Cryopreservation of *Musa* germplasm

Bart Panis and Nguyen Tien Thinh

PRO*MUSA*
A Global Programme for Musa Improvement

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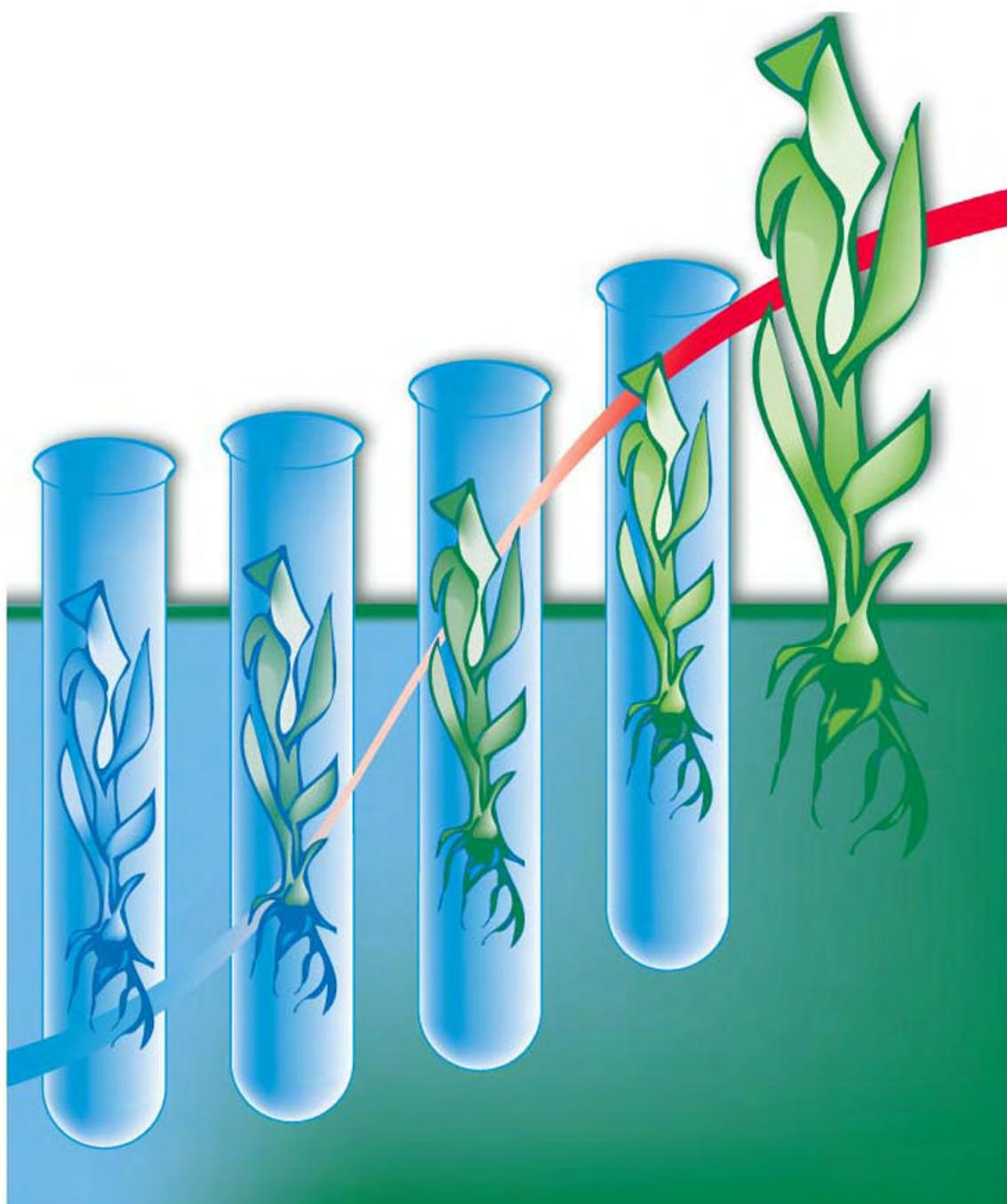
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The Programme has four specific objectives:

- To organize and coordinate a global research effort on banana and plantain, aimed at the development, evaluation and dissemination of improved cultivars and at the conservation and use of *Musa* diversity

- To promote and strengthen collaboration and partnerships in banana-related research activities at the national, regional and global levels

- To strengthen the ability of NARS to conduct research and development activities on bananas and plantains

- To coordinate, facilitate and support the production, collection and exchange of information and documentation related to banana and plantain.

Since May 1994, INIBAP is a programme of the International Plant Genetic Resources Institute (IPGRI).

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The **Technical Centre for Agricultural and Rural Cooperation** (CTA) was established in 1983 under the Lomé Convention between ACP (African, Caribbean and Pacific) group of States and the European Union Member States. Since 2000, it has operated within the framework of the ACP-EC Cotonou Agreement. CTA's tasks are to develop and provide services that improve access to information for agricultural and rural development, and to strengthen the capacity of the ACP countries to produce, acquire, exchange and utilize information in these areas. CTA's programmes are organized around four principal themes: developing information management and partnership strategies needed for policy formulation and implementation; promoting contact and exchange of experience; providing ACP partners with information on demand; and strengthening their information and communication capacities.

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Contents

Foreword

Bananas and plantains (*Musa* spp.) are one of the world's most important crops. Over 400 million people throughout the developing countries of the tropics and subtropics are dependent on this crop, which is both a staple food and an important commodity for marketing locally and internationally.

Bananas and plantains are grown almost exclusively by small-scale farmers and production is based on a wide variety of locally important varieties. In many areas however, this production is being increasingly constrained by pest and disease pressure. In response to this, a number of banana and plantain breeding programmes around the world are working to produce improved pest- and disease-resistant, high-yielding varieties.

The raw materials for banana breeding are the wild *Musa* species and diverse varieties found particularly in Asia, the centre of diversity of *Musa*, but also in Africa and Latin America. These species and cultivars contain the genes necessary for sustainably improved production in the face of pest and disease attack and changing environmental conditions. In order to ensure the availability of these important resources for future breeding and production, it is essential that *Musa* germplasm is safely conserved.

The International Network for the Improvement of Banana and Plantain (INIBAP) is responsible for the world collection of *Musa* germplasm. This consists of over 1100 accessions, both wild species and cultivated varieties, and is held under the auspices of FAO. This collection is presently maintained *in vitro*, under conditions of low light intensity and low temperatures in order to reduce the growth rates of the cultures. Despite these slow-growth conditions, all accessions still need to be recultured on average once per year. The reculturing process is labour-intensive and provides opportunity for accessions to become infected by fungal or bacterial contaminants. Furthermore accessions maintained *in vitro*, even under slow-growth conditions, are liable to somaclonal variation.

In order to overcome these problems and to ensure the safe long-term conservation of *Musa* genetic resources, INIBAP is supporting research on cryopreservation, i.e. storage at ultra-low temperatures, usually that of liquid nitrogen (-196 °C). This is the method of choice for ensuring cost-effective and safe, long-term storage of genetic resources of species which have recalcitrant seeds or are vegetatively propagated, such as *Musa*. This research is being carried out at the *Katholieke Universiteit Leuven*, Belgium (KUL) and the techniques developed are now being used for routine cryopreservation of accessions held by INIBAP.

Cryopreservation techniques are, in principle, applicable to any type of plant tissue with regeneration potential. Such techniques have been developed for more than 112 different plant species cultured in various forms, including cell suspensions, calli, apices, somatic and zygotic embryos (Engelmann 1997). However, the routine use of cryopreservation is still restricted almost exclusively to the conservation of cell lines in research laboratories.

Two types of highly meristematic and regenerative *in vitro* tissues can be obtained from the banana: (i) individual meristems isolated from shoot-tip cultures and (ii) highly proliferating meristem cultures containing 'cauliflower-like' meristem groups. Cryopreservation methods have been developed for both tissue types. In addition, embryogenic cell suspensions of different cultivars belonging to distinct genomic groups are also now being stored in liquid nitrogen (Panis *et al.* 1990, Panis 1995).

The different methods developed at KUL and JIRCAS (Japanese International Research Center for Agricultural Sciences) for cryopreserving *Musa* tissue are described in this publication. The advantages and disadvantages of each method are described and areas where research is still required to further optimize the protocols are identified.

The aim of this publication is to provide information and guidance on cryopreservation methodologies suitable for use on *Musa* germplasm. It is hoped that the detailed descriptions of the methodologies presented will facilitate their adoption and use in different laboratories.

The availability and the type of starting material, the genotypes being cryopreserved and availability of resources will have to be considered to determine which of these methods is most suitable for use in other laboratories.

Introduction

CRYOPRESERVATION PROTOCOLS FOR BANANA MERISTEMS

Until 10 years ago, cryopreservation protocols for plant tissues were mainly based on slow freezing in the presence of cryoprotective mixtures containing DMSO (dimethyl sulphoxide), sugars, glycerol and/or proline. Slow freezing results in a freeze-dehydration, leaving less water in the cells to form lethal ice crystals upon exposure to extreme low temperatures.

During the last decade however, several new cryopreservation procedures have been established. Among them, the protocol termed vitrification involves a treatment with cryoprotective solutions followed by dehydration with highly concentrated vitrification solutions. Vitrification can be defined as the transition of water directly from the liquid phase into an amorphous phase or glass, whilst avoiding the formation of crystalline ice. Vitrification techniques have been developed for different vegetatively propagated tropical crops, including banana (Thinh *et al*. 1999).

INIBAP-supported research at KUL has resulted in the development of two cryopreservation protocols suitable for the long-term storage of meristem cultures of banana. The first method relies on rapid freezing of highly proliferating meristem cultures precultured for 2 weeks on 0.4 M sucrose. The second, and up to now most successful protocol, combines technologies of two previous methods, i.e. vitrification of sucrose precultured, highly proliferating meristem cultures. The labour requirement to cryopreserve accessions, as well as the post-thaw regeneration rates, depend on the cultivar and the method used. Application of the simple freezing protocol and vitrification of proliferating clumps has resulted thus far in the safe storage in liquid nitrogen of accessions belonging to the different genome groups within the genus *Musa*.

DETECTION OF ENDOPHYTIC BACTERIA

During normal meristem culture and storage under limited growth conditions, the presence of endogenous bacteria is rarely observed. If present, such bacteria do not interfere with the growth of the meristem cultures. However, as soon as meristems are subjected to cryopreservation, endogenous bacteria become a problem. As the meristems begin to regrow following cryopreservation, any endogenous bacteria present develop into yellow or white colonies, which overgrow the recovering meristem. Therefore, prior to cryopreservation, cultures are screened for the presence of endophytic bacteria on a bacterial growth medium (BACT medium) containing 23 g/l Difco[®] Bacto nutrient broth, 10 g/l glucose and 5 g/l yeast extract (van den Houwe and

Swennen 2000). These plates are incubated for 3 weeks in the light at 28 °C. Accessions responding positively are discarded.

VIABILITY TESTING

After being stored at least one hour in liquid nitrogen, a small number of replicates of all cryopreserved samples are thawed to evaluate the viability of the material. At KUL, for each new batch of cryopreserved material, a minimum of two cryotubes, each containing 7 to 10 individual meristems or meristem clumps, are thawed immediately to evaluate viability. Thawing is carried out following the methods described below for the different cryopreservation protocols. The evaluation of the percentage of regrowing meristems gives a good indication of the viability of the material which is being stored permanently in liquid nitrogen.

1. Cryopreservation of individual banana meristems

This method is illustrated in Figure 1 and details are given below.

