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Influence of dehydration on cryopreservation of *Musa* spp. germplasm

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Abstract – Cryopreservation is an important technique for the long-term storage of economically important plant germplasm. In this study, an efficient protocol was developed for the long-term conservation of seven economically important *Musa* taxa: *M. acuminata* Colla ssp. *burmannica* N.W. Simmonds, *M. acuminata* Colla ssp. *zebrina* (Van Houtte) R.E. Nasution, *M. balbisiana* Colla, *M. basjoo* Sieb., *M. ornata* W. Roxburgh (St. Lavender), *M. velutina* H. Wendl. et Drude (Velvet Pink Banana), and *M. acuminata* × *balbisiana*. The seeds were dehydrated in a sterile laminar flow cabinet for different exposure times and then they were directly immersed in liquid nitrogen. The critical point was to support the initial germination of cryopreserved seeds and this was achieved by the excision of zygotic embryos after liquid nitrogen treatment that allowed the seed germination. The best moisture content for tolerance to cryopreservation ranged from 15.8% (*M. acuminata* ssp. *zebrina*) to 17.1% (*M. ornata*) and the maximum post-cryopreservation germination rates varied from 86.4% (*M. velutina*) to 55.0% (*M. ornata*). All seedlings derived from seeds germinated after cryopreservation were easily rooted and acclimated to greenhouse conditions.

Keywords: banana, long-term conservation, moisture content, seed desiccation

Introduction

Conservation strategies can be divided into two main categories, *ex situ* and *in situ*, and include many procedures for the preservation of plant biodiversity. *Ex situ* strategies consist of the conservation of biodiversity in an area different from that in which plants have their natural habitat. *In situ* strategies, on the other hand, mean the maintenance of biodiversity as the domestication and/or cultivation of species, in developed artificial media (Engelmann 2012).

Conservation of agrobiodiversity and plant biodiversity is important for food security and the sustainability of plant genetic resources. Genetic diversity increases the options for improvement through crop selection and breeding, including higher yields and greater resistance to adverse environmental conditions (Rao 2004). The banana is the one of most important nutrition sources in the world and many efforts have been made to support the conservation of *Musa* germplasm (FAO 2010, Langhe et al. 2018).

Banana and plantain germplasm has conventionally been preserved in field genebanks, but there are many other different preservation strategies and methods with specific advantages and disadvantages for short-, medium and long-term conservation, depending on different situations, such as plant material and the method to be applied (Sipen et al. 2011). The basic procedure for seed conservation in seed banks consists of dehydration and storage of seeds at low temperatures (below 0 °C). However, active collections are stored above 0 °C. Additionally, different types of genebank, such as *in vitro* and field collections, complement each other for *Musa* spp. germplasm conservation and seed storage, and clonal collections need further complementary conservation procedures (Chin 1996).

The embryo metabolism of orthodox seeds is suspended as a result of maturation drying and seedswith low moisture contents (~10–15%) can not germinate until their water content becomes fully restored, when they are also not dormant (Bewley and Nonogaki 2003). The long-term storage of the seeds of orthodox species in genebanks requires certain conditions: a temperature between –15 °C and –20 °C and a

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moisture content between 3% and 7%. Orthodox seeds can maintain germination and viability under these conditions for long periods of time, a hundred years or more (Hintum and Menting 2003).

Musa spp. seeds are classified as orthodox and can be dehydrated and conserved for long periods of time at low temperatures (Vineesh et al. 2015, Kaya 2016) and they are also known as very dormant. After harvesting, these seeds can easily germinate, however, they usually stay dormant when they lose their moisture content (Chin 1996). The hard and thick seed coat of *Musa* spp. seeds can prevent the water and oxygen uptake that are essential for germination under natural conditions (Puteh et al. 2011). The very small embryo of the seed is under a seed lid named an operculum which blocks water uptake for dried seeds (Ellis et al. 1985, Graven et al. 1996).

