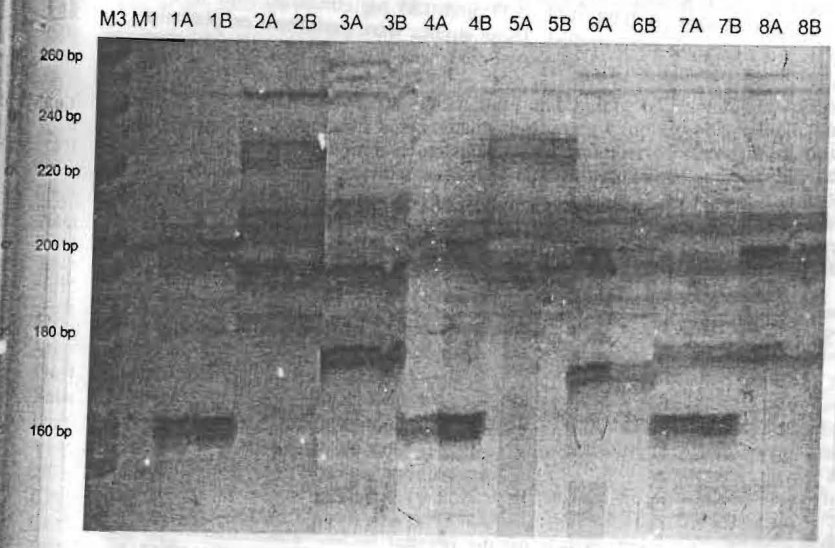

Fig. 2. Analysis of SSR products of HIGT4 loci in 5% polyacrylamide denaturing (8 M urea) gel. M1 – 100 bp ladder (Promega, Madison, WI, USA), M3 – 20 pb Molecular ruler (BIO-RAD, Hercules, CA, USA). 1 – CZ/K26, 2 – Sladek, 3 – Galena, 4 – Osvald's clone 31, 5 – Harmonie, 6 – Comet, 7 – Southern Promise, 8 – Southern Star, A – before and B – after cryopreservation.

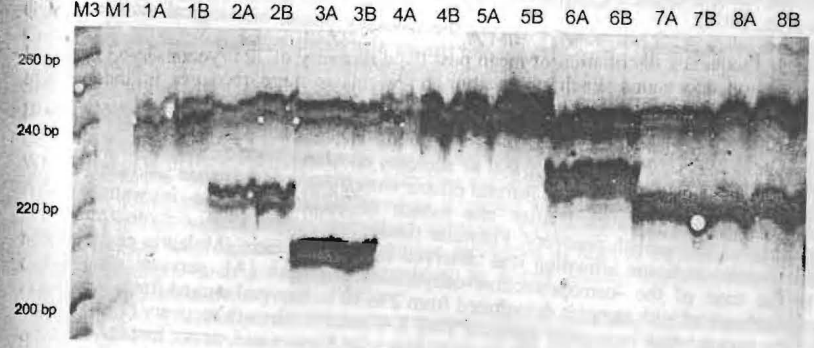

Fig. 3. Analysis of STS products of NDBP1 loci in 5% polyacrylamide denaturing (8M urea) gel. M1 – 100 bp ladder (Promega, Madison, WI, USA), M3 – 20 pb Molecular ruler (BIO-RAD, Hercules, CA, USA). 1 – CZ/K26, 2 – Sladek, 3 – Galena, 4 – Osvald's clone 31, 5 – Harmonie, 6 – Comet, 7 – Southern Promise, 8 – Southern Star, A – before and B – after cryopreservation.

