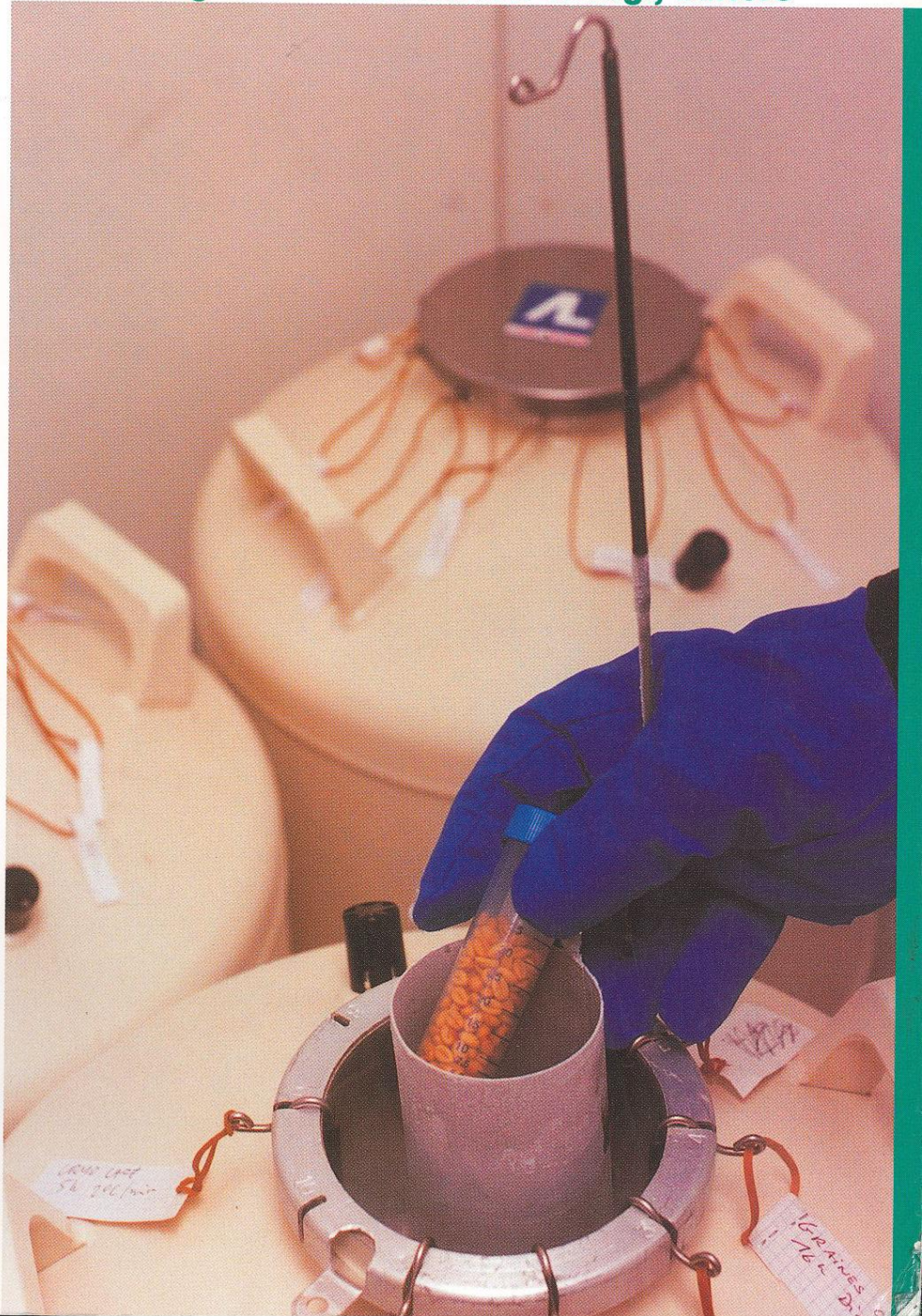




# Cryopreservation of tropical plant germplasm

Current research progress and application

**Florent Engelmann and Hiroko Takagi, editors**



Japan International  
Research Center for  
Agricultural Sciences  
Tsukuba, Japan

## Foreword

Cryopreservation, i.e. the storage of biological material at ultra-low temperature, usually that of liquid nitrogen ( $-196^{\circ}\text{C}$ ), 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.

