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Joana Carolina Costa Santos

IMPROVEMENT OF THE GERMINATION PROTOCOL OF SEEDS OF WILD BANANAS (*MUSA ACUMINATA* COLLA)

Implications for whole seed germination and ex situ germplasm conservation of Crop Wild Relatives of banana (*Musa acuminata* Colla)

Dissertação no âmbito do Mestrado em Biodiversidade e Biotecnologia Vegetal orientada pela Professora Doutora Sílvia Castro e pelo Doutor Filip Vandelook e apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra

04 January, 2024

Acknowledgements

In the first place, I want to express my gratitude to Xavier Scheldeman who welcomed me into the research group of Meise Botanic Garden and gave me the opportunity to work on this project. A special thanks to my supervisor Filip Vandelook for all his support and guidance throughout the work. I am very grateful for the freedom I was given while I always felt supported during my thesis. I also want to thank to Ann Van Vyver for allowing me to work in the Laboratory of the Seed Bank of MBG and for her general kindness.

To my colleagues, Chimene Ghandour, Yannick Coeckelberghs, Lisa Couck and An-Sophie Tas, thank you for your help, affection, for listening to me, for always pulling me up and for the good mood. I also extend my appreciation to the Botanic Garden team and greenhouse-gardeners who contributed to my well-being during my time at the Botanic Garden.

I would like to thank to Bart Panis for allowing me to work with his team in the Laboratory of Tropical Crop Improvement (KU Leuven). A special thanks to Natalia Sleziak, who was always very kind to me, for teaching me how to do ER technique and for being available when I needed assistance.

A heartfelt thanks to Sílvia Castro for her guidance, availability, and willingness to be my supervisor in this project, as well as for attending to all my requests.

I want to declare my gratitude to Tia Lena for giving me the opportunity of living with her in Brussels, for her dedication and patience. I would like to thank to my parents for their never-ending support, and encouragement. My mom, for showing me how to be organized, how to be resilient, and that is always possible to restart. My father, for transmitting me his hunger for knowledge and his courage to set foot into the unknown. I would like to thank my sister Bi and brother Kiko, for being the hidden strength, and my strongest life pillars.

I would also like to extend my gratitude to my partner Jakub for being always by my side, for giving me help and unconditional support, and the providing me the needed distraction from work.

Ultimately, I want to thank to my friends, especially my girls: Ju, Lícia, Babi, Winnie, Mariana, and all “Panadas and Panados” for being present every time I needed them during this (and previous) years.

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Abstract

Bananas (*Musa* spp.) are nowadays the most produced and consumed fruit crop in the world. They comprise commercial dessert, plantain bananas and wild relatives (CWR). Despite many efforts, important commercial Banana varieties face the risk of disappearing as a consequence of pests and diseases, global monoculture production and the present climate crisis.

Meise Botanic Garden, Belgium (MBG) and Leuven University, Belgium (KU Leuven), in association with international banana research centers (as INIBAP, ITC) and *Musa* Consortia investigate seed conservation, *ex situ*, *in vitro* and cryo- conservation of *Musa* resources. Regardless of past efforts, there is still little knowledge on *Musa's* seed germination, dormancy strategy, and storage behavior. Actual germination protocols are not effective, and they require a long time (months to years) to yield results. Seed dormancy appears to be related to integument constraints, for this reason Embryo Rescue (ER) is the prevalent method to germinate banana seeds.

I performed my research in the Seed Bank of MBG and KU Leuven with the goal of contributing to the research on conservation of *Musa* seed resources and developing a feasible germination protocol of CWR of *Musa acuminata*, for future implementation in seed banks. My focus was on quantifying initial viability through morphological analyses of seeds (stored and fresh) and testing combined effects of: 1) different dormancy breaking treatments, 2) temperature oscillation regimes and/or 3) substrates, on diverse seed germination protocols. At the end of the experiments, a Cut test followed by a Tetrazolium Chloride test (TTC) or ER test was made to determine the viability of non-germinated seeds; and TTC and ER results were compared to assess the eligibility of TTC as a reliable post-incubation viability test for *M. acuminata*.

Our findings highlight the importance of considering seed morphology and maturity in the development of effective germination protocols for *M. acuminata*. Despite a substantial number of stored and fresh seeds, and the high number of treatments employed in germination testing, the dormancy-breaking and incubation factors tested proved to be insufficient to effectively overcome the dormancy of *M. acuminata* seeds. The low Final Germination Percentage and the high percentage of

apparent viable seeds after incubation suggest that high dormancy levels are present in both dry and fresh seeds of *M. acuminata*. In both dry and fresh seeds, the ER technique alleviated dormancy constrains, and it revealed that the type of pre-treatment, substrate and temperature regime have a significant impact in embryo viability. I also concluded that the TTC procedure used in this study is not a reliable and precise test for assessing post-incubation viability of *M. acuminata* seeds.

Despite the ineffectiveness demonstrated by the 18 treatments employed in germination testing, the work developed in this project is essential to understand the relationship between seed morphology and initial viability of seeds. Additionally, it contributes to understand the impact of various pre-treatment and incubation variables on post-incubation seed viability. This knowledge is crucial to design innovative germination tests, either by excluding the factors tested in this project or by incorporating them in alternative combinations.

Key-words: Seed Germination, *Musa acuminata,* TTC test, Embryo Rescue, Dormancy release.

// Resumo

As bananas (*Musa* spp.) são atualmente o fruto mais produzido e consumido no mundo. Neste grupo estão incluídas as variedades comerciais de sobremesa, bananas-plantain, e as variedades selvagens (CWR). Apesar dos esforços, importantes variedades comerciais de banana enfrentam o risco de desaparecer como consequência de pragas e doenças, da produção global em monocultura e da atual crise climática.

O Jardim Botânico de Meise (MBG) e a Universidade de Leuven (KU Leuven), ambos na Bélgica, em parceria com centros internacionais de investigação (INIBAP, ITC) e Consorcia em *Musa*, investigam a conservação de recursos genéticos de *Musa* em sementes, *ex* situ -, *in* vitro - e crio- conservação. Existe pouco conhecimento sobre a germinação de sementes de *Musa*, da sua dormência e do seu comportamento em Bancos de Sementes: os atuais protocolos de germinação são ineficientes e levam bastante tempo (meses a anos) a obter resultados. A dormência das sementes de banana aparenta estar relacionada com constrangimentos provocados pelos integumentos; por esta razão, *Embryo Rescue* (ER) (Resgate de Embriões) é o método prevalente de germinação.

Realizado no Banco de Sementes do MBG e na KU Leuven, o meu projeto de tese teve como objetivo contribuir para a investigação na conservação de sementes de *Musa* e desenvolver um protocolo de germinação de CWR de *Musa acuminata*, com perspetivas futuras de implementação em bancos de sementes. O meu foco prendeu-se com a quantificação da viabilidade inicial através de análises morfológicas das sementes (armazenadas e frescas) e testar: 1) tratamentos de quebra de dormência, 2) regimes de oscilação de temperatura, e 3) substratos, em diversos protocolos de germinação. No final de cada experiência, foi executado um *Cut test*, seguido por um teste Tetrazolium Chloride (TTC) ou teste ER de forma a quantificar a viabilidade das sementes não-germinadas. Os resultados de TTC e ER foram comparados para verificar a elegibilidade do teste TTC como um teste de viabilidade pós-incubação para *M. acuminata*.

As nossas descobertas sublinham a importância de considerar a morfologia e maturidade das sementes nos protocolos de germinação de *M. acuminata*. Apesar do elevado número de sementes e dos tratamentos utilizados nos testes de germinação,

os fatores de quebra de dormência e de incubação testados revelaram-se insuficientes para superar a dormência das sementes de *M. acuminata*. A baixa Percentagem de Germinação Final e a elevada percentagem de sementes aparentemente viáveis após a incubação sugerem que as sementes secas e frescas de *M. acuminata* apresentam elevados níveis de dormência. A técnica de ER aliviou a dormência e revelou que o tipo de pré-tratamento, substrato e regime de temperatura tem um impacto significativo na viabilidade dos embriões. Concluímos ainda que o procedimento TTC utilizado neste estudo não é adequado para avaliar a viabilidade pós-incubação das sementes de *M. acuminata*.

Apesar da ineficácia dos 18 tratamentos aplicados no teste de germinação, o trabalho aqui desenvolvido é essencial para perceber o impacto dos vários fatores na viabilidade das sementes; e é crucial para desenhar novos testes de germinação, removendo os fatores testados ou implementando-os em combinações alternativas.

Palavras-chave: Germinação de Sementes, *Musa acuminata,* teste TTC, Resgate de Embriões, quebra de Dormência.

List of Abbreviations

A	Genome A (*Musa acuminata*)
ABA	Abscisic Acid
B	Genome B (*Musa balbisiana*)
BBTV	Banana Bunchy Top Virus
CD	Conditional Dormancy
CWR	Crop Wild Relatives
DCR	Democratic Republic of Congo
DTG	Days taken to Germination
D	Dormancy
ER	Embryo Rescue technique
FAO	Food and Agriculture Organization of the United Nations
FGP	Final Germination Percentage
FOC	*Fusarium oxysporum* f. sp. *cubense*
FOC TR4	*Fusarium oxysporum* f. sp. *cubense* tropical race 4
GA	Gibberellic Acid
IAA	Indole Acetic Acid
KU Leuven	Katholieke Universiteit Leuven, Belgium
M.A.	Temperature regime of monthly alternation of temperature
MBG	Meise Botanic Garden, Belgium
MC	Moisture Content
MD	Morphological Dormancy
MGT	Mean Germination Time
MPD	Morphophysiological Dormancy
MS	Murashige and Skoog medium
MSB	Millennium Seed Bank
MSBP	Millennium Seed Bank Partnership
NaOCl	Sodium hypochlorite
PD	Physiological Dormancy
PY	Physical Dormancy
PY + PD	Combinational Dormancy
RH	Relative Humidity
SB	Seed Bank
SD	Standard deviation
SDGs	Sustainable Development Goals
TTC	2,3,5-Triphenyltetrazolium Chloride test
UN	United Nations
W.A.	Temperature regime of weekly alternation of temperature

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1. Introduction

1.1. Social and economic context of bananas

According to the UN Food and Agriculture Organization (FAO), bananas and plantains (Musaceae) are produced in more than 135 countries (FAO, 2020), and they are the most produced and consumed food crop on a global scale (Statistica, 2023). Bananas (*Musa* spp.) occupy a preeminent position within their native region of South East Asia (Fig. 1) (Robinson & Saúco, 2010), and they hold a status of indispensable dietary constituent in countries like Somalia, Democratic Republic of Congo (DRC), Ethiopia, Sudan and Yemen where hunger and malnutrition persist as an everyday reality (Promusa, 2020a; IFAD, 2023; WFP, 2023).

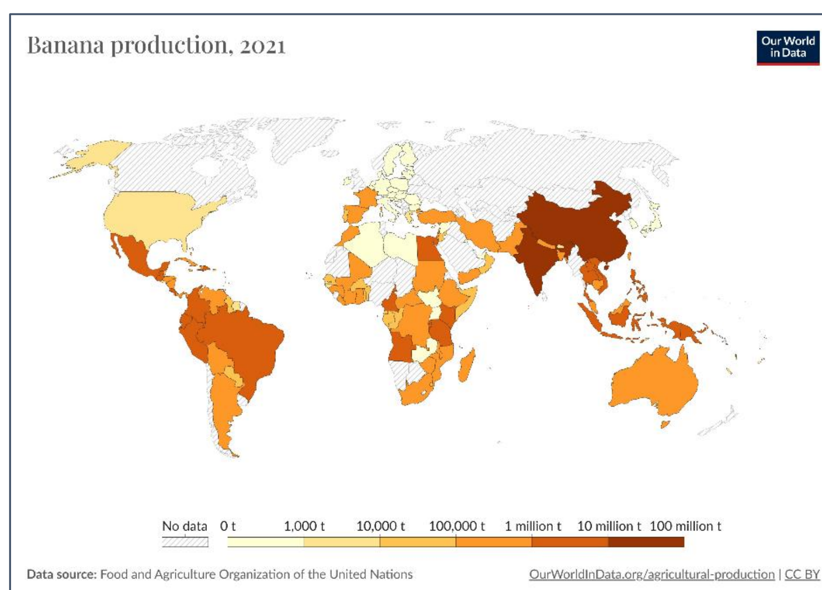

Figure 1. Banana production in tonnes 2021, based on data provided by the FAO (2023).

Although dessert banana's production (*Musa acuminata* Colla) is extensive in low- and middle-income countries, the majority of its production is controlled by multinational companies and exported to countries of the Northern hemisphere (Robinson & Saúco, 2010). This leads to enormous social disparities and deficient food security in countries in Africa, the Middle East and Latin America (Robinson & Saúco, 2010). According to the United Nations (UN), the greater part of the world's population growth is expected to occur in developing countries, until 2050 (United Nations, 2017).

Such population growth can result in considerable threats to human populations in regions most affected by the accelerating Climate Crisis, famine and wide-scale conflicts (United Nations, 2017).

As a reaction to the already present and perceptible social inequalities resulting from this complex crisis, particularly present in less developed countries (WFP, 2023), the UN adopted a policy framework in 2015 called the Sustainable Development Goals (SDGs): a set of targets to be reached until 2030 aimed at eradicating poverty, environmental degradation and ending world hunger (United Nations, n.d.a). The Second Sustainable Development Goal – Zero Hunger (SDG2) was designed to create a world free of hunger before 2030, to "maintain the genetic diversity of seeds, cultivated plants (…) and their related wild species (in) seed and plant banks at the international level" and to "increase investment in plant and livestock gene banks in order to enhance agricultural productive capacity in developing countries" (United Nations, n.d.b).

Advancements in banana research can directly contribute toward the SDG2 by amplifying agricultural output, increasing food production, fostering sustainable agriculture and enhancing the livelihoods of banana farmers (Kallow et al., 2022a). These efforts have a favorable influence on global food security, particularly in areas where bananas are a staple food (Kallow et al., 2022b).

1.2. Banana's ecology

1.2.1. Plant morphology and description

Banana plants (*Musa* sp. Colla) are giant herbaceous perennials that consist of an underground rhizome that produces roots and shoots; a pseudostem, where "tightly overlapped leaf sheaths" give the appearance of a trunk; and large, elongated blade-like structure leaves (Promusa, 2020b). Once the plant is fully mature, a hermaphrodite inflorescence grows from the tip of the pseudostem. This inflorescence is enveloped by a spathe and it contains male flowers at the top, and female flowers towards the bottom. The banana fruit is a pulpous and nutritious berry that grows in clusters called hands, each hand consists of several individual banana fruits, known as fingers (Fig. 2) (Robinson & Saúco, 2010).

In non-domesticated wild species – Crop Wild Relatives (CWR) (Biodiversity International, n.d.) – of banana, the development of fruits occurs after pollination, producing seed-bearing fruits, with numerous, large, black and hard seeds (Renjana et al., 2022). Meanwhile, in commercial varieties, fruit development occurs without fertilization, producing partenocarpic, pulpous, seedless and sterile fruits (Robinson & Saúco, 2010).

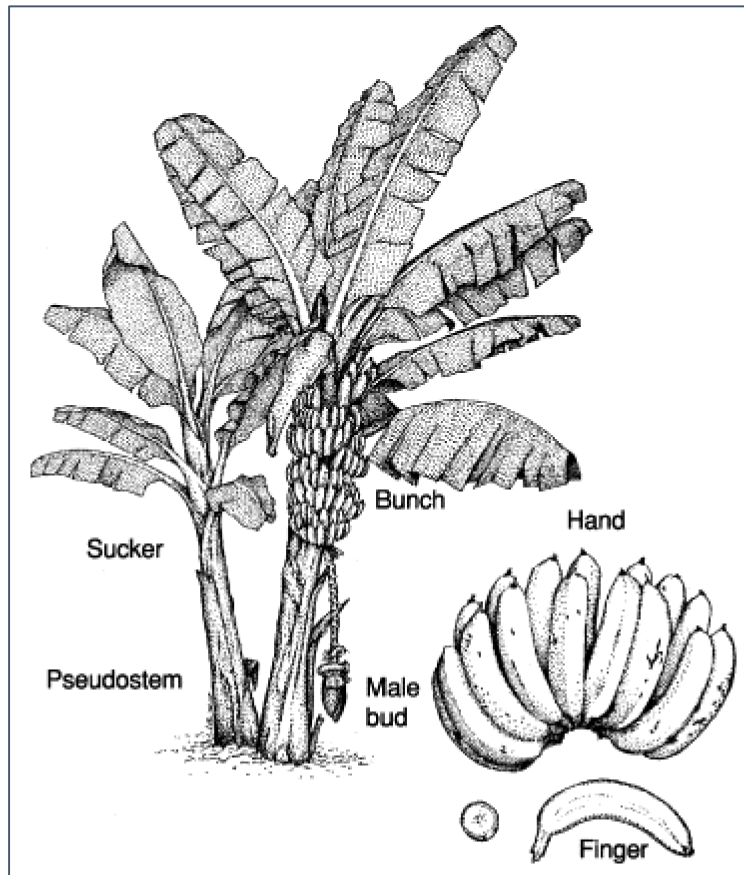

Figure 2. Banana plant, bunch and fruit morphology. Reproduced from Daniells, J.W. (2003). BANANA AND PLANTAINS. In B. Caballero (Ed), Encyclopedia of Food Science and Nutrition *(2nd ed., pp. 372-378). Academic Press. https://doi.org/10.1016/B0-12-227055-X/00080-8*

1.2.2. Origin and domestication of *Musa* sp.

The forests of Malaysia, Indonesia and Pacific regions are believed to be the place of origin of banana plants (Renjana et al., 2022; Singh et al., 2021; Robinson & Saúco, 2010). Bananas are pioneer herbs that can be naturally found in the open canopy of tropical, subtropical and subequatorial regions (Kallow et al., 2020a; Singh et al.,

2021, Robinson & Saúco, 2010). Subtropical and subequatorial areas are characterized by wide temperature fluctuations from day to night and from summer to winter (Robinson & Saúco, 2010), which implies that such temperature fluctuations are necessary for the germination of *Musa*.

The genus *Musa* comprises around 80 species with more than 1000 edible cultivated varieties (Kallow et al., 2020a). *Musa*, *Musella* and *Ensete* are three genera that constitute the Musaseae family, which encompasses both CWR and cultivated plantain, dessert and cooking bananas (Renjana et al., 2022).

Over time, various inedible, seed-bearing, diploid (2n) subspecies of mostly *Musa acuminata* Colla (genome A) and *Musa balbisiana* Colla (genome B) were naturally crossed (Renjana et al., 2022; Kallow et al., 2020b). The production of numerous intra-specific hybrids in the center of origin of all *Musa* resulted in the enlargement of the genetic pool of *Musa*, and it established Southeast Asia as the major center of banana genetic diversity (Renjana et al., 2022).

At some point, local inhabitants discovered that some plants had edible seedless fruits that could be vegetatively propagated (Robinson & Saúco, 2010). This occurrence was the result of natural complex hybridization events and genetic variations that yielded partenocarpic, female sterile, triploid (3n) and polyploid varieties (Renjana et al., 2022; Robinson & Saúco, 2010). This transformative event contributed to the globalization of banana consumption (Drenth & Kema, 2021). The classification of these commercial cultivars is according to the relative genetic contribution of the parent species. Plantains are constituted by 2/3 *M. acuminata* (AAA) and 1/3 *M. balbisiana* (AAB), whereas the Gros Michel and Cavendish cultivars (dessert bananas) are exclusively *M. acuminata* genome (AAA) (Robinson & Saúco, 2010).

1.3. Threats to banana production

As mentioned previously, bananas are one of the most significant fruits in the world and a staple food for millions of people (Nyine & Pillay, 2007). The international banana supply heavily relies on monoculture farming practices, with 47% of the world's banana production dominated by the variety *Musa acuminata* Cavendish (AAA) (Kallow

et al., 2020b). The major threats that significantly impact the global banana supply, the economy of less-developed countries and the sustenance of small banana growers are: 1) climate crisis; 2) monoculture practices and 3) pests and diseases (Kallow et al. 2022a; Drenth & Kema, 2021; Jacobsen et al., 2019).

The conversion of natural ecosystems into banana monoculture fields results in the excessive use of pesticides that triggers in the emergence of new resistant pests and diseases (Promusa, 2021; Robinson & Saúco, 2010; Côte et al., 2009). This also results in the excessive use of chemical fertilizers which pollute soils, rivers and water sources; in the destruction of wide areas of forest; and in habitat loss. Furthermore, the practice of monoculture decreases the natural biodiversity, it shrinks the genetic pool of CWR and it reduces the availability of resistance genes for hybridization (Côte et al., 2009; Renjana et al., 2022).

At the same time, the *Musa acuminata* (AAA) is highly susceptible to pests and diseases. In the 1950's, the Panama Disease also known as *Fusarium* wilt, caused by the fungus *Fusarium oxysporum* (Foc) initiated the disappearing of the most produced and consumed banana variety in the world: *Musa acuminata* Gros Michel (AAA). Nowadays, a new variety of Fusarium (Foc TR4) is threatening the *Musa acuminata* Cavendish (AAA) and it is causing production losses up to 100% (Jacobsen et al., 2019). The large-scale cultivation of a single variety also amplifies the vulnerability of banana plantations to other pests and diseases: Black Sigatoka, a leaf spot disease caused by the fungus *Mycosphaerella fijiensis* Morelet (which affects the photosynthetic capacity, leading to reduced fruit yields and fruit quality); Banana Bunchy Top Virus (BBTV), a viral disease transmitted by aphids (yield losses of up to 100% in severe cases) (FAO, 2020); and Banana weevil (caused by the weevil *Cosmopolites sordidus* Germar, 1824) (Jacobsen et al., 2019). The age-old battle against pests and diseases is still a significant challenge for banana growers, as these pests and diseases can persist in banana crops and in the soil for many years (Jacobsen et al., 2019), whilst it is urgent reduce the amount of chemicals used in agriculture practices (Côte et al., 2009).

The escalating climate crisis has equally devastating effects on banana production (Kallow et al., 2022a). Changes in temperature and rainfall patterns, and extreme weather events frequent in tropical regions (such as hurricanes, flooding,

cyclones, heat waves, storms and tsunamis) impact the growth, development and productivity of banana plantations (Robinson & Saúco, 2010).

The above-mentioned threats place in risk the production of banana worldwide. A decrease in the world supply, or an extinction of important banana cultivars can lead to a disruption in the world economy; and to famine and malnutrition, particularly in tropical and subtropical regions where the banana is an indispensable food (Kallow et al., 2020b). Decelerating the climate crisis and prioritizing the implementation of climate resilience strategies are crucial approaches to guarantee banana sustainability. It is additionally important to change the global agricultural *modus operandi* by promoting sustainable farming practices such as crop rotation and crop diversification; and to implement viable integrated pest management such as the use of appropriate chemical control, controlled application of chemicals based on weather forecasting and the use biological pest-control (Côte et al., 2009; Jacobsen et al., 2019; Promusa, 2021). Just as important, it is essential the implementation of new resistant banana cultivars developed by hybridization techniques between commercial varieties with superior agronomic characteristics and CWR with resistance genes such as disease resistance, drought tolerance, etc. (Renjana et al., 2022).

