IMPROVING GERMINATION OF BANANA ZYGOTIC EMBRYOS

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A THESIS SUBMITTED TO THE DIRECTORATE OF RESEARCH AND GRADUATE TRAINING IN PARTIAL FULFILLMENT OF REQUIREMENTS FOR THE AWARD OF A MASTER OF SCIENCE DEGREE IN CROP SCIENCE OF MAKERERE UNIVERSITY

JULY 2020

DECLARATION

This thesis titled ***improving germination of banana zygotic embryos*** is my original work and has never been submitted for a degree in any university or institution for any academic award whatsoever.

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DEDICATION

To the Almighty God who has enabled me through and to my family for their encouragement and support.

ACKNOWLEDGMENT

With sincere gratitude, I take the pleasure to thank IITA for the funds it availed to me both for tuition and research work, and for letting me use the tissue culture laboratory facilities for the entire study. I also appreciate Makerere University for the opportunity to study. Special thanks to IITA banana breeding leader Prof. Rony Swennen and banana breeder Dr. Allan Brown for accepting me to study as I work. My heartfelt appreciation goes to my research supervisors, Dr. Brigitte Uwimana and Dr. Peter Wasswa, for their incredible support and help throughout my MSc. program. I express great thanks to Dr. Amar Delphine for her expert advice and support in all steps of the research. I am thankful to all the above-mentioned names for their patient guidance, invaluable constructive criticism, and friendly advice during this study. I am sincerely grateful to them for sharing their wisdom and illuminating views on a number of issues related to the research. Further gratitude goes to Ms. Justine Tumushabe and Mr. Francis Kawalya for their help in the laboratory, and the field team – Mr. Godfrey Kasirye, Mr. Elunasani Semitala, Mr. Fred Ndabahire, Mr. Dennis Nteza and Mr. Robert Senteza – for their great support in pollination. Lastly, I am very much indebted to my family for being patient during my educational career.

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LIST OF ABBREVIATIONS

ABA	:	Abscisic acid
BA	:	Benzyladenine
BAP	:	6-Benzylaminopurine
BBrMV	:	Banana bract mosaic virus
BBTV	:	Banana bunchy top virus
BSV	:	Banana streak virus
BXW	:	Banana Xanthomonas wilt
$CaCl_2$ $2H_2O$	:	Calcium chloride dihydrate
$CoCl_2$ $6H_2O$	:	Cobalt chloride
$CuSO_4 \bullet 5H_2O$	:	Copper II sulfate Pentahydrate
EAHB	:	East African Highland Bananas
ER	:	Emershad and Ramming
FAO	:	Food and Agriculture Organization of the United Nations
$FeSO_4$ $7H_2O$	:	Ferrous sulfate
GA_3	:	Gibberellic acid
H_3BO_3	:	Boric acid
HCl	:	Hydrochloric acid
IAA	:	Indole-3-acetic acid
IBA	:	Indole butyric acid
IITA	:	International Institute of Tropical Agriculture
KH_2PO_4	:	Monopotassium phosphate
KI	:	Potassium iodide
KNO_3	:	Potassium nitrate
MD	:	Morphological dormancy
$MgSO_4$ $7H_2O$	:	Magnesium sulfate heptahydrate
$MnSO_4$ $4H_2O$	:	Manganese(II) sulfate
MS	:	Murashige and Skoog
NAA	:	Naphthalene acetic acid
Na_2-EDTA	:	Ethylenediaminetetraacetic acid ferric sodium
Na_2MoO_4 $2H_2O$	:	Sodium molybdate
NARO	:	National Agricultural Research Organisation
NH_4NO_3	:	Ammonium nitrate
NN	:	Nitsch and Nitsch
PD	:	Physiological dormancy
TTC	:	2,3,5-triphenyl-2H-tetrazolium chloride
$ZnSO_4$ $7H_2O$	:	Zinc sulfate

ABSTRACT

Germination of seed is critical for successful banana improvement. However, direct sowing results in poor or no germination, especially for seeds produced from edible triploid bananas. To improve germination, embryos are extracted from seeds and cultured *in vitro*, but with this technique, germination remains low. This study determined the effective soaking time and optimal hormonal concentration of 6-Benzylaminopurine (BAP) and Gibberellic acid (GA_3) triggering embryo germination *in vitro*. The study utilized seeds obtained from 4*x* – 2*x* (1438K-1 – ITC0250 - malaccensis and 1201K-1 – 7197-2) and 2*x* – 2*x* (selfed ITC0249 - Calcutta 4 and selfed ITC1348 - Pisang Serun 404) crosses. Prior to the experiment, seed viability was tested using 2,3,5- triphenyl tetrazolium chloride (TTC). Seeds were soaked in water for 0, 3, 5, 7, 9 days to determine the optimum soaking time. Thereafter, embryos were extracted and cultured on Murashige and Skoog media with 0.0, 0.5, and 1.0mg/l concentrations of BAP and GA_3. Soaking seeds for 3 days significantly increased embryo germination success by 16.2% than all other tests. The addition of BAP and GA_3 hormones into culture medium did not improve embryo growth but positively affected growth parameters. In conclusion, to improve banana embryo germination, the seeds should be soaked for 3 days before embryo extraction, and 1mg/l of BAP should be added to the embryo germination medium, not to improve germination, but to improve the growth of the plantlets after germination.

Keyword: Embryos germination, growth hormones, soaking, seed dormancy, MS medium.

CHAPTER ONE

INTRODUCTION

1.1. General background

Bananas (*Musa* spp.) belong to the order Zingiberales and the family *Musaceae.* The crop's primary center of origin and diversity is in South East Asia (Janssens *et al*., 2016) and has been widely spread to other places by explorers and commercial planters. Bananas are important food and cash crop with a worldwide production of 113.92 million tons (FAOSTAT, 2017). Banana is among the 10 most important crops in the world (Ortiz and Swennen, 2014) and the sixth most important staple crop (FAOSTAT, 2014). About 85% of the cultivated bananas in the world are produced by smallholder farmers for nutrition security amongst other uses (Alakonya *et al.,* 2018). In Uganda, farmers grow a number of banana varieties adapted to specific conditions of production as well as to the varied uses and tastes of the local consumers. The most cultivated banana in Uganda is the cooking type belonging to the East African highland banana (EAHB) subgroup, locally referred to as 'matooke' (Kitavi *et al*., 2016). Other types of cultivated bananas include the sweet banana (dessert type), with the common cultivars being Sukari Ndiizi and Bogoya, and plantains and the beer type (Mbidde) used to produce alcoholic and non-alcoholic beverages (Karamura, 2012).

Cultivated bananas are derived from two *Musa* wild species, namely *Musa acuminata*, or A genome, and *Musa balbisiana*, or the B genome (Ortiz, 2013). Bananas have a diverse combination of ploidy levels and genomic constitutions. In terms of ploidy and genome combinations, there are diploid bananas (AA, AB, BB), triploids (AAA, AAB, ABB) and

tetraploids (AAAA, AAAB, AABB, ABBB). Most cultivated banana varieties are parthenocarpic triploids characterized by low fertility or complete sterility (Batte et al., 2019).

1.2. Constraints to banana production

Banana production in the East and Central Africa region is constrained by several socio-economic, abiotic, and biotic factors (Ssekiwoko *et al*., 2006; Asten *et al*., 2011). Some pests and diseases attack and devastate bananas, greatly reducing yields at harvest, and that lowers the economic value of the crop and frustrates farmers (Swennen *et al*., 2013). Among the pests, nematodes especially *Radopholus similis* and banana weevil *(Cosmopolites sordidus)* cause toppling and snapping of bananas, ultimately reducing plantation lifespan with an estimated mean of yield loss of 40% (Gold *et al*., 2004). Concerning diseases, banana pathogens belong to three groups: fungi, viruses, and bacteria. Among the fungal diseases of banana include black leaf streak caused by *Pseudocercospora (Mycosphaerella) fijiensis* (De Bellaire *et al*., 2010) and yellow Sigatoka caused by *Mycosphaerella musicola* (Nyine and Pillay, 2011). In addition, *Fusarium* wilt affects mostly dessert bananas such as Gros Michel, Sukali Ndiizi, and some ABB triploids, and it is caused by *Fusarium oxysporum* f. sp. *cubense* (Arinaitwe *et al*., 2019). With regard to bacterial diseases, Banana *Xanthomonas* wilt (BXW) caused by *Xanthomonas campestris* pv. *musacearum* restricted to a few countries in Africa, can cause up to 100% of yield loss (Kikulwe *et al*., 2019). Concerning viruses, the most economically important diseases include banana bunchy top virus (BBTV), banana streak virus (BSV), and banana bract mosaic virus (BBrMV) (Tripathi, *et al*., 2016). The integrated BSV into *Musa balbisiana* genome poses a major concern for the international exchange of banana germplasm of the B genome (Kumar *et al*., 2015). In Uganda, the pests (banana weevil and nematodes) as well as diseases (BXW and *Fusarium* wilt) are widespread among smallholder farmers partly due to the clonal propagation

nature of bananas using already infected or infested young suckers culled from the old plantations to establish new fields.

The management of these pests and diseases involves a) use of clean planting material; b) paring and hot water treatment (Tinzaara et al., 2009); c) crop sanitation and trapping (Gold et al., 2001); d) eradication or destruction of diseased plants; e) male bud removal immediately after the last female hand emerges to minimize the spread of the BXW; f) removal of the rhizome; and g) use of resistant cultivars (Kumar et al., 2015).

Breeding for resistance is the most ideal method for managing pests and diseases. Sources of resistance to various pests and diseases have been identified in wild diploid species (Ssebuliba *et al*., 2008). The process involves crossing diverse genotypes to generate new hybrids (Figure 1). The developed hybrids are evaluated for their agronomic performance, resistance to pests and diseases, and table quality. Those that pass those multiple evaluation levels are released as varieties. However, breeding of banana has a number of challenges including polyploidy (Figure 1), parthenocarpic fruit development through seeds, low female fertility, prolonged lifecycle, narrow range of genetic variability, limited seed set and poor embryo germination (Ortiz and Swennen 2014). Embryo culture has been utilized to improve germination but germination rates have remained low, ranging from 7.4% in tetraploid by diploid ($4x - 2x$) to approximately 22.8% in inter-diploid crosses (Batte *et al.,* 2019). The low germination rate results in low hybrid recovery. Thus, embryo/seed germination remains a bottleneck in the banana breeding pipeline.

The success of any breeding programme for banana improvement depends on the suitability and compatibility of the parents used in pollination, the production of large numbers

of viable seeds, and the ability of embryos from these seeds to regenerate into hybrid plantlets (Uma *et al*., 2011). The banana programme of the International Institute of Tropical Agriculture (IITA), together with the National Agricultural Research Organization (NARO) in Kawanda, is currently developing superior banana varieties for evaluation and release to farmers (Batte *et al*., 2019). The programme focuses on incorporating the useful traits (mostly resistance to pests and diseases) from wild and cultivated diploids into existing triploid varieties. New hybrids are developed from triploid East African Highland Banana (EAHB) through crossing triploid landraces with wild diploids to produce tetraploids. The resultant tetraploids are selected and crossed with improved diploids to obtain the final, sterile secondary triploid product (Figure 1, Brown *et al.,* 2017). The improved diploids used in IITA-NARO breeding scheme resulted from intercrossing of superior diploids until a targeted improved diploid was produced with useful traits originating from the wild relatives. These improved diploids are being used for the improvement of many different types of bananas (Escalant and Jain, 2004).

