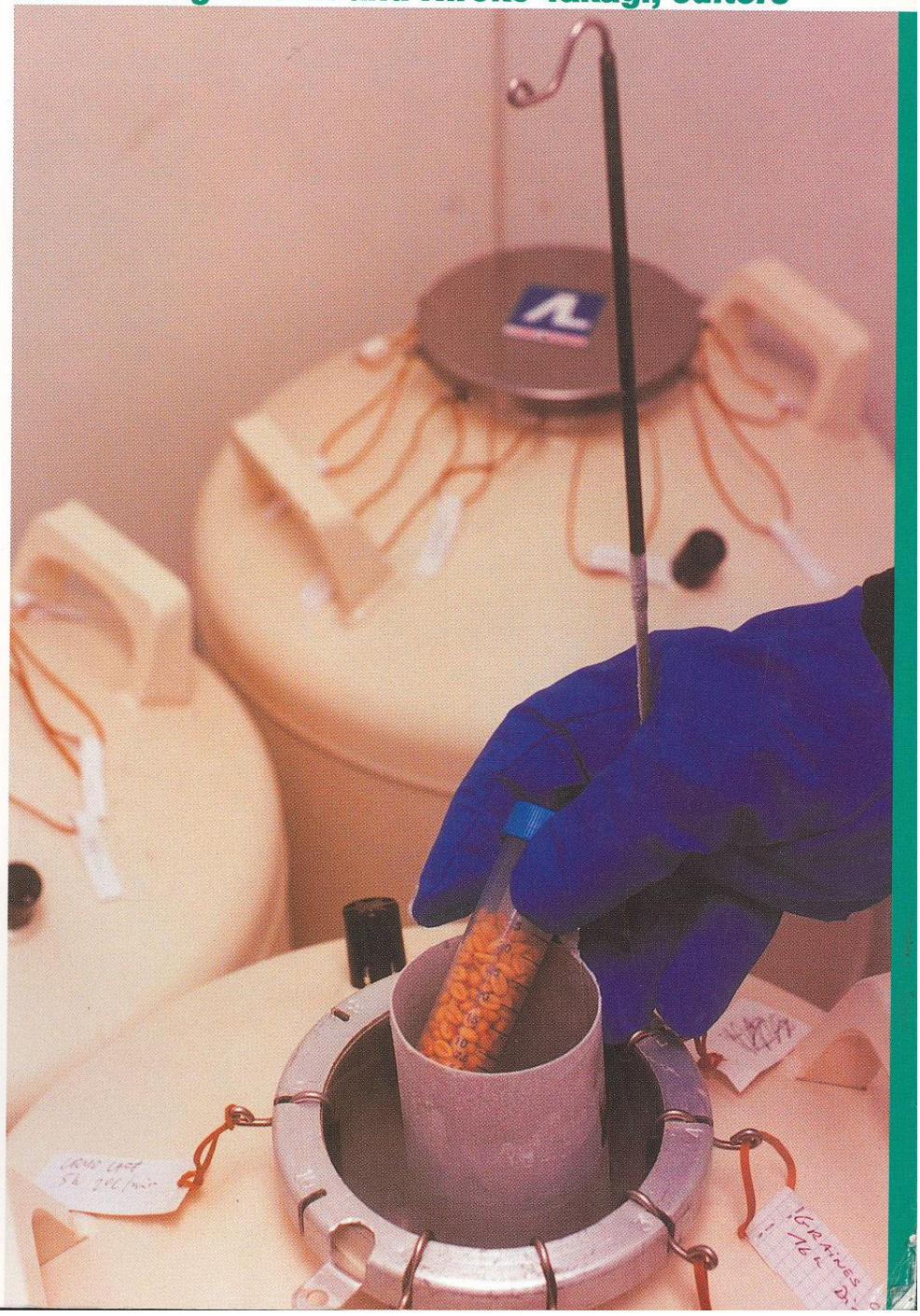
IPGRI

Cryopreservation of tropical plant germplasm

Current research progress and application

Florent Engelmann and Hiroko Takagi, *editors*



JIRCAS JAPAN

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Foreword

Cryopreservation, i.e. the storage of biological material at ultra-low temperature, usually that of liquid nitrogen (–196°C), is the only method currently available to ensure the safe and cost-effective long-term conservation of genetic resources of species that have recalcitrant seeds or are vegetatively propagated.

Dramatic progress has been made over the last 10 years in this area with the development of cryopreservation techniques for well over 100 plant species. Cryopreservation protocols are increasingly becoming available for routine application in genebanks. However, much of the work to date has been done on temperate species, with research on tropical and subtropical species lagging behind. This is of particular concern given the large number of tropical species that are either vegetatively propagated or that produce recalcitrant seeds.

