Cryopreservation of *Musa* germplasm

Bart Panis

2nd edition

The geographical designations employed and the presentation of material in this publication do not imply the expression of any opinion whatsoever on the part of Bioversity or the CGIAR concerning the legal status of any country, territory, city or area or its authorities, or concerning the delimitation of its frontiers or boundaries. Similarly, the views expressed are those of the authors and do not necessarily reflect the views of these organizations.

Mention of a proprietary name does not constitute endorsement of the product and is given only for information.

Citation: Panis B. 2009. Cryopreservation of *Musa* germplasm: 2nd edition. Technical Guidelines No. 9 (F. Engelmann and E. Benson, eds). Bioversity International, Montpellier, France.

INIBAP ISBN 978-2-910810-86-3

Cryopreservation of *Musa* germplasm

2nd edition

Bart Panis

Laboratory of Tropical Crop Improvement, Katholieke Universiteit Leuven, Leuven, Belgium

Edited by Erica Benson[1] and Florent Engelmann[2]

[1] Damar Research Scientists, Damar, Drum Road Cupar Muir, Fife, Ky15 5RJ, Scotland.

[2] IRD, UMR DIAPC, BP 64501 34394 Montpellier cedex 5, France and Bioversity International, Via dei Tre Denari 472/a, 00057 Maccarese (Fiumicino), Rome, Italy

partageons les connaissances au profit des communautés rurales

sharing knowledge, improving rural livelihoods

Acknowledgements

Cover illustration: Roberto Hamm, Crayon & Cie

Contents

Foreword

Bananas and plantains (*Musa* spp.) are among the world's most important crop-groups. Over 400 million people throughout the developing countries of the tropics and subtropics are dependent on these crops, as 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 range 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 attacks 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.

Bioversity International (previously INIBAP) is responsible for the world collection of *Musa* germplasm. This currently consists of over 1185 accessions, both wild species and cultivated varieties, and is held under the auspices of FAO. This active collection is presently maintained *in vitro*, under conditions of low light intensity and low temperature in order to reduce the growth rate of the cultures. Despite these slow-growth conditions, all accessions still need to be subcultured on average once per year. The subculturing 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, Bioversity is supporting research on cryopreservation, i.e. storage at ultra-low temperature, 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 (KULeuven) and the techniques developed are now being used for routine cryopreservation of accessions held by Bioversity. Almost half of the collection is currently safely preserved in liquid nitrogen for the long term. This cryopreserved collection is considered as complementary to the *in vitro* collection and serves as a safe back-up in case accessions are lost due to contamination, somaclonal variation and human errors during subculture events.

Cryopreservation techniques are, in principle, applicable to any type of plant tissue with regeneration potential. Such techniques have now been developed for more than 200 different plant species cultured in various forms, including cell suspensions, calli, apices, somatic and zygotic embryos (Reed 2008).

Two types of 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 clusters. 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, Panis et al. 2005b). The main aim of preserving embryogenic cell suspensions of banana for the long term is not the conservation of banana diversity. As some banana accessions are recalcitrant toward the establishment of embryogenic cell suspensions and moreover this process is extremely time consuming (up to 15 months), cryopreservation should be considered in this case as an aid for biotechnological applications such as genetic engineering (Strosse et al. 2003).

The different methods currently used at KULeuven for cryopreserving *Musa* cultures 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 will facilitate their adoption and standard 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.

1. Cryopreservation protocols for banana meristems

1.1 INTRODUCTION

Until 20 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 freeze-dehydration, leaving less water in the cells to form lethal ice crystals upon exposure to extreme low temperatures.

During the last 20 years, however, several new cryopreservation procedures like vitrification, encapsulation-dehydration, preculture-dehydration, and encapsulation/vitrification have been established, which are all based on vitrification. 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. Cryopreservation protocols based on vitrification techniques have been developed for different vegetatively propagated crops, including banana (Sakai and Engelmann 2007).

Bioversity-supported research at KULeuven has resulted in the development of three 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 medium with 0.4 M (136.8 g/L) sucrose. The second also uses highly proliferating meristem cultures that are precultured on sucrose but they receive an additional vitrification treatment. The third and most generally applicable protocol is vitrification of apical meristems excised from rooted *in vitro* plants. The labour requirement to cryopreserve accessions, as well as the post-re-warming regeneration percentages, depend on the cultivar and the method used (Panis et al. 2007). Application of the aforementioned protocols has resulted thus far in the safe storage in liquid nitrogen of 655 accessions (situation end 2008) 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, often such bacteria do not interfere with the growth of meristem cultures. However, as soon as meristems are subjected to cryopreservation,

growth of endogenous bacteria becomes a problem. As the meristems begin to regrow following cryopreservation, endogenous bacteria present can develop into yellow or white colonies, which overgrow the recovering meristem. Therefore, prior to cryopreservation, cultures are always 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 (Hamill et al. 2005, Thomas et al. 2008, Van den Houwe and Swennen 2000).

1.2 CRYOPRESERVATION OF APICAL BANANA MERISTEMS

Cryopreservation of *in vitro* grown shoot-tips of banana through vitrification was originally reported by Thinh and coworkers (Thinh et al. 1999). Using this method, regeneration percentages were often low and unpredictable. Therefore, the technique was further improved and adapted at KULeuven to make it applicable to a wide variety of cultivars (Panis et al. 2005a).

This method is illustrated in Figure 1 below.
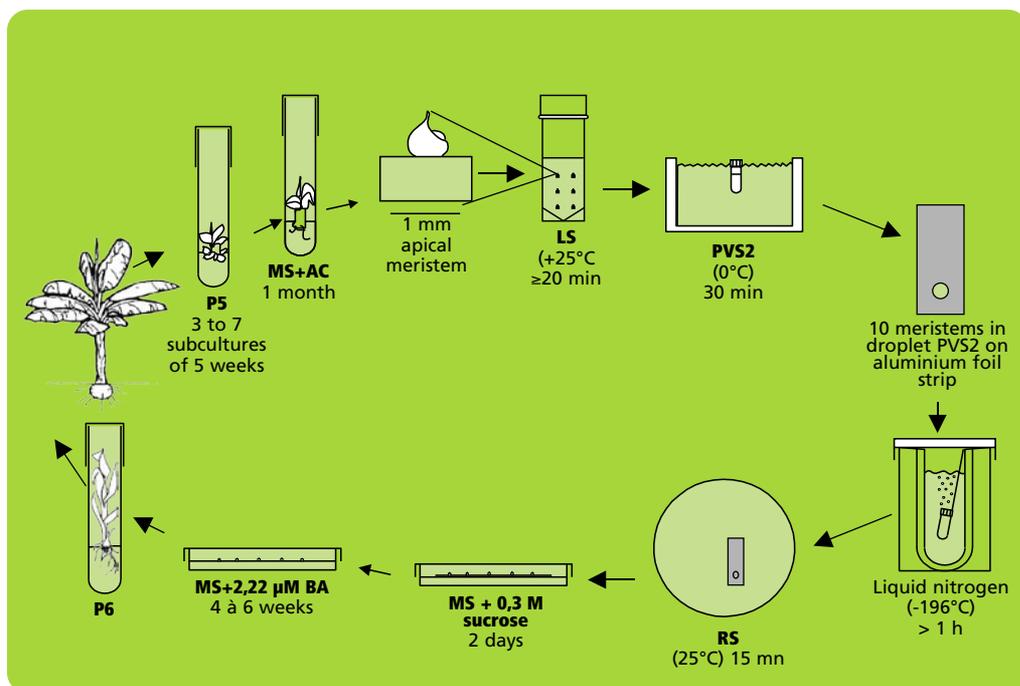
Figure 1. Cryopreservation of individual meristems.

