MALE FERTILITY AND ZYGOTIC EMBRYO GERMINATION IN BANANAS

BY

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DECLARATION

This thesis titled **Male fertility and zygotic embryo germination in bananas** 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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To the Almighty God who has enabled me through and to my family for their encouragement and support.

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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
NaCRRI	:	National Crops Resources Research Institute
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
FCR		Fluorochromatic reaction
MC		Manual counting

ABSTRACT

Crop improvement through crossbreeding relies on the generation of new hybrids from seeds. Therefore, seed set and seed germination are key determining factors for a successful breeding program. In banana breeding, both seed set and seed germination are low, especially in triploid and tetraploid bananas. To improve germination, embryos are extracted from seeds and cultured *in vitro*. Seed set is determined by the quantity and viability of pollen for the male parents, or male fertility, and by the receptibility of the female for the pollen, or female fertility.

In this study, we tested the male fertility in terms of pollen quantity and viability of the main diploid genotypes (Calcutta 4, Malaccensis 250, TMB2x 7197-2, SH 3217, 10969S-1, Opp Zebrina, cv. Rose, TMB2x 5265-1, SH 3362, TMB2x 8075-7, Kokopo and TMB2x 9128-3) used as male donors of resistance to pest and disease resistance in banana breeding. Pollen quantity was determined by digital imaging and subsequent image analysis of pollen grains using imageJ software. Pollen viability was tested by soaking the pollens in 2,3,5- triphenyl tetrazolium chloride (TTC) stain followed by counting the stained pollen. We also investigated the optimal soaking time by soaking seeds in water for 0, 3, 5, 7, 9 days. 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. The effect of hormones on the germination of banana embryos was also determined using 6-Benzylaminopurine (BAP) and Gibberellic acid (GA_3) *in vitro*. 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.

The results indicated highly significant ($p<0.001$) difference among the genotypes for both pollen quantity and viability. Genotype and month interaction significantly ($p<0.001$) influenced both pollen quantity and viability. The pollen mean quantity was generally high in all genotypes

ranging from 2588 to 28252 pollen grains per three anthers of a plant. The month influenced pollen quantity in SH 3217 and cv. Rose. Pollen viability (mean percentage) ranged from 42.69 to 99.67%. The genotypes less affected in terms of pollen viability were Calcutta 4, cv Rose, Kokopo, Opp Zebrina, SH 3362 and TMB2x 7197-2. Soaking seeds for 3 days significantly increased embryo germination success by 16.2% than in other tests. The addition of BAP and GA_3 hormones into culture medium did not improve embryo growth but positively affected growth parameters in all genotypes. In conclusion, the observed differential responses in pollen quantity and viability for individual genotypes could be attributed to changes in physiological processes in order to cope with the constant fluctuations in the prevailing environmental conditions a term called genotypes by environment interactions. 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: Bananas, embryos germination, growth hormones, MS medium Pollen quantity, pollen viability, seed dormancy, soaking.

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 is an 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 lower the economic value of the crop, which 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 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 caused by *Fusarium oxysporum* f. sp. *cubense* affects mostly dessert bananas such as Gros Michel, Sukali Ndiizi, and some ABB triploids (Arinaitwe *et al*., 2019). With regard to bacterial diseases, Banana *Xanthomonas* wilt (BXW) caused by *Xanthomonas campestris* pv. *musacearum* is restricted to a few countries in Africa. The disease 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 first steps in conventional cross breeding in the banana programs are hybridization and selection of recombinants at the diploid level (Novak, 1992). The selected diploids which are disease resistant and pollen fertile (improved) are involved in the process of 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), poor male and female fertility, parthenocarpic fruit development through seeds, 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 improved 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). In order to successfully generate hybrids, it is important to ascertain the pollen fertility by determining the quality and quantity of the male diploids and the factors affecting them to understand the variation in seed set observed during conventional crossbreeding schemes (Stone *et al.,* 1995).

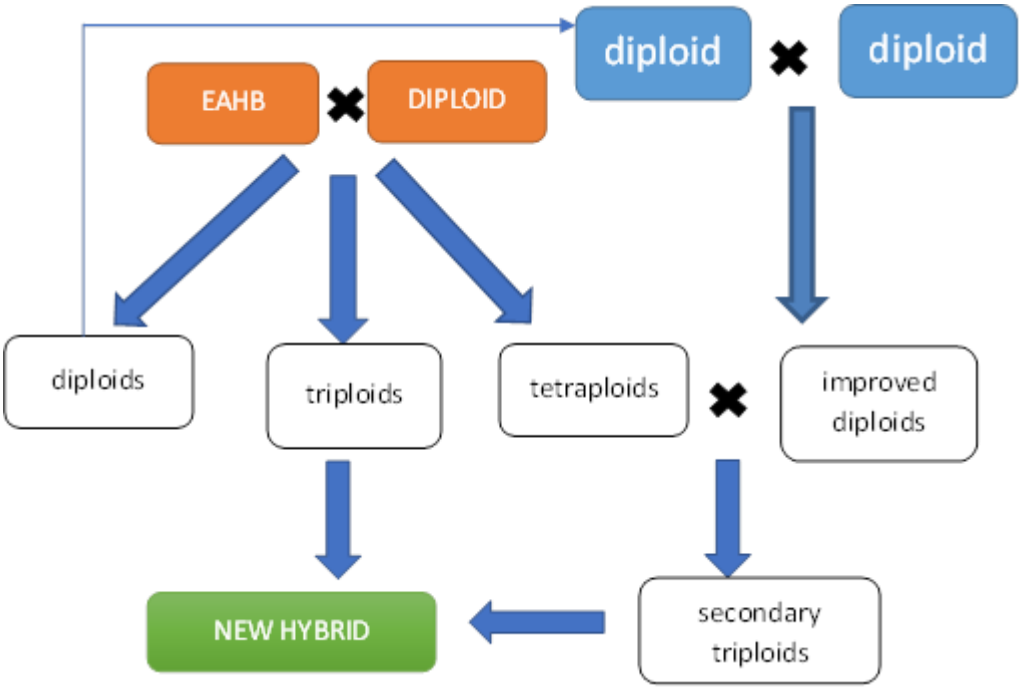


Figure 1: Banana breeding process Source: Brown *et al*., 2017

1.3 Problem statement

For seed set to take place in banana, fertilisation has to occur. This can be enhanced by high pollen quality and quantity for successful fertilisation. Hence pollen grains should have the ability to deliver the sperm cells to the embryo sac. Some studies have been conducted on the *in vitro* germination of banana pollen especially in diploid species (Nyine and Pillay, 2007). However, little information is available on the quantity and viability of pollen from the improved diploids used in the breeding scheme at IITA and on the effect of varying weather on the quantity and viability of this pollen. Furthermore, seeds formed after fertilisation do not easily germinate due to factors like dormancy. Embryo 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 also 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 estimating the quantity and viability of pollen amongst the improved diploids and also to understand 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

Conventional banana breeding involves making crosses between selected genotypes in order to generate seeds which germinate and grow to become hybrids. However, for fertilisation to take place after pollination, the pollen should be viable and of good quantity. Breeders are dedicated to producing new varieties that are resistant to pests and diseases using improved diploids as male parent in the breeding programme. However, efforts of breeders can be realized with greater efficiency if the pollen used in pollination are viable and the resulting seeds germinate into plantlets to become hybrids (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 assessing pollen quality and quantity of the main diploid male parents and enhancing the germination rate of banana zygotic embryos.

