In Vitro Conservation of Vegetatively Propagated Crops

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Abstract

Germplasm preservation in vegetatively propagated crops is usually achieved by using propagules or by maintaining field collections. Since these methods are costly and prone to heavy losses, *in vitro* techniques have been developed to overcome some of the problems of maintaining field collections. Many species can now be successfully stored from a period of a few months to over 3 years, depending on the species. Storage techniques include reduction in incubation temperature, use of a slow growth culture medium, modification of the gaseous environment, or a combination of any of the above methods. Collections of potatoes (*Solanum* spp.), sweet potatoes (*Ipomoea* spp.) and cassava (*Manihot* spp.) are preserved by the International Research Centers in South America for distribution to breeding programs in different countries. Most countries have also established facilities for *in vitro* collections. In Chile, a joint project between INIA and JICA led to the construction of tissue culture facilities which enable to maintain collections of potatoes, sweet potatoes, *Fragaria* spp., *Vaccinium* spp., *Allium* spp., and other species. The use of this technique has contributed significantly to the improvement of germplasm exchange among countries, providing a safe method not only for the introduction of germplasm, but also for the rapid micropropagation of selected cultivars. Also, it has now become possible to effectively screen for virus and obtain plants free from virus and other diseases.

Introduction

The need for preserving genetic diversity for future use is well documented (Frankel,1971). The vast majority of plant species can be stored using seed or pollen. However, a group of economically important species can not be preserved using these reproductive structures, because they are obligate apomicts, produce few, short-lived or recalcitrant seeds, or are sterile. A number of fruits and vegetables, and several of the world's staples, are examples where alternative and/or complementary methods of preservation are needed to effectively preserve their genetic diversity. Furthermore, in breeding such species, there is often a need for preserving the exact genotype of selected individuals. These vegetatively propagated species are usually preserved by storing, under appropriate conditions, tubers, roots, bulbs and cuttings or by maintaining collections in the field. These methods are costly and prone to heavy losses due to disease and pest attacks or are subject to environmental and political threat.

It is now possible to use safer, and generally more economic, *in vitro* culture techniques for short- and medium-term preservation of vegetatively propagated species, as an alternative or as a complement to field collections. Furthermore, biotechnology and plant molecular biology now offer additional tools for multiplication, distribution, characterization and evaluation of these materials.

This paper describes the procedures put forward in Chile by a joint project of the Japan International Cooperation Agency (JICA) and the Chilean Institute of Agricultural Research (INIA) for the introduction, multiplication, and distribution of germplasm of vegetatively propagated species relevant to the country's agriculture.

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Germplasm introduction

Tissue culture techniques can be a safeguard to improve the phytosanitary aspects of plant introduction. The system has a built-in capability for detecting pest and pathogens, because if the growing media remain uncontaminated with fungi and bacteria, the system must be free of insectes, mites, nematodes, and pathogens (Kahn,1976). Viruses and certain fungi, with specific requirements not satisfied by the composition of the medium used for plant growth, are not detected by the system. Therefore, when plants are introduced to a country using *in vitro* culture techniques, there is a minimal risk of introducing their associated pests and pathogens, but special precautions should be taken to prevent spreading of viruses and certain fungal diseases.

Chile is a virtual sanitary island due to its isolation by mountains to the East, a desert to the North and oceans to the South and West. Therefore, germplasm introduction of vegetatively propagated crops using *in vitro* culture techniques is highly recommended if the sanitary patrimony of the country is to be preserved. *In vitro* germplasm introduced by INIA in recent years is listed in Table 1 and includes potatoes, sweet potatoes, blueberries, raspberries, strawberries, kiwifruit, pears, grapes, pineapple, banana, taro, garlic, and asparagus.

In most cases materials were introduced from pathogen-tested collections kept by International Reseatch Centers in Latin America, like the International Potato Center (CIP) or the International Center for Tropical Agriculture (CIAT). Also, germplasm has been introduced from other countries like Japan, the U. S. A. and Europe.