1.4. Conservation of *Musa* genetic resources

The conservation of *Musa* genetic diversity is crucial for reaching the SDG2, and for ensuring the sustainability and resilience of banana production in face of the emerging challenges (Kallow et al., 2022a). There are worldwide non-profit research centers like International Network for the Improvement of Banana and Plantain (INIBAP), International Transit Centre (ITC), Biodiversity International (KU Leuven, Belgium), and Seed Bank of Meise Botanic Garden (Meise, Belgium) that focus on enhancing the conservation of *Musa* genetic resources (Kallow et al., 2022a; Plantentuin Meise, n.d.b; Promusa, 2019; Ruas et al., 2017; Subrahmanyeswari & Gantait, 2022). Consortia like the Global *Musa* Genomics Consortium, Musa Germplasm Information System (MGIS) and MusaNet improve the distribution of knowledge in banana research

and develop important fields such as mapping the banana genome (Kallow et al., 2022a; MusaNet, n.d.; Promusa, 2020c; Ruas et al., 2017; Singh et al., 2021).

Efforts to conserve *Musa* genetic resources include the work done in genebanks. Genebanks play a crucial role in collecting, conserving, and distributing banana germplasm of different commercial varieties and wild species (CWR) in the form of clones (plantlets, adult plants and tissue culture) or seeds (Kallow et al., 2022b; Ruas et al., 2017; Subrahmanyeswari & Gantait, 2022). These institutions develop conservation actions including *in situ* and *ex situ* clonal conservation and seed collections in seed banks (Kallow et al., 2020a).

1.4.1. Clonal conservation

Clonal conservation can be done *in situ*, where plants are maintained in their natural habitat; or *ex situ*, in which plants are stored outside their natural living environment (in field collections, greenhouses, *in vitro* conditions or cryopreserved) (Love & Spaner, 2007). Each method has its advantages and disadvantages:

In situ conservation and *ex situ* field collections can serve as valuable models for the ecological dynamics of plant species within their natural habitat and plantations. (Bohra et al., 2020; Love & Spaner, 2007). Nevertheless, these methods entail susceptibility to biotic stresses, weather events, and they require intensive labor for upkeep (Kallow et al., 2022a; Quaghebeur, 2021).

In vitro and cryopreserved collections use limited space and they furnish a reservoir of uncontaminated propagation material. However, in contrast to *in situ* and field collections, they possess constraints in the number of genetically unique individuals that can be effectively preserved, and they demand a considerable investment of labor and specialized facilities and equipment. Furthermore, *in vitro* collections are also prone to somaclonal variation (Kallow et al., 2022a; Quaghebeur, 2021; Subrahmanyeswari & Gantait, 2022). The cryopreservation technique can also be applied to preserve excised embryos and whole seeds, but it is labor and energy demanding (Singh et al., 2021).

1.4.2. Seed conservation

An alternative cost-efficient approach to store banana genetic resources involves the utilization of seed collections (Li & Pritchard., 2009). This method obviates the occurrence of repetitive clonal replication, facilitates the storage of large numbers of accessions within in a collection, and substantially reduces the likelihood of pathogenic contamination (Engels & Ebert, 2021).

International Seed Banks follow a comprehensive guideline that involves: 1) the collection and seed processing (cleaning, drying and documenting vital information); 2) the storage of seeds under controlled conditions and duplication of accessions; 3) monitoring accessions' viability with periodic sample germination and viability testing; 4) the management of Seed Bank data; and 5) seed distribution for conservation and research purposes (MSBP, 2022). The Seed Bank of Meise Botanic Garden (MBG) is an important pillar within the domain of banana research (Kallow et al., 2022a). Since 2016, the Seed Bank of MBG manages a collection of wild banana seeds originating from 20 distinct species, alongside approximately 150 accessions sourced from diverse locations such as Democratic Republic of Congo and Papua New Guinea (Kallow et al., 2022a; Plantentuin Meise, n.d.b).

The establishment of a Seed Bank of superior quality hinges on the acquisition of specific comprehensive knowledge (Li & Pritchard., 2009) concerning optimal methodologies for: 1) seed collection, 2) storage, 3) sample viability monitoring, and 4) seed germination. However, this foundational knowledge about *Musa* sp. remains deficient as the behavior and viability of stored seeds, dormancy characteristics and germination responses remain poorly understood (Coenen, 2022; Quaghebeur, 2021; Kallow et al., 2022a). Consequently, no gene banks have thus far reported effective strategies for the conservation of seeds belonging to wild *Musa* species.

1.5. *Musa acuminata*'s seed

An essential pre-requisite for seed conservation and plant regeneration is systematic data on 1) seed ecology, morphology and maturation; 2) storage behavior; and 3) dormancy, and dormancy-breaking factors.

1.5.1. Ecology, Morphology and Maturation

In the wild ranges, after pollination, several dozens of seeds start developing inside of a singular fruit (Renjana et al., 2022). The appealing sweet taste of ripe banana fruits attracts birds and mammals. These animals are thought to be the main dispersers of banana seeds, involving processes of endozoochory and/or external seed dispersal (Bohra et al., 2020; Graven et al., 1996; Tang et al., 2007; Tézenas Du Montcel et al., 1996).

The external morphology of fully developed *Musa* seeds consists of a cylindrical shape with a planar base, and a hard, dark brown-black, rugous testa with an operculum surrounds the whole seed (Renjana et al., 2022) (Fig. 3). The seed coat is a hard layer formed by silica crystals. This adaptation serves as a protective mechanism against physical abrasion by animals through mastication, as well as a protection to the passage through the digestive tract (Graven et al., 1996). Furthermore, it confers the capacity for long-term viability of seeds buried in soil over extended periods of time (Chin, 1996). The truncate-conical shape operculum comprises both the micropyle, the micropylar plug and the hilum (Fig. 3) (Graven et al., 1996). The operculum most likely to be area for water uptake (Puteh et al., 2011), and it is expelled during germination through the abscission layer (Graven et al., 1996; Renjana et al., 2022). Nonetheless, previous studies show that the manual excision of the operculum does not confer improved germination results (Graven et al., 1996; Vineesh et al., 2015; Singh et al., 2021).

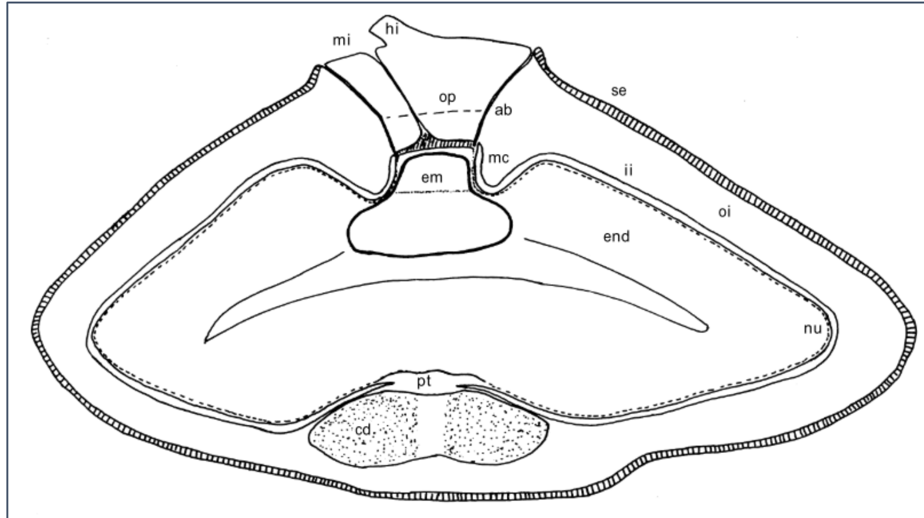

Figure 3: Illustrative representation of a transverse section of a Musa seed*: abscission layer (ab), chalazal disk (cd), embryo (em), haustorium (hau), endosperm (end), hilum (hi), inner integument (ii), micropylar collar (mc), micropyle (mi), nucellar tissue or perisperm (nu), outer integument (oi), operculum (op), parenchymatic tissue between chalaza and nucellus (pt), silicified exotestal (se). (Graven et al., 1996).*

Internally, *Musa* seeds have two chambers: a large chamber that incorporates the embryo (em) and the surrounding starchy endosperm (end); and a small chamber (cd) (Fig. 3). The outer integument (oi) is placed underneath the testa (se) and it encircles the whole seed; while the large chamber is enclosed by an inner integument (ii) (Fig. 3) (Renjana et al., 2022). Located in the first chamber, embryos of *M. acuminata* are described as white, firm, and capitate, with a distinct haustorium located at the basal region (Kallow et al., 2020b), that facilitates the absorption of nutrients from the endosperm. Present in *Musaceae*, the second chamber is an uncommon compartment in other angiosperm seeds, that integrates the chalazal mass/disk (cd) (Fig. 3). The chalazal mass is formed by thin-walled cells densely arranged and replete with water, alongside a fragile and hygroscopic reddish-brown material. During the desiccation of the seeds, the chalazal mass undergoes contraction, resulting in the formation of a cavity (Chin, 1996). Upon re-hydration, the chalazal turns into a gelatinous mass (Kallow et al., 2022b).

Seed maturation plays a crucial role in shaping the desiccation tolerance and germination potential (Kallow et al., 2022b). The timing of seed maturation can be ascertained by tracking the number of days since the pollination (Uma et al., 2011); or by accessing the morphological character of the seed and the embryo (Renjana et al.,

2022), through the execution of a Cut test performed during the collection phase. Relying solely on the count of days post-pollination is an imperfect metric, as the time of maturation and seed morphology are influenced by various factors including environmental conditions and genetic factors (Renjana et al., 2022). Furthermore, such approach may be infeasible in natural contexts. In contrast, a Cut test offers a more reliable criterion for assessing seed maturity and viability (Renjana et al., 2022). Seeds are considered mature when the seed coat has a dark brown-black appearance (Fig. 4) (Renjana et al., 2022). The endosperm of full mature seeds assumes a white dry, powdery texture, as opposed to wet or milky consistency (Fig. 4) (Kallow et al., 2020a). Well-developed embryos are characterized by a compact white color mass, and a mushroom-like, capitated shape (Fig. 4) (Kallow et al., 2020a). Mature viable seeds tend to sunk when placed in water (Uma et al., 2011; Arun et al., 2013).

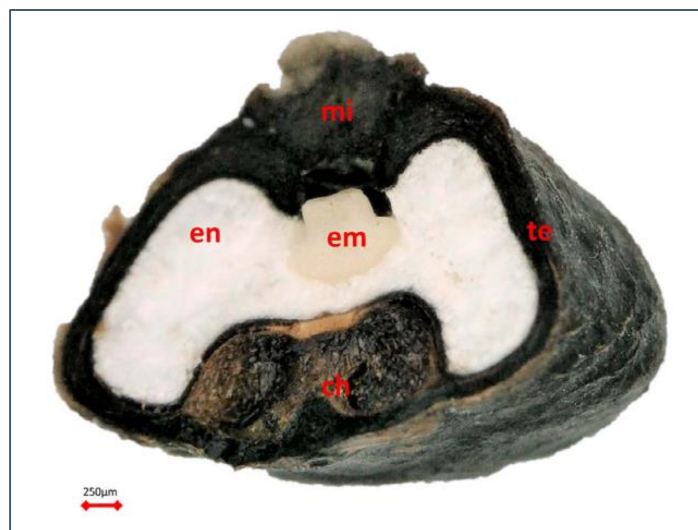

Figure 4. Photography of a transverse section of a mature Musa acuminata seed: micropyle (mi), embryo (em), endosperm (en), testa (te), chalazal mass (ch). (Kallow et al., 2022b).

1.5.2. Storage behavior

In general, seeds are categorized based on their storage behavior into three main groups: orthodox, recalcitrant, or intermediate. Seeds known as 'orthodox' can tolerate desiccation and they remain viable when dried to 3-10% of moisture content (MC). In contrast, seeds that exhibit recalcitrance are highly susceptible to desiccation and typically have a short lifespan. The 'intermediate' storage behavior falls in between

these two categories. These seeds exhibit orthodox and recalcitrant characteristics, and they can tolerate moderate levels of desiccation (10-20% MC), but excessive drying beyond this point reduces their viability (Singh et al., 2021).

Literature on seed storage behavior of *Musa* wild species has indicated a high degree of variation in the desiccation response between and within species (Singh et al., 2021, Kallow et al., 2022b). The heterogeneity present in *Musa* challenges a uniform characterization, although, it is accepted to be placed between orthodox and intermediate storage behavior (Chin, 1996; Kallow et al., 2020a; Bohra et al., 2020; Singh et al., 2021; Renjana et al., 2022).

Upon an evaluation of the behavioral attributes of *Musa* seeds, and in accordance with the guidelines of the Millennium Seed Bank Partnership (MSBP), collected *Musa* seeds are stored as soon as possible, upon reaching moisture equilibrium of 15% ± 3% MC. These seeds are then preserved within hermetically sealed containers under 15 ^{o}C ± 3 ^{o}C within 6 months, or at -20 ^{o}C ± 3 ^{o}C for extended-term storage (MSBP, 2022).

The collection and preservation of mature wild *Musa* seeds are vital for future breeding efforts (Renjana et al., 2022), and characterizing seed responses to drying is a crucial step to effectively utilize them in conservation practices (Kallow et al., 2022b). Therefore, further research into the storage behavior of different *Musa* species and varieties is needed (Renjana et al., 2022; Kallow et al., 2020a).

1.5.3. Seed Dormancy in *Musa*

Seed dormancy (See Annex 1: Seed Dormancy) is an adaptive strategy used by many flowering plant species, and it is characterized by the failure of seeds to germinate under conditions that would typically allow germination (Baskin & Baskin, 2004). This resistance strategy ensures that seeds only germinate when specific and favorable environmental conditions for seedling establishment prevail, and it also contributes to the formation of a persistent soil seed bank (Gubler et al., 2005; Kallow et al., 2021).

The dormancy of *Musa* seeds is a complex and poorly understood phenomenon (Arun et al., 2013; Kallow et al., 2020a; Pancholi et al., 1995). Heterogeneous, incompatible and even contradictory results of seed dormancy in *Musa* are found in the

literature (Chin, 1996; Pancholi et al., 1995; Puteh et al., 2011; Burgos-Hernández, 2014; Kallow et al., 2020a; Kallow et al., 2020b; Kallow et al., 2021; Singh et al., 2021).

Different research emphasizes that dormant *Musa* seeds require specific environmental conditions and dormancy breaking factors to overcome seed dormancy (Chin, 1996; Puteh et al, 2011; Burgos-Hernández, 2014). Soaking *M. balbisiana* seeds prior to embryo culture has been suggested as an effective method to stimulate germination by Afele & De Langhe (1991). Puteh et al. (2011) support the idea that imbibition activates the germination process in *M. acuminata*. Diurnally alternating temperatures are suggested to be almost completely essential for germination of *Musa acuminata* by Kallow et al. (2020b); and in 2021, they have demonstrated that stored dormant seeds lose their dormancy when buried in soil, suggesting a role for stratification in dormancy removal.

Ongagna et al. (2020) reported that dormancy factors in *M. acuminata* are primarily located in the seed integument, including chalaza and albumen, rather than in the embryo itself. Notably, Embryo Rescue (ER) techniques have been shown to effectively alleviate dormancy constrains and promote *Musa* germination (Pancholi et al., 1995; Sharma et al., 1996; Uma et al., 2011; Arun et al., 2013; Burgos-Hernández, 2014; Vineesh et al., 2015; Wang et al., 2016; Kallow et al., 2020a; Ongagna et al., 2020; Singh et al., 2021; Kallow et al., 2022a).

The complexity around *Musa* dormancy and germination emanates from the fact that dormancy in *Musa* is species-specific: different *Musa* species exhibit varying dormancy classes (Chin, 1996; Burgos-Hernández, 2014). Furthermore, within a singular inflorescence, heterogeneity is observed in the dormancy of *Musa* seeds (Kallow et al., 2020b).

1.6. Seed testing in Seed Banks

Seed testing is a process routinely used in Seed Banks to assess the quality and viability of seeds (MBSP, 2022). It involves: 1) Viability tests (Cut test and TTC test) used to assess and monitor the viability of new and stored seed collections; and 2)

Germination tests (Seed germination and Embryo Rescue) used to both monitor the viability of seed collections, and for obtaining plants (Davies et al., 2015a).

Germinating and evaluating the viability of *Musa* seeds can be challenging, primarily due to the limited knowledge for overcoming seed dormancy in *Musa* species (Puteh et al., 2011).

1.6.1. Cut test

A quick and elementary way to estimate seed viability is to bisect the seed with a knife, and visually estimate its viability. As mentioned above, viable mature seeds typically have a white dry endosperm; and a white, mushroom-like shaped embryo (Fig. 4) (Kallow et al., 2020a; Renjana et al., 2022). It is important to note that this method may overestimate seed viability in *Musa,* as some seeds appear healthy yet lack the capacity to germinate due to deep dormancy (Puteh et al., 2011; Pedrini & Dixon, 2020).

1.6.2. TTC test (2,3,5-triphenyltetrazolium chloride)

In this experimental procedure, the embryo is extracted from the seed and immersed in a buffered solution containing 2,3,5 triphenyltetrazolium chloride (TTC) in the dark for 48 h (Quaghebeur, 2021). The activity of dehydrogenases from metabolically active tissues facilitates the reduction of TTC, resulting in the formation of formazan (2,3,5-triphenylformazan). The living parts of the seed manifest a red coloration that serves as an indicator of embryo's viability (Fig. 5) (França-Neto & Krzyzanowski, 2019).

The TTC test is used to analyze a large number of seeds for their viability in a relatively short time and it allows the assessment of viability without breaking seed dormancy (França-Neto & Krzyzanowski, 2019). This approach can be used during all phases of seed processing (França-Neto & Krzyzanowski, 2019) and it is frequently used to evaluate the viability of non-germinated seeds (Lopez Del Egido et al., 2017).

However, this method has some limitations. First, it requires a comprehensive understanding of seed anatomy and training in interpreting tetrazolium results (França-Neto & Krzyzanowski, 2019); and second, it is uncertain if strongly dormant seeds exhibit

similar coloration response (Busso et al., 2015). It is also important to note that this method is not optimized for *Musa* species (Quaghebeur, 2021).

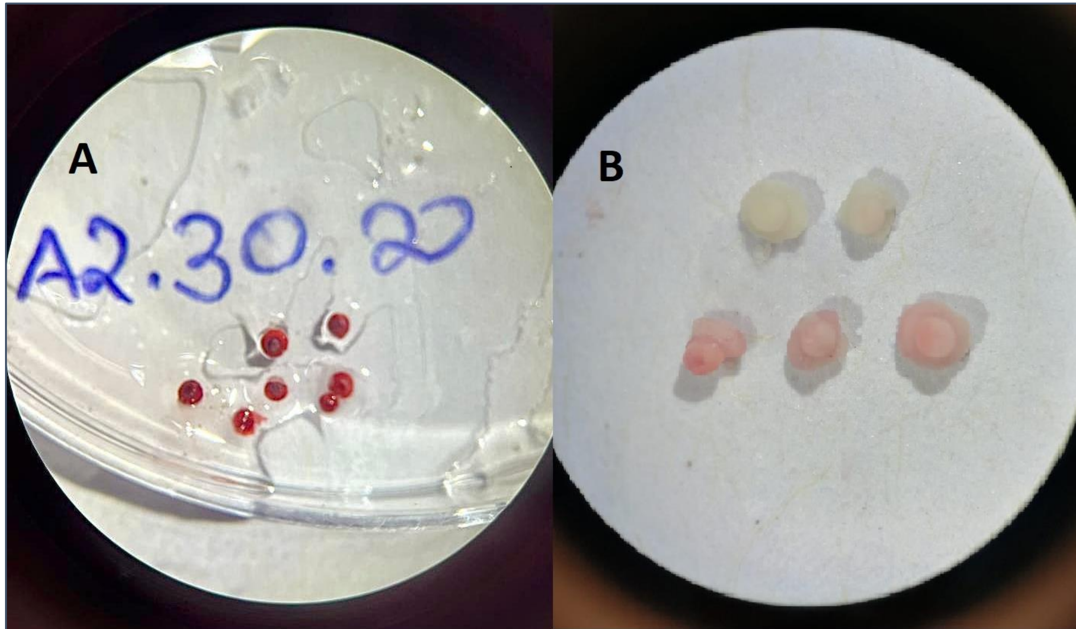

Figure 5. Possible TTC results: Red (A) embryos are considered viable; white (B, top) and pink (B, bottom) embryos are unviable.

1.6.3. Germination testing

Germination testing is used to monitor the viability of seed collections, and to evaluate the ability of seeds to produce healthy seedlings under favorable controlled conditions (Davies et al., 2015a; Plantentuin Meise, n.d.a). This test is the last and crucial step of seed conservation (see section 1.4.2) (Kallow et al., 2022b; Plantentuin Meise, n.d.a), and it is a relatively simple method: seeds are sown in a substrate under appropriate environmental conditions, and the number of germinated seeds is counted at predefined time intervals (Davies et al., 2015a; Pedrini & Dixon, 2020).

In dormant seeds, the germination process is influenced by: 1) the initial seed viability, which concerns the right seed maturity (see section 1.5.1), post-harvest handling, moisture content, drying conditions (see section 1.5.2), seed age, etc.; 2) the seed dormancy (class and level) and the employment of proper species-specific dormancy-release factors (see section 1.5.3 and Annex 1: Seed Dormancy); and 3) a species-specific range of environmental conditions, such as water, oxygen, temperature,

and in some cases also light and nitrate (Finch-Savage & Leubner-Metzger 2006; Baskin & Baskin, 2014; Davies et al., 2015b; Plantentuin Meise, n.d.a).

For orthodox and intermediate storage behavior species (where *Musa* sp. is included), seed germination starts with the imbibition of water by the seed and the embryo (Puteh et al., 2011). This triggers the elongation of the embryogenic axis, culminating in the emergence of the radicle (growing root), that penetrates the adjacent tissues. The conclusion of the germination process is determined by the outgrowth of the radicle (Finch-Savage & Leubner-Metzger, 2006; Davies et al., 2015a).

In dormant seeds, the germination process is distinguished into two phases: *dormancy release*, followed by *germination stimulation*. However, making a clear separation between these two outcomes is often challenging since the method to evaluate them is the actual seed sprout (Finch-Savage & Leubner-Metzger, 2006).

