunged into LN. The optimal time was obtained at 45 min (data not shown).

The recovery rate of apices excised at different ages (10 to 18 days old) of donor plantlets were compared. The apices sampled from the 12-day-old plantlets resumed growth within one week after reculture and developed normal shoots without intermediary callus formation. This simple micropropagation method enabled the production of a large number of relatively homogeneous and adequate apices in terms of size and physiological state and growth response, thereby increasing the chance of positive and uniform responses to subsequent cryogenic treatments and recovery growth. This mono-nodal cutting culture allowed some standardization of the apices used for cryopreservation, as the axillary buds began to develop into terminal ones, though no marked elongation on the stem was noticeable (Bachiri *et al.*, 2001).

The vitrification protocol was tested for ten other cultivars of cassava, six of which produced high rates of recovery growth (70-90%), with an average recovery rate of about 70% **(Table 1).**

Table 1. Rates of recovery growth of ten cultivars of cassava after being cryop reserved by virtification.

	% shoot formation	
Cultivars	–LN[2)]	+ LN[2)]
CM3401-2	90.0	43.3 ± 12.5
Hanatee	80.0	60.0 ± 0.0
MInd 9	90.9	43.3 ± 17
CM323-52	91.7	90.2 ± 7.5
AMM22	90.9	86.7 ± 12.5
CM3281-4	90.9	86.7 ± 4.7
MKUC	90.0	75.6 ± 6.2
Rayong 1	80.0	73.3 ± 4.7
KU50	90.9	70.0 ± 8.2
Rayong 90	90.9	31.9 ± 7.3

[1)] Excised apices were precultured with 0.3 M sucrose for 16 h, and osmo-protected with 2 M glycerol and 0.4 M sucrose for 20 min. Thereafter they were dehydrated with PVS2 for 45 min at 25°C befored being plunged into LN. Ten apices were tested for each of the three replications in every cultivar.

[2)] -LN: without being immersed in liquid nitrogen, + LN: being immersed in liquid nitrogen.

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