1.2.1 Plant materials

Preparation of *in vitro* plantlets

All accessions were obtained from Bioversity *Musa* germplasm *in vitro* collection (KULeuven, Belgium). This collection contains edible banana cultivars as well as wild relatives. Shoot cultures are grown in 25 x 150 ml test tubes on 25 ml p5 medium. P5 medium contains semi-solid Murashige and Skoog (MS) medium supplemented with 30 g/L sucrose, 10 µM BA and 1 µM IAA and solidified with 2 g/L gelrite (Banerjee and de Langhe 1985). They are cultured at 25 ± 2°C under continuous 50 µE m^{-2} s^{-1} illumination provided by 36 W Osram cool-white fluorescent tubes. The pH is adjusted to 5.8 prior to autoclaving. From these multiplying cultures, shoots of 3-5 cm in length are separated and transferred to rooting medium in test tubes. The rooting medium has the same composition as p5 but it is devoid of plant growth regulators and supplemented with 0.5 g/L active charcoal. After one month, robust and well rooted *in vitro* plants are obtained with a corm diameter of 5 to 8 mm that forms an appropriate source for the excision of apical meristems (Figure 2).

Dissection and selection of apical meristems

Like many other monocots, banana apical meristems are tightly covered with several layers of whitish, tubular, immature leaves. Individual apical meristems are excised under a binocular microscope. Leaves are removed one by one until the apical dome is visible but still partially

Figure 2. Production of robust, rooted *in vitro* plants of banana cv. 'Williams'.

covered by one to two young leaf primordia (see Figures 3 and 4) . The leaf base (corm tissue) is 1 mm in diameter. In order to excise corm pieces of exactly this size, a millimetre grid graph paper is placed underneath the transparent sterile plastic Petri dish in which the meristems are excised. The dissected meristems are transferred into the loading solution (in the dark at room temperature). Tips that are slightly damaged or are not in the correct stage (i.e. the meristem is too much or too little covered by leaf primordia) are excluded from cryopreservation. A skilful and trained technician can isolate a maximum of 10 meristems an hour (roughly, 6 minutes/isolated meristem).
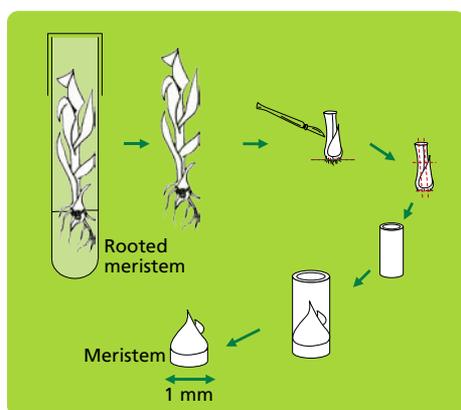
Figure 3. Illustration of meristem isolation. Leaves are removed one by one until the apical dome is visible but still partially covered by 1 to 2 young leaf primordia.

Figure 4. Partly-covered apical meristems of banana cv. 'Williams'.

1.2.2 Cryopreservation through droplet vitrification

Loading, dehydration and rapid freezing

The filter sterilised loading solution contains 2 M glycerol and 0.4 M (= 136.8 g/L) sucrose dissolved in MS medium (pH 5.8). The excised meristems are left in the loading solution in a 20 ml plastic vessel until all of them are dissected. The exposure time thus varies between 20 min and 5 h. Previous research has shown that regrowth of banana meristems is not influenced by loading solution exposure time (Panis et al. 2005a). 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).

After loading, the solution is replaced by ice-cooled PVS2 solution. The PVS2 solution consists of 30% (w/v) (3.26 M) glycerol, 15% (w/v) (2.42 M) ethylene glycol (EG), 15% (w/v) (1.9 M) DMSO and 0.4 M (= 136.8 g/L) sucrose (Sakai et al. 1990). All these compounds are dissolved in MS medium, pH adjusted to 5.8 followed by filter sterilization. The meristems are subjected to the PVS2 solution for 30 to 40 min. at 0°C. Five min. before the end of the treatment, 10 meristems are transferred individually to one droplet of PVS2 solution (of about 15 µl) on a strip of aluminium foil (5x20 mm) with a 2 ml plastic Pasteur pipette (Figure 5). To keep the temperature of the strip around 0°C during the manipulations, the aluminium strip is placed in a plastic Petri dish placed on top of a frozen cooling element. After the PVS2 treatment, the aluminium strip is plunged into liquid nitrogen with a fine forceps. For permanent cryostorage, the frozen foil is quickly transferred to a 2 ml cryotube filled with liquid nitrogen and closed.

Figure 5. Transfer of meristems to a droplet of PVS2 solution (of about 15 µl) on a strip of aluminium foil (5x20 mm) with a 2 ml plastic Pasteur pipette.

Storage, re-warming and unloading

The meristems are kept in liquid nitrogen for at least 20 min. For re-warming, aluminium foil strips are rinsed in 10 ml unloading solution in a small Petri dish at room temperature. After a few seconds, meristems are released from the strip of aluminium foil and kept for another 15 min in the unloading solution. As such, the toxic PVS2 solution is removed whilst re-warming and replaced by a less-toxic unloading solution. The unloading solution consists of 1.2 M (= 410.4 g/L) sucrose dissolved in MS medium (pH 5.8).

Recovery

Unloaded meristems are placed onto two sterile filter papers on top of semi-solid hormone-free MS medium containing 0.3 M (=102.6 g/L) sucrose. After two days, the meristems are transferred onto regeneration medium without filter papers. The first week of culture always takes place in the dark. Four to six weeks following cryopreservation, four types of reactions can be distinguished:

(i)   white shoot-tips resulting from an immediate death of the tissue without blackening;

(ii) completely or partially black shoot-tips, indicating that there was enzymatic reaction following cryopreservation (production and oxidation of polyphenols);

(iii) unorganized callus growth, representing the outgrowth of small isolated areas of the apical dome and/or primordial tissues; and

(iv) shoot-tip regeneration resulting from the survival of a substantial part of the apical dome (Figures 6a-d).
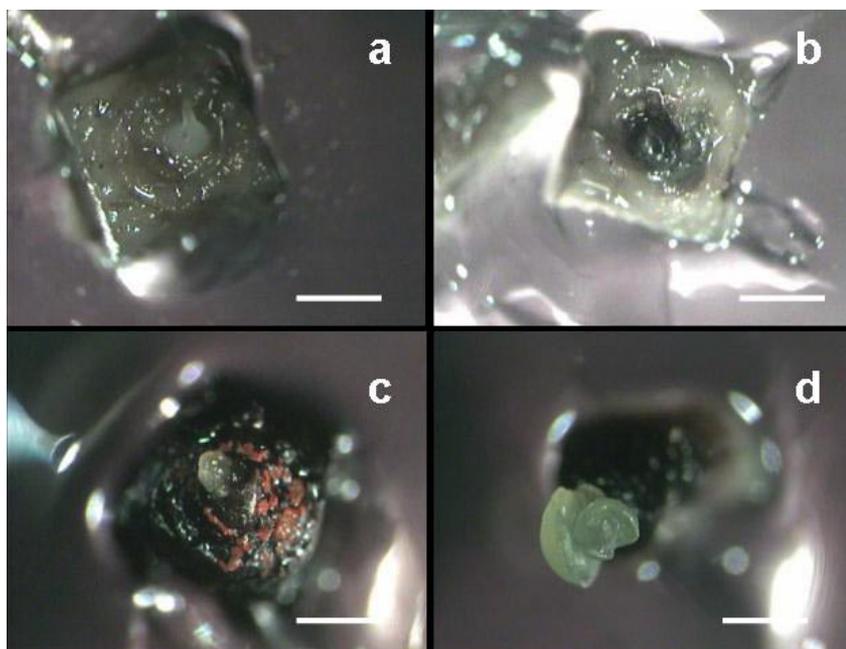
Figure 6. Reaction of apical meristems towards cryopreservation 30 days after re-warming (a) No growth; the meristem remains white; (b) Blackening without further growth; apical dome reacts by formation of polyphenolic compounds that oxidize; (c) Callus formation; watery, non-morphogenic callus; and (d) Shoot regeneration (bar = 600 µm).