Because only the wild *Musa* and plantain species produce large numbers of seeds, the storage of seeds of commercial varieties has limited applicability. While orthodox seeds can be dehydrated and stored for long periods at low temperatures, seed dormancy is frequently a problem after drying and cold treatment processes. For development of an efficient way to start the initial germination, further work on understanding the mechanisms of germination is required. Additionally, more information is needed about the optimization of storage conditions, effective germination and the handling of these seeds for succesful achievement of long-term storage of *Musa* spp. seeds (Kaya 2016).

Cryopreservation has a significant role in global programs to preserve plant genetic resources (Reed 2008). Seeds stored in seed banks are divided into three main groups according to dehydration responses: orthodox (dehydration-tolerant); intermediate (relatively dehydration-tolerant); and recalcitrant (dehydration-sensitive). Orthodox seeds accumulate sugars and proteins in their cytoplasm to achieve vitrification at positive temperatures and to resist damage to their cells and membranes by desiccation at low temperature (Kaya et al. 2017). For this reason, such seeds are cryopreserved easily by being directly plunged into liquid nitrogen after desiccation, without any substantial reduction of seed viabilityor germination rate upon thawing (Gakhova et al. 2006).

Furthermore, *Musa* seed cryopreservation may have applications beyond the conservation of germplasm and can be a valuable tool for breeding programs. Some crosses generate a large number of seeds and the rescue of embryos becomes long and labor-intensive. For the transfer of considerable properties to exclusive varieties, the fertile and viable seeds of *Musa* spp. are important germplasms for breeding programs. This provides an advantage for sterile vegetatively propagated clones which is available as trading varieties. The possibility of cryopreserving these seeds allows the work to be done within the capacity of the laboratory. The aim of this study was to develop a protocol for the long-term conservation of *Musa* seeds using dehydration/direct immersion in liquid nitrogen.

Materials and methods

Plant material

M. acuminata Colla ssp. *burmannica* N.W. Simmonds, *M. acuminata* Colla ssp. *zebrina* (Van Houtte) R.E. Nasution, *M. balbisiana* Colla, *M. basjoo* Sieb., *M. ornata* W. Roxburgh (St. Lavender), *M. velutina* H. Wendl. et Drude (Velvet Pink Banana) and *M. acuminata* × *balbisiana* (Hybrid) seeds were provided by the genebank of the Berlin-Dahlem Botanical Garden.

Decontamination of *Musa* spp. seeds

Musa spp. seeds belonging to the seven taxa were surface sterilized by treatment for 5 minutes in 70% ethanol, 5 minutes in 10% H_2O_2 and two times 10 minutes in 20% concentrated commercial bleach (Domestos®). After each step, the seeds were rinsed in sterile distilled water (Kaya 2016).

Embryo germination media and culture conditions

Zygotic embryos obtained from seeds of the seven *Musa* spp taxa were extracted for *in vitro* germination from all of control (before and after dehydration of seeds) and liquid nitrogen groups (after cryopreservation of seeds) under a microscope (Fig. 1A, B) before being transferred to semi-solid MS (Murashige and Skoog 1962) germination medium supplemented with 20 g L^{-1} of sucrose as carbon source, 1.5 g L^{-1} of phytagel and 3.5 g L^{-1} of agar as solidifying agents, and 0.1 μM of gibberellic acid (13 mg L^{-1}) as dormancy breaker (Fig. 1C). Until the start of germination, the zygotic embryos were kept in the dark at 27 ± 2 °C and then, they were transferred to the standard culture conditions (16/8 h photoperiod, with cool daylight fluorescent lamps rated at 50 $\mu mol^{-1} m^{-2} s^{-1}$).