1.1 PLANT MATERIALS

Preparation of *in vitro* plantlets

In vitro single or multiple shoots of banana are established by culturing isolated shoot tips (dissected from field suckers) on semi-solid MS medium (Murashige and Skoog 1962) supplemented with 30 g/l sucrose and 0.2 or 4 mg/l BA (6-benzylaminopurine). Established shoots, each 3-4 cm long, are cultured for 30-45 days on MS medium supplemented with 60-90 g/l sucrose to form adventitious roots. During this rooting stage, it is recommended to place cultures at 25±2 °C under continuous light or a 16 hours light/8 hours dark photoperiod, with a light intensity of at least 90 mE μ^{-2} s^{-1} (cool white fluorescent lamps). Healthy rooted plantlets with a base diameter of approximately 1 cm form an appropriate source for the excision of apical meristems.
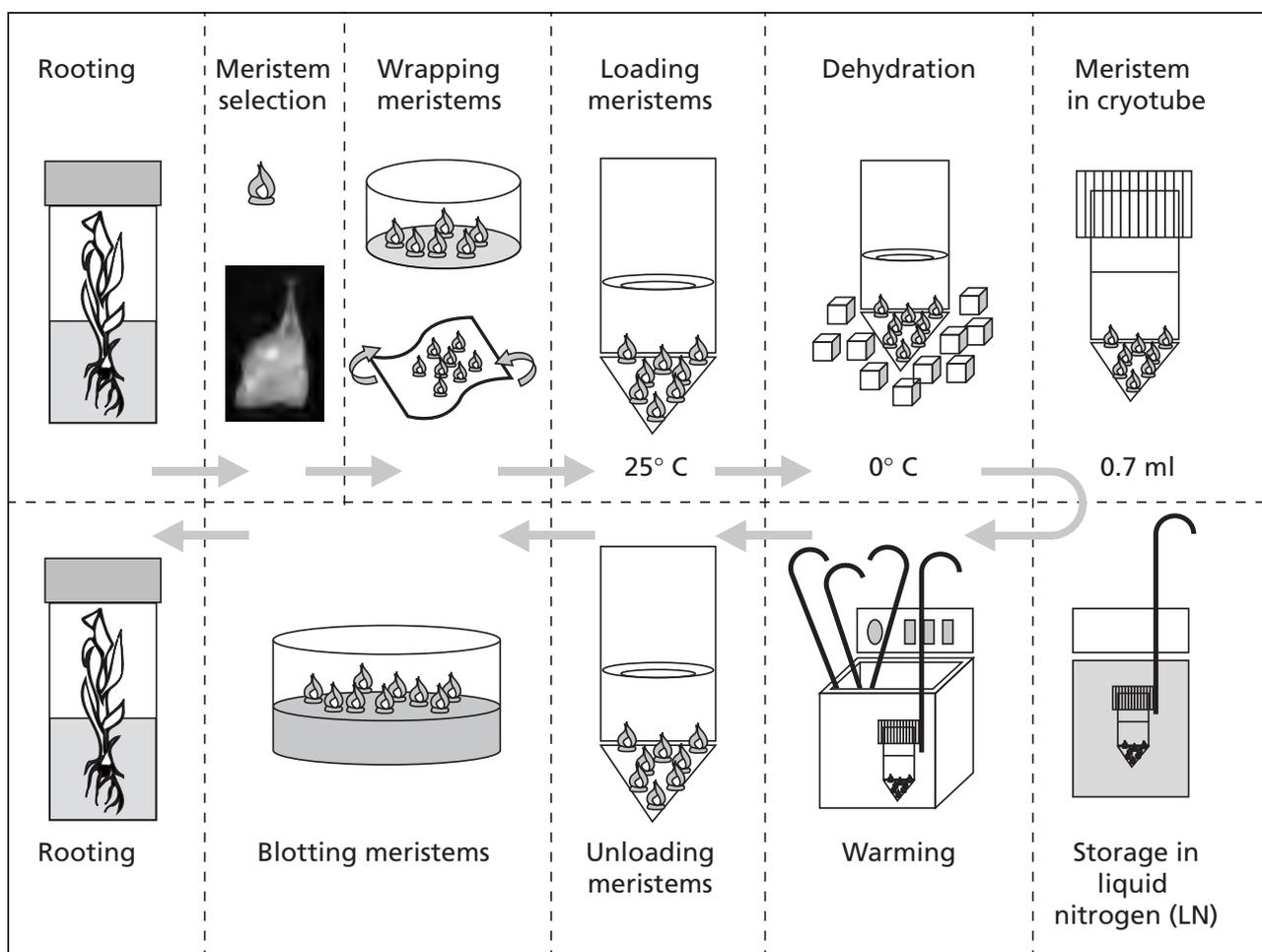
Figure 1. Cryopreservation of individual meristems.

Dissection and selection of apical meristems

Like many other monocots, banana apical meristems are tightly covered with several layers of whitish, tubular, immature leaves. Using a binocular microscope and fine dissection tools, leaves may be carefully and patiently removed one by one until the glassy apical dome of the meristem can be clearly observed. The meristem is then isolated from the main shoot by sharp cuts to its base (Figure 2). Ideal meristems for cryopreservation are those where the apical dome is just partly covered by the bases of the first outer leaf primordia, i.e. PC meristem as shown in Figure 3. These meristems are generally about 0.8-1 mm long.
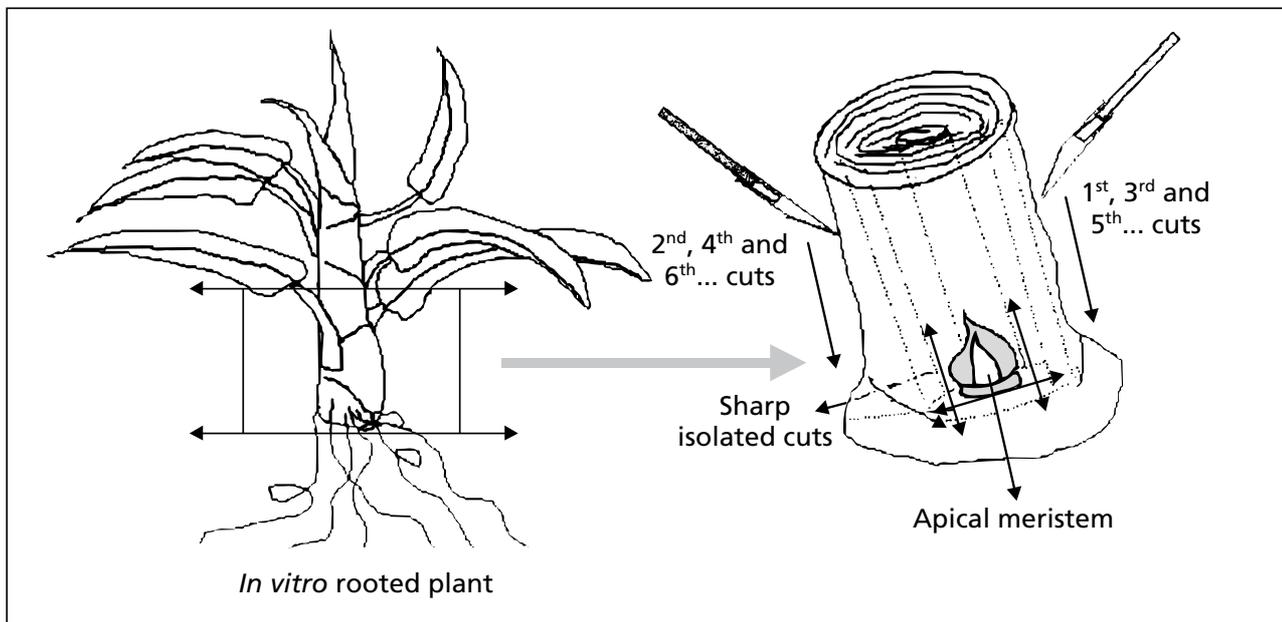
Figure 2. Illustration of meristem isolation in banana.
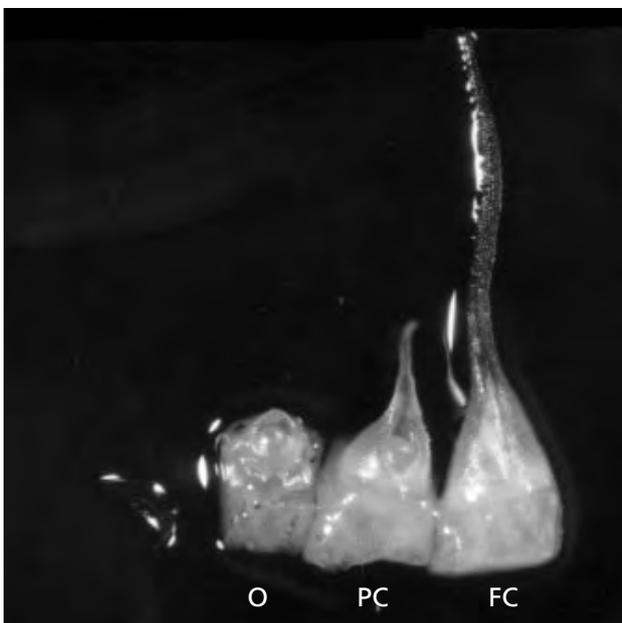
Figure 3. From left to right, freshly isolated open (O), partly-coverd (PC) and fully-covered (FC) apical meristems of bananas cv. Cavendish.

To prevent desiccation, freshly dissected meristems are immediately placed on the surface of MS medium supplemented with 0.1 M sucrose in a Petri dish. All possibly damaged meristems (due to handling errors) should be discarded. A skilful technician can isolate 6-8 meristems an hour (roughly, 7-10 minutes/isolated meristem).

1.2 CRYOPRESERVATION OF INDIVIDUAL MERISTEMS

Loading

Although the precise mechanism of loading is not yet fully understood, it has been proven for different plant species that loading can dramatically enhance the tolerance of isolated meristems to dehydration by the vitrification solution (Matsumoto *et al*. 1994, Takagi *et al*. 1997).

The loading of banana meristems is carried out as follows:

• A piece of tissue paper (1.5-2 x 1.5-2 cm) is placed in a Petri dish and moistened with some drops of loading solution, L1 (see Appendix 1).

• Seven to 10 meristems are wrapped in this tissue paper and immediately immersed for 20 minutes at room temperature (ca. 22-25 °C) in 6 ml L1 solution in a small Petri dish (Δ=6 cm).

• The folded tissue paper containing loaded meristems (hereafter referred to as TPLM) is placed for about 1 minute on a sterile filter paper to drain out excessive L1 solution.

Dehydration

To dehydrate the meristems, the TPLM is immersed in a small Pyrex glass test tube (screwed cap type) with 10 ml of chilled PVS2 solution (see Appendix 1 for preparation of the solution). The tubes are then placed in an ice bath for 20 minutes.

Rapid freezing

After 20 minutes in the ice bath, the TPLM are inserted into small (vol. 0.7 ml) plastic cryotubes filled with fresh, ice-chilled PVS2 solution. After screwing the tube cap, a layer of Teflon tape is applied to seal the edge between the cryotube cap and body. Within a few seconds, the cryotube is rapidly immersed into liquid nitrogen (LN) in a small Dewar flask.

Storage

After 30 minutes of storage in liquid nitrogen, the cryotubes containing the TPLM are transferred from the Dewar flask to the permanent tank of liquid nitrogen. To ensure a rapid transfer (a few seconds) from the Dewar flask to the

long-term storage tank, the two containers should be placed as close together as possible.

Because liquid nitrogen is continuously evaporating, a regular supply of new LN to the tank is necessary to ensure that the cryotubes are continuously exposed to the liquid phase of LN.

Thawing

After being stored at least one hour in liquid nitrogen, samples (cryotubes with TPLM) are thawed to evaluate the viability of the cryopreserved material.

After storage, cryotubes are quickly transferred to an LN-containing Dewar flask and moved to a clean bench for the rewarming step:

The frozen cryotubes are rapidly (within a second) transferred from LN to a 40 °C holding water bath and vigorously shaken for 90 seconds.

Next, the envelope containing the meristems is removed from the tube and placed for 30 seconds on the surface of a sterile filter paper to partly drain out the excess of PVS2 solution.

Unloading

The envelope is then transferred to a 6 cm-diameter Petri dish containing 6 ml of liquid MS medium supplemented with 1.2 M sucrose. Each 5 minutes this unloading solution is refreshed for a total period of 10 minutes.

The envelope is unwrapped and the meristems remain in suspension in the unloading solution for an additional 5 minutes.

Recovery

With the help of a binocular microscope, unloaded meristems are picked up from the unloading solution and placed on a sterile filter paper which is laid over the surface of a fresh semi-solid MS medium supplemented with 0.3 M sucrose in a Petri dish.

After 12 to 18 hours, the meristems are transferred onto semi-solid MS medium supplemented with 0.1 M sucrose and 0.5 mg/l BA (6-benzylaminopurine).

