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Cryoconservation of some wild species of *Musa* L.

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Abstract

The present work was undertaken to devise suitable medium for induction of proliferating meristems in five wild species of *Musa* (*M. acuminata*, *M. balbisiana*, *M. basjo, M. jackeyi* and *M. textilis*) and their subsequent cryopreservation by modification of the standard droplet vitrification protocol. It was found that BAP (100 ìM) alone was not sufficient to induce high proliferation rates and inclusion of TDZ (1-10 ìM) was essential. The duration of pre-growth desiccation (9-14 d) and dehydration with cryoprotectant (PVS2, 90-120 min) varied with each species. The optimized protocol for each species yielded 46-58% shoot recovery after cryopreservation. In terms of ease of explant generation, percentage shoot recovery and plantlet formation, *M. jackeyi > M. basjoo > M. textilis> M. acuminata>M. balbisiana.*

Key words : Crop wild relatives, cryopreservation, *Musa,* vitrification

Introduction

Crop wild relatives (CWR) comprise an enormous reservoir of genetic variation useful in plant breeding, important for meeting the challenges of a rapidly growing world population and accelerated climate change [1]. By definition, CWR comprise wild plant taxon that have indirect use derived from their relatively close genetic relationship to a crop [2].There is an urgent need for systematic efforts to introgress broad subsets of CWR diversity into crop plants to incorporate useful adaptations for traits such as disease resistance, abiotic stress tolerance, and other agronomic challenges required to increase the resiliency and productivity of agriculture in the 21st century [3]. The CWR occur in a wide range of habitats, but due to rapid loss or degradation of such niches many species face risk. There is, thus, urgent need to conserve them both in the wild (*in situ*) and in genebanks (*ex situ*) while the genetic diversity they contain is still available.

Ex situ conservation of some CWR presents major challenges for genebank managers both from technical and management aspects. Often, the storage conditions which have been established mainly for major crops are not well adapted for some of their wild relatives, on which limited research has been undertaken to refine their conservation *ex situ* [4]. Cryopreservation, which encompasses storage of biological tissue at ultra-low temperatures, has gained tremendous importance for conservation of genetic resources of crops that are vegetatively propagated, recalcitrant seeded, rare/ endangered and crop wild relatives (CWR) [5]. It involves the storage of a range of plant tissues (meristems, zygotic/somatic embryos, cell suspensions or callus tissues) at ultra-low temperatures in liquid nitrogen (LN) either at -135°C in the vapour phase or -196°C in the liquid phase [5]. Threatened species and CWR require reliable long-term storage methods such as cryopreservation, while awaiting their utilisation in restoration and breeding projects.

India is one of the major centres of origin and diversity for both wild and cultivated bananas (*Musa* L. spp.), especially for *balbisiana*-derived hybrids [6]. Besides *Musa acuminata* Colla and *Musa balbisiana* Colla, the progenitors of present day bananas, a number of peripheral species contributing to the total diversity occur in India [7-12]. In India the family Musaceae is represented by 31 taxa under two genera*, Musa* L. and *Ensete* L. and largely distributed in North-eastern States, the Western Ghats, Eastern Ghats and Andaman and Nicobar Islands [11]. Many of these wild relatives and species of *Musa* potentially have genes that can contribute for alleviating both biotic and abiotic stresses.

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For example 'Calcutta 4' (*Musa acuminata* spp. *burmannicoides*) a wild, non-edible diploid banana has been used as a source of resistance in banana hybrids to black Sigatoka leaf spot disease, the most serious constraint to banana production globally caused by the fungus *Mycosphaerella fijiensis* [13]. However, these species urgently require protection, due to habitat loss. For example, plants of 'Calcutta 4' (Indian origin), have disappeared under natural conditions [6]. These are only available in *ex situ* genebanks, including the *In Vitro* Genebank at NBPGR, India. Some of these wild species, such as *Musa chunii* Häkkinen, have been given the status of 'critically endangered' due to their occurrence in specific fragile ecological niches that harbour very few individuals in a population [7].