The results of seed germination testing are mostly quantified as the Final Germination Percentage (FGP), which represents the final percentage measured after a predetermined germination interval (Davies et al., 2015a). High FGP signifies healthy and viable seeds; while diminished germination results may either imply unmet dormancy-release factors and/or environmental conditions, as well as/or suboptimal seed quality or damage (Davies et al., 2015a; Pedrini & Dixon, 2020).

1.6.3.1. Factors influencing seed germination in Musa

The study of banana germination by seeds is constrained, primarily due to the low natural germination rates (less than 1% in natural habitats); additionally, the common and effective vegetative propagation limits the incentive for investigating seed germination in bananas (Bohra et al., 2020; Renjana et al., 2022). Furthermore, the lack of knowledge in critical elements like ecological dynamics, storage behavior and dormancy mechanisms is an obstacle in understanding *Musa* seed germination (see section 1.5).

It is recognized that the factors that influence the success of *Musa* seed germination are both present in *dormancy release* and *germination stimulation* phases. Thus, factors influencing seed germination can be grouped as: 1) Pre-incubation factors, and 2) Incubation factors.

Pre-incubation factors can also be designated as a pre-treatment or priming treatment (Arun et al., 2013). These procedures are employed to seeds before sowing (incubating) them, with the goal of releasing seed dormancy. Some pre-treatments applied to different *Musa* species are explained bellow:

- *Hydropriming*: consists of soaking whole seeds in water at pre-defined temperature for a certain period of time, to soften the seed testa. Afele & De Langhe (1991), and Arun et al. (2013) reported that soaking seeds prior to ER technique enhanced embryo germination. Bohra et al. (2020) showed that a pre-treatment of hot water (40°C for 20 minutes) exhibits higher germination results than room-temperature water priming.
- *Hormonal*: consists of imbibing whole seeds in a solution of water and hormones at a pre-defined concentration, for a certain period of time. Arun et al. (2013) reported that soaking seeds in hormonal solutions of BAP, GA and IAA for three days have a highly significant effect on embryo germination, compared to hydropriming and "Among the three growth regulators, GA_3 was found to be the more effective and efficient". Bohra et al. (2020) showed similar results: a pre-treatment with GA_3 exhibits higher germination results than non-pre-treated seeds.
- *Scarification*: consists of weakening or cutting the seed integuments (testa) by mechanical processes. Puteh et al. (2011) demonstrated that scarifying the testa of dry seeds contributes to better water imbibition rates.

Incubation factors are factors related with environmental conditions such as *light, substrate, and* t*emperature* (Baskin & Baskin, 2014; Davies et al., 2015a; Davies et al., 2015b). Uma et al. (2011) proved that *light* is not a limiting factor in *Musa* germination, since *Musa* embryos both germinate in light and dark conditions.

S*ubstrate* seems to have a significant impact on *Musa* seed germination (Bohra et al., 2020):

- *Agar medium* is highly suitable for germination as it retains moisture and it enables easy monitoring (Davies et al., 2015a).
- *Agar medium* combined with GA_3 is reported to have a positive influence on seed germination (Davies et al., 2015a), however no optimal hormonal concentration is stated for *Musa* spp. (Quaghebeur, 2021).
- Chin (1996), Vineesh et al. (2015), Kallow et al. (2020b), and Kallow et al. (2021) reported that a high germination percentage can be achieved using *Sand* as a substrate.
- Other substrates like *Filter paper* is also widely used in germination testing (Davies et al., 2015a).

Temperature alternation is essential for germination of *Musa acuminata* (Kallow et al., 2020b) (see section 1.5.4). *Musa*, being a pioneer species found in open canopies, rely on day and night temperature fluctuations at the soil surface (Chin, 1996; Quaghebeur, 2021). Kallow et al. (2021), demonstrated that the diurnal cycles of 35/20 °C for 6/18h have a significant positive effect on the germination of *Musa acuminata*. Quaghebeur (2021) suggests that a higher temperature than 35 °C could provide a higher FGP, and they show that one hour of dry 45 °C *per* week significantly increased germination results.

1.6.4. ER test

Embryo rescue (ER) is a technique employed in the context of germination testing: 1) to prevent degeneration of weak, immature or hybrid embryos; 2) when conventional seed germination protocols fail to generate plantlets in a short period of time; and/or 3) when dormancy-related factors are associated to seed integuments or the endosperm (Sharma et al., 1996; Uma et al., 2011). However, the implementation of ER requires comprehensive understanding of seed anatomy, training in the technique of embryo excision, it demands substantial manual labor, and relies on access to costly facilities and materials (Sharma et al., 1996).

In the context of *Musa*, ER is widely practiced as it overcomes challenges associated with seed dormancy (Pancholi et al., 1995; Sharma et al., 1996; Uma et al., 2011; Arun et al., 2013; Burgos-Hernández, 2014; Vineesh et al., 2015; Wang et al., 2016; Kallow et al., 2020a; Ongagna et al., 2020; Singh et al., 2021; Kallow et al., 2022a).

Similarly to seed germination (see section 1.6.3), ER success in *Musa* is influenced by factors such as the initial seed viability, maturity stage, pre-incubation factors, and culture medium. Afele & De Langhe (1991) demonstrated that embryo orientation also has a significant effect in embryo germination. Uma et al. (2011) proved that basal Murashige and Skoog (MS) medium is sufficient for direct mature embryo regeneration. Notably, unlike seed germination, a warm constant temperature (between 26 °C and 32 °C) is sufficient for a successful regeneration of excised embryos (Afele & De Langhe, 1991; Neves et al., 2001; Uma et al., 2011; Arun et al., 2013; Burgos-Hernández, 2014; Dayarani et al., 2014; Vineesh et a., 2015; Kallow et al., 2020a; Ongagna et al., 2020).

2. Objectives

2.1. Design a feasible germination protocol for *Musa acuminata* seeds (stored and fresh seeds)

The main goal of this project is to design a feasible germination protocol of CWR of *M. acuminata*, for future implementation in seed banks. As described previously in this document, the success of germination is influenced by the initial viability of the batch, which is determined, amongst other factors, by seed morphology and maturity, and storage conditions. The germination of dormant seeds also implies the employment of suitable species-specific dormancy-release factors and proper environmental requirements.

Within this main goal, I purposed the following aims:

1) Quantify seed viability through morphological analyses of seeds and embryos, and the ability of embryos to absorb water: Seed structure and morphology were assessed to correlate weight, diameter and area with initial apparent seed viability. The embryo's ability to absorb water without coat scarification was also evaluated.

2) Test different germination protocols, including various dormancy-release and germination-stimulation factors, using wild *M. acuminata* seeds from different storing methods. The significance of pre-treatments, and the effect of temperature regimes and substrates during incubation were investigated; by conducting various germination experiments in defrosted and fresh wild *M. acuminata* seeds.

3) Assess viability of non-germinated seeds after different treatments. In the end, to acknowledge the impact of the pre-treatments and incubation methods, the viability of apparent viable seeds was determined by performing a Cut test, followed by an ER test in the first sample and a TTC test in the second sample of each experiment.

2.2. Determine the eligibility of TTC as a viability test for banana

The second goal of this project is to determine the reliability and precision of the Tetrazolium Chloride (TTC) test compared to Embryo Rescue (ER). Using non-germinated seeds from the previous whole seed germination tests, a comparison was

made between the results obtained from TTC staining with the actual germination results achieved through ER.

3. Materials and Methods

Whole seed incubation experiments were performed at the Laboratory of the Seed Bank of Meise Botanic Garden (MBG) (Meise, Belgium), and Embryo Rescue and TTC tests were performed at the Laboratory of Tropical Crop Improvement (University of Leuven, Belgium).

3.1. Plant material

3.1.1. Origin of seeds

Two batches of wild *Musa acuminata* seeds, originating from Yangambi, Democratic Republic of Congo (DRC), were used in the procedures.

The first batch – **Batch 20171632-94** – consisted of seeds from different plants of the same population, collected on 31 July 2017. Seeds were dried-stored in identified aluminum-sealed bags in freezers of the Seed Bank of MBG at -20 °C and 15% relative humidity (RH). To be used in the procedures, the seeds were air-defrosted and placed at a constant temperature of 15 °C and 15% RH (Fig. 6).

Figure 6. Defrosted Musa acuminata seeds in a Petri dish (batch 20171632-94).

For the second batch – **Batch 20230003** – fresh bananas fruits at different ripeness stages (Fig. 7) were collected on 26 January 2023 and transported to the laboratory of the Seed Bank of MBG. Six days later, fruits were opened, the pulp was

extracted, and seeds were rinsed and cleaned with tap water. The fresh seeds were air-dried for 3 days and then packed in plastic zip-lock bags to maintain humidity.

Figure 7. Musa acuminata fruits, at different maturity stages, used in the batch 20230003.

3.1.2. Seed selection

Batch **20171632-94** was sub-divided in six lots, correlated with each original plant. Seeds were removed from the hermetically sealed plastic containers within the Seed Bank of MBG and placed into labeled paper bags for utilization in subsequent procedures. An initial ER test was made in a 10 embryo-sample, in order to determine the viability of stored seeds (Table 1). Only the lots S/N.3 and S/N.4 (Table 1) were used in subsequent germination procedures.

Table 1. Plant material: Overview of batch 20171632-94.

Lot	Initial no. seeds	Viability in ER test
S/N.1 - 43391	10	Not tested
S/N.2 - 48243	763	0%
S/N.3 - 48244	**708**	**30%**
S/N.4 - 48245	**371**	**30%**
S/N.5 - 48246	514	10%
S/N.6 - 48247	538	20%

Seeds from batch **20230003** were extracted from fruits at different maturity stages. To distinguish seeds by maturity, seeds were primarily grouped by color: *black, brown, light brown,* and *white-ish;* and grouped again by its density in water through a

floating-test (results: *sink*, or *float)*. Based on previous experience, seeds from groups *Black Sink* and *Brown Sink* were identified as the most mature seeds. As embryo maturity is a key factor for seed germination (Kallow et al., 2022b), 3360 seeds from these two groups were used in subsequent germination procedures (Table 2). Moisture content was measured using a moisture hydrometer before seed testing.

Table 2. Plant material: Initial seed content of batch 20230003.

Groups	Initial no. of seeds
Black Sink	**2251**
Black Float	512
Brown Sink	**1109**
Brown Float	410
Light Brown Sink	118
Light Brown Float	24
White-ish Sink	8
White-ish Float	30

3.2. Seed characterization (initial apparent viability)

As mentioned previously (see section 1.6), assessing seed viability prior to germination testing and monitoring the viability of seed collections are routine procedures realized in Seed banks (MSBP, 2022). After collection, seeds are clean and stored as a Seed Bank accession.

To primarily discard unviable seeds (as explained in the section 3.1), a first test based on color is made (results: *black*, *brown*, *light-brown,* or *white-ish*), followed by a floating test (results: *sink*, or *float*). However, a great number of unviable seeds could not be perceived. In order to optimize this step (of primarily discarding non-viable seeds), and understand the relation between seed morphology and seed viability, the weight, diameter and area of seeds was compared to *apparent seed viability* of stored (batch **20171632-94**) and fresh seeds (batch **20230003**).

During this step, seed viability was defined as *apparent seed viability*, to differentiate from true seed viability confirmed by TTC and ER tests.

Apparent seed viability of stored seeds - Batch 20171632-94

To assess seed weight, diameter and area from batch **20172632-94**, a random sample of 100 seeds from the lot S/N.6 was weighted with a precision balance (0.001 g) and photographed. Seed diameter and area were assessed using collected photos and the *Image J* 1.54d program.

Apparent seed viability was estimated by a Cut test made after assessing the morphology. Seeds were classified as *Intact* when they show a white-dry endosperm (Fig. 8) and a white mushroom-like shaped embryo (Fig. 4); or *Unviable*, if they show abnormal seed morphology or a lack of embryo.

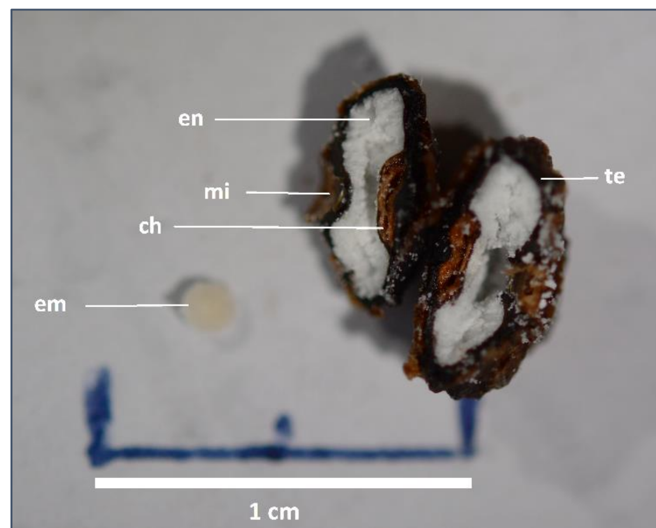

Figure 8. Photography of a bisected Intact Musa acuminata seed: chalazal mass (ch), white and mushroom shaped embryo (em), white and dry endosperm (en), micropyle (mi), testa (te).

Apparent seed viability of fresh seeds - Batch 20230003

Initially, the moisture content was measured using a HygroPalm moisture meter (Gold, K. & Manger, K., 2022). Seeds with a moisture content of MC = 8.9% (eRH = 26.6%, Temp. = 16.7 °C) were used in the procedures.

To characterize the seed morphology (weight, diameter and area), a random sample of 20 seeds from each Group (except *White-ish Sink,* 8 seeds were used) was evaluated as described in the previous section.

Seeds from the groups Black Sink and Brown Sink were classified as *apparent mature viable seeds*; in contrast to the other groups, that were classified as *Unviable*.

3.2.1. Water uptake studies

To confirm the ability of seeds to absorb water without removing or scarifying seed integuments, the seed sample used previously to assess morphology (lot S/N.6) was divided into two groups of 50 seeds each (Group A, Group B).

Seeds from *Group A* were not submitted to any procedure. Seeds from *Group B* were soaked in water for two days at room temperature: B.0 designates seeds from *Group B* on day 0 (zero); B.2 designates seeds from *Group B* on day 2 (two). After assessing weight, diameter and area, seeds from *Group A* were longitudinally cut using a scalpel and tweezers to separate the embryo from the seed. Embryos were classified as *Intact*, when they showed an apparent viability in the form of a white coloration with a prominent haustorium and stalk, and a white starchy endosperm; or *Unviable* if they appear as a deformed, dark or absent embryo, and/or half of the endosperm content. Apparent viable embryos were placed in a small flask, counted and weighted together.

After assessing weight, diameter and area, seeds from *Group B* were sterilized using 1% NaOCl for 20 minutes, rinsed three times with de-mineralized water and placed in individual identified 25 ml flasks with de-mineralized water at room temperature. After 2 days (*Group B.2*), the excess of water was removed using kitchen-paper (Arun et al., 2013) and seed morphology was assessed one more time. Seeds were dissected, following the same procedure, to remove the embryo. Apparent viable embryos were placed in a small flask, counted and weight together.

To assess the ability of the seed and the embryo to absorb water, it was compared: 1) the seed weight, diameter and area of non-soaked seeds and seeds soaked for two days in water at room temperature; and 2) the total embryo weight of *Group A* (non-soaked seeds) and *Group B.2* (seeds soaked for two days in water at room temperature).

3.3. Germination testing

The germination testing procedure was organized in three hierarchical levels – treatment, experiment and replicate; thus, a treatment contains experiments and replicates, and an experiment contains only replicates.

The procedure includes 18 treatments (identified from A to R), and a variable number of experiments (example of an experiment I.D.: 2017.A.30.20 and 2023.H.35P.30.20; from batch 20171632-94 and 20230003, respectively). Experiments from Treatments A to M were composed of two replicates (example: 2017.A1.30.20, 2017.A2.30.20). Treatments N and O were composed of two experiments, with four replicates of the first experiment, and six replicates of the second experiment. Treatments P to R were composed of two experiments, with four replicates of both experiments (Table A3.1, Table A3.2).

Each replicate was conducted in a Petri dish, where 20 seeds were distributed in lines of 4 x 5 and placed in a plastic hermetically-sealed bag to prevent dehydration (Fig. 9). Small contaminations were periodically controlled by cleaning seeds with de-mineralized water, drying and placing them in a new Petri dish medium.

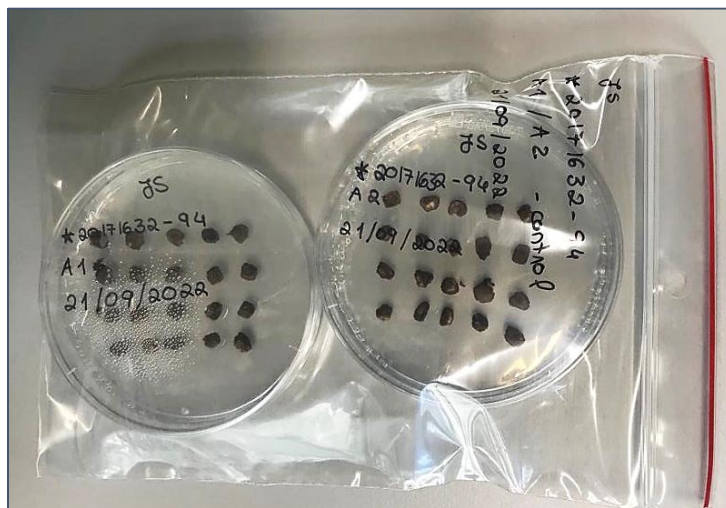

Figure 9. Example of an experiment comprised by two replicates of 20 seeds each and placed in a plastic zip-lock bag.

The procedure used for whole seed germination testing consisted of three main steps: *Disinfection*, *Pre-treatment*, and *Incubation*.

3.3.1. Disinfection

Before each experiment, seeds were disinfected in a 100 mL Erlenmeyer and submerged in 50 mL ethanol 70%. After 3 minutes, the ethanol was removed and 150

mL of sodium hypochlorite (NaOCl) 1% was added for 20 minutes, with an occasional shaking of the Erlenmeyer. After that time, the NaOCl was pour out and seeds were rinse 3 times with demineralized water (demi-water).

3.3.2. Pre-treatment

After disinfection, seeds were submitted to 14 pre-treatments, organized as: 1) *none;* 2) *thermal hydropriming*: 20 °C, 35 °C or 100 °C; 3) *hormonal priming*: indole-3-acetic acid (IAA), gibberellic acid (GA_3), 6-benzylminopurine (BAP) or combinational; or 4) *scarification* with medium sand paper grit or fine sand paper grit.

Pre-treatments lasted for 48 h to 72 h, except for the *scarification* and *100 °C hydropriming* (30 seconds or 60 seconds).

1) No pre-treatment

A total of 920 seeds from batch 20171632-94 and batch 20230003 were incubated without pre-treatment. These seeds are identified as *None* in Pre-treatment in Table A3.1 and Table A3.2 of Annex 3: Germination Testing.

2) Thermal hydropriming

20°C hydropriming

A total of 360 seeds from batch 20230003 were placed in a disinfected glass recipient and 20 °C demi-water was added until all seeds were submerged. The glass recipient was placed in an incubator at a constant temperature of 20 °C for 48 h to 72 h. These seeds are identified as *20 °C water* in Pre-treatment in Table A3.2 of Annex 3: Germination Testing.

35°C hydropriming

A total of 2120 seeds from both batches were placed in a disinfected glass recipient and 35 °C demi-water was added until all seeds were submerged. The glass recipient was placed in an incubator at constant temperature of 35 °C for 48 h to 72 h. These seeds were identified as *35 °C water* in Pre-treatment in Table A3.1 and Table A3.2 of Annex 3: Germination Testing.

100°C hydropriming

A group of 80 seeds from batch 20230003 was boiled in 100 °C demi-water for 30 seconds (Experimental coding: 2023.A.BOIL.30.20); and a second group of 80 seeds from the same batch was boiled for 60 seconds (Treatment: Q – Experimental coding: 2023.B.BOIL.30.20).

3) Hormonal priming

A total of 520 seeds from both batches was submitted to hormonal pre-treatments. Seeds were placed in a disinfected glass recipient with a hormonal solution of indole-3-acetic acid (IAA), gibberellic acid (GA_3) or 6-benzylminopurine (BAP) at 1000ppm, 1000ppm and 500ppm, respectively, or a combination of them (IAA + GA_3; IAA + BAP; GA_3 + BAP; IAA + GA_3 + BAP). The glass recipient was placed in an incubator at constant 20 °C temperature for 48 h to 72 h. These seeds were identified as *IAA, GA, BAP*, or combinational in Pre-treatment in Table A3.1 and Table A3.2 of Annex 3: Germination Testing.

4) Scarification

A group of 80 seeds from batch 20230003 was scarified in the planar base of the seed using a fine grit sand paper (Treatment R – Experimental coding: 2023.A.SCAR.30.20), and a second group of 80 seeds from the same batch was scarified using a medium grit sand paper (Treatment R – Experimental coding: 2023.B.SCAR.30.20).

3.3.3. Incubation conditions

After the pre-treatment, seeds were rinsed 3 times with de-mineralized water to clean any chemical compounds released by seeds. The *Incubation* step was divided in 2 parts: Substrate and Temperature.

3.3.3.1. Substrate

For the *Incubation* step, five kinds of substrate were tested: 1) *Agar-Agar*; 2) *Agar with GA_3*; 3) *Perlite*; 4) *Sand*; and 5) *Filter Paper*.

1) Agar medium (AGAR)

In each Petri dish, 25 mL of Agar medium containing 1% agar-agar (Davies et al., 2015a) was distributed; and the Petri dishes were then hermetically sealed in plastic bags and stored in a fridge at 7 °C until use. A set of 3240 seeds were incubated in Agar medium. These seeds were identified as *AGAR* in Substrate in Table A3.1 and Table A3.2 of Annex 3: Germination Testing.

2) AGAR + GA_3

Agar medium 1% agar-agar with gibberellic acid (GA_3) was also tested. GA_3 at 250 mL/L was added to the boiling demineralized water before the agar-agar was added (Davies et al., 2015a). A set of 440 seeds from batch 20171632-94 were incubated at Agar medium with GA_3. These seeds were identified as *AGAR + GA* in Substrate in Table A3.1 of Annex 3: Germination Testing.

3) River Sand

River sand was first disinfected by placing it in an electrical oven at 90 °C for 30 to 45 minutes (Quaghebeur, 2021). After cooling, 70 g of sand was distributed over the Petri dishes, and 15 mL of demineralized water was added to make the substrate sufficiently moist (Fig. 10). A set of 200 seeds from batch 20230003 were incubated in Sand substrate. These seeds, belonging to Treatment N, were identified as *SAND* in Substrate in Table A3.2 of Annex 3: Germination Testing.

Figure 10. Seed distribution of Treatment N (SAND Substrate).

