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In Vitro Conservation of Banana: Medium-term Storage and Prospects for Cryopreservation

I. Van den houwe[1] and B. Panis[2]

BANANAS: IMPORTANCE AND USES

For many people in the tropics, bananas are an essential component in their daily diet. The fruits, parthenocarpically produced, are eaten raw, cooked, brewed into an alcoholic beverage or processed into chips. The leaves are used for wrapping food, the terminal bud of the inflorescence cooked as a vegetable, fibers used for ropes and corm tissue (*Ensete* spp.) fermented. Bananas are native to Southeast Asia and evolved from inter-and intraspecific hybridization between two wild diploid species of the genus *Musa*, i.e., sp. *acuminata* (AA) and sp. *balbisiana* (BB) (Simmonds and Shepherd, 1955). Because of the different contributions of the two main subspecies, edible cultivars of the following genomic configuration are currently cultivated : diploids AA and AB ; triploids AAA, AAB, and ABB; and the tetraploids AAAA, AAAB, AABB, and ABBB. Triploids are more widely cultivated than diploids, while tetraploids are very rare.

The total world production in 1995 was about 85 million tons (FAO, 1995), of which 36% consisted of plantains. Plantains, mostly locally consumed, form a subgroup within the triploid AAB bananas which are used for cooking. The export trade deals almost entirely with dessert bananas (AAA Cavendish type) and accounts for only 10% of the world production.

Bananas production is threatened by many pests and diseases caused

[1] INIBAP*Musa* Germplasm Transit Centre, c/o Laboratory of Tropical Crop Improvement, K.U. Leuven, K. Mercierlaan 92, B-3001 Heverlee, Belgium.
[2] Laboratory of Tropical Crop Improvement, K.U. Leuven, K. Mercierlaan 92, B-3001 Heverlee, Belgium.

by fungi, viruses, bacteria, nematodes and insects. Especially the fungal diseases, black Sigatoka and the Panama disease, respectively caused by *Mycosphaerella fijiensis* and *Fusarium oxysporium* are very destructive. It was the Panama disease that destroyed in the beginning of this century the Gros Michel-based export trade and forced its replacement by Cavendish-type bananas.

Most cultivated, but also recently collected bananas, have neither been taxonomically described nor evaluated for their yield potential, resistance to pests and diseases, and adaptation to new habitats. Bananas are jungle weeds and since deforestation in Asia is proceeding at an alarming rate, edible cultivars as well as their wild ancestors need to be collected and subsequently stored for use in classical breeding programs (Swennen and Vuylsteke, 1993), modern biotechnology (Sagi et al. 1995), and as short-term replacement of disease-affected banana cultivars (Hahn et al., 1990).

EX SITU GERMPLASM CONSERVATION

Introduction

The conservation of *Musa* through seeds is feasible but restricted to wild germplasm, as edible bananas do not produce seeds. It is a cheap alternative for field genebanks but slow germination of the banana seeds might be a hindrance for its routine application. Chin (1995) proved that *Musa* seeds could be stored for a period up to one year, provided their moisture content was reduced to 10%.

Until the 1970s, the only means of conserving cultivated forms *ex situ* was in field genebanks, where the material is maintained in the vegetative stage. Field genebanks are most valuable for germplasm characterization and evaluation but have some significant drawbacks. They are most vulnerable to diseases, pests, and adverse weather conditions. Even if these risks are strictly minimized, not all cultivars of one crop can be kept in optimal conditions at one ecological site since they are adapted to different ecological habitats. For example, beer and cooking bananas of the AAA group are exclusively grown in East Africa where they thrive in the highlands at altitudes between 1,200 and 1,900 m above sea level. Therefore, it is unlikely that one field genebank would represent the entire variation of the crop's genepool. Bananas are perennial giant herbs that occupy a considerable amount of space and need to be transplanted periodically. Moreover, banana field collections demand vast areas of land and a large input of labor and careful management. In addition, the supply of samples from a field genebank involves the risk of spreading diseases and is time consuming because

of the low multiplication rate of bananas and plantains by the conventional method.

Considering the disadvantages of *Musa* field genebanks, *in vitro* collections can be viewed as valuable complements. The risk of losing germplasm *in vitro* is much lower, the method allows conservation in minimal space with fewer inputs and cultures are protected from pests and diseases. Thanks to the technique of *in vitro* multiplication, requests for plants can be met rapidly. Under the appropiate storage conditions the duration of *Musa* shoot-tip cultures can be extended to approximately one year requiring less labor input. A major impediment of tissue culture collections is the occurrence of somaclonal variation. Such variation can be useful as a source of new variation for breeding but forms a threat for the conservation of distinct genotypes.

In Vitro Conservation: The INIBAP *Musa* Germplasm Transit Centre Collection

History

In the 1970s, a new *in vitro* technique for the propagation of *Musa* using shoot tips was developed (Berg and Bustamante, 1974). Shoot-tip culture was successfully applied for the micropropagation of 'Cavendish' bananas (Ma and Shii, 1972) and since then a wide range of *Musa* genotypes has been found amenable to this *in vitro* technique. In the early 1980s, shoot-tip culture was adopted at the Laboratory of Tropical Crop Improvement of the Catholic University, Leuven in Belgium (Banerjee and De Langhe, 1985) to maintain a small *in vitro* working collection of nearly 20 banana accessions. In 1984, the International Network for the Improvement of Bananas and Plantain (INIBAP) was established and the Laboratory of Tropical Crop Improvement chosen to host its Germplasm Transit Centre (ITC) and *in vitro* active collection. INIBAP was integrated with the International Plant Genetic Resources Institute (IPGRI) subsequently in 1994.

At present, this international genebank contains more than 1,000 accessions, representing most of the genetic diversity within the genus *Musa.* The composition of this collection is shown in Table 1. Organizations such as IPGRI, in collaboration with national and international institutions, have played a key role in the assembly of the collection. Accessions were acquired from existing field and *in vitro* collections among which are the collections of FHIA (Fundacion Hondurena de Investigation Agricola) at La Lima, Honduras, IRAZ (Institute de Recherche Agronomique et Zootechnique de la Communauté Economique des Pays des Grands Lacs) at Gitega, Burundi, BPI (Bureau of Plant Industry) at Davao, Philippines, CRBP (Centre Regional Bananiers et Plantains) at Njombe,

Table 1. INIBAP *in vitro* collection (tentative classification)

Classification Genus	Section	Species/group	Number of accessions
Ensete			
	Ensete		
		gilletii	1
		ventricosum	2
		unknown	1
Musa			
	Australimusa		
		Fe'i	9
		jackeyi	1
		lolodensis	2
		maclayi	7
		peekelii	5
		textilis	4
	Callimusa		
		coccinea	2
	Eumusa		
		acuminata	86
		balbisana	23
		basjoo	1
		beccarii	1
		boman	1
		schizocarpa	12
		acuminata x schizocarpa	5
		AA	230
		AAA	155
		AAAA	17
		AAAB	26
		AAB	313
		AABB	3
		AAS	1
		AB	7
		ABB	60
		ABBB	4
		BBB	1
		BBBB	1
		unknown	69
	Eumusa x Australimusa		
		AAT	5
	Rhodochlamys		
		laterita	2
		Ornata	3
		sanguinea	1
		velutina	2
	Unknown		
		unknown	3
Unknown			1

Cameroon, CIRAD (Centre de Coopération Internationale en Recherche Agronomique pour le Dévelopment), Guadeloupe and IITA (International Institute for Tropical Agriculture), Nigeria, from botanical gardens, and from national and regional breeding programs in more than 50 locations in the world. INIBAP participated in the collection of more than 250 edible diploids in Papua New Guinea in 1987–1989, organized by QDPI with the cooperation and support of IPGRI (International Plant Genetic Resources Institute) (Sharrock, 1990). Most of these valuable accessions were deposited as duplicates in the genebank in Leuven.

In 1995, accessions in the collection were designated to FAO and the international organization INIBAP received the mandate to conserve these genetic resources in trust for the International Community and for future generations. At present INIBAP is the repository for the world *Musa* germplams collection, with a total of 1,067 introductions, including 4 accessions of the genus *Ensete.* Although the collection could be considered fairly representative for the genus *Musa,* a continuous search is underway for resistance genes for the control of banana endangered by pests, diseases and environmental stresses. These useful genes may be found in traditional landraces and their wild relatives that still remain to be collected and conserved in areas where *Musa* diversity occurs. In the years to come, the INIBAP *in vitro* collection is expected to be further increased with elite germplasm developed by breeding programs, unique natural cultivars as well as wild forms obtained from collecting missions (botanical expeditions) to unexplored regions in the centers of origin, Southeast Asia and East Africa. In 1994 and 1995, over 100 new accessions were collected during 5 collecting missions in Vietnam. They are conserved in national field and *in vitro* genebanks in Vietnam and duplicates kept at the ITC.

The genebank also plays an active and key role in the exchange of *Musa* germplasm throughout the world: virus-tested accessions are freely accessible for fundamental and applied research, field evaluation, direct farmed utilization and for breeding.

Culture Media

A wide spectrum of tissue culture media have been tested for *in vitro* propagation of *Musa* spp. Most of the media formulations are based upon the Murashige and Skoog salt mixture (Murashige and Skoog, 1962), usually slightly modified and adapted to the specific needs of the plant tissue in a particular developmental stage. Modifications mostly involve organic compounds (carbon source, vitamins, growth regulators). Media used at the ITC are described in Table 2. The composition of mineral salts is identical to the one proposed by Murashige and Skoog, except for the doubled phosphate concentration.

