UNIVERSITI PUTRA MALAYSIA

EFFECTS OF CHEMICAL TREATMENTS ON SEED GERMINATION AND DESICCATION OF WILD BANANA (Musa acuminata Colla ssp malaccensis AND truncata) SEEDS FOR CRYOPRESERVATION

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the degree of Master of Science

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Malaysia is one of the centers of diversity for bananas and plantain. Most of the edible banana available to date originated from the wild type namely *Musa acuminata* which is non-edible due to the large number of seeds within the pulp. It contains valuable genes and should be conserved. Although there are various methods of conservation, wild bananas produce seeds freely, thus conservation based on seeds would be the most cost effective. Despite being classified as orthodox, i.e., can tolerate desiccation to low moisture content and freezing the seeds are highly dormant, resulting in very low and erratic germination. This study was conducted to determine the effect of gibberellic acid (GA_3), ethrel and acetone on wild banana seed germination. Secondly, this study determined the effect of desiccation on germination of wild banana seeds followed by the effects of desiccation and cryoprotectants on survival of wild banana embryos after cryopreservation. In the first part of the study, chemical treatments were used to break the dormancy and also to improve seed germination. Two ssp. namely *Musa acuminata*, *malaccensis* (low altitude type) and *truncata* (high altitude type) were studied. Freshly harvested wild banana seeds were pretreated with thirteen treatments namely control (no pretreatment, T1), ethrel (v/v); 0.01% (T2), 0.05% (T3), 0.1% (T4) and 0.5% (T5), GA_3 (w/v); 0.02 %, (T6), 0.04 % (T7), 0.06% (T8) and 0.1 % (T9) and acetone (v/v); 10% (T10), 20% (T11), 30% (T12) and 40% (T13). Data on percentage of germination (G), mean germination time (MGT), germination index (GI) and percentage of embryo viability using tetrazolium test were recorded. There was no significant effect among the treatments on all parameters at $P<0.05$ for *Musa acuminata* ssp *malaccensis*. Higher germination percentage (26.5%) was observed when seeds were treated with 40% acetone (T13). The shortest time (MGT) for ssp *malaccensis* to germinate, was 11.93 weeks when treated with 0.1% ethrel (T4) and the longest time (MGT) to germinate was for T13, 20.72 weeks, almost 5 months. Speed of germination (GI), was higher when treated with 0.05% ethrel (T3), 5.19.

However, there was a significant difference in the germination percentage of *Musa acuminata* ssp *truncata*, whereby seeds treated with ethrel 0.01% (T2) improved germination up to 93%, an increment of 26% compared to control. The shortest mean germination time (MGT), was 2.99 weeks when treated with 30% acetone (T12) and the longest MGT was for 0.1% ethrel (T4), 4.64 weeks. Speed of germination (GI) was higher when treated with 0.01% ethrel (T2), 99.44. In the second experiment, wild banana seeds were desiccated to understand the sensitivity of wild banana seeds to desiccation. Freshly harvested seeds of *Musa acuminata* ssp *malaccensis* and ssp *truncata* were desiccated to six target moisture contents (TMC); 40, 30, 20, 15, 10 and 5% followed by germination in sand and seed moisture content (MC), percentage of seed germination (G), mean germination time (MGT), germination index (GI) and percentage of survival for the embryo culture were recorded. The germination of *Musa acuminata ssp. truncata* seeds was relatively high, with more than 84%. However, excessive desiccation to below 10% moisture, reduced germination to 58.5%. Finally, in the third experiment, as an alternative the embryo was removed from the surrounding chalazal mass to obtain high germination. This option allowed the use of zygotic embryo for conservation of wild banana *via* cryopreservation. The zygotic embryos were excised aseptically and subjected to three cryoprotectants (MS-basal, 0.5 v/v% DMSO and 0.5 w/v% sucrose), a control without any cryoprotectant was also included in the experiment, prior to liquid nitrogen exposure. Upon thawing, the embryos were cultured onto MS medium and evaluated for survival. Pretreatment with only basal media was sufficient to enhance desiccation tolerance and survival in LN up to 80% as compared to without cryoprotectant, which had 50% survival. *Musa acuminata* ssp *malaccensis* was tolerant to desiccation but hindrance to germination prevails. This study demonstrates the germination potential and feasibility of long term preservation of *M. acuminata* ssp. *malaccensis* and *M. acuminata* ssp. *truncata* embryo by cryopreservation. In conclusion, *Musa acuminata* ssp. *malaccensis* is very dormant and chemical enhancer is not effective in overcoming their dormancy. Germination of *Musa acuminata* ssp. *truncata* was significantly high with control giving 74%, while addition of ethrel at 0.01% improved germination up to 90%. *Musa acuminata* ssp. *truncata* is tolerant to desiccation. Embryo rescue technique improved germination of *Musa acuminata* ssp. *malaccensis*. Desiccation is an important factor, which can determine the survival of banana embryos cryopreserved in liquid nitrogen. Pretreatment with only basal media can enhance desiccation tolerance and survival in liquid nitrogen up to 80%.