4) Perlite

Perlite was first disinfected by placing it in an electrical oven at 90 °C for 30 to 45 minutes. After cooling, 5 g of perlite was distributed over the Petri dishes, and 30 mL of demineralized water was added to make the substrate sufficiently moist (Fig. 11). A set of 200 seeds from batch 20230003 were incubated in Perlite substrate. These seeds, belonging to Treatment O, were identified as *PERLITE* in Substrate in Table A3.2 of Annex 3: Germination Testing.

Figure 11. Seed distribution of Treatment O (PERLITE substrate).

5) Filter Paper

Two sheets of filter paper were placed in the Petri dish and demineralized water was added to make the paper humid, but not moist. A set of 160 seeds from batch 20230003 were incubated in Filter Paper substrate. These seeds, belonging to Treatment P, were identified as *PAPER* in Substrate in Table A3.2 of Annex 3: Germination Testing.

3.3.3.2. Temperature

To test the influence of temperature in CWR of *M. acuminata* germination, four incubators at different temperatures were used: 1) Incubator 1 (PHCbi MLR-352-PE), at constant 20 °C for 24 h; 2) Incubator 2 (LMS coolsystems), at 30/20 °C for 8/16 h; 3) Egg incubator 1 (HHD 12 egg incubator), 40 °C at a variable time (Fig. 12); and 4) Egg incubator 2 (HHD 12 egg incubator), 50 °C at a variable time (Fig. 12).

Figure 12. Incubators (HHD 12 egg incubator) used for temperatures of 40 °C and 50 °C, at variable times.

Using a combination of these four incubators, eight temperature regimes were designed: 1) Daily constant; 2) Daily oscillation; 3) Monthly alternation; 4) Weekly alternation; 5) 40 °C + daily oscillation; 6) 50 °C + daily oscillation; 7) 40 °C + daily constant; 8) 50 °C + daily constant.

1) Daily constant

This method consists of incubating seeds at a constant temperature of 20 °C for 24 h (Incubator 1). A group of 240 seeds from both batches were submitted to this

method. These seeds are identified as *20 °C* in Temperature in Table A3.1 and Table A3.2 of Annex 3: Germination Testing.

2) Daily oscillation

This method consisted of incubating seeds at a temperature of 30/20 °C for 8/16 h daily (Incubator 2). A total of 1840 seeds from both batches were submitted to this method. Seeds from these experiments are identified as *30/20 °C* in Temperature in Table A3.1 and Table A3.2 of Annex 3: Germination Testing.

3) Monthly alternation (M.A.)

This method consisted of alternating the seeds between Incubator 1 (constant temperature) and Incubator 2 (daily temperature oscillation) for six months (24 weeks). A group of 240 seeds from lot S/N.4 - 48245 (Batch 20171632-94) were submitted to this method. During this treatment, seeds were incubated, following the pattern explained in the Table 3:

Table 3. Temperature variation pattern for temperature regime Monthly Alternation.

Temperature I.D.	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
M.A.C	20°C	20°C	20°C	30/20°C	30/20°C	30/20°C
M.A.D	30/20°C	30/20°C	30/20°C	20°C	20°C	20°C
M.A.E	30/20°C	30/20°C	20°C	20°C	30/20°C	30/20°C
M.A.F	20°C	20°C	30/20°C	20°C	20°C	30/20°C
M.A.G	20°C	30/20°C	30/20°C	20°C	30/20°C	30/20°C
M.A.H	20°C	30/20°C	20°C	30/20°C	20°C	30/20°C

These seeds are identified as *M.A.* in Temperature in Table A3.1 of Annex 3: Germination Testing.

4) Weekly alternation (W.A.)

During three months (12 weeks), 720 seeds from batch 20230003 were submitted to a weekly alternation between Incubator 1 and Incubator 2, following the pattern explained in the Table 4:

Table 4. Temperature variation pattern for temperature regime Weekly Alternation.

Temperature I.D.	Week 1 & 2	Week 3 &4	Week 5 & 6	Week 7 & 8	Week 9 & 10	Week 11 & 12
W.A.C	20°C	20°C	20°C	30/20°C	30/20°C	30/20°C
W.A.D	30/20°C	30/20°C	30/20°C	20°C	20°C	20°C
W.A.E	30/20°C	30/20°C	20°C	20°C	30/20°C	30/20°C
W.A.F	20°C	20°C	30/20°C	20°C	20°C	30/20°C
W.A.G	20°C	30/20°C	30/20°C	20°C	30/20°C	30/20°C
W.A.H	20°C	30/20°C	20°C	30/20°C	20°C	30/20° C

These seeds are identified as *W.A.* in Temperature in Table A3.1 and Table A3.2 of Annex 3: Germination Testing.

5) 40 °C + daily oscillation

To understand the influence of high temperatures in triggering the germination response, 360 seeds from batch 20171632-94 and from batch 20230003 were incubated at daily oscillation of 30/20 °C (8/16 h) and exposed to a temperature of 40 °C for 2 h or 4 h, at different frequencies *per* week (1, 3 or 5 times), during six weeks (Table 5).

Table 5. Temperature variation pattern for temperature regime 40 °C + daily oscillation, and related experiments.

Experiment I.D.		Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
2017.B.35P.40	2023.B.35P.40	30/20°C	30/20°C	30/20°C	40°C (4h) + 30/20°C	30/20°C	30/20°C	30/20°C
2017.C.35P.40	2023.C.35P.40	30/20°C	40°C (4h) + 30/20°C	30/20°C	40°C (4h) + 30/20°C	30/20°C	40°C (4h) + 30/20°C	30/20°C
2017.D.35P.40	2023.D.35P.40	30/20°C	40°C (4h) + 30/20°C	40°C\ (4h) + 30/20°C	40°C (4h) + 30/20°C	40°C (4h) + 30/20°C	40°C (4h) + 30/20°C	30/20°C
	2023.E.35P.40	30/20°C	30/20°C	30/20°C	40°C (2h) + 30/20°C	30/20°C	30/20°C	30/20°C
	2023.F.35P.40	30/20°C	40 (2h) + 30/20	30/20°C	40°C (2h) + 30/20°C	30/20°C	40°C (2h) + 30/20°C	30/20°C
	2023.G.35P.40	30/20°C	40°C (2h) + 30/20°C	40°C (2h) + 30/20°C	40°C (2h) + 30/20°C	40°C (2h) + 30/20°C	40°C (2h) + 30/20°C	30/20°C

6) 50 °C + daily oscillation

To understand the influence of high temperatures in triggering the germination response, 360 seeds from both batches were incubated at daily oscillation of 30/20 °C

(8/16 h) and exposed to a temperature of 50 °C for 2 h or 4 h, at different frequencies *per* week (1, 3 or 5 times), during six weeks (Table 6).

Table 6. Temperature variation pattern for temperature regime 50 °C + daily oscillation, and related experiments.

Experiment I.D.		Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
2017.B.35P.50	2023.B.35P.50	30/20°C	30/20°C	30/20°C	50°C (4h) + 30/20°C	30/20°C	30/20°C	30/20°C
2017.C.35P.50	2023.C.35P.50	30/20°C	50°C (4h) + 30/20°C	30/20°C	50°C (4h) + 30/20°C	30/20°C	50°C (4h) + 30/20°C	30/20°C
2017.D.35P.50	2023.D.35P.50	30/20°C	50°C (4h) + 30/20°C	50°C (4h) + 30/20°C	50°C (4h) + 30/20°C	50°C (4h) + 30/20°C	50°C (4h) + 30/20°C	30/20°C
	2023.E.35P.50	30/20°C	30/20°C	30/20°C	50°C (2h) + 30/20°C	30/20°C	30/20°C	30/20°C
	2023.F.35P.50	30/20°C	50°C (2h) + 30/20°C	30/20°C	50°C (2h) + 30/20°C	30/20°C	50°C (2h) + 30/20°C	30/20°C
	2023.G.35P.50	30/20°C	50°C (2h) + 30/20°C	50°C (2h) + 30/20°C	50°C (2h) + 30/20°C	50°C (2h) + 30/20°C	50°C (2h) + 30/20°C	30/20°C

7) 40 °C + daily constant

To understand if an abrupt change of temperature would influence the germination response, 240 seeds from batch 20230003 were incubated in Incubator 1 and exposed to a temperature of 40 °C for 2 h or 4 h, at different frequencies *per* week (1, 3 or 5 times), during six weeks (Table 7).

Table 7. Temperature variation pattern for temperature regime 40 °C + daily constant, and related experiments.

Experiment I.D.	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
2023.B.35P.40.a	20°C	20°C	20°C	40°C (4h) + 20°C	20°C	20°C	20°C
2023.C.35P.40.a	20°C	40°C (4h) + 20°C	20°C	40°C (4h) + 20°C	20°C	40°C (4h) + 20°C	20°C
2023.D.35P.40.a	20°C	40°C (4h) + 20°C	40°C (4h) + 20°C	40°C (4h) + 20°C	40°C (4h) + 20°C	40°C (4h) + 20°C	20°C
2023.E.35P.40.a	20°C	20°C	20°C	40°C (2h) + 20°C	20°C	20°C	20°C
2023.F.35P.40.a	20°C	40°C (2h) + 20°C	20°C	40°C (2h) + 20°C	20°C	40°C (2h) + 20°C	20°C
2023.G.35P.40.a	20°C	40°C (2h) + 20°C	40°C (2h) + 20°C	40°C (2h) + 20°C	40°C (2h) + 20°C	40°C (2h) + 20°C	20°C

8) 50 °C + daily constant

To understand if an abrupt change of temperature would influence the germination response, 240 seeds from batch 20230003 were incubated in Incubator 1 and exposed to a temperature of 50 °C for 2 h or 4 h, at different frequencies *per* week (1, 3 or 5 times), during six weeks (Table 8).

Table 8. Temperature variation pattern for temperature regime 50 °C + daily constant, and related experiments.

Experiment I.D.	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
2023.B.35P.50.a	20°C	20°C	20°C	50 °C (4h) + 20°C	20°C	20°C	20°C
2023.C.35P.50.a	20°C	50°C (4h) + 20°C	20°C	50°C (4h) + 20°C	20°C	50°C (4h) + 20°C	20°C
2023.D.35P.50.a	20°C	50°C (4h) + 20°C	50°C (4h) + 20°C	50°C (4h) + 20°C	50°C (4h) + 20°C	50°C (4h) + 20°C	20°C
2023.E.35P.50.a	20°C	20°C	20°C	50°C (2h) + 20°C	20°C	20°C	20°C
2023.F.35P.50.a	20°C	50°C (2h) + 20°C	20°C	50°C (2h) + 20°C	20°C	50°C (2h) + 20°C	20°C
2023.G.35P.50.a	20°C	50°C (2h) + 20°C	50°C (2h) + 20°C	50°C (2h) + 20°C	50°C (2h) + 20°C	50°C (2h) + 20°C	20°C

3.4. Post-incubation viability testing

At the end of each treatment, the Final Germination Percentage (FGP) and the percentage of non-germinated seeds were assessed.

To understand the impact of each factor of the procedures used in the germination testing, a viability test was performed in apparently viable but non-germinated seeds. The viability test consisted of a Cut test followed by Embryo Rescue (ER) in the first half of the replicates of each experiment, and a TTC test in the second half. The results of post-incubation viability were counted using the actual germination achieved through the ER test. The results achieved through the TTC test were used to determine the accuracy of the TTC test compared to the ER test.

3.4.1. Cut test

The Cut test was performed under a binocular microscope (specific protocol requirements for ER and TTC are explained below), where seeds were placed in a sterile

paper and longitudinally bisected using a scalpel and forceps. Each seed was fixed between the forceps with the micropyle facing up, an incision was made on both sides. The embryo was gently detached from the endosperm using the scalpel (Quaghebeur, 2021).

Seeds were identified as: 1) *apparent viable seeds*, when showing intact white embryos and firm, starchy and white endosperm; 2) *non-viable seeds,* exhibiting signs of contamination, abnormal morphology or clear signs of being nonviable; or 3) *no embryo*, for seeds devoid of embryo.

Only apparent viable seeds were evaluated in ER and TTC procedures.

3.4.2. ER procedure

The medium used in the Embryo Rescue test was ½ MS (Murashige & Skoog, 1962): all the components (Table 9) were dissolved in distilled water (except phytagel) using an electrical stirred. The pH was measured by a calibrated pH meter and set to 6.12 by adding NaOH or HCl using a pipet. Subsequently, 3 g/L of Phytagel was added and the mixture was heated on an electrical heater, while continuous stirring until the mixture was clear. The medium was transferred to test tubes; subsequently the tubes were closed with caps and autoclaved for 20 minutes at 121 °C. The sterile tubes were identified and stored in the dark until needed (Quaghebeur, 2021; Coenen, 2022).

Table 9. Components of ½ MS medium (1 L).

Component	Quantity
Murashige & Skoog Medium (1/2 conc. FeNaEDTA)	2.16 g
Murashige & Skoog Vitamin Mixture	0.10 g
Sucrose	20.00 g
Phytagel	3.00 g
Ascorbic acid stock solution(10^{-3} M in destilled water)	1.00 mL

Seeds submitted to ER were always manipulated in sterile conditions in a laminar air flow chamber. Seeds were first sterilized in a 100 mL sterile Erlenmeyer and submerged in ethanol 96%, with a Petri dish closing the lid. After 3 minutes, the ethanol was removed and seeds were immersed in a solution of sodium hypochlorite (NaOCl) 1% with a drop of soap for 20 minutes, with an occasional shaking of the Erlenmeyer.

After that time, the NaOCl was poured out, while seeds remained in the Erlenmeyer to be rinsed three times with sterile demineralized water (Quaghebeur, 2021; Coenen, 2022).

After sterilization, seeds were placed on a sterile paper under a binocular microscope, where embryos were carefully removed from seeds using sterile forceps and a sterile scalpel (see section 3.4.1). The embryo was carefully transferred, using long forceps, to a test tube containing sterile ½ MS medium. The haustorium of the embryo was in close contact with the medium, and its tip was facing upwards. The test tubes were grouped in identified racks, placed in plastic boxes and incubated for two weeks in the dark at 26 ± 1 °C. Then, a first evaluation was made, and the racks were removed from the boxes and transferred to the light (24 h photoperiod) for another two weeks. A second evaluation was made after two weeks of light exposure, and a final evaluation was made four weeks after light exposure. The ER germination was evaluated under the following categories: germination; callus; dark; no reaction; or contamination (Quaghebeur, 2021; Coenen, 2022).

3.4.3. TTC procedure

The TTC buffer was first prepared by weighting on an analytical scale 3.402 g of (0.05 M) KH_2PO_4 (Potassium dihydrogen phosphate) and dissolving it together with 4.355 g of (0.05 M) K_2HPO_4 (Dipotassium hydrogen phosphate) in 500 mL of demineralized water; afterwards, the pH was equilibrated to 7. With 50 mL of the previously made solution, 0.25 g of 0.5% TTC was added while wearing gloves. The centrifuge tube with the solution was wrapped in aluminum foil to prevent photo-oxidation and stored in a fridge at 5 °C until use (Kallow et al., 2020b; Quaghebeur, 2021; Coenen, 2022).

Under a binocular microscope, embryos were carefully removed from seeds using forceps and a scalpel (section 3.4.1). A maximum of 20 *apparent viable* embryos were submerged in the previously made solution in a labeled 2 mL Eppendorf tube. The tube was gently shaken, covered by aluminum foil to avoid light and kept at 27 °C for 48 h. The staining pattern was evaluated under the following categories: red, pink, white (Fig. 5 of section 1.6.2). Embryos that completely stained red (dark red, or pink with a

red coloration at the tip) were considered viable; pink or white embryos were considered unviable (Kallow et al., 2020b; Quaghebeur, 2021; Coenen, 2022).

3.5. Statistical analysis

The morphological seed characterization was described qualitatively and quantitatively. Quantitative data were compiled and analyzed using Microsoft Excel 2010 and GraphPad Prism 8.0.1 (244).

Morphological data (seed weight, diameter and area) from stored seeds (batch 20171632-94) was analyzed using a paired t-test to assess differences between apparently viable and unviable seeds.

Morphological data (seed weight, diameter and area) from fresh seeds (batch 20230003) was analyzed through a One-way ANOVA to assess differences between seed groups (Black Sink, Black Float, Brown Sink, Brown Float, Light-Brown Sink, Light-Brown Float, White-ish Sink, and White-ish Float).

Seed water uptake was evaluated using changes in seed morphological data (seed weight, diameter and area) before (Group B.0) and after (Group B.2) seed-soaking for two days in water (batch 20171632-94) using a paired t-test.

All seed germination tests resulted in very low Final Germination Percentage (FGP), making it impossible to analyze them statistically.

The post-incubation viability (ER and TTC tests) of seeds subjected to different treatments was analyzed through a Chi-Square test.

To compare the post-incubation viability of both batches, the results of ER test from stored and fresh seeds were analyzed using an unpaired t-test.

The accuracy between the viability tests TTC and ER was calculated through a paired t-test.

4. Results

4.1. Morphology and initial apparent viability

Stored seeds (batch 20171632-94)

Musa acuminata stored seeds were black-brown in color; they had a regular or irregular globose shape with a flat bottom and they exhibited a warty hard seed coat (Fig. 13.A; Fig. 13.B; Fig. 13.C). Seed diameter ranged from 0.559 cm to 0.799 cm; seed area ranged from 0.202 cm^2 to 0.343 cm^2; the average seed weight was 0.057 g; and individual seed weight ranged from 0.044 g to 0.080 g (only data from apparent viable seeds was integrated here) (Table A2.1; Table A2.2). Fourteen *unviable* embryos were found, meaning that 14% of this sample of defrost seeds was not viable (Table A2.1; Table A2.2).

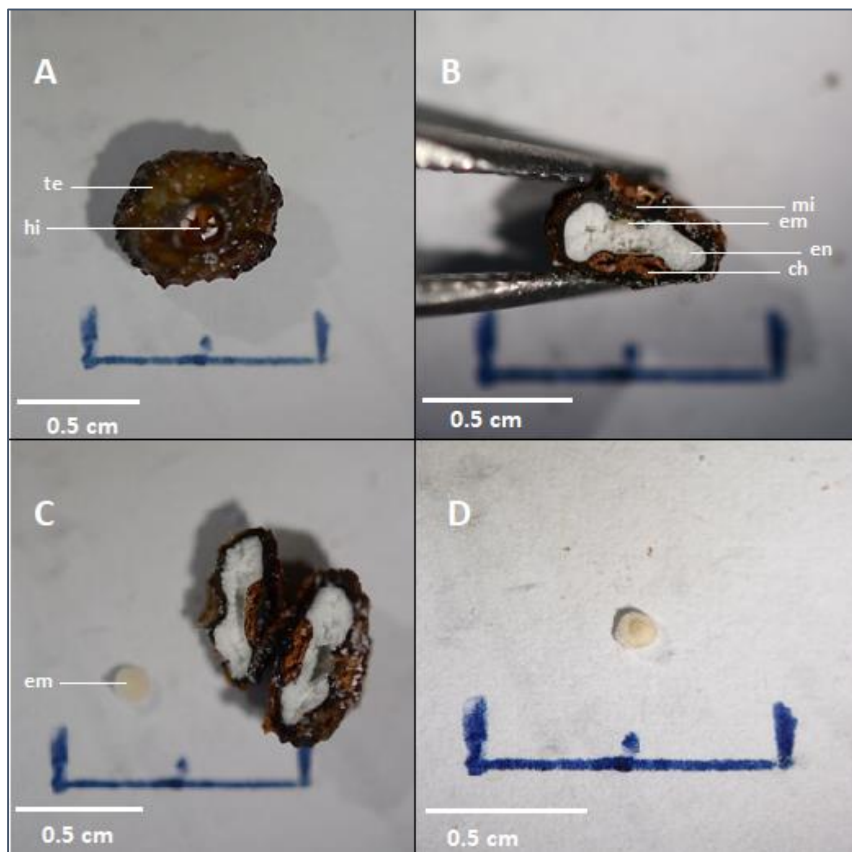

Figure 13. Morphology and structutre of M. acuminata seeds from batch 20171632-94: (A) top-view of a globose-shaped seed; (B) longidutinal section of the seed with embryo; (C) longidutinal section of the seed and extracted embryo; (D) embryo. Chalazal mass (ch); embryo (em); endosperm (en); hilum (hi); micropyle (mi); testa (te). White bar: 0.5 cm.

Unviable seeds exhibited a significantly lower seed weight (t = 8.066, P < 0.001) (Fig. 14.A) and seed area (t = 2.652, P = 0.020) (Fig. 14.C) compared to apparent viable (*Intact*) seeds (P < 0.05). Seed diameter did not show significant differences between *Intact* and *Unviable* seed groups (t = 1.753, P = 0.103) (Fig. 14.B) (P < 0.05) (Table A2.1; Table A2.2)

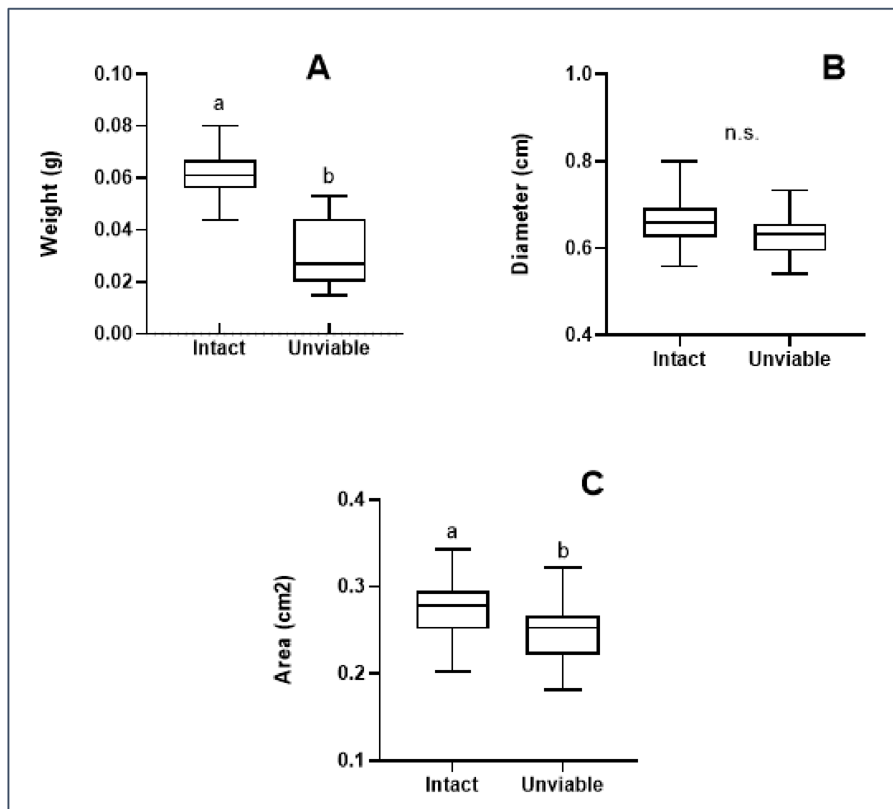

Figure 14. Comparison between apparent seed viability and seed measurements from 100 seeds of batch 20171632-94: (A) seed weight, (B) seed diameter, and (C) seed area. Different lowercase letters in the same graphic mean significant difference between groups (P < 0.05); n.s. denote non-significant differences at P > 0.05. Error bars stand for SD (standard deviation).