Table 2. Composition of the modified Murashige and Skoog nutrient medium used for tissue culture of bananas and plantains at INIBAP Transit Centre

Compounds		Concentration	
		mg l^{-1}	μmol l^{-1}
Macronutrients	NH_4NO_3	1,650	20,630
	KNO_3	1,900	18,810
	$CACl_2.2H_2O$	440	2,990
	$MgSO_4.7H_2O$	370	1,500
	KH_2HPO_4	400	2,970
Micronutrients	H_3BO_3	6.18	100
	$MnSO_4.H_2O$	16.90	100
	$ZnSO_4.7H_2O$	8.60	30
	KI	0.83	5
	$Na_2MoO_4.2H_2O$	0.24	1
	$CoCl_2.6H_2O$	0.024	0.1
	$CuSO_4.5H_2O$	0.025	0.1
Iron	$FeSO_4.7H_2O$	27.80	100
	$Na_2.EDTA.2H_2O$	37.22	100
Vitamins	Glycine	2.0	26.6
	Thiamine hydrochloride	0.1	0.30
	Nicotinic acid	0.5	4.06
	Pyridoxine hydrochloride	0.5	2.43
Antioxidant	Ascorbic acid	10.0	56.78
Carbon source	Sucrose	30,000	87,642
Gelling agent	Gelrite	2,000	–
Growth regulators	N^6-benzylaminopurine		
		2.25	10*
		0.225	1**
	Indole-3-acetic acid	0.175	1

*For multiplication/storage **For regeneration

As a carbon source, sucrose in a concentration of 30–40 g l^{-1} medium is usually applied. Generally, media are agar-solidified (5–8g l^{-1}); however, for germplasm storage Gelrite (2–4 g l^{-1}) is preferred as clear gels are formed which allow detection of microbial contamination.

Banana tissue cultures often suffer from excessive blackening caused by oxidation of polyphenolic compounds released from wounded tissue. Therefore, most investigators include antioxidants such as ascorbic acid or citric acid in concentrations ranging from 10-150 mg l^{-1} or 2–40 mg l^{-1} L-cysteine. Alternatively, blackening can be reduced by dipping the explant in an antioxidant solution prior to transferring the tissue to the culture medium.

Other essential supplements of the culture medium are growth regulators, the cytokinin and auxin. Their concentration and ratio determines the growth and morphogenesis of the banana cultures. BA

(6-benzyladenine) is the preferred cytokinin in banana tissue culture as it gives better results for the shoot proliferation *in vitro* than kinetin or zeatin. Incorporation of this growth regulator in a concentration of 0.1–10 mg l^{-1}, promotes the growth of axillary buds and reduces apical dominance of any one main shoot. To initiate rhizogenesis several auxins have been reported in the literature to be incorporated in the culture medium. The most commonly used auxins are IAA (indole-3-acetic acid), NAA (α-naphthalene-acetic acid) or IBA (indole-3-butyric acid). They are added in concentrations up to a maximum of 0.2 mg l^{-1}.

Sometimes organic additives such as malt extract, casein hydrolysate and coconut milk are incorporated in the medium to stimulate growth. Although they may have a beneficial effect on the culture growth, they are often omitted from the media because their exact composition is undefined. The pH of the medium is adjusted to 5.8 prior to autoclaving during 10 min at 121°C and a pressure of 1.05 kg cm^{-2}.

Culture Initiation

Explants for tissue culture initiation can be derived from all apex-containing parts of the banana plant. This includes the apices of the parental pseudostem, small suckers, peepers, lateral buds and eyes, as well as the apex of the inflorescence and flower buds.

At the ITC, shoot tips are isolated preferably from young, vigorous, and healthy looking suckers of 40–100 cm in height with a corm diameter of about 10 cm. These should be collected from a flowering mother plant to guarantee trueness to-type. A selected sucker is trimmed to a cube of tissue containing the apical meristem. This clump of tissue is dipped in 70% ethanol for 10 seconds and then surface sterilized in a 2% sodium hypochlorite solution (containing 0.1% Tween 20). After 20 min incubation time the cube is rinsed three times in sterile water for 10 min. Subsequently, the shoot tip measuring about 3 by 5 mm is aseptically isolated. Several variants exist on this decontamination protocol. They differ in explant type and size, disinfestation method (single or double disinfestation) (Hamill et al., 1993), type of disinfestant (calcium hypochlorite instead of NaOCl) and its concentration, and duration of the treatment (Wong, 1986).

After surface disinfection, the tip, consisting of the apical dome covered with several leaf primordia and a thin layer of corm tissue at the base, is directly placed on a proliferation-inducing culture medium, described in Table 2. Subsequently, the culture is incubated under normal growth conditions at a temperature of 28 ± 2°C and a Photo-synthetic Photon Flux (PPF) of 63 $\mu E\ m^{-2}\ s^{-1}$, using cool-white fluorescent lights (24 h).

When first placed on the culture medium, shoot tips release excessively exudates which oxidize and form a barrier around the tissue preventing nutrient uptake. Therefore, fresh shoot tips need to be transferred every 1–2 weeks to a new medium during the first 4–6 weeks in culture.

The optimal size of the explant depends on the purpose to be met. For rapid multiplication a relatively large explant (0.3–1.0 cm) is desirable, whereas for virus elimination small meristems (1 mm) are preferably applied. The excision of meristems is slow, labor intensive, and a question of good skills. Initially, meristem cultures also show a high mortality rate and their growth is very slow whereas larger explants are more susceptible to blackening and contamination.

Multiplication

Shoot-tip cultures are stimulated to form multiple buds and shoots when cultured on a multiplication medium characterized by a relatively high concentration of cytokinin. Approximately 2–3 months after culture initiation, new axillary and adventitious buds/shoots may arise directly from the shoot-tip explant. These can be separated, trimmed and subcultured every 4–6 weeks.

The morphogenetic response of the explants differs among different cultivars and can be influenced by the composition of the culture medium. Experiments by Banerjee and Sharma (1988) revealed that cultivars having only an A genome have a lower proliferation capacity than cultivars having one or two B-genomes when grown on the same culture medium. Diploid and triploid cultivars tend to produce single shoots to a cluster of a few miniature shoots, wheras tissue cultures of AAB- and ABB-cultivars form a cluster of multiple shoots and/or numerous tiny buds in which outgrowth of leaves is limited. In general, multiplication of shoot-tips of genotypes, showing low levels of proliferation, can be greatly improved by increasing the concentration of cytokinins up to 20 mgl^{-1}. Higher concentrations have been found to have adverse effects on the multiplication rate and morphology of the culture.

In Vitro *Conservation*

Normal growth conservation: Cultures at normal growth require frequent subculturing. *Musa* spp. kept at an optimal temperature of 28 ± 2°C need to be subcultured every 2–4 months, involving an increased risk of losing valuable germplasm through accidental contamination, while subculturing, and mixing up varieties through mislabeling of changes in genotype through somaclonal variation (Scowcroft, 1984). Conservation of germplasm under short-term storage conditions can be most useful to breeders or researchers who wish to maintain a small working collection

of breeding products during the course of their evaluation, testing, selection and hybridization work, or to maintain products of *in vitro* manipulation and plants which cannot be conveniently conserved in any other way.

Slow-growth conservation: Slow-growth conditions are most suitable for the conservation of valuable germplasm if the material requires to be readily available for regeneration, multiplication and distribution. The technique has the benefit of limiting the number of subcultures, making significant savings in labor input and reducing the risk of mutations compared to germplasm kept under normal growth conditions.

Suppression of growth can be achieved by various modifications of the physical and/or chemical tissue culture environment. Reduction of the growth temperature from the optimum, imposition of osmotic and nutritional stresses (Roca et al, 1983; Staritsky, 1986; Bessembinder et al., 1993), application of exogenous growth inhibitors (Jarret and Gawel, 1991a, b), adaptation of the type (Reed, 1991), and size of culture container (Roca et al., 1983) have been tried out and applied with success to reduce *in vitro* growth of several plant species.

For practical reasons, low temperature as the growth-reducing factor has been widely adopted in the *in vitro* active genebanks of the genus *Musa* (Schoofs, 1989; Vuylsteke, 1990). Although temperature reduction has been shown to be most effective, it is a costly option in the tropics (Withers, 1980). Alternative slow-growth storage methods are being investigated but, to date, little standard work has been published.

Mora et al. (1988) tested 20 combinations of sucrose (0, 1.5, 3, 6, and 9%) and mannitol (0, 2, 4, and 6%) concentrations in the culture medium of banana cultivars Curare (AAB), Pelipita (ABB), and Gran Enano (AAA). Cultures were incubated at 27 ± 1°C and 2,500 lux (16 h). Combinations of 3% or 6% sucrose and 4% mannitol resulted in 50% growth reduction and had a positive effect on storage capacity at this temperature compared to the reference treatment (3% sucrose). The storage capacity of these cultures grown on the mannitol-enriched medium was extended to at least 6 months whereas cultures on the control medium started to deteriorate after 4 months. Higher concentrations of mannitol caused undesirable effects on plant morphology and loss of viability.

Ko et al. (1991) investigated the possibility of tissue culture storage by means of a new technique which involves the incubation of tissue on a support saturated with a sugar solution in the absence of nutrients and combined with low temperature. In a preliminary experiment, pieces of tissue comprising 1–2 newly emerged buds were excised from one-month-old meristem-tip cultures of Cavendish. The tissues were placed on 1.5 g of cotton, covered with a layer of cheesecloth, and saturated

with 20 ml of various kinds of sugar (ribose, fructose, glucose, sucrose, lactose) solutions at 3% in a culture vessel. The banana meristem-tip tissues were incubated at 17°C and under continuous light (47 μE m^{-2} s^{-1}). The results of this experiment showed that all sugars tested could improve survival. The live tissues produced whitish adventitious buds in various sizes and numbers. Ribose was the most effective in prolonging the storage duration, followed by sucrose, glucose, fructose and lactose. After 16 and 21 months, respectively 83% and 33% of the tissue survived on 3% ribose.