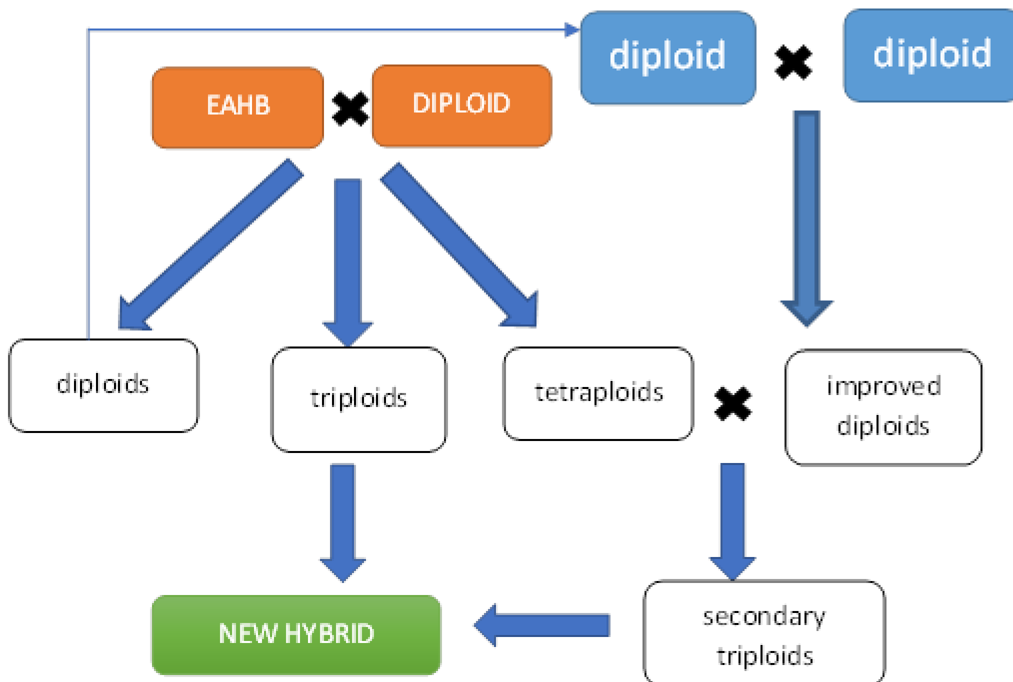

Figure 1: Banana breeding process Source: **Brown *et al*., 2017**

1.3. Problem statement

In banana, seed soaking in distilled water prior to embryo excision has been recommended as it was observed to improve the germination of embryos from *Musa balbisiana* (Afele and De Langhe, 1991). *M. balbisiana* produces highly viable seeds that germinate easily even when directly sown in the soil. However, limited information is available regarding embryo germination with the soaking technique, especially with seeds generated from tetraploid by diploid crosses. Plant hormones such as gibberellic acid (GA_3) have been reported to improve seed germination in tomato (Balaguera.Lopez, 2009), guayule (Dissanayake *et al.,* 2010) and rye (Abdullah and Abdulrahman, 2017). The addition of GA_3 to Murashige and Skoog (MS) media improved the germination of citrus embryos (Kurt and Ulger, 2014), but has not been tested yet in bananas. Furthermore, cytokinins such as 6-Benzylaminopurine (BAP) are known to promote cell division in actively growing plant tissues (Pereira et al., 2018). The use of BAP to improve the germination of *Musa acuminata* embryos has been studied but results showed limited improvement (Vineesh *et al.,* 2015). However, the effect of combining GA_3 and BAP on embryo germination has not been studied. Therefore, this research focused on understanding the effect of seed soaking and GA_3/BAP concentration on banana embryo germination rates, with the aim of improving banana breeding efficiency.

1.4 Justification of the study

Cultivated banana varieties have low genetic variation, hence increases their susceptibility to pests and diseases, resulting in reduced production. Breeders are dedicated to producing new varieties that are resistant to pests and diseases. However, efforts of breeders can be realized with greater efficiency after increasing seed set and embryo germination rates (Burgos-Hernández, et al., 2014). Hybrid embryos give rise to genetic diversity/variation, which is required in banana breeding programs to select new varieties. The rate of zygotic embryo germination is lower than

that of somatic embryos obtained from banana calli (Sadik, 2014), yet somatic embryos do not give rise to genetic diversity. Therefore, optimising soaking of seeds and supplementation of MS medium with hormones will improve banana seed embryo germination, resulting in an increased number of hybrids from which selection can be made.

1.5. Aim of the study

To contribute to improved banana breeding efficiency by enhancing the germination rate of banana zygotic embryos.

1.5.1. Specific objectives:

i. To determine the optimum soaking period of the banana seeds in distilled water prior to embryo excision for improved germination rate of zygotic embryos of banana seeds
ii. To establish the optimal hormonal concentration of BAP and GA_3 for the germination and growth parameters of banana zygotic embryos

1.6. Hypothesis

This study was based on the hypotheses that:

i. There is no difference in the germination rate of zygotic embryos from soaked seeds.
ii. The addition of GA_3 or BAP or both combined in the culture medium does not improve banana zygotic embryo germination.
iii. The addition of GA_3 or BAP or both combined in the banana culture medium does not enhance embryo growth parameters.

CHAPTER TWO

LITERATURE REVIEW

2.1. Origin and classification of bananas

Bananas are perennial monocotyledonous herbs grown in tropical and sub-tropical zones. The plants belong to the order Zingiberales, family Musaceae. The edible bananas originated from several hybridizations of two seeded progenitors *Musa acuminata* and *Musa balbsiana* (Bakry *et al*., 2009). The *M. acuminata* and its hybrids characterized with parthenocarpy and sterility (Ortiz, 2013) originated from Malaysia and Indonesia (Asif *et al*., 2001; Daniells *et al*., 2001), whereas *M. balbisiana* and its hybrids characterized with hardness, starchiness, drought tolerance and disease resistance (Pillay *et al*., 2002) originated from India (Robinson, 1996).

Bananas are classified according to ploidy – i.e. depending on the number of chromosomes in somatic cells – into diploids (2*x*), triploids (3*x*), and tetraploids (4*x*). Recently, using genome constitutions, bananas are classified into genomic groups designated by the letters A and B to represent *M. acuminata* (AA) and *M. balbisiana* (BB) (Sipen *et al*., 2011). In Uganda, bananas are classified based on the use of the fruits (Karamura, 1998, Gold *et al*., 2002) into cooking type (AAA-EAHB), roasting type (AAB - plantains), dessert types such as Sukali Ndiizi (AAB) and Bogoya (AAA), and the beer types, for example, Kayinja (ABB) and Kisubi (AB), and 'Mbidde' (AAA-EAHB).

EAHB are triploid bananas comprising cooking and beer types. Based on their morphology, the cultivars are subdivided into five clone sets, namely Nfuuka, Musakala, Nakabululu, Nakitembe and Mbidde (Karamura, 1998; Karamura and Pickersgill, 1999). EAHB

are seedless bananas obtained from ancestral bananas in Malaysia and introduced in East Africa (Kitavi *et al*., 2016). In Eastern Africa, Uganda is the main producer of EAHB, locally known as "tooke" (singular) or "matooke" (plural) in Luganda. They are the most preferred type of bananas, because they are harvested green and steamed to make a dish of the same name "matooke", with an appropriate pleasant taste, good texture for the desired purpose and yellowish color. However, they are susceptible to black Sigatoka, BXW, weevils and nematodes, though resistant to banana *Fusarium* wilt (*Fusarium oxysporum* f. *cubense* race 1). As such, new EAHB varieties have been bred through cross-hybridization to produce pest and disease-resistant hybrids that grow well in a range of conditions and meeting consumers' taste. Hence, numerous banana varieties with different ploidy levels and genome combinations have been produced (Sipen *et al*., 2011).

2.2 Banana improvement

Banana breeding involves improving the agronomic characteristics of the diploid male parents through intercrossing diploids until an elite improved diploid is selected. On the other hand, cultivated varieties (EAHB) are crossed with diploids to produce tetraploids which are further crossed with improved diploids to produce triploids (Figure 1; Brown *et al*., 2017). The whole process involves the production of seeds that are germinated to obtain hybrids.

2.2.1 Seed germination to produce hybrids as a way of improving banana

A seed is a small embryonic plant covered by a seed coat comprising some stored food. The seed has three primary parts: the seed coat consisting of multilayer integuments, the endosperm acting as a source of stored food, and the embryo, which is the earliest form of a plant (Steinbrecher and Leubner-Metzoar, 2017). Meanwhile, germination can be described as the development and emergence of essential structures including the radicle and plumula. In banana,

the embryo is small and located under the operculum (Graven et al., 1996). During germination, the micropylar plug of an embryo is displaced by the elongating radicle-hypocotyl axis (Figure 2; McGahan, 1961).

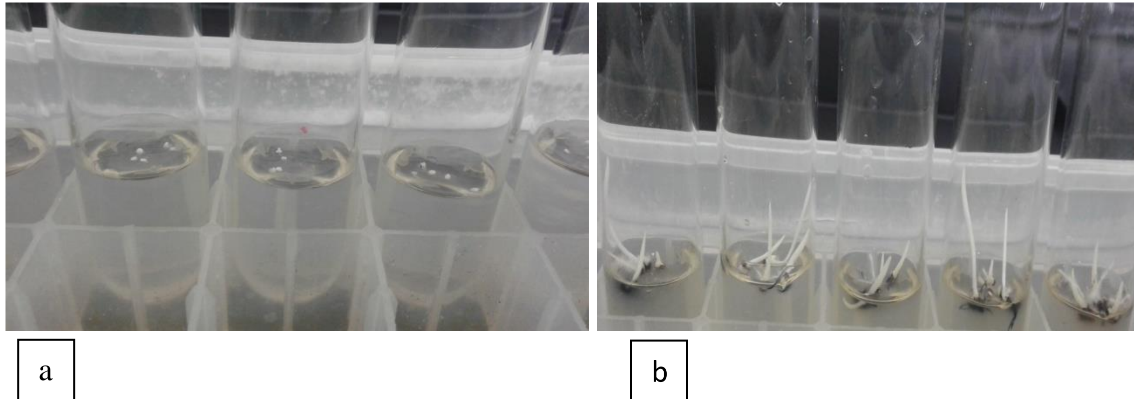

Figure 2: The banana embryos on media (a) and the germinating embryos (b)

Source: **Luyiga Jane, IITA, 2019**

Banana seeds from wild diploids, which are highly viable, take at least four months to germinate when directly sown in the soil (Burgos-Hernández, 2014). The maximum germination success reported was over 50% in *M. balbisiana* under optimum moisture, temperature, and oxygen (Afele and De Langhe, 1991). The low germination rates of banana seeds were attributed to the hard seed coat (Debeaujon *et al*., 2010) which contributes to seed dormancy as an adaptation to the harsh tropical environment. This led to the development of embryo culture techniques (Sharma *et al*., 1996). The immature embryos are excised from the ovary and cultured on artificial nutrient medium such as MS (Murashige and Skoog, 1962). Afele and De Langhe (1991) reported 94 % germination success in 5 days of soaking whereas without soaking, only 56% germination was obtained when mature *M. balbisiana* embryos were excised and cultured on MS medium. However, banana embryos may fail to germinate even if they are extracted at

full maturity (Chin, 1996). The factors affecting seed germination are categorized into external mainly environmental conditions and internal majorly dormancy.

2.2.1.1 External factors affecting banana seed germination

External factors are mainly environmental-related factors, including moisture content, which hydrates the vital activities (metabolism) of the seed. Water also softens the seed coat increasing permeability and thus facilitating imbibition and rapture of the seed coat and converting the insoluble food into soluble form for translocation to the embryo. Oxygen is also necessary for respiration and growth. The vital activities of protoplasm are also affected by specific temperatures. Germination can take place over a wide range of temperatures, with the optimum temperature for banana seeds ranging from 25°C to 30°C (Uma *et al*., 2011; Dayarani *et al*., 2014).

2.2.1.2 Internal factors affecting banana seed germination

Internal factors affecting germination are related to the seed mechanisms that may enhance seed dormancy. Seed dormancy is the failure of seeds to germinate under favorable environmental conditions (Bewley, 1997; Finch Savage and Leubner-Metzger, 2006) or status of inactivity of seeds to overcome unfavorable conditions (Finkelstein *et al*., 2008). Therefore, during inactivity or dormancy period, the seeds remain viable. As such, seeds produced by mature plants should pass through a period of inactivity prior to germination (Langens-Gerrits *et al*., 2003). Dormancy can be categorized as seed coat based dormancy and embryo-based dormancy. Seed coat-based dormancy is further divided into physical, mechanical, and chemical dormancy. Physical dormancy is a result of an impermeable layer formed during maturation and drying of the seed or fruit (Offord and Meagher, 2009). The impermeable layer prevents water uptake and gas exchange by the seed, leading to reduced germination or complete germination

failure (Bentsink and Koornneef, 2008; Baskin *et al*., 2000). In mechanical dormancy, the seed coat is too hard so it is cracked to allow moisture or water to help in embryo expansion during germination. In the chemical dormancy, the embryo is surrounded by cells that seem to block water uptake during germination (Finch-Savage and Leubner-Metzger, 2006).

Embryo-based dormancy is also divided into morphological dormancy (MD) and physiological dormancy (PD) (Finch-Savage and Footitt, 2017). Under morphological dormancy, the seeds have underdeveloped but contain differentiated embryos at fruit ripening. In this case, the embryo remains dormant until maturation and it germinates. Physiological dormancy is further subdivided into three levels: (i) deep PD where the excised embryos fail to germinate. However, when germination occurs, the subsequent seedlings are abnormal in growth or appearance (e.g. *Acer platanoides)*, (ii) intermediate PD where the excised embryos germinate and produce normal seedlings e.g. banana embryos. Intermediate PD can be broken by hormones e.g. Gibberellic acid (GA_3) and scarification. Indeed, banana seeds germinate immediately after extraction from the ripe fruit (Pillay and Tenkouano, 2011) but may experience secondary dormancy due to drying (Chin, 1996). However, primary dormancy is induced by the presence of abscisic acid (ABA) during seed development (Bewley, 1997). In bananas, there is a need to know the viability of the embryo before applying methods to break dormancy.

2.3 Determining seed viability

Seed viability is the ability of the embryo to germinate. The status of seed viability is governed by environmental conditions e.g. heat, drought, and the seed age. Hence the duration of seed viability (longevity) is dependent on the genotype and environment. Seed vigor is the result of a combination of these properties which determine the level of activity and the performance of the seed during germination and seedling emergence. The process, called physiological aging (or

deterioration), starts before harvest and continues during harvest, processing, and storage. Physiological aging progressively reduces performance capabilities, due to changes in cell membrane integrity, enzyme activity, and protein synthesis. The endpoint of this deterioration is ultimate seed death (i.e. complete loss of germination). However, seeds lose vigor before they lose the ability to germinate. That is why seed lots that have similar high germination values can differ in their physiological age (the extent of deterioration) and so differ in seed vigor and therefore the ability to perform (Shaban, 2013).