Fresh seeds (batch 20230003)

Fresh seeds from batch 20230003 exhibit a pronounced heterogeneity with regard to seed color and their capacity to sink or float (as observed previously - Table 2; Fig. 7).

It is noticeable that both the *Black Sink* and *Brown Sink* groups (considered mature groups) have significantly higher ($P < 0.05$) mean seed weight (ranging from 0.037 g to 0.08 g), in comparison to the remaining groups (ranging from 0.007 g to 0.034 g) (Table 10; Fig. 15.A; Table A2.3; Table A2.4). *Black Float, Brown Float* and *White-ish Sink* show intermediate and low values in seed weight (Table 10; Fig. 15.A; Table A2.3; Table A2.4).

The mean diameter of *Brown Sink* group is significantly higher ($P < 0.05$) than the remaining groups, except the *White-ish Sink* group that exhibits intermediate values (Table 10; Fig. 15.B; Table A2.3; Table A2.4).

The *Brown Sink* group has the highest mean seed area, significantly different ($P < 0.05$) from the remaining groups, except *Black Sink* that exhibits intermediate values ($P < 0.05$) (Table 10; Fig. 15.C; Table A2.3; Table A2.4).

There were significant differences for the three studied variables among all the seed groups, between morphological characteristics (Weight: $F = 172.7$, $P < 0.001$; Diameter: $F = 4.978$, $P < 0.001$; Area: $F = 4.939$, $P < 0.001$).

Table 10. Measurements of seeds from batch 20230003.

Groups	Weight (g) Min – Max	Diameter (cm) Min – Max	Area (cm^2) Min – Max
Black Sink	**0.042 – 0.079**	0.574 – 0.756	0.196 – 0.321
Black Float	0.011 – 0.034	0.528 – 0.703	0.183 – 0.321
Brown Sink	**0.037 – 0.080**	**0.590 – 0.785**	0.208 – 0.378
Brown Float	0.007 – 0.029	0.566 – 0.711	0.164 – 0.319
Light-Brown Sink	0.007 – 0.015	0.524 – 0.695	0.160 – 0.276
Light-Brown Float	0.007 – 0.016	0.574 – 0.680	0.181 – 0.294
White-ish Sink	0.007 – 0.016	0.576 – 0.649	0.167 – 0.253
White-ish Float	0.008 – 0.019	0.515 – 0.744	0.188 – 0.330

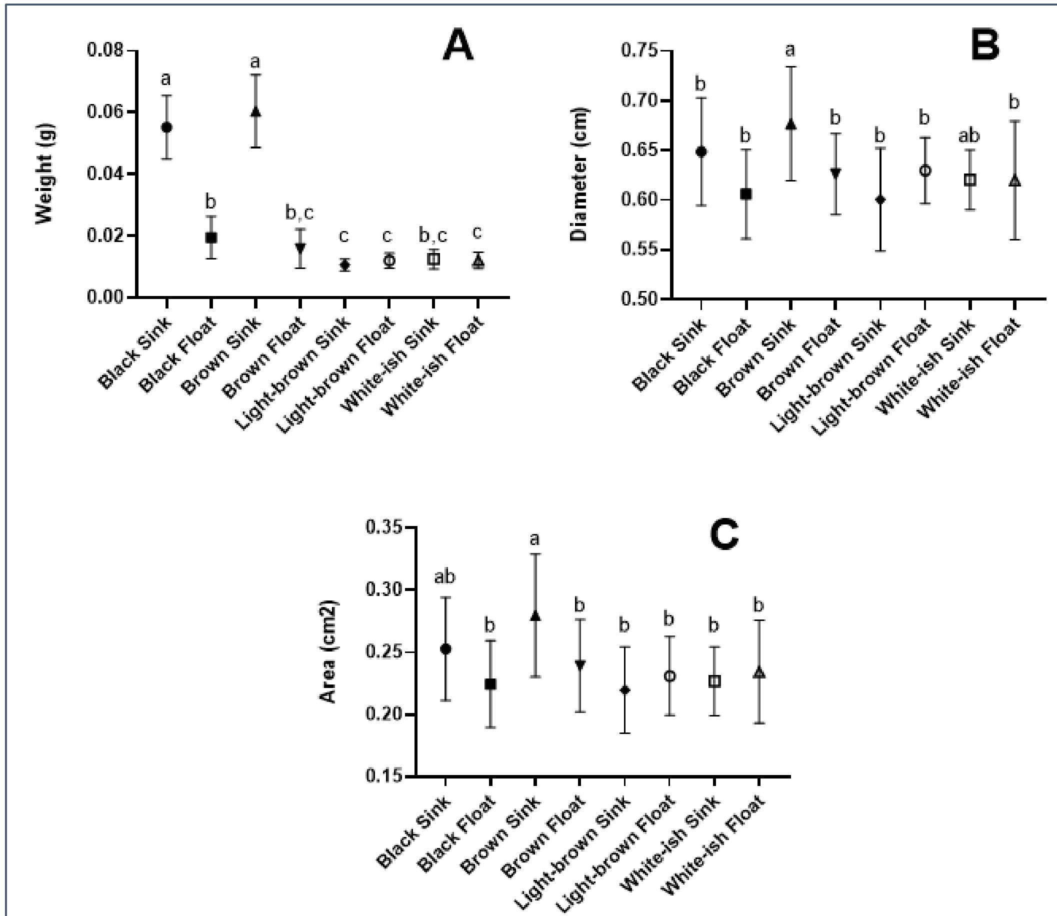

Figure 15. Mean values of measurements from different groups of batch 20230003: (A) seed weight, (B) seed diameter, and (C) seed area. Different lowercase letters in the same graphic mean significant difference between groups ($p < 0.05$). Error bars stand for SD (standard deviation).

4.1.1. Water uptake studies

Seeds from *Group B* (i.e., seeds soaked in water) showed a significant increase in weight (t = 5.834, P < 0.001), diameter (t = 2.262, P = 0.026) and area (t = 2.972, P < 0.004) from day 0 to day 2 (Fig. 16). The mean embryo weight of 50 non-soaked seeds (*Group A*), was inferior (21 mg, 43 embryos), compared to the mean embryo weight of *Group B.2* (33 mg, 43 embryos) (Table A2.1; Table A2.3).

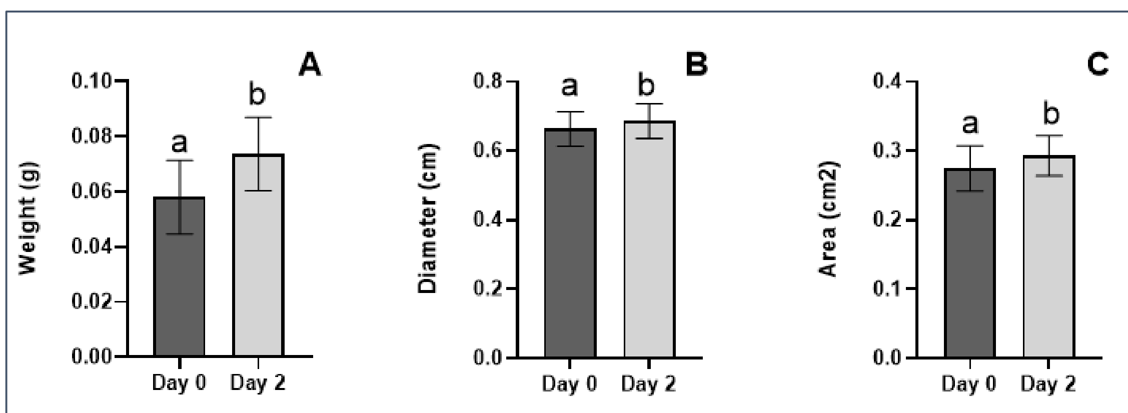

Figure 16. Comparison of measurements between day 0 and day 2 from Group B, batch 20171632-94: (A) seed weight; (B) seed diameter; and (C) seed area. Different lowercase letters in the same graphic mean significant difference between groups ($p < 0.05$). Error bars stand for standard deviation.

4.2. Germination testing

4.2.1. Stored seeds (batch 20171632-94)

The Final Germination Percentage (FGP) of the five treatments made in batch 20171632-94 was 0% or near 0% (Treatment A: 1.25%). Despite the low FGP, 55.94% to 75.00% of seeds were apparently viable (Table 11).

Table 11. Results of Seed Germination testing on Treatments A to E of batch 20171632-94.

Treatment I.D.	No. incubated seeds	FGP	% of Non-germinated seeds		
			Apparent viable	Contaminated/ Abnormal/Dead	No embryo
A	320	1.25	55.94	39.69	3.12
B	160	0.00	70.00	29.38	0.62
C	160	0.00	73.75	23.75	2.50
D	120	0.00	75.00	21.67	3.33
E	120	0.00	59.17	40.00	0.83

4.2.2. Fresh seeds (batch 20230003)

The Final Germination Percentage (FGP) of the thirteen treatments made in batch 20230003 was 0% or extremely low (Treatment G: 0.31%). Despite the low FGP,

85.63% to 97.50% of seeds were apparently viable (Table 12). Experiment R (pre-treatment: scarification) exhibited 100% of seed damage (Table 12).

Table 12. Results of Seed Germination testing on Treatments F to R of batch 20230003.

Treatment I.D.	No. incubated seeds	FGP	% Non-germinated seeds		
			Apparent viable	Contaminated/ Abnormal/Dead	No embryo
F	320	0.00	94.69	4.69	0.62
G	320	0.31	94.38	4.38	0.93
H	320	0.00	95.00	4.38	0.62
I	280	0.00	90.36	8.57	1.07
J	280	0.00	86.43	12.14	1.43
K	280	0.00	95.00	5.00	0.00
L	280	0.00	96.00	4.29	0.71
M	400	0.00	94.50	3.75	1.75
N	200	0.00	97.50	2.50	0.00
O	200	0.00	92.50	5.50	2.00
P	160	0.00	96.25	3.75	0.00
Q	160	0.00	85.63	13.75	0.62
R	160	0.00	0.00	100.00	0.00

4.3. Post-incubation viability testing

4.3.1. Stored seeds (batch 20171632-94)

4.3.1.1. ER testing

The results obtained for the ER viability test from multiple experiments in 250 apparent viable but non-germinated seeds from batch 20171632-94 (stored seeds) are described below.

Influence of Pre-treatment in plant regeneration

The ER test revealed significant differences in post-incubation plant regeneration among various pre-treatments (Chi-square = 126.3, $P < 0.001$) (Table 13).

The highest FGP value in ER was achieved with *no pre-treatment* (83.75%), compared to *35 °C water* and *hormonal pre-treatment* (25.93% and 10.11%, respectively). The *hormonal pre-treatment* exhibits the lowest FGP value (10.11%) and

the highest Dark and Contamination percentage (42.70 and 47.19%, respectively) (Table 13).

Table 13. Results of ER testing on seeds from batch 20171632-94 submitted to different Pre-treatments. (Contamin.: contamination; no.: number; react.: reaction).

Factor	No. tested embryos	FGP	% Callus	% Dark	% No react	% Contamin
None	80	83.75	0.00	10.00	0.00	6.25
35 °C water	81	25.93	0.00	38.27	6.17	29.63
Hormonal	89	10.11	0.00	42.70	0.00	47.19

Influence of Substrate in plant regeneration

The ER test revealed significant differences in the FGP among the substrates *Agar* and *Agar + GA* (Chi-square = 33.33, P < 0.001) (Table 14).

The *Agar* substrate exhibits the highest FGP (59.66%), and the lowest % Dark and % Contaminated embryos (19.33% and 21.01%, respectively) (Table 14).

Table 14. Results of ER testing on seeds from batch 20171632-94 submitted to different Substrates. (Contamin.: contamination; no.: number; react.: reaction).

Factor	No. tested embryos	FGP	% Callus	% Dark	% No react	% Contamin
AGAR	119	59.66	0.00	19.33	0.00	21.01
AGAR + GA_3	131	19.85	0.00	41.22	3.82	35.11

Influence of Temperature in plant regeneration

The ER test revealed significant differences in FGP among various temperature regimes (Chi-square = 632.9, P < 0.001) (Table 15).

The temperature regime *daily constant* exhibited the highest FGP (100%), followed by regimes *M.A.E* (91.67%), *M.A.F* (90.00%) and *M.A.D* (88.89%). Temperature regimes *M.A.H, M.A.C, M.A.G* display an embryo germination of 66.67%, 75.00% and 76.92%, respectively. A viability of 40.00%, 33.33% and 25.00% was achieved through the temperature regimes *40 °C + daily oscillation (3x), (1x) and (5x)*, respectively. The 126 embryos submitted to a *daily temperature oscillation* between 30 °C and 20°C

display a post-incubation viability of 23.81%. The temperature regime *50 °C + daily oscillation* exhibits the lowest post-incubation embryo viability (between 0% and 14.29%) (Table 15).

Table 15. Results of ER testing on seeds from batch 20171632-94 submitted to different Temperature regimes. (Contamin.: contamination; no.: number; react.: reaction).

Factor		No. tested embryos	FGP	% Callus	% Dark	% No react	% Contamin
Daily constant		7	100.00	0.00	0.00	0.00	0.00
Daily oscillation		126	23.81	0.00	34.13	2.38	39.68
M. A.	C	4	75.00	0.00	25.00	0.00	0.00
	D	9	88.89	0.00	11.11	0.00	0.00
	E	12	91.67	0.00	8.33	0.00	0.00
	F	10	90.00	0.00	10.00	0.00	0.00
	G	13	76.92	0.00	15.38	0.00	7.69
	H	9	66.67	0.00	0.00	0.00	33.33
40 °C + daily oscillation	1x	12	33.33	0.00	50.00	16.67	0.00
	3x	10	40.00	0.00	50.00	0.00	10.00
	5x	8	25.00	0.00	37.50	0.00	37.50
50 °C + daily oscillation	1x	8	0.00	0.00	62.50	0.00	37.50
	3x	14	14.29	0.00	50.00	0.00	35.71
	5x	8	12.50	0.00	25.00	0.00	62.50

4.3.1.2. TTC testing

The results obtained for the TTC viability test from multiple experiments in 269 apparent viable but non-germinated seeds from batch 20171632-94 (stored seeds) are described below.

Influence of Pre-treatment in embryo viability

A TTC test revealed significant differences in post-incubation embryo viability among various pre-treatments (Chi-square = 11.06, P = 0.004) (Table 16).

In stored seeds, *pre-treatment of 35 °C water* for three days exhibits higher embryo viability (84.54%, Table 16), compared to *none pre-treatment* and *hormonal pre-treatment*, with the latter showing intermediate values.

Table 16. Results of TTC testing on seeds from batch 20171632-94 submitted to different Pre-treatments. (No.: number).

Factor	No. tested embryos	%. Red (Viability %)	% Pink	% White
None	99	65.66	34.34	0.00
35 °C water	97	84.54	11.34	4.12
Hormonal	73	68.06	19.44	12.50

Influence of Substrate in embryo viability

A TTC test revealed no significant differences in post-incubation embryo viability among various substrates (Chi-square = 0.641, P = 0.423) (Table 17).

The substrate *Agar* with and without *GA3* produced similar post-incubation viability results (70.67% and 76.27%, Table 17).

Table 17. Results of TTC testing on seeds from batch 20171632-94 submitted to different Substrates. (No.: number).

Factor	No. tested embryos	% Red (Viability %)	% Pink	% White
AGAR	150	70.67	26.66	2.67
AGAR + GA_3	119	76.27	16.10	7.63

Influence of Temperature in embryo viability

A TTC test revealed significant differences in post-incubation embryo viability among various temperature regimes (Chi-square = 400.0, P < 0.001) (Table 18).

Temperature regimes *M.A.F* and *50 °C + daily oscillation (1x)* exhibit highest embryo viability (100%), followed by regimes *M.A.H* (92.31%) and *40 °C + daily oscillation (1x)* (91.67%). Temperature regimes *M.A.E*, *M.A.G*, *40 °C + daily oscillation (3x), and 40 °C + daily oscillation (5x)* produced a viability of 76.92%, 80.00%, 83.34%, and 88.89%, respectively. The 112 embryos submitted to a *daily temperature oscillation* between 30 °C and 20 °C display a post-incubation viability of 72.97%. A viability of 62.50%, 63.64%, and 69.23% was achieved through the temperature regimes *M.A.C*, *50 °C + daily oscillation (3x)*, and *50 °C + daily oscillation (5x)*, respectively. The *daily constant* temperature regime exhibits a viability percentage of 46.67%. The

temperature regime *M.A.D* exhibits the lowest post-incubation embryo viability (7.69%, Table 18).

Table 18. Results of TTC testing on seeds from batch 20171632-94 submitted to different Temperature regimes. (No.: number).

<table>
<tr><th colspan="2">Factor</th><th>No. tested embryos</th><th>% Red (Viability %)</th><th>% Pink</th><th>% White</th></tr>
<tr><td colspan="2">Daily constant</td><td>15</td><td>46.67</td><td>53.33</td><td>0.00</td></tr>
<tr><td colspan="2">Daily oscillation</td><td>112</td><td>72.97</td><td>16.22</td><td>10.81</td></tr>
<tr><td rowspan="6">M.A.</td><td>C</td><td>8</td><td>62.50</td><td>37.50</td><td>0.00</td></tr>
<tr><td>D</td><td>13</td><td>7.69</td><td>92.31</td><td>0.00</td></tr>
<tr><td>E</td><td>13</td><td>76.92</td><td>23.08</td><td>0.00</td></tr>
<tr><td>F</td><td>11</td><td>100.00</td><td>0.00</td><td>0.00</td></tr>
<tr><td>G</td><td>15</td><td>80.00</td><td>20.00</td><td>0.00</td></tr>
<tr><td>H</td><td>13</td><td>92.31</td><td>7.69</td><td>0.00</td></tr>
<tr><td rowspan="3">40 °C + daily oscillation</td><td>1x</td><td>12</td><td>91.67</td><td>8.33</td><td>0.00</td></tr>
<tr><td>3x</td><td>12</td><td>83.34</td><td>8.33</td><td>8.33</td></tr>
<tr><td>5x</td><td>9</td><td>88.89</td><td>11.11</td><td>0.00</td></tr>
<tr><td rowspan="3">50 °C + daily oscillation</td><td>1x</td><td>12</td><td>100.00</td><td>0.00</td><td>0.00</td></tr>
<tr><td>3x</td><td>11</td><td>63.64</td><td>36.36</td><td>0.00</td></tr>
<tr><td>5x</td><td>13</td><td>69.23</td><td>30.77</td><td>0.00</td></tr>
</table>

4.3.2. Fresh seeds (batch 20230003)

4.3.2.1. ER testing

The results obtained for the ER viability test from multiple experiments in 1457 apparent viable but non-germinated seeds from batch 20230003 (fresh seeds) are described below.

Influence of Pre-treatment in plant regeneration

The ER test revealed significant differences in post-incubation plant regeneration among various pre-treatments (Chi-square = 97.03, $P < 0.001$) (Table 19).

The highest FGP value in ER was achieved with *20 °C water* (59.28%), followed by 53.99% embryo germination achieved through *no pre-treatment*. The pre-treatments *hormonal* and *30 °C water* exhibited a FGP of 46.57% and 30.11%, respectively. The *100 °C water pre-treatment* exhibits the 0.00% FGP value and 100% Contamination (Table 19).

Table 19. Results of ER testing on seeds from batch 20230003 submitted to different Pre-treatments. (Contamin.: contamination; no.: number; react.: reaction).

Factor	No. tested embryos	FGP	% Callus	% Dark	% No react	% Contamin
None	276	53.99	0.36	21.38	6.52	17.75
20 °C water	167	59.28	1.20	34.13	1.80	3.59
35 °C water	807	30.11	0.25	35.19	14.62	19.83
100 °C water	76	0.00	0.00	0.00	0.00	100.00
Hormonal	131	46.57	0.00	22.90	3.05	27.48

Influence of Substrate in plant regeneration

The ER test revealed significant differences in the FGP among the tested substrates (Chi-square = 29.62, $P < 0.001$) (Table 20).

The substrate *River sand* exhibits the highest FGP (60.42%), followed by *Perlite* (51.19%). A viability of 35.97% and 25.00% was achieved through the substrates *Agar* and *Filter Paper*, respectively (Table 20).

Table 20. Results of ER testing on seeds from batch 20230003 submitted to different Substrates. (Contamin.: contamination; no.: number; react.: reaction).

Factor	No. tested embryos	FGP	% Callus	% Dark	% No react	% Contamin
AGAR	1201	35.97	0.42	34.05	8.41	21.15
River sand	96	60.42	0.00	2.08	15.63	21.88
Perlite	84	51.19	0.00	7.14	13.10	28.57
Filter Paper	76	25.00	0.00	17.11	21.05	36.84

Influence of Temperature in plant regeneration

The ER test revealed significant differences in FGP among various temperature regimes (Chi-square = 966.0, $P < 0.001$) (Table 21).

Temperature regime *W.A.F* exhibited the highest FGP (75.45%), followed by regimes *W.A.G* (74.14%), *W.A.E* (65.45%) and *W.A.H* (59.14%). Temperature regimes *W.A.D* and *W.A.C* display an embryo germination of 56.14% and 51.85%, respectively. The *daily constant temperature* regime exhibits a FGP of 44.57%. The 606 embryos

submitted to a *daily temperature oscillation* between 30 °C and 20 °C display a post-incubation viability of 40.10%. A viability of 40.00%, 37.50%, 33.33%, 31.58% was achieved through the temperature regimes *40 °C + daily constant (5x, 2h)*, *40 °C + daily oscillation (1x, 4h)*, *40 °C + daily oscillation (5x, 4h)*, *50 °C + daily oscillation (1x, 2h)*, respectively. The remaining temperature regimes exhibited a FGP inferior to 18.75% (Table 21).

Table 21. Results of ER testing on seeds from batch 20230003 submitted to different Temperature regimes. (Contamin.: contamination; no.: number; react.: reaction).