One month after re-warming, a 0.5 cm long shoot can be observed (Figure 7). Calluses never produced shoots.

Figure 7. Recovered shoots from cryopreserved apical meristems of banana cv. 'Williams' 1 month after re-warming.

1.3 CRYOPRESERVATION OF BANANA MERISTEM CLUSTERS (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 clusters. 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, Strosse et al. 2006).

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

• Simple freezing method (which involves sucrose preculture) (Panis et al. 1996, Panis et al. 2002),

• Droplet-vitrification of 'cauliflower-like' meristem clusters (combining preculture with droplet-vitrification) (Panis et al. 2000b, Agrawal et al. 2004).

1.3.1 Plant material

Production of 'cauliflower-like' meristem clusters

Preliminary experiments revealed that re-growth 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 one to two months, the material is subcultured and small clumps of 'cauliflower-like' meristems are selected and transferred to fresh medium (Strosse et al. 2006). The high BA concentration (up to 100 µM) in the P4 medium suppresses outgrowth of meristems, thereby favouring the formation of numerous white apical domes (Figure 8). Repeated subculturing is necessary and can take 4 to 12 months.

Nakitengwa	Williams	Bluggoe	Nakitengwa	Williams	Bluggoe
p5 medium (10 µM BA)			p4 medium (100 µM BA)		
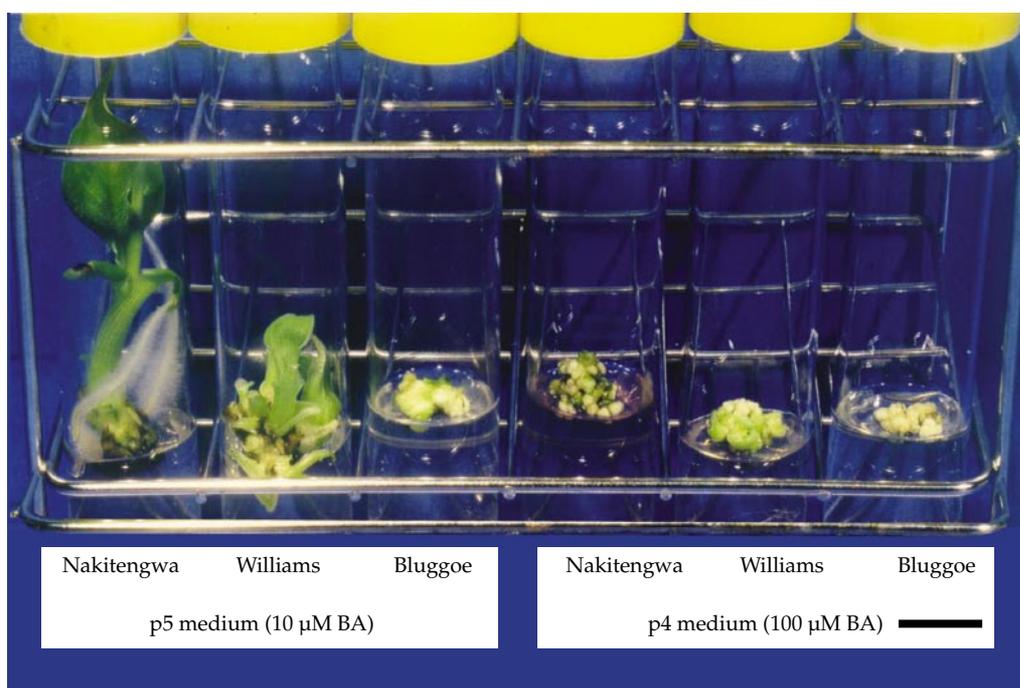
Figure 8. Meristem cultures of banana cultivars Nakitengwa (AAA highland banana), Williams (AAA group) and Bluggoe (ABB group) on (left) p5 medium (containing 10µM BA) and (right) p4 medium (containing 100µM BA)
(bar = 2 cm).

Preculture of meristem clumps

Following the appearance of 'cauliflower-like' clusters, and four 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 preculture medium (P5 + 0.4 M (= 136.8 g/L) sucrose) for four weeks. They are cultured at 25°C ± 2°C in darkness.

1.3.2 Simple freezing method

This method is illustrated in Figure 9.
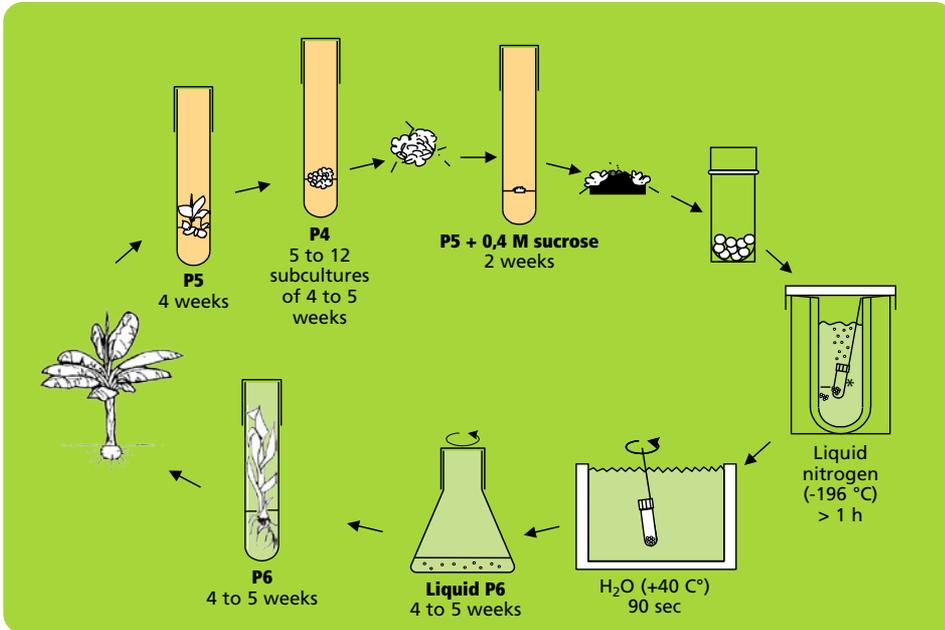
Figure 9. Simple freezing protocol.

Cryopreservation

Small white meristematic clumps weighing 5-15 mg (2-3 mm diameter), containing three to six meristematic domes, are excised from the precultured clumps (Figure 10). Brown tissues are removed and only the healthiest parts, as indicated by a white-yellowish colour, are retained. The clumps are transferred to sterile cryotubes (2 ml) without any liquid
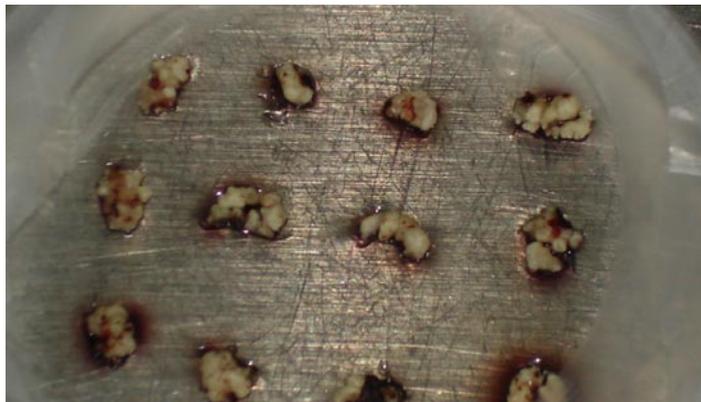
Figure 10. Precultured meristem clumps of "*Musa schizocarpa*"

solution 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 takes place rapidly (within a few seconds), thereby preventing slow lethal re-warming of samples.