Viability is important for embryo quality, although not all viable embryos germinate. Viability seed testing can be determined using a) the Rolled Paper Towel Test which involves placing seeds in a row on a paper towel, which is subsequently rolled, moistened, inserted into a tube and incubated at the specific temperature for a defined period; b) the Excised Embryo Test which involves excising and removing hard water-impermeable seed coat and other fruit parts to expose the embryo, which is subsequently cultured on a specific media. This procedure requires good sanitary conditions since the exposed embryo is very susceptible to microbial attack. In dicotyledonous plants, seeds are viable if the cotyledons turn green and spread apart. c) The Chemical Test which involves the use of Tetrazolium 2,3,5-tri-phenyltetrazolium chloride (TTC) to stain living tissues. TTC is a clear-water soluble compound that can differentiate between viable and non-viable seeds. Highly viable embryos will stain red, while less viable embryos will stain pink, and dead or non-viable embryos will not react with the solution (Marcos-Filho, 2015). The reaction is based on the activity of dehydrogenase enzymes that reduce the TTC in the living tissues to the red compound called 1,3,5-triphenylformazan, indicating respiratory activity (França Neto, 1999). This reduction occurs as a consequence of hydrogen ions donated to the TTC upon dehydrogenase activity in metabolically active tissues, such as in the seed embryo

(Junillon *et al*., 2014). Tetrazolium has been used to test seed viability of castor beans (Gaspar-Oliveira *et al*., 2009), barley (Grzybowski *et al*., 2012), sorghum (Carvalho *et al*., 2014), triticale (Souza *et al*., 2010), Barbados nut (Pinto *et al*., 2009), wheat (Carvalho *et al*., 2013), sunflower (Silva *et al*., 2013), crambe (Rezende *et al*., 2015) and forage turnip (Nery *et al*., 2015). It is a useful technique for assessing the potential viability of banana seed embryos (Burges-Hernandez *et al*., 2014) and many other plant species (Dissanayake *et al*., 2010). Moreover, it is an efficient viability test for processing, handling, storing and marketing large quantities of seed in a short time, testing dormant seed lots, and assigning vigor rating of seed lots. It is noteworthy that viability test does not show the degree of dormancy but the number of embryos that are likely to germinate. Since not all viable embryos are capable of germinating, methods of breaking dormancy should be considered.

2.4 Methods of breaking dormancy

There are several ways of breaking dormancy depending on the cause and these include stratification, light, and scarification. Stratification involves exposing the seeds to a low temperature to break their dormancy. In this dormancy breaking process, the seeds are allowed to imbibe water first followed by exposure to low temperatures. When exposed to cold temperatures under the stratification process, (Sharma, 1996). In the laboratory, stratification can be achieved by exposing seeds to cold temperatures in the fridge, simulating a short winter. According to Dayarani *et al*., (2014), embryos are best kept between 3 and 5ºC and then grown at 28ºC ± 2. However, other species need higher temperatures to break dormancy.

Some light-sensitive seeds require a specific wavelength of light to induce germination (Dissanayake *et al*., 2010). Tobacco, guayule, and tomato seeds are examples of light-sensitive seeds. The seeds should be exposed to white light to break down germination inhibitors and

promote the germination process. Seeds respond to light only after imbibition, but light becomes ineffective under dry conditions (Costa, 2016). Other seeds from wild flowers require exposure to very low light intensity for a short duration of 1-2 minutes to overcome dormancy. In other seeds, the red part of the white light of wave length 660 µm is very effective for germination (Dissanayake *et al*., 2010), but with a higher wavelength of 730 µm inhibits germination.

Seeds with a hard seed coat are treated by scarification either by mechanical or chemical means. The germination inhibitors can be absent in seed but seeds still fail to germinate due to a hard seed coat that is impermeable to water e.g. Givotia seeds (Jetti *et al*., 2017). In such cases, mechanical scarification is done using: (i) punctured or mechanically damaging the seed with a knife or sandpaper to allow water penetration, and (ii) aseptically removing seed coat and allowing the embryo to germinate under *in vitro* conditions, a process called embryo culture which is the case in banana (Bakry, 2008). In the chemical scarification, the seeds are treated with sulphuric acid for a very short (1 – 5 min) so that the embryo is not killed (Aliero, 2004). The seed coat softens hence, germination takes place e.g. sweet potato seeds (Nair *et al*., 2017). This imitates the soil, where the microorganisms act on the hard seed coat making it soft and thus promoting germination.

Soaking seeds is another chemical scarification method where water is used to soften the hard seed coat and allows moisture inside to enhance seed germination (Afele and De Langhe, 1991; Nadjafi *et al*., 2006; Arowoseghe, 2016). Water also washes out germination inhibitors present in the seed, thus promoting germination (Afele and De Langhe, 1991). In addition, phytohormones such as gibberellic acid and kinetin can replace the red light requirements and promote germination especially in lettuce seed (Mousavi *et al*., 2011). In the presence of hormones, seeds can germinate in total darkness. Seeds containing an inhibitory hormone, such

as abscisic acid is suppressed by GA_3 (Miransari and Smith, 2014). GA_3 is a promoter while ABA is an inhibitor hormone. Seeds can be soaked in water containing GA_3 which replaces the chilling requirements to increase the germination rate.

Negative photoblastic seeds can only be stimulated to germinate when placed in complete darkness (Uma *et al*., 2011). The red-far-red reversibility and phytochrome cannot play any role in breaking dormancy in such seeds. Double dormancy is overcome by several methods, for example, the seeds are stratified and then exposed to light. Some seeds are soaked in water and thereafter put in cold temperatures.

2.5 Methods of improving banana seed germination

Improving banana seed germination can be achieved through water soaking, use of hormones and embryo culture.

2.5.1 Water soaking

The first step in germination is the absorption of water into the seed, resulting in the expansion and elongation of the seed embryo (Miransari, 2014). Germination starts with water imbibition followed by physiological changes in the seed and is completed with the appearance of the radicle (Nonogaki *et al.,* 2010). Therefore, water imbibition has positive effects on removing dormancy by washing away ABA and other compounds that have negative effects on germination (Mousavi, 2011).

The seed coat is impermeable to water and this is a limiting factor in germination. Water helps to soften the seed coat and boosts the moisture content around the seed which signals to the seed that it is now safe to grow. When okra seeds imbibe water, a chain of reactions is triggered (metabolic reactions) resulting in seedling development (Musara *et al*., 2015). Soaking seeds in

water helps to reduce the time required for germination and improves germination percentage. Some types of seeds actually contain germination inhibitors such as ABA that are designed to prevent a seed from germinating inside the fruit in unfavorable environmental conditions (e.g. drought). Such inhibitors are leached out before a seed can germinate especially when seeds are soaked in water which helps to speed up the process.

Water enters the seed through different sites depending on the species. In cowpea, water enters the seed though the hilum (Hu *et al.,* 2009), while in banana, it is reported that the operculum blocks water from entering into the embryo (Graven *et al.,* 1996). The more water the seed takes in during soaking, the higher could be the degree of its physical dormancy (Baskin and Baskin, 2004). Soaking seeds in water has been reported to reduce germination time and improve germination percentage through leaching germination inhibitors (Afele and De Langhe, 1991).

Several studies reported that soaking seeds in water for different crops enhanced germination, with great success in banana (Afele and De Langhe, 1991; Shareef *et al*., 2016), litchi (Zhang *et al*., 2015), okra (Musara *et al*., 2015), and tomato (Sabongari and Aliero, 2004). However, limited knowledge is known about the relationship between water uptake and testa or associated structures surrounding the hilum region (Puteh *et al*., 2011).

2.5.2 Use of hormones

Hormones or growth regulators are signal molecules produced within plants in extremely low concentrations required to trigger growth. The plant regulators belong to four broad classes: auxins, cytokinins, gibberellins, and abscisic acid (Fathi and Jahani, 2012). The auxins including indole-3-acetic acid (IAA), indole butyric acid (IBA), 2,4- dichlorophenoxyl acetic acid (2,4D)

and naphthalene acetic acid (NAA) are used in embryo culture media (Pinto *et al.*, 2002) to stimulate callus production and cell growth, initiate shoot and root development, induce somatic embryogenesis and stimulate growth from shoot apices. The cytokinins consisting of 6-benzylaminopurine or benzyladenine (BAP or BA) e.g. gibberellic acid and abscisic acid (ABA) stimulate cell division, induce shoot formation, and axillary shoot proliferation but retard root formation. Therefore, abscisic acid is added to the media to promote distinct developmental pathways such as somatic embryogenesis as well as stimulating callus growth, enhancing shoot or bud proliferation, but inhibits cell division. Meanwhile, gibberellic acid (GA_3) is added to the media to promote the growth of low-density cell cultures, enhance callus growth and elongate dwarfed or stunted plantlets.

Since seed dormancy is regulated by the balance between germination promoters and inhibitors (Dissanayake *et al.* 2010) application of exogenous growth regulators alters the balance to induce germination. Indeed, the application of exogenous GA_3 reverses the effect of ABA (Bewley, 1997; Miransari and Smith, 2009). Apart from breaking dormancy, GA_3 also promotes germination, inter-nodal length, hypocotyl growth, and cell division in the cambial zone. Gibberellic acid stimulates hydrolytic enzymes needed for degradation of the cells surrounding the radicle and thus speeding up germination by promoting seedling elongation (Abdullah and Abdulrahman, 2017). In intact seeds, GA_3 is also known for its ability to remobilize nutrient reserves from the endosperm making them readily available for the germinating embryo, hence its role in embryogenic tissue development (Jones and Stoddard, 1977; Kurti and Ulger, 2014). Treatment of citrus seeds with GA_3 promoted germination (Dilip *et al.*, 2017). Similarly, soaking turf grass seeds in five different GA_3 concentrations (0, 50, 100, 200 and 400 mg/l), resulted in a positive correlation between the germination rate and the

concentration of GA_3 (Abdullah and Abdulrahman, 2017). Likewise, the treatment of GA_3 and BAP were effective on *Echinacea angustifolia* seed germination (Chuanren *et al*., 2004). However, gibberellic acid did not have an effect on *Musa velutina* embryo germination (Pancholi *et al*. 1995).

2.5.3 Embryo culture

Embryo culture is an *in vitro* technique used to grow embryos aseptically excised from seeds with a goal of obtaining viable plants. Plant breeders have used embryo culture techniques since the eighteenth century. The first successful embryo culture under aseptic conditions was done in the nineteenth century by Hannig in 1904. Laibach (1925) subsequently emphasized the potential applications of embryo culture in rescuing embryos from interspecific hybrids. In 1933, cherry embryos were successfully cultured (Tukey, 1933). Furthermore, Charles Bonnet worked on *Phaseolus* and *Fagopyrum* (Schopfer 1943; Sharma *et al*. 1996). To date, embryo culture is widely used in seedless breeding, triploid breeding and interspecific breeding of various fruit crops such as apple (Dantas *et al*., 2006; Druart, 2000), citrus (Viloria *et al.*, 2005), mango (Krishna and Singh, 2007), muskmelon (Ezura *et al*., 1994; Nun˜ez- Palenius *et al*., 2006), peach (Pinto *et al*., 1994; Anderson *et al*., 2002), persimmon (Hu *et al*., 2013), watermelon (Tas¸kın *et al*., 2013) and banana (Bakry, 2008; Uma *et al*., 2011) among others. In these fruit crops, embryo culture is applied for various purposes such as seedless breeding, triploid breeding, and interspecific breeding.

Banana seeds have a very hard seed coat and the endosperm is made of powdery granules that protect the embryo from adverse conditions. Therefore, embryo culture is a technique widely used in bananas because the seeds do not readily germinate when sown directly in the soil (Bakry, 2008; Uma *et al*., 2011). Some seeds can take up to one year before they germinate

depending on the environmental conditions (Purseglove, 1972). Fruit breeding programs exploit interploidy hybridizations to combine desirable genetic traits of complementary parents at the triploid level for the purpose of developing improved seedless fruits (Shen *et al*., 2011). Crossing related species from wild plants enables access to a wider range of genes required for genetic improvement of plants such as banana (Tripathi *et al*., 2007). Seeds from wide or interspecific crosses may fail to develop to full maturity due to embryo abortion and/or endosperm degeneration (Laibach, 1925). Hence embryo culture techniques help in the breeding work to successfully create hybrids with high genetic diversity by germinating banana embryos from wide or interspecific crosses (Lulsdorf *et al*., 2014). The removal of the seed coat helps the embryo to be exposed to optimal temperature and growth regulators in the media for easy germination (Sharma *et al*., 1996), eliminates seed germination inhibitors localized in the endosperm and seed coat (Pierik, 1987) and allows faster uptake of water to reactivate the metabolic and catabolic processes.

