TISSUE CULTURE METHODS FOR GERMPLASM CONSERVATION*

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INTRODUCTION

The need to maintain selected clones and promising hybrids in a disease-free condition for distribution to national programs stimulated some of the early studies on *in vitro* germplasm maintenance of potatoes (*Solanum*) and cassava (*Manihot*).

Immediate utilization of the improved germplasm was the major force behind these early *in vitro* conservation efforts with potatoes and cassava (Schilde-Rentschler and Roca 1987, and Roca, 1985). At about the same time Morel (1975) and Henshaw (1975) advocated *in vitro* culture techniques for plant genetic resource conservation, in contrast to merely holding collections for immediate use in plant breeding. Also during this period, successful work on the conservation of plant tissues by cryopreservation took place at the University of Nottingham under the late Professor Street (1973). It was only in 1980 that the potential of *in vitro* culture methods for the conservation of "difficult" plant species was recognized (Withers and Williams 1985). These included vegetatively propagated crop plants and those whose seed was unresponsive to orthodox seed conservation conditions such as low temperature and

* Paper presented at the OAS/SRG Biotechnology Conference, "Tissue Culture Technology for Improved Farm Production", Sept. 30-Oct. 3, 1991, Kingston, Jamaica, W.I.

decreased water content, which is typical of many tropical perennial fruit species and various palms. In the early 1980s, following a report on global *in vitro* conservation by Withers (1979), the International Board for Plant Genetic Resources (IBPGR) established a working group of scientists to look at all aspects of *in vitro* plant conservation. The following series of events occurred (Withers 1980; Bajaj 1977; International Board for Plant Genetic Resources 1983); (i) the basic conceptual framework for *in vitro* conservation was clarified; (ii) species requiring priority attention for *in vitro* conservation were identified; and (iii) the following critical areas of research in *in vitro* conservation were identified: the problem of genetic stability of cultures, the need for parameters for characterization of accessions entering *in vitro* storage, the need for disease indexing techniques at the *in vitro* level, development of techniques for *in vitro* germplasm collection and exchange, and development of an efficient documentation and data management system for *in vitro* conservation.

In vitro conservation has to be considered part of the overall conservation strategy for a particular plant species, as a valuable adjunct to genetic resource conservation. In some cases, *in vitro* storage would be the only strategy for a given species, for example, for some tropical fruit trees such as cacao (*Theobroma*). Tuber and root crops like potatoes, cassava, and sweet potatoes (*Impomoea*) would probably be stored as seed and *in vitro* methods being prefered for the conservation of genotypes (cultivars, hybrids, elite clones, etc.). For other tropical tuber and root crop genotypes and fruit species (e.g., *Musa*) that seldom produce seed and are totally sterile, *in vitro* storage and *ex situ* field gene banks would probably be complementary. In the case of tropical species with recalcitrant seed, *in vitro* collection and exchange should play a basic role.

IN VITRO CONSERVATION

The range of crop species whose conservation is potentially achievable through *in vitro* techniques is wide. In addition to the species listed above, several genera of asexually propagated-plants can be included, such as *Colocasia, Xanthosoma, Vitis,* and *Saccharum.* Furthermore, genera traditionally propagated by sexual means but whose seed is recalcitrant or presents sterility problems are also potential candidates for the *in vitro* approach. This range of species includes *Theobroma, Citrus, Coffea, Cocos,* and *Elais.*

Conservation of plant genetic resources by means of *in vitro* culture methods can be achieved by changing the culture environment to slow down or totally suppress the growth of cells and tissues, the objective being to either increase the culture transfer period as much as possible or extend it indefinitely.

Two types of *in vitro* gene-bank conservation have been proposed (Withers and Williams 1985): (i) the *in vitro* active gene bank (IVAG), where cultures are maintained under slow growth and (ii) the *in vitro* base gene bank (IVBG), where cultures are cryopreserved. The IVAG is being developed, to a large extent, for cassava, potatoes, sweet potatoes, banana, and sugar cane and constitutes a working collection. Its counterparts would be field collection and sexual seed collection for short-term storage. The IVBG constitutes a base collection; cryopreservation is still not fully developed for a given crop. The latter approach enables germplasm genotypic stability to be fully maintained, and its counterpart would be sexual seed collection for long-term storage.