Cryopreservation as a Tool for the Management of Coconut Germplasm

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Keywords: *Cocos nucifera* L., conservation and exchange of germplasm, encapsulation-dehydration, osmoprotection, plant growth recovery, plumule, zygotic embryo

Abstract

We present hereafter a review of different collaborative research studies carried out on the cryopreservation of coconut plumules excised from the zygotic embryo. Plumule (shoot apical meristem and leaf primordia) tissues have shown different degrees of success in cryopreservation depending on the combination of alginate encapsulation and osmotic or evaporative dehydration used. The percentage of regrowth was progressively improved from vitrification (0%), to osmoprotection (10%), and subsequently the encapsulation-dehydration technique allowed 20% regrowth level into leafy shoots. Addition of abscisic acid (20 to 40 µM) boosted recovery growth after freezing (up to 40%). Histological studies have clearly shown that the addition of lower amounts of ABA (10 µM) allows cells to maintain the structural characteristics of control cells for immersion into liquid nitrogen without dehydration. The effect of plant material conditioning, for transport from collecting site to laboratories, was studied to identify possible effects of uncontrolled factors on tissue tolerance to cryopreservation. Three conditioning methods [embryo set in endosperm core (ALB); embryo transferred onto of solidified agar (SW); embryo immersed into KCl solution (KCl)] were used. SW performance is clearly more efficient when combined with dehydration and freezing, giving 40% recovery. These results are interesting as they show that the medium surrounding the embryo (endosperm or medium supply) can be replaced by agar alone, without nutritive factors. This approach should also facilitate germplasm exchange. As a result of the absence of phloem vascular bundles in the plumular tissues, the approach described here should increase the scope for obtaining material free of pathogenic agents, an essential prerequisite for the conservation and exchange of germplasm. This approach should be a strong point in the fight against Lethal Yellowing Disease, the most serious disease affecting coconut plantations.

INTRODUCTION

The development of strategies for coconut (*Cocos nucifera* L.) germplasm conservation and exchange is of great importance because of the biological characteristics of the seed. Its large size (one of the largest of the plant kingdom) and physiology (no dormancy and recalcitrance to storage) make its conservation and exchange a difficult problem to resolve.

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Up to the 80s, conservation strategies were achieved through field genebanks and coconut germplasm exchange in the form of seeds. In the field, germplasm conservation offers a satisfactory approach to the conservation of genetic resources which can be easily observed and evaluated. However, this strategy is subject to various environmental and human factors; it requires huge land areas and it is not cost-effective because of considerable inputs in the form of land, labour, management and materials (Engelmann et al., 2005).

Since the 90s, because of these limiting factors and in addition to plant disease considerations, conservation strategies have focused more on ex situ conservation using in vitro techniques, whereas exchange of coconut germplasm was carried out only through zygotic embryos and pollen (Frison et al., 1993; Ramanatha Rao and Batugal, 1998; Malaurie, 2001).

In this context, in vitro culture techniques have a great potential for the exchange and conservation of plant germplasm. These techniques are of great importance for recalcitrant seeds such as those of coconut (Engelmann et al., 2005).

Up to now, coconut in vitro technology was focused on three research areas: somatic cell manipulation; zygotic embryo in vitro culture (including conservation and slow growth culture) which is routinely applied in numerous laboratories (Engelmann et al., 2005), and cryopreservation. Previous cryopreservation experiments have been performed on three types of material: pollen (Frison et al., 1993; Engelmann, 1999; Hocher et al., 2004); zygotic embryos (Chin et al., 1989; Assy-Bah and Engelmann, 1992a, b); and plumules (Hornung et al., 2001). In 1989, only one single coconut embryo was reported to have survived 15 days after freezing using cryoprotection with DMSO plus slow freezing (Chin et al., 1989). Whereas in 1992, the work of Assy-Bah et al. gave more optimistic results from 4 different coconut genotypes [hybrid PB121; Cameroon Red Dwarf (CRD); Rennell Tall (RT); and Indian Tall (IT)] with high recovery of frozen mature embryos (93, 36, 33, and 48% respectively) using the pregrowth/ desiccation technique (Assy-Bah and Engelmann, 1992b). Previous work on plumule cryopreservation dates back to 2001, and focused on obtaining embryogenic callus as a basis for the cloning of elite coconut genotypes (Hornung et al., 2001).