2.6 Culture medium for *in vitro* growth of plants

Several media formulations are available for cell and tissue culture work (Table 1). These include Murashige and Skoog (MS, Murashige and Skoog, 1962) and Gamborg B5 (Gamborg, 1968) commonly used as basal media for embryo rescue/culture studies (Bridgen, 1994), the Schenk and Hilderbrandt used for *in vitro* callus culture of monocotyledonous and dicotyledonous plants (Schenk and Hilderbrandt 1972), Nitsch and Nitsch used for *in vitro* anther callus culture of *Nicotiana* (Nitsch and Nitsch, 1969), and Chu (N6) (Chu, 1975) utilized *in vitro* anther culture of *Oryza sativa*. The types and concentration of media supplements required depend greatly on the stage of development of the embryo (Reed, 2005). Murashige and Skoog's (MS) medium, and Gamborg's B-5 medium are all high in macronutrients, while the other media

formulations contain considerably fewer macronutrients. MS media is used for micropropagation, organ, callus, and cell suspension culture. It was established by Murashige and Skoog (1962) for *in vitro* callus culture of *Nicotiana tabacum* and is the most frequently used culture medium in plant tissue culture due to its success with many plant species. The MS culture medium also provides all essential macro elements, micro-elements, and vitamins.

Table 1. Types of media and their composition

Components	**Amount (mg l^{-1})**				
	White's White's medium (1963)	**Murashige and Skoog (MS)** Murashige and Skoog, (1962)	**Gamborg (B5)** Gamborg *et al.*(1968)	**Chu (N6)** Chu *et al.*, (1975)	**Nitsch's** Nitsch and Nitsch (1969)
Macronutrients					
$MgSO_47H_2O$	750	370	250	185	185
KH_2PO_4	-	170	-	400	68
$NaH_2PO_4H_2O$	19	-	150	-	-
KNO_3	80	1900	2500	2830	950
NH4NO3	-	1650	-	-	720
$CaCl_22H_2O$	-	440	150	166	-
$(NH_4)_2SO_4$	-	-	134	463	-
Micronutrients					
H3BO3	1.5	6.2	3	1.6	-
$MnSO_44H_2O$	5	22.3	-	4.4	2.5
$MnSO_4H_2O$	-	-	10	3.3	-
$ZnSO_47H_2O$	3	8.6	2	1.5	10
$Na_2MoO_42H_2O$	-	0.25	0.25	-	0.25
$CuSO_45H_2O$	0.01	0.025	0.025	-	0.025
$CoCl_26H_2O$	-	0.025	0.025	-	0.025
Kl	0.75	0.83	0.75	0.8	-
$FeSO_47H_2O$	-	27.8	-	27.8	27.8
$Na_2EDTA_2H_2O$	-	37.3	-	37.3	37.3
Organic supplements					
Vitamins					
Thiamine HCl	0.01	0.5	10	1	0.5
Pyridoxine (HCl)	0.01	0.5	1	0.5	0.5
Nicotinic acid	0.05	0.5	1	0.5	5
Myoinositol	-	100	100	-	100
Others					

Glycine Folic acid Biotin	3 - -	2 - -	- - -	- - -	2 0.5 0.05
Sucrose (g)	20	30	20	50	20
pH	5.8	5.8	5.5	5.8	5.8

Source: http://www.biologydiscussion.com/

2.7 Media components for *in vitro* growth of embryos

Growth of embryos *in vitro* is governed by the composition of the culture medium i.e. the levels of macro and micronutrients, vitamins, amino acids or other nitrogen supplements, sugar(s), other organic supplements, solidifying agents or support systems, and growth regulators (Saad and Elshahed, 2012). The macronutrients provide the six elements; nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S) required for both structural and functional roles in the plant cell or tissue growth. N and S are required for protein synthesis while P, N and S for nucleotide synthesis, Ca for cell wall synthesis and Mg is used for membrane integrity. The optimum concentration of each macronutrient for achieving maximum growth rates varies considerably among species. The essential micronutrients include iron and manganese for photosynthesis, zinc, copper, and molybdenum acts as co-factor, and boron for lignin formation.

The most preferred carbohydrate (carbon and energy source) in embryo culture media is sucrose, however, this can be substituted with glucose and fructose. Glucose is as effective as sucrose but fructose is somewhat less effective. Carbon must be supplied to the culture medium because few plant cell lines have been isolated that are fully autotrophic, e.g. capable of synthesizing their own carbohydrate by CO_2 assimilation during photosynthesis. Sugar also serves as an osmotic stabilizer in culture media (Sharma *et al*., 1996).

Vitamins are required by plants as catalysts in various metabolic processes. When plant cells and tissues are grown *in vitro*, some vitamins may become limiting factors for cell growth. The vitamins most frequently used in cell and tissue culture media include thiamin (B1), nicotinic acid, pyridoxine (B6) and myo-inositol. Thiamin is a universal vitamin required by all cells for growth.

Amino acids are particularly important for establishing cell cultures and protoplast cultures. Amino acids provide plant cells with an immediately available source of nitrogen, which generally can be taken up by the cells more rapidly than inorganic nitrogen. Examples of amino acids that enhance cell growth are glycine and glutamine. Other media compositions commonly used are solidifying agents including agar, phytagel and gel rite. Hormones are also added to the media.

CHAPTER THREE

MATERIAL AND METHODS

3.1 Site description

This study was carried out at the International Institute of Tropical Agriculture (IITA), at the Sendusu substation – banana breeding programme – which works on the improvement of EAHB. Sendusu is at about 28 km North-East of Kampala in Central Uganda at 1,200 m above sea level; 0°32′N and 32°34′E, and receives an annual rainfall of about 1,377 mm. The banana pollination plots of the programme were used for crosses. The seed germination study was conducted under standard tissue culture laboratory conditions at the IITA banana breeding laboratory at Sendusu.

3.2 Material selection, pollination and seed collection procedure

Four female-male combinations were used in this study, including two $2x$ selfing crosses and two $4x$ - $2x$ crosses (Table 2). Tetraploids and diploids were used as females while only diploids served as male parents. Hand pollination was carried out in the field including six diploid self-pollinations and ten cross-pollination events per cross-type. Pollination was carried out in September, April, and May respectively for $4x – 2x$, while for $2x – 2x$, it was carried out in April, and May.

Table 2. Banana crosses (parents) used in the study

Parents	**Cross 1**	**Cross 2**	**Cross 3**	**Cross 4**
Female	ITC0249 - Calcutta 4 (2*x*)	ITC1348 - Pisang serun 404 (2*x*)	1438K-1 (4*x*)	1201K-1 (4*x*)
Male	ITC0249 - Calcutta 4 (2*x*)	ITC1348 - Pisang serun 404 (2*x*)	ITC0250 - Malaccensis (2*x*)	TMB2x7197-2 (2*x*)

The newly opened inflorescence of a female-designated banana plant was pollinated with pollen from a diploid male-designated plant after which the bunch was covered with a bag to avoid unwanted pollen. For self-pollinating Calcutta 4 (ITC0249) and Pisang Serun 404 (ITC1348), one mat was designated as a female parent, and another mat of the same genotype was designated as the male parent. Pollination was carried out between 7 a.m. and 9 a.m. Mature fruit bunches were harvested 3.5 months and 5 months after pollination for diploid x diploid and tetraploid x diploid crosses, respectively. The harvested bunches were then stored in a ripening room until the fruits were completely ripened. Seeds were hand-extracted from the ripe fruits and washed thoroughly under running tap water to remove all fruit pulp and dried at room temperature for 10 min. Seeds were sorted based on the hardness of the integument by pressing each one of them using a thumb and only hard seeds were selected for embryo excision.

3.3 Seed viability test

To ensure that the extracted embryos used for the experiment were viable and of high quality, a viability test was performed. Thirty embryos from each of the four crosses were subjected to viability tests using TTC (Bhardwaj *et al.*, 2016). To obtain the embryos, seeds were cracked under the laminar flow and the embryos were carefully excised from the seed to avoid damage. Excised embryos were submerged in a 0.5% TTC solution and incubated for 2 days at 27°C in the dark. The TTC solution was drained and the embryos were rinsed three times with sterile distilled water. The staining patterns of the embryos were studied under a dissecting microscope. The microscope specifications were eyepiece: 10x, and objective: 0.8x. The percentage of viable embryos from each cross was determined using the formula in Equation 1.

$$Viability = \frac{Red\ embryos + Pink\ embryos}{Total\ number\ of\ embryos} \qquad \text{Eq. 1}$$

3.4 Study 1: Determine the effect of soaking seeds in distilled water prior to embryo excision on the germination rate of zygotic embryos of banana seeds

Seeds were subjected to five soaking treatments: 0 days (no soaking), 3, 5, 7 and 9 days of soaking in sterile water at 4ºC. Three replicates, each of 100 seeds from each cross were used. Seeds were then transferred to the laminar flow and surface-sterilized with absolute ethanol for 3 minutes followed by 15 % (0.00525% w/v) sodium hypochlorite solution, mixed with 2 drops of TWEEN® 20 for 20 minutes and rinsed three times with sterilized distilled water. Embryos were aseptically excised from the seed using a pair of forceps and a scalpel in the laminar flow hood. In each test tubes (25 mm) containing sterilized MS basal medium (without any hormone), five excised embryos were inoculated . The medium was prepared and sterilized as follows before embryos initiation. Powdered basal medium of 4.4g (Murashige and Skook premix from Caisson laboratories, inc. Research Park Way, North Logan, UT 84341 U.S.A) was weighed and dissolved in a litre of distilled water. Vitamins and ascorbic acid (Table 3) were added to the dissolved water, followed by 30g/l sucrose. The pH was adjusted to 5.8 using 1N HCl or 1N NaOH. Phytagel (1.5g/l) was used as a solidifying agent. Twenty-five ml medium was dispensed into each test tube before autoclaving at 121˚C for 15 min. The cultured embryos were maintained under complete darkness at 28±2°C and embryo germination data were collected weekly for 8 weeks. The experiment was laid in a completely randomised design.

Viability was used to adjust the germination rate using the following equation.

$$Adjusted\ germination\ (\%) = \frac{Number\ of\ germinated\ embryos}{Number\ of\ cultured\ embryos\ x\ viability}\ x\ 100 \qquad \text{Eq. 2}$$

Table 3. Vitamins and ascorbic acid stocks and working concentrations added to the powered basal medium used in this study

	Final medium concentration (mg/L)	**Stock (20x in 100ml)**	**Stock quantity to dispense / L**
Vitamins			
Glycine	2mg	0.0400g	5ml
Thiamine-HCL	0.4mg	0.0080g	5ml
Pyridoxine-HCL	0.5mg	0.0100g	5ml
Nicotinic Acid	0.5mg	0.0100g	5ml
Ascorbic acid	20mg	0.4000g	5ml

3.5 Study 2: Establishing the optimal BAP and GA_3 hormonal concentration for germination of banana zygotic embryos from water-soaked seeds

Water-soaked treated seeds from study one were sterilised and embryos excised as described in study one. Extracted embryos were immediately transferred to Petri-dishes with a moist sterile double layer of filter paper (Whatman no. 40) and exposed in the laminar flow for 5 minutes to blow off ethylene emitted during ripening of the banana fruit. Embryos were then transferred to MS basal medium with composition as described in study one, but supplemented with different concentration combinations of BAP and GA_3 of 0 (no hormones), 0.5 and 1 mg/l (Table 4). Forty embryos per treatment per cross were used. The optimal days of soaking from study one was used for different hormonal combinations with 0 days (no soaking) as control. Cultures were kept in the dark at 28±2°C for a maximum of two months. The experiment was laid in a completely randomised design. As in the first study, germinated embryos were counted weekly for 8 weeks. Germination percentage was calculated and adjusted to the viability of the seed lot as in Equation 2.

Table 4. Concentration combination of growth hormones used in MS basal medium for banana embryo culture

Treatment combinations	BAP (mg/l)	GA (mg/l)
1	0.0	0.0
2	0.5	0.0
3	1.0	0.0
4	0.0	0.5
5	0.0	1.0
6	0.5	0.5
7	0.5	1.0
8	1.0	0.5
9	1.0	1.0

3.6 Data collection

For the two experiments, germination was recorded when shoots emerged to about 1cm above the medium. The number of germinated embryos was recorded weekly until no further germination occurred for a maximum of eight weeks. Growth parameters like plantlet height, girth, and number of roots for a one week germinated embryo were also recorded.

3.7 Statistical analysis

The number of germinated embryos was adjusted using the viability test and subjected for analysis. Analysis of variance (ANOVA) was carried out at 95% confidence interval using GenStat 14th Edition to determine if significant differences existed between treatments. Multiple comparisons of treatment means were done using Fisher's least significant difference (LSD).

To determine the effect of soaking on seed embryo germination from different crosses, the following linear model was used;

$$y_{ij} = \mu + c_i + s_j + cs_{ij} + e_{ij} \qquad \text{Eq.3}$$

Where y_{ij} is the observed embryo germination rate of the ith cross under jth soaking treatment, μ is the overall mean of germination rate, c_i is the effect of the ith cross, s_j is the effect of the jth

level of soaking, cs_{ij} is the effect of the interaction between cross and soaking and e_{ij} is the random error

The effect of different hormone concentration combinations on embryo germination was determined using the linear model below.

$$y_{ijkl} = \mu + c_i + b_j + g_k + bg_{ij} + e_{ijk} \qquad \text{Eq.4}$$

Where y_{ijk} is the observed embryo germination rate of the ith cross, inoculated on a medium with j^{th} BAP and k^{th} GA concentrations, μ is the overall germination mean rate, $c_{i.}$ is the effect of the ith cross, b_j is the effect of the jth BAP concentration, g_k is the effect of the kth GA_3 concentration, bg_{jk} is the effect of interaction between BAP and GA, and e_{ijk} is the random error.