Nobuyoshi Maeno, Director General  
Japan International Research Center for  
Agricultural Sciences (JIRCAS)  
Tsukuba, Japan

Geoff Hawtin, Director General  
International Plant Genetic  
Resources Institute (IPGRI)  
Rome, Italy

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## Cryopreservation and cassava germplasm conservation at CIAT

William M. Roca, D. Debouck, Roosevelt H. Escobar, Graciela Mafla and M. Fregene

CIAT, Cali, Colombia

### Introduction

Cassava is a staple food crop for 500 million people in tropical countries of Africa, Asia, Latin America, the Caribbean and Oceania. The crop is a chief source of calories and constitutes a unique food security resource, particularly when production of other crops has failed owing to edaphoclimatic problems like drought. In recent years, cassava has entered the commodity market as a feed, and has demonstrated potential for the starch industry. Cassava is grown on 16 million ha, with a total production of 150 million MT (FAO 1995); around 60% of the production is in Africa, 25% in Asia and 15% in Latin America.

### Cassava genetic resources

The genus *Manihot* comprises some 100 species of which *M. esculenta* is the only cultivated species; wild materials include herbs, shrubs and trees. *Manihot* species originated in tropical America, and domestication and selection of cassava preferred large storage roots and vegetative propagation. Modern cassava is monoecious, highly heterozygous and outcrossed. Vigour and productivity are maintained through vegetative reproduction. Seed production is generally low owing to poor flowering and poor seed set, but genotypes with high seed production and high yield have been selected in cassava breeding programmes like those at CIAT, IITA and several National Programs. From its primary centre of diversity in tropical America, cassava was taken by Portuguese and Spanish to Africa and Asia between the mid-1500s and mid-1800s. Consequently secondary centres of diversity are found, particularly in Africa.

Because of collecting efforts, principally between the early 1970s and early 1980s, nearly 20000 clonal accessions of cassava (principally landraces) have been assembled in germplasm collections in nearly 40 countries (Table 1).

Table 1. National and international cassava collections assembled to 1997<sup>†</sup>

| Geographic area         | No. countries | No. clonal accessions |
|-------------------------|---------------|-----------------------|
| South America           | 6             | 9538                  |
| Central America         | 4             | 466                   |
| Caribbean               | 2             | 541                   |
| South Africa            | 5             | 1685                  |
| West and Central Africa | 11            | 4710                  |
| Asia and Oceania        | 11            | 2747                  |
| Total                   | 39            | 19687                 |

<sup>†</sup> Bonierbale et al. 1997.

In the CGIAR system, CIAT and IITA share the responsibility for cassava genetic resources conservation, with CIAT having the global mandate and IITA the mandate for Africa. The CGIAR international cassava collections, including over 9000 clonal accessions in total, are maintained in trust under an agreement signed with FAO in 1994.