The long-term conservation of *Musa* germplasm, comprising both bananas and plantains, is being carried out at NBPGR for the past one decade, using vitrification-based cryopreservation techniques [14, 15]. Three different methods of cryopreservation (pre-growth desiccation, vitrification and droplet-vitrification) have been tested and suitably applied on genotypes of various genomic groups like AA, AB, ABB, AAB, AAA. However, as wild species were reported to be somewhat recalcitrant to cryopreservation using the standard protocol [16], it was considered important to devise specific protocols for a few wild species. The major impediment for cryobanking of *Musa* germplasm is generation of adequate and appropriate meristem explants, which can be frozen in LN. The explants most amenable for cryopreservation of *Musa* and also easy to isolate are *in vitro* proliferating meristems (PM), in contrast to individual meristems from *in vitro* rooted plants (IVM) (15). The standard medium for obtaining such PM reported earlier [14, 15] was found to be sub-optimal or ineffective for wild species of *Musa*. The present work was undertaken to devise suitable medium for induction of PM in five wild species of *Musa (M. acuminata* Colla, *M. balbisiana* Colla, *M. basjoo* Siebold & Zucc. ex Iinuma, *M. jackeyi* W.Hill and *M. textilis* Née.) and their subsequent cryopreservation by modification of the standard droplet vitrification protocol [16] in terms of preculture duration, cryoprotection duration and recovery medium.

Materials and methods

Five important *Musa* species (6 accessions) available as elongated *in vitro* shoot cultures in the *In Vitro* Genebank of NBPGR, New Delhi, were used as experimental material (Table 1). Shoot explants (each containing three to four shoots smaller than 0.5 cm) were cultured for 4 weeks on standard p5 medium comprising Murashige and Skoog's (1962) medium (MS) supplemented with 10 μM 6-benzylaminopurine (BAP), 1 μM indole-3-acetic acid (IAA), 10 mg/l ascorbic acid, 3% sucrose and 0.25% gelrite (15). All the cultures were maintained at 25±2°C with 40 μE $m^{-2}s^{-1}$ photon flux density 16 h light/8h dark. After 2 culture cycles on p5 medium, multiple shoots were grown on p4 medium (p5 medium with 100 μM BAP) in darkness [16]. Subsequently, the smallest shoots with clusters of meristems at their leaf bases were selected and subcultured on p4 medium, until groups of proliferating meristems (about 0.5 cm in diameter) could be cultured separately. In addition to p4 medium, the following growth regulators were tested for induction of shoot proliferation:

Table 1. List of wild species of *Musa* used for cryopreservation

Accession no.	Section	Name of species
EC 653539	*Australimusa*	*M. basjoo*
EC 653577	*Australimusa*	*M. textilis*
EC 653582	*Australimusa*	*M. jackeyi*
EC 653544	*Eumusa*	*M. balbisiana*
EC 653579	*Eumusa*	*M. balbisiana* type butuhan, intermediate apex
EC 653563	*Eumusa*	*Musa acuminata* type Kluai Thong Det

P7 medium: BAP (10 μM) + TDZ (5 μM); P8 medium: BAP (20 μM) + TDZ (5 μM); P9 medium: BAP (20 μM) + TDZ (1 μM); P10 medium: BAP (50 μM) + TDZ (10 μM) and P11 medium: TDZ (1 μM).

Pregrowth-desiccation of meristematic clumps (6-10 mm diam.) was carried out by transferring the clumps to C0 medium (P5 medium + 0.4 M sucrose) in the dark for 8 to 15 days, depending on the species. On the day of the experiment, explants (2-3 mm dia.) were excised from the sucrose-pretreated meristematic cultures, and immersed in loading solution (LS): 2 M glycerol + 0.4 M sucrose in MS medium (pH 5.8) for 20 min at 25°C. After decanting the LS, 5 ml of Plant Vitrification Solution 2 (PVS2): 30% (3.26 M) glycerol + 15% (2.42 M) ethylene glycol +15% (1.9 M) dimethylsulfoxide (DMSO) + 0.4 M sucrose in MS medium (pH 5.8)] was added. Explants were incubated for 90, 120 and 150 min in PVS2 at 0°C. Five minutes before the completion of the incubation period, 10 explants were transferred to a drop