To determine the effect of hormones on the growth parameters, the linear model was used for individual parameters;

$$y_{ij} = \mu + b_i + g_j + bg_{ij} + e_{ij} \qquad \text{Eq.5}$$

where yi is the observed growth parameter (plant height, number of roots and stem girth) on a medium with i^{th} BAP and j^{th} GA concentration, μ is the overall germination mean rate, b_i is the effect of the ith BAP concentration, g_j is the effect of the jth GA concentration, bg_{ij} is the effect of the interaction between BAP and GA, and e_{ij} is the random error.

CHAPTER FOUR

RESULTS

4.1 Effect of seed soaking on embryo germination rate from different crosses

Crosses differed significantly (*P*<0.001, Table 5) in germination rate. However, the interaction between cross and soaking regime did not significantly affect embryo germination (*P*>0.05, Table 5). Zero (0) days of soaking seeds in water gave the lowest embryo germination while 3 days of soaking in water gave the highest germination rates in all crosses (Figure 3). Soaking for 5 days gave slightly lower germination than soaking for 3 days, but the difference was not significant (Figure 3). However, increasing the time of soaking to 9 days had a negative impact on embryo germination rate for all the cross types used in the study. Soaking for 3 days on average improved germination by 16.2%, and the highest germination was observed in Calcutta 4 – Calcutta 4 embryos which recorded 47.3% germination rate, while the lowest germination was recorded in a 1201K-1 – 7197-2 at 32.0% (Figure 3).

Table 5. ANOVA result on germination rate of banana embryos as affected by time of soaking in distilled water and cross-type

Source of variation	**d.f.**	**Mean squares**	**F pr.**
Treatments	4	1420.59	<.001
Cross	3	661.73	<.001
Treatments.Cross	12	40.17	0.927
Residual	40	87.48	
Total	59		

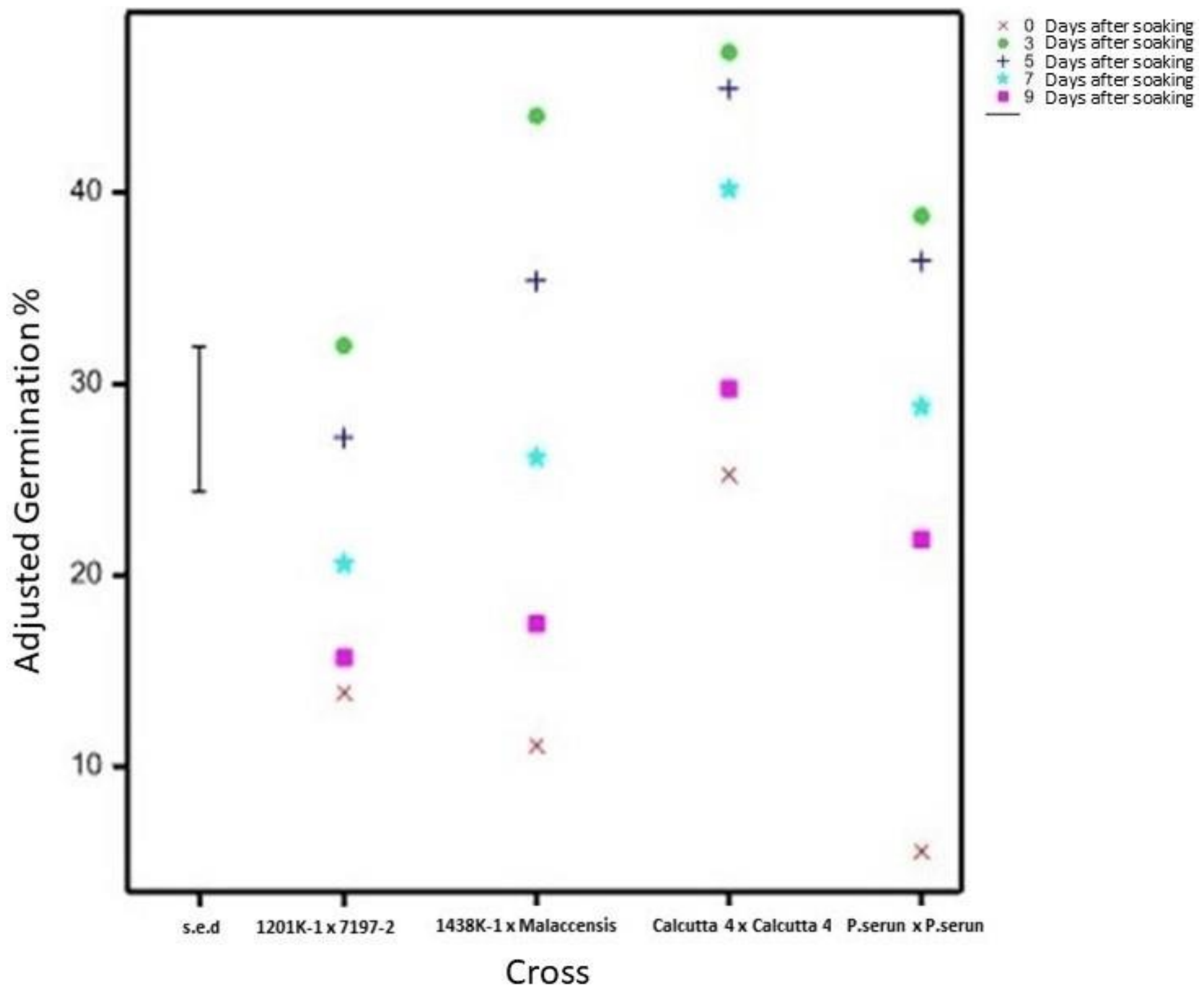

Figure 3: Effect of crosses on embryo germination rates at different levels of soaking duration (days)

4.2 Effect of BAP and GA_3 concentration in the MS medium on the germination rate of embryos crosses

After establishing the optimum days of the soaking period, only the embryo extracted after 3 days of soaking were used in the different treatments of this experiment. Considering a cross of tetraploids by improved diploid parents, supplementing MS with varying concentrations of BAP and GA_3 did not significantly ($P>0.05$) affect the growth of embryos while the crosses were significantly different ($P<0.05$) (Table 6).

Table 6. ANOVA result on the germination rate of embryos from 4*x* - 2*x* crosses as affected by BAP and GA_3 concentration

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cross	1	3962.8	3962.8	5.59	0.020
BAP	2	1377.1	688.5	0.97	0.382
GA_3	2	1392.3	696.2	0.98	0.377
BAP.GA_3	4	6442.3	1610.6	2.27	0.065
Residual	125	88626.8	709.0		
Total	134	101801.3			

Considering diploids crosses, there was a significant difference ($P<0.05$) among crosses. However, hormones did not significantly affect embryo germination (Table 7).

Table 7. ANOVA result on the germination rate of embryos from 2*x* - 2*x* crosses as affected by BAP and GA_3 concentration

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cross	1	8832.2	8832.2	17.30	<.001
BAP	2	901.1	450.5	0.88	0.421
GA_3	2	1359.3	679.7	1.33	0.275
BAP.GA_3	4	446.7	111.7	0.22	0.927
Residual	44	22462.5	510.5		
Total	53	34001.7			

4.3 The effect of BAP and GA_3 concentration on growth parameters of banana embryos

Supplementing the MS medium with different concentrations of BAP and GA_3 resulted in significant differences in height, number of roots, and stem girth. Among 2*x* – 2*x* crosses, significant effects of BAP ($P \leq 0.05$) and GA_3 ($P<0.001$) were observed for height (Table 8). BAP also significantly ($P<0.01$) affected number of roots but GA_3 did not have significant effect on number of roots (Table 8). Both BAP and GA_3 did not significantly affect stem girth among 2*x* – 2*x* crosses (Table 8). A siginificant effect of BAP and GA_3 interaction was observed for height ($P<0.05$), number of roots ($P<0.05$) and stem girth ($P<0.001$) (Table 8).

Table 8. ANOVA result on germination parameters from 2*x* - 2*x* as affected by BAP and GA_3

Source of variation	Mean squares and significance		
	Height	**No. of roots**	**Stem girth**
BAP	75.30^{*}	11.299^{**}	0.2934^{ns}
GA_3	134.51^{***}	4.424^{ns}	0.7153^{ns}
$BAP.GA_3$	49.91^{*}	5.580^{*}	2.2153^{***}
Residual	17.00	1.695	0.3024

ns: not significant ($P>0.05$), *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

Among 4*x* – 2*x* crosses, significant effects of BAP ($P \leq 0.01$) and GA_3 ($P<0.001$) were observed for height (Table 9). BAP significantly ($P<0.01$) affected number of roots but did not have significant effect on stem girth while GA_3 significantly ($P<0.001$) affected stem girth but did not have significant effect on number of roots (Table 9). A siginificant effect of BAP and GA_3 interaction was observed for height ($P<0.05$), number of roots ($P<0.05$) and stem girth ($P<0.001$) (Table 9).

Table 9. ANOVA result on germination parameters from 4*x* - 2*x* as affected by BAP and GA_3

Source of variation	Mean squares and significance		
	Height	No. of roots	Stem girth
BAP	105.15^{**}	11.299^{**}	0.5365^{ns}
GA	173.52^{***}	4.424^{ns}	2.3802^{***}
BAP.GA_3	58.51^{*}	5.580^{*}	1.7135^{***}
Residual	21.31	1.695	0.3208

ns: not significant (P>0.05), *P≤0.05, **P≤0.01, ***P≤0.001

The height of germinated embryos ranged from 6.4 mm at 0mg/l BAP and 0mg/l GA_3 to 15.6 mm at 1mg/l BAP and 0.5mg/l GA_3 in tetraploids. In diploid, the range was 7.7mm at 0mg/l BAP and 0mg/l GA_3 to 15.8mm at 1mg/l BAP and 0.5mg/l GA_3 (Table 10). The highest number of roots was 1.9 at 0mg/l BAP and 0.5mg/l GA_3, while the lowest number was 0 at 1mg/l BAP and 0.5mg/l GA_3, and at 1 mg/l BAP and 1 mg/l GA_3 for both crosses (4*x* – 2*x* and 2*x* – 2*x*). The stem girth had the highest value as 1.9 mm (4*x* – 2*x*) and 1.7 mm (2*x* – 2*x*) at 1mg/l BAP and 0mg/l GA_3, while the lowest was 0.9 mm at 1mg/l BAP and 0.5mg/l GA_3 for both crosses (4*x* – 2*x* and 2*x* – 2*x*). The plants with the highest height of 15.6 mm (4*x* – 2*x*) and 15.8 mm (2*x* – 2*x*) had no roots and had 0.9 mm as the lowest stem girth. On the other hand, the plants with the lowest height was 6.4 mm (tetraploids) and 7.7 mm (2*x* – 2*x*) which also had 0.3 mm of roots, and had stem girth of 1.4 mm (4*x* - 2*x*) and 1.0 mm (2*x* – 2*x*). The plants with the highest no of roots (1.9) had 9.1 mm (4*x* - 2*x*) and 9.8 mm (diploids) of height and 1.2 mm (tetraploids) and 0.9 mm (2*x* - 2*x*) of stem girth. The plant with the biggest stem girth (1.9 mm) for tetraploids had a height of 9.1 mm (4*x* – 2*x*) and 1.7 mm for diploids had a height of 9.6 mm (2*x* - 2*x*) and 0.1 mm of roots for both 2*x* – 2*x* and 4*x* – 2*x* crosses (Table 10). It was observed that a combination of 1mg/l of BAP and 0.5mg/l of GA_3 was the best for plant height, 1mg/l of BAP and no GA_3 was good for stem girth, and 0.5mg/l of GA_3 without BAP was good for the production of roots, for both 2*x* – 2*x* and 4*x* – 2*x* crosses.

Table 10. Effect of different hormone concentrations on growth parameters of one-week-old banana embryos

Hormones		Height (mm)		No. of Roots		Stem Girth (mm)	
BAP	GA_3	2x x 2x	4x x 2x	2x x 2x	4x x 2x	2x x 2x	4x x 2x
0	0	7.7^{c}	6.4^{c}	0.3^{c}	0.3^{c}	1.0^{c}	1.4^{bc}
0	0.5	9.8^{bc}	9.1^{bc}	1.9^{a}	1.9^{a}	0.9^{c}	1.2^{bcd}
0	1	10.6^{b}	9.9^{b}	0.3^{c}	0.3^{c}	1.6^{a}	1.6^{ab}
0.5	0	10.2^{bc}	9.3^{bc}	1.3^{ab}	1.3^{ab}	1.5^{ab}	1.7^{ab}
0.5	0.5	11.9^{b}	11.4^{b}	0.9^{bc}	0.9^{bc}	1.5^{ab}	1.6^{ab}
0.5	1	10.9^{b}	10.3^{b}	0.6^{bc}	0.6^{bc}	1.2^{bc}	1.3^{bcd}
1	0	9.6^{bc}	9.1^{bc}	0.1^{c}	0.1^{c}	1.7^{a}	1.9^{a}
1	0.5	15.8^{a}	15.6^{a}	0.0^{c}	0.0^{c}	0.9^{c}	0.9^{d}
1	1	10.1^{bc}	9.4^{bc}	0.0^{c}	0.0^{c}	1.1^{c}	1.1^{cd}
Grand mean		10.72	10.06	0.60	0.60	1.28	1.42
LSD		2.88	3.23	0.91	0.91	0.38	0.40

NB: BAP = Benzylaminopurine; GA_3 = Gibberellic acid; LSD: Least significant difference
Means followed by the same letter in the same column are not significantly different using the LSD test at 5% significance level.

