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Research article/Araştırma makalesi

Long-term germplasm conservation of two economical important *Musa* species via cryopreservation-dehidration technique

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Abstract

An important alternative technique for plant germplasm conservation is offered by a biotechnology-based approache of cryopreservation and it refers to storage of plant material at ultra-low temperatures in liquid nitrogen. A procedure for cryopreservation of seeds was improved for two different *Musa* sp. in current study. Seed dehydration was performed at different exposure times, in the sterile conditions of a laminar flow-hood. The tested cultivars showed the highest tolerance to cryopreservation when the seeds were dehydrated to a moisture content of 16.7% (*M. velutina* H. Wendl. & Drude) to 16.3% (*M. acuminata* Colla subsp. *burmanica* Simmonds) and the post-cryopreservation germinability also ranged respectively from 84.3% to 64.2%. Dehydration was beneficial for germination of all seeds of the tested *Musa* species after the liquid nitrogen exposure and the cryopreserved seedlings had well-formed shoot and roots, and their acclimatization to greenhouse conditions was easy.

Key words: Cryopreservation, *Musa* spp., viability and germination, seed moisture content, dehydration

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Kriyoprezervasyon-dehidratasyon tekniği kullanılarak ekonomik öneme sahip iki Musa türünün germplazmasının uzun süreli saklanması

Özet

Bitki germplazmasının korunmasında önemli, alternatif ve biyoteknoloji tabanlı bir teknik, sıvı azot içerisinde bitki materyallerinin ultra düşük sıcaklıklarda saklanması anlamına gelen kriyoprezervasyondur. Mevcut çalışmada iki farklı Musa sp.'ye ait tohumların kriyoprezervasyonu için bir yöntem geliştirilmiştir. Steril koşullarda, yatay akımlı kabin içerisinde farklı uygulama sürelerinde tohum dehidratasyonu gerçekleştirilmiştir. Test edilen kültür çeşitlerinde tohum nem içerikleri % 16.7 (M. velutina H. Wendl. & Drude için) ve % 16.3 (M. acuminata Colla subsp. burmanica için) oranlarına düşürüldüğünde, en yüksek kriyoprezervasyon toleransı görülmüştür ve ayrıca kriyoprezervasyon sonrası çimlenme de sırasıyla % 84.3 ve % 64.2 aralığında olmuştur. Dehidratasyon, sıvı azot uygulaması sonrası test edilen Musa türlerine ait tüm tohumlarının çimlenmesi üzerinde olumlu etkiye sahipti, kriyoprezervasyon sonrası elde edilen fidelerden gelişen sürgün ve kökler iyi formdaydı ve sera koşularına iklimlendirilmesi kolay oldu.

Anahtar kelimeler: Kriyoprezervasyon, Musa spp., canlılık ve çimlenme, tohum nem içeriği, dehidratasyon

1. Introduction

Plant genetic resources preservation is important for food guarantee and agro-biodiversity. Plant biodiversity makes options to develop through selection and breeding of new and more economical important crops, resistant to biological and enviromental stresses "Rao, 2004; Alao, 2009". *Musa* spp. are the fourth most important global food commodity after rice, wheat and corn in terms of gross value of production "Tribe, 1994". Numerous developments in the past decade have changed the scenario for genetic conservation of *Musa*, particularly the establishment of INIBAP (International Network for the Improvement of Banana and Plantain). Many varieties of banana and plantain exist all

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over the world, and it is believed that many problems such as disease and pest susceptibilty can be solved by the selection of existing resistant varieties and the breeding of new varieties. Improvement of *Musa* has, so far, been relying on a very narrow genetic base and broad germplasm collections are required "Chin, 1996".