1.5.1 Specific objectives:

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

1.5.2 Hypothesis

i. Pollen quantity and viability are high in the male diploid banana genotypes
ii. Soaking of banana seeds in distilled water prior to embryo excision can enhance the germination rate of zygotic embryos.
iii. The addition of either GA3 or BAP or a combination of both in the culture medium may either improve banana zygotic embryo germination or 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) 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).

Most flowering plants have hermaphrodite flowers, i.e. each flower has female and male organs, both of which are functional. While a banana flower also has both female and male organs, the relative development of these organs determines whether the flower is female and develops into fruit, or male and produces pollen (Kirchoff, 2017). Banana female flowers have a massive style, stigma and stamens which are reduced to staminodes that do not produce pollen grains. The style

and stigma function in fertilization, which leads to the formation of the banana fruit. The stigma is the receptive tip of the carpel, which receives pollen and on which the pollen grains germinates. When the pollen grains land on the stigma, it germinates into the pollen tube guided down the stylar canal to one of the locules where the ovules are located. The pollen tube enters the micropylar to fertilize the ovules. In the wild species, the fruits contain seeds if the flower is pollinated. In edible banana, the ovary develops into seedless fruit by parthenocarpy. The tiny black dots in the centre of the fruit are the remnants of the ovules.

A male banana flower is composed of a slender style and stigma, and well developed anthers which contain pollen especially in the wild species while in the edible bananas, the amount of pollen is absent or reduced. The anther and the filament form a stamen. A male banana flower typically has five stamens (Buddenhagen, 2008). The male flowers are contained in the male bud in which they are arranged in clusters called hands (Figure 2b). Each hand is enfolded by a bract that lifts when the flowers have finished developing (Figure 2a). Male flowers usually fall to the ground a short time after flowering.

Figure 2: Image of a male bud (a), cluster of male flower (b) and a male flower with pollen grains (c) Source: Jane Luyiga, IITA, 2018

East African Highland Bananas are triploid bananas comprising of 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 (pollen donators) to produce triploids (Figure 1; Brown *et al*., 2017). The whole process involves acquiring pollen grains, pollination, fertilization and the production of seeds that are germinated to obtain hybrids.

2.2.1 Pollen grains in bananas cultivars

The critical factors that affect fruit setting in the plant kingdom include pollination and fertilization (Oselebe *et al*., 2014). For fertilization to be effective, pollen grains should have the ability to perform their functions of delivering the sperm cells to the embryo sac following compatible pollination (Shivanna *et al*., 1991). The barriers affecting fertilization may include variability in pollen production, poor or malfunctioning of pollen grains among genotypes. The ability of pollen grains to effect fertilization is dependent on their viability. Diploid bananas for example Calcutta 4 and Malaccensis are found to have resistant traits to diseases and they have been used in the breeding programs (Pillay *et al*., 2012) however their agronomic traits are not favorable to the farmer's need because of the bunch size and fruit quality. It is important to know information on pollen viability and quantity in diploids used in the research program. It is imperative to know the extent of viability and quantity of the pollen sample to be used for pollination.

2.2.2 Test for pollen viability

Pollen viability is the ability of pollen to complete post pollination events and to effect fertilization. Direct and indirect methods are used to determine pollen viability either *in vitro* (Peng *et al*., 2015) or in vivo (Soares *et al*., 2014; Souza *et al*., 2015). The indirect method is through staining (Melloni *et al*., 2013; Souza *et al*., 2015). Staining with non-vital stains may be useful to determine the degree of pollen viability in plants (Alexander 1969, 1980). The first method for testing pollen involves determining viability by staining. Fresh pollen and dead pollen from plants can be tested using different stains. For instance, X-gal test to determine the content of β-galactosidase, an enzyme involved in the lactose degradation (Atiaksheva *et al*., 2000). The X-gal test consists of a solution of 1 mg Xgal (5-bromo-4-chloro-3-indoyle-β-galactoside) that is dissolved in 50 μL N,N-dimethyl formamide and 1 mL acetate buffer (50

mmol with pH 4.8). Viable pollen turns blue. 2,3,5-triphenyltetrazolium chloride (TTC) test is also used to estimate pollen viability and it closely mimics *in vitro* germination (Bolat and Pirlak, 1999; Huang *et al*., 2004; Abdelgadir *et al*., 2012). The assessment of viability using TTC is based on the color change of the tissues in the presence of a salt solution of 2,3,5-triphenyltetrazolium chloride, which is reduced by dehydrogenase respiratory enzymes in live tissues, resulting in a red carmine-colored compound called formazan (Beyhan and Serdan, 2008) and either dead or non-viable tissues that are not dyed (Figure 3). Several studies show that staining with TTC allows a more reliable estimate of pollen viability, nearer to that provided by *in vitro* germination tests (Huang et al., 2004; Abdelgadir et al. 2012). The fluorochromatic reaction (FCR) test can also be used to determine the esterase activity and the intactness of the cell membrane. The fluorochromatic reaction test consists of fluorescein diacetate dissolved in acetone (2 mg mL-1) and is used on 10-6 mol L-1 in 0.8 mol L-1 sucrose. The pollens are then investigated under a fluorescence microscope (Wang *et al*., 2004).