During the first 10-15 days, cultures are incubated in darkness or dim light, then they are placed at 25±2 °C under continuous light or a 16 hours light/ 8 hours dark photoperiod with an intensity of at least 90 μE m^{-2} s^{-1} (cool white fluorescent lamps). Successfully cryopreserved meristems will form small shoots after 40-60 days (Figures 4, 5 and 6; see next page and page 21).
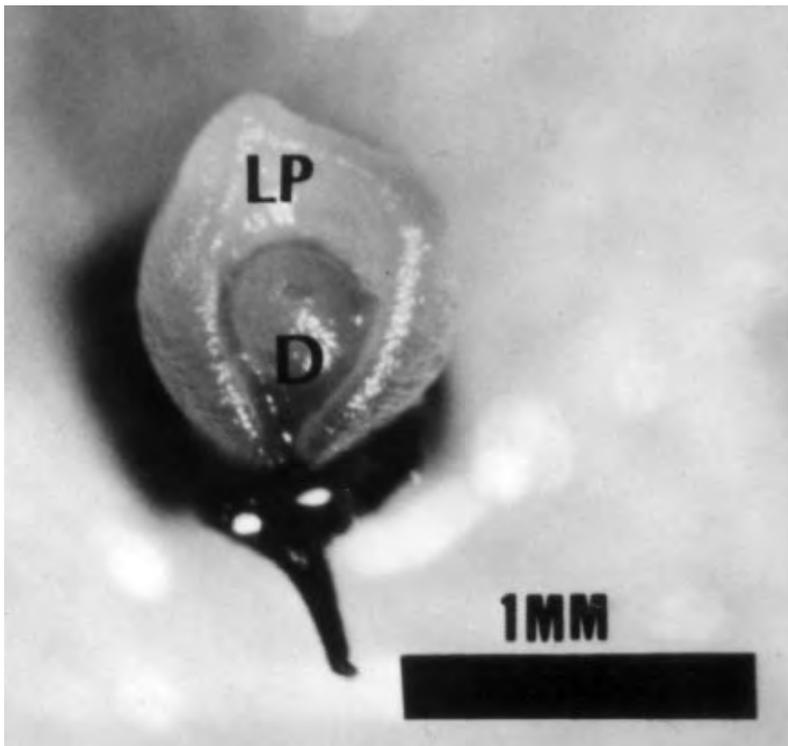
Figure 4. Fully recovering apical meristem after exposure to LN, 5 days after rewarming (D = apical dome, LP = growing leaf primordium).

2. Cryopreservation of banana meristem clumps (cauliflower-like structures)

A second type of regenerative meristematic tissue in banana which has been successfully cryopreserved is the highly proliferating (sometimes called cauliflower-like) meristem clump. This tissue type was originally produced as a starting material to initiate embryogenic cell suspension cultures in bananas (Dhed'a *et al.* 1991, Schoofs 1997).

Two cryopreservation techniques applied to highly proliferating 'cauliflower-like' meristem cultures are described below:

• simple freezing method (which involves a sucrose preculture) (Panis *et al.* 1996),

• combined cryopreservation method (combining preculture with vitrification) (Panis *et al.* 2000b).

2.1 PLANT MATERIAL

Production of 'cauliflower-like' meristem clumps

Preliminary experiments revealed that regrowth of meristematic clumps after cryopreservation can only succeed with 'cauliflower-like' clusters. To produce this kind of material in all *Musa* accessions, meristem cultures are transferred to a medium containing a high concentration of BA (P4 medium, see Appendix

1). Every 1 to 2 months, the material is subcultured and small clumps of 'cauliflower-like' meristems are selected and transferred to fresh medium. The high BA concentration in the P4 medium suppresses outgrowth of meristems, thereby favouring the formation of numerous white apical domes (Figure 7; see page 22).

Preculture of meristem clumps

Following the appearance of 'cauliflower-like' clumps, and six weeks after the last subculture, white meristematic clumps of about 4 mm diameter, each containing at least four apical domes, are excised and transferred onto a preculture medium (P5 + 0.4 M sucrose) for 2 weeks. They are cultured at 25 °C ± 2 °C under continuous 50 μE m^{-2} s^{-1} illumination provided by cool-white fluorescent tubes.

2.2 SIMPLE FREEZING METHOD

This method is illustrated in Figure 8.

Cryopreservation

Small white meristematic clumps of 5-15 mg, containing 3 to 6 meristematic domes, are excised from the preculturedclumps. Brown tissues are removed
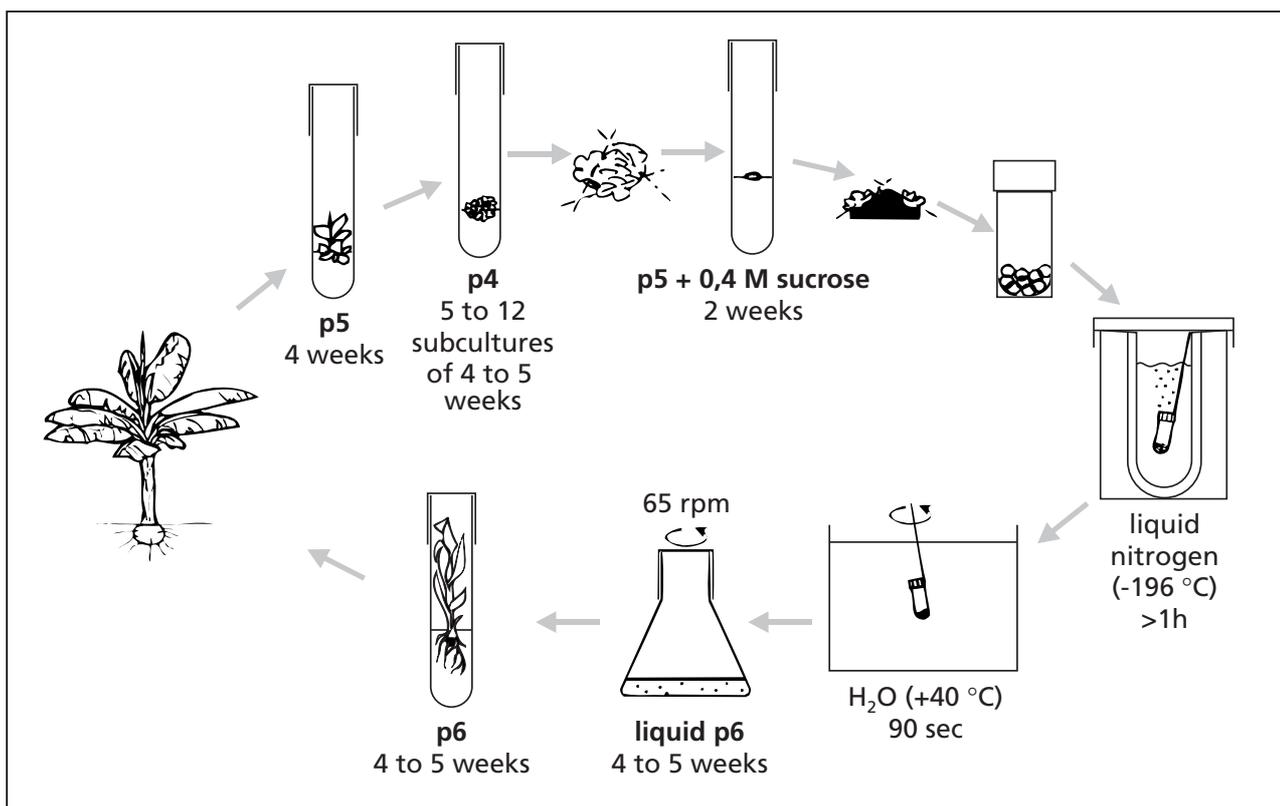
Figure 8. Simple freezing.

and only the most healthy, as indicated by a white-yellowish colour, are retained. The clumps are transferred to sterile cryotubes (2 ml) without any liquid solution, sealed with a layer of Teflon tape and plunged directly into a Dewar flask containing liquid nitrogen. Each cryotube contains 7 to 10 clumps. At this stage, samples can be stored for the long term by transferring the cryotubes to the liquid nitrogen tank, ensuring that their transfer from one container to the other happens rapidly (within a few seconds), thereby preventing slow lethal thawing of samples.

Thawing and recovery

After storage, rapid thawing takes place by stirring the frozen cryotube in a water bath at 40 °C for 90 seconds.

Regeneration of the frozen meristems can be carried out in two different ways:

• Meristems are transferred to 9 cm Petri dishes containing semi-solid regeneration medium (P6) and sealed with parafilm.

• Alternatively, regeneration can be executed in liquid medium. Thawed meristems are transferred to 100 ml Erlenmeyer flasks containing 30 ml of liquid regeneration medium (P6 without solidifying agent) and placed on a rotary shaker at 70 rpm.

After 1 week of culture in dark conditions, Petri dishes and flasks are transferred to continuous light at 50 μE m^{-2} s^{-1}. Cultures are kept at all times at 25 $\pm$ 2 °C.

Three weeks after transferring to the regeneration medium, frozen meristem regrowth is determined under a binocular microscope. Two types of surviving tissues are distinguished, i.e. shoots and non-regenerating callus. All calluses are systematically discarded and only recovered shoots (Figure 9; see page 22) are transferred to test tubes with regeneration medium to promote further development of whole plants. As soon as rooted plants are sufficiently developed, they are planted in soil.

2.3 COMBINED CRYOPRESERVATION METHOD

This method is illustrated in Figure 10.

Loading

Meristem clumps are transferred to 5 ml of a loading solution (Appendix 1), in a 20 ml plastic vessel where they are kept for 20 minutes at room temperature.
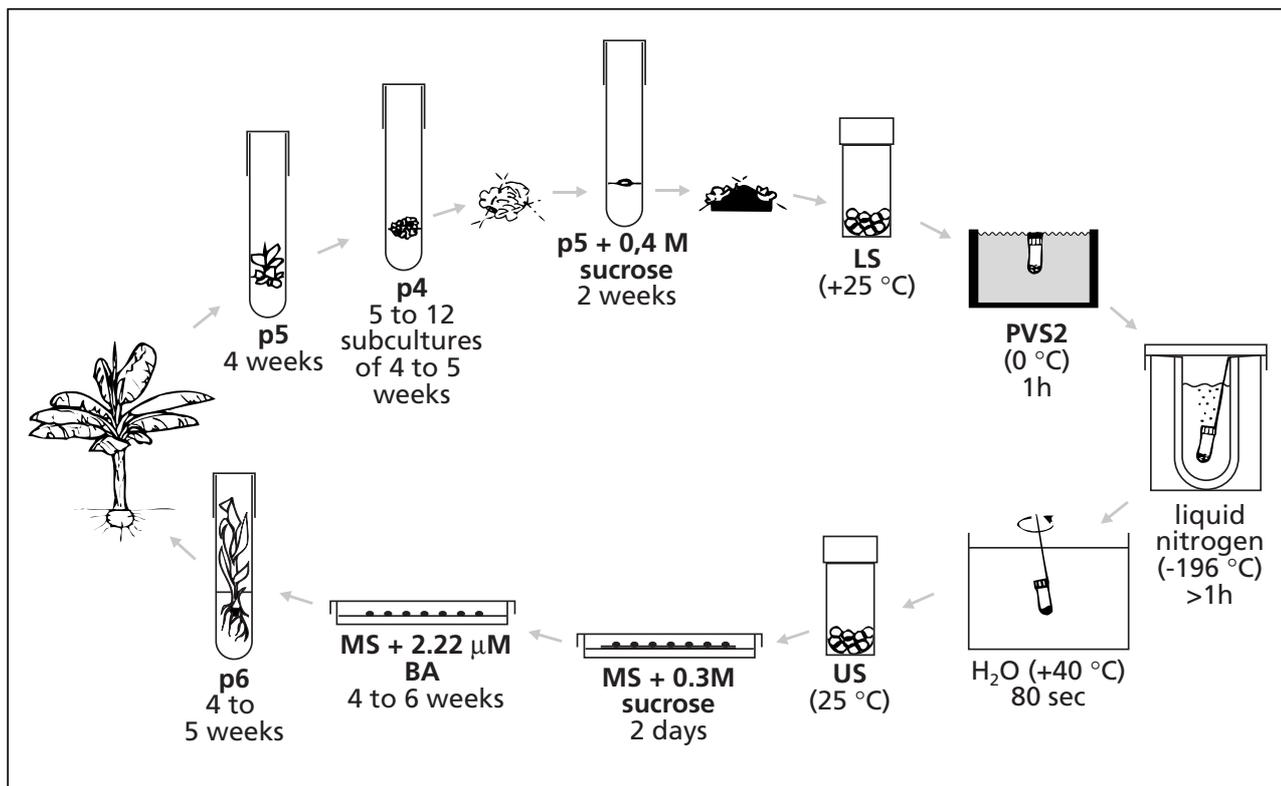
Figure 10. Vitrification of proliferating material.