Factor			No. tested embryos	FGP	% Callus	% Dark	% No react	% Contamin
Daily constant			92	44.57	0.00	41.30	1.09	13.04
Daily oscillation			606	40.10	0.00	18.32	8.25	33.33
W. A.	C		54	51.85	0.00	38.89	7.41	1.85
	D		57	56.14	1.75	26.32	3.51	12.28
	E		55	65.45	3.64	20.00	0.00	10.91
	F		57	75.45	1.75	21.05	1.75	0.00
	G		58	74.14	1.72	15.52	5.17	3.45
	H		59	59.33	0.00	33.90	1.69	5.08
40 °C + daily oscillation	1x	4h	16	37.50	0.00	62.50	0.00	0.00
		2h	16	18.75	0.00	37.50	37.50	6.25
	3x	4h	17	17.65	0.00	29.41	35.29	17.65
		2h	15	6.67	0.00	53.33	6.67	33.33
	5x	4h	18	33.33	0.00	50.00	16.67	0.00
		2h	14	7.14	0.00	71.43	14.29	7.14
50 °C + daily oscillation	1x	4h	16	18.75	0.00	62.50	18.75	0.00
		2h	19	31.58	0.00	5.26	47.37	15.79
	3x	4h	14	14.29	0.00	85.71	0.00	0.00
		2h	16	6.25	0.00	0.00	68.75	25.00
	5x	4h	14	0.00	0.00	71.43	28.57	0.00
		2h	16	0.00	0.00	56.25	31.25	12.50
40 °C + daily constant	1x	4h	20	15.00	0.00	45.00	30.00	10.00
		2h	20	5.00	0.00	70.00	5.00	20.00
	3x	4h	18	5.56	0.00	11.11	22.22	61.11
		2h	20	10.00	0.00	40.00	5.00	45.00
	5x	4h	16	12.50	0.00	25.00	37.50	25.00
		2h	20	40.00	0.00	30.00	5.00	25.00
50 °C + daily constant	1x	4h	18	0.00	0.00	61.11	11.11	27.78
		2h	19	10.53	0.00	52.63	0.00	36.84
	3x	4h	19	0.00	0.00	52.63	15.79	31.58
		2h	20	0.00	0.00	40.00	15.00	45.00
	5x	4h	18	0.00	0.00	72.22	0.00	27.78
		2h	20	0.00	0.00	40.00	20.00	40.00

4.3.2.2. TTC testing

The results obtained for the TTC viability test from multiple experiments in 1500 apparent viable but non-germinated seeds from batch 20230003 (fresh seeds) are described below.

Influence of Pre-treatment in embryo viability

The TTC test revealed significant differences in post-incubation embryo viability among various pre-treatments (Chi-square = 207.1, P < 0.001) (Table 22).

In fresh seeds, *none*, *20 °C water* and *hormonal* pre-treatments, exhibited the highest embryo viability (80.22%, 80.36% and 80.92%, respectively). The pre-treatment *35 °C water* showed intermediate values (68.03%), and the pre-treatment *100°C water* exhibited 0% of viability (Table 22).

Table 22. Results of TTC testing on seeds from batch 20230003 submitted to different Pre-treatments. (No.: number).

Factor	No. tested embryos	% Red (Viability %)	% Pink	% White
None	283	80.22	11.31	8.47
20 °C water	168	80.36	16.07	3.57
35 °C water	857	68.03	12.60	19.37
100 °C water	61	0.00	0.00	100.00
Hormonal	131	80.92	6.87	12.21

Influence of Substrate in embryo viability

The TTC test revealed significant differences in post-incubation embryo viability among various substrates (Chi-square = 8.931, P < 0.030) (Table 23).

The substrate *Perlite* exhibits the highest post-incubation viability (83.33%), followed by *River sand* (77.55%). A viability of 68.51% and 68.84% was achieved through the substrates *Agar* and *Filter Paper*, respectively (Table 23).

Table 23. Results of TTC testing on seeds from batch 20230003 submitted to different Substrates. (No.: number).

Factor	No. tested embryos	% Red (Viability %)	% Pink	% White
AGAR	1229	68.51	12.12	19.37
River sand	98	77.55	8.16	14.29
Perlite	96	83.33	7.29	9.38
Filter Paper	77	68.84	15.58	15.58

Influence of Temperature in embryo viability

The TTC test revealed significant differences in post-incubation embryo viability among various temperature regimes (Chi-square = 1076, P < 0.001) (Table 24).

Temperature regime *40 °C + daily constant (3x, 2h)* exhibits highest embryo viability (100%), followed by regimes *40 °C + daily constant (1x, 2h)* (94.74%), *W.A.E* (92.98%) and *40 °C + daily oscillation (3x, 2h)* (89.47%). The regimes *40 °C + daily constant (1x, 4h) and (3x, 4h)* produced similar effects in embryo viability (88.89%), followed by 88.24% viability achieved through *40 °C + daily constant (5x, 4h).* Temperature regimes *W.A.G*, *W.A.F* and *W.A.C*, showed a viability of 79.63%, 80.36% and 81.36%, respectively. The *daily constant* temperature regime exhibits a viability percentage of 79.12%, followed by 78.95% and 77.78% viability achieved through the temperature regimes *40 °C + daily oscillation (5x, 2h)* and *40 °C + daily oscillation (5x, 4h)*, respectively. A viability of 75% was achieved through the temperature regimes *40 °C + daily oscillation (1x, 2h)* and *40 °C + daily constant (5x, 2h)*, followed by the temperature regime *W.A.D* (74.58%). The 615 embryos submitted to a *daily temperature oscillation* between 30 °C and 20 °C display a post-incubation viability of 71.54%, followed by the temperature regime *40 °C + daily oscillation (1x, 4h)* (70% viability). The regimes *W.A.H* and *40 °C + daily oscillation (3x, 4h)* exhibit a viability of 68.42%. The temperature regimes *50 °C + daily oscillation* and *50 °C + daily constant* exhibit the lowest post-incubation embryo viability (between 0.00% and 68.42%) (Table 24).

Table 24. Results of TTC testing on seeds from batch 20230003 submitted to different Temperature regimes. (No.: number).

Factor			No. tested embryos	% Red (Viability %)	% Pink	% White
Daily constant			91	79.12	9.89	10.99
Daily oscillation			615	71.54	8.46	20.00
W.A.	C		59	81.36	11.86	6.78
	D		59	74.58	25.42	0.00
	E		57	92.98	5.26	1.75
	F		56	80.36	14.29	5.35
	G		54	79.63	18.52	1.85
	H		57	68.42	29.82	1.75
40 °C + daily oscillation	1x	4h	20	70.00	0.00	30.00
		2h	20	75.00	15.00	10.00
	3x	4h	19	68.42	15.79	15.79
		2h	19	89.47	0.00	10.53
	5x	4h	18	77.78	16.67	5.56
		2h	19	78.95	5.26	15.79
50 °C + daily oscillation	1x	4h	20	20.00	35.00	45.00
		2h	19	47.37	26.32	26.31
	3x	4h	18	27.78	16.67	55.55
		2h	17	29.41	17.65	52.94
	5x	4h	19	0.00	0.00	100.00
		2h	17	23.53	5.88	70.59
40 °C + daily constant	1x	4h	18	88.89	11.11	0.00
		2h	19	94.74	0.00	5.26
	3x	4h	18	88.89	0.00	11.11
		2h	20	100.00	0.00	0.00
	5x	4h	17	88.24	0.00	11.76
		2h	20	75.00	15.00	10.00
50 °C + daily constant	1x	4h	19	68.42	10.53	21.05
		2h	20	65.00	35.00	0.00
	3x	4h	19	10.53	15.79	73.68
		2h	19	68.42	15.79	15.79
	5x	4h	18	0.00	11.11	88.89
		2h	20	55.00	20.00	25.00

4.3.3. Stored seeds VS Fresh seeds

The Stored seeds batch exhibit a lower mean post-viability incubation value (31.30%) compared to Fresh seeds batch (35.83%) (Fig. 17), however, no significant differences were found between them (t = 0.327, P = 0.748) (Fig. 17).

Figure 17. Comparative analysis of post-incubation viability using ER test in stored and fresh seed batches. Same lowercase letters in the graphic mean no significant difference between groups (P < 0.05). Error bars stand for SD (standard deviation).

4.4. Accuracy of Viability tests (ER & TTC)

A comparative analysis of embryo viability outcomes revealed significant differences between ER and TTC methods in the analyses of both batches (Fig. 18.A: t = 2.918, P = 0.008; Fig. 18.B: t = 9.028, P < 0.001), with the TTC method revealing significantly higher viability values than the ER method.

Overall, experiments (represented as empty circles in Fig. 18) exhibit high heterogeneity in embryo viability (Viability %) after ER and TTC tests in both batches (Fig. 18). When the results from ER and TTC of the same experiment are connected (through lines in Fig. 18), it is noticeacle that the outcomes of ER and TTC are not accurate – they produce different results with different directions, i.e., increasing or decreasing depending on the method. In graph A, the majority of experiments that manifest low viability values from ER are linked to high values of viability % from TTC, and the opposite is also observed. In graph B, this pattern is also perceptible, although

in some experiments similar viability values were observed in both ER and TTC tests (Fig. 18).

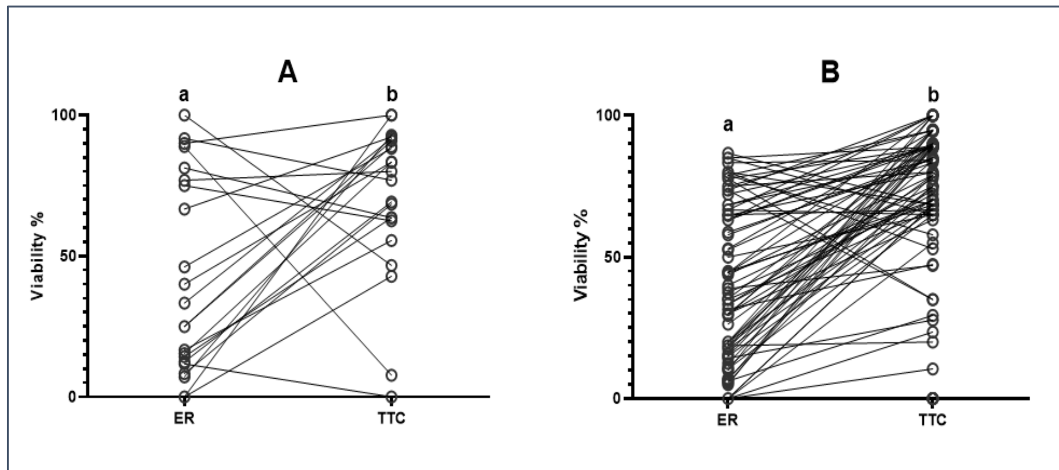

Figure 18. Comparative analysis of embryo viability outcomes using ER and TTC tests in different treatments in both batches: (A) Stored seeds (batch 20171632-94); (B) Fresh seeds (batch 20230003). Different lowercase letters in the same graphic mean significant difference between groups (P < 0.05). Error bars stand for SD (standard deviation).

5. Discussion

5.1. Designing a feasible germination protocol for *Musa acuminata* seeds

The main goal of this thesis was to develop a feasible germination protocol of CWR of *M. acuminata* from different storing methods, with the intention of future implementation in seed banks. The germination of dormant *Musa* seeds is influenced by various factors such as storage conditions, seed morphology and maturity, seed storage and dormancy behavior, and the application of dormancy-release factors and optimal environmental requirements. To accomplish this goal, the quantification of seed viability through morphological analyses was conducted; followed by the measurement of several factors in dormancy release and germination stimulation, through germination testing; and finally, the impact of different combinations of pre-treatments and incubation methods in the viability of post-incubated seeds was quantified via ER testing. Below I discuss the results obtained.

Morphological analyses and apparent seed viability

The results revealed that seeds stored in the Seed Bank of MBG (batch 20171632-94) were apparently mature: seeds were black-brown in color, had a regular or irregular globose shape with a flat bottom and exhibited a hard seed coat. These results are in accordance with the results obtained by Arun et al. (2013) in *M. acuminata* ssp*. burmannicoides* studies, and by Renjana et al. (2022) in *Musa acuminata* var. *flava*. Morphological analyses of a 100 seed-sample from lot S/N.6 revealed significant differences in weight and area between intact and unviable seeds. Seed diameter, however, did not show significant differences between groups (see section 4.1. Stored seeds). Fourteen unviable seeds detected through a Cut test exhibited significantly lower weight and area compared to intact seeds. These findings are in accordance with Renjana et al. (2022) which proposed that the assessment of morphological characteristics can determine the viability and quality of seeds. This way, we suggest discarding smaller and lighter seeds prior to storage and germination testing. The observed low ER outcomes of all seed lots (0 – 30%; Table 1) highlight the importance

of conducting a preliminary viability test for establish the minimum number of seeds to utilize in a germination test.

Fresh seeds (batch 20230003) originated from different ripeness-stage fruits from the same mother plant and presented high heterogeneity in seed morphology. The pronounced heterogeneity with regard to seed color and its capacity to sink or float is related to seed maturity (Uma et al., 2011; Arun et al., 2013). Mature groups (namely, *Black Sink* and *Brown Sink*) exhibited a higher mean seed weight and area, and *Brown Sink* group exhibited a higher mean diameter, compared to the remaining groups. Uma et al. (2011) and Arun et al. (2013) suggested that floating seeds are either devoid of embryo or have a partially developed embryo or endosperm, confirming once again the importance of conducting morphological analyses prior to germination testing. These observations are in accordance with the morphological results obtained in the previous batch, emphasizing the importance of discarding smaller and lighter (in regard to both weight and color) seeds prior storage and germination testing.

Our results from imbibition studies showed that seeds soaked in water increase in weight, diameter and area from day 0 to day 2 (see section 4.1.1). These results are accordance with Puteh et al. (2011), Arun et al. (2013) and Kallow et al. (2020b), which demonstrated that *Musa* seeds have the ability to absorb water without scarification, suggesting physiological dormancy in *Musa acuminata*.

The results obtained here combined with previous studies underscore the importance of considering seed morphology and maturity in the development of effective germination protocols for *M. acuminata*. The selection of seed lots with higher viability, based on morphology (weight and area), color, density criteria and ER viability contribute to increase the successful germination in *Musa acuminata*. Despite acknowledging the natural morphological heterogeneity of seeds, this study also supports additional research to correlate seed morphology with apparent seed viability.

Germination experiments

Some studies have reported that *Musa* seeds germinate readily upon collection but enter into dormancy once they dry (Chin, 1996; Pancholi et al., 1995; Kallow et al., 2021). Desiccation is suggested as the cause of physiological dormancy in *Musa* seeds

by Kallow et al. (2020a), however, our findings suggest that the storage method did not influence seed germination as no major differences were observed in the response between fresh and stored seeds. The low FGP and the high percentage of apparent viable seeds suggest that high dormancy levels are both present in dry and fresh seeds of *Musa acuminata*. Furthermore, none of the factors tested were sufficient to effectively overcome the dormancy of *M. acuminata* seeds.

Out of 880 stored incubated seeds, only four seeds from Treatment A germinated (Table 11; Table A3.1). Both seeds from experiment 2017.C.30.20 germinated in the beginning of Month 4 (data not shown) (see Table 3, M.A.C). Seeds from experiment 2017.H.30.20 were first exposed to a *constant daily temperature of 20 °C* during one month, and they germinated a few days after being transferred to a temperature regime of *30/20 °C* (8h light/ 16h dark) (see Table 3, M.A.H) (data not shown). Out of 3360 fresh seeds incubated, only one seed from experiment 2023.C.20P.30.20 germinated (Table 12; Table A3.2). This experiment was defined by a pre-treatment *hydro-priming at 20 °C* for three days; followed by incubation in *Agar* medium and exposition to Temperature regime *W.A.C* (see Table 4). The germination of this seed also occurred a few days after the transference from *daily constant temperature* to *daily oscillation temperature* (data not shown). Germinated seeds from both batches were first incubated in *Agar* at a *constant temperature of 20 °C* following by being exposed to a temperature regime of *30/20 °C* (8h light/ 16h dark), these results are in accordance with results obtained by Kallow et al. (2020b), in which diurnally alternating temperatures were completely essential for germination of *M. acuminata*. However, other experiments using the same factors also resulted in contrasting outputs with no germination being recorded. Thus, no major conclusions can be ascertained regarding the role of diurnally alternating temperatures in breaking seed dormancy. In 2021, Kallow et al. have also demonstrated that stored dormant seeds lose their dormancy when buried in soil, suggesting a role for stratification in dormancy removal. A possible additional explanation for the variable germination outcome is the heterogeneous dormancy found within the same inflorescence (Kallow et al., 2020b).

It is important to note that, despite being disinfected like other seeds, seeds subjected to scarification with sandpaper (Treatment R) exhibited signs of severe

contamination and damage, leading to a loss of viability before germination can occur (Table 12). The seed damage derived from the scarification of the planar base of the seed was also observed by Burgos-Hérnandez (2014), however, they also indicated that a 100% survival rate was obtained through chemical scarification. Base on the results obtained here, a more delicate scarification using sandpaper or a scalpel, on the lateral side of the seed coat, within a sterile environment is proposed, and may potentially lead to better results.

The low germination results of stored seeds (Table 11) can be partly explained by its low initial viability (30%, Table 1), and by the high dormancy present in both fresh and stored seeds. We also acknowledge that seed germination experiments were conducted for six to twenty-four weeks in the first batch, and six to twelve weeks in the second batch; therefore, it is possible that an extended testing period would have led to germination of additional seeds. Despite the fact that dormancy release factors and environmental conditions tested were not met, several studies (e.g., Afele & De Langhe, 1991; Chin, 1996; Arun et al., 2013; Burgos-Hernández, 2014; Vineesh et al., 2015; Bohra et al., 2020; Kallow et al., 2020b; Kallow et al, 2021) underscore the importance of the pre-treatment and incubation factors tested in this study to break dormancy and stimulate germination. This way a different combination of these factors is suggested in a future research.

Post-incubation viability testing

As expected, the Embryo Rescue (ER) technique alleviated dormancy constrains and promoted germination. However, both stored and fresh seeds exhibited similar low post-incubation viability (31.30% and 35.83% in stored and fresh seeds, respectively) (Fig. 17). Although no significant differences in embryo viability were found between storage methods, the ER post-incubation test revealed significant differences among various pre-treatments, substrates and temperature regimes, in both stored and fresh seeds. It is important to highlight that, based on the Technical Information sheet from MSBP (Davies et al., 2015a), “high viability” is defined when germination outcomes exceed 85%.

In stored seeds, the pre-treatment that achieved highest FGP values was *no pre-treatment* (83.75%, Table 13), and both *hydro*- and *hormonal-priming* deteriorated embryo viability. The results derived from this study conflict with the findings of Afele & De Langhe (1991), Arun et al. (2013) and Bohra et al. (2020), who reported that a pre-treatment prior to ER technique enhanced embryo germination. The *Agar* substrate revealed a better result over *Agar + GA* (Table 14), indicating that gibberellic acid (GA_3) at 250 mL/L is unsuitable for the incubation of *Musa acuminata* seeds. The temperature regime *daily constant* exhibited the highest FGP (100%), however it is important to note that only 7 embryos were apt of being tested through ER. Temperature regimes of *monthly alternation* between *daily oscillation* (30/20 °C) and *daily constant* (20 °C) produced high viability results of 91.67%, 90.00% and 88.89% for *M.A.E, M.A.F* and *M.A.D*, respectively (Table 15). Despite that, the temperature regime of *daily oscillation* displays reduced post-incubation viability (FGP: 23.81%, Table 15). The observed decrease in viability can be attributed to the low viability associated with other factors (e.g. *hormonal* pre-treatment and *Agar + GA* substrate). The high temperature exposition of *40 °C + daily oscillation* reduced embryo viability compared to temperature regimes of *20 °C* and *30/20 °C*; however, an exposition to *40 °C*, three times *per* week, for four hours showed a post-incubation viability of 40% (Table 15). The temperature regime of *50 °C + daily oscillation* exhibits the lowest post-incubation embryo viability, making it incompatible for the incubation of *M. acuminata* seeds (Table 15).

It is noteworthy that a greater number of pre-incubation and incubation factors were tested using fresh seeds than stored seeds. Regardless of the low results achieved using *35 °C water* as pre-treatment in both batches, a *hydro-priming* at a temperature of *20 °C water* reached the highest FGP value in ER (59.28%, Table 19), followed by 53.99% embryo germination achieved without pre-treatment (Table 19). This supports the idea proposed by Puteh et al. (2011) and Arun et al. (2013) that imbibition activates the germination process in *M. acuminata* and enhances germination. The substrate *River sand* exhibits the highest FGP (60.42%), keeping up with the previous research (Chin, 1996; Vineesh et al., 2015; Kallow et al., 2020b; Kallow et al., 2021). This outcome was followed by *Perlite* substrate (51.19%), revealing it as a valuable factor to include in future research. *Agar* substrate denoted a reduced post-incubation viability (35.97%),

but it is crucial to acknowledge that a greater number of seeds was incubated using this substrate. The observed decrease in viability from stored seeds to fresh seeds may be attributed to the low viability associated with other factors (e.g. high temperature regimes) (Table 20). A similar value of post-incubation viability was achieved using both temperature regimes of *daily constant* and *daily oscillation* (44.57% and 40.10%). Nonetheless, temperature regimes of *weekly alternation* between *daily oscillation* (30/20 °C) and *daily constant* (20 °C) manifest higher germination results, from 51.85% to 75.45% (Table 21). Exposing seeds to high temperatures temperature regimes: *40 °C + daily oscillation* and *50 °C + daily oscillation,* impact negatively the embryo viability. It is interesting to note that in the regime *40 °C + daily oscillation,* a longer exposure to *40 °C* (4 hours exposure) reaches higher FGP results than a shorter *40 °C* exposure (2 hours exposure) (Table 21). One possible explanation for these results may be that during the transference from Incubator 2 (30/20 °C) to Egg Incubator 1 (40 °C), the seeds were exposed to rapid fluctuations of temperature, leading to seed damage. The employment of abrupt changes in temperature (temperature regimes: *40 °C + daily constant and 50 °C + daily constant)* have a detrimental impact on the viability of *M. acuminata* seed; however, an exposition to *40 °C*, five times *per* week, for two hours, showed a post-incubation viability of 40% (Table 21).

Despite the ineffectiveness of the 18 treatments employed in germination testing, the work developed in this project is essential to understand the relation between seed morphology and initial seed viability, and to understand the impact of various pre-treatment and incubation factors on post-incubation seed viability. This knowledge is crucial to design new germination tests, either by removing the factors tested in this project or by implementing them in alternative combinations.

5.2. Determine the eligibility of TTC as a viability test for banana

The second goal of this project was to acknowledge the eligibility of Tetrazolium Chloride test (TTC) as a post-incubation viability test for *Musa acuminata*. The reliability and precision of the TTC was compared to Embryo Rescue (ER), by assessing the post-

incubation viability results obtained from the TTC staining with the actual germination results achieved through ER.

Both comparisons of TTC and ER results in stored and fresh seeds demonstrated significant differences: post-incubation TTC test tend to show higher viability than the actual germination achieved though ER (Fig. 18). These results are in accordance with Kallow et al. (2020b), who also demonstrated no linear relationship between these two viability-testing methods. Our findings could indicate that the ER procedure used in this study does not completely overcome embryo dormancy of *M. acuminata*; however, Kallow et al. (2020a) asserted ER as the most effective measure of *Musa* seed viability.