of fresh, chilled PVS2 solution placed on a strip of aluminium foil (20 × 5 mm) and plunged directly into LN. Thereafter, the aluminium foil strips were removed and rinsed in 10 ml of recovery solution (RS) 1.2 M sucrose dissolved in MS medium (pH 5.8). For regeneration of meristems, explants were placed onto a stack of two sterile filter papers on top of semi-solid growth regulator-free MS medium containing 0.3 M sucrose. The Petri plates containing meristems were incubated in the dark for 48 h, after which the meristems were transferred to semi-solid or liquid regeneration medium (MS + 2.22 µM BAP and 0.09 M sucrose). Regrowth (shoot regeneration and/or callus formation) of explants was recorded under a binocular microscope (Olympus) at weekly intervals up to 10 wk. The regrowth rate was calculated as the percentage of meristems or meristematic clumps that survived the cryopreservation treatment and produced shoots or shoot buds. For each experiment, 30-120 explants were used for LN treatment. The experiments were performed three times for each accession. Results are presented as means (%) ± standard error of means. Data were subjected to Duncan's Multiple Range Test to test for significant differences at $P \leq 0.05$ using SPSS ver. 10.0.

Results and discussion

Proliferating meristems in most cultivated bananas have been reported to develop by the use of high BAP (100 µM) in the medium [14-17]. However, amongst the wild species investigated in the present study (except *M. basjoo*), BAP (100 µM) alone was not sufficient to induce high proliferation rates (Table 2). It was found that inclusion of TDZ was essential for multiple shoot formation at low concentration (1 µM) in *M. textilis* and *M. acuminata* and at medium concentration (5 µM) in *M. jackeyi. Musa balbisiana* was very difficult for induction of shoot proliferation and required use of both BAP (50 µM) and TDZ (10 µM) for yielding meristematic clumps (Table 2). One an average it took at least 10-16 subcultures at monthly intervals to obtain sufficient quantity and quality of proliferating shoots suitable for cryopreservation. In an earlier report, meristematic clumps in cultivated triploid cooking bananas (ABB group), plantains (AAB group) and Cavendish banana (AAA group) were obtained after, respectively, 5-9, 7-11 and 8-10 monthly cycles on BAP (100 µM) medium [17]. Present work has shown that BAP alone was not sufficient for induction of proliferating meristems, and TDZ (1-5 µM) was additionally required for the wild species tested (except in *M. basjoo*). This is in contrast to the report on shoot proliferation of *M. balbisiana* 'Kluai Hin' (BBB) where BAP was found to induce more shoot multiplication than TDZ (18). TDZ, a synthetic phenyl urea derivative, increases shoot formation of several plant species more efficiently than adenine derivatives such as BAP. The differential responses to TDZ concentrations among banana genotypes is documented and ascribed to their difference in cytokinin uptake, translocation to the meristematic region and degradation [19].

In most cryopreservation protocols on cultivated triploid bananas, the pregrowth desiccation of meristematic clumps in high sucrose medium (0.4 M) is given for 2 wk [15]. However, in the present study it was observed that 2 wk culture led to necrosis of tissue in *M. textilis* and *M. acuminata,* where 8-9 days incubation was sufficient (Table 2). In one accession of *M. balbisiana* correlation was observed between pre-growth for 14 days with reduced requirement for PVS2 incubation. *M. basjoo* and *M. jackeyi* could tolerate desiccation for the standard 14 days. Most previous reports on *Musa* cryopreservation have highlighted the critical role of sucrose dehydration of meristems prior to their cryopreservation [14-16, 20-22]. Only one report has shown that glucose and fructose are better than sucrose, mannitol or trehalose for pregrowth desiccation in case of four *Musa* cultivars namely Pisang Mas AA, Pisang Nangka AAA, Pisang Berangan AAA and Pisang Awak ABB (23). In fact 0.5 M trehalose, or 0.4 and 0.5 M sucrose or mannitol were extremely toxic to meristems, and shoot regeneration from 5 - 48% was achieved in cryopreserved meristems dehydrated with glucose and fructose (0.4-0.5 M).