Typically, materials used for germplasm exchange consist of plantlets in "stage 2" or "stage 3" of the micropropagation scheme (Debergh and Maene, 1981). Plantlets in stage 2 are in their multiplication phase while those in stage 3 are already rooted and ready to be acclimatizated, *viz.*, ready to be transferred to a green house or to the field. Other propagules can also be used for germplasm exchange. In potatoes, microtubers produced *in vitro* (Tovar *et al.*, 1985) have been used, and in garlic microbulbs induced *in vitro* have proved effective in certain genotypes (Moriconi *et al.*, 1991). These types of propagules are preferred, when germplasm is received in locations where tissue culture or acclimatization facilities do not exist. This was demonstrated when an experimental exchange of selected garlic clones was carried out using *in vitro* plantlets (Muñoz *et al.*, 1988). The method was effective for receptors that had tissue culture facilities, but totally inadequate for those lacking them, because acclimatization was very difficult, and a large number of plants died during this process.

Table 1 Germplasm introduced to Chile by INIA using *in vitro* culture techniques

Species	Number of accessions	Origin	Year of introduction
Potatoes	163	Perú, USA, Brazil	1990-93
Sweet potatoes	27	Perú	1991-92
Blueberries	29	USA	1988-92
Raspberries	3	USA, Italy	1991
Strawberries	25	USA, Japan, Argentina	1990-92
Kiwifruit	2	Italy	1990
Pears	2	USA	1992
Grapes	14	Japan	1991
Pineapple	2	Costa Rica	1990
Banana	2	Israel	1990
Taro	4	Costa Rica	1990
Garlic	8	Argentina	1991
Asparagus	2	Japan	1992-93

Virus testing

When plants do not originate from pathogen-tested collections it is advisable to screen for the presence of virus in the introduced materials. Procedures to screen for viruses include examination of visual symptoms, infectivity tests employing indicator plants, serological tests, electron microscope observation, and direct detection of RNA using molecular biology techniques. In our laboratory we have extensively used this latter technique to non-specifically detect the presence or absence of viruses in introduced materials. We are now routinely using this method as the first step for assessing the sanitary status of imported vegetatively propagated plant materials. The techique most commonly used consists of the extraction, isolation and characterization of double-stranded RNA (ds-RNA) which is produced by most plant viruses during their replication phase (Dodds *et al*., 1984). Thus, the presence of ds-RNA's in a sample strongly suggests a virus infection. In order to further pursue the identification of a virus, this non-specific virus detection method should be followed by serological, electron microscopy and/or other known methods.

Micropropagation

After introduction it is often necessary to propagate the introduced materials in order to use them in breeding or to directly evaluate the materials under field conditions in different environments. Furthermore, once selected clones are ready for farmer's use, an efficient and sanitary effective propagation tool is required.

Micropropagation is a very useful means to rapidly propagate true-to-type clones. Protocols for *in vitro* culture of a number of species are available (George *et al*., 1987) and the list of the genotypes that can be micropropagated is increasing rapidly. Despite the multiple advantages of micropropagation, which includes a year-round operation, a geometric multiplication rate of the plant materials, enhancement of the propagation ability due to rejuvenation of the propagation materials (Swartz *et al*., 1981), and the sanitary quality of the plants produced, there are a number of disadvantages which should be considered before a micropropagation scheme is applied in a particular crop.

One of the problems is that *in vitro* micropropagation can result in the production of off-type plants. This may be an advantage when additional variation is needed in a population, but it is an obvious disadvantage for clonal multiplication. This permanent genetic variation, which has been referred to as somaclonal variation (Larkin and Scowcroft, 1981), usually originates from preexisting variation in the source plant or can be induced by the *in vitro* process (Swartz, 1991) and should be avoided if true-to-type plants are to be produced.