Treatment with vitrification solution (dehydration) and freezing

The loading solution is replaced by 5 ml of ice-cooled PVS2 solution and the plastic vessels containing the meristems are placed on ice. The meristems are subjected to the PVS2 solution for 60 to 180 minutes at 0 °C.

During the PVS2 treatment, meristems are transferred from the plastic vessels into cooled 2 ml sterile cryotubes which contain 1 ml of fresh ice-cold PVS2.

The cryotubes are sealed with a layer of Teflon tape and plunged directly into a Dewar flask containing liquid nitrogen.

Thawing and unloading

After storage, meristems are rapidly thawed by stirring the tubes in a warm water bath at 40 °C for 90 seconds. Directly after thawing, the toxic PVS2 solution is removed from the cryotubes and replaced by the 'unloading' solution (see Appendix 1). The meristems are unloaded for 15 minutes at room temperature.

Regeneration

Frozen meristems are taken from the unloading solution and placed in 9 cm plastic Petri dishes on 2 sterile filter papers on top of about 25 ml semi-solid hormone-free MS medium containing 0.3 M sucrose.

After two days, the filter paper is removed and the meristems are transferred to Petri dishes with an MS medium supplemented with 2.22 μM BA. The first week of culture always takes place in the dark. After a maximum of 6 weeks, meristems are transferred to test tubes with P6 medium for further development of whole plants (Figure 11; see page 23).

3. Cryopreservation of banana embryogenic cell suspensions

3.1 INTRODUCTION

Since most of the cultivated banana varieties are highly sterile, classical breeding programmes are very slow and labour-intensive. Furthermore, no sources of resistance are available in the banana genepool against some pathogens, such as banana viruses. Genetic engineering therefore offers a welcome alternative for the genetic improvement of bananas. In monocots, embryogenic cell suspensions are often the material of choice for transformation, particularly in sterile crops like banana where zygotic embryos are not available. Embryogenic cell suspensions are presently the only source of regenerative protoplasts in banana (Panis *et al*. 1993). When subjected to electroporation, protoplasts derived from embryogenic cell suspensions give rise to a high frequency of transient expression of introduced marker genes (Sagi *et al*. 1994). Walled suspension cells can be successfully transformed by means of particle bombardment (Sagi *et al*. 1995) and *Agrobacterium* (Hernandez *et al*. 1998). In this way genes coding for new types of anti-fungal proteins as well as virus resistance have been introduced into bananas.

The main bottleneck for transformation remains the initiation of cell suspensions of good quality, i.e. homogeneous embryogenic cell suspensions with high regeneration frequency. The initiation of these suspension cultures is difficult and time-consuming, irrespective of the starting material used (immature male flowers, immature zygotic embryos or proliferating *in vitro* meristems). Once established, these valuable cell suspensions are subject to somaclonal variation and microbial contamination. Moreover, a prolonged culture period may result in a decrease and eventually a total loss of morphogenic capacity.

In 1990, a cryopreservation technique for 'ideal' cell suspensions was developed which involves cryoprotection with 7.5% DMSO (dimethyl sulphoxide) for 1 hour at 0 °C, followed by slow freezing at 1 °C/minute to -40 °C and plunging into liquid nitrogen. An 'ideal' embryogenic cell suspension contains a high proportion of cells that are isodiametric and characterized by a relatively large nucleus, small multiple vacuoles and tiny

starch and protein grains (Figure 12; see page 23). Later on, this cryopreservation protocol was optimized in order to apply it to less 'ideal' but also highly regenerative banana cells (Panis *et al.* 2000a). Less 'ideal' suspensions are more heterogeneous and can contain, besides embryogenic cell clumps, cells which are highly vacuolated and elongated, cells with very dense but granular cytoplasm and cells with large starch grains and/or organized globules.

Recently, banana cell suspensions were recovered after 5 years storage in liquid nitrogen. The ability to produce somatic embryos remained intact. Also embryogenic cell suspensions could again be initiated from the frozen material.

3.2 PLANT MATERIAL

Starting material

The more recent studies published on the cryopreservation of banana cell suspensions show some differences in the procedure depending on the tissue used as starting material to initiate the cell suspensions. In these guidelines, two different types of suspensions will be considered: (i) suspensions derived from male flowers (Côte *et al.* 1996) and (ii) suspensions derived from proliferating meristem cultures (Schoofs 1997).

Cell suspensions derived from proliferating meristem cultures

Cell suspensions are kept in ZZ liquid medium (Appendix 1) on a rotary shaker at about 70 rpm and at 25 ± 2 °C under continuous light conditions of $25\ \mu$E m^{-2} s^{-1} PAR (photosynthetic active radiation).

Cell suspensions derived from male flowers

Cell suspensions are kept in MA2 liquid medium (Appendix 1).

3.3 CRYOPRESERVATION OF CELL SUSPENSIONS

Preculture

This step is recommended only for cell suspensions derived from male flowers. Cells are cultured for 24 hours in liquid MA2 supplemented with 180g/l sucrose.

Cryoprotection

Cell suspensions are always cryopreserved when they are in their exponential growth phase. Exponential cell growth usually takes place 7 to 10 days after the last subculture.

Cells are allowed to settle in a graduated centrifuge tube and the old medium is removed.

New liquid ZZ medium[1] with 180 g/l sucrose is added until a final settled cell volume of 30% is obtained.

An equal volume of sterile ZZ[1] +180 g/l sucrose medium containing 15% dimethylsulfoxide (DMSO) is gradually transferred to the concentrated cell suspension over a period of one hour at room temperature.

As such, the final cryoprotective solution, in suspensions derived both from male flower or meristematic cultures, contains 7.5% DMSO and 180 g/l sucrose.

Freezing and storage

Slow freezing in methanol bath

Samples of 1.5 ml of the cryoprotected cell suspensions are transferred to 2 ml cryotubes, sealed with Teflon tape and placed in a stirred methanol bath (Cryocool CC-60, Exatrol and agitainer from Neslab, Portsmouth, New Hampshire, USA). This methanol bath cools at a rate of 1 °C/minute.

As soon as a temperature of -7.5 °C is obtained, cryotubes are immersed for 3 seconds in liquid nitrogen to initiate ice crystallization. Then they are further cooled to -40 °C. After 30 minutes at -40 °C, cryotubes are plunged in liquid nitrogen (-196 °C) for further storage.

Slow freezing using Nalgene™ cryo 1 °C freezing container

The cryotubes containing 2 ml of cryoprotected cell suspensions are placed in a Nalgene™ cryo 1 °C Freezing Container. This simple freezing device consists of a plastic container holding 250 ml of iso-propanol. Its transfer into a freezer (–80 °C) allows a cooling rate of about 1 °C/minute.

In both cases, the temperature decrease inside the cryotube is followed using a temperature probe which is placed in a control cryotube.

Thawing and recovery

After storage, cryotubes are rapidly thawed in a beaker filled with sterile water at 40 °C for about 1.5 to 2 minutes until most of the ice melted.

Cell suspensions derived from proliferating meristem cultures

Thawed cells are plated on semi-solid medium ZZ or RD1 medium (Appendix 1) in 90 mm Petri dishes. RD1 medium is employed when regenerated plantlets are required from the cryopreserved material. Semi-solid ZZ medium is used when an embryogenic cell suspension culture must be

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1 ZZ medium is replaced by MA2 medium when cells of male flower origin are used.

re-established. During the first week following cryopreservation, Petri dishes are always placed in the dark (Figures 13 A, B, C; see page 24).

Cell suspensions derived from male flowers

Thawed cells are plated on semi-solid medium MA2 for 24 hours in 90 mm Petri dishes. After 24 hours, cells are transferred to MA3 medium for further development of the somatic embryos regenerated from the cell suspension or to MA2 medium when an embryogenic cell suspension culture must be re-established.

Viability test of the cell suspensions

Cell viability is determined by the fluorescein diacetate (FDA) test (Widholm 1972) whereby surviving cells fluoresce very brightly under ultraviolet illumination (Figure 14; see page 24).

If no fluorescent microscope is available, the 2,3,4-triphenyl tetrazolium chloride (TTC) reduction test (Dixon 1985) can be applied. Surviving cells convert the colourless TTC into red formazan crystals, which can be observed using an ordinary microscope.

Figure 5. Development of cryopreserved apical meristems of banana cv. 'Chuoi Huong' 30 days after thawing.