The present paper summarizes the different work carried out in our laboratory or in association with collaborating research teams and advances made in coconut cryopreservation research with its possible impact on the management of coconut germplasm. It then describes the investigation of several cryopreservation techniques, the optimization of plumule cryopreservation by dehydration/encapsulation as confirmed by histological studies and investigations towards finding the best way to transport and store zygotic embryos of coconut. The use of plumules as the main plant material for cryopreservation work depends mainly on its structure (presence of cells in a meristematic state). This makes its use viable for the obtaining of a safe (i.e. disease-free) material, as previously observed with this type of explant (Morel and Martin, 1952).

MATERIALS AND METHODS

One type of plant material was used for cryopreservation purposes: plumule tissues (shoot meristem surrounded by leaf primordia) excised from zygotic embryos. Two types of zygotic embryos were used, namely the Malayan Yellow Dwarf/ Nain Jaune Malais zygotic embryos (MYD/NJM) at 10 to 12 months after the pollination (N'Nan et al., 2004; Engelmann et al., 2005), and the Sri Lankan Tall (SLT) -which was used at a 12 to 14 months after pollination (Malaurie et al., 2006).

Investigations into plumule cryopreservation led to the use of several techniques, all based on plumule encapsulation in alginate beads. These techniques differed from each other either by their pretreatment step (liquid or solid medium) or by the dehydration step (osmotic or evaporative dehydration): i) encapsulation/osmoprotection/dehydration [A] (Sakai et al., 2000; Malaurie et al., 2002); ii) encapsulation/osmoprotection/vitrification [B] (Sakai et al., 2000; Malaurie et al., 2002, 2004); iii) encapsulation/dehydration (Dereuddre et al., 1990; N'Nan, 2004; N'Nan et al., 2008).

To optimize plumule recovery after cryopreservation via encapsulation/ dehydration (N'Nan, 2004; N'Nan et al., 2008), the effects of abscisic acid (ABA) (0, 10, 20, and 40 µM) and sorbitol (0, 0.03, 0.11, 0.27, and 0.55 M) combined with 0.75 M sucrose and 16 h dehydration were studied. These studies were performed on plumules excised from embryos isolated in an endosperm core, which had been confined before excision for one week of transport and storage (Malaurie et al., 2006), or directly after albumen core collection in a tissue culture division Laboratory (CRI, Sri Lanka), without any conditioning for transport (Bandupriya et al., 2007).

In order to identify the most suitable method to transport/store mature zygotic embryos to allow delayed excision of plumules for further cryopreservation, three temporary storage condition were evaluated over a period of 7 days: i) embryo maintained in endosperm core; ii) embryo transferred onto solidified agar [0.45% (w/v) agar]; iii) embryo immersed in KCl solution (16.2 g L^{-1}) (Malaurie et al., 2006; Bandupriya et al., 2008).

RESULTS AND DISCUSSION

Plumule Cryopreservation

The most efficient plumule cryopreservation protocol (20% recovery) was reported after encapsulation/dehydration (N'Nan et al., 2008). This perhaps low result is nevertheless quite remarkable for a plant recognized as having highly recalcitrant storage behaviour (Chin and Roberts, 1980). Moreover, this result can be considered as satisfactory in the context of genetic resource conservation (Dussert et al., 2003).

Histological Studies

Histological studies showed that for the optimal sucrose concentration, longer dehydration durations have a stronger effect on cells compared with the effect of increasing sucrose concentrations at the optimal dehydration duration. When using optimal conditions (1 M sucrose and 16 h), we showed that dividing cells could be observed after freezing (+LN). These results are in accordance with observations made of morphogenetic development (N'Nan et al., 2003, 2008).