Determination of moisture content and evaluation of germination rate

After the *Musa* spp. seeds were weighed (initial weight), they were placed in sterile Petri dishes in a laminar flow cabinet at room temperature. Additionally, to test the initial germination rate, ten zygotic embryos removed from non-dehydrated seeds were directly transferred to germination medium, while the other seeds were kept in the laminar flow cabinet (up to 9 hours) to measure the seed weight every hour for determination of the water loss rate. The environmental conditions (temperature and relative humidity) of the laminar flow cabinet were recorded during the dehydration procedure and ten seeds were also transferred to germination medium to test the effect of dehydration on germination after each hour. In all, 200 seeds (20 seeds for each hour of dehydration time from 0 to 9 hours, ten for moisture content measurement and the others for germination testing) were used. Each test was repeated at least three times. Seed moisture content was calculated using the formula of Pixton (1966): % Moisture Content (MC) = [(Initial weight of seeds – dry wight of seeds) / Initial weight of seeds] × 100.

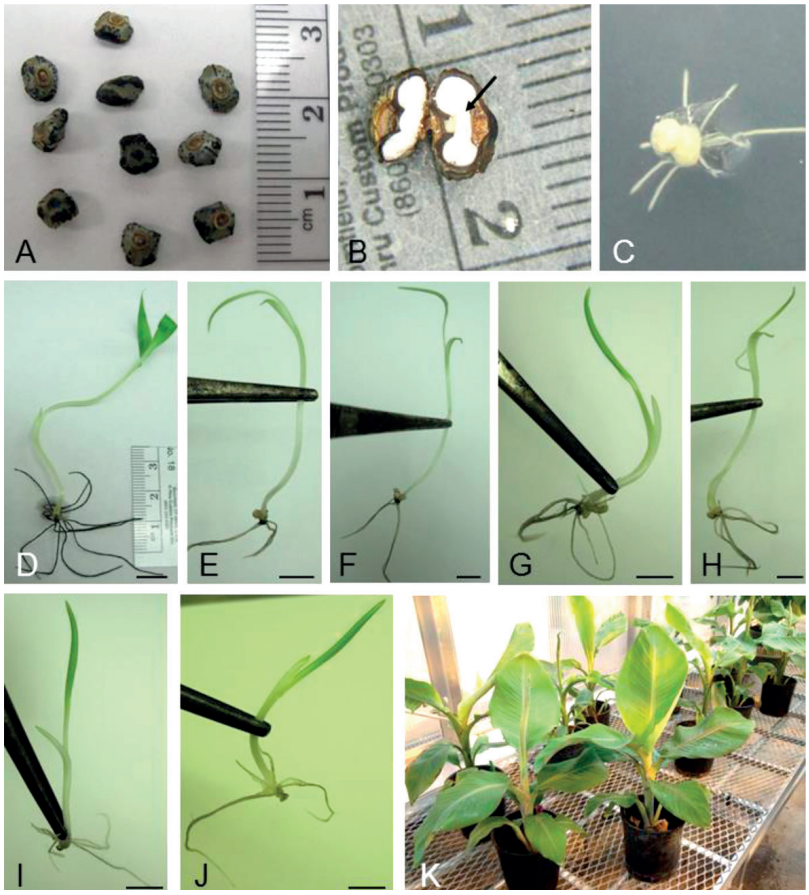


Fig. 1. *In vitro* germination of zygotic embryos excised from *Musa* spp. after cryopreservation: seeds of *M. velutina* (A), embryo of *M. ornata* (arrow indicates embryo in seed) (B), embryo germination of cryopreserved seed of *M. acuminata* ssp. *burmannica* in semi-solid germination medium after four weeks incubation (C). Seedlings having well-formed shoots and strong roots derived from embryos of cryopreserved seeds of *M. acuminata* ssp. *burmanica* (D), *M. acuminata* ssp. *zebrina* (E), *M. balbisiana* (F), *M. basjoo* (G), *M. ornata* (H), *M. velutina* (I), *M. acuminata' balbisiana* (J), All *Musa* spp. cryopreserved seeds were very easily acclimated to greenhouse conditions (K). Scale bars: 1 cm.