Temporal alterations in plant performance, which are epigenetic, can also cause problems when micropropagated plants are used. Flowering is often delayed and plant morphology resembles that of seedling plants (Swartz, 1991). Although most plants will eventually recover from this condition, fruit bearing will be delayed which may have significant economic effects.

To avoid unexpected genetic variability, tissue dedifferentiation and redifferentiation should always be prevented during the propagation scheme and microcuttings including axillary buds should be selected as explant material. Also, extensive field testing of *in vitro*-propagated plants is absolutely essential to prevent problems associated with epigenetic changes like induced juvenility and rejuvenation or habituation.

Our laboratory has been considerably involved in the development of micropropagation protocols for a number of species. Most of them have been directly adopted from published data, but for a number of species protocols had to be developed. We have created or improved protocols for the micropropagation of several of the North American *Vaccinium* species and for South American species like pepino (*Solanum muricatum*) and pitaya (*Cereus triangularis*). Field tests are also under way in order to evaluate the performance of these micropropagated species.f

Germplasm preservation

Although *in vitro* culture techniques were initially developed for the propagation and genetic manipulation of plant species, it is now possible to use these methods for conserving plant genetic resources (Kartha, 1982; Withers *et al*., 1990; Roca *et al*., 1991; Dodds, 1991). There are two basic systems for *in vitro* germplasm conservation. One of them is cryopreservation, which totally suppresses growth and cellular metabolism. The other, only limits, the growth rate of the preserved plant materials. This paper will consider only this last method of preservation.

Protocols for preserving plant material *in vitro* are available for successful storage of almost 50 plant species which can be stored for a few months to over 3 years depending on the species (Ng and Ng, 1991). The strategy for conserving germplasm using *in vitro* culture techniques is to reduce the growth rate of the plant material to a minimum in order to extend the period between subcultures, without affecting the survival. This can be achieved by reducing the incubation temperature, by using a slow-growth culture medium, by modifying the gaseous environment, or by a combination of any of the above methods (Ng and Ng, 1991).

A decrease in the incubation temperature is the method most commonly used to reduce the growth rate of the cultures. Normal incubation temperatures are in the range of 25°C, whereas temperatures ranging from 1 to 22°C can effectively reduce the growth rate of most species. Storage temperatures depend on the cold tolerance of particular crops. Species originating from temperate zones, like *Fragaria* spp. (Mullin and Schlegel, 1976) and *Malus* spp. (Lundergan and Janick, 1979) can withstand the lowest storage temperatures, while those originating from tropical areas like *Musa* spp. (Banerjee and De Langhe, 1985) should be stored at higher temperatures (15°C).

Often reduced incubation temperatures are used together with a reduced-growth medium to further decrease the growth rate of plantlets. A reduced-growth medium generally consists of lower salt or sucrose concentrations (Monette, 1986), a high osmoticum (Espinoza *et al*., 1984), or a medium to which a growth retardant has been added (Roca *et al*., 1991).

Modification of the gaseous environment can be accomplished by using culture containers of different sizes, different sealing systems (George and Sherrington, 1984) or by the addition of mineral oil to the culture medium (Caplin, 1959).

Light is another factor that can limit plant growth rate. *In vitro* cultures are generally incubated under an irradiation of 38 $\mu Em^{-2}seg^{-1}$, but incubation under lower irradiation (Banerjee and De Langhe, 1985) or under complete darkness (Mullin and Schlegel, 1976) has been reported as to be efficient systems for germplasm storage in various species.

It has already been mentioned that *in vitro* tissue culture can result in genetic variability. This variability can be due to preexisting chromosome alterations in the explant or the expression of mutations associated with the large number of stresses that explants were subject to during the different phases of the culturing process (Scowcroft, 1984). This variability can be a serious drawback if clones are to be conserved for direct use, but may have no consequences if the total variability within a population is to be preserved.