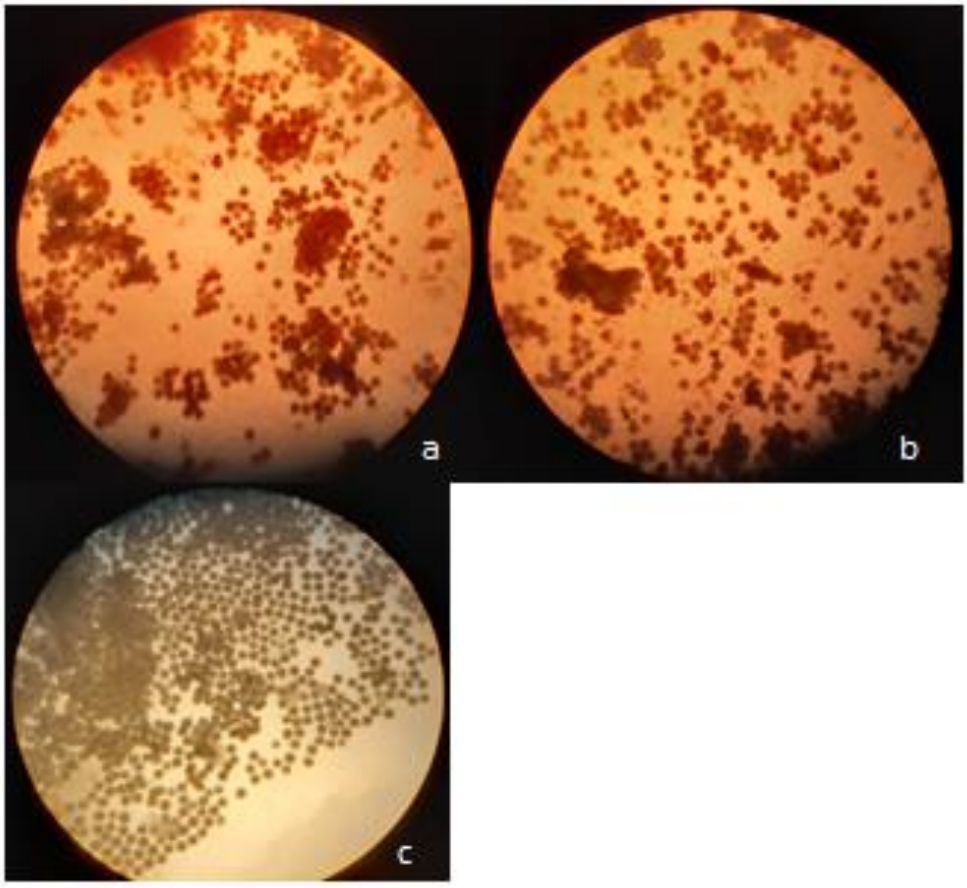


Figure 3: Image showing stained pollen grains (a and b) and non-stained pollen grains(c)

Source: Jane Luyiga, IITA, 2018

The pollen grains can be stained with non-vital stains such as acetocarmine, iodine in potassium iodide, and a test with aniline blue. Acetocarmine is a dye which reacts with chromosomes by binding them to give a red colour when viable and the others unstained unviable. The specific nuclear stain for example feulgen reacts with chromosomes to give them the colour components of the cytoplasm of certain cells a less intense red. This test measures the integrity of the cytoplasm; the pollen grains get stained red when the cytoplasm membrane is integral (Ordoñez, 2014). The Lugol solution consisting of iodine and potassium iodide can also be used to test pollen viability. Lugol solution detects starch content in the pollen and viable pollen turns black.

Aniline blue can be used for detection of callose in pollen walls and tubes. The aniline blue in lactophenol solution contains 5 mL 1% aqueous aniline blue, 20 mL phenol, 20 mL lactic acid, 40 mL glycerine and 20 mL water. Viable pollen turns distinct blue. However, aniline blue in lactophenol essentially imparts color to the contents of the pollen in fresh as well as dead pollens. It is therefore necessary to carefully chose stains and apply different tests for a given pollen system and find out the one that reflects true viability.

2.2.3 Germination test

This method considers the germination of pollen tube germination. There are two major tests, which can be divided in two different parts. In *in vitro* germination, pollen is grown on a specific media. In *in vivo* germination pollen is grown on the stigma of the plant.

2.2.3.1 In vitro germination for pollen viability

In vitro is a method that simulates the conditions of style-stigma inducing germination and growth of the pollen tube (Soares *et al.*, 2008). The success of *in vitro* pollen germination depends on the genotype, environmental conditions, pollen maturity, nutritional state, season of the year, time of collection, photoperiod, temperature, collection method, incubation period and culture medium, besides adjustments in the composition of the medium for each species (Sharafi, 2010; Moura *et al.*, 2015). The procedure requires much less time than the in vivo approach and is adapted to routine screening of many samples. However, its potential ability to predict pollen performance depends heavily on optimization of the germination medium and temperature and on the availability of an adequate amount of pollen in the tested sample (Heslop-Harrison *et al*., 1984). Fresh harvested pollen is grown on a medium containing sucrose, boric acid and calcium nitrate. These compounds have been shown to be very important for pollen germination in

different species. The pollen is considered mature when the pollen tube length is longer than the diameter of the pollen grain (Wang *et al*., 2004).

The composition of a germination medium to obtain optimal responses has to be empirically formulated for each species. For many pollen systems only three constituents, namely sucrose, boric acid and Calcium nitrate are sufficient whereas the optimum concentration of sucrose required varies with the species, 100mg/l, boric acid and 300mg/l Calcium nitrate are optimal for most species studied. Some of the pollen culture medium standardized by different investigations were Brewbaker and Kwack's medium (Brewbaker and Kwack, 1963); found suitable for some 86 species, Robert's medium (Roberts *et al.,* 1983); standardized for Brassica oleracea and Hodgkin and Lyon's medium (Hodgkin and Lyon, 1986) also standardized for Brassica.

2.2.3.2 In vivo germination for pollen viability

The in vivo approach involves placing the pollen on the stigmas of emasculated flowers and determining the number of pollen tubes in squashed styles (van Koot and van Rovestijin, 1963; Abdalla and Verkerk, 1968; Dempsey, 1970) or the number of seeds in the mature fruit (McGuire, 1952; El Ahmadi and Stevens, 1979; Maisonneuve and Philouze, 1982). These methods are time-consuming and therefore impractical for testing many samples. However, the advantage is that they consider all natural conditions of pollen grains germination on stigma (Albert *et al*., 2018). Furthermore, seed set may depend not only on fertilization, but also on the post-pollination development of the ovary, pistil receptivity, and incompatibility reactions (Heslop-Harrison *et al*., 1984; Berry and Uddin, 1988).

2.2.4 Test for pollen quantity

The quantity of pollen can be obtained using different methods including manual count, electronic particle counter and imaging using software.

2.2.4.1 Manual counting (MC) of pollen grains

The number of pollen grains is counted using a haemocytometer (a slide with grids where a specified volume of the sample is put) under a microscope. This method gives highly repeatable and accurate results but it is extremely time consuming and laborious (Mudd and Arathi, 2012). The sample requires to be counted twice, first count for viable pollen and the second one for non-viable.

2.2.4.2 Electronic particle counters

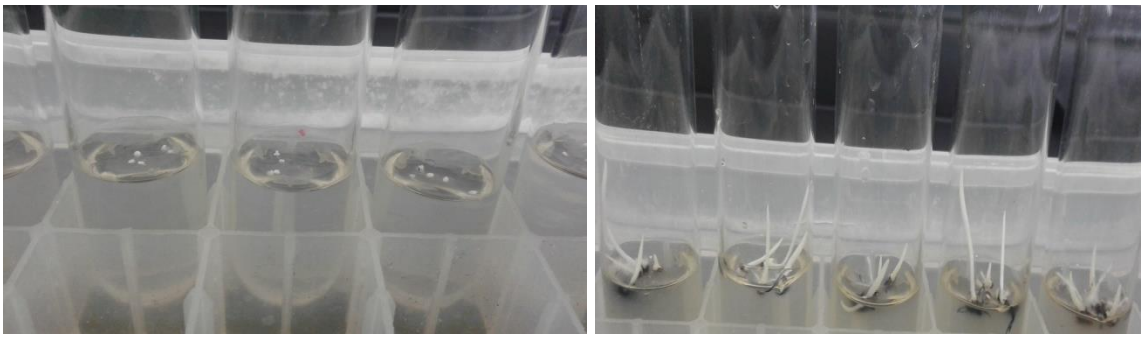
The use of particle counters for distinguishing viable from non-viable pollen grains skips the need for staining of pollen grains because the counters can differentiate objects based on size. This method however, has to be preceded by a preliminary study that measures the size of viable and inviable pollen in each of the target species by staining and manual counting (Kelly et al., 2002). This preliminary step generates two distinct size distributions for viable and non-viable pollen and determines the extent of overlap in their diameters. The counters require a pre-set aperture for a given shape and a narrow size range and can therefore only distinguish pollen based on the diameter. Thus, particle counters are likely to miss many pollen grains, especially the inviable ones that do not fit the defined size measures.