The potential use of growth retardants for the conservation of plantlets of *Musa* has been reported by Reuveni and Golubowicz (1993). Antigibberellins (uniconazole, paclobutrazol, and ancymidol) or abscisic acid (ABA) added in relatively low concentrations (1–5 mg l^{-1}), and p-chlorophenoxy isobutyric acid (PCIB), daminozide, and chlormequat in relatively high concentrations (>100 mg l^{-1}) successfully reduced growth of shoot-tip cultures of the cultivar Williams (AAA). The effects of the growth retardants were measured on the *in vitro* grown plantlets after 6 weeks. It was found that plant height was effectively reduced. Cultures treated with antigibberellins in particular showed in addition very compact structures and a reduction in leaf size. They remained compact and continued to form small leaves for a period of four months in culture. Further studies to determine optimal concentration of growth retardants, optimal combinations of treatments, e.g., reduced temperature in conjunction with a chemical growth retardant, effects over longer periods and several subculture cycles are needed to evaluate the usefulness of growth-suppressing substances in extending the storage life of *Musa* shoot-tip cultures.

INIBAP active in vitro collection: Low temperature storage of shoot cultures. At the ITC, the collection is conserved under minimal growth conditions achieved by lowering the ambient temperature. The cultures are preincubated under normal growth conditions at an optimal growth temperature of 28 ± 2°C and PPF of 63 μE m^{-2} s^{-1} (24 h) for 2 to 3 weeks. Once the cultures are established they are visually checked for contaimination, sealed with parafilm and transferred to slow-growth conditions at a temperature of 16±1°C. In the storage room the PPF is reduced to 25 μE m^{-2} s^{-1} (24 h).

The collection is kept in one medium-term storage room, which has the capacity to hold a total of 1,404 accessions, represented by 20 replicates each. The room can accommodate 660 tubes per m^2 (Fig. 1). Proliferating shoot cultures (Fig. 2) are grown in test tubes of 15 cm high and 2.5 cm diameter filled with 20 ml proliferation medium as described above. They are closed with a plastic cap and firmly sealed with parafilm to preclude desiccations of the culture medium.

Fig. 1. INIBAP *Musa* genebank at the Catholic University, Belgium.

It was demonstrated that under these conditions storage duration of *Musa* species is nearly one year on average. However, the frequency of subculture varies for the different genomic (sub) groups and even for cultivars within the same genomic subgroup. Experience at the INIBAP Transit centre showed that East-African highland bananas and nonplantain bananas can be stored significantly longer under the same conditions.

Fig. 2. One-year-old proliferating shoot culture of banana stored under slow-growth conditions.

The storage period for wild *Musa balbisiana* (BBw) is significantly shorter (275 days) than for any genomic (sub) group of parthenocarpic bananas (Table 3). Some accessions can be stored for a period of up to 615 days (Lady Finger-Pome, AAB), whereas others require subculturing

Table 3. Storage duration (days) influenced by genome configuration and temperature

Genome configuration	Storage temperature 16 ± 1°C		Storage temperature 22 ± 3°C		Difference in storage duration (days)
	N[1]	Storage duration (days) [2]	N[1]	Storage duration (days) [3]	
BBw	10	275±110 d[4]	–	–	–
AAw	40	300±113 cd	–	–	–
AA	69	331±115 bc	16	274±60	57
AAA	49	343±105 b	–	–	–
AAA*	32	390±77 a	–	–	–
AAB	25	386±111 a	6	303±19	83
AAB**	146	324±73 bc	19	149±59	175
ABB	30	345±101 b	–	–	–
All genotypes	401	334±79	41	220±87	114

1: number of observations.
2: each observation is the average of 2 storage times per clone.
3: each observation is one storage time per clone.
4: mean and standard deviation; means are highly significantly different following GLM (General Linear Model) (P > F: < 0.0001); means followed by the same letter are not significantly different based on the Duncan multiple range test at the 5% level.
W: wild.
*: East-African highland bananas (Lujugira/Mutika subgroup).
**: plantain subgroup.

every 60 days (SF215, a parthenocarpic derivate of *Musa acuminata* sp. *banksii*) (Van den houwe et al., 1995).

Experiments at the ITC indicated that average storage duration per genotype could be extended from 57 days (for AA edible cultivars) to 175 days (for the plantains) when stored at 16 ± 1°C instead of 22 ± 3°C (Table 3). At temperatures below 14°C it is generally accepted that *in vitro* shoot-tip cultures of *Musa* lose viability (Withers and Williams, 1986).

The need for subculturing is determined by visual examination of individual cultures under slow growth conditions. They are periodically checked for deterioration, including microbial contamination, necrosis, excessive blackening, bleaching of leaves, and hyperhydricity. Unsuitable cultures are immediately rejected from the collection. Once the minimum number of 12 healthy looking cultures is reached, the clone is always removed from storage and subcultured for reasons of safety. To limit the risk of somaclonal variation, shoot tips are taken from all remaining replicates to obtain 20 new subcultures.

If all cultures of one single clone accidentally get contaminated during storage or at subculturing, *in vitro* plants are regenerated from the contaminated cultures on a medium, eventually supplemented with anti-biotics. These plantlets are transferred to the greenhouse for a

3-9-month period and aseptic shoot tips are isolated again and tissue cultures reinitiated.

Duplication

INIBAP also maintains a duplicate of its Transit Centre *in vitro* collection for reasons of safety. To date, 40% of the accessions have been duplicated and placed in TBRI (Taiwan Banana Research Institute), Taiwan as an insurance against losses. They are maintained under similar slow-growth conditions as those applied at the ITC. Duplication of germplasm at CATIE (Centro Agronomico Tropical de Investigiación y Enseñenza), Costa Rice is currently ongoing.

Prospects for Cryopreservation

Although *in vitro* collections under normal or limited growth conditions have proven their value, the occurrence of somaclonal variation, i.e., the genetic variability generated during tissue culture (Scowcroft, 1984), remains a serious impediment (Vuylsteke et al., 1991; Côte et al., 1993). Long-term tissue cultures are also known to show a decline or even a complete loss of morphogenic potential (Bajaj, 1995). In addition, there is always the risk of losing germplasm due to contamination or human error during subculture. Lastly, conservation of a large collection is still a very labor-intensive endeavor. Alternatively, once cells or tissues are stored through cryopreservation at ultralow temperatures (–196°C), growth is totally terminated due to nonavailability of liquid water and the inhibition of all chemical reactions. Hence, cryopreserved tissue is not subject to somaclonal variation. There remains only the extreme low risk for mutation by free radical damage from background radiation (Whittingham et al., 1977; Benson, 1990). Since no chemical repair mechanism exists at ultralow temperatures, damaging events causing point mutations may accumulate during time. Nevertheless, there is no reason to believe that during long-term conservation of plant tissue cultures, viability should be lost or should lead to unwanted genetic aberrations since animal cell cultures have been stored in a similar manner for over 30 years without problems (Fort-Lloyd and Jackson, 1986).

In theory, any regenerable material or tissue is suitable for storage of germplasm through cryopreservation. For banana, this requirement is met by seed, zygotic embryos, regenerable callus, somatic embryos, embryogenic cell suspensions, and meristem cultures. Cryopreservation protocols have been developed for seed and zygotic embryos. Banana seed properly dried to a moisture content below 10% were able to survive freezing in liquid nitrogen (LN) (Chin, 1995). Zygotic embryos excised from seed of *M. balbisiana* and *M. acuminata* were also subjected to freezing (Abdelnour Esquivel et al., 1992). Drying to a moisture content

of about 15% prior to freezing again appeared essential for obtaining high levels of postthaw survival. Although simple and very efficient, these methods only offer promise for the long-term conservation of wild, seed-producing banana varieties. In addition, because bananas are cross pollinators, the use of seed and zygotic embryos is handicapped by the lack of knowledge of the genetic make-up of the preserved germplasm. Regenerable callus and somatic embryos are seldom available at sufficient quantities.

At the Laboratory of Tropical Crop Improvement of K.U. Leuven, Belgium, the potential of embryogenic cell suspensions and meristem cultures for cryopreservation is being investigated.

Cryopreservation of Embryogenic Cell Suspension Cultures

Embryogenic cell suspensions are isolated from proliferating meristem cultures (Dhed'a et al., 1991). These suspensions are the source of regenerable protoplasts (Panis et al., 1993: Panis et al., 1994a) which can be transformed through electroporation (Sagi et al., 1994). Direct transformation of these cells using particle bombardment resulted in the world's first transgenic banana plants (Sagi et al., 1995). However, the initiation of embryogenic banana suspensions is very time consuming and once initiated subject to contamination, somaclonal variation, and physiological changes.

The protocol developed for cryopreservation of embryogenic banana cell suspensions involves the following steps (Panis et al., 1990):

0) Embryogenic cell suspensions are maintained in half-strength MS medium supplemented with 5 μM 2,4–D, 1 μM zeatin, standard MS vitamins, 100 mg l^{-1} ascorbic acid and 30 g l^{-1} sucrose.
1) They are frozen 2 weeks after their last subculture, in their exponential growth phase.
2) The susupension is cryoprotected with 7.5% DMSO (dimethyl sulfoxide) for 1 h.
3) The cryoprotected suspension is transferred to a 2 ml cryotube and slowly frozen at a rate of 1°C min^{-1} to –40°C. Optimal freeze-dehydration is only obtained when during the slow-freezing process ice-crystal formation is initiated at – 7.5°C.
4) When –40°C is obtained, cryotubes are plunged into LN for storage.
5) To prevent lethal ice-recrystallization, thawing must proceed rapidly by plunging the tubes in a stirred water bath of 40°C.
6) Thawed suspensions are plated onto a semisolid regeneration medium (half-strength MS supplemented with 1 μMBA).

After this freeze-thaw cycle a chemical viability test with fluorescein diacetate (FDA) is executed. This test revealed that only typical

embryogenic cells survive freezing. Typical embryogenic cells are small (30 mm diameter), isodiametric, have a relatively large nucleus and contain a dense cytoplasm with many tiny vacuoles. Large elongated cells and organized structures (like globular structures and proembryos) do not survive the freezing process. As such, freezing results in a selection of the most embryogenic cells. The best proof of the integrity of frozen cells is to test their regenerability. Through somatic embryogenesis hundreds of plantlets can be regenerated from 1 ml of frozen suspensions.