Using well established *in vitro* preservation techniques, International Research Centers in South America now keep extensive clonal collections of certain crops for distribution to breeding programs in different countries. Over 3,000 accessions of potatoes (*Solanum tuberosum* and related species) and 2,300 of sweet potatoes (*Ipomoea batata*) are kept by the International Potato Center (CIP) in Perú, and over 5,000 accessions of cassava (*Manihot esculenta*) are stored at the *in vitro* active bank of the International Center for Tropical Agriculture (CIAT) in Colombia.

Most countries are also constructing facilities for keeping *in vitro* collections of selected species. In Chile, a 5 millon dollar project between INIA, the National Institute for Agricultural Research and JICA, the Japan International Cooperation Agency, built facilities and trained personnel to operate 1 seed base bank, 3 active banks, a biotechnology laboratory and quarantine facilities to contribute to the introduction, preservation, characterization and use of plant genetic resources (Suzuki and Cubillos, 1991).

The biotechnology laboratory includes tissue culture facilities, where *in vitro* preservation work is starting. At the momoent, 145 accessions of potatoes (*Solanum* spp.), 27 of sweet potatoes (*Ipomoea* spp.),

20 of strawberries (*Fragaria* spp.), 44 of blueberries (*Vaccinium* spp.), 10 of garlic (*Allium* spp.) and various accessions of other species are kept in our tissue culture facilities.

In Table 2 the conditions used for storing these materials are presented. For potatoes (Espinoza *et al*., 1984) and sweet potatoes (Lizarraga *et al*., 1990) storage conditions recommended by CIP are adopted. For the rest of the species the micropropagation media used at our laboratory, have been slightly modified, and no attempts have been made, so far, to optimize a preservation medium for these particular species.

Also, available facilities allow karyotypic and biochemical characterization of the stored germplasm. For example, selected garlic clones were karyotypically characterized (Toyao *et al*., 1993) and wheat genotypes are being characterized using protein markers, like high molecular weight glutenins and gliadines (P. Hinrichsen and N. Hewstone, personal communication).

The project is expected to further extend its activities both to other species and to increase the collection of plant materials with particular emphasis on native species like *Fragaria chiloensis, Solanum* spp. and other vegetatively propagated species for which Chile has large germplasm reservoirs.

Table 2 Species and methods used for conserving germplasm using *in vitro* culture techniques at the National Agricultural Research Center (INIA) of Chile

Species	Plant material	Basic salts	Culture meida supplement	Temperature	Light	Storage between subcultures
Potatoes[1] *Solanum* spp.	Plantlets	MS	Mannitol 4 % Sucrose 0.5%	8 °C	37μE. $m^{-2} \cdot s^{-1}$	1 year
Sweet Potatoes[2] *Ipomoea batatas*	Plantlets	MS	GA_3 0.1mg/1 Glucose 2 % Sorbitol 1.5% Putrescine HCI 20ppm Agar 0.4%	25°C	37μE. $m^{-2} \cdot s^{-1}$	1 year
Strawberries *Fragaria* spp.	Plantlets	1/2 MS	Sucrose 3 % phloroglucinol 162 mg/1 Activated charcoal 5.0 g/1 Agar 0.4%	4 °C	Darkness	6 months
Blueberries *Vaccinium* spp.	Shoots	WPM	2 ip 5 mg/1 Sucrose 2 % Agar 0.4%	25°C	37μE. $m^{-2} \cdot s^{-1}$	6 months
Garlic *Allium* spp.	Micro-bulbs	B_5	NAA 0.2 mg/1 IBA 3.0 mg/1 Fe-Citrate 30 mg/1 Sucrose 2 % Agar 0.5%	25°C	37μE. $m^{-2} \cdot s^{-1}$	3 months

[1] Espinoza, N. Estrada, R., Tovar, P., Bryan, J. and Dodds. J. H. (1984)
[2] Lizarraga, R., Panta, A., Espinoza, N y Dodds, J. H. (1990)

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