Re-warming and recovery

After storage, rapid re-warming takes place by stirring the frozen cryotube in a water-bath or a beaker containing water at 40°C for 90 s.

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. Re-warmed 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 one 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 ± 2°C.

Three weeks after transfer to the regeneration medium, frozen meristem re-growth is determined under a binocular microscope. Two types of surviving tissues are distinguished, i.e. shoots and non-regenerative callus. All calluses are systematically discarded and only recovered shoots (Figure 11) are transferred to test tubes on regeneration medium

Figure 11. Petri dish containing control (left) and frozen (right) meristematic clumps of cv. Bluggoe (ABB group), 8 weeks after cryopreservation (bar = 1 cm).

to promote further development of whole plants. As soon as rooted plants are sufficiently developed, they are planted in the soil.

1.3.3 Droplet vitrification of 'cauliflower-like' meristem clusters

This method is illustrated in Figure 12. Loading, dehydration, rapid freezing, storage, re-warming and unloading are almost identical to the droplet vitrification described in paragraph 1.2. Therefore, only the essential (and differential) steps are indicated in detail below.
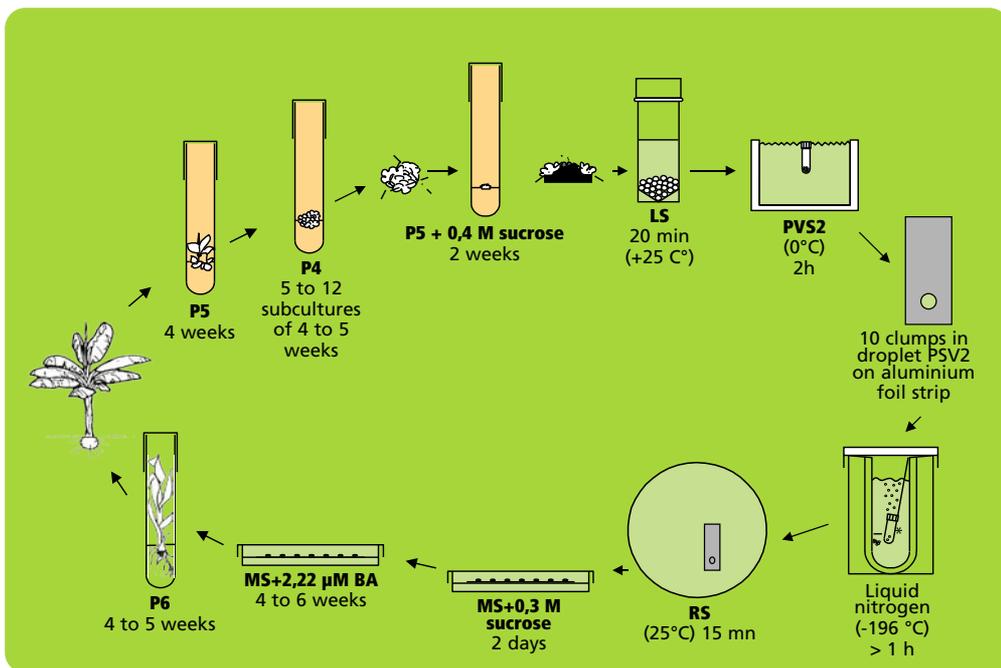
Figure 12. Droplet vitrification protocol of meristem clumps.

Loading, dehydration and rapid freezing

The excised meristem clumps are left in the loading solution (Appendix 1) in a 20 ml plastic vessel until all of them are dissected. The exposure time thus varies between 20 min. and 3 h.

After loading, the solution is replaced by ice-cooled PVS2 solution (Appendix 1). The meristem clumps are subjected to the PVS2 solution for 2 h at 0°C. Five min before the end of the treatment, about 10 meristem clumps are transferred to one droplet of PVS2 solution on a strip of aluminium foil (5x20 mm) with a forceps and a 2 ml plastic Pasteur

pipette (Figure 13). To keep the temperature of the strip around 0°C during the manipulations, it is placed in a plastic Petri dish placed on top of a frozen cooling element. After the PVS2 treatment, the aluminium strip is plunged into liquid nitrogen with a fine forceps. For permanent cryostorage, the frozen foil is quickly transferred to a 2ml cryotube filled with liquid nitrogen and closed.

Figure 13. Meristem clumps in a droplet of PVS2 solution (of about 15 µl) on a strip of aluminium foil (5x20 mm).

Storage, re-warming and unloading

The meristem clumps are kept in liquid nitrogen for at least 20 min. For re-warming, aluminium foil strips are plunged in 10 ml unloading solution (Appendix 1) in a small Petri dish at room temperature. After a few seconds, meristem clumps are released from the aluminium foil and kept for another 15 min in the unloading solution. As such, the toxic PVS2 solution is removed whilst re-warming and replaced by a less-toxic unloading solution.

Recovery

Frozen meristem clumps are taken from the unloading solution and placed in 9 cm plastic Petri dishes on two sterile filter papers on top of about 25 ml semi-solid hormone-free MS medium containing 0.3 M (= 102.6 g/L) sucrose.

After two days, the filter paper is removed and the meristem clumps are transferred to Petri dishes with MS medium supplemented with 2.22 µM BA. The first week of culture always takes place in the dark. After a maximum of six weeks, meristem clumps are transferred to test tubes with P6 medium for further development of whole plants (Figure 14).

Figure 14. Regenerating shoots from one control and three frozen proliferating meristems of the cultivars 'Kisubi' (AB group) 'Dominico Harton' (AAB plantain), 'Bluggoe' (AAB group) and 'Williams' (AAA group) 3 months after cryopreservation.

2. Cryopreservation of banana embryogenic cell suspensions

2.1 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 welcomed 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, Remy et al. 2005). 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 the decrease and eventually the total loss of morphogenic capacity (Strosse et al. 2006, Strosse et al. 2003).

In 1990, a cryopreservation technique for 'optimal' cell suspensions was developed which involves cryoprotection with 7.5% (v/v) DMSO (dimethyl sulphoxide) for 1 h at 0°C, followed by slow freezing at 1°C/min 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 15). 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, or cells with very dense but granular cytoplasm, or cells with large starch grains and organized globules. Recently, banana cell
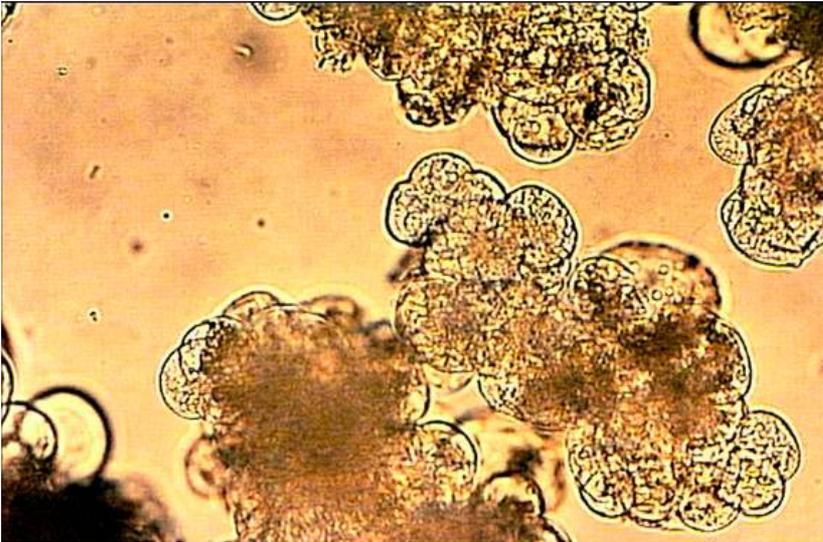
Figure 15. Embryogenic cell suspensions of the cultivar Bluggoe (ABB group).

suspensions were recovered after 15 years storage in liquid nitrogen (unpublished results). The ability to produce somatic embryos remained intact. Also, embryogenic cell suspensions could again be initiated from the frozen material. These re-initiated suspensions proved to retain their transformation competence comparable to non-frozen controls (Panis et al. 2005b).