2.2.4.3 Labeling and imaging

Pollen is suspended in a small volume of medium and laid on microscope slides for imaging. The solutions used during this process are prepared before use and there is no exposure to light For acquiring the images. A Nikon Eclipse E400 epi-fluorescent microscope with B-2A filters and a Nikon Digital Sight DS-U1 camera system are used. Images are taken at a resolution of 640×480 pixels and $100 \times$ magnification, from three microscope slides for each taxon and pooled together for the following analysis.

2.3 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). 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 4; McGahan, 1961).



a b

Figure 4: The banana embryos on media (a) and the germinating embryos (b)

Source: Jane Luyiga, 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.

2.3.1 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^{o}C to 30^{o}C (Uma *et al*., 2011; Dayarani *et al*., 2014).

2.3.2 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.

2.4 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. 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.

2.5 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.

2.6 Methods of improving banana seed germination

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

2.6.1 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).

2.6.2 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).

2.7 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.

2.7.1 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					
$MgSO_4 7H_2O$	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
$CaCl_2 2H_2O$	-	440	150	166	-
$(NH_4)_2SO_4$	-	-	134	463	-
Micronutrients					
H3BO3	1.5	6.2	3	1.6	-
$MnSO_4 4H_2O$	5	22.3	-	4.4	2.5
$MnSO_4H_2O$	-	-	10	3.3	-
$ZnSO_4 7H_2O$	3	8.6	2	1.5	10
$Na_2MoO_4 2H_2O$	-	0.25	0.25	-	0.25
$CuSO_4 5H_2O$	0.01	0.025	0.025	-	0.025
$CoCl_2 6H_2O$	-	0.025	0.025	-	0.025
Kl	0.75	0.83	0.75	0.8	-
$FeSO_4 7H_2O$	-	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	3	2	-	-	2
Folic acid	-	-	-	-	0.5
Biotin	-	-	-	-	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/

2.7.2 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.

In conclusion, bananas are improved through different processes among them selecting male and female parents with good traits, collecting pollen and pollinating at the right time, to enable the embryos to germinate as many as possible to generate new hybrids which are later evaluated and screened for further advancement. To study the pollen production and its viability is essential for breeders to determine the seed set from different diploids used in breeding and their capability to germinate. The methods used in this study to evaluate pollen production are pollen imaging and for viability, is staining using TTC. To improve embryo germination, soaking banana seeds prior to excision and the use of BAP and GA incorporated into media was also studied.

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. The programme works on the improvement of EAHB. Sendusu is located about 28 km North-East of Kampala in Central Uganda, between 0°32′N and 32°34′E, at an altitude of 1,200 m above sea level. The place receives an annual rainfall of about 1,377 mm, bi-modally distributed; the main wet season being March-May and September-November with dry season being June-August, and minimum and maximum temperature of 16 ^{o}C and 28 ^{o}C respectively (IITA, 1991). The pollen used in this study was collected from the banana pollination plots of the programme that were established for use in cross pollination. The pollen quantity and viability and the seed germination studies were conducted under standard tissue culture laboratory conditions at the IITA banana breeding laboratory at Sendusu.

3.2 Study 1: Determining the pollen viability and quantity of diploid banana genotypes

The pollen study was carried out between May and August 2018. Initially, twelve diploid genotypes that are frequently used as males in banana breeding pipeline were studied for pollen quantity and viability. The criterion for choosing the male parents was based on important traits including resistance to pests and diseases, yield traits, quality traits and stature (Table 2). To investigate pollen quantity and viability among the twelve diploid banana genotypes, three plants per genotype were randomly selected from the diploid pollination field. Male flower clusters were collected from each of the three random plants per genotype between 8:00 am and 10:00am and immediately taken to the laboratory to prevent the sticky pollen grains from drying out from

the sun heat. The study was executed at the end of the first rainy season and during dry season from May to August 2021 (Figure 5). For each month, samples were collected from individual genotypes at approximately mid-months over three days. The daily weather records of temperature, rainfall and relative humidity were obtained from the meteorological department, Namulonge station (NaCRRI) (Figure 5)

Table 2 Male diploid banana genotypes used in the pollen study

Genotype	ITC code[a]	Ploidy	Key traits[b]	Type	Pedigree
Calcutta 4	ITC0249	2x	Resistant to banana weevil, nematodes, black Sigatoka and high level of male fertility	Wild	-
Malaccensis 250	ITC0250	2x	Resistance to black sigatoka and high level of male fertility	Wild	-
TMB2x 7197-2	-	2x	Resistance to Black sigatoka, resistance to nematode and big bunch.	Improved diploid	SH 3362 x Long Tavoy
SH 3217	-	2x	Big bunch.	Improved diploid	SH 2095 x SH 2766
10969S-1	-	2x	Black sigatoka tolerance, has matooke qualities.	Improved diploid	376K-7 x TMB2x 5105-1
Opp Zebrina (IITA hybrid 2145/1320)	ITC1448	2x	Dwarfism.	Cultivar	-
cv. Rose	ITC0712	2x	Parthenocarpic fruits, resistance to fusarium wilt race 1 and tolerance to black sigatoka.	Cultivar	-
TMB2x 5265-1	-	2x	Black sigatoka resistance	Improved diploid	Tjau lagada x Calcutta 4

SH 3362	-	2x	Big bunch, resistance to nematodes and pathenocarpic fruits.	Improved diploid	SH 3217 x SH 3142
TMB2x 8075-7	-	2x	Resistance to black sigatoka, resistance to nematode resistance (Radopholus similis) and big bunch.	Improved diploid	SH 3362 x Calcutta 4
Kokopo	ITC1243	2x	Yellow palp colour, early maturity, short stature and pathenocarpic fruits.	Cultivar	-
TMB2x 9128-3	ITC1437	2x	Big bunch and pathenocarpic fruits, bunch, resistance to fusarium wilt race1.	Improved diploid	Tjau lagada x Pisang lilin

[a]International Musa Germplasm Transit Centre, https://www.crop-diversity.org/mgis/,
[b]Reference: IITA banana breeding progamme, Sendusu/Namulonge

Figure 5: Daily weather records on rainfall and temperature condition from March to August 2021 (Uganda National Meteorological Authority – Station Namulonge). The arrows show the exact sampling time

3.2.1 Pollen quantification test

Pollen grains from the three anthers of the middle flower of the cluster per plant were scraped off using a scalpel blade and immediately put onto a microscope slide. A drop of detergent solution (two drops of tween 20 dissolved into 250 ml of distilled water) was added to disperse the pollen grains. For each slide, an image was captured at X4 (PL4/0.10) magnification using a digital GXCAM-U3-18 camera connected to the GXML2800 compound microscope. The camera was connected to the laptop, with the installed camera settings. The images (JPEG format) were imported into imageJ software for pollen quantification following the methodology described by Rasband (1997-2009) which provides reliable counts of pollen grains numbers (Costa and Yang, 2009). Each image was individually processed to remove noise and enhance individual pollen grains for accurate counting.