Still conventionally *Musa* spp. has been preserved in arable field genebanks, all techniques are available for the preservation of such material. Each technique has spesific advantages and disadvantages in relation to the type of material to be preserved and the goals of the preservation programme "Sharrock and Engels, 1997; Kaya et al., 2013". For a seed-propagated crop, a base collection in genebank will consist of seeds dried and stored at subzero temperatures. An active collection consists of seeds stored at above 0ºC. In the case of *Musa* genetic conservation, different types of genebanks (*in vitro*, field collection) are utilized wich complement each other. There is a need to further complement the clonal collections by seed storage "Chin, 1996".

Cryopreservation is a biotechnological method for conservation of plant germplasm and it plays an important role in conservation of the world's genetic resources "Bajaj, 1995; Benson, 1999". Orthodox seeds are protected against low-temperature damage by dehydration and accumulation of sugars and proteins that vitrify their cytoplasm at positive temperatures. Cryopreservation of such seed presents no problems: they are just placed into liquid nitrogen without protectants. This does not appreciably harm seed viability upon thawing "Gonzalez-Benito et al., 2004; Kaya et al., 2016"; sometimes the seed germination ability is even improved, as shown for hard seed of wild legumes "Chetverikova, 2008". Orthodox seeds are deposited in long-term cryobanks "Gakhova et al., 2006".

The aim of this study was to evaluate time of desiccation and moisture content in seed that would maintain the highest germination after cryopreservation of *M. velutina* and *M. acuminata* seeds.

2. Materials and methods

2.1. Plant Materials

M. velutina and *M. acuminata* seeds were provided by USDA, ARS, Tropical Agriculture Research Station (Mayaguez, Puerto Rico; Figure 1A-F).

2.2. Decontamination of Musa spp. seeds

Seeds of *M. velutina* and *M. acuminata* were surface sterilized by soaking in 70% ethanol for 5 min and disinfected by two times 10-min treatment with 20% commercial bleach, with consecutive rinses in sterile dH_2O after each step.

2.3. Seed Germination Media

Excised embriyos from seeds of *M. velutina* and *M. acuminata* were germinated *in vitro* by placing them to Petri dishes (100 x 15 mm) on semi-solid (1.5 gl^{-1} phytagel, Sigma; 4 gl^{-1} agar, Sigma 7002) MS medium (Murashige and Skoog, 1962, MS-519, Sigma) supplemented with 0.1uM GA (2.7ul Phytotech GA solution G362, 13mgl^{-1}) and 20g l^{-1} sucrose (Phytotech) maintained under the standard culture conditions. During germination, the embriyos were kept at 27±2°C in the dark (The culture conditions; 27±2°C temperature, 16-h photoperiod, with light provided by cool daylight fluorescent lamps 50 $\mu mol^{-1}m^{-2}s^{-1}$).

2.4. Determination of the Moisture Content (MC)

Ten of non dehydrated seeds were directly tested for germinability by transferring them to Petri dishes (100 x 15 mm) on semi-solid germination medium. Afterwards, in a preliminary trial, the seeds were placed top of open Petri dishes at room temperature, in the sterile air current of a laminar flow hood, and the MC of ten of seeds was determined every hour (up to 9 hours) and until a MC below 20% was reached. During dehydration, the environmental conditions of the laboratory were monitored for temperature (77 ± 2 ºF) and relative humidity (RH, 17 ± 1%). Moisture contents of *M. velutina* and *M. acuminata* seeds and embriyos were determined by using formula "Pixton, 1966" which describes below;

Moisture content (%) = [(AC-BD) /AC] x100

Where **A**, original weight of portion of sample; **B**, weight after 1st stage drying; **C**, initial weight of ground sample for 2nd stage, and **D**, final weight of dried ground sample.