Long-term storage via cryopreservation

After calculation of seeds' MC in the dehydration test, the dehydrated seeds (from each dehydration time in hours) were placed in 2 mL cryovials, five seeds per vial, and were directly plunged into a storage tank containing liquid nitrogen. The thawing process was performed by placing the seeds in a sterile laminar flow cabinet at room temperature for 5 minutes after storage of 24 hours at −196 °C. The zygotic embryos removed from the cryopreserved and thawed seeds were transferred to germination medium and kept in the dark at 27 ± 2 °C, and then transferred to culture media under the conditions stated above.

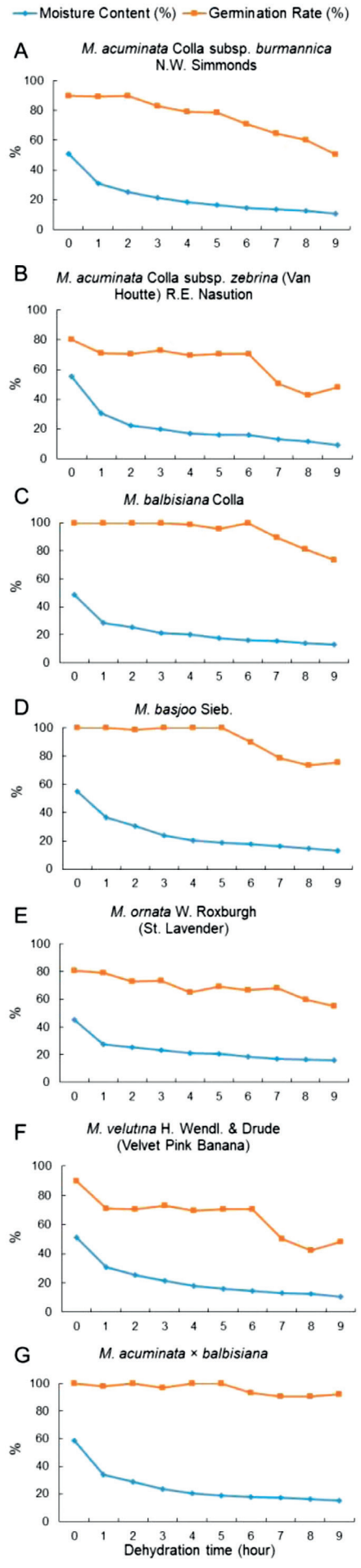
Evaluation of data and statistical analyses

Zygotic embryo germination of the control and liquid nitrogen group was evaluated with respect to dormancy for three and four weeks (for control group) and six and eight weeks (for liquid nitrogen group) after being transferred to germination medium. There were the germination time differences between control group and liquid nitrogen group. Germination of the liquid nitrogen group took longer than the control group. Zygotic embryos of both groups producing at least one morphologically normal seedling were accepted as successful germinations. Thirty *Musa* spp. seeds were used for each treatment step for MC determination. The treatments of all control and liquid nitrogen groups were repeated at least three times. Embryo germination porcentage was compared by multiple X^2 test by the SPSS program (IBM SPSS Statistics 21.0) and statistical analysis was also performed with ANOVA, followed by the LSD test at $P \leq 0.05$ (Marascuilo and McSweeney 1977).

Results

Relation between seed moisture content and embryo germination percentage

After treatment of successful surface sterilization (which obtained approximately 90% sterile material), the seeds presented normal morphology. Initial moisture content of all seven *Musa* taxa ranged between 45.1% (*M. ornata*) and 58.5% (*M. acuminata* × *balbisiana*) and embryo germination percentage ranged between 80% (*M. acuminata* ssp. *zebrina*) and 100% (*M. balbisiana*, *M. basjoo* and *M. acuminata* × *balbisiana*). The moisture content of seeds was reduced by dehydration in the laminar flow cabinet, to values between 9.2%