3.2.2 Pollen viability test

One flower was picked from the middle part of each cluster. Pollen grains were removed from three anthers with the help of the scalpel blade onto a glass slide. 1% 2,3,5- TTC was used as the staining agent. Two droplets of the stain were added and a cover slip mounted on to the glass slide. The slides were incubated for about two hours at room temperature. The slides were later observed under bright-field illumination (x 4 magnification) using Olympus AX70 compound microscope (Olympus optical CO. Ltd, Taiwan).

3.2.3 Statistical data analysis

The data of pollen quantity and pollen viability were analysed using GenStat 20th Edition. For both traits, the data sets were subjected to normality test. The data was subjected to two-way analysis of variance (ANOVA) using the model below (Eq1). Multiple comparisons of treatment means were done using Fisher's least significant difference (LSD) test.

$$y_{ijk} = \mu + n_i + m_j + p_k + n/p_{ik} + nm_{ij} + e_{ijk} \quad \text{Eq.1}$$

Where y_{ikj} is the observed pollen quantity and viability, μ is the overall mean, n_i is the main effect of the genotype, m_j is the main effect of the month, p_k is the main effect of plant, n/p_{ik} is the main effect of plant nested within the genotype, nm_{ij} is the interaction effect of genotype and month and e_{ijk} is the random error.

3.3 Study 2: Determining the effect of soaking seeds in distilled water prior to embryo excision on the germination rate of zygotic embryos of banana seeds

3.3.1 Cross-pollination to generate seeds used in the study

Four female-male combinations were used in this study, including two 2*x* selfing crosses and two 4*x* - 2*x* crosses (Table 3). Tetraploids and diploids were used as females while only diploids served as male parents. Hand pollination was carried out in the field including three diploid self-pollinations and three cross-pollination events per cross-type. Pollination was carried out between September, 2018 and May, 2019 for all crosses.

Table 3 Banana crosses used to generate seeds for 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 4 months and 6 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.

3.3.2 Seed viability test before the soaking experiment

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 2.

$$Viability\ (\%) = \frac{Red\ embryos + Pink\ embryos}{Total\ number\ of\ embryos}\ x\ 100 \qquad \text{Eq. 2}$$

3.3.3 Seed soaking treatment in distilled water

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 tube (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 4) 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. The experiment was laid in a completely randomized design.

3.3.3.1 Data collection and statistical analysis

Germinated embryos were recorded when shoots emerged to about 1cm above the medium. The number of germinated embryos was cumulatively recorded weekly for a maximum of eight weeks. Germination percentage was calculated and adjusted according to the embryo viability by using the equation below;

$$Adjusted\ germination\ (\%) = \frac{Number\ of\ germinated\ embryos}{Number\ of\ cultured\ embryos\ x\ viability}\ x\ 100 \qquad \text{Eq. 3}$$

The data were subjected to analysis of variance (ANOVA) using the model below in Eq 4. Fisher's least significant difference (LSD) test was used to compare means.

$$y_{ij} = \mu + c_i + s_j + cs_{ij} + e_{ij} \qquad \text{Eq.4}$$

Where y_{ij} is the observed embryo germination rate, μ is the overall mean of germination rate, c_i is the main effect of the cross, s_j is the main effect of the level of soaking, cs_{ij} is the effect of the interaction between cross and soaking and e_{ij} is the random error.

3.4 Study 3: Establishing the optimal BAP and GA_3 hormonal concentration for germination of banana zygotic embryos

Banana seeds were soaked in water using the optimum days of soaking determined from study two. The seeds were sterilised and embryos excised as described in study two. 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 two, 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 two 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.

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

3.4.1 Data collection and statistical analysis

As in the first study, germinated embryos were counted weekly for eight weeks. The number of germinated embryos was recorded weekly for a maximum of eight weeks. The growth parameters (plantlet height, girth, and number of roots) of germinated embryos were measured and recorded. Germination percentage was calculated and adjusted to the viability of the seed lot as in Equation 3. The data was subjected to analysis of variance (ANOVA) function as applied in GenStat 20th Edition. Multiple comparisons of treatment means were done using Fisher's least significant difference (LSD) test. The linear model below was used in the analysis;

$$y_{ijkl} = \mu + c_i + b_j + g_k + bg_{ij} + e_{ijk} \quad \text{Eq.5}$$

Where y_{ijk} is the observed embryo germination, μ is the overall germination mean rate, $c_{i.}$ is the main effect of the cross, b_j is the main effect of the BAP concentration, g_k is the main effect of the 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;

$$y_{ij} = \mu + b_i + g_j + bg_{ij} + e_{ij} \quad \text{Eq.6}$$

where yi is the observed growth parameter (plant height, number of roots and stem girth) on a medium with ith BAP and jth 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 Pollen quantity among male diploid genotypes

In this study, results indicated highly significant ($p<0.001$) difference among the genotypes for pollen quantity. However, months of sampling had no significant ($p=0.186$) effect on the quantity of the pollen. The interaction between genotype and month significantly ($p<0.001$) influenced pollen quantity (Table 5). Across sampling periods, pollen was observed to degrade easily especially for Zebrina OPP. The observed degradation was observed in about two minutes of exposure to the detergent solution. This was observed after recording the data and never affected our recorded results for Zebrina OPP. Nevertheless, the highest value for pollen quantity was recorded for Calcutta 4 (22,929) and Malaccensis 250 (21,420) and they were statistically in the same range. The lowest pollen quantity was recorded for the improved diploids 10969-1 (9,135), TMB2x 9128-3 (8,608) and TMB2x 5265-1 (6,730). However, these were not significantly different from SH 3362, Kokopo, SH 2317 and OPP Zebrina (Figure 5). Individual genotypes influenced by months were SH 3217 and cv. Rose. The results indicated that Calcutta 4 and Malaccensis 250 produce three times as much pollen as TMB2x 5265-1 and three times as much as TMB2x 9128-3. The pollen of Calcutta 4, cv. Rose, TMB2x 8075-7, SH 3362 and Malaccensis 250 consistently demonstrated high pollen quantity (Figure 5).