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KESAN RAWATAN KIMIA TERHADAP PERCAMBAHAN DAN PENGERINGAN KE ATAS BIJI BENIH PISANG LIAR (*Musa acuminata* Colla ssp *malaccensis* DAN *truncata*) UNTUK KRIOAWETAN

Oleh

ZAITIALIA BINTI MOHLISUN

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Malaysia merupakan salah satu pusat kepelbagaian pisang. Kebanyakan pisang yang boleh dimakan yang ada sehingga kini berasal daripada jenis liar iaitu *Musa acuminata* yang tidak boleh dimakan kerana bilangan biji yang banyak dalam pulpanya. Ia mengandungi gen yang sangat berharga dan harus dipelihara. Walaupun terdapat pelbagai kaedah pemuliharaan, pisang liar menghasilkan benih secara bebas. Oleh itu pemuliharaan berasaskan benih akan menjadi kaedah yang paling berkesan dari segi kos. Walaupun diklasifikasikan sebagai ortodoks, iaitu, boleh bertoleransi dengan keadaan pengeringan dan pembekuan, masalahnya biji benih didapati dorman dan percambahan tidak menentu serta sangat rendah. Kajian ini dijalankan untuk menentukan kesan asid giberelik, ethrel dan aseton terhadap percambahan biji benih pisang liar, untuk menentukan kesan pengeringan terhadap percambahan biji benih pisang liar dan untuk menilai kesan pengeringan dan kriopelindung terhadap kemandirian embrio pisang liar untuk krioawetan. Pada bahagian pertama kajian, rawatan kimia digunakan untuk memecahkan dormansi dan juga untuk meningkatkan percambahan benih. Dua jenis subspesis pisang liar iaitu *Musa acuminata* ssp. *malaccensis* (jenis tanah rendah) dan *truncata* (jenis tanah tinggi) telah dikaji. Biji benih pisang liar yang baru dituai telah dirawat dengan ethrel, asid giberelik (GA^3), dan aseton. Tiga belas rawatan digunakan dalam kajian ini terdiri daripada kawalan (tanpa prarawatan, T1), ethrel (v/v); 0.01% (T2), 0.05% (T3), 0.1% (T4) dan 0.5% (T5), GA^3 (w/v); 0.02 %, (T6), 0.04 % (T7), 0.06% (T8) dan 0.1 % (T9) dan aseton (v/v); 10% (T10), 20% (T11), 30% (T12) dan 40% (T13) dan data peratusan percambahan (G), min masa percambahan (MGT), indeks percambahan (GI) dan peratus kemandirian embrio menggunakan ujian tetrazolium telah direkodkan. Daripada pemerhatian, tiada kesan yang signifikan terhadap rawatan kimia ke atas biji benih pada semua parameter pada $P<0.05$ bagi *Musa acuminata* ssp *malaccensis*. Peratusan percambahan yang lebih tinggi (26.5%) ditunjukkan