Recent experiments (Panis, et al., 1999a) revealed that the cryopreservation technique originally developed for suspensions of the cv. Bluggoe was also applicable to embryogenic suspensions of other cultivars provided that the sucrose concentration in the cryoprotective mixture was increased to 180 gl^{-1}.

Cryopreservation of Meristem Cultures

Unlike cell suspensions, for which slow freezing in the presence of a cryoprotective agent such as DMSO is a widely applied technique, no standard protocols are available for the cryopreservation of organized cultures such as meristems.

In concordance with embryogenic cell suspensions, we tested slow freezing in the presence of cryoprotective solution. Various preculture treatments, freezing rates, and cryoprotective solutions were applied–all without success. Application of highly concentrated vitrification solutions (Sakai et al., 1990) followed by rapid freezing also never resulted in postthaw survival. Like vitrification, the technique of encapsulation-dehydration developed by Fabre and Derreudra (1990) is currently employed for many plant species. The unique feature of this method is that synthetic seeds (alginate beads) are produced. When applied to proliferating banana meristems, however, only 7% postthaw survival could be obtained. Unlike other plant species, banana meristems proved very sensitive to high sucrose concentrations as well as drying-two treatments essential for precluding lethal ice crystallization (Panis et al., 1994b).

Because of the inefficiency of previously described methods, we thus had to look for alternatives. This resulted in a simple cryopreservation system which involves the following steps (Panis, 1995; Panis et al., 1996).

0) Of uttermost importance is the starting material. Best survival is obtained using "cauliflower-like" structures, which can be obtained by culturing meristems on MS medium containing high concentrations (up to 100 μM) of benzyladenine (BA) (Fig. 3).

1) Scalps (the upper and most meristematic part of the cauliflower-like buds) of 5 mm diameter, each containing 5 to 10 meristematic domes, are excised and inoculated onto a proliferation medium (MS medium with 10 μM BA) containing a high (0.3 to 0.5 M) sucrose concentration.
2) Due to the high sucrose concentration, growth of the meristems is retarded and a part of the cultures will even become black and die (Fig. 3). This reaction is very cultivar dependent. After 2 to 4 weeks, clumps of 3–5 mm diameter of surviving meristems are isolated and transferred to 2 ml cryotubes.
3) The cryotubes are plunged directly into LN for storage.
4) Following storage, the meristems are thawed by plunging the cryotubes in a stirred water bath at 40°C, then transferred to a semisolid regeneration medium (MS with 1 μM BA and 1 μM IAA). After one week of culture in darkness, cryotubes are transferred to continuous light at 50 mE m^{-2} S^{-1} illumination.

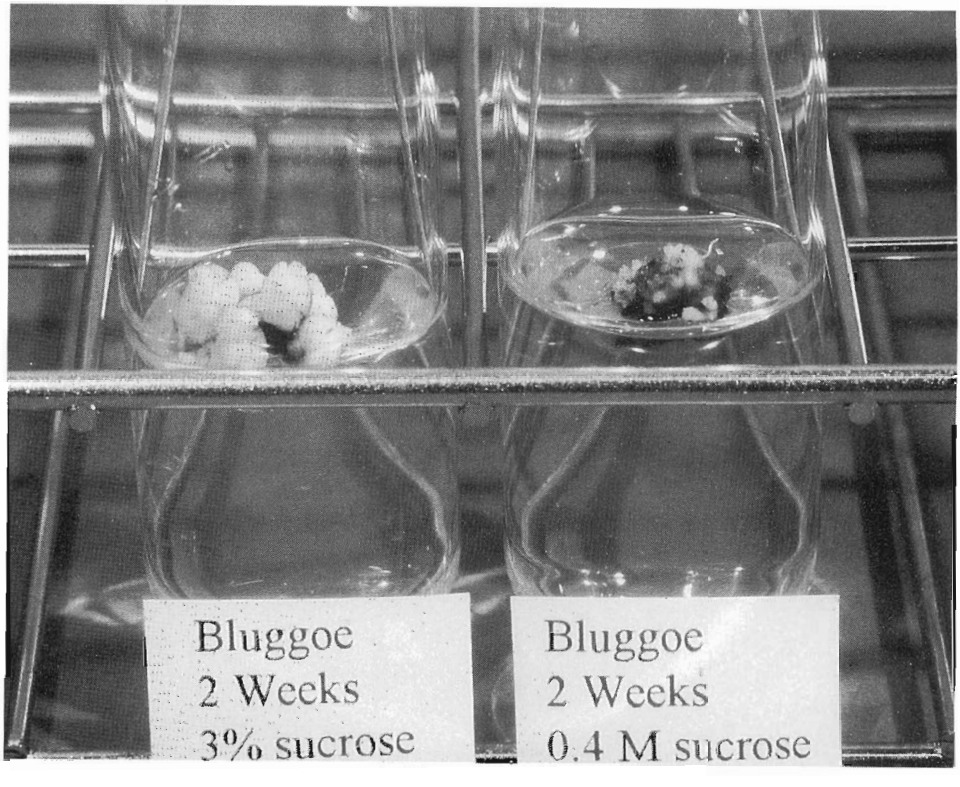

Fig. 3. Proliferating "cauliflower-like" meristem cultures of the cv. "Bluggoe" (ABB group) after 2 weeks culture on proliferation medium with 3% (left) or 0.4 M (right) sucrose.

5) Pregrown and frozen meristems regenerate directly without an intermediate callus phase. This is an important observation in view of avoiding somaclonal variation. After 6 weeks, first shoots appear on the explant. They are excised, regenerated into rooted plantlets, and transferred to the greenhouse to grow normally (Fig. 4).

This cryopreservation protocol was developed and optimized for the cv. Bluggoe and yielded survival rates ranging from 35 to 55%. Since the final aim of cryopreservation is the development of germplasm collection in LN, we screened 15 cultivars belonging to 7 different genomic groups for their ability to withstand cryopreservation (Fig. 5). The first and most important observation is that all the cultivars tested responded positively. The viability rates varied considerably according to the genomic group. Within the ABB group 43 to 64% survived the freeze protocol but only 7 to 19% of the AAA cultivars. Other groups, the AAB plantains and AAB nonplantains showed large differences. To establish a germplasm collection, not the survival rate, but rather the reliability of the system

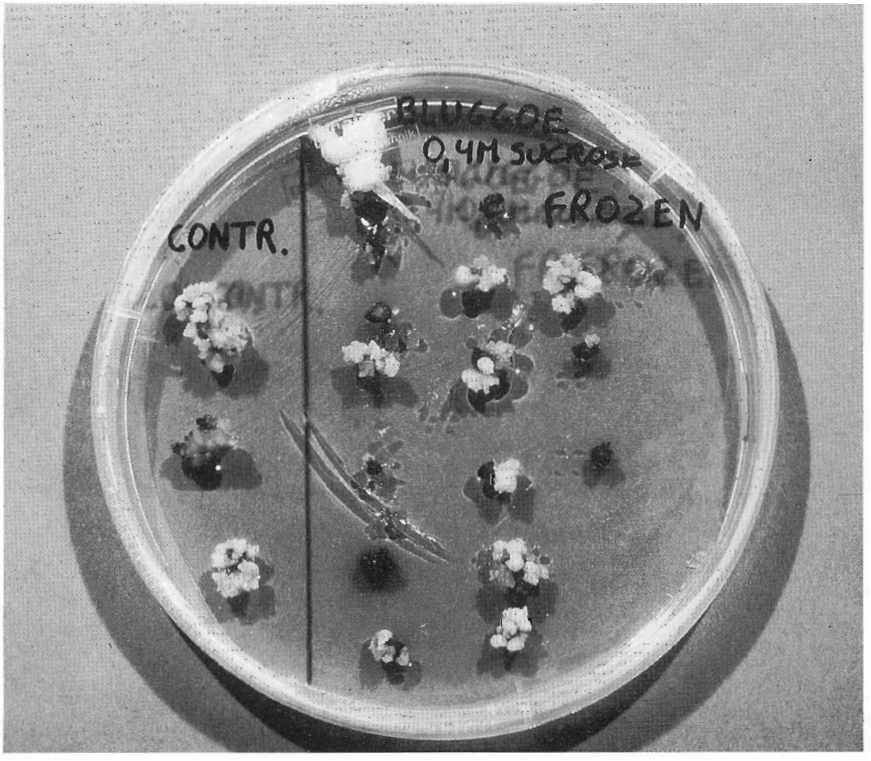

Fig. 4. Petri dish containing control (left) and frozen (right) meristematic clumps of the cv. "Bluggoe" (ABB group), 6 weeks after cryopreservation.