### Cassava genetic resources conservation at CIAT

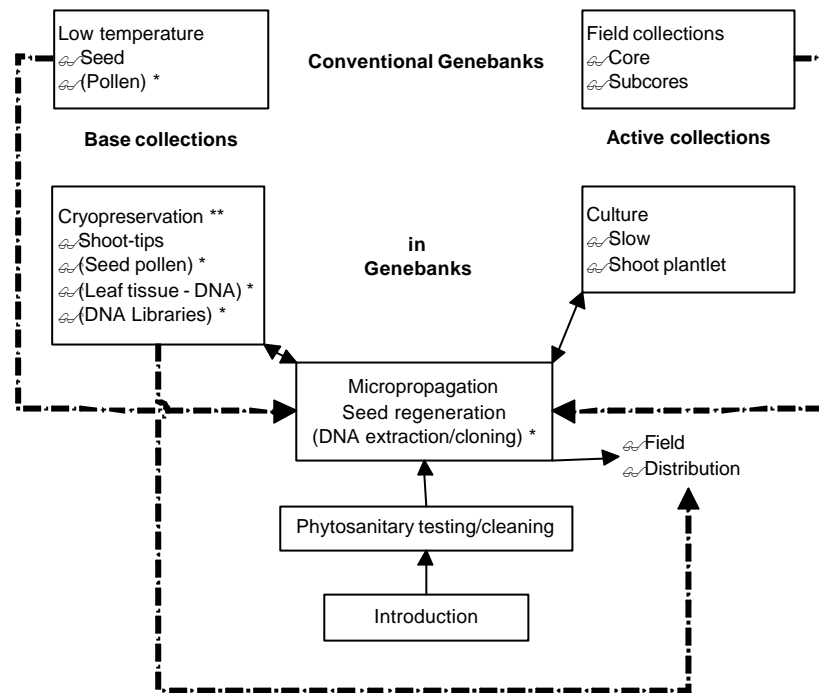
CIAT's activities in cassava conservation should be seen in the context of the Center's overall research programme on cassava genetic resources, which is designed to address key questions like the kind and amount of genetic resources to be conserved, the most appropriate methods of conservation, and the efficient utilization of cassava genetic resources. CIAT research on cassava genetic resources comprises the three areas and research activities, and the methods and techniques available at the Center to tackle the respective research areas, as indicated below.

| Research type   | Methods/tools   |
|---|---|
| <b>Research on genetic diversity</b><br>Spatial/geographic distribution<br>Genepool structure<br><i>Ex situ</i> collections vs. <i>in situ</i> diversity<br>Comparative genome mapping                  | GIS, molecular markers, botany, ecology, participatory research   |
| <b>Research on conservation</b><br>Rationalization of collections<br>– Identification of redundancies<br>– Development of core collections<br>Conservation methodologies<br>– Active and base genebanks | Morphology, agronomy, ecology, molecular markers, seed storage, <i>in vitro</i> storage, cryopreservation |
| <b>Research on genetic base</b><br>Measure diversity within/between species<br>Trait introgression  | Molecular markers, morphology, agronomy, wide crossing, transformation                                    |

Research on cassava genetic diversity has been critical for developing scientific criteria for improving conservation strategies, *ex situ* and *in situ*, including *in vitro* and cryopreservation methodologies, and for enhancing utilization of cultivated and wild genetic resources in cassava improvement.

### Integrated conservation of cassava genetic resources at CIAT

The genetic resources available for cassava improvement at CIAT include: collected landraces, advanced breeding lines and the crop's wild relatives. Figure 1 shows the overall scheme for a genus-wide conservation approach for cassava. The various conservation components shown in the diagram are considered complementary. They provide an integral conservation strategy for *Manihot* both for the long term and for immediate practical utilization of the international collections. Each of the conservation modalities shown in the scheme should be considered dependent on the dynamics of the research activities shown above, in particular on those under genetic diversity and conservation research.



**Fig. 1.** Integral, complementary, conservation methods for cassava genetic resources. (\*) Indicates components of conservation scheme not yet implemented; cryopreservation is entering the implementation phase.

The conventional and *in vitro* genebanks are complementary, as well as the active and base collections. It is envisaged that only the core and subcore collections will be maintained in the field, at any one time, while all clonal accessions will be represented all the time in the *in vitro* slow growth collections. Cassava germplasm can also be conserved in the base collections as seed and pollen, particularly as a means of maintaining genebanks. The role of cryopreservation in the cassava base collection scheme is considered extremely critical since all types of germplasm and material can be maintained, including all clonal accessions as shoot-tips, genebanks as seed and pollen, frozen leaf tissue of any material as sources of DNA, and DNA libraries. In addition, cryopreservation, once fully developed, should also provide an expeditious and cheaper means to duplicate the base collection for safety reasons, as well as for the distribution of germplasm sets to other countries/continents. Cassava being preferentially reproduced by vegetative means, the role of micropropagation is also key to the management of the integral conservation strategy. Improvement of current micropropagation through the implementation of bioreactor technology for both shoot-tip based and somatic embryogenesis methods will be essential. Finally, current advances in cassava molecular biology will contribute to implementing DNA banks and their utilization through molecular screening and DNA cloning in cassava improvement.