2.2 PLANT MATERIAL

2.2.1 Starting material

The studies published on 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, Strosse et al. 2006, Strosse et al. 2003).

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.

Cell suspensions derived from male flowers

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

2.3 CRYOPRESERVATION OF CELL SUSPENSIONS

2.3.1 Preculture

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

2.3.2 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% (v/v) is obtained.

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

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

2.3.3 Freezing and storage

For slow freezing, some laboratories would use electronic programmable freezers for which the coolant is liquid Nitrogen. Since the author is not aware of laboratories that have applied this equipment to banana cells, only the use of the methanol bath and the Nalgene™ cryo 1°C Freezing Container are discussed below.

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

Slow freezing in methanol bath

Samples of 1.5 ml of the cryoprotected cell suspensions are transferred to 2 ml cryotubes 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/min from room temperature to -40°C.

As soon as the temperature of -7.5°C is obtained, cryotubes are immersed for 3 s in liquid nitrogen to initiate ice crystallization. Then they are further cooled to -40°C. After 30 min 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 (Figure 16). Its transfer into a freezer (-80°C) allows a cooling rate of about 1°C/min.

In both cases, the temperature decrease inside the cryotube inside the Freezing Container is monitored with temperature probe which is placed in a control cryotube containing 1.5ml cryoprotectant medium.

Figure 16. Slow freezing using Nalgene™ cryo 1°C Freezing Container with temperature probe.

2.3.4 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 min until most of the ice has melted.

Cell suspensions derived from proliferating meristem cultures

Thawed cells are plated on semi-solid ZZ or RD1 medium (Appendix 1) in 90 mm Petri dishes. RD1 medium is employed when plants are required from the cryopreserved material. Semi-solid ZZ medium is used when an embryogenic cell suspension culture must be re-established. During the first week following cryopreservation, Petri dishes are always placed in the dark (Figures 17 A, B, C).
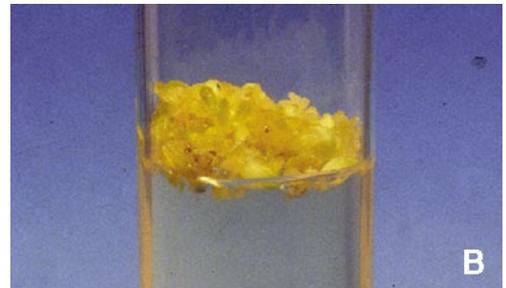
Figure 17. (A) Regrowth after 4 weeks on semi-solid medium of unfrozen (left) and frozen (right) embryogenic cell suspensions of 'Bluggoe' (ABB group); (B) Mass of somatic embryos originating from a frozen cell culture; (C) Greenhouse plants derived from a frozen cell suspension.

Cell suspensions derived from male flowers

Thawed cells are plated on semi-solid MA2 medium for 24 h in 90 mm Petri dishes. After 24 h, 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.

2.3.5 Viability test of the cell suspensions

Chemical cell viability is determined by the fluorescein diacetate (FDA) test (Widholm 1972) whereby surviving cells fluoresce very brightly under ultraviolet illumination (Figure 18).

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.
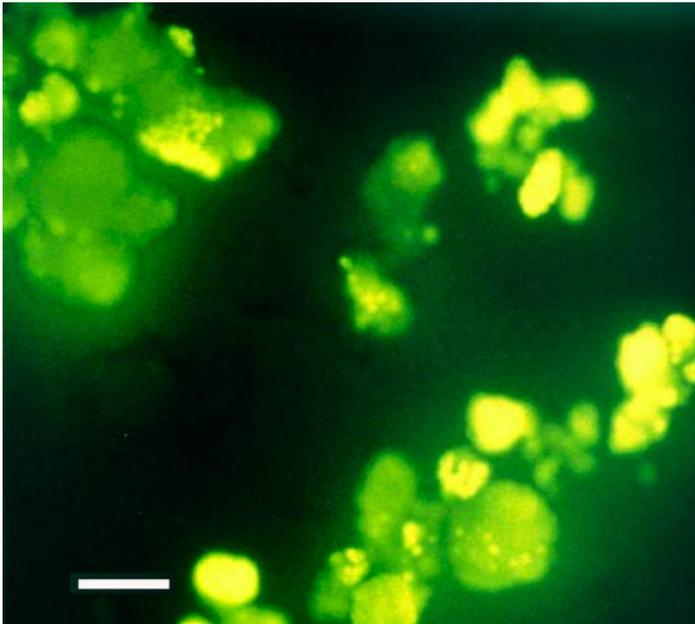
Figure 18. Embryogenic cell suspension of the cv. 'Bluggoe' (ABB group), cryoprotected with 5% (v/v) DMSO, frozen in liquid nitrogen, stained with FDA and observed with ultra violet light. The small surviving embryogenic cells fluoresce very brightly while larger structures show more diffuse fluorescence (bar = 100 μm).

3. Cryopreservation of banana zygotic embryos

3.1 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% (v/v) commercial bleach for 5 min 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 19).
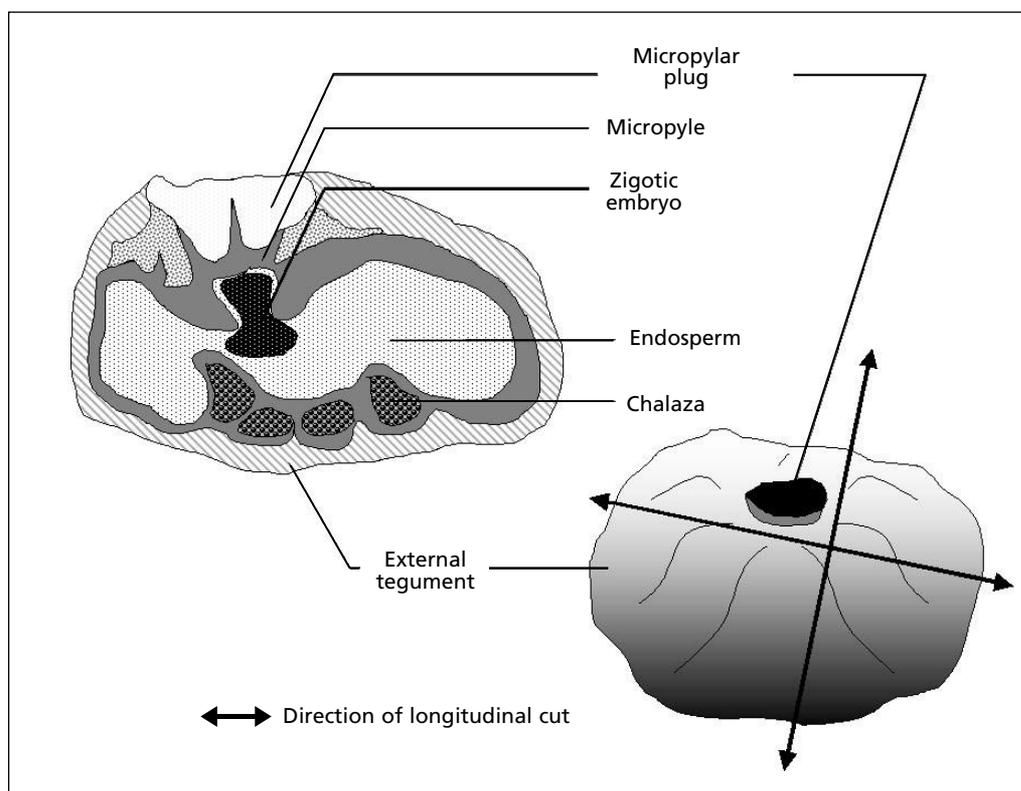
Figure 19. Graphic representation of a longitudinal cut of a banana seed.