apabila benih dirawat dengan 40% aseton (T13). Masa yang lebih singkat (MGT) untuk ssp *malaccensis* bercambah ialah 11.93 minggu apabila dirawat dengan 0.1% ethrel (T4) dan masa yang paling lama (MGT) benih untuk bercambah ialah T13, 20.72 minggu iaitu hampir 5 bulan. Kelajuan percambahan (GI), lebih tinggi apabila dirawat dengan 0.05% ethrel (T3), 5.19. Walau bagaimanapun, terdapat perbezaan yang ketara dalam peratus percambahan *Musa acuminata* ssp *truncata*, di mana apabila menggunakan ethrel 0.01% (T2) percambahan meningkat sehingga 93%, peningkatan sehingga 26% berbanding kawalan. Purata masa percambahan (MGT) yang lebih pendek iaitu 2.99 minggu apabila dirawat dengan 30% aseton (T12) dan masa paling lama untuk MGT ialah 0.1% ethrel (T4), 4.64 minggu. Kelajuan percambahan (GI) lebih tinggi apabila dirawat dengan 0.01% ethrel (T2), 99.44. Dalam eksperimen kedua, biji benih pisang liar telah dikeringkan untuk memahami kesan biji benih pisang liar terhadap pengeringan. Benih *Musa acuminata* ssp *malaccensis* dan ssp *truncata* yang baru dituai telah dikeringkan kepada enam kelembapan secara bersasar (TMC); 40, 30, 20, 15, 10 dan 5% diikuti dengan percambahan dalam pasir dan kandungan lembapan biji benih (MC), peratusan percambahan biji benih (G), min masa percambahan (MGT), indeks percambahan (GI) dan peratusan kemandirian untuk kultur embrio direkodkan. Walau bagaimanapun, percambahan *Musa acuminata* ssp. biji *truncata* agak tinggi, dengan lebih daripada 84%. Walau bagaimanapun, pengeringan yang berlebihan kepada kelembapan di bawah 10%, menurunkan percambahan kepada 58.5%. Seterusnya dalam eksperimen akhir, sebagai alternatif embrio telah dikeluarkan dari biji benih untuk mendapatkan percambahan yang tinggi. Pilihan ini membenarkan penggunaan zigotik embrio untuk pemuliharaan pisang liar melalui krioawetan. Embrio zigotik telah dipotong secara aseptik dan dirawat menggunakan tiga bahan krioawetan (0.5 v/v% DMSO, 0.5 w/v% sukrosa dan MS-basal). Kawalan tanpa sebarang rawatan juga dimasukkan dalam eksperimen, sebelum embrio dimasukan ke dalam cecair nitrogen. Selepas pencairan, embrio telah dikulturkan ke atas medium MS dan data kemandirian embrio direkod. Prarawatan dengan hanya menggunakan media basal adalah mencukupi untuk meningkatkan toleransi pengeringan dan kelangsungan hidup dalam cecair nitrogen sehingga 80% berbanding tanpa bahan krioawetan, yang hanya mampu hidup sebanyak 50%. *Musa acuminata* ssp *malaccensis* tahan terhadap pengeringan tetapi berlaku halangan dalam percambahan. Kajian ini menunjukkan potensi percambahan dan pemuliharaan untuk jangka panjang bagi embrio *M. acuminata* ssp. *malaccensis* dan *M. acuminata* ssp. *truncata* menggunakan kaedah krioawetan. Sebagai kesimpulan, *Musa acuminata* ssp. *malaccensis* adalah dorman dan rawatan kimia tidak berkesan untuk mengatasi dormansi. *Musa acuminata malaccensis* dan ssp. *truncata* tahan terhadap kesan pengeringan. Teknik menyelamat embrio meningkatkan percambahan *Musa acuminata* ssp. *malaccensis*. Pengeringan adalah faktor yang sangat penting yang boleh menentukan kebolehupayaan embrio pisang yang diawetkan dalam cecair nitrogen. Prarawatan dengan hanya media basal boleh meningkatkan toleransi pengeringan dan kemandirian embrio dalam cecair nitrogen sehingga 80% hidup.

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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%	Percentage
°C	degree Celsius
µL	micro liter
ABA	abscisic acid
ANOVA	Analysis of variance
cm	centimetre
CRD	Completely Randomized Design
df	degree of freedom
DMRT	Duncan Multiple Range Test
DMSO	dimethyl sulfoxide
g	gram
g/L	gram per litre
G	Percentage of germination
GA_3	gibberellic acid
GI	germination index
ha	hectare
ISTA	International Seed Testing Association
LN	liquid nitrogen
m	meter
M	molar
MC	moisture content
mg/L	miligram per litre
MGT	mean germination time

Min	minute
mL	millilitre
MGT	mean germination time
mm	millimeter
MS	Murashige and Skoog
NaOH	natrium hydroxide
n.s	non significant
per mL	per millilitre
p.s.i	pound-force per square inch
P-value	probability value
RH	relative humidity
SAS	Statistical Analysis Software
SE	Standard Error
ssp	subspecies
S.V	source of variation
UPM	Universiti Putra Malaysia
TMC	target moisture content
TTC	tetrazolium test
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Bananas are an important crop in the tropics. Banana is cultivated in more than 130 countries in the world with a yearly production of 129 million tons (Panis, *et al*., 2015; FAO, 2018). It is a multipurpose plant with great versatility and potential as a food security crop and as well as a plant genetic resources. In Malaysia, banana is the second largest fruit crop cultivated after durian and is available throughout the year. In the year 2020, around 17% (26,210 ha) of the total fruit hectarage was cultivated with banana contributing a total production of 313, 811 metric tonnes (DOA, 2020). However, bananas production of Malaysia reduced by 3.83 % from 325,447 tonnes in 2019 to 312,968 tonnes in 2020. Since the 13.24 % increment in 2017, bananas production dropped by 10.71 % in 2020 (Knoema, 2022).

Despite the importance of edible banana, the industry is threatened by various factors such as devastating diseases, high cost of production, lack of high yielding variety, unorganised production and as well as climate change (Mohamad Roff *et al.,* 2012; FAO, 2020; Fones *et al*., 2020). The main constraints in the production of banana in Malaysia includes the Moko disease, Panama disease, Sigatoka leaf spots and virus disease (Mohamad Roff *et al.,* 2012).