Figure 6. Recovered shoots from cryopreserved apical meristems of banana cv. 'Sanjaku' 60 days after thawing.
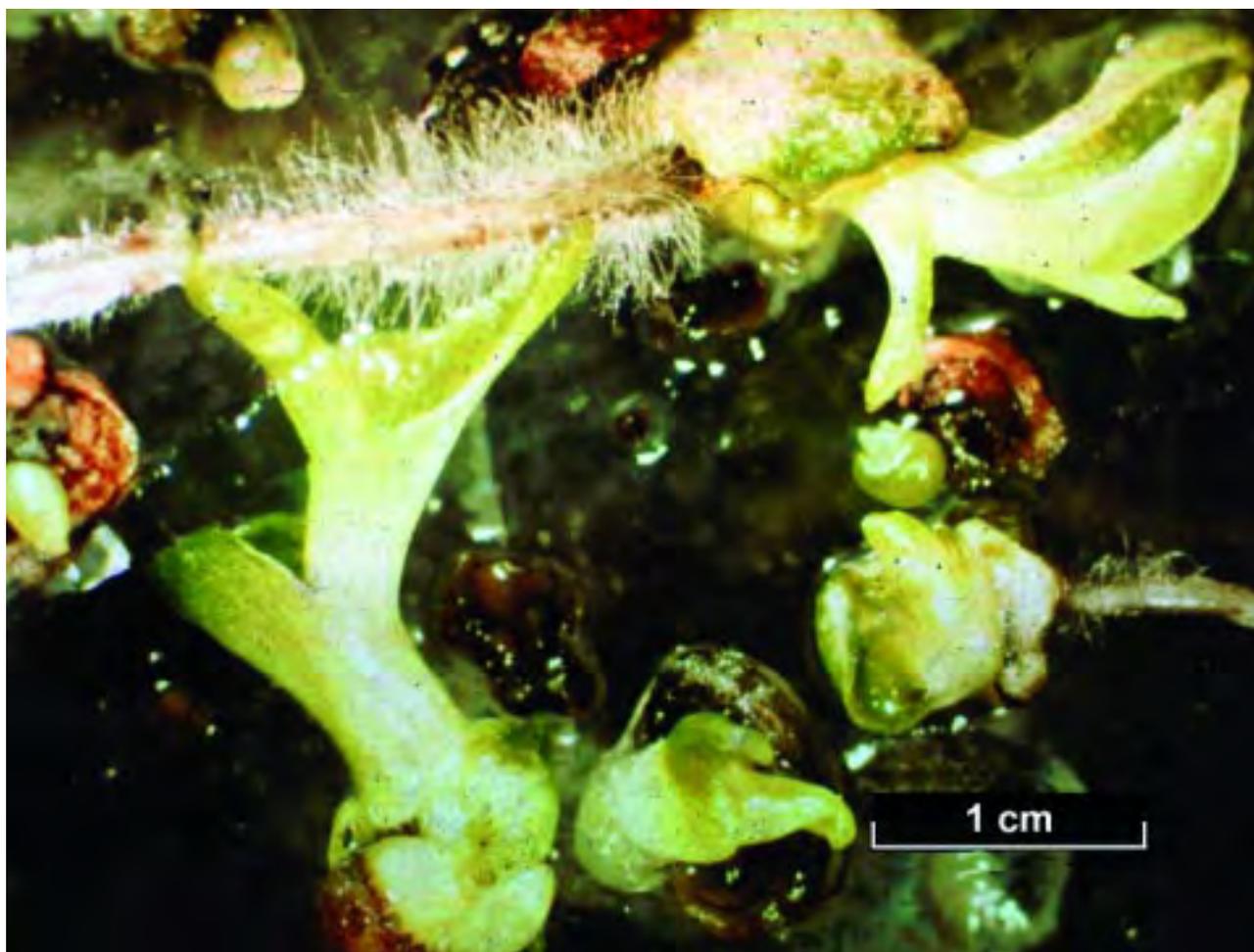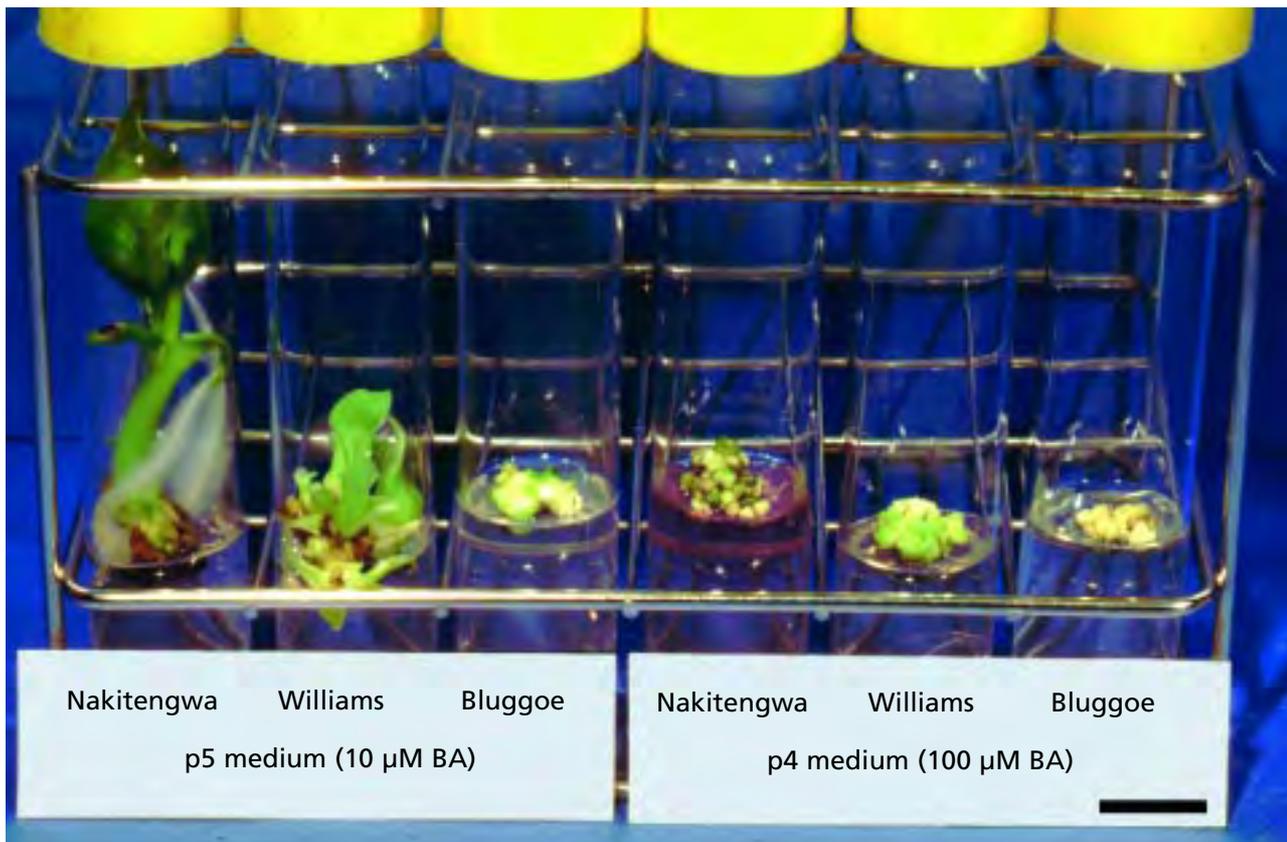
Figure 7. Meristem culture of banana cultivars Nakitengwa (AAA highland banana), Williams (AAA group) and Bluggoe (ABB group) on (left) p5 medium (containing 10µM BA) and (right) p4 medium (containing 100µM BA). (bar = 1 cm).
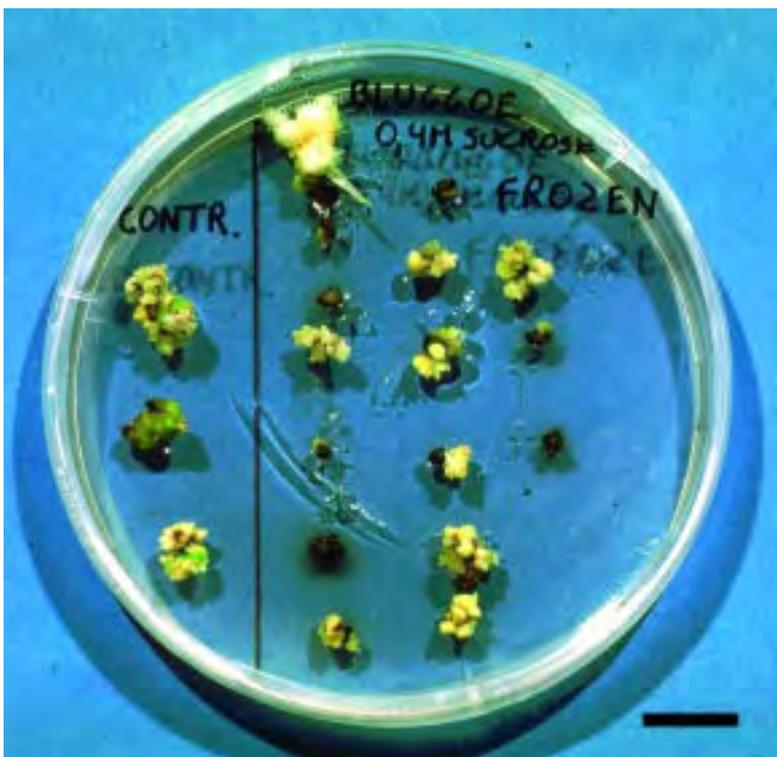
Figure 9. Petri dish containing control (left) and frozen (right) meristematic clumps of the cv. Bluggoe (ABB group), 8 weeks after cryopreservation (bar = 1 cm).

| Control | Frozen | Frozen | Frozen | Control | Frozen | Frozen | Frozen |

Kisubi (AB group)   Dominico Harton (AAB plantain)
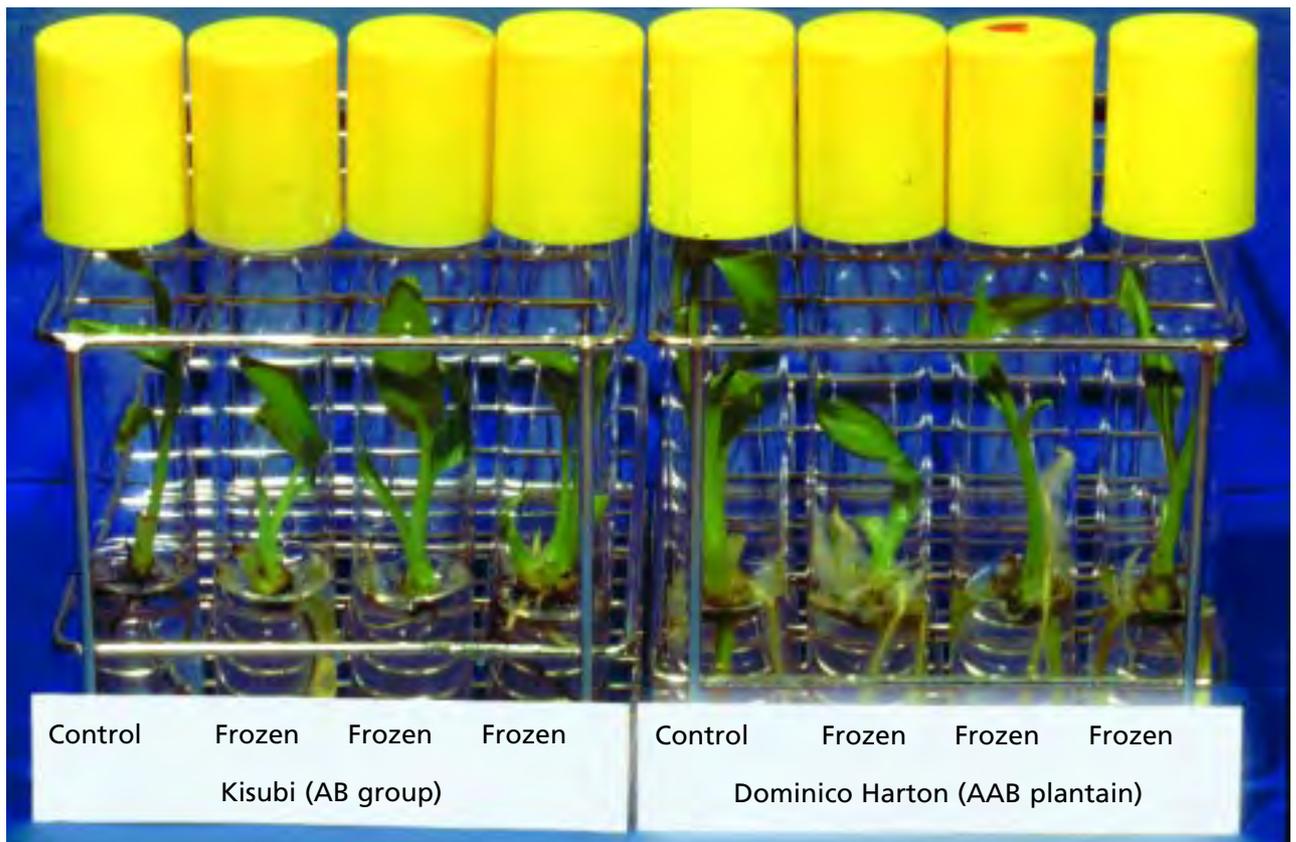
Figure 11. Regenerating shoots from 1 control and 3 frozen proliferating meristems of cultivars 'Kisubi' (AB group) and 'Dominico Harton' (AAB plantain), 3 months after cryopreservation.
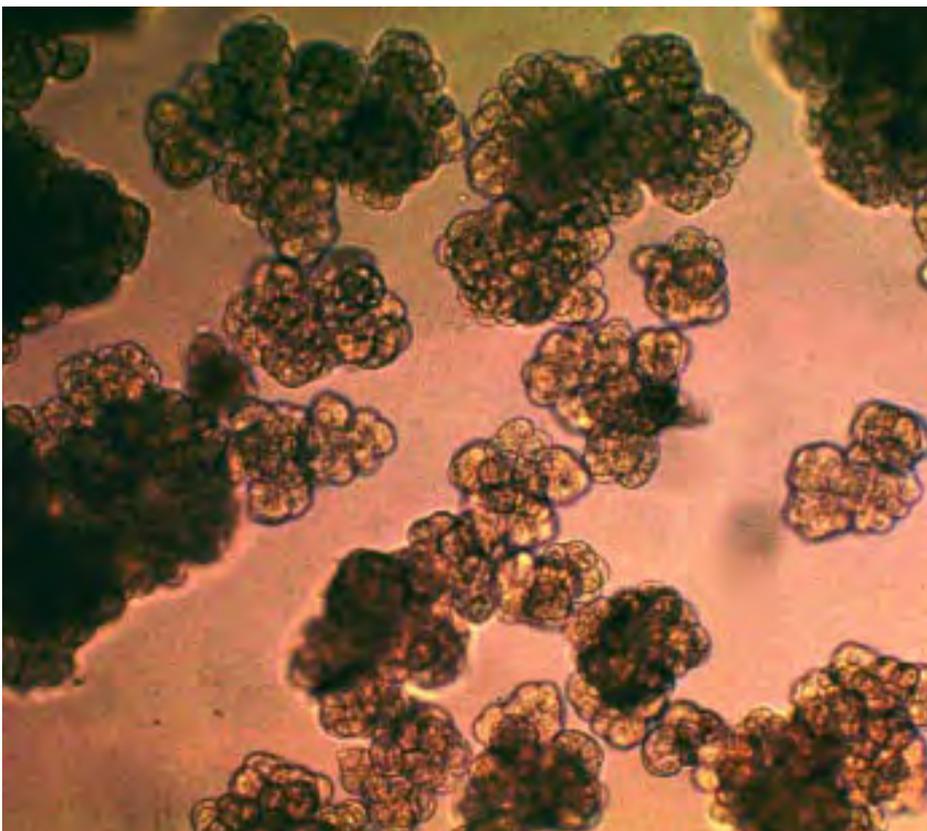
Figure 12. Embryogenic cells suspensions of the cultivar Three Hand Planty (AAB Plantain).
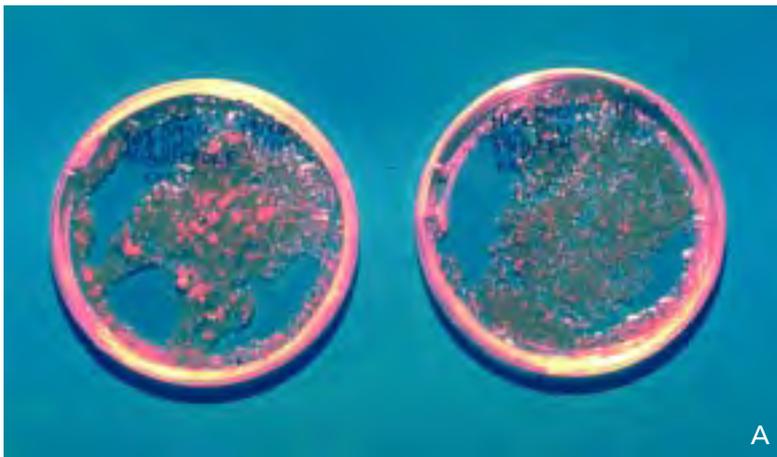
Figure 13. (A) Regrowth after 4 weeks on semi-solid medium of unfrozen (left) and frozen (right) embryogenic cell suspensions of 'Bluggoe' (ABB group). (B) Mass of somatic embryos originating from a frozen cell culture. (C) Greenhouse plants derived from a frozen cell suspension.