The addition of ABA has a positive effect on the preservation of cellular structures after dehydration and freezing, due to the beneficial effects of ABA on tolerance to dehydration and freezing stress. The supply of 10 µM even allows to the cells to keep the structural characteristics of control cells (without pre-treatment/-Dehydration/-LN, and with 0.75 M/+Dehydration) for immersion conditions into LN without dehydration (Malaurie et al., 2006).

In relation to what we observe during the recovery phase, histological studies allow us to define the nature of the cellular and tissues alterations which are compatible with this recovery and which could therefore be reversible (Malaurie et al., 2006).

Techniques Using Osmoprotection

Preliminary experiments were performed with encapsulation combined with osmoprotection (LS: 2 M glycerol and 0.4 M sucrose) or vitrification (LS, and PVS2 solution) (Malaurie et al., 2002, 2004). Several effects were observed on tissues following osmoprotectant/vitrifiant treatment after one month of culture, such as browning, swelling, survival and growth recovery. Plumular tissues were affected by dehydration and freezing stress as tissue browning was observed for 79% of them (Malaurie et al., 2002). In the case of the -osmoprotection/-dehydration approach [A], survival after freezing was observed with samples dehydrated from 2 to 10 h. Survival ranged from 3 to 15%, and the best survival rates were recorded after 3, 4 and 5 h. Growth recovery (10%) was observed only once with samples dehydrated for at least 3 hours and, or not frozen.

In the case of the vitrification approach [B], all samples turned brown after cryopreservation and no growth recovery was observed, irrespective of the duration of exposure (20, 60 and 100 min.). In contrast, 15% of the unfrozen control exposed to 20

min of dehydration showed growth recovery (Malaurie et al., 2003; N'Nan, 2004).

Optimization of the Recovery Frequency

It was showed that adding ABA to the pretreatment medium boosts recovery growth. We report that the most significantly favourable treatment prior to the dehydration and freezing is 20 µM ABA, which allow 38% growth recovery (Malaurie et al., 2006). Other authors for whom which material processing was different (no transport and storage in endosperm core) reported that 40 µM ABA was the optimal concentration for recovery (39%) (Bandupriya et al., 2007).

These results show that ABA addition to the pretreatment media improves significantly the recovery after cryopreservation, and reveals the importance of ABA action on tolerance to desiccation and to the freezing of plumules of coconut embryos.

Effect of Transport or Storage Conditions on Plumule Cryopreservation Performance

The effect of plant material conditioning, for transport from the collecting site to laboratories was studied to identify possible impacts of uncontroled factors on tissue further tolerance to cryopreservation (Malaurie et al., 2006; Bandupriya et al., 2008).

This work focused on three forms of conditioning used for coconut zygotic embryo shipping (Assy-Bah et al., 1987; N'Nan, 2004; S. Fernando, pers. commun.). It was showed that the three conditioning forms [embryo set in endosperm core (ALB), embryo transferred onto of solidified agar (SW); embryo immersed into KCl solution (KCl)] do not affect the capacity of the tissues of the plumule to respond to dehydration and freezing treatments. It also revealed that SW and KCl are compatible with further cryopreservation experiments. Indeed, we noted an effect of conditioning on survival rate as well as on recovery. SW and KCl survival rates are greater than those obtained with ALB for 0.75 M (9%). In the case of 1 M sucrose, SW performance is clearly more marked in the case of dehydration and freezing with 40% recovery.

Observation of water contents between the ALB and SW-KCl groups reinforces the idea that the conditioning has allowed a reduction in water content down to a threshold more suited to regrowth after cryopreservation. These results are interesting as they show that the medium surrounding the embryo (endosperm or medium supply) can be replaced by agar alone, without nutritive factors. Agar can ensure good maintenance of the embryos and good reactivity of the plumules used in the different pretreatments. These findings should also facilitate germplasm exchange (Malaurie et al., 2006).