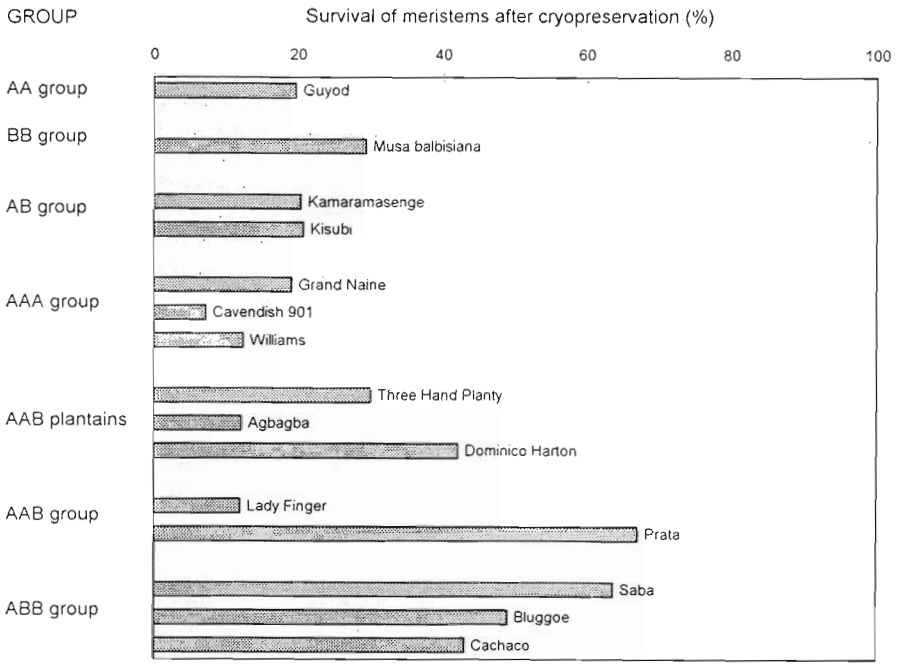

Fig. 5. Regeneration frequencies after cryopreservation of different banana cultivars belonging to different genomic groups.

is important. Low viability rates can be counterbalanced by freezing larger quantities of the material. Recent experiments (Panis et al., 1999b) indicated that postthaw regeneration rates of the precultured meristem clumps increased considerably if a vitrification step (Sakai et al., 1990) is included. Thinh and coworkers (1999) reported that meristems excised from rooted in vitro plants could also be successfully cryopreserved using the vitrification technique.

The importance of sucrose in the pregrowth media was shown by histocytological studies, dry weight measurements, and the fact that sucrose could not be replaced by mannitol. The proposed modes of action of a sucrose preculture in enhancing freeze resistance are numerous. Freeze resistance results from a slow reduction in moisture content (Uragami, 1991; Engelmann and Duval, 1986) due to osmosis and sucrose uptake, which depress the freezing point and the amount of freezable water. Sucrose can maintain the liquid crystalline state of the membrane bilayers and stabilize proteins under frozen conditions (Kendall et al., 1993). An indirect effect of sucrose, which exerts osmotic stress on the tissue, could be the accumulation of water-stress protective compounds, e.g., proline (Delvallée et al., 1989).

The technique developed for cryopreservation of embryogenic cell suspensions is particularly interesting as a tool in genetic engineering since desirable cultures can be stored in a safe and stable state. In view of the time-consuming and not always reliable initiation procedure of embryogenic cell suspensions, preference must be given to meristem cultures for the establishment of long-term germplasm collections. Meristem cultures are readily available and what is more the cryopreservation technique which we developed for this tissue type is simple, rapid, and applicable to all the banana accessions screened thus far.

DISEASE INDEXING AND ERADICATION

In vitro cultures initiated from explants, surface sterilized prior to culture initiation, are considered as free from insects, nematodes, and most microorganisms. However, viruses and some microbial contaminants can still occur in tissue cultures since these organisms are able to pass through *in vitro* cultures without expressing symptoms on the plant tissue or visible signs in the medium. In view of the long-term conservation of genetically important plant material, safe international exchange and use of *in vitro* cultures in modern biotechnology, effective testing and disease elimination procedures are required to guarantee a high-health status of the germplasm maintained.

Four main viruses are reported in banana. The most important virus disease of banana is bunchy top disease, caused by the banana bunchy top virus (BBTV), and is found in Asia, Australia, some African countries, and some Pacific Islands. The virus is transmitted by aphids and the symptoms are expressed by dwarfing of plants and the absence of bunches. In extreme cases, yield losses may be complete. Cucumber mosaic cucumovirus (CMV) is a second virus affecting banana. It is transmitted in a nonpersistent manner by aphids and causes banana mosaic or infectious chlorosis. The disease has a worldwide distribution but is not considered a major problem in *Musa.* Young plants infected with CMV may become stunted and yield may be significantly reduced but, generally, the disease does not cause major losses. When plants are infected at a later stage symptoms range from mild chlorotic mosaics to severe leaf distortion. The CMV heart-rot strain causes severe yellowing and necrosis of the leaves followed by rotting of the heart-leaf and the central cylinder. A third viral disease is banana streak caused by the banana streak virus (BSV) and transmitted in a semi-persistent manner by a mealy bug, *Planococcus citri.* The distribution of BSV is known to be widespread and has gained importance in recent years. Plants affected by the disease show leaf striping symptoms which later develop into necrotic streaks. They have reduced plant growth and vigor, and smaller

bunches. Considerable yield losses ranging from 7% to 90% have been reported in the Poyo cultivar in Côte d'Ivoire (Jones and Lockhart, 1993). Banana bract mosaic virus (BBMV), causing banana bract mosaic disease, is an aphid transmitted virus strictly confined to the Philippines, India and Sri Lanka. Symptoms develop as dark-colored streaks on the bracts of the male flower buds, and dark-colored stripes or mosaics on the pseudostem. Losses up to 40% have been reported in the Philippines.

Ensuring the supply of healthy germplasm, entries at the ITC are systematically checked for the presence of these viruses. From the plant material of the new accession one single shoot tip is used for initiation of tissue cultures. From the initiated cultures, one newly formed meristem is picked to produce a subclone of 7 cultures. Five out of the 7 cultures are regenerated as *in vitro* plantlets for testing. The 2 remaining cultures are kept at the ITC for further multiplication and distribution, subsequently by medium-term storage, if the indexing results are negative.

The indexing tests are undertaken at one of the three Virus Indexing Centres (VICs), operated by INIBAP and located at CIRAD (France), QDPI (Australia) and TBRI (Taiwan). The VICs follow virus testing procedures described in the FAO/IPGRI Technical Guidelines for the Safe Movement of *Musa* Germplasm (Diekmann and Putter, 1996). The plants are grown under quarantine conditions for six months, observed regularly, and tested for virus pathogens. CMV and BBTV can be reliably detected by ELISA (enzym-linked immunosorbent assay) but for the detection of BSV and BBMV, ISEM (immunosorbent electron microscopy) is required.

As new plant material of certain introductions is not always easy to access, clones found to carry virus or virus-like particles are retained in the collection. Their maintenance is continued while awaiting the development of suitable therapeutic techniques for virus eradication.

Elimination of CMV and BBTV by means of meristem culture or in combination with heat treatment has been reported in banana, although the application of these techniques is variable in outcome and has been successful in achieving only some virus-free plants.

Berg and Bustamante (1974) heat treated CMV infected rhizomes (of at least 20 cm in diameter) of the Cavendish AAA subgroup of banana cultivars at 35–43°C. The lateral buds which developed after 100 days of treatment were subjected to meristem culture. Approximately 75% of the plants regenerated from the tissue cultured meristems were found to be free from CMV. Gupta (1985) obtained more successful results by heat treating suckers of various AAA and AAB plantain cultivars at 38–40°C for 14 days, prior to meristem culture. Eradication of CMV was achieved for approximately 100% of the plants regenerated.

Experiments conducted by Drew et al. (1992) revealed that virus-

free plants derived from BBTV-infected plants of Mons Mari (Cavendish AAA subgroup) could be obtained simply through rapid multiplication and subculturing axillary buds. The mircopropagated plants were tested serologically and 27% proved to be virus free. At the National Taiwan University, Taiwan, Wu and Su (1991) succeeded in freeing banana tissue culture plantlets from BBTV at high temperature. Infected cultures were grown for 3 months at a temperature of 35°C. Five of the 11 treated cultures produced one or more healthy, virus-free plants, suggesting that the high temperature treatment inhibits multiplication of BBTV and that some primordial cells can be freed from the virus, possibly associated with its uneven distribution. Similar treatments at 40°C (16 h/24 h) for 5 and 12 weeks failed to free the cultures from BBTV.

At present no technique for virus elimination in *Musa* collections is applied on a routine base. More research in this area and in particular with regard to chemo- and thermotherapeutic methods, size of the explant, and culture method is required.

The occurrence of endophytic bacteria is a considerable source for concern in all aspects of plant cell, tissue, and organ culture. These microorganisms are not necessarily pathogenic or harmful to plants in the field but they may represent a serious problem during *in vitro* culture as surface disinfectants do not reach the internal parts of the explant and fail to control these contaminants. They may survive in the plant material with no visible sign in the culture medium during several subculture cycles over an extended period of time.

Control and elimination of these nonviral pathogens can be possible by using explants from a source plant unlikely to harbor microorganisms–an appropriate tissue culture procedure, i.e., meristem culture and screening explants for asepsis prior to tissue culture.

At the ITC, plant material introduced in the genebank is tested for internal bacteria on a selective medium after surface disinfestation. Prior to placing each shoot tip on culture medium, the base is streaked onto a bacterial growth medium, i.e., Diffco-Bacto nutrient agar, in 55 x 15 mm petri plates. The streaked plants are incubated for 1 to 6 weeks in light at 28°C. Explants that show bacterial growth in their streak are discarded. This procedure is currently an integral part of the tissue culture activities related to the initiation of cultures for the genebank.

Antibiotics have been used to control microbial contamination in many plant tissue cultures. However, their use is limited for the following reasons: (1) their mode of action is biostatic rather than biocidal; (2) it is difficult to achieve effective levels to the antibiotic at the site of infection without damaging the plant tissue; (3) their application may be a hindrance in the international exchange of germplasm as they are restricted or forbidden by legislation in some countries; (4) prophylactic

use of antibiotics to control unidentified populations of microorganisms should be discouraged, since it is unlikely to control all contaminants and it may lead to the development of resistant strains of microorganisms. Therefore, antibiotics should be applied only for limited time periods and to control known contaminants.