We conclude that the TTC procedure used in this study is not a reliable and precise test for assessing post-incubation viability of *M. acuminata* seeds.

6. Conclusion and Future research

Both stored (dry) and fresh seeds of CWR of *Musa acuminata* presented high dormancy levels, and the combination of factors tested in this thesis was insufficient to effectively overcome the dormancy and germinate *M. acuminata* seeds. However, a stratification at 20 °C followed by diurnally alternating temperatures were completely essential for seed germination. Agar, River sand and Perlite led to good post-incubation viability results, revealing them as valuable factors to include in future research. Exposing seeds to high temperatures temperature regimes of *40 °C* and *50 °C* was incompatible with the germination of *M. acuminata* seeds as they impact negatively in the post-incubation embryo viability. In conclusion, to develop a successful germination protocol of CWR of *M. acuminata* it is important to select seed lots with high viability, based on: 1) seed morphology (weight and area): heavier and bigger seeds, 2) seed color: black and dark-brown seeds, 3) seed weight: seeds that sink in water, and 4) initial ER viability: to establish the minimum number of seeds. Based on the information provided through this document and on the results obtained from this thesis, mechanical physiological dormancy seems the dormancy type most adequate to define the dormancy of mature *M. acuminata* seeds. This way, Embryo Rescue technique is the most effective germination protocol for *M. acuminata,* as it overcomes the lack of knowledge of *M. acuminata*'s seed dormancy, storage behavior and environmental germination requirements.

The procedure of the viability test TTC used in this study demonstrated to be unreliable for assessing the post-incubation viability of *M. acuminata* seeds. This way, to assess post-incubation viability of *M. acuminata* seeds, we suggest employing ER technique or to use an optimized TTC procedure.

Despite acknowledging the natural morphological heterogeneity of seeds, this study supports additional research to correlate seed weight and area with apparent seed viability. In regard to seed germination, we encourage additional research in pre-treatments and incubation factors that can soften seed coat. Bioactive compounds and chemical composition of banana fruits can be clues to essential factors for *Musa* seed germination, as *Musa acuminata* fruits and seeds are naturally indehiscent. Ascorbic acid (vitamin C), folic acid (vitamin B), or other gentle acids, could enable the embryo to

sprout more easily. It would also be important to change the medium every day, as seeds can release a wide range of chemicals that can affect the germination of neighboring seeds (Quaghebeur, 2021). A delicate scarification using sandpaper or a scalpel on the lateral side of the seed coat within a sterile environment; a dry or humid, and cold or warm stratification could also be pre-incubation factors to test in the future. Substrates with different proportions of sterile clay, sand, perlite, organic matter, silica sand, coco coir, vermiculite, potting mix or Agar at pH < 7, can be factors to include in future research. It could be interesting to include heat mats in the incubation process. Moreover, reducing the number of seeds used in each Petri dish could improve germination results, as inadequate spacing can prevent germination due to the release of chemicals by the seeds.

Research on banana seeds, although challenging, it is of great importance for the sustenance of worldwide banana production and supply. The limited genetic diversity observed in commercial varieties and the global circumstances that threat bananas emphasize the need to preserve wild genetic resources. Research on *Musa* spp. seeds is crucial for breeding programs focused on developing cultivars resistant to pests and diseases and to the escalating climate crisis, by incorporating desirable traits found in CWR. Additionally, it contributes to the conservation of Crop Wild Relatives, thereby enhancing the resilience, sustainability and productivity of banana crops.

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Annex 1: Seed Dormancy

Seed dormancy is an inherent characteristic of seeds that defines the specific parameters in which the seed is able to germinate. Its manifestation is determined by genetics with a significant environmental impact; and it is internally controlled by the plant hormones abscisic acid (ABA) and gibberellins. Any external stimulus that widens the environmental requirements for germination is recognized as a Dormancy-release Factor (Finch-Savage & Leubner-Metzger, 2006).

Seed dormancy can be classified based on the time of induction as:

- *Primary dormancy:* occurs when the seed enters a dormant state while still maturing on the mother plant (Baskin & Baskin, 2004), and it is induced by the production of ABA by the embryo itself (Gubler et al., 2005).
- *Induced dormancy:* occurs when seeds are non-dormant at the time of dispersal and they transit to dormant state (Soltani et al., 2018).
- *Secondary dormancy:* refers to the re-entry of a non-dormant seed into dormancy (Baskin & Baskin, 2004), often triggered by unfavorable environmental conditions like high temperatures, anoxia, or desiccation (Gubler et al., 2005).
- *Conditional dormancy:* encompasses a *continuum* spectrum between non-dormancy and complete dormancy, and it is characterized by a narrower range of conditions to germination relatively to non-dormant seeds (Baskin & Baskin, 2004).

Based on Nikolaeva's (1977) work, Baskin & Baskin (2014) classified seed dormancy into 5 classes (see below). Some of these classes are further divided into different levels of depth (e.g. *deep*, *intermediate*, or *non-deep dormancy*).

- Physiological dormancy (PD): is the most abundant form of seed dormancy. A diverse range of factors (Fig. A1.1) can influence the PD of seeds. Each of these factors alleviates successive impediments to germination, necessitating a sequential order for it to work (Finch-Savage & Leubner-Metzger 2006).

Table 2. Characteristics of dormancy in seeds with deep, intermediate and non-deep physiological dormancy (from information in Baskin and Baskin, 1998)

Deep
- Excised embryo produces abnormal seedling
- GA does not promote germination
- Seeds require *c*. 3–4 months of cold stratification to germinate

Intermediate
- Excised embryo produces normal seedling
- GA promotes germination in some (but not all) species
- Seeds require 2–3 months of cold stratification for dormancy break
- Dry storage can shorten the cold stratification period

Non-deep
- Excised embryo produces normal seedling
- GA promotes germination
- Depending on species, cold (*c*. 0–10°C) or warm (≥15°C) stratification breaks dormancy
- Seeds may after-ripen in dry storage
- Scarification may promote germination

Figure A1.19. Summary of aspects of Physiological Dormancy' levels and related dormancy-breaking factors. Reproduced from Baskin & Baskin, 2004.

Physiological dormancy can be sub classified into:

- Chemical dormancy: is characterized by the presence of inhibitory chemicals in seeds (Baskin & Baskin, 2004), accumulated during fruit and/or seed development. It can be overcome by leaching seeds with water (Nikolaeva, 1977).
- Mechanical dormancy: results from the physical resistance by internal structures like the endosperm and/or perisperm that impede radicle growth. Mechanical dormancy can be overcome by weakening these structures (Baskin & Baskin, 2004) with warm and/or cold stratification, removing seed coverings, or removing the embryo from the seed (Onganga et al., 2020).

- Morphological dormancy (MD): occurs when the embryo is not fully developed. Germination is primarily time-dependent and it does not require a dormancy breaking agent (Baskin & Baskin, 2004). However, embryo development can be accelerated by chilling (exposure to < 15 °C), alternating temperatures, and/or treatments containing GA_3 (Quaghebeur, 2021).

- Morphophysiological dormancy (MPD): is associated with an underdeveloped embryo and physiological barriers that impede germination (Baskin & Baskin, 2004). For germination to occur, it is indispensable dormancy-breaking pre-treatment. In these seeds, embryo growth and radicle emergence

takes a considerable longer period of time in comparison seeds with MD (Baskin & Baskin, 2004).

- Physical dormancy (PY): results from impermeable seed coats (Baskin & Baskin, 2004).
- Combinational (PY + PD): these seeds show a physical restriction (seed and/or fruit coat is water impermeable) in combination with a physiological dormant embryo (Baskin & Baskin, 2004).

Annex 2: Morphology and Initial seed viability

Stored seeds (batch 20171632-94)

Table A2. 1 Measures of 50 seeds from Group A (lot S/N.6).

Seed No.	Diameter (cm)	Area (cm^2)	Weight (g)	Observations
1	0.689	0.296	0.070	
2	0.632	0.284	0.061	
3	0.572	0.211	0.020	Unviable
4	0.569	0.205	0.063	
5	0.650	0.280	0.064	
6	0.687	0.318	0.072	
7	0.686	0.283	0.068	
8	0.619	0.274	0.062	
9	0.622	0.251	0.068	
10	0.648	0.245	0.060	
11	0.657	0.263	0.063	
12	0.650	0.261	0.057	
13	0.714	0.331	0.071	
14	0.661	0.280	0.056	
15	0.692	0.301	0.067	
16	0.712	0.287	0.071	
17	0.593	0.264	0.061	
18	0.727	0.324	0.064	
19	0.696	0.303	0.051	
20	0.628	0.248	0.064	
21	0.596	0.228	0.050	
22	0.560	0.206	0.056	
23	0.651	0.266	0.029	Unviable
24	0.614	0.233	0.046	
25	0.598	0.237	0.060	
26	0.541	0.182	0.017	Unviable
27	0.615	0.275	0.067	
28	0.715	0.250	0.059	
29	0.577	0.212	0.015	Unviable
30	0.699	0.267	0.065	
31	0.763	0.343	0.061	
32	0.673	0.288	0.062	
33	0.645	0.260	0.059	
34	0.633	0.241	0.066	
35	0.670	0.252	0.052	
36	0.692	0.255	0.062	
37	0.599	0.245	0.025	Unviable
38	0.593	0.257	0.051	
39	0.596	0.202	0.049	
40	0.631	0.236	0.047	
41	0.648	0.286	0.057	
42	0.738	0.307	0.059	
43	0.637	0.281	0.061	
44	0.632	0.272	0.044	Unviable
45	0.657	0.245	0.060	
46	0.700	0.286	0.063	
47	0.733	0.322	0.046	Unviable
48	0.669	0.242	0.056	
49	0.697	0.285	0.070	
50	0.640	0.282	0.061	

Table A2. 2. Measures of 50 seeds from Group B.0 (lot S/N.6).

Seed No.	Diameter (cm)	Area (cm^2)	Weight (g)	Observations
1	0.603	0.232	0.020	Unviable
2	0.643	0.292	0.061	
3	0.667	0.261	0.023	Unviable
4	0.709	0.298	0.060	
5	0.666	0.241	0.025	Unviable
6	0.694	0.298	0.073	
7	0.754	0.315	0.078	
8	0.755	0.329	0.071	
9	0.616	0.272	0.054	
10	0.728	0.317	0.080	
11	0.665	0.278	0.069	
12	0.606	0.252	0.057	
13	0.640	0.273	0.062	
14	0.616	0.220	0.048	
15	0.631	0.274	0.053	
16	0.688	0.288	0.059	
17	0.631	0.207	0.052	
18	0.653	0.265	0.048	Unviable
19	0.663	0.245	0.058	
20	0.675	0.278	0.068	
21	0.559	0.206	0.048	
22	0.632	0.222	0.044	
23	0.618	0.225	0.041	Unviable
24	0.680	0.308	0.067	
25	0.709	0.319	0.069	
26	0.777	0.295	0.066	
27	0.626	0.285	0.053	
28	0.679	0.257	0.062	
29	0.685	0.292	0.061	
30	0.605	0.253	0.048	
31	0.673	0.266	0.058	
32	0.693	0.267	0.063	
33	0.639	0.269	0.050	
34	0.622	0.216	0.059	
35	0.708	0.303	0.069	
36	0.688	0.320	0.072	
37	0.687	0.330	0.076	
38	0.647	0.282	0.065	
39	0.799	0.316	0.079	
40	0.569	0.234	0.060	
41	0.719	0.321	0.056	
42	0.683	0.292	0.059	
43	0.670	0.298	0.064	
44	0.624	0.286	0.068	
45	0.638	0.288	0.056	
46	0.636	0.260	0.053	Unviable
47	0.600	0.278	0.052	
48	0.622	0.236	0.057	
49	0.734	0.291	0.072	
50	0.635	0.267	0.035	Unviable

Water uptake studies

Table A2. 3. Measures of 50 seeds from Group B.2 (lot S/N.6).

Seed No.	Diameter (cm)	Area (cm^2)	Weight (g)	Observations
1	0.689	0.298	0.038	Unviable
2	0.688	0.297	0.078	
3	0.733	0.305	0.049	Unviable
4	0.703	0.301	0.073	
5	0.702	0.270	0.045	Unviable
6	0.682	0.319	0.089	
7	0.744	0.329	0.087	
8	0.782	0.339	0.088	
9	0.707	0.310	0.067	
10	0.743	0.344	0.096	
11	0.663	0.309	0.084	
12	0.634	0.279	0.071	
13	0.670	0.291	0.080	
14	0.636	0.237	0.060	
15	0.620	0.264	0.065	
16	0.723	0.322	0.074	
17	0.641	0.254	0.067	
18	0.648	0.289	0.059	Unviable
19	0.682	0.281	0.074	
20	0.683	0.309	0.084	
21	0.575	0.226	0.062	
22	0.627	0.248	0.060	
23	0.621	0.279	0.052	Unviable
24	0.717	0.314	0.088	
25	0.692	0.337	0.089	
26	0.794	0.321	0.082	
27	0.669	0.305	0.072	
28	0.715	0.293	0.077	
29	0.680	0.284	0.078	
30	0.644	0.272	0.059	
31	0.684	0.275	0.071	
32	0.714	0.279	0.075	
33	0.645	0.262	0.059	
34	0.675	0.235	0.073	
35	0.728	0.317	0.087	
36	0.718	0.348	0.085	
37	0.723	0.338	0.089	
38	0.685	0.301	0.081	
39	0.857	0.312	0.096	
40	0.642	0.274	0.075	
41	0.713	0.333	0.075	
42	0.698	0.285	0.078	
43	0.655	0.287	0.080	
44	0.653	0.287	0.082	
45	0.688	0.297	0.077	
46	0.713	0.286	0.066	Unviable
47	0.632	0.280	0.067	
48	0.594	0.250	0.076	
49	0.712	0.294	0.090	
50	0.665	0.272	0.049	Unviable

Fresh seeds (batch 20230003)

Table A2. 4. Diameter (cm), area (cm2) and weight (g) measurements of seeds from groups Black and Brown. Each row of each group represents one single seed.

Black Sink			Black Float			Brown Sink			Brown Float		
Diameter (cm)	Area (cm^2)	Weight (g)	Diameter (cm)	Area (cm^2)	Weight (g)	Diameter (cm)	Area (cm^2)	Weight (g)	Diameter (cm)	Area (cm^2)	Weight (g)
0.574	0.196	0.042	0.528	0.183	0.011	0.590	0.208	0.037	0.566	0.164	0.007
0.578	0.207	0.044	0.553	0.185	0.012	0.601	0.217	0.038	0.566	0.192	0.008
0.579	0.208	0.044	0.559	0.185	0.013	0.607	0.219	0.046	0.575	0.196	0.010
0.587	0.214	0.046	0.565	0.187	0.013	0.609	0.235	0.049	0.595	0.206	0.010
0.587	0.220	0.047	0.572	0.194	0.014	0.628	0.245	0.053	0.601	0.209	0.011
0.593	0.221	0.048	0.578	0.202	0.014	0.633	0.248	0.054	0.602	0.215	0.011
0.624	0.221	0.048	0.580	0.209	0.016	0.641	0.255	0.056	0.606	0.218	0.012
0.631	0.225	0.051	0.591	0.211	0.016	0.652	0.257	0.057	0.613	0.232	0.013
0.638	0.230	0.051	0.597	0.212	0.016	0.665	0.257	0.060	0.617	0.235	0.013
0.648	0.241	0.052	0.598	0.213	0.016	0.667	0.272	0.060	0.618	0.238	0.014
0.657	0.247	0.054	0.605	0.216	0.018	0.670	0.276	0.063	0.618	0.240	0.014
0.664	0.252	0.055	0.610	0.227	0.019	0.689	0.282	0.065	0.624	0.250	0.015
0.664	0.262	0.056	0.611	0.229	0.020	0.692	0.284	0.066	0.625	0.252	0.017
0.675	0.271	0.058	0.618	0.239	0.020	0.717	0.286	0.066	0.632	0.253	0.020
0.692	0.275	0.060	0.622	0.240	0.023	0.722	0.291	0.069	0.651	0.255	0.021
0.697	0.298	0.063	0.628	0.248	0.026	0.731	0.327	0.069	0.661	0.266	0.021
0.699	0.314	0.065	0.639	0.261	0.029	0.732	0.346	0.073	0.667	0.274	0.021
0.713	0.315	0.066	0.677	0.263	0.029	0.746	0.352	0.073	0.684	0.276	0.022
0.718	0.317	0.075	0.683	0.265	0.031	0.758	0.357	0.074	0.693	0.293	0.028
0.756	0.321	0.079	0.703	0.321	0.034	0.785	0.378	0.080	0.711	0.319	0.029

Table A2. 5. Diameter (cm), area (cm2) and weight (g) measurements of seeds from groups Light-Brown and White-ish. Each row of each group represents one single seed.

Light-Brown Sink			Light-Brown Float			White-ish Sink			White-ish Float		
Diameter (cm)	Area (cm^2)	Weight (g)	Diameter (cm)	Area (cm^2)	Weight (g)	Diameter (cm)	Area (cm^2)	Weight (g)	Diameter (cm)	Area (cm^2)	Weight (g)
0.524	0.160	0.007	0.574	0.181	0.007	0.576	0.167	0.007	0.515	0.188	0.008
0.532	0.164	0.008	0.582	0.192	0.010	0.579	0.211	0.009	0.541	0.190	0.009
0.544	0.171	0.009	0.591	0.201	0.010	0.606	0.223	0.012	0.543	0.191	0.009
0.554	0.174	0.009	0.596	0.204	0.010	0.624	0.232	0.012	0.564	0.194	0.010
0.564	0.200	0.009	0.603	0.204	0.010	0.634	0.239	0.014	0.580	0196	0.010
0.568	0.200	0.009	0.603	0.205	0.010	0.647	0.240	0.015	0.583	0.204	0.011
0.570	0.209	0.010	0.607	0.217	0.011	0.648	0.248	0.015	0.583	0.204	0.011
0.571	0.211	0.010	0.618	0.219	0.011	0.649	0.253	0.016	0.595	0.208	0.011
0.580	0.211	0.010	0.627	0.224	0.011				0.609	0.212	0.011
0.582	0.218	0.010	0.628	0.224	0.011				0.610	0.215	0.011
0.588	0.219	0.011	0.633	0.224	0.012				0.627	0.221	0.012
0.609	0.230	0.011	0.635	0.227	0.012				0.630	0.233	0.013
0.615	0.237	0.011	0.636	0.233	0.013				0.636	0.251	0.013
0.621	0.239	0.011	0.646	0.239	0013				0.638	0.265	0.013
0.627	0.239	0.012	0.647	0.242	0.014				0.656	0.267	0.013
0.633	0.245	0.012	0.664	0.265	0.014				0.661	0.270	0.014
0.664	0.261	0.012	0.670	0.265	0.015				0.686	0.273	0.014
0.679	0.261	0.013	0.676	0.265	0.015				0.688	0.276	0.015
0.690	0.267	0.013	0.679	0.293	0.016				0708	0.298	0.016
0.695	0.276	0.015	0.680	0.294	0.016				0.744	0.330	0.019

Annex 3: Germination testing results

Stored seeds (batch 2017162-94)

Table A3. 1 Summary of seed germination tests done in batch 20171632-94. (mo.: months; no.: number; treat.: treatment).

Lot	Treat. I.D.	Experimental coding	Pre - treatment	Substrate	Temperature	Duration of incubation	Initial no. seeds	No. seeds germinated
S/N. 4 – 48245	**A**	2017.A.30.20	*None*	*AGAR*	*20ºC*	6 mo. (24 weeks)	40	0
		2017.B.30.20	*None*	*AGAR*	*30/20ºC*		40	0
		2017.C.30.20	*None*	*AGAR*	*M.A.C*		40	**2**
		2017.D.30.20	*None*	*AGAR*	*M.A.D*		40	0
		2017.E.30.20	*None*	*AGAR*	*M.A.E*		40	0
		2017.F.30.20	*None*	*AGAR*	*M.A.F*		40	0
		2017.G.30.20	*None*	*AGAR*	*M.A.G*		40	0
		2017.H.30.20	*None*	*AGAR*	*M.A.H*		40	**2**
S/N.3 – 48244	**B**	2017.A.35P.40	*35ºC water*	*AGAR + GA*	*30/20ºC*	1.5 mo. (6 weeks)	40	0
		2017.B.35P.40	*35ºC water*	*AGAR + GA*	*30/20ºC + 1x(4h40ºC)*		40	0
		2017.C.35P.40	*35ºC water*	*AGAR + GA*	*30/20ºC + 3x(4h40ºC)*		40	0
		2017.D.35P.40	*35ºC water*	*AGAR + GA*	*30/20ºC + 5x(4h40ºC)*		40	0
S/N.3 – 48244	**C**	2017.A.35P.50	*35ºC water*	*AGAR + GA*	*30/20ºC*	1.5 mo. (6 weeks)	40	0
		2017.B.35P.50	*35ºC water*	*AGAR + GA*	*30/20ºC + 1x(4h50ºC)*		40	0
		2017.C.35P.50	*35ºC water*	*AGAR + GA*	*30/20ºC + 3x(4h50ºC)*		40	0
		2017.D.35P.50	*35ºC water*	*AGAR + GA*	*30/20ºC + 5x(4h50ºC)*		40	0
S/N.3 – 48244	**D**	2017.A.IAA.30.20	*IAA*	*AGAR*	*30/20ºC*	6 mo. (24 weeks)	40	0
		2017.B.GA.30.20	*GA*	*AGAR*	*30/20ºC*		40	0
		2017.C.IAA.GA.30.20	*IAA + GA*	*AGAR*	*30/20ºC*		40	0
S/N. 3 – 48244	**E**	2017.A.IAA.30.20.GA	*IAA*	*AGAR + GA*	*30/20ºC*	6 mo. (24 weeks)	40	0
		2017.B.GA.30.20.GA	*GA*	*AGAR + GA*	*30/20ºC*		40	0
		2017.C.IAA.GA.30.20.GA	*IAA + GA*	*AGAR + GA*	*30/20ºC*		40	0
					TOTAL		880	4

Fresh seeds (batch 20230003)

Table A3. 2. Summary of seed germination tests done in batch 20230003. (mo.: months; no.: number; treat.: treatment).