Both JIRCAS and IPGRI are heavily involved in cryopreservation research. In the framework of its Visiting Fellowship Programme, JIRCAS has carried out a project specifically to develop techniques for the long-term preservation of vegetatively propagated crop germplasm. During the project, visiting scientists from developing countries have developed cryopreservation protocols for selected tropical crops. For more than 15 years, IPGRI and its predecessor IBPGR has supported cryopreservation research in collaboration with partners in Asia, the Pacific and Oceania, Africa, the Americas and Europe.

As a result of their experience in the field of cryopreservation, JIRCAS and IPGRI, in October 1998, jointly organized an international workshop to assess the current state of the science, to explore cryopreservation applications and to examine outstanding problems. The focus of the workshop was on the use of cryopreservation to conserve the germplasm of tropical plant species. An additional objective was to identify priority areas for collaborative research, technology development, transfer and application. The workshop was attended by a large number of cryopreservation experts from both developing and developed countries who presented their latest research results and contributed to the discussions.

This publication of the proceedings of the workshop thus presents a comprehensive overview of current knowledge concerning the biological and physical mechanisms involved in cryopreservation, and the status of the development of protocols for new species and their application in genebanks. We trust that it will help to stimulate further collaborative research and thus contribute to the wider application of cryopreservation for the safe long-term and cost-effective conservation of genetic resources of tropical species.

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Development of cassava cryopreservation

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Introduction

Vegetative propagation, bulkiness of planting material and risk of genetic erosion make cassava an ideal candidate for the application of innovative germplasm conservation techniques. Cryopreservation is considered as the most economical and safest method for the long-term conservation of cassava genetic resources.

Our efforts at CIAT, in collaboration with IBPGR/IPGRI, started with the development of a basic cassava cryopreservation protocol which was established in 1990, and consisted of the application of chemical dehydration of shoot-tips using a moderate concentration of sorbitol, DMSO and sucrose. Several factors were considered critical for the process. Cassava shoot-tips are affected by osmotic agents in different ways according to concentration and type of compound. High concentration of sorbitol changes the morphogenic response of shoot-tips, callus induction being the most typical. When sucrose was used in combination with 1M sorbitol, shoot-tips did not grow, and DMSO reduced callus formation. The best combination of osmotic agents tested with cassava shoot-tips consisted of 1M sorbitol, 0.1M DMSO and 0.11 M sucrose (medium C4) during 3 days.

Other factors appeared to be critical, including the level of tissue dehydration and the size of shoot-tips. We found that dehydration for 1 h on filter paper could improve shoot recovery after freezing; dehydration at room temperature (26–28°C) favoured elimination of water from the explant surface, allowing a gradual loss of water from within the tissue as well.

A relationship between the extent of cell damage due to freezing and the size of explants was established. Small shoot-tips (1–2 mm) gave better results than larger ones (3–4 mm).

Based on these parameters we established a basic protocol for cassava (Escobar *et al.* 1997) (Fig. 1).

By preculturing shoot-tips on C4 medium for 3 days, and using a two-step cryoprotective treatment, it was possible to recover viable cassava plants from frozen shoot-tips with cv. MCol 22 (50–60% as shoot formation); thereafter the method was reproduced with several cultivars representing a wide geographic distribution. Some cultivars were sensitive to osmotic concentration applied during the preculture medium. It seems that for some cassava cultivars, the edaphoclimatic origin affected response after freezing (Escobar *et al.* 1997). Sakai and Sugawara (1973) found a narrow correlation between the hardening behaviour and geographic origin of the plant, and Reed (1990) did not observe this relationship with *Pyrus* meristems. By adjusting the osmotic concentration in the preculture medium (0.25–0.5M sorbitol, $1x10^{-3}$– $1x10^{-4}$ M DMSO and 0.11–0.25M sucrose for 5 days) it was possible to increase shoot recovery in low responding cassava cultivars like MVen 232 and MMex 71 (Fig. 2). In cassava

there exists a close relationship between osmotic concentration, exposure time and response after freezing. Preculture media containing high osmotic concentration for long periods reduces shoot recovery, even prior to freezing (Escobar *et al.* 1997).