The effect of duration of PVS2 for 60, 90 and 120 min. on cryopreserved meristematic clumps of the five tested species is shown in Fig 1. All species except *M. balbisiana* could tolerate PVS2 dehydration up to 120 min, where a shorter time (90 min) of PVS2 incubation was optimal (Fig. 1, Table 2). Shorter duration of 60 or 90 min in all other species was found to be insufficient for good regeneration rates. The effects of duration of PVS2 exposure on *Musa* have been investigated earlier, and correlation to genotype and explant type have been shown [14, 22].

In general, all the wild species exhibited a prolonged recovery period after LN treatment, ranging from 8-12 wk. In previous reports with cultivated bananas, recovery response can be obtained within 3-6 wk (14-16). Phenolic leachates were very high in all species and callus formation also occurred in few explants. Table 2 provides shoot recovery (mean±SE) obtained after LN treatment of the meristems in all the

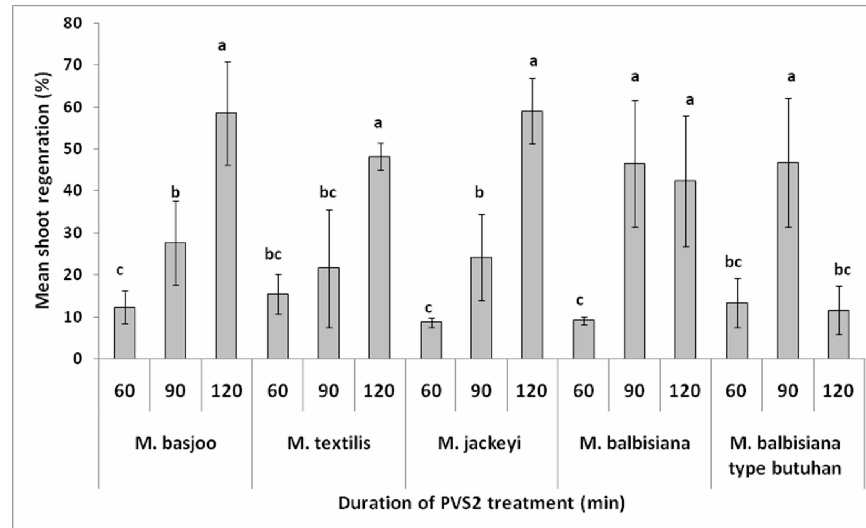

Fig. 1. Effect of PVS2 duration on shoot regeneration of cryopreserved meristematic clumps of the five wild species tested. Bars marked with different letters are significantly different for shoot regeneration using DMRT at *P* = 0.05. *Y*-bars represent standard deviation

Table 2. Response of wild species of *Musa* to various parameters tested for cryopreservation

Name of species	Optimum media (no. of monthly subculture cycles)	Optimum duration of pre-growth desiccation (days)	Optimum duration for PVS2 dehydration (min)	Shoot regrowth of cryopreserved meristems (mean ± SE)
M. basjoo (ITC 0061)	P5 (2); P4(8)	14	120	57.89 ± 3.2
M. textilis (ITC 0539)	P5 (2); P11 (1); P4 (9)	7	120	49.18 ± 4.31
M. jackeyi (ITC 0588)	P5 (6); P4 (1); P7(2)	14	120	58.65 ± 7.21
M. balbisiana (ITC 0094)	P5 (2); P11 (1); P4 (11)	9, 14	120, 90	44.97 ± 9.52
M. balbisiana type butuhan (ITC 0565)	P5 (2); P7 (1); P8 (1), P9 (1); P4 (4)	14	90	46.58 ± 8.65
Musa acuminata type Kluai Thong Det (ITC0404)	P5 (2); P11 (1); P4 (8)	8	120	54.11 ± 3.24

species tested. Use of liquid recovery medium gave significantly better results than semi-soil medium (data not shown). In general *M. balbisiana* was found to be the most difficult to cryopreserve. In terms of ease of explant generation, percentage shoot recovery and plantlet formation, *M. jackeyi* > *M. basjoo* > *M. textilis*> *M. acuminata*>*M. balbisiana.* Thus, protocol for *Musa* wild species cryopreservation could be improved by use of TDZ in the proliferation medium for generation of explants, optimizing the dehydration duration for individual species and use of liquid regeneration medium for recovery. The optimized protocols have been applied to cryobank germplasm of the tested accessions at NBPGR. The protocol can be further extend for use in other wild species of *Musa*.

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