SOMACLONAL VARIATION

One of the criteria for efficient *in vitro* storage of germplasm is the maintenance of original genotypes over longer periods of time. Although organized cultures (meristem, shoot and root-tip cultures) are believed to be genetically more stable than disorganized cultures (cell suspensions, protoplasts, callus, dedifferentiated cells, etc.) (Scowcroft, 1984), variation appears to be relatively widespread in micropropagated plants. Variation observed in tissue-culture-produced plants can be of three types (Swartz, 1991): (1) variation preexisting in the explant, e.g., chimeras, expressed through rapid and adventitious proliferation *in vitro;* (2) variation affecting the genome of the *in vitro* propagated plant. This is most commonly due to changes in chromosomal structure, particularly deletions and changes in chromosome number (polyploidy, aneuploidy). This type of variation is mostly deleterious from the conservator's point of view but can be great value to mutation breeders. In some cases, somaclonal variation is used as an approach to generate new cultivars with improved desirable characters such as disease resistance. In Taiwan, the national breeding program has applied this approach to select Cavendish plants with resistance to *F. oxysporum* sp. *cubense* race 4 (Hawang and Ko, 1987). (3) Changes due to epigenetic or physiologic effects. In some cases it is likely that a carryover effect of stress-imposing factors, such as relatively high concentrations of growth regulators used in the culture medium, are responsible for nonheritable, reversible changes in the phenotype. Although this effect might be disturbing it is not so serious or important for a genetic conservator.

Factors known to influence the occurrence of somaclonal variation are culture mode, time in culture, number of subculture cycles, genotype, and composition of the culture medium.

Somaclonal variation in *Musa* is typically expressed in plant stature (dwarfism, giantism), abnormalities in the foliage (deformation, drooping, erect, variegated or mosaic patterns), variation in pseudostem coloration and variation affecting inflorescence and fruit (prolonged juvenility, small bunch, shortened fingers). Variations due to *in vitro* culture mostly occur *in vivo* as well, although at much lower frequencies (Vuylsteke et al., 1991). The type and frequency of variation in micropropagated banana plants is known to be genotype and cultivar dependent. For example,

different degrees of dwarfism and to a lesser extent foliage abnormalities, such as mosaic patterns are the most common types of variation in the Cavendish subgroup (AAA). These types of variation account for approximately 90% and 9% of the somaclonal variation occurring in this subgroup. Percentages of variation observed range from 2.4% to 25% in Giant Cavendish and Grande Naine (Israeli et al., 1991) and up to 31.7% in tissue-cultured plants of Williams (AAA) (Smith, 1988).

Vuylsteke et al., (1991) studied the phenomenon of somaclonal variation in the plantain subgroup (*Musa* sp. AAB). Seven plantain cultivars, representing the range of cultivar variability within the plantain subgroup (*Musa* spp, AAB group), were micropropagated through shoot-tip culture and screened for somaclonal variation at the field-grown stage. The observed variations were foliage abnormalities and changes in inflorescence morphology, associated female fertility, fruit shape, pseudostem, petiole and bract color, and plant stature. Percentages of stable inflorescence offtypes observed ranged from 0.5 to 69.1%, depending on the cultivar. The study revealed that somaclonal variation is strongly influenced by the intrinsic factor of genetic stability of each cultivar since 25-100% of the variation observed showed similarity to the variability occurring naturally. The frequency of this variation, however, seems to be greatly amplified *in vitro.*

It appears that the rate of somaclonal variation is positively related to the number of tissue culture cycles. Reuveni and Israeli (1990) evaluated Williams (AAA) *in vitro* plants obtained after one, two, and five subculture cycles. Dwarfism and leaf-offtypes counted for 3.7% and 0.7% respectively after one cycle *in vitro* and increased to 6.1% and 1.9% respectively after 5 cycles *in vitro.*

There is no evidence that growth regulators routinely used in tissue culture induce mutagenesis *per se* (Scowcroft, 1984; Reuveni and Israeli, 1990). The use of relatively high concentration of growth substances in culture media may result in abnormalities in the plant morphology but apparently these changes are mostly epigenetic and reversible (George and Sherrington, 1984); however, prudence is in order.

Important measures to reduce the risk of producing somaclones *in vitro* is the selection of true-to-type explant material, the choice of a low-risk culture mode, and appropriate culture media, preferably with low levels of plant growth regulators. The duration of *in vitro* growth and the number of subcultures should be limited, although this measure is difficult to practice for *in vitro* genebanks.

Monitoring of genetic stability by visual examination in the *in vitro* stage is very difficult and inadequate as the morphology of the culture changes and differs from the performance of the plant *in vivo.* Intergenomic and intragenomic differences are morphologically not distinguishable *in*

vitro. It has been demonstrated that a few types of variation can be detected visually *in vitro.* Israeli et al. (1991) reported the detection of extreme dwarfism and leaf variegation in Cavendish at the test-tube stage. However, most of the common dwarf and mosaic variants are detected at the nursery stage at the earliest.

Development of morphological, biochemical, and molecular techniques for early detection and identification of somaclonal variants would be very helpful in order to enhance the security of conservation of an identified genepool and to give one a better insight into the observed variation. Reuveni and Israeli (1990) measured the reaction of dwarf and normal regenerated plants of the cultivar Williams to gibberellic acid (GA_3). Several weeks after culturing on a GA_3-containing medium, normal plants showed elongation while dwarf offtypes remained insensitive to GA_3. Reuveni and Israeli (1990) also found a significant difference in number and structure of stomata in the leaves of normal and offtype plants *in vitro.* Both criteria could contribute to the early screening and elimination of somaclonal variants.

The ability of molecular techniques such as RAPD (random amplified polymorphic DNA; Ford-Lloyd et al., 1993; Howell et al., 1994) and DNA-fingerprinting (Kaemmer et al., 1992) for assessing genetic variation in tissue cultures of banana is currently under study.

CHARACTERIZATION AND EVALUATION

Traditionally, characterization and evaluation of banana germplasm is based on morphological descriptions. However, confusion may arise in morphotaxonomic identification of clones when phenotypic changes occur due to environmental influences or somatic mutations (Swennen, 1990; Horry, 1990). Neither can genetic variation in such altered phenotypes be correlated to morphological characteristics.

In recent years, a wide range of molecular marker techniques have received attention as a complementary aid in taxonomic studies, evaluating genetic diversity, clonal identification, and characterization of somaclonal variants in banana. Isozymes have been reported as reliable biochemical markers to discriminate between clones belonging to different genomic groups and subgroups (Bath et al., 1992a, b; Jarret and Litz, 1986; Horry; 1990; Lebot et al., 1993). However, the technique may fail when it comes to the identification of closely related cultivars, as demonstrated for the AAA Cavendish subgroup. Jarret and Litz (1986) found the Cavendish cultivars Robusta, Giant Cavendish, Dwarf Cavendish, and Pisang masak hijau (Lakatan) to be monomorphic in 15 enzyme systems tested. They believe that individual clones could be identified when additional enzyme systems are analyzed. The discriminating ability of

isozymes is limited to the number of polymorphic loci which can be identified and characterized (Jarret and Gawel, 1995), and although isozyme markers may represent only a small fraction of the genome, they are of great value for detecting variation in *Musa.*

At the DNA level various markers have recently been tried for *Musa.*

1) RFLP (restriction fragment length polymorphism). The RFLP technique offers a greater potential for the study of genetic diversity between and within *Musa* groups since the number of markers is considerably greater than the known biochemical markers. Actually, unlimited sources of genetic polymorphism may be detected (Carreel et al., 1994).

2) RAPD (random amplified polymorphic DNA). Polymorphism is detected by arbitrary primers in combination with PCR also called DNA amplification fingerprinting. Although the technique is considered as less reliable than RFLP, it has a high potential to complement other marker techniques. Investigators at the University of Birmingham, for example, found the technique can be used to characterize dwarfism, detected in a plant of the cultivar Uunapope (AA), that was obtained from the ITC collection. Using two different primers, two band differences were found between the original cultivar and the dwarf offtype (Ford-Lloyd et al., 1993; Howell et al., 1994). Kaemmer et al., (1992) identified a mutant of Grande Naine (GN60A) obtained through irradiation, by means of this technique.

3) The finger-printing method. It recognizes polymorphic simple repetitive DNA sequences (variable minisatellite tandem repeats and/or oligonucleotide sequences). The technique has been successfully used to differentiate between cultivars belonging to various genomic groups but may also be applied in the recognition of genetic diversity at a very sensitive level: cultivar identification, assessment of somaclonal variation, and identification of duplicates (Kaemmer et al., 1992).

USE OF THE COLLECTION: ACCESSIBILITY AND GERMPLASM EXCHANGE

If the world collection of *Musa* is to serve a useful purpose, accessions should be readily available, in a healthy state, and propagated in such a way that genetic integrity is guaranteed.

One of INIBAP's roles is to offer to the International Community all sources of *Musa* germplasm. Samples of virus-tested clones in its collection are freely available for distribution, attracting a wide range of users throughout the world (Table 4). Since 1985, nearly 4,000 accessions (Table 5) have been provided to research scientists, plant breeders, and other users in national agricultural research centers (NARS), international

Table 4. Number of accessions exported from ITC to various regions

	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	Total	%
AM	0	0	13	127	88	111	68	99	155	183	905	23	
W/C AFR	20	27	154	105	109	54	79	0	24	158	41	771	19
E AFR	0	0	0	105	69	42	21	48	66	19	93	463	12
AS/PAC	12	0	3	5	48	68	50	83	75	154	436	934	24
EUR	0	0	10	14	34	122	103	104	179	119	183	868	22
Total	32	27	180	356	348	397	321	296	443	605	936	3941	100

AM: America
W/C AFR: West and Central Africa
E AFR: East Africa
As/PAC: Asia and the Pacific
EUR: Europe

Table 5. *Musa* germplasm accessions distributed by the INIBAP Transit Centre

Year	NARS	IARC	Others	Total of acc. distributed
1985	12	20	0	32
1986	0	27	0	27
1987	76	94	10	180
1988	167	114	75	356
1989	219	106	23	348
1990	221	44	132	397
1991	255	40	26	321
1992	239	3	54	296
1993	318	19	106	443
1994	524	14	67	605
1995	825	22	89	936
Total	2,856	503	582	3,941

agricultural research centres (IARC), and other organizations in developed and developing countries.