Figure 14. Embryogenic cell suspension of the cv. 'Bluggoe' (ABB group), cryoprotected with 5% DMSO, frozen in liquid nitrogen, stained with FDA and observed with ultra violet light. The small surviving embryogenic cells fluoresce very brightly while larger structures show more diffuse fluorescence (bar = 100 μm).
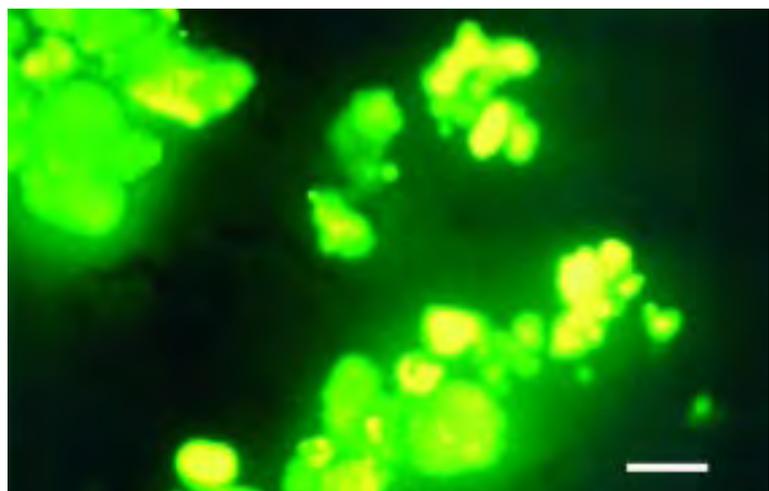
4. Cryopreservation of banana zygotic embryos

4.1 MATERIAL AND METHODS

This cryopreservation method requires fully developed zygotic embryos excised from mature *Musa* seeds. The fruit is brushed and washed with tap water and liquid soap. Subsequently, it is sterilized with 20% commercial bleach for 5 minutes and rinsed three times with sterile water.

The banana is peeled under sterile conditions using a laminar flow cabinet. Seeds are extracted and the zygotic embryo is isolated using a stereoscopic microscope. Because the embryo is located just beneath the 'opercule' it is important to use the scalpel and cut longitudinally near-around the 'micropylar plug' (Figure 15).

Preculture

The embryos are transferred for 5 hours onto the medium described by Escalant and Teisson (1987) which consists of the mineral nutrients of Murashige and Skoog with macro-elements at half strength, Morel vitamins,
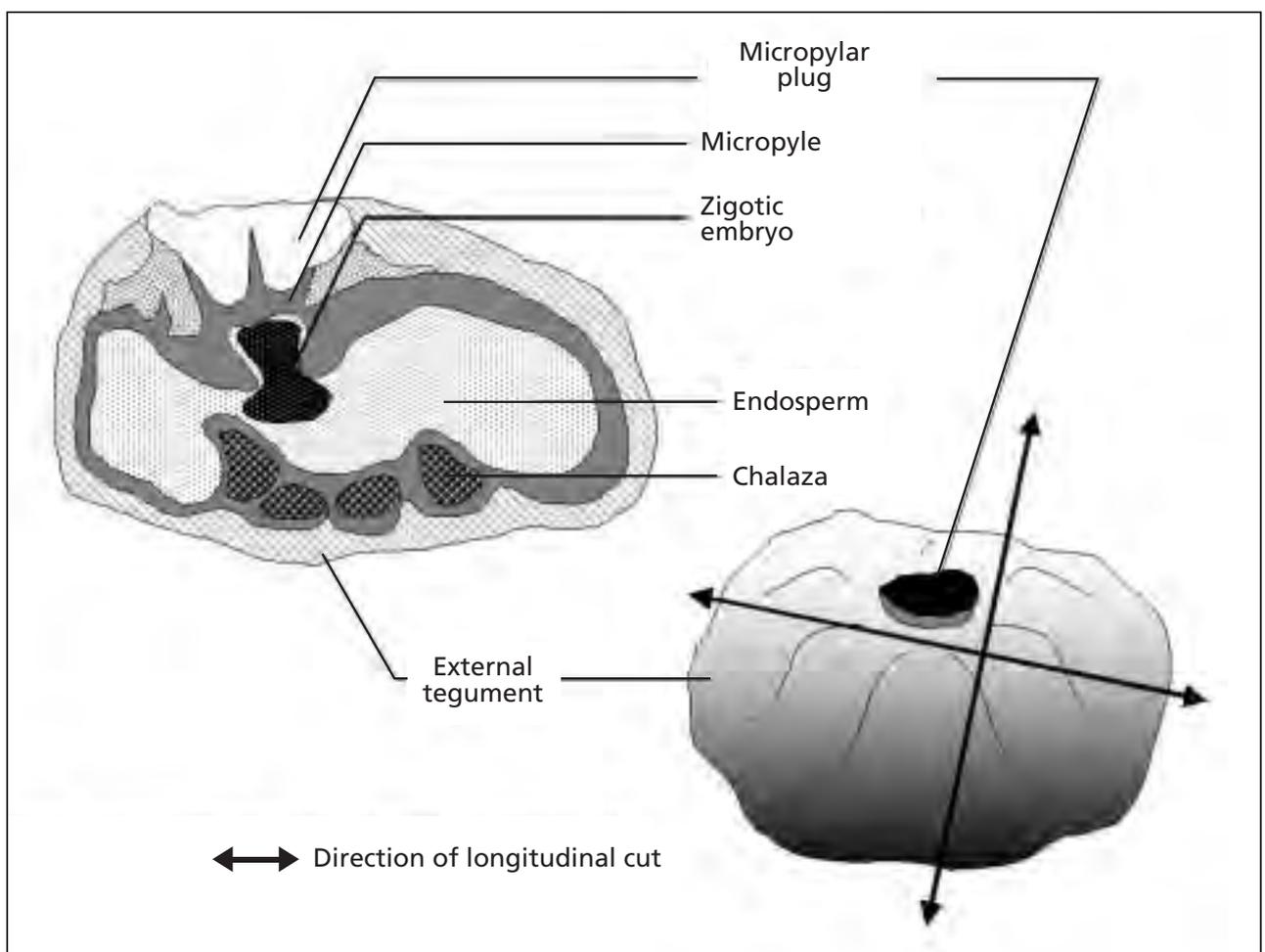

Figure 15. Graphic representation of a longitudinal cut of a banana seed.

60 g/l sucrose, and 2 mg/l gelrite. The pH is adjusted to 5.8 prior to autoclaving.

Dehydration

Embryos are then dehydrated under the sterile air of a laminar flow cabinet (Abdelnour-Esquivel *et al.* 1992). However, depending on both the laboratory conditions and the banana species, the desiccation period may require some adjustment (see Table 1). It is therefore recommended to have an estimate of the time required to obtain a water content of around 14% (% of fresh weight) in the embryos since this proved to be optimal water content resulting in the highest post-thaw recovery. For *Musa acuminata* and *Musa balbisiana*, this water content is reached after 1.5 and 2 hours of dehydration respectively.

Table 1. Evolution of water content of excised embryos of *Musa acuminata* and *M. balbisiana* as a function of dehydration duration.

	Water content (% of fresh weight)*	
Time (h)	*M. acuminata*	*M. balbisiana*
0	64	79
0.5	26	37
1.0	20	28
1.5	15	19
2.0	11	14
2.5	8	11
3.0	6	9

(*) Average of 3 experiments with 3 replicates of 12 embryos each.

Freezing

Freezing of the embryos is carried out in 2 ml cryotubes by direct immersion in liquid nitrogen.

Thawing and recovery

The embryos are quickly thawed by warming the sample vials in a 40 °C water bath for about 2 minutes. For regeneration, embryos are placed on the medium described above supplemented with 0.5 mg/l BA. Cultures are kept in the dark for a period of approximately 4 weeks. Germinated embryos are then transferred to the rooting and development medium (MS medium without hormones).

4.2 Discussion

In view of its efficiency and simplicity, the cryopreservation technique established for *M. acuminata* and *M. balbisiana* embryos might be usefully applied in the future for long-term germplasm storage of fertile diploids of *Musa.*

5. Discussion and perspectives

5.1 CRYOPRESERVATION OF BANANA MERISTEMS

Cryopreservation of individual meristems

A number of different *Musa* genotypes have been cryopreserved using this protocol (Thinh *et al.* 1999). Microscopic observation of the recovery of cryopreserved meristems has revealed that: (i) the whole dome of the isolated meristem survives exposure to liquid nitrogen, and (ii) no post-cryopreservation callus is formed. Therefore, it would appear that somaclonal variation is unlikely to occur.

The main limitations to this procedure are as follows:

• Post-cryopreservation viability/regeneration rates vary according to the operator (at least considerable variation was observed between JIRCAS and KUL).

• Considerable experience in the dissection of tiny and fragile banana apical meristems is required before this cryopreservation protocol can be applied.

• Only a certain type of meristems (PC type, Figure 3) can be used as explants. Some *in vitro* plantlets (ca. 20%) do not contain meristems of the PC type.

• Many meristems survive but become black and are not able to form regenerating shoots. Therefore, regeneration conditions need to be optimized.

Cryopreservation of meristem clumps

The most labour-intensive part of cryopreservation of this type of material is the production of the highly proliferating cultures. Currently, these can only be obtained by using BA at extremely high, nearly toxic, concentrations (100 μM). Prolonged culture on 100 μM BA containing media, therefore, often results in a quality decrease (loss of the typical 'cauliflower-like' characteristics) of the cultures. Moreover, the reaction towards this medium is very cultivar-dependent. Therefore, it is believed that other cytokinins or cytokinin-like plant growth regulators should be screened for their potential to induce highly proliferating meristem cultures.

Simple freezing method

Best results (up to 70% post-thaw regrowth) have been obtained with the ABB cultivars like Bluggoe, Cachaco and Monthan. Intermediate results (around 25% regrowth) were reached with AAA dessert and AAB bananas. AAB plantains and diploids generally respond poorly. For all cultivars under investigation belonging to these genomic groups, plants were regenerated and

grown in the greenhouse. However, none of the AAA Highland bananas was able to survive simple freezing.

Often, the lack of reproducibility is a factor that limits routine application of cryopreservation (Benson *et al.* 1996). However, researchers at INIFAT (*Instituto Nacional de Investigación Fundamental en Agricultura Tropical,* Cuba) and FONAIAP (*Fondo Nacional de Investigaciones Agropecuarias,* Venezuela) (Surga *et al.* 1999), have successfully applied the simple freezing method. At INIFAT, a post-thaw survival rate of 34% was obtained for the local cultivar Burro Criollo (IPGRI 1996).

Combined cryopreservation method

It has been found that post-thaw regrowth rates of sugar pregrown meristems are higher compared to those grown on normal P4 medium. A sucrose preculture seems to increase tolerance of meristems not only towards the PVS2 solution but also towards freezing. The frequency of callus regrowth, however, is still high. If the results from the combined cryopreservation method are compared with those obtained using the simple freezing method, an increase in post-thaw viability rates for almost all cultivars has been observed.

The increase in post-thaw regeneration for the ABB bananas is limited. Post-thaw recovery remains between 50 and 70%. For AAA dessert and AAB bananas, regeneration rates amount to 30-50%, while for plantains 20-30% is reached. AAA Highland bananas which proved to be recalcitrant towards cryopreservation using simple freezing gave 5-15% survival using the combined cryopreservation method.