CHAPTER FIVE

DISCUSSION

Soaking of seeds and germinating them in a hormone-supplement medium is known to improve seed germination (Arun *et al*., 2013). In this study, different periods of seed soaking in distilled water and the supplementation of different levels of concentration for 6-Benzylaminopurine and Gibberellic acid to improve the germination of banana zygotic embryos were tested.

There was a significant difference in the viability of crosses and batches. The date of pollination (season) affected viability; the 1st, 2nd, and 3rd batches of all crosses were pollinated in September, April, and May and these months occurred during the two rainy seasons. All batches of diploids (batches 1, 2 and 3 were only pollinated in April and May (Calcutta 4 – Calcutta 4 and P. serun – P. serun). Batch 4 was pollinated in May and June, while batch 5 and 6 were pollinated in July. Batch 7 was pollinated in June and November. Batches from banana plants pollinated in the rainy season had higher viability as opposed to the ones pollinated in dry seasons. Low viability was because pollination took place in periods during which there were no rains.

Germination rates (adjusted for seed viability) of zygotic embryos from fresh seeds of the four crosses varied between 28.7% and 72.9% of water-soaked seeds treated with hormones. Vuylsteke and Swennen (1993) reported a 15% germination rate of zygotic embryos from *M. acuminata* while Pancholi *et al* (1995) reported 69.9% germination success of *M. velutina* seed embryos. However, in both experiments, the rate was far below the high levels of 94% germination rate recorded in *M. balbisiana* (Afele and De Langhe, 1991), 90.8% in *M.*

acuminata ssp. (Asif *et al*., 2001), and 92% in *M. ornata* (Burgos-Hernández, 2014). Seed dormancy and species or genotype differences could explain the variation in embryo germination percentages. Physical dormancy was reported in *Musaceae* by Baskin and Baskin (1998) and the different degrees of dormancy were defined by Baskin and Baskin (2004). In *Musa* seed dormancy was reported by Asif *et al.* (2001), Finch-Savage and Leubner-Metzger (2006), and Uma *et al*. (2011), although the wild bananas, *M. acuminata* and *M. balbisiana* were reported to germinate easily (Simmonds, 1959).

The imbibition process occurred when seeds were soaked in water and enabled rapid embryo germination. We found that soaking time positively affected the germination of banana embryos with the average percentage germination of 39.5% for the 3 days after soaking (DAS) treatment as compared to 0 DAS for both 4*x* – 2*x* and 2*x* - 2*x* crosses. These results are in agreement with the findings of Afele and De Langhe (1991) when banana seeds were soaked for 5 days. Soaking seeds also affects other crops, for instance, soaking seeds of guava in distilled water for 48hr significantly increased the germination percentage, compared with the soaking period of 24hr (Bhanuprakash *et al*., 2008). Soaking seeds of coconut in water for two weeks resulted in higher germination percentage and better growth of seedlings than soaking in water for one week (Thomas, 1974). An optimal level of soaking of tomato seeds enhanced effects on germination and growth (Sabongari and Aliero, 2004). Higher germination is possibly due to the leaching out of water-soluble inhibitors when seeds are soaked (Rajendrakumar, 2017). These observations confirm the positive effect of soaking on embryo germination. However, the decline of germination in our study when seeds were soaked for more than 3 days may be due to the rotting of the embryo, though Simmonds (1952) reported the reason to be unknown. It appears

that the seeds in excess of water get serious injuries and this increases with incease in time of soaking (Zhang *et a*l., 2015).

Variations in embryo germination on different hormonal combinations across crosses may be attributed to genetic differences in cultivars (Fathi and Janani, 2012). Hormonal combinations had no significant effect on the germination of banana embryos but it appeared to promote growth. Plant growth regulators generally play a small role in embryo culture. Instead, hormones slightly promote embryo growth (Fathi and Jahani, 2012) which was also observed in our study.

The basal MS medium is sufficient for mature embryo germination and regeneration process (Uma *et al.*, 2011). However, in the present study, not all mature embryos germinated into plantlets. Yet a matured embryo is considered to be a miniature plant that should develop into a normal plant without any requirement of a plant growth regulator (Dayarani *et al*., 2014). The results of the present study are in close agreement with the findings of Patel *et al.* (2016) in mango and Shabaq (2013) in loquat.

Results from this study revealed that the combination of hormones had a significant effect on growth parameters. The maximum seedling height of 15.6 mm with 0 roots and 0.9 mm of girth was obtained with 1 mg/l BAP and 0.5 mg/l GA_3 treatment. The highest stem girth was obtained with a combination of 1 mg/l BAP only with 1.9 mm of girth, 9.1 mm height, and 0.1 mm of roots. However, BAP hindered root growth, consistent with findings of Musara *et al*. (2015). It was noted that the highest girths were obtained with 1mg/l and no GA_3. This is because the girth increased due to greater cell division and elongation at the stem portion. The highest number of roots was found when the media was supplemented with 1 mg/l of GA_3 and

no BAP. This improvement in root parameters due to GA_3 treatment might have resulted from increased production of enzymes for assimilation and redistribution of materials within the embryo (Vachhani *et al*., 2014; Pandiyan *et al*., 2011). These results are in close agreement with Anburani and Shakila (2010) in papaya; Vasantha *et al.* (2014) in tamarind; Swamy *et al*. (1999) in Jamun; Meshram *et al.* (2015) in acid lime and Parmar *et al*., (2016) in custard apple.

The hormonal combination which promoted the tallest plant height was obtained from 1mg/l BAP combined with 0.5mg/l GA_3. The increase in height with GA_3 was due to the fact that this hormone increased uptake of nutrients, causing cell elongation, while BAP improved cell division and thus increased height (Shanmugavelu, 1966). Among the three parameters, i.e. stem height, stem girth and number of roots, we can argue that stem girth is more important as the plantlets prepare to proliferate and undergo subsequent sub-culturing, while root formation and plant height become important at the last cycle of the plants in tissue culture, as the plants get ready to be weaned.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusion

Seed soaking in water improved zygotic embryo germination, and supplementing the MS medium with BAP and GA_3 increased the vigor of the plantlets after germination. The highest germination rate was observed when tetraploid by diploid and diploid by diploid seeds were soaked for 3 days. The germination increase obtained were 15.6%, 34.3%, 10.1% and 4.7% from self-pollinated ITC0249 - Calcutta 4, selfed ITC1348 - Pisang serum 404, 1201K-1 – TMB2x 7197-2 and 1438K-1 – ITC0250 - Malaccensis respectively. The observations revealed that supplementing MS medium with 1mg/l BAP and 0mg/l GA_3 improved plant vigor when compared with the control medium (MS without BAP or GA_3).

6.2 Recommendation

To improve the germination of zygotic embryos, banana seeds should be soaked for 3 days before excising out the embryos and the germination MS medium should be supplemented with 1 mg/l BAP to improve the vigor of the plantlets after germination. But because these hormones are expensive, more studies should be carried out using intervals of 0.1mg/l of the hormone to determine the exact optimal concentration which may be either slightly lower or higher than 1mg/l.

REFERENCES

Abdullah, B. S. and Abdulrahman, Y. A. (2017). Effect of different concentrations of gibberellic acid on seeds germination and growth in different turf grass genera. *Kufa Journal for Agricultural Sciences*, *9*(2), 226-247.

Afele, J. C. and De Langhe, E. (1991). Increasing in vitro germination of Musa balbisiana seed. *Plant Cell, Tissue and Organ Culture*, 27(1), 33-36.

Alakonya, A. E., Kimunye, J., Mahuku, G., Amah, D., Uwimana, B., Brown, A. and Swennen, R. (2018). Progress in understanding Pseudocercospora banana pathogens and the development of resistant Musa germplasm. *Plant Pathology*, 67(4), 759-770.

Aliero, B. L. (2004). Effects of sulphuric acid, mechanical scarification and wet heat treatments on germination of seeds of African locust bean tree, *Parkia biglobosa*. *African Journal of Biotechnology*, *3*(3), 179-181.

Anburani, A. and Shakila, A. (2010). Influence of seed treatment on the germination and seedling vigour of papaya. Acta hort., 85: 295-298.

Anderson, J. E., Kono, T. J., Stupar, R. M., Kantar, M. B. and Morrell, P. L. (2016). Environmental association analyses identify candidates for abiotic stress tolerance in Glycine soja, the wild progenitor of cultivated soybeans. *G3: Genes, Genomes, Genetics*, *6*(4), 835-843.

Anderson, N., Byrne, D. H., Sinclair, J. and Burrell, A. M. (2002). Cooler temperature during germination improves the survival of embryo cultured peach seed. *HortScience*, 37(2), 402-403.

Arinaitwe, I. K., Teo, C. H., Kayat, F., Tumuhimbise, R., Uwimana, B., Kubiriba, J. Swennen, R., Harikrishna, J.A. and Othman, R. Y. (2019). Evaluation of banana germplasm and genetic analysis of an F 1 population for resistance to Fusarium oxysporum f. sp. cubense race 1. *Euphytica*, 215(10), 175.

Arowosegbe, S. (2016). Studies on methods of breaking seed dormancy and germination enhancement in Senna alat a (l.) Roxb., a plant with great medicinal value. *International Research Journal of Natural Sciences*, *4*(2), 31-40.

Arun, K., Uma, S., Saraswathi, S., Backiyarani, S. and Durai, P. (2013). Effects of whole seed priming on the in vitro germination of hybrid banana embryos (Musa spp.). *Seed Science and Technology*, 41(3), 439-451.

Asif, M. J., Mak, C. and Othman, R. Y. (2001). In vitro zygotic embryo culture of wild Musa acuminata ssp. malaccensis and factors affecting germination and seedling growth. *Plant cell, Tissue and Organ Culture*, 67(3), 267-270.

Bakry, F. (2008). Zygotic embryo rescue in bananas. *Fruits*, 63(2), 111-115.

Bakry, F., Carreel, F., Jenny, C. and Horry, J. P. (2009). Genetic improvement of banana. In *Breeding plantation tree crops: tropical species* (pp. 3-50). Springer, New York, NY.

Balaguera-López, H. E., Deaquiz, Y. A. and Álvarez-Herrera, J. G. (2009). Tomato seedlings (*Solanum lycopersicum* L.) from seeds embedded in different solutions of gibberellins (GA3). *Colombian Agronomy* , 27 (1), 57-64.

Banana production volume worldwide statista. 2017. FAOSTAT

Baskin, J. M. and Baskin, C. C. (2004). A classification system for seed dormancy. *Seed science research*, 14(1), 1-16.

Baskin, J. M., Baskin, C. C. and Li, X. (2000). Taxonomy, anatomy and evolution of physical dormancy in seeds. *Plant species biology*, 15(2), 139-152.

Batte, M., Swennen, R., Uwimana, B., Akech, V., Brown, A., Tumuhimbise, R., Hovmalm, H.P., Geleta, M. and Ortiz, R. (2019). Crossbreeding East African highland bananas: lessons learnt relevant to the botany of the crop after 21 years of genetic enhancement. *Frontiers in plant science*, 10, 81.

Bentsink, L. and Koornneef, M. (2008). Seed dormancy and germination. *The Arabidopsis Book/American Society of Plant Biologists*, *6*.

Bewley, J. D. (1997). Seed germination and dormancy. *The plant cell*, 9(7), 1055.

Bhanuprakash, K., Yogeesha, H. S., Vasugi, C., Arun, M. N. and Naik, L. B. (2008). Effect of pre-soaking treatments and temperature on seed germination of guava (*Psidium guajava L.*). *Seed Science and Technology*, 36(3), 792-794.

Bhardwaj, A. K., Kapoor, S., Naryal, A., Bhardwaj, P., Warghat, A. R., Kumar, B. and Chaurasia, O. P. (2016). Effect of various dormancy breaking treatments on seed germination, seedling growth and seed vigour of medicinal plants. *Tropical Plant Research*, 3(3), 508-16.

Bridgen, M. P. (1994). A review of plant embryo culture. *HortScience*, 29(11), 1243-1246.

Brown, A., Tumuhimbise, R., Amah, D., Uwimana, B., Nyine, M., Mduma, H., Talengera, D., Karamura, D., Kuriba, J. and Swennen, R. (2017). Bananas and plantains (Musa spp.). In *Genetic improvement of tropical crops* (pp. 219-240). Springer, Cham.

Burgos-Hernández, M., Castillo-Campos, G., Mata-Rosas, M., González, D., Vovides, A. P. and Murguia-González, J. (2014). Seed germination of the wild banana Musa ornata (Musaceae). *Seed Science and Technology*, 42(1), 16-27.