The ITC also plays a crucial role in the provision of plant materials within various specific INIBAP-programs. As a part of the IMTP, International *Musa* Testing Program, advanced cultivars from breeding programs as well as some land races and wild species are multiplied and supplied for evaluating *Musa* disease resistance and agronomic performance on a multilocation basis. Within the framework of the *Musa* Germplasm Information System (MGIS), a set of 30 reference cultivars is being distributed from the ITC to evaluate environmental effects on the morphotaxonomic characters.

Usually proliferating tissue cultures for further multiplication are provided. Cultures are removed from storage and subcultured in plastic culture vessels containing 10 ml proliferation medium and sealed with a screw cap. They are incubated at 28°C for 14 days, labeled and

sealed in plastic shock-absorbent bags, packed in a cardboard box and mailed by air courier. In general, five samples per accession are provided.

If the requestor is not able to handle clusters of multiple shoot or bud cultures, *in vitro* rooted plantlets ready for soil planting are supplied. From the propagules in storage unrooted shoots are produced on a regeneration medium. The composition of the regeneration medium differs from the medium used for culture initiation, multiplication and storage in cytokinin concentration. The BA concentration is reduced to 1 μM (Table 2). The shoots are aseptically packed in gas-permeable plastic tissue culture bags containing a semisolid root-inducing culture medium, containing the MS macronutrients at half strength, MS vitamins, enriched with 15 g l^{-1} sucrose and 1 μM of the auxin IBA (indolebutyric acid) to promote root formation. On this medium roots usually appear within 10 days. In general, 5 samples per accession are provided. *Musa* tissue cultures dispatched from the ITC are accompanied by passport information and a germplasm health statement, containing the results of virus tests undertaken. A receiving report requesting information on culture viability and contamination is enclosed in each shipment. An original phytosanitary certificate or plant passport (for shipments within the EC), issued by the Belgian Ministry of Agriculture and, if required by the quarantine service of the germplasm introducing country, a plant importation permit accompany the exported plant material.

References

Abdelnour-Esquivel, A., Mora, A. and Villalobos, V., 1992. Cryopreservation of zygotic embryos of *Musa acuminata* (AA) and *Musa balbisiana* (BB). Cryo-Lett., 13: 159–164.

Bajaj, Y.P.S., 1995. Cryopreservation of plant cell, tissue, and organ culture for the conservation of germplasm and biodiversity. In: Y.P.S. Bajaj (Ed.), Biotechnology in Agriculture and Forestry, Vol 32. Cryopreservation of Plant Germplasm. Springer-Verlag, Berlin, pp. 3–28.

Banerjee, N. and De Langhe, E., 1985. A tissue culture technique for rapid clonal propagation and storage under minimal growth conditions of *Musa* (Banana and Plantain). Plant Cell Rep., 4: 351–354.

Banerjee, N. and Sharma, A.K., 1988. *In vitro* response as a reflection of genomic diversity in long-term cultures of *Musa.* Theor. Appl. Gen., 76: 733–736.

Benson, E.E., 1990. Free radical damage in stored plant germplasm. IBPGR, Rome, 128 pp.

Berg, L.A. and Bustamante, M., 1974. Heat treatment and meristem culture for the production of virus-free bananas. Phytopathology, 64: 320–322.

Bessembinder, J.J.E., Startisky, G. and Zandvoort, E.A., 1993. Long-term *in vitro* storage of *Colocasia esculenta* under minimal growth conditions. Plant Cell, Tissue and Organ Cult., 33: 121–127.

Bhat, K.V., Bhat, S.R and Chandel, K.P.S., 1992a. Survey of isozyme polymorphism for clonal indentification in *Musa.* I, Esterase, acid phosphatase and catalase. J. Hort. Sci., 67: 501–507.

Bhat, K.V., Bhat, S.R and Chadel, K.P.S., 1992b. Survey of isozyme polymorphism for clonal identification in *Musa.* II. Peroxidase, superoxide dismutase, shikimate dehydrogenase and malate dehydrogenase, J. Hort. Sci., 67: 737–744.

Carreel, F., Fauré, S., Gonzalez de Leon, D., Lagoda, P.J.L., Perrier, X, Bakry, F., Tezenas du Montcel, H., Lanaud, C. and Horry, J.P., 1994. Evaluation de la diversité génétique chez les bananaiers diploïdes (*Musa* sp.). Gen. Sel. Evol. 26: Suppl. 1, 125s–136s.

Chin, H.F., 1995 Germination and storage of banana seeds. In: Proc. International Banana Breeding Workshop 'New Frontiers in Resistance Breeding for Nematode, Fusarium and Sigatoka'. MARDI/INIBAP, Serdang, Selangor, Malaysia, October 2–5, 1995, 242 pp.

Côte, F.X., Perrier, X. and Teisson, C., 1993. Somaclonal variation in *Musa* sp.: theoretical risks and risk management. Future research prospects. In: Biotechnology Applications for Banana and Plantain Improvement. Proc. of INIBAP Workshop, San José, Costa Rica, Jan 27–31, 1992, pp. 192–199.

Cronauer, S. and Krikorian, A.D., 1984, Rapid multiplication of banana and plantains by *in vitro* shoot tip culture. HortScience, 19: 234–235.

Delvallée, I., Guillaud, J., Beckert, M. and Dumas, C., 1989. Cryopreservation of immature maize embryos after freeze-hardening in the ear and in vitro. Plant Sci., 60: 129–136.

Dhed'a, D., Dumortier, F., Panis, B., Vuylsteke, D. and De Langhe, E., 1991. Plant regeneration in cell suspension cultures of the cooking banana cv. 'Bluggoe' (*Musa* spp., ABB group). Fruits 46: 125–135.

Diekmann, M. and Putter, C.A.J. (Eds.), 1996. FAO/IPGRI Technical Guidelines for the Safe Movement of Germplasm. No. 15. *Musa* (2nd ed.). Food and Agriculture Organization of the United Nations, Rome/International Plant Genetic Resources Institute, Rome, 26 pp.

Drew, R., Smith, M.K. and Anderson, D.W., 1992. Field evaluation of micropropagated bananas from plants containing Banana Bunchy-Top Virus. Plant Cell, Tissue and Organ Cult., 28: 203–205.

Engelmann, F. and Duval, Y., 1986. Cryopreservation of oil palm somatic embryos (*Elaeis guineensis* Jacq.): Results and application prospects. Oléagineux, 41: 169–174.

Fabre, J. and Dereuddre, J., 1990. Encapsulation-dehydration: A new approach to cryopreservation of *Solanum* shoot tips. Cryo-Lett., 11: 413–426.

FAO, 1995. Production Year Book 1995. FAO UN, Rome, 291 pp.

Ford-Llyod, B. and Jackson, M., 1986. Plant genetic resources: An Introduction to Their Conservation and Use. Edward Arnold Ltd., London, 146 pp.

Ford-Llyod, B.V., Howell, E. and Newbury, H.J., 1993. An evaluation of Random Amplified Polymorphic DNA (RAPD) as a tool for detecting genetic instability in *Musa* germplasm stored *in vitro.* In: Breeding Banana and Plantain: Proc. Internat. Symp. Genetic Improvement of Bananas and Resistance to Diseases and Pests. Montpellier, France, September 7–9, 1992. CIRAD-FLHOR, INIBAP, Montpellier (France), 393 pp.

George, E.F. and Sherrington, P.D., 1984. Plant Propagation by Tissue Culture: Handbook and Directory of Commercial Laboratories. Exegetics Ltd., Basingstoke, England, 709 pp.

Gupta, P., 1985. Eradication of mosaic disease and rapid clonal multiplication of bananas and plantains through mersitem tip culture. Plant Cell, Tissue and Organ Cult., 6: 33–39.

Hahn, S., Vuylsteke, D. and Swennen, R., 1990. First reactions to ABB cooking bananas distributed in southeastern Nigeria. In: R.A. Fullerton and R.H. Stover (Eds.), Sigatoka Leaf Spot Diseases of Banana. INIBAP, Montpellier, France, pp. 306–315.

Hamill, S.D., Sharrock, S.L. and Smith, M.K., 1993. Comparison of decontamination methods used in intiation of banana tissue cultures from field-collected suckers. Plant Cell, Tissue and Organ Cult., 33: 343–346.

Horry, J.P., 1990. Biochemical approaches to banana characterization. In: *Musa* Conservation and Documentation. Proc. Workshop, Leuven, Belgium, December 11–14 1989, pp. 48–52.

Howell, E.C., Newbury, H.J., Swennen, R., Withers, L.A. and Ford-Lloyd, B.V., 1994. The use of RAPD for identifying and classifying *Musa* germplasm. Genome, 37: 328–332.

Hwang, S.C. and Ko, W.H., 1987. Somaclonal variation of bananas and screening for resistance to Fusarium wilt. In: G.J. Persley and E.A. De Langhe (Eds.), Banana and Plantain Breeding Strategies, ACIAR Proc. No. 21, Canberra, pp. 151–156.

Israeli, Y., Reuveni, O. and Lahav, E., 1991. Qualitative aspects of somaclonal variations in banana propagated by *in vitro* techniques. Sci. Hortic., 48: 71–88.

Jarret, R.L. and Litz R.E., 1986. Isozymes as genetic markers in bananas and plantains. Euphytica, 35: 539–547.

Jarret, R.L. and Gawel, N., 1991a. Abscisic induced-growth inhibition of sweet potato (*Ipomoea batatas* L.) *in vitro*. Plant Cell, Tissue and Organ Cult., 24: 13–18.

Jarret, R.L. and Gawel, N., 1991b. Chemical and environmental growth regulation of sweet potato (*Ipomoea batatas* L.) *in vitro*. Plant Cell, Tissue and Organ Cult., 25: 153–159.