FACTORS INFLUENCING *IN VITRO* CONSERVATION

Tissue culture capability

Regeneration of whole plants from cell culture systems is often the limiting step in applying *in vitro* culture techniques to plant species that cannot be propagated by means of preformed meristems. Despite the ability to initiate callus from a variety of tissues and organs in many crop species, reproducible regeneration of whole plants remains problematic. Adventitious regeneration via somatic embryogenesis is highly desirable, as the process affords high multiplication rates and results in propagules that possess both root and shoot axes (Stamp and Henshaw 1987). Somatic embryos may develop from single cells, and plants with more stable genotypes may be recovered (Evans et al. 1981).

Tissue culture viability

The evaluation of viability of *in vitro* cultures should be carried out systematically. Under slow-growth conditions, in which the cycle term is extended for several months or years, evaluation of cultures should be more frequent than with potentially indefinite conservation under liquid nitrogen, for example. The most important features to be evaluated under slow-growth storage of shoot tip derived cultures are (i) contamination, (ii) leaf senescence (the ratio of green leaves to dead leaves), (iii) number of green shoots (for further micropropagation), (iv) presence or absence of roots, (v) occurrence of callus, etc.

Genotypic stability

Genetic stability in cultures has long been a matter of concern in the potential application of *in vitro* techniques for germplasm conservation. The material retrieved from *in vitro* conservation should genetically represent the material accessed.

In vegetatively propagated crops, morphological criteria have been applied to characterize genotypes, but such differences are difficult to detect in cultures propagated *in vitro*. I has been pointed out that genetic variation due to chromosomal rearrangement can occur in tissue culture (D'Amato 1964). A correlation exists between the time during which the plant material is grown as callus and the probability of chromosome changes. This could cause a shift to a variant type in *in vitro* propagation (Schilde-Rentschler and Roca 1987). The use of axillary buds as a base for multiplication reduces the probability of genetic variation. Therefore, the formation of callus and adventitious buds should be avoided. The stability record of cryopreserved material from higher plant cultures is only now being tested in this respect (Whiters 1988; Schilde-Rentschler and Roca 1987).

Monitoring the genetic stability of *in vitro* plant cultures of crop species is becoming of great interest. Morphological, biochemical, and molecular criteria for the detection of genetic changes have been proposed (Withers and Williams 1985). Techniques to assess isozyme variability in small samples of tissue obtained from *in vitro* cultures should be developed as a complementary method of stability evaluation. Furthermore, monitoring genetic stability of cultures by molecular techniques, e.g., variation in fragment lengthy polymorphisms and use of genetic fingerprinting should be tested.

Data management

An *in vitro* gene bank should maintain and continuously update a computerized data base of information related to all aspects of *in vitro* conservation and associated research areas. The data base should include a large amount of information related to each of the accessions, such as passport data, genotypic characterization, disease indexing, micropropagation, international exchange, equipment and supplies, and other logistics of an *in vitro* conservation operation.

IN VITRO CONSERVATION OF CASSAVA

Cassava (*Manihot esculenta* Crantz) is one of the most important staple food crops of the lowland tropics. It constitutes a major source of calories for nearly 500 million people in over 60 countries. Present world cassava output reaches 130 million tons, most being produced in southeast Asia, Africa and tropical Latin America. Cassava is mainly utilized for human consumption and is available in the fresh market or processed into flour.

The potential danger of genetic erosion of both cultivated and wild *Manihot* germplasm resources and the requirement of genetic variability for improvement of the crop justify cassava germplasm conservation efforts.

Conventional maintenance of cassava germplasm collections is carried out by continuous vegetative field cultivation. New germplasm plantings often use freshly cut stakes from old fields. In addition to high costs, field maintenance often exposes the valuable germplasm to insect attack, disease, and soil or climatic problems. Freshly cut stakes can only be stored for a short time because of premature sprouting and insect or microbial attack.

Vegetative propagation often exposes the cassava crop to a wide range of pests and diseases which can be transmitted via the stakes through successive generations. For instance, cassava bacterial blight, African mosaic disease, superelongation disease, and frog-skin disease can produce great losses.

Maintenance of cassava germplasm by meristem culture can be done through a combination of cryogenic techniques and minimum-growth storage conditions.