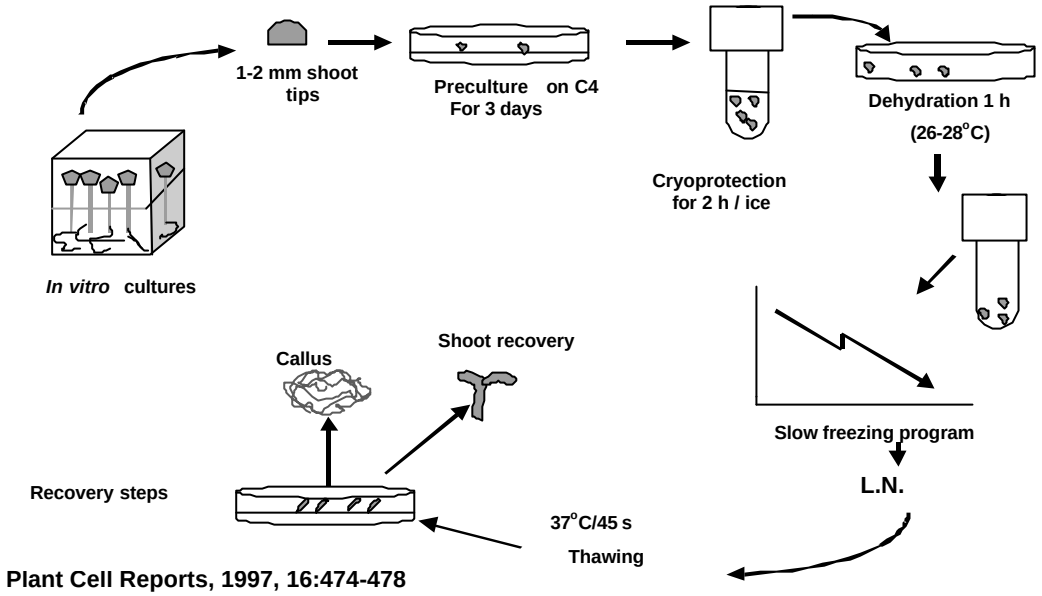

Fig. 1. Protocol established for cassava cryopreservation (from Escobar *et al.* 1997).

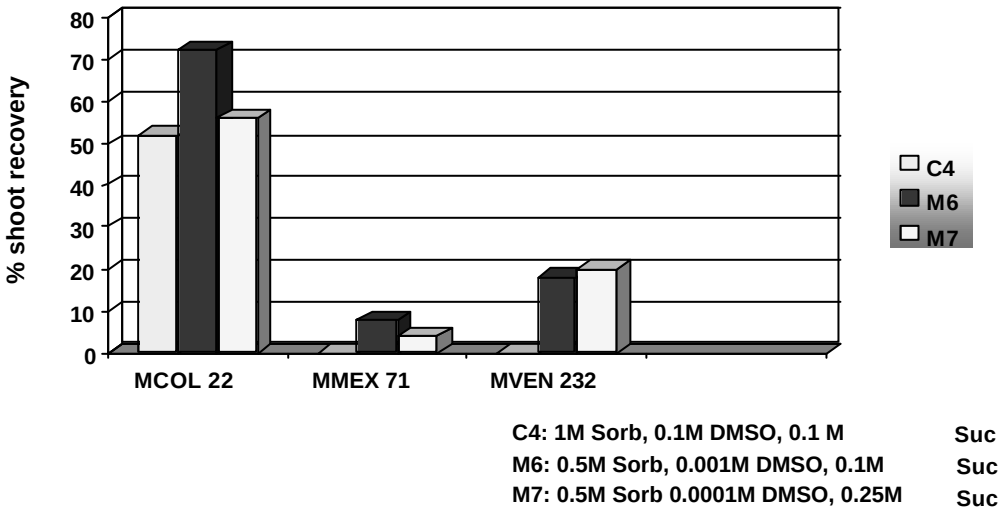

Fig. 2. Preculture medium and shoot recovery of recalcitrant cassava cultivars.

In order to increase the recovery rate of viable plants, and to minimize genotypic differences, we found that modifications of pre- and post-freezing conditions affected the recovery of shoots. Adjustment of growth conditions of donor cultures can influence shoot recovery after freezing. Lower temperature (21–23°C) and higher illumination (75 µE m^{-2} s^{-1}) of cassava donor cultures increased shoot recovery after freezing consistently (50–60% rate). Benson *et al.* (1989) found the same response with shoot-tips of *Solanum tuberosum*, with different pre- and post-freeze light regimes. We found that younger tissues (3–4 months of *in vitro* culture) were more reactive than older ones, and pregrowing shoots on CIAT–4E medium (Roca 1984) for 3 days before freezing resulted in a significant increase in survival. Medium 4E induces a higher amount of meristematic cells, able to support desiccation and with good response after freezing. Henshaw *et al.* (1985) showed that a pregrowth period is critical in determining the survival of cryopreserved shoot-tips of *S. tuberosum*.