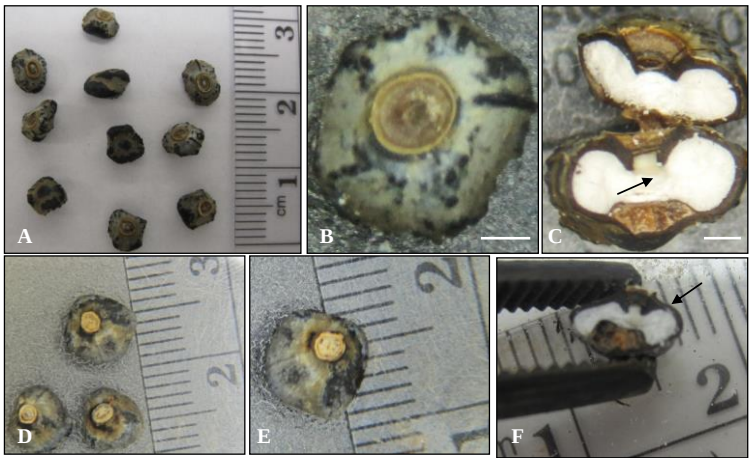

Figure 1. Seeds of *M. velutina* (**A, B**), *M. acuminata* (**D, E**)**,** an embriyo of *M. velutina* (**C**), *M. acuminata* (**F**), (Bars 1.5 mm).

2.5. Long-term conservation

After the evaluation of results from the preliminary dehydration trial, each of dehydration times were tested for cryopreservation of *M. velutina* and *M. acuminata* seeds, in order to obtain seeds with MCs between 30 and 15%. For cryostorage, the seeds were placed in 1.5 ml cryovials (CORNING®), five seeds per cryovial, which were directly plunged into liquid nitrogen. After at least 24 hr of storage at -196ºC, the seeds were rewarmed by waiting of the cryovials in laminar flow hood at room temparature for 15 min. Then, embriyos were excised from the seeds were transferred to the germination medium, under the climatic conditions stated above. Germinability was evaluated six weeks after recovering of embriyos from liquid nitrogen. Embriyos which produced at least one morphologically-normal seedling were considered germinated. Percentages of germination and the average numbers of seedlings per embriyos were calculated on the basis of 10-15 seeds per treatment, and each experiment was repeated at least three times. Levels of germination were compared by multiple x^2 test using by SPSS (12.0 for Windows) and statistical analysis performed by ANOVA, followed by LSD test at $P \leq 0.05$.

3. Results

3.1. Moisture Content of M. velutina and M. acuminata Seeds and Embriyos

Initial moisture content (MC) of *M. velutina* and *M. acuminata* seeds (respectively, 48.6% and 45.3%) and moisture content of embriyos of *M. velutina* seeds (52.5%) was high for cryopreservation (Because of moisture content of *M. velutina* seeds and embriyos are similar, we didn't measure moisture content of embriyos of *M. acuminata* seeds). After nine hours in the laminar flow hood moisture content of seeds was quite reduced to about 15% and 11.4% (Table 1). The moisture content of *M. velutina* seeds was decreased under 20% end of the fifth hour but the moisture content of embriyos was decreased rapidly under 20% end of the seventh hour. The results were showed that moisture content of seeds and embryos approximately were similar for *M. velutina* seeds (Table 1, Figure 2).

Table 1. Moisture content of *M. velutina* and *M. acuminata* seeds and also embriyos for *M. veluntina* (enviromental conditions, temperature, 77±2 ºF; relative humudity, 17±1%)

Dry Time in the Laminar Flow Hood	Moisture Content (%)		
	M. velutina		*M. acuminata*
	Seed (% ± SE*)	Embriyo (% ± SE)	Seed (% ± SE)
0	48.6 ± 0.1	52.5 ± 0.0	45.3 ± 0.4
1	27.9 ± 0.8	44.1 ± 0.0	30.9 ± 1.3
2	25.5 ± 0.5	40.3 ± 0.0	25.4 ± 0.5
3	23.9 ± 0.3	32.4 ± 0.0	21.3 ± 0.1
4	22.3 ± 0.3	28.4 ± 0.0	19.2 ± 0.1
5	20.7 ± 0.2	27.9 ± 0.0	16.3 ± 0.1
6	19.3 ± 0.1	27.1 ± 0.0	14.7 ± 0.1
7	18.0 ± 0.2	26.7 ± 0.0	13.3 ± 0.3
8	16.7 ± 0.3	17.4 ± 0.2	12.4 ± 0.2
9	15.9 ± 0.2	15.1 ± 0.3	11.4 ± 0.1