It is important to note that edible bananas originated from the diploid seeded wildtype, namely *Musa acuminata* (AA) and *Musa balbisiana* (BB). *Musa acuminata* Colla is highly variable in Malaysia and are very important donors of valuable genes for the improvement of cultivated banana species for food security. The wild bananas have been reported to be tolerant to fusarium wilt, a soil-borne disease (Panis *et al.,* 2020). Therefore, the wild bananas are crucial germplasm and must be conserved. Hence breeding for new materials which are resistant to environmental factors and diseases are an ongoing process.

In the case of wild bananas, seed storage would be the most practical in terms of safety and is a relatively inexpensive method to conserve plant genetic resources. The number of seeds in a fruit is large; thousands of seeds can be obtained from a bunch. However, a major constraint with storage of banana seeds for conservation appears to be its ability to germinate. Little is known about the factors that affect seed germination in banana, except that germination is extremely variable and relatively difficult to obtain under natural conditions (Asif *et al*., 2001). Some authors have indicated that mature wild banana seeds are highly dormant (Purseglove, 1975; Chin, 1996) and the degree of dormancy varies between species and type of banana.

In order to establish a suitable long term conservation for wild banana seeds, the dormancy issue has to be addressed. Seed dormancy can be defined as a condition in which seeds are prevented from germinating even under the favourable environmental conditions for germination including, temperature, water, light, gas, seed coats, and other mechanical restrictions (Hilhorst, 1995; Bewley, 1997). According to Baskin and Baskin, (2004) dormancy maybe due to physical, morphological and physiological factors or combination of these factors. Some members in the family Musaceae have shown a physical seed dormancy (Baskin and Baskin, 1998). This is probably due to the hard and rigid nature of the seed coat found in banana seed (Puteh *et al*., 2011). Hard seed coat or physical dormancy is associated with a barrier to water imbibition in some seeds which subsequently leads to a reduced germination or complete germination failure (Baskin *et al*., 2000).

Wild banana seeds show varying degrees of dormancy, and they respond differently to various dormancy breaking treatments including chemical treatments. Chemical treatment breaks the dormancy by dissolving the water impermeable seed coat using any organic or inorganic compound to allow imbibition of water, hence a better germination (Hopkinson and Paton, 1993). Though many studies on the effects of these chemical treatments have been reported to effectively trigger germination on other species, yet very few papers of the use of chemical treatments showed a positive effect in treating banana seeds. According to Nadjafia *et al*., (2006), plant hormones such as gibberellic acid (GA_3) is a known compound proposed to control primary dormancy by inducing germination while ethylene (C_2H_4) regulates germination and dormancy via a complex hormonal signalling work (Arc *et al*., 2013). Another chemical, acetone, can carry chemicals through the seed coats to reach the embryo to promote germination (Tao and Khan, 1974; Amritphale *et al*., 1993). These three compounds have yet to be tested on wild banana seeds.

Banana seeds have been classified as orthodox, they can be dried and stored for long periods at reduced temperatures (Roberts, 1973; Ellis *et al*., 1985; INIBAP, 1997) with minimal loss in viability. In seed conservation, desiccation is very important. Quality and desiccation tolerance information is important, not only for conservation purposes, but also for medium and long-term propagation strategies (Calderon-Hernandez and Perez-Martinez, 2018). However, in some cases desiccation can induce secondary dormancy or the existing dormancy at harvest prevails after desiccation and storage, preventing germination. Consequently, failing this method of conservation, which is probably the scenario for bananas. Hence the influence of desiccation on storage and viability and germination of wild banana seeds is necessary.

In the event where viable seeds cannot be triggered to germinate, other alternatives have to be considered. According to Chin (1995), despite the low and erratic germination, when the zygotic embryo of stored seeds (more than one year) is removed from the surrounding chalazal mass both for fresh and

desiccated seeds, there was a high germination. Thus, the use of zygotic embryo may be an option for conserving the wild banana via cryopreservation. Cryopreservation is a long term conservation method of biological material at ultra-low temperature of liquid nitrogen. Cryopreservation is the only viable alternative for long-term storage of germplasm under conditions favouring high genetic stability and minimal maintenance requirements (Engelmann, 1997). According to Vineesh *et al*., (2015), success in cryopreservation can only be achieved if intracellular ice crystal formation is blocked during freezing treatment, otherwise will cause damage to membrane structure resulting in the death of the cell. To avoid this, the optimum moisture content in the cell is critical for achieving good results and can be only achieved by proper desiccation process.

In view of the above facts, this study aimed to develop a conservation protocol for the wild banana, *Musa acuminata* spp. *malaccensis* and *Musa acuminata* spp. *truncata*. The objectives of the study are as below:

1. To determine the effects of gibberellic acid, ethrel and acetone on germination of wild banana seed.
2. To determine the effect of desiccation on germination of wild banana seed.
3. To evaluate the effects of desiccation and cryoprotectants on survival of wild banana embryos after cryopreservation.

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