In the post-freezing phase we found that low agar concentration in the recovery medium affects survival after freezing; it seems that media with low agar concentration (0.35%) have a positive effect on the growth of plants recovered from frozen shoot-tips. Semi-solid medium avoids the formation of gradients, but contributes to hyperhydricity. Low agar concentration in the preculture phase increases damage due to freezing. The response after freezing was affected by the type of cytokinin used and its concentration. Kinetin at 0.5 mg/L was more efficient than 2iP, BAP, TDZ and Adenine. When BAP concentration was increased from 0.04 to 0.5 mg/L, a drastic effect was evident. Reducing NAA (0.01 mg/L) and increasing GA_3 (0.5 mg/L) in the recovery medium diminished callus growth and stimulated elongation of shoots.

Ultra-rapid freezing of shoot-tips resulted in similar or higher recovery rates than slow freezing (Table 1). It would also save time and reduce the cost of introducing the entire CIAT cassava collection into cryopreservation (Escobar and Roca 1997).

The encapsulation-dehydration technique was established as an alternative to slow/rapid freezing cryopreservation (Escobar *et al.* 1998; Palacio 1998). The technique has been shown to be expeditious and consistent; shoot growth from frozen shoot-tips was rapid and direct with less callus formation.

Shoot-tip size, pretreatment with sucrose, and dehydration time to achieve optimal water content of beads were found critical for the success of the encapsulation-dehydration technique. It seems that a gradual increase in sucrose concentration could improve the recovery of osmo-sensible cassava cultivars. Furthermore, increasing cytokinins and reducing auxin level in the recovery media favoured shoot response after freezing and the consistency of response (Fig. 3).

We moved plants from 16 cassava cultivars to the field after recovery from frozen storage and compared them with the respective non-cryopreserved *in vitro* propagated plants. We are monitoring morphological traits and will test possible genetic changes assisted by molecular markers.

Table 1. Effect of freezing technique on cryopreservation of cassava shoots

Genotype	Freezing†	% Viability	% Shoot recovery
MCol 304	Programmed	63.6	18.1
	Rapid	100	54.5
MCol 1389	Programmed	90.9	9.1
	Rapid	90	10
MCol 1468	Programmed	53.8	0
	Rapid	84.6	0
MPar 71	Programmed	84.6	76.9
	Rapid	92.8	71.4
MCol 22	Programmed	76.5	49.75
	Rapid	80.25	55.5

† Programmed protocol, Escobar *et al.* 1997; rapid freezing (direct immersion), Escobar and Roca 1997.

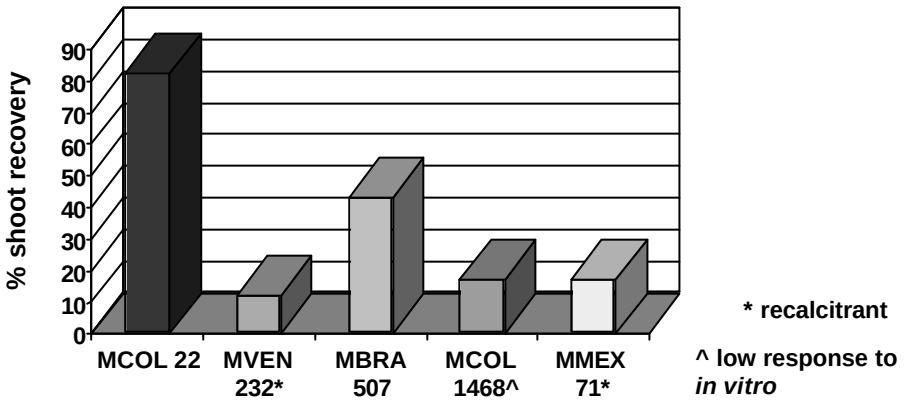

Fig. 3. Response of cassava genotypes to encapsulation-dehydration.

Cryopreservation will allow great reduction of the high costs of maintaining the cassava germplasm collections which are largely due to the labour required in the field as well as in the active *in vitro* bank. Cryopreservation should also reduce the laboratory space needed for a collection as large as the one maintained at CIAT (over 6000 clonal accesions). In addition, cryopreservation of pollen, seeds (Marin *et al.* 1990), somatic embryos and other cells and tissues will be fundamental for achieving the conservation goals, which are basic to the cassava improvement programmes at CIAT and elsewhere.

Conclusion

1. We developed three cryopreservation methodologies for cassava. Viable plants were recovered from all of them.
2. Not all methodologies can be applied to all cultivars. There are varietal differences. Adjusting osmotic concentration is a key factor.
3. Encapsulation-dehydration seems to be the fastest and cheapest method to introduce the cassava collection into cryopreservation.

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