3.1.1 Preculture

The embryos are transferred for 5 h 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, 60 g/L sucrose, and 2 mg/L gelrite. The pH is adjusted to 5.8 prior to autoclaving.

3.1.2 Dehydration

Embryos are then dehydrated under the sterile air of a laminar flow cabinet. However, depending on both the laboratory conditions and the banana species, the desiccation period may require some adjustments (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 (Abdelnour-Esquivel et al. 1992). For *Musa acuminata* and *Musa balbisiana*, this water content is reached after 1.5 and 2 h of dehydration, respectively.

3.1.3 Freezing

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

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.

3.1.4 Thawing and recovery

The embryos are quickly thawed by warming the sample vials in a 40°C water bath for about 2 min. For regeneration, embryos are placed on the medium described above (Escalant and Teisson 1987) supplemented with 0.5 mg/L BA. Cultures are kept in the dark for a period of approximately four weeks. Germinated embryos are then transferred to the rooting and development medium (MS medium without hormones).

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*.

4. Discussion and perspectives

4.1 CRYOPRESERVATION OF BANANA MERISTEMS

4.1.1 Droplet vitrification of apical banana meristems

Different *Musa* genotypes have been cryopreserved using this protocol (Thinh et al. 1999, Panis et al. 2005a). Post re-warming regeneration percentages range between 20 and 85% (with an average of 53%). These recovery percentages are relatively genotype independent. It was, however, observed that genotypes with more B-genome survive significantly better than those with solely the A-genome (Panis et al. 2005a). Microscopic observation of the recovery of cryopreserved meristems (Helliot et al. 2003) has revealed that:

(i) the whole dome of the isolated meristem withstands exposure to liquid nitrogen; and

(ii) no regenerable 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 percentages vary depending on the operator. Considerable experience in the dissection of tiny and fragile banana apical meristems is required before this cryopreservation protocol can be applied (using this method only 60 meristems can be excised and cryopreserved in one day).

- Many meristems survive but become black and are not able to form regenerating shoots. Regeneration conditions could therefore still be optimized.

- Low regrowth may be due to low quality meristems (tips that are slightly damaged or are not at the correct stage or too much or too little covered by leaf primordia). The quality of the donor plants (more light, fewer plantlets in containers) could therefore be improved.

- Using the droplet vitrification method, some researchers have shown some concern with respect to the effects of the direct contact of liquid nitrogen with the plant material. Putative problems like contamination and loss of material were, however, never encountered in the author's laboratory.

This droplet vitrification protocol was recently successfully applied to a wide range of plant species such as potato, ulluco, sweet potato, chicory, strawberry, taro, pelargonium, date palm, thyme, olive and hop and might thus be considered as the first generally applicable protocol (Gallard et al. 2008, Sanchez-Romero and Panis 2008, Sant et al. 2008, Marco et al. 2007, Panta et al. 2006).

4.1.2 Cryopreservation of meristem clumps

The most labour-intensive part of cryopreservation of this type of material is the production of the highly proliferating cultures. The quality of 'cauliflower-like' meristem clumps may be too poor for use in cryopreservation experiments (meristematic tissue *versus* corm tissue is too low and/or the explant shows too much blackening). This can be due to the fact that the cultivar belongs to a 'difficult' genomic group (for example East African highland bananas and many plantains). Previously, proliferation was only 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. Recently, the use of alternative cytokinins like thidiazuron (TDZ) at lower concentration (1 µM) proved to increase proliferation rates (Strosse et al. *in press*).

4.1.3 Simple freezing method

The simple freezing protocol was applied to 36 banana cultivars belonging to 8 genomic groups (Panis et al. 2002). The results were extremely genotype dependent. Best results (up to 70% 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, most of the AAA Highland bananas were not able to withstand simple freezing.

With regard to this simple freezing method, blackening, due to the oxidation of polyphenols is often observed when re-warmed 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. This resulted in regeneration percentages that were up to 20 % higher

4.1.4 Droplet vitrification of 'cauliflower-like' meristem clusters

It has been found that post re-warming regrowth percentages of sugar pregrown meristems are higher compared to those grown on normal P4 medium. Sucrose preculture seems to increase tolerance of meristems not only towards the PVS2 solution but also towards the damaging events taking place during the cooling process. When comparing the results from the droplet vitrification of 'cauliflower-like' meristem clusters with those obtained using the simple freezing method for the same cultivar, an increase in viability percentages for almost all cultivars is observed. The increase in post-thaw regeneration for the ABB bananas is limited. Recovery remains between 50 and 70%. For AAA dessert and AAB bananas, the increase of regeneration percentages amount to 30-50%, while for plantains 20-30% is reached. AAA Highland bananas which proved to be recalcitrant towards cryopreservation using simple freezing give 0-20% survival using the droplet vitrification method.

For most plant species, optimal dehydration of meristematic tissues with PVS2 is obtained after 10 to 30 min 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 min and 7 h, respectively (Plessis and Steponkus 1996, Gonzalez-Arnao et al. 1998). The duration of this treatment has to 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 been observed that optimal post re-warming regeneration percentages are generally obtained after a 2 or 2.5-h PVS2 treatment. Survival after 3 h for most cultivars is considerably lower, probably due to the toxicity of this highly concentrated solution.

4.1.5 Further optimization of protocols

To facilitate the development of even more efficient cryopreservation protocols, a better knowledge of the physico-chemical background of cryopreservation is required. This can only be unravelled through fundamental studies that involve both thermal analysis and a thorough examination of the different parameters that can influence cryo-

behaviour, like endogenous sugars, membrane composition, oxidative stress and protective proteins. In the framework of a European research project (CRYMCEPT, see http://www.agr.kuleuven.ac.be/dtp/tro/CRYMCEPT/) and a European Union COST Action (http://www.agr.kuleuven.ac.be/dtp/tro/cost871/Home.htm) these parameters are investigated for different plant species, and among them banana.

We have shown that a preculture phase on medium containing elevated sucrose concentration is essential to make banana meristem cultures tolerant towards cryopreservation. 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, Zhu et al. 2006) due to its osmotic effect and sucrose uptake, thus depressing the freezing point and the amount of freezable water. Sugars can also 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). In banana, we determined sucrose-preculture induced changes in proteins (Carpentier et al. 2005, 2007), membrane components, sugars and polyamines (Zhu et al. 2006) and this for cultivars with different cryopreservation abilities. These analyses revealed that the cryoprotective process induced by sugar preculture is extremely complex: besides changes in sterols, fatty acids, and polyamines and the production of specific protecting proteins, there could still be other parameters or limiting factors involved such as the capacity for free radical eradication. We believe that when the exact mode of action of sucrose preculture with respect to cryoprotection is determined, the different cryopreservation protocols will be optimized more efficiently.

Often, the lack of reproducibility is a factor that limits routine application of cryopreservation (Benson et al. 1996, Reed et al. 2001, Reed et al. 2004). 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 percentage of 34% was obtained for the local cultivar Burro Criollo (IPGRI 1996). Also the droplet-vitrification protocol was

successfully applied to banana at NBPGR (National Bureau for Plant Genetic Resources), New Delhi, India (Agrawal et al. 2004).

4.1.6 Cryopreservation of the banana collection

Currently, droplet-vitrification of apical meristems as well as droplet-vitrification of 'cauliflower-like' meristem clusters are both applied to the *Musa* collection. The simple freezing method is not applied anymore for these purposes in view of the relatively low post-thaw regeneration percentages obtained for most genomic groups. Table 2 compares the labour requirement for the two methods that are now applied.

Table 2. Comparison of labour requirement for two cryopreservation protocols.