Slow-growth storage

Recent research on cassava conservation at CIAT has provided means to store *in vitro* cultures under minimal conditions of growth. It has been shown (Roca 1984, 1985) that storage at 23-24°C decreases the rate of shoot elongation to about one-fifth of that of cultures kept at 28-30°C. Storage temperatures lower than 18°C were detrimental to a number of cassava varieties if the illumination was kept high. However, culture viability could be maintained by lowering the illumination to less than 500 lx. Increasing benzyl adenine (BA) from 0.04 to 0.11 mM and sucrose from 0.050 to 0.12 M also decreased shoot elongation but maintained 90% viability. However, if low-temperature storage was combined with high BA and sucrose levels, the growth of cultures was arrested to such a degree that most of them deteriorated after 3 months. Recent findings indicate that culture growth is decreased when the total nitrogen content of the medium is lowered to 20mM at 27-28°C and 40mM at 20-22°C. Mannitol at 5-25 mM was found to be effective not only in arresting growth but in decreasing tissue viability at the lowest storage temperature. However, if mannitol was added to the medium, along with 0.088 and 0.18 M sucrose, culture viability significantly increased at both low and high storage temperatures.

Nearly 5000 varieties from the world cassava germplasm collection assembled at CIAT have been put into *in vitro* storage under minimal conditions of growth. Depending on the variety, these cultures can be maintained for 15-18 months without being transferred to fresh media. Varieties differ in their tolerance to reduced temperature and in their rate of growth. Furthermore, old cultures of certain varieties tend to deteriorate as a result of the oxidation of phenolic-type exudates from the roots. Throughout storage, the cultures produce axillary buds, and the number of axillary buds is directly related to culture viability and hence to micropropagation potential upon retrieval from storage. At the end of each storage cycle the axillary buds are transferred to fresh medium to initiate a new cycle. The *in vitro* germplasm bank (a room measuring 5 x 6 x 2 m) can potentially hold 6000 cassava accessions, which would otherwise require 6 ha of land.

Sample cultures are retrieved at random from storage once per year, micropropagated, and grown in the field along with stake-propagated plants. Evaluation of phenotypic stability is underway, using morphoagronomic and biochemical criteria (Ramirez et al. 1987). Information collected to date indicates that in general, the plants look true to type. Narrowing of leaf lobes has been observed in a few varieties following retrieval from storage and growth in the field, but reversion to the wide-lobe type began after the second vegetative growth cycle.

Cryopreservation

Through a collaborative project between CIAT and IBPGR a basic protocol for the cryopreservation of cassava shoot tips in liquid nitrogen was recently developed (Biotechnology Research Unit, CIAT, 1991). Two-three mm shoot tips were cryopreserved with DMSO, sorbitol and sucrose, and freezed at 0.5°C/min to -35°C and then immersed directly into liquid nitrogen (-196°C). After rapid thawing, up to 90%

viable tissues and up to 50% shoot growth was obtained with the cassava cv. M. Col 22. Although success of cryopreservation is still variable according to the cassava genotype, the results obtained so far has opened the way to the development of a long-term base, gene bank, for cassava vegetative material.

CONCLUSION

Slow-growth storage is now a viable method for maintaining large collections in a small space free of pests and risk of disease. Currently, *in vitro* active gene banks exist for *Manihot*, *Solanum*, and *Ipomoea*, and work is advanced with *Colocasia, Xanthosoma, Musa,* and *Saccharum.* The future of genetic resource cryopreservation is promising and there are efforts at present underway with cassava, potato, and banana.

The establishment of an *in vitro* active germplasm bank has become important for cassava germplasm at CIAT, and recent advances in cryopreservation promises the establishment of a base gene bank for this important crop plant.

Plant regeneration of a number of cassava cultivars by somatic embryogenesis is possible. Secondary embryogenesis has been established, permitting plant regeneration from long-term embryogenic cultures (Szabados et al. 1987). On the other hand, construction of a molecular map of cassava based on RFLP and RAPD markers has recently been initiated at CIAT (Biotechnology Research Unit, CIAT, 1991). These developments will help the future use of cellular and molecular genetic methods in cassava germplasm characterization and crop improvement. The availability of comprehensive, readily accessible *in vitro* germplasm collections, under both slow-growth and cryogenic conditions, will be crucial to that effort.

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