### Cassava collections assembled and conserved at CIAT

Table 2 shows the overall current status of the international cassava germplasm collections held in trust at CIAT. Over 6000 clonal accessions from 23 countries are maintained in the field and *in vitro* genebanks, including cultivated cassava, mostly landraces, selected improved materials, and wild *Manihot* genotypes representing 32 species. The greater size of the *in vitro* collection than the field collection is because many introductions *in vitro* have moved directly to the *in vitro* active collection (Fig. 1) and await phytosanitary testing before moving to the field for evaluation. Another reason is that a number of duplicates (identified through morphological and biochemical/molecular methods) have been eliminated from the field collection, to be maintained only in the *in vitro* collection.

**Table 2.** Current status of the cassava germplasm collections assembled and conserved at CIAT (CIAT 1998)<sup>†</sup>

| Germplasm                        | Type              | Number   |          |
|----------------------------------|-------------------|----------|----------|
|                                  |                   | In field | In vitro |
| Cultivated material <sup>‡</sup> | Clonal accessions | 4750     | 6017     |
| Wild material <sup>§</sup>       | Genotypes         | 10–15    | 353      |

<sup>†</sup> From 23 primary diversity countries, mostly Latin American and Caribbean.

<sup>‡</sup> *Manihot esculenta*.

<sup>§</sup> 32 wild *Manihot* spp.

### *In vitro* collections

Between the late 1970s and early 1980s apical meristem and shoot-tip based micropropagation of cassava was implemented at CIAT and implications in genetic resources conservation established (Roca 1984; Roca *et al.* 1989). The techniques utilize the MS basal medium supplemented with gibberellic acid (GA), benzyl aminopurine (BAP) and naphthalene acetic acid (NAA), and proved to be universally applicable for virus elimination (associated with thermotherapy and for micropropagation (through shoot-tip and nodal microcuttings). Modifications of these methodologies, involving media composition and osmotic concentration, and physical *in vitro* conditions of culture, allowed the gradual implementation of an active genebank for the cassava collection. Currently, more than 6000 clonal accessions comprise the *in vitro* active genebank at CIAT. Each accession is represented by six 18 x 25 mm test tubes, and the entire collection occupies a 50-m<sup>2</sup> room. Temperature and illumination have been set at 22–23°C and 1500 and 2000 lux, respectively. Under these conditions, subculture interval ranges between 12 to 18 months, depending upon the cassava genotype. More than 1500 accessions have been pathogen-tested, including the core collection (630 accessions). The CIAT cassava *in vitro* genebank comprises mostly landraces collected in Latin America, a set of advanced breeding lines and 325 wild *Manihot* genotypes. Unlike the landraces, the latter cultures derive from zygotic embryos because the shoot-tip culture of wild *Manihot* is still not well developed. After 10 years of slow growth storage, involving 10–20 subculture cycles, the genotypic

stability of seven varieties was assessed using molecular markers. No changes were observed in DNA fingerprintings vis-à-vis non-conserved controls (Angel *et al.* 1996).

### **The Pilot *In Vitro* Active Gene Bank Project**

Although the cassava *in vitro* collection has been operative at CIAT since the early 1980s, several aspects of the management of such a genebank were not established, particularly those that relate to scaling-up of the process. In order to tackle this issue, between 1988 and 1991, CIAT and IPGRI conducted a pilot project to assess the technical and logistical aspects of establishing and running an *in vitro* active genebank (IVAG) using cassava as a model (IPGRI/CIAT 1994). A condensed, representative sample of 100 genotypes from the cassava world collection assembled at CIAT was used for storage under slow growth conditions, and the following parameters were monitored throughout the process: phytosanitary status of cultures, culture viability, genetic stability, sample size and subculture interval. Also, the study determined needs of laboratory, equipment facilities, consumable items and staffing. A database system was designed for the management of *in vitro* genebanks. The project provided guidelines and testing parameters for establishing and running an IVAG (IPGRI/CIAT 1994).