*SE: Standard Error

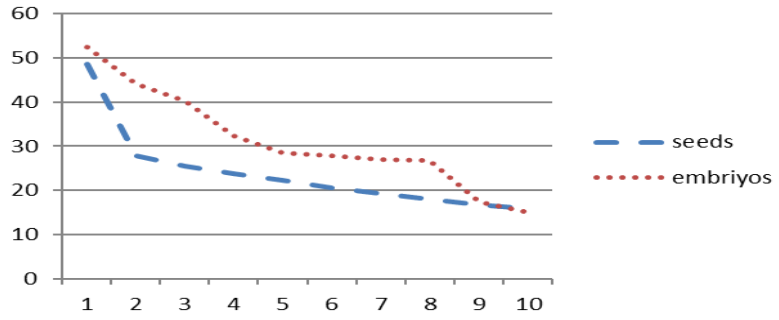

Figure 2. Moisture content change of *M. velutina* seeds and embriyos (enviromental conditions, temperature, 77±2 ºF; relative humudity, 17±1%)

3.2. Cryopreservation of M. velutina and M. acuminata Seeds

Effect on germination rates of different preiods of dehydration, followed or not by direct plunging of *M. velutina* and *M. acuminata* seeds into liquid nitrogen shown at Table 3. The best results for *M. velutina* obtained from 8 hour dryed seeds (84.3% germination rate; MC: 16.7%) and for *M. acuminata* obtained from 5 hour dryed seeds (64.2% germination rate; MC: 16.3%) (Table 3, Figure 3).

Table 3. Effect of different periods of dehydration in a laminar flow hood, followed or not by direct plunging of *M. velutina* and *M. acuminata* seeds into liquid nitrogen, on seeds and embriyos moisture content (% of FW) and germination (%). (LN, plunging into liquid nitrogen, MC, moisture content)

Dehydration Period (hr)	*M. velutina*			*M. acuminata*		
	Seed MC (%)	Germination (%)		Seed MC (%)	Germination (%)	
		Dry	Dry + LN		Dry	Dry + LN
1	27.9 ± 0.8	100 ± 0.0a	0.0	30.9 ± 1.3	89.5 ± 0.1c	0.0
2	25.5 ± 0.5	100 ± 0.0a	0.0	25.4 ± 0.5	90.0 ± 1.5bc	0.0
3	23.9 ± 0.3	96.7 ± 0.8ab	0.0	21.3 ± 0.1	50.0 ± 0.0i	4.2 ± 0.2m
4	22.3 ± 0.3	76.7 ± 0.7e	0.0	19.2 ± 0.1	59.3 ± 1.8h	23.3 ± 0.4k
5	20.7 ± 0.2	86.7 ± 0.7cd	0.0	16.3 ± 0.1	68.9 ± 0.2f	64.2 ± 0.5g
6	19.3 ± 0.1	83.3 ± 1.8d	70.0 ± 0.5f	14.7 ± 0.1	50.7 ± 2.7i	48.5 ± 0.4i
7	18.0 ± 0.2	86.7 ± 0.7cd	75.2 ± 0.8e	13.3 ± 0.3	64.8 ± 1.7g	60.0 ± 0.5h
8	16.7 ± 0.3	93.3 ± 0.6b	84.3 ± 0.2d	12.4 ± 0.2	60.0 ± 1.8h	30.0 ± 0.3j
9	15.9 ± 0.2	96.7 ± 0.6ab	83.3 ± 0.7d	11.4 ± 0.1	49.3 ±2.2i	30.7 ± 0.5j

*Statistical analysis performed by ANOVA, followed by LSD test at P≤0.05; **SE: Standard Eror