Treat I.D.	Experimental coding	Pre - treatment	Substrate	Temperature	Duration of incubation	Initial no. seeds	No. seeds germinated
F	2023.A.0P.30.20	*None*	*AGAR*	*20ºC*	3 mo. (12 weeks)	40	0
	2023.B.0P.30.20	*None*	*AGAR*	*30/20ºC*		40	0
	2023.C.0P.30.20	*None*	*AGAR*	*W.A.C*		40	0
	2023.D.0P.30.20	*None*	*AGAR*	*W.A.D*		40	0
	2023.E.0P.30.20	*None*	*AGAR*	*W.A.E*		40	0
	2023.F.0P.30.20	*None*	*AGAR*	*W.A.F*		40	0
	2023.G.0P.30.20	*None*	*AGAR*	*W.A.G*		40	0
	2023.H.0P.30.20	*None*	*AGAR*	*W.A.H*		40	0
G	2023.A.20P.30.20	*20ºC water*	*AGAR*	*20ºC*	3 mo. (12 weeks)	40	0
	2023.B.20P.30.20	*20ºC water*	*AGAR*	*30/20ºC*		40	0
	2023.C.20P.30.20	*20ºC water*	*AGAR*	*W.A.C*		40	**1**
	2023.D.20P.30.20	*20ºC water*	*AGAR*	*W.A.D*		40	0
	2023.E.20P.30.20	*20ºC water*	*AGAR*	*W.A.E*		40	0
	2023.F.20P.30.20	*20ºC water*	*AGAR*	*W.A.F*		40	0
	2023.G.20P.30.20	*20ºC water*	*AGAR*	*W.A.G*		40	0
	2023.H.20P.30.20	*20ºC water*	*AGAR*	*W.A.H*		40	0
H	2023.A.35P.30.20	*35ºC water*	*AGAR*	*20ºC*	3 mo. (12 weeks)	40	0
	2023.B.35P.30.20	*35ºC water*	*AGAR*	*30/20ºC*		40	0
	2023.C.35P.30.20	*35ºC water*	*AGAR*	*W.A.C*		40	0
	2023.D.35P.30.20	*35ºC water*	*AGAR*	*W.A.D*		40	0
	2023.E.35P.30.20	*35ºC water*	*AGAR*	*W.A.E*		40	0
	2023.F.35P.30.20	*35ºC water*	*AGAR*	*W.A.F*		40	0
	2023.G.35P.30.20	*35ºC water*	*AGAR*	*W.A.G*		40	0
	2023.H.35P.30.20	*35ºC water*	*AGAR*	*W.A.H*		40	0
I	2023.A.35P.40	*35ºC water*	*AGAR*	*30/20ºC*	1.5 mo. (6 weeks)	40	0
	2023.B.35P.40	*35ºC water*	*AGAR*	*30/20ºC + 1x(4h40ºC)*		40	0
	2023.C.35P.40	*35ºC water*	*AGAR*	*30/20ºC + 3x(4h40ºC)*		40	0
	2023.D.35P.40	*35ºC water*	*AGAR*	*30/20ºC + 5x(4h40ºC)*		40	0
	2023.E.35P.40	*35ºC water*	*AGAR*	*30/20ºC + 1x(2h40ºC)*		40	0
	2023.F.35P.40	*35ºC water*	*AGAR*	*30/20ºC + 3x(2h40ºC)*		40	0
	2023.G.35P.40	*35ºC water*	*AGAR*	*30/20ºC + 5x(2h40ºC)*		40	0
J	2023.A.35P.50	*35ºC water*	*AGAR*	*30/20ºC*	1.5 mo. (6 weeks)	40	0
	2023.B.35P.50	*35ºC water*	*AGAR*	*30/20ºC + 1x(4h50ºC)*		40	0
	2023.C.35P.50	*35ºC water*	*AGAR*	*30/20ºC + 3x(4h50ºC)*		40	0
	2023.D.35P.50	*35ºC water*	*AGAR*	*30/20ºC + 5x(4h50ºC)*		40	0
	2023.E.35P.50	*35ºC water*	*AGAR*	*30/20ºC + 1x(2h50ºC)*		40	0

	2023.F.35P.50	*35°C water*	*AGAR*	*30/20°C + 3x(2h50°C)*		40	0
	2023.G.35P.50	*35°C water*	*AGAR*	*30/20°C + 5x(2h50°C)*		40	0

K	2023.A.35P.40.a	*35°C water*	*AGAR*	*20°C*	1.5 mo. (6 weeks)	40	0
	2023.B.35P.40.a	*35°C water*	*AGAR*	*20°C + 1x(4h40°C)*		40	0
	2023.C.35P.40.a	*35°C water*	*AGAR*	*20°C + 3x(4h40°C)*		40	0
	2023.D.35P.40.a	*35°C water*	*AGAR*	*20°C + 5x(4h40°C)*		40	0
	2023.E.35P.40.a	*35°C water*	*AGAR*	*20°C + 1x(2h40°C)*		40	0
	2023.F.35P.40.a	*35°C water*	*AGAR*	*20°C + 3x(2h40°C)*		40	0
	2023.G.35P.40.a	*35°C water*	*AGAR*	*20°C + 5x(2h40°C)*		40	0

L	2023.A.35P.50.a	*35°C water*	*AGAR*	*20°C*	1.5 mo. (6 weeks)	40	0
	2023.B.35P.50.a	*35°C water*	*AGAR*	*20°C + 1x(4h50°C)*		40	0
	2023.C.35P.50.a	*35°C water*	*AGAR*	*20°C + 3x(4h50°C)*		40	0
	2023.D.35P.50.a	*35°C water*	*AGAR*	*20°C + 5x(4h50°C)*		40	0
	2023.E.35P.50.a	*35°C water*	*AGAR*	*20°C + 1x(2h50°C)*		40	0
	2023.F.35P.50.a	*35°C water*	*AGAR*	*20°C + 3x(2h50°C)*		40	0
	2023.G.35P.50.a	*35°C water*	*AGAR*	*20°C + 5x(2h50°C)*		40	0

M	HP.2023.A.0P.30.20	*None*	*AGAR*	*30/20°C*	3 mo. (12 weeks)	40	0
	HP.2023.B.20P.30.20	*20°C water*	*AGAR*	*30/20°C*		40	0
	HP.2023.C.35P.30.20	*35°C water*	*AGAR*	*30/20°C*		40	0
	HP.2023.D.IAA.30.20	*IAA*	*AGAR*	*30/20°C*		40	0
	HP.2023.E.BAP.30.20	*BAP*	*AGAR*	*30/20°C*		40	0
	HP.2023.F.GA.30.20	*GA*	*AGAR*	*30/20°C*		40	0
	HP.2023.G.IAA.GA.30.20	*IAA + GA*	*AGAR*	*30/20°C*		40	0
	HP.2023.H.BAP.GA.30.20	*BAP + GA*	*AGAR*	*30/20°C*		40	0
	HP.2023.I.IAA.BAP.30.20	*IAA + BAP*	*AGAR*	*30/20°C*		40	0
	HP.2023.J.IAA.BAP.GA.30.20	*IAA + BAP + GA*	*AGAR*	*30/20°C*		40	0

N	2023.A.0P.SAND.30.20	*None*	*SAND*	*30/20°C*	3 mo. (12 weeks)	80	0
	2023.B.35P.SAND.30.20	*35°C water*	*SAND*	*30/20°C*		120	0

O	2023.A.0P.PERLITE.30.20	*None*	*PERLITE*	*30/20°C*	3 mo. (12 weeks)	80	0
	2023.B.35P.PERLITE.30.20	*35°C water*	*PERLITE*	*30/20°C*		120	0

P	2023.A.0P.PAPER.30.20	*None*	*PAPER*	*30/20°C*	3 mo. (12 weeks)	80	0
	2023.B.35P.PAPER.30.20	*35°C water*	*PAPER*	*30/20°C*		80	0

Q	2023.A.BOIL.30.20	*30 sec. boil*	*AGAR*	*30/20°C*	3 mo. (12 weeks)	80	0
	2023.B.BOIL.30.20	*60 sec. boil*	*AGAR*	*30/20°C*		80	0

R	2023.A.SCAR.30.20	*fine sandpaper*	*AGAR*	*30/20°C*	3 mo. (12 weeks)	80	0
	2023.B.SCAR.30.20	*medium sandpaper*	*AGAR*	*30/20°C*		80	0

TOTAL	3360	1

Annex 4: Post-incubation viability testing

Stored seeds (batch 20171632-94)

TTC results

Table A4. 1. Results of TTC viability testing in non-germinated seeds from batch 20171632-94. (mo.: months; no.: number; treat.: treatment).

Treat. I.D.	Experimental coding	No. tested embryos	No. red	No. pink	No. white	Viability %
A	2017.A.30.20	15	7	8	0	46.67
	2017.B.30.20	11	7	4	0	63.64
	2017.C.30.20	8	5	3	0	62.50
	2017.D.30.20	13	1	12	0	7.69
	2017.E.30.20	13	10	3	0	76.92
	2017.F.30.20	11	11	0	0	100.00
	2017.G.30.20	15	12	3	0	80.00
	2017.H.30.20	13	12	1	0	92.31
B	2017.A.35P.40	11	10	0	1	90.91
	2017.B.35P.40	12	11	1	0	91.67
	2017.C.35P.40	12	10	1	1	83.33
	2017.D.35P.40	9	8	1	0	88.89
C	2017.A.35P.50	17	15	0	2	88.24
	2017.B.35P.50	12	12	0	0	100.00
	2017.C.35P.50	11	7	4	0	63.64
	2017.D.35P.50	13	9	4	0	69.23
D	2017.A.IAA.30.20	18	15	1	2	83.33
	2017.B.GA.30.20	14	13	1	0	92.86
	2017.C.IAA.GA.30.20	19	13	4	2	68.42
E	2017.A.IAA.30.20.GA	7	3	3	1	42.86
	2017.B.GA.30.20.GA	5	0	5	0	0.00
	2017.C.IAA.GA.30.20.GA	9	5	0	4	55.56

ER results

Table A4. 2. Results of ER viability testing in non-germinated seeds from batch 20171632-94. (mo.: months; no.: number; treat.: treatment).

Treat. I.D.	Experimental coding	No. tested embryos	No. germinated embryos	No. callus	No. dark	No. no reaction	No. contamination	FGP
A	2017.A.30.20	7	7	0	0	0	0	100.00
	2017.B.30.20	16	13	0	2	0	1	81.25
	2017.C.30.20	4	3	0	1	0	0	75.00
	2017.D.30.20	9	8	0	1	0	0	88.89
	2017.E.30.20	12	11	0	1	0	0	91.67
	2017.F.30.20	10	9	0	1	0	0	90.00
	2017.G.30.20	13	10	0	2	0	1	76.92
	2017.H.30.20	9	6	0	0	0	3	66.67
B	2017.A.35P.40	8	2	0	1	1	4	25.00
	2017.B.35P.40	12	4	0	6	2	0	33.33
	2017.C.35P.40	10	4	0	5	0	1	40.00
	2017.D.35P.40	8	2	0	3	0	3	25.00
C	2017.A.35P.50	13	6	0	2	2	3	46.15
	2017.B.35P.50	8	0	0	5	0	3	0.00
	2017.C.35P.50	14	2	0	7	0	5	14.29
	2017.D.35P.50	8	1	0	2	0	5	12.50
D	2017.A.IAA.30.20	13	2	0	6	0	5	15.38
	2017.B.GA.30.20	14	1	0	5	0	8	7.14
	2017.C.IAA.GA.30.20	12	1	0	4	0	7	8.33
E	2017.A.IAA.30.20.GA	15	0	0	9	0	6	0.00
	2017.B.GA.30.20.GA	17	2	0	10	0	5	11.76
	2017.C.IAA.GA.30.20.GA	18	3	0	4	0	11	16.67

Fresh seeds (batch 20230003)

TTC results

Table A4. 3. Results of TTC viability testing in non-germinated seeds from batch 20230003. (mo.: months; no.: number; treat.: treatment).

Treat. I.D.	Experimental coding	No. tested embryos	No. red	No. pink	No. white	Viability %
F	2023.A.0P.30.20	18	16	0	2	88.89
	2023.B.0P.30.20	20	19	0	1	95.00
	2023.C.0P.30.20	19	18	0	1	94.74
	2023.D.0P.30.20	20	18	2	0	90.00
	2023.E.0P.30.20	19	16	2	1	84.21
	2023.F.0P.30.20	18	16	2	0	88.89
	2023.G.0P.30.20	17	9	8	0	52.94
	2023.H.0P.30.20	18	16	2	0	88.89
G	2023.A.20P.30.20	17	8	6	3	47.06
	2023.B.20P.30.20	19	16	3	0	84.21
	2023.C.20P.30.20	20	17	2	1	85.00
	2023.D.20P.30.20	19	19	0	0	100.00
	2023.E.20P.30.20	18	17	1	0	94.44
	2023.F.20P.30.20	18	16	2	0	88.89
	2023.G.20P.30.20	19	18	0	1	94.74
	2023.H.20P.30.20	20	7	13	0	35.00
H	2023.A.35P.30.20	19	18	0	1	94.74
	2023.B.35P.30.20	19	11	8	0	57.89
	2023.C.35P.30.20	20	13	5	2	65.00
	2023.D.35P.30.20	20	7	13	0	35.00
	2023.E.35P.30.20	20	20	0	0	100.00
	2023.F.35P.30.20	20	13	4	3	65.00
	2023.G.35P.30.20	18	16	2	0	88.89
	2023.H.35P.30.20	19	16	2	1	84.21
I	2023.A.35P.40	20	13	3	4	65.00
	2023.B.35P.40	20	14	0	6	70.00
	2023.C.35P.40	19	13	3	3	68.42
	2023.D.35P.40	18	14	3	1	77.78
	2023.E.35P.40	20	15	3	2	75.00
	2023.F.35P.40	19	17	0	2	89.47
	2023.G.35P.40	19	15	1	3	78.95
J	2023.A.35P.50	18	15	1	2	83.33
	2023.B.35P.50	20	4	7	9	20.00
	2023.C.35P.50	18	5	3	10	27.78
	2023.D.35P.50	19	0	0	19	0.00
	2023.E.35P.50	19	9	5	5	47.37
	2023.F.35P.50	17	5	3	9	29.41
	2023.G.35P.50	17	4	1	12	23.53
K	2023.A.35P.40.a	20	18	0	2	90.00
	2023.B.35P.40.a	18	16	2	0	88.89
	2023.C.35P.40.a	18	16	0	2	88.89
	2023.D.35P.40.a	17	15	0	2	88.24
	2023.E.35P.40.a	19	18	0	1	94.74

	2023.F.35P.40.a	20	20	0	0	100.00
	2023.G.35P.40.a	20	15	3	2	75.00
L	2023.A.35P.50.a	17	12	3	2	70.59
	2023.B.35P.50.a	19	13	2	4	68.42
	2023.C.35P.50.a	19	2	3	14	10.53
	2023.D.35P.50.a	18	0	2	16	0.00
	2023.E.35P.50.a	20	13	7	0	65.00
	2023.F.35P.50.a	19	13	3	3	68.42
	2023.G.35P.50.a	20	11	4	5	55.00
M	HP.2023.A.0P.30.20	19	15	1	3	78.95
	HP.2023.B.20P.30.20	18	17	0	1	94.44
	HP.2023.C.35P.30.20	19	19	0	0	100.00
	HP.2023.D.IAA.30.20	19	19	0	0	100.00
	HP.2023.E.BAP.30.20	18	13	0	5	72.22
	HP.2023.F.GA.30.20	20	18	0	2	90.00
	HP.2023.G.IAA.GA.30.20	18	12	2	4	66.67
	HP.2023.H.BAP.GA.30.20	19	13	4	2	
	HP.2023.I.IAA.BAP.30.20	18	12	3	3	66.67
	HP.2023.J.IAA.BAP.GA.30.20	19	19	0	0	100.00
N	2023.A3.0P.SAND.30.20	20	17	1	2	85.00
	2023.A4.0P.SAND.30.20	19	13	3	3	68.42
	2023.B4.35P.SAND.30.20	19	14	2	3	73.68
	2023.B5.35P.SAND.30.20	20	16	0	4	80.00
	2023.B6.35P.SAND.30.20	20	16	2	2	80.00
O	2023.A3.0P.PERLITE.30.20	19	15	2	2	78.95
	2023.A4.0P.PERLITE.30.20	19	13	3	3	68.42
	2023.B4.35P.PERLITE.30.20	19	17	2	0	89.47
	2023.B5.35P.PERLITE.30.20	19	17	0	2	89.47
	2023.B6.35P.PERLITE.30.20	20	18	0	2	90.00
P	2023.A3.0P.PAPER.30.20	20	14	4	2	70.00
	2023.A4.0P.PAPER.30.20	18	12	2	4	66.67
	2023.B3.35P.PAPER.30.20	20	15	3	2	75.00
	2023.B4.35P.PAPER.30.20	19	12	3	4	63.16
Q	2023.A3.BOIL.30.20	10	0	0	10	0.00
	2023.A4.BOIL.30.20	12	0	0	12	0.00
	2023.B3.BOIL.30.20	19	0	0	19	0.00
	2023.B4.BOIL.30.20	20	0	0	20	0.00

ER results

Table A4. 4. Results of ER viability testing in non-germinated seeds from batch 20230003. (mo.: months; no.: number; treat.: treatment).

Treat. I.D.	Experimental coding	No. tested embryos	No. germinated embryos	No. callus	No. dark	No. no reaction	No. contamination	FGP
F	2023.A.0P.30.20	18	14	0	1	0	3	77.78
	2023.B.0P.30.20	17	3	0	9	0	5	17.65
	2023.C.0P.30.20	17	5	0	10	1	1	29.41
	2023.D.0P.30.20	20	6	0	9	0	5	30.00
	2023.E.0P.30.20	19	13	0	2	0	4	68.42
	2023.F.0P.30.20	18	13	1	4	0	0	72.22
	2023.G.0P.30.20	20	16	0	2	1	1	80.00
	2023.H.0P.30.20	20	17	0	3	0	0	85.00
G	2023.A.20P.30.20	18	7	0	11	0	0	38.89
	2023.B.20P.30.20	18	15	0	2	1	0	83.33
	2023.C.20P.30.20	19	11	0	6	2	0	57.89
	2023.D.20P.30.20	19	12	1	4	0	2	63.16
	2023.E.20P.30.20	17	9	0	6	0	2	52.94
	2023.F.20P.30.20	19	13	0	6	0	0	68.42
	2023.G.20P.30.20	19	15	1	3	0	0	78.95
	2023.H.20P.30.20	19	14	0	5	0	0	73.68
H	2023.A.35P.30.20	16	12	0	4	0	0	75.00
	2023.B.35P.30.20	19	15	0	3	0	1	78.95
	2023.C.35P.30.20	18	12	0	5	1	0	66.67
	2023.D.35P.30.20	18	14	0	2	2	0	77.78
	2023.E.35P.30.20	19	14	2	3	0	0	73.68
	2023.F.35P.30.20	20	17	0	2	1	0	85.00
	2023.G.35P.30.20	19	12	0	4	2	1	63.16
	2023.H.35P.30.20	20	4	0	12	1	3	20.00
I	2023.A.35P.40	14	7	0	7	0	0	50.00
	2023.B.35P.40	16	6	0	10	0	0	37.50
	2023.C.35P.40	17	3	0	5	6	3	17.65
	2023.D.35P.40	18	6	0	9	3	0	33.33
	2023.E.35P.40	16	3	0	6	6	1	18.75
	2023.F.35P.40	15	1	0	8	1	5	6.67
	2023.G.35P.40	14	1	0	10	2	1	7.14
J	2023.A.35P.50	17	10	0	5	0	2	58.82
	2023.B.35P.50	16	3	0	10	3	0	18.75
	2023.C.35P.50	14	2	0	12	0	0	14.29
	2023.D.35P.50	14	0	0	10	4	0	0.00
	2023.E.35P.50	19	6	0	1	9	3	31.58
	2023.F.35P.50	16	1	0	0	11	4	6.25
	2023.G.35P.50	16	0	0	9	5	2	0.00
K	2023.A.35P.40.a	20	4	0	10	1	5	20.00
	2023.B.35P.40.a	20	3	0	9	6	2	15.00
	2023.C.35P.40.a	18	1	0	2	4	11	5.56
	2023.D.35P.40.a	16	2	0	4	6	4	12.50
	2023.E.35P.40.a	20	1	0	14	1	4	5.00
	2023.F.35P.40.a	20	2	0	8	1	9	10.00

	2023.G.35P.40.a	20	8	0	6	1	5	40.00
L	2023.A.35P.50.a	20	4	0	12	0	4	20.00
	2023.B.35P.50.a	18	0	0	11	2	5	0.00
	2023.C.35P.50.a	19	0	0	10	3	6	0.00
	2023.D.35P.50.a	18	0	0	13	0	5	0.00
	2023.E.35P.50.a	19	2	0	10	0	7	10.53
	2023.F.35P.50.a	20	0	0	8	3	9	0.00
	2023.G.35P.50.a	20	0	0	8	4	8	0.00
M	HP.2023.A.0P.30.20	19	5	0	7	1	6	26.32
	HP.2023.B.20P.30.20	19	3	0	14	0	2	15.79
	HP.2023.C.35P.30.20	20	4	0	13	2	1	20.00
	HP.2023.D.IAA.30.20	18	12	0	3	0	3	66.67
	HP.2023.E.BAP.30.20	18	6	0	9	0	3	33.33
	HP.2023.F.GA.30.20	17	6	0	0	2	9	35.29
	HP.2023.G.IAA.GA.30.20	20	13	0	3	0	4	65.00
	HP.2023.H.BAP.GA.30.20	20	9	0	5	1	5	45.00
	HP.2023.I.IAA.BAP.30.20	20	7	0	6	1	6	35.00
	HP.2023.J.IAA.BAP.GA.30.20	18	8	0	4	0	6	44.44
N	2023.A1.0P.SAND.30.20	19	10	0	0	2	7	52.63
	2023.A2.0P.SAND.30.20	20	16	0	1	1	2	80.00
	2023.B1.35P.SAND.30.20	20	6	0	0	4	10	30.00
	2023.B2.35P.SAND.30.20	17	11	0	1	3	2	64.71
	2023.B3.35P.SAND.30.20	20	15	0	0	5	0	75.00
O	2023.A1.0P.PERLITE.30.20	16	7	0	1	2	6	43.75
	2023.A2.0P.PERLITE.30.20	15	13	0	0	2	0	86.67
	2023.B1.35P.PERLITE.30.20	16	3	0	2	4	7	18.75
	2023.B2.35P.PERLITE.30.20	18	8	0	2	3	5	44.44
	2023.B3.35P.PERLITE.30.20	19	12	0	1	0	6	63.16
P	2023.A1.0P.PAPER.30.20	18	8	0	2	4	4	44.44
	2023.A2.0P.PAPER.30.20	20	3	0	8	4	5	15.00
	2023.B1.35P.PAPER.30.20	18	2	0	3	3	10	11.11
	2023.B2.35P.PAPER.30.20	20	6	0	0	5	9	30.00
Q	2023.A1.BOIL.30.20	17	0	0	0	0	17	0.00
	2023.A2.BOIL.30.20	19	0	0	0	0	19	0.00
	2023.B1.BOIL.30.20	20	0	0	0	0	20	0.00
	2023.B2.BOIL.30.20	20	0	0	0	0	20	0.00