### **Cassava cryopreservation at CIAT**

As show in Figure 1, cryopreservation is considered to play a critical role in the integral, complementary, conservation strategy for cassava. The technical developments in cassava cryopreservation have been described in these proceedings by Escobar *et al.* (this vol., p. 222). The major events in the evolution of cassava cryopreservation research at CIAT can be summarized chronologically as follows:

- |               |   |
|---------------|---|
| 1985          | First discussions on cryopreservation feasibility with Prof. A. Sakai and Dr K. Kartha in Hokkaido, Japan.  |
| 1988–<br>1990 | A collaborative project between CIAT and IBPGR, with the participation of the PBI, Saskatoon, was carried out. As a result, a technique for cassava true seed cryopreservation was developed (Marin <i>et al.</i> 1990), and the first viable shoot-tips of cassava variety M Col22 were recovered. The classical slow freezing technique was used, with a programmable freezing apparatus donated by the Japanese government.          |
| 1991–92       | Cryopreservation research continued with CIAT's core resources.   |
| 1993–98       | During this period, viable shoot-tips and plants were recovered using the slow freezing technique with a range of cassava varieties (Escobar <i>et al.</i> 1997), and fully grown plants were moved to the field for assessing genotypic stability. On the other hand, using a rapid freezing technique, shoot regrowth rates comparable to the slow freezing technique, were obtained. Interestingly, high genotypic response occurred |

with cassava landraces adapted to subtropical or high drought areas. The encapsulation-dehydration cryotechnique was also implemented in this period, with obvious advantages gained over the former two methods, in simplicity and rapidity of the technique. In addition, genotypic response was high (CIAT 1998). Informal cooperation with IPGRI took place in this period.

### **Future plans**

The assessment of the technical and logistical aspects of establishing and running a base genebank under cryopreservation will be the main CIAT activity in the period 1999–2001. Prior to this, the vitrification technique, and combinations with encapsulation, will be tested with cassava. This developmental work will be carried out as a pilot project using a subcore of the 630 cassava core accessions maintained in the *in vitro* active genebank. Important parameters such as monitoring genotypic stability, culture viability, sample size per clonal accession, and number of replicates, will be key components of the project. Integration with other CGIAR-mandated vegetatively propagated crops like potato, yams and banana/plantains is considered highly appropriate, and thus a collaborative effort between CIAT, CIP, IITA and INIBAP is being planned.

### **The role of research in cassava genetic diversity**

CIAT has developed a repertoire of molecular genetic markers for cassava including RAPDs, RFLPs, AFLPs and SSRs, both nuclear and cytoplasmic, which are integrated with morphological, edaphoclimatic and ecological data. This information can be used for assessing diversity at inter- and intraspecies level, drawing phylogenetic relationships between *Manihot* spp. and cassava and describing structural relationships between unimproved (landraces) and improved (elite material) germplasm. And through GIS, we can describe the geographic distribution of diversity. Diversity data will serve to quantify similarities among accessions, define core and subcore collections, for short- and long-term cassava conservation.

It would be possible also to relate molecular diversity and geographic origin to *in vitro* and cryogenic response of cassava landraces, and define sets of wild species for *in situ* and/or *ex situ* conservation. For example, recent analysis of cassava relationships with wild *Manihot* species using molecular markers has provided support to the existence of three subspecies: the domesticated *M. esculenta* subsp. *esculenta*, and its two wild forms *M. esculenta* subsp. *peruviana* and *M. esculenta* subsp. *flabellifolia* (Roa *et al.* 1996; Bonierbale *et al.* 1997). This information will find direct application not only in designing genus-wide conservation strategies for cassava, but also for enhancing the genetic improvement of the crop. Cassava molecular markers such as AFLPs and SSRs, and the recent development of the cassava molecular genetic map at CIAT (Fregene *et al.* 1997), provide powerful tools for monitoring and assessing the genotypic stability of *in vitro* and cryopreserved cassava germplasm, and for enhancing the utilization of wild *Manihot* spp. in crop improvement.

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