(*M. acuminata* ssp. *zebrina*) and 15.9% (*M. velutina*) after nine hours. The germination percentage decreased as the water content declined. The seeds most sensitive to dehydration belonged to *M. ornata*, because germination decreased to 55.2% when the moisture content declined to 15.8% after nine hours of dehydration. On the other hand, seeds that had almost the same moisture content (between 15.8% and 16.2% comparing four taxa of *Musa*), such as 16.0% (*M. balbisiana*, 6 h), 15.9% (*M. velutina*, 9 h), 16.2% (*M. acuminata* × *balbisiana*, 8 h) and 15.8% (*M. acuminata* ssp. *zebrina*, 6 h) had good germination rates, of 100%, 86.2%, 84.7% and 70.3%, respectively (Tab. 1, Fig. 2). These dehydration time differences were caused by the different shapes and sizes of the different *Musa* taxa seeds used in the study.

Cryopreservation of seeds

The best cryopreservation results, ranging from germination of 55% (*M. ornata*) to 86.4% (*M. velutina*) were obtained from seeds that had moisture content reduced to between 15.9% (*M. acuminata* ssp. *zebrina*) and 17.1% (*M. ornata*) (Tab. 1, Fig. 2). There was a positive correlation between moisture content and germination percentage in the control group. On the other hand, there was a negative correlation after cryostorage. Another significant result was that most of the *Musa* spp. seeds with a moisture content of 20% or more did not germinate, the exceptions being *M. acuminata* subsp. *burmanica* and *M. ornata*, with 21.3% and 20.8% moisture content respectively, although the germination percentage were very low (4.2% for *M. acuminata* ssp. *burmannica* and 10% for *M. ornata*).

Furthermore, the blocking effect of liquid nitrogen delayed the *in vitro* germination compared to control groups. For example, the zygotic embryos removed from cryopreserved seeds started to germinate after approximately six or eight weeks for all the *Musa* spp., while the germination times of embryos removed from control group seeds were shorter (maximum four weeks) (Fig. 1C).

Finally, all seedlings obtained from zygotic embryos of cryopreserved seeds had well-formed shoots and strong roots (Fig. 1D-J) and they were very easily acclimated to greenhouse conditions (Fig. 1K).

Discussion

Although *Musa* spp. seeds are classified as orthodox, they have been found to be dormant after preservation, depending on the different morphological, physical and physiological characteristics among species (Chin 1996, Burgos-Hernández et al. 2014). Their hard and strict seed coat causes physical dormancy by reducing water intake, which is crucial for germination, thus decreasing or completely preventing germination (Baskin et al. 2000). *Musa* spp. seed endosperms are surrounded by a tegmen and testa. The first one is a weak inner integument and the other is a multilayer outer integument. The *Musa* seed zygotic embryos are very soft, smooth and small, and are located under a seed lid called the operculum (Graven et al. 1996) and in some seeds,

Fig. 2. Effect of varying moisture content on *Musa* spp. seed germination: A – *M. acuminata* ssp. *burmannica*, B – *M. acuminata* ssp. *zebrina*, C – *M. balbisiana*, D – *M. basjoo*, E – *M. ornata*, F – *M. velutina*, G – *M. acuminata* × *balbisiana* (enviromental conditions during seed dehydration: temperature 23.04 ± 0.06 °C; relative humudity, 46 ± 2.65%).

Tab. 1. Germination percentages of control group and cryopreserved seeds belonging to seven *Musa* taxa: 1: *M. acuminata* Colla ssp. *burmannica* N.W.Simmonds; 2: *M. acuminata* Colla ssp. *zebrina* (Van Houtte) R.E. Nasution; 3: *M. balbisiana* Colla; 4: *M. basjoo* Sieb.; 5: *M. ornata* W. Roxburgh (St. Lavender); 6: *M. velutina* H. Wendl. et Drude (Velvet Pink Banana; 7: *M. acuminata* × *balbisiana* (Cont. – control group, LN+ – liquid nitrogen group). Values in bold indicated the best germination percentages obtained from cryopreserved seeds. Germination percentage ± standard error values were statistically analyzed by a nonparametric test, the post hoc multiple comparisons test (Marascuilo and McSweeney 1977). Statistical analysis performed by ANOVA, followed by LSD test at $P \leq 0.05$. Different letters at apex of values indicate statistically significant differences between the values obtained from treatment results, $P \leq 0.05$.