Carvalho, T. C. D., Grzybowski, C. R. D. S., Ohlson, O. D. C. and Panobianco, M. (2014). Adaptation of the tetrazolium test method for estimating the viability of sorghum seeds. *Journal of Seed Science*, 36(2), 246-252.

Carvalho, T. C. D., Krzyzanowski, F. C., Ohlson, O. D. C. and Panobianco, M. (2013). Tetrazolium test adjustment for wheat seeds. *Journal of Seed Science*, 35(3), 361-367.

Chin, H. F. (1996). Germination and storage of banana seeds. *New Frontiers in Resistance Breeding for Nematodes, Fusarium and Sigatoka (EA Frison, JP Horry & D. De Waele, eds.). INIBAP, Montpellier, France*, 218-227.

Chu, C. C., Wang, C. C., Sun, C. S., Hsu, C., Yin, K. C. and Bi, F. Y. (1975). Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Scientia Sinica*, 18(5), 659-668.

Chuanren, D., Bochu, W., Wanqian, L., Jing, C., Jie, L. and Huan, Z. (2004). Effect of chemical and physical factors to improve the germination rate of *Echinacea angustifolia* seeds. *Colloids and Surfaces B: Biointerfaces*, 37(3-4), 101-105.

Costa, A., Dias, A. S., Grenho, M. G. and Dias, L. S. (2016). Effects of dark or of red, blue or white light on germination of subterranean clover seeds. *Emirates Journal of Food and Agriculture*, 853-864.

Daniells, J., Jenny, C., Karamura, D. and Tomekpe, K., (2001). *Musalogue: a catalogue of musa germplasm.* Diversity in the genus Musa (E. Arnaud and S. Sharrock, compil.). International Network for the Improvement of Banana and Plantain (INIBAP), Montpelier, France, pp213.

Dantas, A. C. D. M., Boneti, J. I., Nodari, R. O. and Guerra, M. P. (2006). Embryo rescue from interspecific crosses in apple rootstocks. *Pesquisa Agropecuária Brasileira*, 41(6), 969-973.

Dayarani, M., Dhanarajan, M. S., Arun, K., Uma, S. and Narayani, P. (2014). Embryo culture and embryo rescue studies in wild Musa spp.(Musa ornata). *Journal of Applied Horticulture*, 16(2), 126-130.

De Bellaire, L. D. L., Fouré, E., Abadie, C. and Carlier, J. (2010). Black Leaf Streak Disease is challenging the banana industry. *Fruits*, 65(6), 327-342.

Debeaujon, I., Lepiniec, L., Pourcel, L. and Routaboul, J. M. (2018). Seed coat development and dormancy. *Annual Plant Reviews online*, 25-49.91

Dilip, W. S., Singh, D., Moharana, D., Rout, S. and Patra, S. S. (2017). Effect of gibberellic acid (GA) different concentrations at different time intervals on seed germination and seedling growth of Rangpur Lime. *J Agroeco Nat Resource Management*, 4, 157-165.

Dissanayake, P., George, D. L. and Gupta, M. L. (2010). Effect of light, gibberellic acid and abscisic acid on germination of guayule (Parthenium argentatum Gray) seed. *Industrial Crops and Products*, 32(2), 111-117.Druart PH (2006). Aneuploidy, a source of genetic diversity for fruit species. *Acta Hortic.* 725.

Druart P (2000) Aneuploids and variants of apple (*Malus domestica* Borkh.) through in vitro culture techniques. Acta Hortic 520:301–307

Escalant, J. V. and Jain, S. M. (2004). Banana imrovement with cellular and molecular biology, and induced mutations: Future and perspectives.

Ezura, H., Kikuta, I., & Oosawa, K. (1994). Production of aneuploid melon plants following in vitro culture of seeds from a triploid x diploid cross. *Plant cell, tissue and organ culture*, 38(1), 61-63.

Fathi, H. and Jahani, U. (2012). Effect of various dormancy breaking treatments on seed germination, seedling growth and seed vigour of medicinal plants Ashwani Kumar Bhardwaj, Sahil Kapoor, Avilekh Naryal, Pushpender Bhardwaj, Ashish Rambhau Warghat, Bhuvnesh Kumar and Om Prakash Chaurasia. Annals of Biological Research, 3 (9):4276-4281

Finch-Savage, W. E. and Footitt, S. (2017). Seed dormancy cycling and the regulation of dormancy mechanisms to time germination in variable field environments. *Journal of experimental botany*, 68(4), 843-856.

Finch-Savage, W. E. and Leubner-Metzger, G. (2006). Seed dormancy and the control of germination. *New phytologist*, *171*(3), 501-523.

Finkelstein, R., Reeves, W., Ariizumi, T. and Steber, C. (2008). Molecular aspects of seed dormancy. *Annu. Rev. Plant Biol.*, 59, 387-415.

Food and Agriculture Organization of the United Nations, 2014. Banana market review and banana statistics 2012–2013. http://www.fao.org

França Neto, J. B. (1999). Teste de tetrazólio para determinação do vigor de sementes. *Vigor de sementes: conceitos e testes. Londrina: ABRATES*, 1-7.

Gamborg, O. L., Miller, R. and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental cell research*, 50(1), 151-158.

Gaspar-Oliveira, C.M., Martins, C.C. and Nakagawa, J. (2009). Method of preparing castor bean seeds (*Ricinus communis L.*) for the tetrazolium test. *Revista Brasileira de Sementes* , 31 (1), 160-167.

Gold, C. S., Kagezi, G. H., Night, G. and Ragama, P. E. (2004). The effects of banana weevil, Cosmopolites sordidus, damage on highland banana growth, yield and stand duration in Uganda. *Annals of Applied Biology*, 145(3), 263-269.

Gold, C.S., Kiggundu, A., Abera, A.M.K. and Karamura, D. 2002. Selection criteria of *Musa* cultivars through a farmer participatory appraisal survey in Uganda. Experimental Agriculture 38:29-38.

Graven, P., De Koster, C. G., Boon, J. J. and Bouman, F. (1996). Structure and macromolecular composition of the seed coat of the Musaceae. *Annals of Botany*, 77(2), 105-122.

Grzybowski, C.R.S., Ohlson, O.C., Silva, R.C. and panobianco, M. 2012. Viability of barley seeds by the tetrazolium test., Revista Brasileira de Sementes v.34, p.47-54.

Hu, D., Tang, X., Zhang, Q. and Luo, Z. (2013). Cross compatibilities of oriental persimmon '*Mopanshi*' and *'Luotian-tianshi*' with 'Zenjimaru'. In *V International Symposium on Persimmon* 996 (pp. 165-170).

Hu, X. W., Wang, Y. R., Wu, Y. P. and Baskin, C. C. (2009). Role of the lens in controlling water uptake in seeds of two Fabaceae (*Papilionoideae)* species treated with sulphuric acid and hot water. *Seed Science Research*, 19(2), 73-80.

Janssens, S. B., Vandelook, F., De Langhe, E., Verstraete, B., Smets, E., Vandenhouwe, I. and Swennen, R. (2016). Evolutionary dynamics and biogeography of Musaceae reveal a correlation between the diversification of the banana family and the geological and climatic history of Southeast Asia. *New Phytologist*, 210(4), 1453-1465.

Jetti, A., Jetti, J. and Perla, R. (2017). Treatments to Break Seed Dormancy in *Givotia rottleriformis Griff. Adv Crop Sci Tech*, 5(273), 2.

Jones, R.L and Stoddard, J.L. 1977. Gibberellins and seed germination. In: Khan, A.A. (ed.) *The Physiology and Biochemistry of Seed Dormancy and Germination,* North-Holland Pub. Co..pp. 77-109.

Junillon, T., Morand, L. and Flandrois, J. P. (2014). Enhanced tetrazolium violet reduction of Salmonella spp. by magnesium addition to the culture media. *Food microbiology*, 42, 132-135.

Karamura, D. A. (1998). Numerical Taxonomic Studies of the East African Highland Bananas (Musa AAA-East Africa) in Uganda. Ph.D. thesis, The University of Reading, Reading

Karamura, D. A., Karamura, E. B. and Tinzaara, W. (2012). Banana cultivar names, synonyms and their usage in Eastern Africa, Bioversity International, Uganda. *Cover photo: DA Karamura Bioversity International–Headquarters Via dei Tre Denari 472a*, *57*.

Karamura, D. and Pickersgill, B. (1999). A classification of the clones of East African highland bananas (Musa) found in Uganda. *Plant Genetic Resources Newsletter (IPGRI/FAO) Bulletin des Ressources Phytogenetiques (IPGRI/FAO) Noticiario de Recursos Fitogeneticos (IPGRI/FAO)*.

Kikulwe, E. M., Kyanjo, J. L., Kato, E., Ssali, R. T., Erima, R., Mpiira, S., Ocimati, W., Tinzaara, W., Kubiriba, J., Gotor, E. and Stoian, D. (2019). Management of Banana Xanthomonas Wilt: Evidence from Impact of Adoption of Cultural Control Practices in Uganda. *Sustainability*, 11(9), 2610.

Kitavi, M., Downing, T., Lorenzen, J., Karamura, D., Onyango, M., Nyine, M., Ferguson, M. and Spillane, C. (2016). The triploid East African Highland Banana (EAHB) genepool is genetically uniform arising from a single ancestral clone that underwent population expansion by vegetative propagation. *Theoretical and applied genetics*, 129(3), 547-561.

Krishna, H, and Singh, S. K. (2007). Biotechnological advances in mango (*Mangifera indica L.*) and their future implication in crop improvement—a review. *Biotechnology Advances*, 25(3), 223-243.

Kumar, P. L., Selvarajan, R., Iskra-Caruana, M. L., Chabannes, M. and Hanna, R. (2015). Biology, etiology, and control of virus diseases of banana and plantain. In *Advances in virus research* (Vol. 91, pp. 229-269). Academic Press.

Kurt, S. and Ulger, S. (2014). Production of common sour orange× Carrizo citrange hybrids using embryo rescue. *International journal of fruit science*, 14(1), 42-48.

Laibach, F. (1925). The deafness of bastard seeds and the artificial rearing of early dying bastard embryos. Journal Bot. 17: 417-459. 1929. Ectogenesis in plants. *Jour. Hered* , 20 , 201-208.

Langens-Gerrits, M. M., Miller, W. B., Croes, A. F. and De Klerk, G. J. (2003). Effect of low temperature on dormancy breaking and growth after planting in lily bulblets regenerated in vitro. *Plant growth regulation*, 40(3), 267-275.

Leubner-Metzger, G. (2006). Hormonal interactions during seed dormancy release and germination. *Handbook of seed science and technology*, *303*.

Lulsdorf, M. M., Ferrie, A., Slater, S. M. and Yuan, H. Y. (2014). Methods and role of embryo rescue technique in alien gene transfer. *In Alien Gene Transfer in Crop Plants, Volume* 1 (pp. 77-103). Springer, New York, NY.

Marcos Filho, J. (2015). Physiology of seeds of cultivated plants 2.ed. *Londrina: abrates,* 660p..

McGahan, M.W. (1961). Studies on the seed of banana. Ii. The anatomy and morphology of the seedling of M*usa balbisiana*. *American Journal of botany* 48(7), 630-637.

Meshram, P. C., Joshi, P. S., Bhoyar, R. K. and Sahoo, A. K. (2015). Effect of different plant growth regulators on seedling growth of acid lime. *Res. Envrn. Life Sci*, 8(4), 725-728.

Miransari, M. (2014). Plant signaling under environmental stress. In *Oxidative Damage to Plants* (pp. 541-555). Academic Press.

Miransari, M. and Smith, D. (2009). Rhizobial lipo-chitooligosaccharides and gibberellins enhance barley (*Hordeum vulgare L.*) seed germination. *Biotechnology*, 8(2), 270-275.

Miransari, M. and Smith, D. L. (2014). Plant hormones and seed germination. *Environmental and Experimental Botany*, 99, 110-121.

Mousavi, S. R., Rezaei, M. and Mousavi, A. (2011). A general overview on seed dormancy and methods of breaking it. *Advances in environmental biology*, 5(10), 3333-3337.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, 15(3), 473-497.

Musara, C., Chitamba, J. and Nhuvira, C. (2015). Evaluation of different seed dormancy breaking techniques on okra (*Abelmoschus esculentus L.*) seed germination. *Afr J Agric Res*, 10(17), 1952-1956.

Nadjafi, F., Bannayan, M., Tabrizi, L. and Rastgoo, M. (2006). Seed germination and dormancy breaking techniques for *Ferula gummosa* and *Teucrium polium*. *Journal of Arid Environments*, 64(3), 542-547.

Nair, A. G., Vidya, P., Ambu, V., Sreekumar, J. and Mohan, C. (2017). Genetic diversity studies in cultivated sweet potato (Ipomoea batatas (L.) Lam) revealed by simple sequence repeat markers. *International Journal of Advanced Biotechnology Research*, 7(1), 33-48.

Nery, M. C., Nery, F. C. and de Oliveira Pires, R. M. (2015). Tetrazolium test to evaluate the viability of oil radish seeds. *Bioscience Journal*, 31(3), p.663-671

Nitsch, J. P. and Nitsch, C. (1969). Haploid plants from pollen grains. *Science*, *163*(3862), 85-87.