CONCLUSION

These present review demonstrates the great potential of cryopreservation for the long-term conservation of coconut genetic resources. Germplasm conservation through cryopreservation is feasible with plumule tissues excised from zygotic embryos, and transfer of zygotic embryos is now mastered and feasible between areas free of virus and phytoplasma (MLO) diseases. Temporary storage conditioning for transport of zygotic embryos can have a substantial effect on subsequent behaviour of the tissues. 'Solid agar conditioning' should be preferred as it greatly increases the reactivity of the plumules when excised for cryopreservation purposes. Cryopreservation of coconut plumules is already well mastered with percentage recoveries as high as 20%. The addition of other chemicals such as ABA is promising as it can greatly improve tissue tolerance to dehydration and cryopreservation.

Coconut germplasm conservation strategies cannot be safely developed using only the cryopreservation approach, and have to be combined with the ex situ multi-site International Coconut Genebanks in five host-countries (Brazil, Côte d'Ivoire, India, Indonesia, and Papua New-Guinea) developed as Centers of Excellence, as well as with the in situ and on-farm conservation practices being developed through a farmer participatory approach (Batugal, 2005). The cryopreservation approach using plumule tissue should increase the scope for obtaining material free of pathogenic agents, an essential prerequisite for the conservation and exchange of germplasm. This approach should be a strong point in the fight against Lethal Yellowing Disease.

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Cryopreservation of Mint – Routine Application in a Genebank, Experience and Problems

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Abstract

The genebank of the IPK contains 216 mint accessions, which are maintained in the field or in vitro. In 2005, routine cryopreservation of seed-sterile mint was started, using axillary shoot tips from cold-acclimated nodal segments and a simple droplet-vitrification method with PVS 2 (Senula et al., 2007). At present, 35 accessions from 12 species are cryopreserved by this method. The average regeneration rate was 65% however, there was high variation in regeneration % (between 13 to 100%), both between accessions and between two repetitions of the same accession. This was correlated to the genotype and endophytes in the in vitro donor plants that were previously found to be free of visible infections. After rewarming, bacterial infections often broke out of and reduced or even inhibited regeneration of plantlets. A screening of endophytes using single donor plants prior to multiplication and cryopreservation was executed. Since bacteria are not homogeneously distributed in the plants, the results of the screening test reflected only the given situation in the tested material proper, but not that in the whole plant. Therefore, the use of these pre-tests alone is not conclusive. Furthermore, some endophytes do not grow on culture media alone, which makes detection difficult. The preventive use of antibiotics in the regeneration medium after cryopreservation resulted in higher plant regeneration, when the donor plants were infected. The antibiotics so far tested (Cefotaxim, Ticarcillin, Vancomycin) delayed the regeneration process, but did not reduce regeneration rates.

INTRODUCTION

The maintenance of vegetatively propagated plants is a special challenge for genebanks. It requires a lot of space in the field and high labour input. Furthermore, the risk of losses of accessions through plant diseases and unfavourable weather exists.

The IPK mint collection consists of 216 accessions, which are maintained in field plots or in pots. Sixty-eight percent of these accessions can be maintained only vegetatively because of the purely clonal nature of some taxa, such as *Mentha* x *piperita* L. and also because not enough seeds for storage is formed under local conditions in some accessions. In addition to field maintenance, in vitro storage and cryopreservation can be used to maintain the collection. In vitro storage of mint is easy and widely distributed in genebanks. The collection of the National Clonal Germplasm Repository at Corvallis, Oregon, USA, consists of 493 accessions, 157 of which are maintained in vitro (GRIN, 2009), and the collection of the genebank of the Brazilian Agricultural Research Corporation EMBRAPA consists of 54 accessions, 24 being maintained in vitro (Silva et al., 2006).

The in vitro maintenance of mint accessions has been routine at the IPK genebank since 1997. At present, the mint in vitro collection comprises 148 accessions, which are maintained at 2 and 10°C (IPK-GBIS, 2009). In vitro culture has several advantages compared to field maintenance, such as less space requirement and the protection against plant diseases, weather and intermixing of the accessions. Nevertheless subculturing and