Table 5: ANOVA to test pollen quantity among male diploid banana genotypes for four months

Source of variation	d.f.	m.s.
Genotype	11	3.105E+08***
Month	3	4.133E+07^{ns}
Genotype/Plant	24	4.768E+07*
Genotype x Month	33	6.395E+07***
Residual	69	2.506E+07
CV%		36.6

d.f=degree of freedom, m.s = Mean squares, CV = Coefficient of variation, x = Interaction between month and genotypes

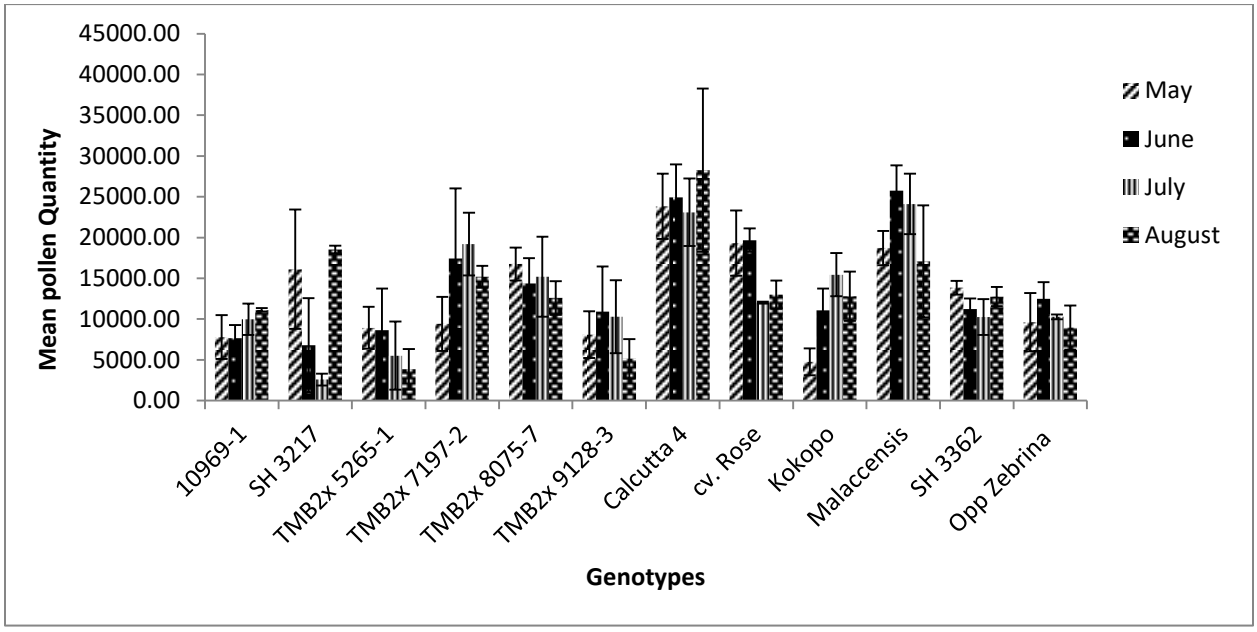


Figure 6: Mean pollen quantity of the twelve male diploid banana genotypes in different months

4.2 Pollen quality among male diploid genotypes

In our study, the time (months) of sampling pollen from genotypes as well as genetic differences significantly ($p<0.001$) influenced pollen viability. Also, the interaction between genotype and month significantly ($p<0.001$) influenced pollen viability (Table 6). The overall percentage viability was significantly higher in June (92.97) and significantly lower in August (76.17). Percentage pollen viability in May (83.89) and July (83.25) was significantly intermediate

between June and August (Figure 7). Based on the four viability categories [high (85-100%), Moderate (70-84%), Lower (50-69%) and poor (<50%)], genotypes generally performed highly in May, June and July, with a general decline in August (Figure 6). June had the highest viability with two categories (high and moderate), followed by July with three categories (High, moderate and lower), yet May and August had all the categories. Based on our results, it was evident that pollen viability was generally above 70% across genotypes except genotypes SH 3217 and TMB2x 8075-7 that generally recorded viability that was below 70% between May and August. Individual genotypes that were less influenced by months were TMB2x 7197-2, Calcutta 4, cv. Rose, Kokopo, Malaccensis, Opp Zebrina and SH 3362. It was difficult to ascertain the effect of weather patterns on pollen viability during the study period. From May to August, trace amounts of rainfall were recorded and temperatures fluctuated more frequently (Figure 5 Chapter 3).

Table 6: ANOVA for pollen viability among male diploid banana genotypes for four months

Source of variation	**d.f.**	**m.s.**
Genotype	11	3900.17***
Month	3	6836.04***
Genotype/Plant	24	105.12^{ns}
Genotype x Month	33	1579.65***
Residual	504	74.24
CV%		10.2

d.f=degree of freedom, m.s = Mean squares, CV = Coefficient of variation, x = Interaction between month and genotypes

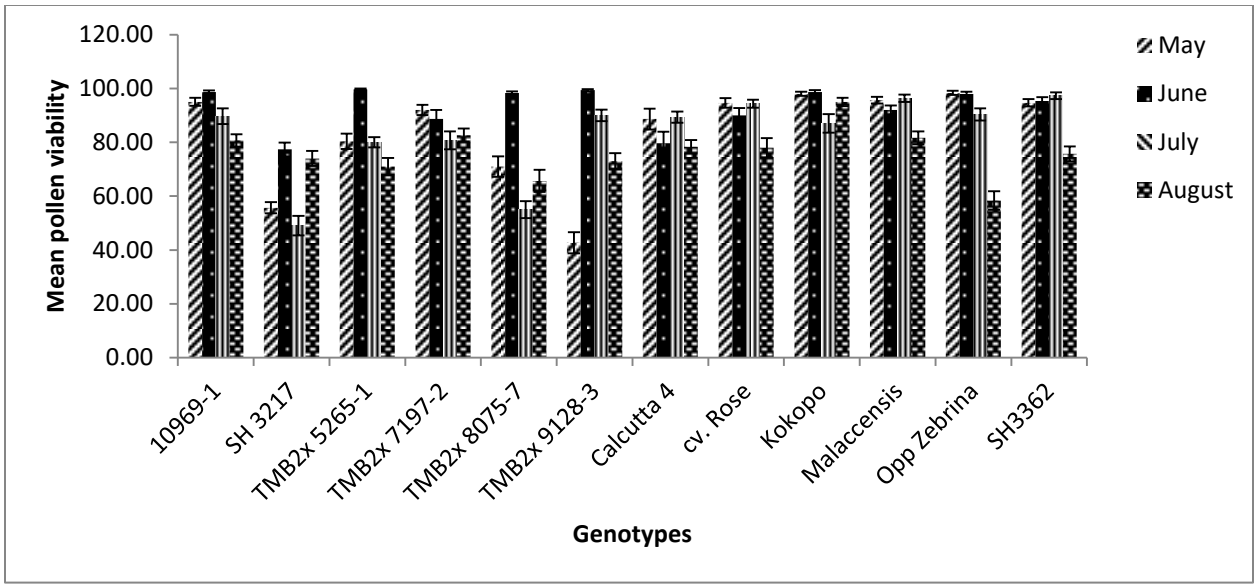


Figure 7: Mean pollen viability of male banana diploid genotypes of different months

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

Crosses and soaking highly differed significantly (P<0.001, Table 7) in germination rate. However, the interaction between cross and soaking regime did not significantly affect embryo germination (P=0.927, Table 7). 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 8). Soaking for 5 days gave slightly lower germination than soaking for 3 days, but the difference was not significant (Figure 8). However, increasing the time of soaking to 9 days had a negative impact on embryo germination rate for the entire 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 8).