Freezing method	Labour time (hours)[1]	cvs/person (person/year)[2]	Time needed (months)[3]	Cultivars
Vitrification of proliferating clumps	30 to 40	59 to 44	12 to 13	ABB AAB some AAB plantains…
Vitrification of individual meristems	60 to 70	29 to 25	8 to 9	AAAh bananas *Musa acuminata* some AAB plantains…

[1] Calculated time needed to prepare the culture media and meristem cultures followed by cryopreservation. For each cultivar 3 repetitions are made with for each repetition at least 6 cryotubes containing 10 (vitrification of individual meristems) to 20 (vitrification of proliferating clumps) explants. For each repetition at least 3 representative samples (tubes) are re-warmed and recovery screened.

[2] 1 year = 220 working days (8 h/day).

[3] Starting from 2 tubes until this accession is safely stored in liquid nitrogen (3 repetitions)

The most labour intensive method (vitrification of apical meristems excised from rooted *in vitro* plants) will only be applied to the cultivars which are difficult to cryopreserve with the other method (for example AAA highland bananas). For banana accessions belonging to the ABB group and AAB (non-plantain) bananas, droplet-vitrification of proliferating clumps is always applied, while the preferred method is droplet-vitrification of apical meristems for *Musa acuminata* and the East African highland bananas. For all other accessions, the method of choice is based on (i) the proliferation degree of the meristem clumps that are obtained after three subculture cycles on a medium containing high cytokinin concentrations and (ii) survival of the proliferating meristem clumps after a preliminary cryopreservation trial.

To decide whether a cryopreservation experiment is successful or not, we use the mode of calculation developed by Dussert and coworkers (2003). These calculations have been applied to all our data. An experiment is considered as successful provided that the chance to regenerate at least one shoot from the stored material is more than 95%. This probability is depending on:

(i)   the number of explants stored in liquid nitrogen (ranging for the long-term between 30 and 50);

(ii) the number of explants re-warmed (ranging between 16 and 50); and

(iii) the post re-warming regeneration percentage (%).

An experiment leading to a lower probability level will not be considered as successful, irrespective of its regeneration percentage. In such cases, a new repetition will be executed.

We consider a banana accession as "safely" preserved if three independent (and successful) experiments are completed. According these requirements we are currently storing 655 accessions belonging to 30 different genomic banana groups in liquid nitrogen (situation end 2008).

4.2 CELL SUSPENSIONS

4.2.1 Pretreatments

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 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 osmotic dehydration with 6% (w/v) mannitol for 2 or 7 days does not affect viability after cryopreservation (unpublished results). For a male bud-derived cell suspension of *Musa acuminata*, however, preculture with 180 g/L sucrose for 24 h proved to be beneficial (Côte et al. 2000).

4.2.2 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% (v/v)), glycerol (at 5, 10 and 15% (v/v)), proline (at 10% (v/v)), and

a cryoprotective mixture (containing 0.5 M Glycerol, 0.5 M DMSO and 1 M (=342 g/L) sucrose). Although all treatments resulted, according to the FDA viability test, in the survival of frozen cells, only DMSO at 5, 7.5 and 10% (v/v) gave satisfactory post-thaw regrowth. The addition of higher sucrose levels (180 g/L) to the cryoprotective solution had, for most suspensions, a positive effect on FDA viability percentage and, more importantly, on post-thaw regrowth. This was also observed in sugarcane embryogenic callus (Martinez-Montero et al. 1998).

4.2.3 Freezing

Comparable post-thaw regrowth is obtained using the methanol bath and the Nalgene™ cryo 1°C Freezing Container, provided that the cryotubes are transferred to liquid nitrogen as soon as a temperature of -40°C is reached. If the Nalgene™ cryo 1°C Freezing Container is left overnight in the -80°C freezer, no post-thaw recovery is observed. The use of the Nalgene™ cryo 1°C Freezing Container also proved to be very efficient for banana embryogenic cell suspensions initiated from male flowers (Côte et al. 2000). Its main advantage is that no expensive equipment (except for a -80°C freezer) for controlled slow freezing is required.

4.2.4 Post-thaw treatments

The removal of the potentially toxic cryoprotection solution directly after thawing and its replacement by cryoprotection-free liquid medium, before transfer to semi-solid medium, results in complete loss of regrowth capacity and the cells becoming white. Direct transfer of cells to 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 solution, are directly transferred to semi-solid medium.

Using the optimized cryopreservation protocol described above, KULeuven is now storing in liquid nitrogen more than 2700 cryotubes containing embryogenic cell suspensions belonging to 19 different banana cultivars. Recently, banana cell suspensions were recovered after 15 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 has also been 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 banana 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 rather than of practical value.

Appendices

Appendix 1. Media and solution composition

MS medium (Murashige and Skoog 1962)

MS components Inorganic salts	Concentration (mg/L)
Calcium Chloride	332.02
Ammonium Nitrate (NH_4NO_3)	1650
Magnesium Sulfate	80.70
Boric Acid (H_3BO_3)	6.2
Cobalt Chloride ($CoCl_2 \cdot 6H_2O$)	0.025
Cupric Sulfate ($CuSO_4 \cdot 6H_2O$)	0.025
Manganese Sulfate ($MnSO_4 \cdot H_2O$)	16.90
Potassium Iodide (KI)	0.83
Potassium Nitrate (KNO_3)	1900
Potassium Phosphate (KH_2PO_4)	170
Sodium Molybdate ($Na_2MoO_4 \cdot 2H_2O$)	0.25
Zinc Sulfate ($ZnSO_4 \cdot 7H_2O$)	8.60

Iron source	
Sodium EDTA ($Na_2 \cdot EDTA$)	37.26
Ferric Sulfate ($FeSO_4 \cdot 7H_2O$)	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 30 g/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 (=136.8 g/L).

P6 Regeneration medium

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

Loading solution

MS medium components diluted in water supplemented with 2 M glycerol and 0.4 M (=136.8 g/L) sucrose, pH adjusted to 5.8. The solution is sterilized through a filter (0.22 µm).

PVS2 solution

Consists of 30% (w/v) (3.26 M) glycerol, 15% (w/v) (2.42 M) ethylene glycol (w/v) (EG), 15% (w/v) (1.9 M) DMSO and 0.4 M (= 136.8 g/L) 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 (0.22 µm) unloading solution consists of 1.2 M (410.4 g/L) sucrose dissolved in MS medium. (pH 5.8).

ZZ medium

Half strength MS macroelements 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
NH4H2PO4	300
$MnSO_4 \cdot H_2O$	10
H_3BO_3	5
$ZnSO_4 \cdot 7H_2O$	1
KI	1
$CuSO_4 \cdot 5H_2O$	0.2
$NaMoO_4 \cdot 2H_2O$	0.1
CoCl2	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

Tools (forceps and scalpels)

Liquid nitrogen (LN) source

Cryotanks   – 1 for liquid nitrogen storage

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

Polystyrene boxes

Dewar vessels

Sterile 2 ml cryotubes and cryovial holder (12 x 12)

Stirred methanol bath (-40°C) *or* Propanol container Alcohol bath (e.g. Nalgene™ cryo 1°C Freezing Container.) *or* Programmable freezer (only for research purpose or large-scale application) *or* (-70°C or -80°C freezer + Mr Freeze)

Chemicals (DMSO, PEG, etc.)

Binocular stereoscopic microscope (with good light source)

Fluorescence microscope (optionally)

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

Water bath (or warm water in plastic beaker)

Thermometer

Timer

Crushed ice and ice packs

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). CryoLetters 13:159-164.

Agrawal A., R. Swennen & B. Panis. 2004. A comparison of four methods for cryopreservation of meristems in banana (*Musa* spp.). CryoLetters 25:101-110.

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

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

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. CryoLetters 17:119-128.