Optimization of protocols

With regard to the simple freezing method, blackening, due to the oxidation of polyphenols is often observed when thawed meristems are placed on semi-solid medium. This can cause cytotoxic effects and may also result in the recovering clumps being surrounded by an impermeable layer, thereby preventing nutrient uptake for further outgrowth. One method to overcome this problem is to use liquid regeneration media in order to dilute the released polyphenols.

For most plant species, optimal dehydration of meristematic tissues with PVS2 is obtained after 10 to 30 minutes at room temperature (Takagi 2000). Among the exceptions are shoot apices of sweet potato and pineapple which need to be treated with PVS2 for 100 minutes and 7 hours respectively (Plessis and Steponkus 1996; Gonzalez-Arnao *et al.* 1998). The duration of this treatment should be optimized case by case since enough dehydration must take place to

avoid the formation of lethal ice crystals during freezing. At the same time care has to be taken to prevent the treatment with the potentially toxic solution from irreversibly damaging the tissue. In the case of sucrose precultured proliferating banana cultivars, it has recently been observed that optimal post-thaw regeneration rates are generally obtained after a 2 or 2.5-hour PVS2 treatment. The survival rates after 3 hours for most cultivars were considerably lower, probably due to the toxicity of this highly concentrated solution.

A preculture phase on media containing elevated sucrose concentrations is essential to make banana meristem cultures tolerant towards the simple freezing method. Also vitrification of proliferating meristems is more successful after sucrose preculture. The plausible modes of action for sucrose preculture in enhancing freeze resistance are numerous. Sucrose preculture results in a slow reduction in moisture content (Uragami 1991, Engelmann and Duval 1986) due to its osmotic effect and sucrose uptake, thus depressing the freezing point and the amount of freezable water. Histocytological studies reveal the accumulation or synthesis of a sugar-like compound inside the cytoplasm after preculture (Panis *et al*. 1996). This is confirmed by detailed sugar analysis. A direct correlation between sugar content and post-thaw survival, however, has not been found (Panis *et al*. 1998). Sugars also 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 exercises a mild osmotic stress to the tissue, could come from the accumulation of water stress protective compounds, like proline (Delvallée *et al*. 1989). Recently, it has been discovered that sucrose preculture also results in increased total protein content and changes in the membrane lipid (Panis *et al*. 1998), polyamine and sterol composition (unpublished results). If the exact mode of action of the sucrose preculture with respect to cryoprotection could be determined, the different cryopreservation protocols could be optimized more efficiently.

Currently 39 different banana accessions belonging to 7 different genomic groups have been put into long-term storage in liquid nitrogen at the INIBAP Transit Center (ITC), Leuven, Belgium. Regeneration rates vary between 10 and 80% depending on the cultivar and the cryopreservation protocol followed. ABB cultivars are generally stored using the simple freezing method, while the combined cryopreservation protocol is applied to the other genotypes.

5.2 Cell suspensions

Pretreatment

Often in cryopreservation protocols, a pregrowth phase is included to increase the freezing tolerance of tissue cultures. Osmotically active compounds like sorbitol or mannitol are added in the medium to reduce the intracellular water content before freezing, thus reducing the amount of water available for lethal ice formation (Withers and Street 1977). In the case of banana cells, it has been found that an osmotic dehydration with 6% mannitol for 2 or 7 days does not affect viability after cryopreservation. For a male flower-derived cell suspension of *Musa acuminata*, however, preculture with 180 g/l during 24 hours proved to be beneficial (Côte *et al.* 2000).

Cryoprotection

Different cryoprotective solutions have been tested, consisting of MS medium with 30 g/l sucrose added with DMSO (at 2.5, 5, 7.5, 10 and 15%), glycerol (at 5, 10 and 15%), proline (at 10%), and a cryoprotective mixture (containing 0.5 M Glycerol, 0.5 M DMSO and 1 M sucrose). Although all treatments result, according to the FDA viability test, in the survival of frozen cells, only DMSO at 5, 7.5 and 10% gave satisfactory post-thaw regrowth. The addition of higher sucrose levels (180 g/l) to the cryoprotective solution has, for most suspensions, a positive effect on FDA viability rates and, more importantly, on post-thaw regrowth. This is also observed in sugarcane embryogenic callus (Martinez-Montero *et al.* 1998).

Freezing

Comparable post-thaw regrowth is obtained using the methanol bath and the Nalgene boxes, provided that the cryotubes are transferred to liquid nitrogen as soon as a temperature of –40 °C is reached. If the Nalgene boxes are left overnight in the –80 °C freezer, no post-thaw recovery is observed. A possible reason that might account for this lack of regrowth is that cells become too severely dehydrated, since dehydration proceeds during freezing down to -70 °C and during the holding period at this temperature before immersion of cryotubes in liquid nitrogen. The use of Nalgene boxes also proves to be very efficient for banana embryogenic cell suspensions initiated from male flowers (Côte *et al.* 2000). The main advantage of using Nalgene boxes is that no expensive equipment for controlled slow freezing is required.

Post-thaw treatment

The removal of the potentially toxic cryoprotectant solution directly after thawing and its replacement by cryoprotectant-free liquid medium, before transfer to a semi-solid medium, results in a complete loss of regrowth capacity

and the cells becoming white. Direct transfer of cells to a liquid medium, which subjects the cells to similar post-thaw wash stresses, likewise results in growth failure. Regrowth can only be achieved when cells, still suspended in the cryoprotective solutions, are directly transferred to semi-solid medium.

Using the optimized cryopreservation protocol described above, KUL is now storing in liquid nitrogen 50 lines of embryogenic cell suspensions belonging to 9 different banana cultivars. Recently, banana cell suspensions were recovered after 5 years storage. The ability to produce somatic embryos remained intact and embryogenic cell suspensions could again be established from the frozen material.

The fact that some banana cell suspensions are not able to withstand cryopreservation might be considered a reason for further optimization of the cryopreservation protocol. In addition to the more conventional procedure involving slow freezing in the presence of a cryoprotective solution often containing DMSO, successful cryopreservation of cell suspensions is also reported after vitrification (Watanabe *et al*. 1995, Huang *et al*. 1995, Nishizawa *et al*. 1993, Sakai *et al*. 1990), encapsulation dehydration (Bachiri *et al*. 1995; Swan *et al*. 1998), encapsulation vitrification (Gazeau *et al*. 1998), encapsulation combined with slow freezing (Gazeau *et al.* 1998) and vitrification combined with slow freezing (Wu *et al*. 1997). However, since the cell suspensions, which are recalcitrant to the above-described cryopreservation protocol, are not regenerative, they will not be used in genetic engineering. Therefore, their preservation might be more of scientific than of practical value.

Appendix 1. Media and solution composition

MS MEDIUM

(Murashige and Skoog 1962)

MS components	Concentration (mg/l)
Inorganic salts	
Calcium chloride (CaCl$_2$)	332.02
Ammonium nitrate (NH$_4$NO$_3$)	1650
Magnesium sulfate (MgSO$_4$)	80.70
Boric acid (H$_3$BO$_3$) 6.2 Cobalt chloride (CoCl$_2$ · 6H$_2$O)	0.025
Cupric sulfate (CuSO$_4$ · 6H$_2$O)	0.025
Manganese sulfate (MnSO$_4$ · H$_2$0)	16.90
Potassium iodide (KI)	0.83
Potassium nitrate (KNO$_3$)	1900
Potassium phosphate (KH$_2$PO$_4$)	170
Sodium molybdate (Na$_2$MoO$_4$ · 2H$_2$O)	0.25
Zinc sulfate (ZnSO$_4$ · 7H$_2$O)	8.60
Iron source	
Sodium EDTA (Na$_2$ · EDTA)	37.26
Ferric sulfate (FeSO$_4$ · 7H$_2$O)	27.80
Vitamins	
Myo-inositol	100
Nicotinic acid	0.5
Pyridoxine HCL	0.5
Thiamine HCl	0.5
Glycine (free base)	2.00

VITAMINS OF MOREL

(Morel 1950)

Calcium panthotenate	1
Myo-inositol	100
Nicotinic Acid	1
Pyridoxine HCL	1
Thiamine HCl	1
Biotine	0.01

P5 MEDIUM

MS medium supplemented with sucrose 30g/l , 10 μM BA, 1 μM IAA, 2 g/l gelrite or 5 g/l agar (Banerjee and De Langhe 1985). (pH: 5.8).

P4 MEDIUM

P5 medium with a 10-fold higher (100 μM) BAP concentration.

PRECULTURE MEDIUM (PCM)

This medium contains all P5 elements but the sucrose level is increased to a final concentration of 0.4 M.

P6 REGENERATION MEDIUM

P5 medium with a 10-fold lower BAP concentration (1 μM).

LOADING SOLUTION L1

MS medium components diluted in water supplemented with 2 M glycerol and 0.4 M sucrose, pH adjusted to 5.8. The solution is sterilized by filtration (0.22 μm).

PVS2 SOLUTION

Consists of 30% (3.26 M) glycerol, 15% (2.42 M) ethylene glycol (EG), 15% (1.9 M) DMSO and 0.4 M sucrose (Sakai *et al.* 1990). All these compounds are dissolved in MS medium, pH adjusted to 5.8 followed by filter sterilization (0.22 μm).

UNLOADING SOLUTION

The filter sterilized (0.22μ) unloading solution consists of 1.2 M sucrose dissolved in MS medium. (pH: 5.8).

ZZ MEDIUM

Half strength MS macro elements and iron, MS microelements, 5 μM 2,4-D, 1 μM zeatine, standard MS vitamins, 10 mg/l ascorbic acid, and 30 g/l sucrose. (pH: 5.8).

RD1 MEDIUM

MS macroelements and iron, MS microelements, 1 μm BA, standard MS vitamins, 100 mg/l myo-inositol, 10 mg/l ascorbic acid, 30 g/l sucrose and 2 g/l gelrite. (pH: 5.8).

MA2 MEDIUM

MS macro- and micro-elements, biotine 1 mg/l, glutamine 100 mg/l, malt extract 100 mg/l, 2,4-D 1 mg/l and sucrose 45 g/l. (pH:5.3).

MA3 MEDIUM

Inorganic salts	Concentration (mg/l)
KNO_3	2500
$CaCl_2 \cdot 2H_2O$	200
$MgSO_4 \cdot 7 H_2O$	400
$NH_4H_2PO_4$	300
$MnSO_4 \cdot H_2O$	10
H_3BO_3	5
$ZnSO_4 \cdot$	7
H_2O 1 KI	1
$CuSO_4 \cdot 5H_2O$	0.2
$NaMoO_4 \cdot 2H_2O$	0.1
$CoCl_2$ 0.1	

Iron source	
$FeSO_4 \cdot 7H_2O$	15
Na_2DTA 20	

MS vitamins

Other components	
ANA	0.2
Zeatine	0.05
2iP	0.2
Kinetine	0.1
Lactose	10 g/l
Sucrose	45 g/l
Agarose	7 g/l

pH 5.3

Appendix 2. Basic equipment required

Liquid nitrogen source

Cryotanks   1 for liquid nitrogen storage

              1 to store plant material with racks or boxes to store cryotubes (2 for safety)

Dewar vessels (2)
2 ml Cryotubes
Cryocanes

Stirred methanol bath (-40 °C)

Alcohol bath (e.g. Mr Freeze NalgeneTM)

Programmable freezer (only for research purposes or large-scale application) or (-70 °C or –80 °C freezer + Mr Freeze)

Chemicals (DMSO, PEG, etc.)

Binocular stereoscopic microscope and fluorescent microscope

Safety equipment: gloves and goggles (for manipulation of liquid nitrogen).

Appendix 3. List of abbreviations

2,4 D	2,4—dichlorophenoxyacetic acid
BA,	BAP 6-benzylaminopurine
DMSO	dimethyl sulfoxide
EG	ethylene glycol
FDA	fluorescein diacetate
IAA	indoleacetic acid
LN	liquid nitrogen
MS	medium Murashige and Skoog medium
PEG	polyethylene glycol
TTC	triphenyl tetrazolium chloride

References

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

Bachiri Y., C. Gazeau, J. Hansz, C. Morisset & J. Dereuddre. 1995. Successful cryopreservation of suspension cells by encapsulation-dehydration. Plant Cell, Tissue Org. Cult. 43:241-248.

Banerjee N. & E. De Langhe. 1985. A tissue cultures technique for rapid clonal propagation and storage under minimal growth conditions of *Musa* (banana and plantain). Plant Cell Rep. 4:351-154.