Table 7 ANOVA to test the effect of time of soaking in distilled water and cross-type on germination rate of banana embryos

Source of variation	d.f.	m.s.
Cross	3	1420.59***
Treatments	4	661.73***
Cross x Treatments	12	40.17^{ns}
Residual	40	87.48
Total	59	

[a]degrees of freedom, [b]mean squares, [c]F test probability

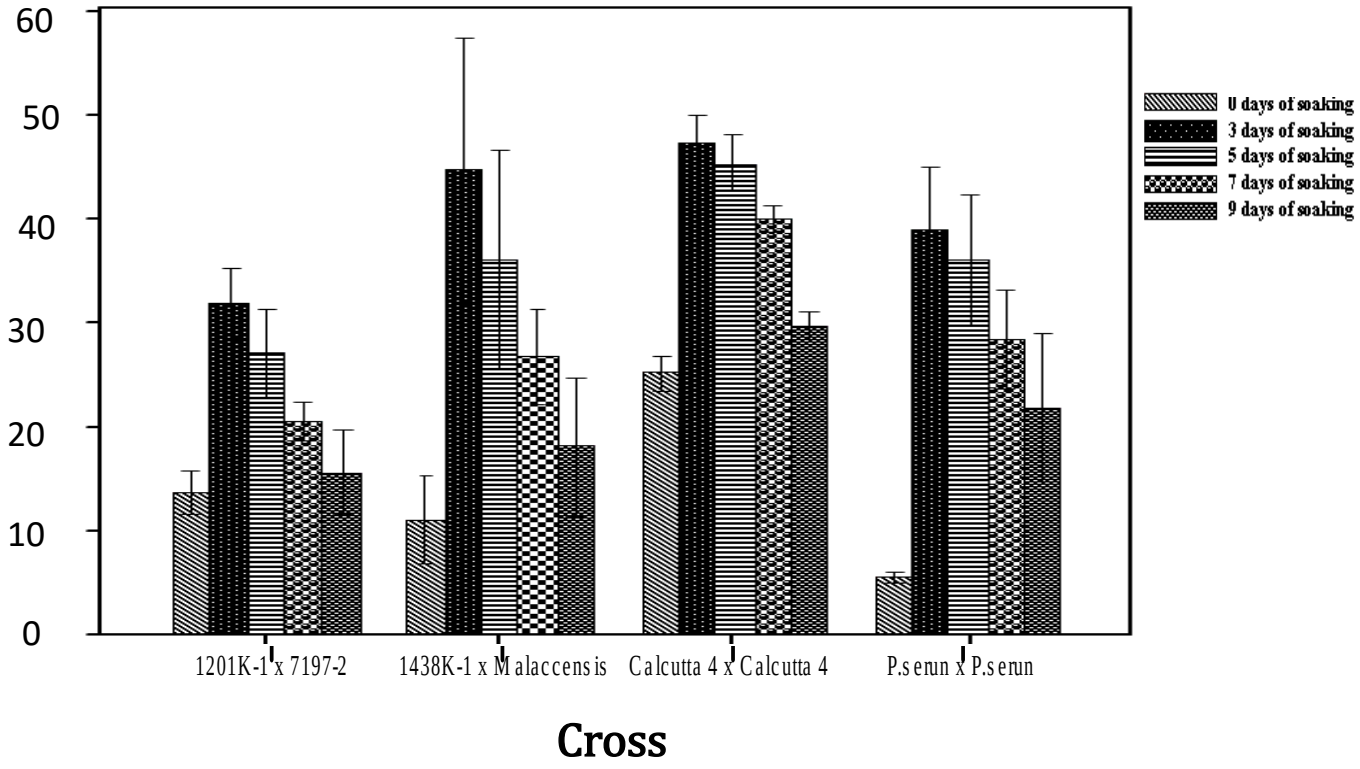


Figure 8: Effect of cross on embryo germination rates at different levels of soaking duration (days)

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

The crosses were significantly different (P<0.05) while the interaction between BAP and GA3 did not have significant (P>0.05) effect (0.065) on embryo growth (Table 8).

Table 8 ANOVA to test the effect of BAP and GA_3 concentration on the germination rate of embryos from 4x - 2x crosses

Source of variation	d.f.	m.s.
Cross	1	3962.8*
BAP	2	688.5^{ns}
GA	2	696.2^{ns}
BAP.GA	4	1610.6^{ns}
Residual	125	709
Total	134	

There was a significant difference ($P<0.001$) among crosses. However, hormones did not significantly affect embryo germination (Table 9).

Table 9: ANOVA to test the effect of BAP and GA_3 concentration on the germination rate of embryos from 2x - 2x crosses

Source of variation	**d.f.**	**m.s.**
Cross	1	8832.2***
BAP	2	450.5^{ns}
GA_3	2	679.7^{ns}
$BAP.GA_3$	4	111.7^{ns}
Residual	44	510.5
Total	53	

4.5 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 10). BAP also significantly ($P < 0.01$) affected number of roots but GA_3 did not have significant effect on number of roots (Table 10). Both BAP and GA_3 did not significantly affect stem girth among 2*x* – 2*x* crosses (Table 10). 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 10).

Table 10: ANOVA to test the effect of BAP and GA_3 concentration on germination parameters from 2x - 2x cross

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 11). 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 11). A significant 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 11).

Table 11: ANOVA to test the effect of BAP and GA_3 concentration on germination parameters from 4x - 2x

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 ($P>0.05$), $*P\leq0.05$, $**P\leq0.01$, $***P\leq0.001$

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. 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. The stem girth had the highest value as 1.9 mm 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. The plants with the highest height of 15.6 mm had no roots and had 0.9 mm as the lowest stem girth. On the other hand, the plants with the lowest height were 6.4 mm which also had 0.3 mm of roots, and had stem girth of 1.4 mm. The plants with the highest no of roots (1.9) had 9.1 mm of height and 1.2 mm of stem girth. The plant with the biggest stem girth (1.9 mm) had a height of 9.1 mm and 0.1 mm of roots (Table 12). 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.

Table 12: Effect of different hormone concentrations on growth parameters of one-week-old banana embryos in 4x x 2x

BAP	GA_3	Height (mm)	No. of Roots	Stem Girth (mm)
0	0	6.4^{c}	0.3^{c}	1.4^{bc}
0	0.5	9.1^{bc}	1.9^{a}	1.2^{bcd}
0	1	9.9^{b}	0.3^{c}	1.6^{ab}
0.5	0	9.3^{bc}	1.3^{ab}	1.7^{ab}
0.5	0.5	11.4^{b}	0.9^{bc}	1.6^{ab}
0.5	1	10.3^{b}	0.6^{bc}	1.3^{bcd}
1	0	9.1^{bc}	0.1^{c}	1.9^{a}
1	0.5	15.6^{a}	0.0^{c}	0.9^{d}
1	1	9.4^{bc}	0.0^{c}	1.1^{cd}
Grand mean		10.06	0.60	1.42
LSD		3.23	0.91	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.