Carpentier S., E. Witters, K. Laukens, H. Van Onckelen, R. Swennen & B. Panis. 2007. Banana (*Musa* spp.) as a model to study the meristem proteome: acclimation to osmotic stress. Proteomics 7:92-105.

Carpentier S., E. Witters, K. Laukens, P. Deckers, R. Swennen & B. Panis. 2005. Preparation of protein extracts from recalcitrant plant tissues: An evaluation of different methods for two-dimensional gel electrophoresis analysis. Proteomics 5:2497-2507.

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. CryoLetters 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.

Dussert S., F. Engelmann & M. Noirot. 2003. Development of probalistic tools to assist in the establishment and management of cryopreserved plant germplasm collections. CryoLetters 24:149-160.

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.

Gallard A., B. Panis, N. Dorion, R. Swennen & A. Grapin. 2008. Cryopreservation of *Pelargnonium* apices by droplet-vitrification. CryoLetters 29: 243-251.

Gazeau C., H. Elleuch, A. David & C. Morisset. 1998. Cryopreservation of transformed *Papaver somniferum* cells. CryoLetters 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. CryoLetters 19:375-382.

Hamill S., K. Wasmund, M. Smith, K. Eccleston & D. McKay. 2005. Endogenous bacterial isolated from banana meristems during tissue culture initiation: problems and potential. pp.101-111 *in* Contributing to a Sustainable Future. (Benett, I.J., Bunn, E., Clarke, H., and McComb, J.A., eds). Proc. Australian Branch IAPTC & B. Perth, Western Australia, AU.

Helliot B., R. Swennen, Y. PouMay, E. Frison, P. Lepoivre & B. Panis. 2003. Ultrastructural changes associated with cryopreservation of banana (*Musa* spp.) highly proliferating meristems. Plant Cell Rep. 21:690-698.

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 abscisic acid pretreatment. Plant Cell Rep. 12:89-94.

Marco A., J.L. Casas & B. Panis. 2007. Crioconservación de *Thymus moroderi*. VII Reunion de la Sociedad Española de Cultivo *In Vitro* de Tejidos Vegetales. Alcalá de Henares, Madrid, Spain, 25-27 June 2007.

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. CryoLetters 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.) embryogenetic 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. 201pp.

Panis B., I. Van den houwe, B. Piette & R. Swennen. 2007. Cryopreservation of the banana germplasm collection at the International Transit Centre - Bioversity International. Adv. Hort. Sci. 21: 235-238.

Panis B., B. Piette & R. Swennen. 2005a. Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all Musaceae. Plant Sci. 168:45-55.

Panis B., B. Helliot, H. Strosse, S. Remy, P. Lepoivre & R. Swennen. 2005b. Germplasm conservation, virus eradication and safe storage of transformation competent cultures in banana: The importance of cryopreservation. Acta Hort. 692:51-59.

Panis B., H. Strosse, S. Van den Hende & R. Swennen. 2002. Sucrose preculture to simplify cryopreservation of banana meristem cultures. CryoLetters 23:375-384.

Panis B., H. Schoofs, S. Remy, L. Sági & 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., 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 Hort. 461. ISHS.

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., 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. CryoLetters 11:337-350.

Panta A., B. Panis, C. Ynouye, B. Criel, R. Swennen & W. Roca. 2006. Improvement of potato cryopreservation for the long-term conservation of Andean landraces at CIP. 43rd Meeting of the Society for Cryobiology

in association with the Society for Low Temperature Biology. Hamburg, Germany, 24-27 July 2006.

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

Reed B.M., D. Dumet, J.M. Denoma & E.E. Benson. 2001. Validation of cyopreservation potocols for pant germplasm conservation: a pilot study using Ribes L., Biodivers. Conserv. 10:939-949.

Reed B.M., I. Kovalchuk, S. Kushnarenko, A. Meier-Dinkel, K. Schoenweiss, S. Pluta, K. Straczynska & E.E. Benson. 2004. Evaluation of critical points in technology transfer of cryopreservation protocols to international plant conservation laboratories. CryoLetters 25:341-352.

Reed B.M. 2008. Plant Cryopreservation: A Practical Guide. New York, NY: Springer. 515 pp.

Remy S., E. Thiry, B. Coemans, S. Windelinckx, R. Swennen & L. Sági. 2005. Improved T-DNA vector for tagging plant promoters via high-throughput luciferase screening. BioTechniques 38:763-770.

Sági 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.

Sági 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. & F. Engelmann. 2007. Vitrification, encapsulation-vitrification and droplet-vitrification. CryoLetters 28:151-172.

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.

Sánchez-Romero C. & B. Panis. 2008. Cryopreservation of olive embryogenic cultures. Cryopreservation of crop species in Europe. CRYOPLANET - COST Action 871. Agrifood Research Working papers 153. Oulu, Finland, 20-23 February 2008. 24-25.

Sant R., B. Panis, M. Taylor & A. Tyagi. 2008. Cryopreservation of shoot-tips by droplet vitrification applicable to all taro (*Colocasia esculenta* var. *esculenta*) accessions. Plant Cell Tiss. Org. Cult. 92:107-111.

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

Strosse H., R. Domergue, B. Panis, J.V. Escalant & F. Côte. 2003. Banana and Plantain embryogenic cell suspensions (A. Vézina and C. Picq, eds). INIBAP Technical Guidelines 8. INIBAP, Montpellier, France. 31 pp.

Strosse H., H. Schoofs, B. Panis, E. André, K. Reyniers & R. Swennen. 2006. Development of embryogenic cell suspensions from shoot meristematic tissue in bananas and plantains (*Musa* spp.). Plant Sci. 170: 104-112.

Strosse H., E. André, L. Sági, R. Swennen & B. Panis. (*in press*). Adventitious shoot formation is not inherent to micropropagation of banana as it is in maize. Plant Cell Tissue and Organ Culture.

Surga J., R. Swennen & B. Panis. 1999. Cryoconservation de méristèmes chez le bananier (*Musa* spp.): optimisation de la régénération. 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. CryoLetters 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. CryoLetters 20(3):163-174.

Thomas P., G.K. Swarna, P.K. Roy & P. Patil. 2008. Identification of culturable and originally non-culturable endophytic bacteria isolated from shoot tip cultures of banana cv Grand Naine. Plant Cell Tiss. Org. Cult. 93: 55-63.

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 Hort. 530:69-79.

Watanabe K. & P.L. Steponkus. 1995. Vitrification of *Oryza sativa* L. cell suspensions. CryoLetters 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. CryoLetters 18:317-324.

Zhu G. Y., J. Geuns, S. Dussert, R. Swennen & B. Panis. 2006. Sugar, sterol and fatty acid composition in banana meristem cultures in relation to cryopreservation ability. Physiol. Plant.128:80-94.

FURTHER RECOMMENDED LITERATURE

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

Bajaj Y.P.S. 1995. Biotechnology in Agriculture and Forestry 32: Cryopreservation of Plant Germplasm 1. Springer, Berlin.

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* Proceedings of the workshop on New Frontiers in Resistance Breeding for Nematodes, *Fusarium* and Sigatoka, 2-5 October 1995, Kuala Lumpur, Malaysia (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.

Fuller B.J., N. Lane & E.E. Benson. 2004. Life in the Frozen State. CRC Press, Boca Raton.

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. 1988. Annual Report 1987. International Network for the Improvement of Banana and Plantain. Montpellier, France. 43pp.

INIBAP. 1998. 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: Proceedings of an international workshop held at Cairns, Australia, 13-17 October, 1986. 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.

Towill L.E. & Y.P.S. Bajaj. 2001. Biotechnology in Agriculture and Forestry 32: Cryopreservation of Plant Germplasm 2. Springer, Berlin.

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.

CTA

partageons les connaissances au profit des communautés rurales

sharing knowledge, improving rural livelihoods