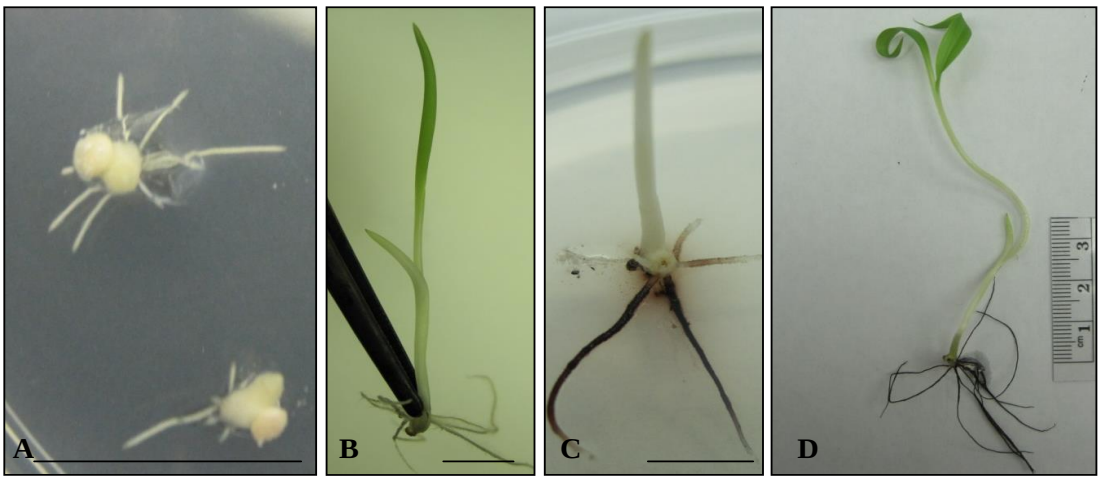

Figure 3. After liquid nitrogen treatment **A.** germinated embriyos of *M. velutina*, **B.** Three weeks later seedling of *M. velutina*, **C.** two weeks, **D.** six weeks later seedling of *M. acuminata* (Bars 1cm)

4. Conclusions and discussion

Cryopreservation, i.e. deep freezing of seeds, meristems, and other plant objects, is used for long-term storage of genetic resources "Tikhonova, 1999". The history of cryobiology, the sequelae of cold stress, the mechanisms of

cryoprotection, etc. have been described extensively " Manuil'skii, 1992; Belous and Grishchenko, 1994". In this study we aimed to long term storage of two different seeds of *Musa* species (*M. velutina* and *M. acuminata*) by dehydration-cryopreservation technique, and we obtained 84.3% germination rate from *M. velutina* and 64.2% germination rate from *M. acuminata* seeds after liquid nitrogen. We realized that another critical point for two different species were moisture content of seeds. Under the 17% moisture content was optimum for germination after the liquid nitrogen for two species (*M. velutina*, 16.7%; *M. acuminata* 16.3%).

Maturing orthodox seed experiences a number of processes that allow them to sustain profound dehydration. Accumulating soluble sugars (sucrose, raffinose, etc.) are supposed to be essential to maintaining the native biopolymer structure. Orthodox seed not only have more sugars than recalcitrant ones, they also have a higher raffinose/sucrose ratio, which prevents sucrose crystallization even at high concentrations in the embryo "Steadman et al., 1996; Yücel et al., 2008".

There are two hypotheses on the protective role of sugars during seed dehydration. One implies that oligosaccharides substitute for water in hydrogen bonding with membrane phospholipids "Crowe et al., 1988". The other considers the involvement of sugars in vitrification of the cell contents. Thus it is supposed "Buitink and Leprince, 2004" that upon dehydration the hypersaturated intracellular solution does not crystallize but converts into an extremely viscous amorphous mass, virtually precluding diffusion and metabolism. The vitreous state has been experimentally shown to stabilize proteins "Sun et al., 1998" and favor seed viability.

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