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. 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.The plants with the highest height of 15.8 mm had no roots and had 0.9 mm as the lowest stem girth. On the other hand, the plant with the lowest height was 7.7 mm which also had 0.3 mm of roots, and had stem girth of 1.0 mm. The plants with the highest no of roots (1.9) had 9.8 mm of height and 0.9 mm of stem girth. The plant with the biggest stem girth 1.7 mm had a height of 9.6 mm (2*x* - 2*x*) and 0.1 mm of roots (Table 13). 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.

Table 13: Effect of different hormone concentrations on growth parameters of one-week-old banana embryos in 2x x 2x

BAP	GA_3	Height (mm)	No. of Roots	Stem Girth (mm)
0	0	7.7^{c}	0.3^{c}	1.0^{c}
0	0.5	9.8^{bc}	1.9^{a}	0.9^{c}
0	1	10.6^{b}	0.3^{c}	1.6^{a}
0.5	0	10.2^{bc}	1.3^{ab}	1.5^{ab}
0.5	0.5	11.9^{b}	0.9^{bc}	1.5^{ab}
0.5	1	10.9^{b}	0.6^{bc}	1.2^{bc}
1	0	9.6^{bc}	0.1^{c}	1.7^{a}
1	0.5	15.8^{a}	0.0^{c}	0.9^{c}
1	1	10.1^{bc}	0.0^{c}	1.1^{c}
Grand mean		10.72	0.60	1.28
LSD		2.88	0.91	0.38

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

Diploid banana genotypes are usually used as the sources of pollen in cross breeding programs to improve East African Highland banana (EAHB). They produce large quantities of fertile pollen compared to other ploidy levels in banana. In this study, we investigated the dynamics in the pollen quantity and viability among twelve genotypes used in our breeding program. Banana breeding involves careful selection of male diploid parents that are genetically diverse in terms of agronomic traits desired by farmers. These traits are introgressed into local varieties by cross breeding to improve their performance and adaptability. Pollen quantity and viability tests should be a prerequisite in male parent selection for successful banana breeding program. Generally, the pollen quantity in the diploid banana genotypes was high. Our results were similar to those obtained by Dumpe and Ortiz, (1996); Fortescue and Turner (2004) and Ssebuliba *et al.* (2008) of using these genotypes as male parents in the breeding. Pollen grains are involved in the process of pollination and subsequent fertilization which are critical factors that determine seed set (Oselebe *et al.*, 2014). Variability in pollen quantity and poor or malfunctioning pollen grains occurring among banana genotypes has been reported to hinder fertilization (Oselebe et al., 2014). Likewise, in this study, the observed pollen disintegration or degradation in some genotypes could be due to pollen maturity or immediate germination. Studies of Ssali et al. (2012) showed that pollen quantity and viability positively correlate with pollen germination. In fertile females, pollen quantity, viability and germination determine the number of seeds. Therefore, the higher the pollen viability and quantity, the higher the seed set.

In this study, pollen viability was generally greater than 50%, with significant variability among genotypes. The results of high viability in diploids presented here corroborate with those

discussed in the previous studies such as (Panda *et al.,* 2019; Fortescue and Turner 2004). The observed variation was due to inherent differences in the genotypes (Ssali *et al,* 2012; Oselebe *et al.,* 2014). The variation in environmental factors such as temperatures, relative humidity and rainfall recorded during the experimental period seemed not to influence pollen production and viability. For instance, trace amount of rainfall together with constantly fluctuating temperatures and relative humidity were recorded during the study from May to August. Moreover, the month of pollination had no significant effect on pollination success rather on seed set between years in EAHB (Batte *et al.,* 2019). On contrary, earlier studies reported that environmental factors influence pollen quantity and quality in banana genotypes (Krishnakumar *et al.,* 1992). Therefore, only genetic factors could have influenced the observed variations in pollen quantity and quality among the genotypes used in our study.

Despite the little or no influence of the recorded climatic factors, the interaction between genotype and months showed to strongly influence pollen quantity and viability. The observed variation in the pollen quantity and viability for individual genotypes at different sampling time points could be explained by genotype by environment (GxE) interactions phenomenon. The term GxE interaction has always been used in banana research to refer to the differential responses of genotypes in different environments including management practices (Nyine *et al.,* 2017) and locations or agro-ecological zones (Kimunye *et al.,* 2021) to assess the phenotypic stability of important traits. In the context of our research, plants were grown in one location under the same management. Therefore, the observed differential responses in pollen quantity and viability for individual genotypes could be attributed to changes in physiological processes in order to cope with the constant fluctuations in the prevailing environmental conditions. According to Begna (2020), genotypes by environment interactions normally influence

quantitative traits that are controlled by many genes with additive effect. Therefore, variations in pollen quantity and viability could be due to the environmental influence on the expression and function of genes controlling the traits. It can therefore be speculated that pollen production and viability are highly sensitive traits that require a stable environment for optimal performance. Nevertheless, genotypes consistently produced variable but large quantities of pollen associated with high viability across the sampling months.

Due to large amounts and high viability of the pollen produced by the studied diploid genotypes, the diploids were used for the second study where many seeds were needed for the experiments. Seeds were obtained following intra-ploidy selfing (2x – 2x) and inter-ploidy crossing (4x – 2x). Variable success in seed set was observed, with higher seed set being recorded in 2x -2x selfing than 4x – 2x crosses. The differences in seed set could have been due to high female fertility and receptivity in diploid banana (Waniale *et al.*, 2021). The seeds resulting from the designed parental mating combinations were further studied to improve their germination. Poor seed or embryo germination has been the major factor limiting hybrid production in banana breeding. The seeds were subjected to different tests such as imbibition and various hormonal combinations.

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).

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 increase in time of soaking (Zhang *et al.,* 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

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The studied genotypes represent most of the male parents used in the breeding program at IITA, Uganda. We found that pollen quality was affected by a strong interaction between the genotype and the environmental conditions, determined by the month. However, environmental conditions alone did not affect pollen but the interactions of genotype by environment did affect pollen quantity. The implication is that diploid genotypes produce high quantities of pollen, with high viability which determined high success rate of seed set in the designed crosses. 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). The results presented here provide important information that could be used for improving our understanding of banana reproduction biology and planning crosses by identifying the best male parents to maximize the potential of production of viable seeds not only at IITA but throughout the world.

6.2 Recommendation

We studied the pollen quantity and viability of the male diploid parents monthly, from May to August. There is need to study these traits throughout the year and for three consecutive years due to greatly variable climate change. Increasing the frequency of sampling from one to twice or thrice a week and sampling space from three plants to five plants per genotype is also recommended. This can help to find out the behavior of genotypes and whether they are affected by time. Some genotypes that produce much pollen can be conserved for further use. The storage of pollen grains permits genetic material sharing and easy transportation to other banana research centers. There is also need to evaluate all the male parents used in the breeding programs in IITA. We suggest that in vivo germination tests should be carried out to correlate pollen viability with parameters of production such as the number of seeds. 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. 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 extremely lower than 1 mg/l.

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