Musa taxon		Dehidration time								
		1st h	2nd h	3rd h	4th h	5th h	6th h	7th h	8th h	9th h
1	Cont.	89.5 ± 0.1^{c}	90.0 ± 0.6^{c}	83.0 ± 1.1^{de}	79.3 ± 1.8^{e}	78.9 ± 0.9^{e}	70.7 ± 1.1^{g}	64.8 ± 0.7^{h}	60.0 ± 0.7^{i}	59.3 ± 0.9^{i}
	LN+	0.0	0.0	4.2 ± 0.3^{r}	23.3 ± 0.5^{p}	**68.3 ± 0.4^{h}**	48.5 ± 0.5^{k}	40.0 ± 1.3^{m}	40.4 ± 0.4^{m}	32.7 ± 0.6^{no}
2	Cont.	71.0 ± 0.0^{g}	70.6 ± 0.2^{g}	73.1 ± 1.3^{f}	69.5 ± 0.6^{g}	70.5 ± 0.6^{g}	70.3 ± 0.5^{g}	50.3 ± 0.3^{k}	42.5 ± 0.7^{lm}	48.0 ± 0.3^{k}
	LN+	0.0	0.0	0.0	6.3 ± 0.3^{r}	48.4 ± 0.3^{k}	**60.9 ± 0.7^{i}**	42.3 ± 0.3^{lm}	0.0	10.5 ± 0.3^{q}
3	Cont.	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}	99.0 ± 0.9^{a}	95.5 ± 0.1^{b}	100 ± 0.0^{a}	89.6 ± 0.1^{c}	81.1 ± 1.1^{e}	73.3 ± 0.1^{f}
	LN+	0.0	0.0	0.0	10.2 ± 0.9^{q}	70.4 ± 0.3^{g}	**79.6 ± 0.1^{e}**	48.4 ± 0.37^{k}	38.3 ± 0.3^{m}	41.7 ± 1.3^{m}
4	Cont.	100 ± 0.0^{a}	98.3 ± 0.1^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}	89.6 ± 0.1^{c}	78.3 ± 1.1^{e}	73.3 ± 0.1^{f}	75.4 ± 0.1^{f}
	LN+	0.0	0.0	0.0	0.0	30.9 ± 0.4^{o}	56.0 ± 0.4^{j}	**71.5 ± 0.1^{g}**	48.4 ± 0.3^{k}	0.0
5	Cont.	79.3 ± 0.5^{e}	73.0 ± 0.3^{f}	73.3 ± 0.2^{f}	65.1 ± 0.2^{h}	69.5 ± 0.4^{g}	66.7 ± 0.5^{h}	68.3 ± 0.3^{h}	60.0 ± 0.5^{i}	55.2 ± 0.3^{j}
	LN+	0.0	0.0	0.0	0.0	10.0 ± 0.3^{q}	48.0 ± 0.7^{k}	**55.0 ± 0.4^{j}**	43.3 ± 0.7^{l}	40.7 ± 0.1^{m}
6	Cont.	100 ± 0.0^{a}	100 ± 0.0^{a}	96.7 ± 0.1^{b}	96.0 ± 0.1^{b}	91.0 ± 1.1^{c}	89.3 ± 0.4^{c}	91.5 ± 0.1^{c}	89.4 ± 0.2^{c}	86.2 ± 0.1^{d}
	LN+	0.0	0.0	0.0	0.0	0.0	34.0 ± 0.2^{n}	48.2 ± 0.3^{k}	**86.4 ± 0.1^{d}**	83.3 ± 0.3^{d}
7	Cont.	98 ± 0.0^{a}	100 ± 0.0^{a}	96.7 ± 0.1^{b}	99.7 ± 0.1^{a}	100 ± 0.0^{a}	93.3 ± 0.3^{b}	90.7 ± 0.1^{c}	90.3 ± 0.1^{c}	92.1 ± 0.1^{c}
	LN+	0.0	0.0	0.0	0.0	51.8 ± 1.1^{k}	64.0 ± 0.2^{h}	77.2 ± 0.8^{ef}	**84.7 ± 0.1^{d}**	75.0 ± 0.3^{f}