Nonogaki, H., Bassel, G. W. and Bewley, J. D. (2010). Germination—still a mystery. *Plant Science*, 179(6), 574-581.

Nuñez-Palenius, H. G., Klee, H. J. and Cantliffe, D. J. (2006). Embryo-rescue culture of the 'Galia'muskmelon (*Cucumis melo L. var. reticulatus Ser*.) male parental line. *Plant cell, tissue and organ culture*, 85(3), 345-352.

Nyine, M. and Pillay, M. (2011). Breeding increases diversity of bananas. *Acta Hort*.

Offord, C. A. and Meagher, P. F. (Eds.). (2009). Plant germplasm conservation in Australia: strategies and guidelines for developing, managing and utilising ex situ collections. Canberra: Australian Network for Plant Conservation.

Ortiz R. 2013. Conventional banana and plantain breeding. Acta Hort; 986: 77–194.

Ortiz, R. (2011). Conventional banana and plantain breeding. In *VII International Symposium on Banana: ISHS-ProMusa Symposium on Bananas and Plantains: Towards Sustainable Global Production* 986 (pp. 177-194). Pillay 2002

Ortiz, R. and Swennen, R. (2014). From crossbreeding to biotechnology-facilitated improvement of banana and plantain. *Biotechnology Advances*, 32(1), 158-169.

Pancholi, N., Wetten, A. and Caligari, P. D. S. (1995). Germination of Musa velutina seeds: Comparison ofin vivo andin vitro systems. *In Vitro Cellular & Developmental Biology-Plant*, 31(3), 127-130.

Pandiyan, R., Manivannan, K. and Kumar, R. A. (2011). Effect of growth regulators and age of root stocks on the propagation of Jack through grafting. *Res. J. Agri. Sci*, 2(2), 214-243.

Parmar, K. P., Patel, M. J., Thakkar, R. M. & Tsomu, T. (2016). Influence of seed priming treatments on germination and seedling vigour of custard apple (*Annona squamosa L.*) cv. Local. Int. Quarterly J. Life Sci., 11(1) : 389-393.

Patel, R. J., Ahlawat, T. R., Singh, A., Momin, S. K. and Gavri, C. (2016). Effect of pre-sowing treatments on stone germination and shoot growth of mango (*Mangifera indica L.*) seedlings. *Int. J. Agric. Sci*, 8(52), 2437-2440.

Pereira, G. A., Santaella, M. B., Alves, L. M. S. M., Silva, E. C., Flenga, A. I. S. and Santos, D. M. A. (2018). Concentrations of 6-Benzylaminopurine (BAP) in micropropagation of banana '*Farta Velhaco*'(AAB). *Comunicata Scientiae*, 9(1), 58-63.

Pierik, R.L.M. 1987. In Vitro Culture of Higher Plants. Martinus Nijhoff Publishers. Pillay, M., Tenkouano, A. and Hartman, J. (2002). Future challenges in Musa breeding. *In: Products Press. New York.* pp. 223-252.

Pillay, M. and Tenkouano, A. (2011). Banana breeding: progress and challenges. CRC Press.

Pillay, M., Tenkouano, A. and Hartman, J., (2002). Future challenges in Musa breeding. In: Kang, M.S. (Ed.), Crop Improvement: Challenges in the Twenty-First Century. Food Products Press

Pinto, T. L. F., Marcos Filho, J., Forti, V. A., Carvalho, C. D. and Gomes, J. (2009). Evaluation of the Jatropha curcas L. seed viability by tetrazolium and X-ray tests. *Revista Brasileira de Sementes*, 31(2), 195-201.

Purseglove, J.W. (1972). Tropical Crops: Monocotyledons, Longman, London. Volume 9, pp. 334 and 273.

Puteh, A. B., Aris, E. M., Sinniah, U. R., Rahman, M., Mohamad, R. B. and Abdullah, N. A. (2011). Seed anatomy, moisture content and scarification influence on imbibition in wild banana (*Musa acuminata Colla*) ecotypes. *African Journal of Biotechnology*, 10(65), 14373-14379.

Rajendrakumar, P.M. (2017). Response of soaking time and chemicals on germination and growth of tamarind (*Tamarindus indica L.*). A thesis submitted to the navsari agricultural university

Reed, S. (2005). Embryo rescue. *Plant development and biotechnology. CRC Press, Boca Raton, Fl*, 235-239.

Rezende, R. G., de Jesus, L. L., Nery, M. C., de Souza Rocha, A., Cruz, S. M. and de Resende Andrade, P. C. (2015). Teste de tetrazólio em sementes de crambe. *Semina: Ciências Agrárias*, 36(4), 2539-2543.

Robinson, J. C. (1996). Bananas and plantains. 240 pp. *CABI, Wallingford, UK*.

Saad, A. I. and Elshahed, A. M. (2012). Plant tissue culture media. *Recent advances in plant in vitro culture*, 30-40.

Sabongari, S. and Aliero, B. L. (2004). Effects of soaking duration on germination and seedling growth of tomato (*Lycopersicum esculentum Mill*). *African Journal of Biotechnology*, 3(1), 47-51.

Sadik K., Arinaitwe, G., Rubaihayo, P.R., Kiggundu, A. and Mukasa S.B. (2014). Tdz and 4-cppu in gamborg b5 salts with ms vitamins doubles Embryogenic response from male flowers of ea-aaa banana. African Crop Science Journal, Vol. 22, No. 3, pp. 191 - 203

Schenk, R. U. and Hildebrandt, A. C. (1972). Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Canadian Journal of Botany*, 50(1), 199-204.

Schopfer, W. H. (1943). Plants and Vitamins, Chronica botanica. *Co., Waltham, Mass*.

Shaban, M. (2013). Effect of water and temperature on seed germination and emergence as a seed hydrothermal time model. *International Journal of Advanced Biological and Biomedical Research*, 1(12), 1686-1691.

Shabaq, M.N.A. (2013). The role of different concetrations of GA_3 on seed germination and seedling growth of loquat (*Eriobotrya japonica L.*). J. *Agric. Vet. Sci.,* 4(5): 03-06.

Shanmugavelu, K. G. (1966). A note on the effects of gibberellic acid on the root and shoot weights of some tree species. *Madras agric. J*, 54, 44-45.

Shareef, H. J., AL-Mayahi, A. M. and Alhamd, A. D. (2016). Effect of desiccation and cold hardening on germination of somatic embryos in date palm (*Phoenix dactylifera L.*) Berhi cultivar in vitro. *Adv. Appl. Sci. Res*, 7, 58-64.

Sharma, D. R., Kaur, R. and Kumar, K. (1996). Embryo rescue in plants—a review. *Euphytica*, 89(3), 325-337.

Shen, X., Gmitter, F. G. and Grosser, J. W. (2011). Immature embryo rescue and culture. In *Plant Embryo Culture* (pp. 75-92). Humana Press.

Silva, R. C., Souza Grzybowski, C. R., Barros França-Neto, J. and Panobianco, M. (2013). Adaptation of the tetrazolium test to assess the viability and vigor of sunflower seeds. *Pesquisa Agropecuária Brasileira* , 48 (1), 105-113.

Simmonds, N.W. (1959). Experiments on the germination of banana seeds. *Tropical Agriculture (Trinidad)*, 36, 259-273.

Simmonds, N.W. 1952. The germination of banana seeds. Trop. Agric. Trin., 37 : 211-221.

Sipen, P., Chubo, J. K., King, P. J., Huat, O. K. and Davey, M. R. (2011). Genetic improvement of banana using conventional and in vitro technologies. *Journal of crop improvement*, 25(6), 697-727.

Souza, C. R. D., Ohlson, O. D. C., Gavazza, M. I. A. and Panobianco, M. (2010). Tetrazolium test for evaluating triticale seed viability. *Revista Brasileira de Sementes*, 32(3), 163-169.

Ssebuliba, R. N., Tenkouano, A. and Pillay, M. (2008). Male fertility and occurrence of 2n gametes in East African Highland bananas (Musa spp.). *Euphytica*, 164(1), 53-62.

Ssebuliba, R., Talengera, D., Makumbi, D., Namanya, P., Tenkouano, A., Tushemereirwe, W. and Pillay, M. (2006). Reproductive efficiency and breeding potential of East African highland (Musa AAA-EA) bananas. *Field Crops Research*, 95(2-3), 250-255. Pillay 2002

Ssekiwoko, F., Tushemereirwe, W. K., Batte, M., Ragama, P. E. and Kumakech, A. (2006). Reaction of banana germplasm to inoculation with *Xanthomonas campestris pv musacearum*. *African Crop Science Journal*, 14(2), 151-155.

Steinbrecher, T. and Leubner-Metzger, G. (2017). The biomechanics of seed germination. *Journal of Experimental Botany*, 68(4), 765-783.

Swamy, G. S. K., Patil, P. B., Athani, S. I. and Prabhushankar, D. S. (1999). Effect of organic and inorganic substances on germination of Jamun (*Syzygium cumini*) seeds. *Advances in Agricultural Research in India*, 11, 89-91.

Swennen, R., Blomme, G., van Asten, P., Lepoint, P., Karamura, E., Njukwe, E., Njukwe, E., Tinzaara, W., Viljoen, A. and Karangwa P. (2013). "Mitigating the impact of biotic constraints to build resilient banana systems in Central and Eastern Africa," in *Agro-Ecological Intensification of Agricultural Systems in the African Highlands*, eds B. Vanlauwe, P. van Asten, and G. Blomme (New York, NY: Routledge), 85–104.

Taşkın, H., Kemal Yücel, N., Baktemur, G., Çömlekçioğlu, S., & Büyükalaca, S. (2013). Effects of different genotypes and gamma ray doses on haploidization with irradiated pollen technique in watermelon (*Citrullus lanatus L.*). *Canadian Journal of Plant Science*, 93(6), 1165-1168.

Thomas, K. M. (1974). Influence of Certain Physical and Chemical Treatments on the Germination and Subsequent Growth of Coconut *Cocos nucifera. L.* Seedlings: A Preliminary Study. *East African Agricultural and Forestry Journal*, 40(2), 152-156.

Tinzaara, W., Kiggundu, A., Gold, C. S., Tushemereirwe, W. K. and Karamura, E. B. (2009). Management options for highland banana pests and diseases in East and Central Africa. *Outlooks on Pest Management*, 20(5), 204.

Tripathi, L., Tripathi, J. N. and Kubiriba, J. (2016). Transgenic technologies for bacterial wilt resistance. In *Banana: Genomics and transgenic approaches for genetic improvement* (pp. 197-209). Springer, Singapore

Tripathi, L., Tripathi, J. N. and Vroh-Bi, I. (2007). Bananas and plantains (*Musa spp.*): Transgenics and biotechnology. *Transgenic Plant Journal*, 1(1), 185-201.

Tukey, H. B. (1933). Artificial culture of sweet cherry embryos. *Journal of Heredity*, 24(1), 7-12.

Uma, S., Lakshmi, S., Saraswathi, M. S., Akbar, A. and Mustaffa, M. M. (2011). Embryo rescue and plant regeneration in banana (*Musa spp*.). *Plant Cell, Tissue and Organ Culture (PCTOC)*, 105(1), 105-111.

Vachhani, K. B., Gohil, J. H., Pandey, R. and Ray, N. R. (2014). Influence of chemicals, PGR's and cow-dung slurry as seed treatment on germiability, growth and development of khirnee (*Manilkara hexandra Roxb*) under net house condition. *Trends in Biosciences*, 7(14), 1641-1643.

van Asten, P. J., Fermont, A. M. and Taulya, G. (2011). Drought is a major yield loss factor for rainfed East African highland banana. *Agricultural water management*, 98(4), 541-552.

Vasantha, P. T., Vijendrakumar, R. C., Guruprasad, T. R., Mahadevamma, M. and Santhosh, K. V. (2014). Studies on effect of growth regulators and biofertilizers on seed germination and seedling growth of tamarind (*Tamarindus indica L.*). *Plant Archives*, 14(1), 155-160.

Viloria, Z., Grosser, J. W. and Bracho, B. (2005). Immature embryo rescue, culture and seedling development of acid citrus fruit derived from interploid hybridization. *Plant cell, tissue and organ culture*, 82(2), 159-167.

Vineesh, P. S., Skaria, R., Mukunthakumar, S., Padmesh, P. and Decruse, S. W. (2015). Seed germination and cryostorage of *Musa acuminata* subsp. burmannica from Western Ghats. *South African Journal of Botany*, 100, 158-163.

Vuylsteke, D. and Swennen, R. (1993). Genetic improvement of plantains: the potential of conventional approaches and the interface with *in-vitro* culture and biotechnology. In Biotechnology Applications for Banana and Plantain Improvement, (ed. INIBAP), pp. 169-176.

White, P. R., 1963. The cultivation of animal and plant cells. The Ronald Press, New York.

Zhang, C., Wu, J., Fu, D., Wang, L., Chen, J., Cai, C. and Ou, L. (2015). Soaking, temperature, and seed placement affect seed germination and seedling emergence of Litchi chinensis. *HortScience*, 50(4), 628-632.