Benson E.E., M. Wilkinson, A. Todd, U. Ekuere & J. Lyon. 1996. Developmental competence and ploidy stability in plants regenerated from cryopreserved potato shoot-tips. Cryo-Letters 17:119-128.

Côte F.X., R. Domergue, S. Monmarson, J. Schwendiman, C. Teisson & J.V. Escalant. 1996. Embryogenic cell suspensions from the male flower of *Musa* AAA cv. 'Grand nain'. Physiol. Plant. 97:285-290.

Côte F.X., O. Goue, R. Domergue, B. Panis & C. Jenny. 2000. In-field behavior of banana plants (*Musa* spp.) obtained after regeneration of cryopreserved embryogenic cell suspensions. Cryo-Letters 21:19-24.

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

Dhed'a D., F. Dumortier, B. Panis, D. Vuylsteke & E. De Langhe. 1991. Plant regeneration in cell suspension cultures of the cooking banana cv. 'Bluggoe' (*Musa* spp., ABB group). Fruits 46(2):125-135.

Dixon R.A. 1985. Isolation and maintenance of callus and cell suspension cultures. Pp. 1-20 *in* Plant cell culture. A practical approach (R.A. Dixon, ed.). IRL Press, Oxford.

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

Engelmann F. 1997. Importance of desiccation for the cryopreservation of recalcitrant seed and vegetatively propagated species. Plant Genet. Res. Newsl. 112:9-18.

Escalant J.V. & C. Teisson. 1987. Comportement *in vitro* de l'embryon isolé du bananier. Fruits 42(6):333-342.

Gazeau C., H. Elleuch, A. David & C. Morisset. 1998. Cryopreservation of transformed *Papaver somniferum* cells. Cryo-Letters 19(3):147-159.

González-Arnao M.T., M. Márquez, C. Urra, M. Martínez-Montero & F. Engelmann. 1998. Cryopreservation of apices from pineapple (*Ananas comosus*) *in vitro* plantlets. Cryo-Letters 19:375-382.

Hernandez J.B., S. Remy, V. Galán Saúco, R. Swennen & L. Sági. 1998. Chemotactic movement and attachment of *Agrobacterium tumefaciens* to single cells and tissues of banana. J. Plant Physiol. 155:245-250.

Huang C.-N., J.-H. Wang, Q.-S. Yan, X.-Q. Zhang & Q.-F. Yan. 1995. Plant regeneration from rice (*Oryza sativa* L.) embryogenic cell suspension cells cryopreserved by vitrification. Plant Cell Rep. 14:730-734.

IPGRI. 1996. Final report of the project 'Refinement of cryopreservation techniques for the long-term conservation of sugarcane and banana in Cuba'. International Plant Genetic Resources Institute, Rome, Italy.

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

Martínez-Montero M., M.T. González-Arnao, C. Borroto-Nordelo, C. Puentes-Diaz & F. Engelmann. 1998. Cryopreservation of sugarcane embryogenic callus using a simplified freezing process. Cryo-Letters 19:171-176.

Matsumoto T., A. Sakai & K. Yamada. 1994. Cryopreservation of *in vitro*-grown apical meristems of wasabi (*Wasabi japonica*) by vitrification and subsequent high plant regeneration. Plant Cell Rep. 13:442-446.

Morel G. 1950. Sur la culture des tissus de deux monocotylédones. C.R. Acad. Sc. Paris 230:1099-1101.

Murashige T. & F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Nishizawa S., A. Sakai, Y. Amnao & T. Matsuzawa. 1993. Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic cells and subsequent plant regeneration by vitrification. Plant Sci. 91:67-73.

Panis B. 1995. Cryopreservation of banana (*Musa* spp.) germplasm. Dissertationes de Agricultura 272. Katholieke Universiteit Leuven, Belgium. 201p.

Panis B., H. Schoofs, S. Remy, L. Sagi & R. Swennen. 2000a. Cryopreservation of banana embryogenic suspensions: an aid for genetic transformation. Pp. 103-109 *in* Cryopreservation of tropical plant germplasm. Current research progress and application (F. Engelmann & H. Takagi, eds). Japan

International Research Center for Agricultural Sciences, Tsukuba, Japan/International Plant Genetic Resources Institute, Rome, Italy.

Panis B., H. Schoofs, N.T. Thinh & R. Swennen. 2000b. Cryopreservation of proliferating meristem cultures of banana. Pp. 238-243 *in* Cryopreservation of tropical plant germplasm. Current research progress and application (F. Engelmann & H. Takagi, eds). Japan International Research Center for Agricultural Sciences, Tsukuba, Japan / International Plant Genetic Resources Institute, Rome, Italy.

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

Panis B., K. Vandenbranden, H. Schoofs & R. Swennen. 1998. Conservation of banana germplasm through cryopreservation. Pp. 515-521 *in* Proceedings of the International Symposium on Biotechnology of Tropical and Subtropical Species, Brisbane, Queensland, Australia, 29 Sept.-3 Oct. 1997 (R.A. Drew, ed.). Acta Horticulturae 461. ISHS.

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

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

Plessis P. & P.L. Steponkus. 1996. Cryopreservation of sweet potato shoot-tips by vitrification. Cryobiology 33:655-656.

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

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

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

Schoofs H. 1997. The origin of embryogenic cells in *Musa*. Dissertationes de Agricultura 330. Katholieke Universiteit Leuven, Belgium. 257p.

Surga J., R. Swennen & B. Panis. 1999. Cryopreservation of banana meristems (*Musa* spp.): optimization of regeneration. Info*Musa* 8(1):23-24.

Swan T.W., E.A. Deakin, G. Hunjan, G.R. Souch, M.E. Spencer, A.M. Stafford & P.T. Lynch. 1998. Cryopreservation of cell suspensions of *Polygonum aviculare* using traditional controlled rate freezing and encapsulation/dehydration protocols, a comparison of post-thaw cell recovery. Cryo-Letters 19:237-248.

Takagi H. 2000. Recent developments in cryopreservation of shoot apices of tropical species. Pp. 178-193 *in* Cryopreservation of tropical plant germplasm. Current research progress and application (F. Engelmann & H. Takagi, eds). Japan International Research Center for Agricultural Sciences, Tsukuba, Japan / International Plant Genetic Resources Institute, Rome, Italy.

Takagi H., N.T. Thinh, O.M. Islam, T. Senboku & A. Sakai. 1997. Cryopreservation of *in vitro*-grown shoot tips of taro (*Colocasia esculenta* (L.) Schott) by vitrification. 1. Investigation of basic conditions of the vitrification procedure. Plant Cell Rep. 16:594-599.

Thinh N.T., H. Takagi & S. Yashima. 1999. Cryopreservation of *in vitro*-grown shoot tips of banana (*Musa* spp.) by vitrification method. Cryo-Letters 20(3):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. & R. Swennen. 2000. Characterization and control of bacterial contaminants in *in vitro* cultivars of banana (*Musa* spp.). Acta Hortic. 530:69-79.

Watanabe K. & P.L. Steponkus. 1995. Vitrification of *Oryza sativa* L. cell suspensions. Cryo-Letters 16:255-262.

Widholm J.M. 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol. 47:189-194.

Withers L.A. & H.E. Street. 1977. Freeze-preservation of cultured plant cells III: The pregrowth phase. Physiol. Plant. 39:171-178.

Wu Y., F. Engelmann, A. Frattarelli, C. Damiano & L.A. Withers. 1997. Cryopreservation of strawberry cell suspensions. Cryo-Letters 18:317-324.

Recommended literature

Argent G.C.G. 1976. The wild bananas of Papua New Guinea. Notes Roy. Bot. Gard. Edinb. 35:77-114.

CGIAR. 1992. Review of CGIAR priorities and strategies, Part 1, section 5.3.5. Consultative Group on International Agricultural Research (CGIAR), Washington DC, USA.

Chin H.F. 1996. Germination and storage of banana seeds. Pp. 218-227 *in* New Frontiers in Resistance Breeding for Nematodes, *Fusarium* and Sigatoka (E.A. Frison, J.P. Horry & D. De Waele, eds.). INIBAP, Montpellier, France.

Dereuddre J., C. Scottez, Y. Arnaud & M. Duro. 1990. Resistance of alginate-coated axillary shoot tips of pear tree (*Pyrus communis* L. cv. Beurré Hardy) *in vitro* plantlets to dehydratation and subsequent freezing in liquid nitrogen: effects of previous cold hardening. C.R. Acad. Sci. Paris, t. 310, Série III:317-323.

Dumet D., F. Engelmann, N. Chabrillange & Y. Duval. 1993. Cryopreservation of oil palm (*Elaeis guineensis* Jacq.) somatic embryos involving a desiccation step. Plant Cell Rep. 12: 352-355.

Engelmann F. & H. Takagi (eds). 2000. Cryopreservation of tropical plant germplasm. Current research progress and application. Japan International Research Center for Agricultural Sciences, Tsukuba, Japan/International Plant Genetic Resources Institute, Rome, Italy.

FAO. 1997. Production Year Book 1997. Food and Agriculture Organization of The United Nations, Rome.

Hahn S., D. Vuylsteke & R. Swennen. 1990. First reactions to ABB cooking bananas distributed in southeastern Nigeria. Pp. 306-315 *in* Sigatoka leaf spot diseases of banana (R.A Fullerton & R.H. Stover, eds). INIBAP, Montpellier, France.

Humphrey J.E. 1896. The development of the seed in Scitamineae. Ann. Bot. Lond. O.S. 10:1-40.

INIBAP. 1987. Annual Report 1987. International Network for the Improvement of Banana and Plantain. Montpellier, France. 43pp.

INIBAP. 1997. Networking Bananas and Plantains. Annual Report 1997. International Network for the Improvement of Banana and Plantain. Montpellier, France. 72pp.

Jamaluddin S.H. 1986. Characterization, evaluation and utilization of the banana germplasm in Malaysia. Pp. 315-329 *in* Prosid. Simp. Buahbuahan Keb. (Y.K Chan & P. Raveendranathan, eds). MARDI, Kuala Lumpur, Malaysia.

Kiew R. 1987. Notes on the natural history of the Johore Banana, *Musa gracilis* Holtum. Malayan Nature Journal 41:239-248.

Persley G.J. & E.A. De Langhe (eds). 1987. Banana and plantain breeding strategies. ACIAR proceedings No. 21. 187pp.

Sagi L., G.D. May, S. Remy & R. Swennen. 1998. Recent developments in biotechnological research on bananas (*Musa* spp.). Biotech. Gen. Eng. Rev. 15:313-327.

Sakai A. 1997. Potentially valuable cryogenic procedures for cryopreservation of cultured plant meristems. Pp. 53-66 *in* Conservation of plant genetic resources *in vitro* (M.K. Razdan & E.C. Cocking, eds). Science Publishers, Inc., Enfield, New Hampshire, USA.

Simmonds N.W. 1952. The germination of banana seeds. Trop. Agriculture, Trin. 29:2-16.

Simmonds N.W. 1955. Wild bananas in Malaya. Malayan Nature Journal, 10:1-8.

Swennen R. & D. Vuylsteke. 1993. Breeding black Sigatoka resistant plantains with a wild banana. Trop. Agric. 70(1):74-77.

Tribe D.E. 1994. Feeding and greening the world. The role of international agricultural research. CABI, Oxon, UK. 274pp.

Uragami A., A. Sakai & M. Nagai. 1990. Cryopreservation of dried axillary buds from plantlets of *Asparagus officinalis* L. grown *in vitro*. Plant Cell Rep. 9:328-331.

Van den Houwe I., K. De Smet, H. Tezenas du Montcel & R. Swennen. 1995. Variability in storage potential of banana shoot cultures under medium-term storage conditions. Plant Cell Tiss. Org. Cult. 42:269-274.

Vuylsteke D., R. Ortiz, R.S. Ferris & J.H. Crouch. 1997. Plantain improvement. Pp. 267-320 *in* Plant Breeding Reviews. Vol. 14 (J. Janick, ed.). John Wiley, New-York, USA.

Wesley-Smith J., C.W. Vertucci, P. Berjak, N.W. Pammenter & J Crane. 1992. Cryopreservation of desiccation-sensitive axes of *Camellia sinensis* in relation to dehydration, freezing rate and the thermal properties of tissue water. J. Plant Physiol. 140:596-604.

Withers L.A. 1992. *In vitro* conservation. Pp. 169-200 in Biotechnology of perennial fruit crops (F.A. Hammerschlag & R.E. Litz, eds.). Biotechnology in agriculture No. 8. CAB International, Wallingford, UK.

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