Jarret, R.L. and Gawel, N. 1995. Molecular markers, genetic diversity and systematics in Musa. In: Gowen, S. (Ed.), Bananas and Plantains. Chapman and Hall, London (UK), pp 66–83.

Jones, D.R. and Lockhard, B.E.L., 1993, *Musa* Disease Fact Sheet No.1. Banana Streak Disease. INIBAP, Montpellier.

Kaemmer, D., Afza, R., Weising, K., Kahl, G. and Novak, F., 1992. Oligonucleotide and amplification fingerprinting of wild species and cultivars of banana (*Musa* spp.). Biotech., 10: 1030–1035.

Kendall, E.J., Kartha, K.K., Qureshi, J.A. and Chermak, P., 1993. Cryopreservation of immature spring wheat zygotic embryos using abscisic acid pretreatment. Plant Cell Rep., 12: 89–94.

Ko, W., Hwang, S. and Ku, F., 1991. A new technique for storage of meristem-tip cultures of 'Cavendish' banana. Plant Cell, Tissue and Organ Cult., 25: 179–183.

Lebot, V., Aradhya, K.M., Manshardt, R. and Meilleur, B., 1993. Isozymes and Classification of Pacific Bananas and Plantains. In: Ganry J. (ed.) Breeding Banana and plantain for Resistance to Diseases and Pests. Montpellier, France.

Ma, S.S. and Shii, C.T., 1972. *In vitro* formation of adventitious buds in banana shoot apex following decapitation. J. Chin. Soc. Hort. Sci., 18: 135–142.

Mora, I., Sandoval, J. and Müller L., 1988. Utilización de efecto osmótico en la conservación *in vitro* de *Musa*. In: Memorias 1986 de la IV Reunion Sobre Agrofisiologia del Banana. ASBANA, San José, Costa Rica, 155 pp.

Murashige, T. and Skoog, T., 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant., 15: 473–497.

Panis, B., 1995. Cryopreservation of banana (*Musa* spp.) germplasm. Diss. Agric. 272. Catholic University of Leuven, Belgium, 201 pp.

Panis, B., Withers, L.A. and De Langhe, E., 1990. Cryopreservation of *Musa* suspension cultures and subsequent regeneration of plants. Cryo-Lett., 11: 337–350.

Panis, B., Van Wauwe, A. and Swennen, R., 1993. Plant regeneration through direct somatic embryogenesis from protoplasts of banana (*Musa* spp). Plant Cell Rep., 12: 403–407.

Panis, B., Sagi, L. and Swennen, R., 1994a. Regeneration of plants from protoplasts of *Musa* species. In: Y.P.S. Bajaj (Ed.), Biotechnology in Agriculture and Forestry, Vol 29. Plant Protoplasts and Genetic Engineering. Springer-Verlag, Berlin, pp. 102–114.

Panis, B., De Smet, K., Van den Houwe, I. and Swennen, R., 1994b. *In vitro* conservation of *Musa* germplasm: Prospects of cryopreservation. In: Proc. XIth Reunion Meeting of ACORBAT. San José, Costa Rica, February, 13–18, 1994. pp. 127–148.

Panis, B., Totté, N., Van Nimmen, K., Withers, L.A. and Swennen, R. 1996. Cryopreservation of banana (Musa spp.) meristem cultures after preculture on sucrose. Plant Sci., 121: 95–106.

Panis, B., Schoofs, H., Remy, S., Sagi, L. and Swennen, R., R., 1999a. Cryopreservation of banana embryogenic cell suspensions: a tool for genetic engineering. In: Proc. JIRCAS/IPGRI Joint International Workshop: Cryopreservation of Tropical Plant Germplasm, Current Research Progress and Applications, Tsukuba, Japan, 20–23 Oct, 1998 (in press).

Panis, B., Schoofs, H., Thinh, N.T. and Swennen, R., 1999b. Cryopreservation of proliferating meristem cultures of banana. In: Proc. JIRCAS/IPGRI Joint International Workshop: Cryopreservation of Tropical Plant Germplasm, Current Research Progress and Applications, Tsukuba, Japan, 20–23 Oct, 1998 (in press).

Reed, B.M., 1991. Application of gas-permeable bags for *in vitro* cold storage of strawberry germplasm. Plant Cell Rep., 10: 431–434.

Reuveni, O. and Israeli, Y., 1990. Measures to reduce somaclonal variation in *in vitro* propagated bananas. Acta Hort., 275: 307–313.

Reuveni, O. and Gobulowicz, S., 1993. Reponse of *in vitro* banana plantlets to growth retardants. In: R.V. Valmayor, S.C. Hwang, S.W. Lee and V.N. Roa (Eds.), Proc.: Internat. Symp. Recent Developments in Banana Cultivation Technology. Taiwan Banana Research Institute, Chiuju, Pingtung, Taiwan, December 14–18, 1992, 314 pp.

Roca, W.M., Reyes, R. and Beltrán, J., 1983. Effect of various factors on minimal growth in tissue culture storage of cassava germplasm. In: Proc. 6th Symp. International Soc. Tropical Root Crops. Lima, Peru, February 21–26, 1983, pp. 441–446.

Sagi, L., Remy. S., Panis, B., Swennen, R. and Volvkaert, G., 1994. Transient gene expression in electroporated banana protoplasts (*Musa* spp. cv. 'Bluggoe' ABB group) isolated from embryogenic cell suspensions. Plant Cell, 13: 262–266.

Sagi, L., Panis, B., Remy, S., Schoofs, H., De Smet, K., Swennen, R. and Cammue, B.P.A., 1995. Genetic transformation of banana and plantain (*Musa* spp.) via particle bombardment. Bio/Technol., 13: 481–485.

Sakai, A., Kobayashi, S. and Oiyama, I., 1990. Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. Plant Cell Rep., 9: 30–33.

Schoofs, J., 1989. The INIBAP *Musa* germplasm Transit Centre. In: INIBAP/ IBPGR *Musa* Conservation and Documentation. Proc. Workshop Leuven, Belgium, pp. 25–30.

Scowcroft, W.R., 1984. Genetic variability in tissue culture: Impact on germplasm conservation and utilization. Rome, Internat. Bd. Plant Gen. Res., Tech. Rep. AGPG: IBPGR/84/152, 41 pp.

Sharrock, S., 1990. Collecting *Musa* in Papua New Guinea. In: R.L. Jarret (Ed.), Identification of Genetic Diversity in the Genus *Musa*. Proc. Internat. Workshop Los Banos, Philippines, September 5–10, 1988. IBPGR Publ., Rome, pp. 140–157.

Simmonds, N.W. and Shepherd, K., 1955. The taxomony and origins of the cultivated bananas. J. Linn. Soc. Lond. Bot., pp. 302–312.

Smith, M.K., 1988. A review of factors influencing the genetic stability of micropropagated bananas. Fruits, 43: 219–223.

Staritsky, G., 1986. *In vitro* conservation of arid germplasm at reduced temperatures and under osmotic stress. In: L.A. Withers and P.G. Alderson (Eds.), Plant Tissue Culture and Its Agricultural Application. Butterworths, London, pp. 285–290.

Swartz, H.J., 1991. Post culture behavior: genetic and epigenetic effects and related problems. In: Debergh, P.C. and Zimmerman, R.H. (Eds.), Micropropagation: Technology and Application. Kluwer Dordrecht (Netherlands), pp. 95–121.

Swennen, R., 1990. Limits of morphotaxonomy: Names and synonyms of plantains in Africa and elsewhere. In: R.L. Jarret (Ed.), Genetic Diversity in the Genus *Musa*. INIBAP, Montpellier, France, pp. 172–210.

Swennen, R. and Vuylsteke, D., 1993. Breeding black sigatoka resistant plantains with a wild banana. Trop. Agric. (Trinidad), 70 (1): 74–77.

Thinh, N.T., Takagi, H. and Yashima, S., 1999. Cryopreservation of in vitro grown shoot tips of banana (*Musa* spp.) by vitrification method. Cryo-Lett., 20: 163–174.

Uragami, A., 1991. Cryopreservation of asparagus (*Asparagus officinalis*) cultured *in vitro*. Res. Bul. Hokkaido Natl. Agr. Exp. Stn., 156: 1–37.

Van den houwe, I., De Smet, K., Tezenas du Montcel, H. and Swennen, R., 1995. Variability in storage potential of banana shoot cultures under medium term storage conditions. Plant Cell, Tissue and Organ Cult., 42: 269–274.

Vuylsteke, D.R., 1990. Shoot-tip culture for the propagation, conservation and exchange of *Musa* germplasm. IBPGR, Rome, 56 pp.

Vuylsteke, D., Swennen, R. and De Langhe, E., 1991. Somaclonal variation in plantain (*Musa* spp., AAB group) derived from shoot-tip culture. Fruits, 46: 429–439.

Vuylsteke, D., Swennen, R., Wilson, G.F. and De Langhe, E., 1988. Phenotypic variation among *in vitro* propagated plantain (*Musa* sp. cultivar 'AAB'). Sci. Hort., 36: 79–88.

Whittingham, D.G., Lyon, M.F. and Glenister, P.H., 1977. Long term storage of mouse embryos at –196°C: The effect of background radiation. Genet. Resources, 29: 171–181.

Withers, L.A., 1980. Tissue culture storage for genetic conservation. IBPGR, Rome, 91 pp.

Withers, L.A. and Williams, J.T., 1986. *In vitro* conservation. IBPGR, Highlights 1984–1985, 21 pp.

Wong, W.C., 1986. *In vitro* propagation of banana (*Musa* spp.): Initiation, proliferation and development of shoot tip cultures on defined media. Plant Cell, Tissue and Organ Cult., 6: 159–166.

Wu, R.Y. and Su, H.J., 1991. Regeneration of healthy banana plantlets from banana bunchy top virus-infected tissues cultured at high temperature. Plant Pathol., 40: 4–7.