the cells surrounding them can inhibit water uptake during germination (Finch-Savage and Leubner-Metzger 2006). In this study, to overcome this problem, the zygotic embryos were removed from all the dehydrated seeds before transfer to the germination medium. In this way, they germinated easily (germination start of the control seeds took three to four weeks, while for the liquid nitrogen group it took six to eight weeks). Many treatments aimed at breaking the dormancy of *Musa* seeds have been described, such as mechanical scratching, treatment with gibberellic acid, removal of zygotic embryos, chemical exposure (treatment with sulfuric acid with different exposure times), but only removal of zygotic embryos, as in this study, and/or chemical exposure have shown successful germination (Puteh et al. 2011, Uma et al. 2011, Burgos-Hernández et al. 2014).

The capability of orthodox seeds to survive significant dehydration tends to be obtained during seed maturation at which seeds reach highest dry weight, or earlier, based upon the environment conditions to which they have been subjected (Hong and Ellis 1992). This capability can be lost during seed germination. The dried seeds turn out to be sensitive to dehydration if it is permitted to develop too far (Koster and Leopold 1988). Loss of dehydration tolerance tends to overlap with radicle emergence (Senaratna and McKersie 1983). Dehydration sensitivity of intermediate and recalcitrant seeds have also been shown to be decreased during the development, but not to the same extent as in orthodox seeds (Ellis et al. 1985).

Cryopreservation (storage at ultra-low temperatures) of different types of plant materials (seeds, meristems, buds, embryos, calli) is used for long-term conservation of plant genetic resources (Reed 2008). The main aim of this study was to optimize a protocol for long-term storage of seven different *Musa* taxa using a one-step freezing technique (dehydration and direct immersion in liquid nitrogen). The results were successful, with germination percentage of embryos from cryopreserved seeds between 55% and 86.4%. The other critical result was that optimum germination was obtained with seed moisture contents between 15.9% and 17.1% (Tab. 1, Fig. 2). It can be deduced that for long-term cryopreservation of *Musa* spp seeds in liquid nitrogen, the moisture content should be less than 17% for optimal viability and germination. Panis (2009) cryopreserved zygotic embryos of *M. acuminata* and *M. balbisiana* and obtained an average germination rate of 53% from zygotic embryos having 14% moisture content. In another study, Kaya (2016) used two different *Musa* species (*M. velutina* and *M. acuminata*) for cryopreservation and obtained an optimum germination rate (up to 84.3%) from seeds with moisture content of less than 17%. Our results corroborate these earlier findings.

However, some orthodox seeds have critical problems such as dormancy as seen in the current study. They are still very suitable material for successful cryopreservation, because they have strong sugar reserves and their zygotic embryos have high sucrose/raffinose contents, preventing sucrose crystallization at high concentrations. These properties have a protective role during seed desiccation (Steadman et al. 1996). One explanation for this supposition is that sugars cause the vitrification of cell cytoplasm. Thus, the intracellular matrix becomes concentrated via dehydration and does not form ice crystals (Buitink and Leprince 2004). Another possible mechanism is that oligosaccharides change with water bound to membrane phospholipids via hydrogen bonding (Crowe et al. 1988).

This study showed a variation in the response of the different *Musa* spp. seeds to dehydration and cryopreservation. However, despite this, the presented technique can be applied to the cryopreservation of Musa orthodox seeds. As for the problem of identified dormancy, it can be overcome by the excitation of the embryo and its cultivation in culture medium.

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