EFFECTS OF ANTIFUNGAL TREATMENTS ON SOME RECALCITRANT SEEDS

By

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Submitted in fulfillment of the academic requirement for degree of Doctor of Philosophy in the School of Life Sciences, University of KwaZulu-Natal, Westville campus, South Africa

June 2017

PREFACE

The experimental work described in this thesis was carried out in the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Westville, from January 2008 to August 2014, under the supervision of Professor Norman Pammenter and the late Professor Patricia Berjak.

These studies represents original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it duly acknowledged in the text.

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ANELISWA PHUMZILE MAKHATHINI

June, 2017

DECLARATION 1 – PLAGIARISM

I……..ANELISWA PHUMZILE MAKHATHINI……, declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
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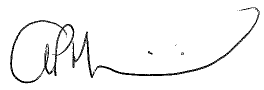

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AP MAKHATHINI

DECLARATION 2 – PUBLICATION

Details of contribution to publication that form part and/or include research presented in this thesis (include publication submitted and give details of the contributions of each author to the experimental work and writing of publication)

Publication 1

A.P. Makhathini[1], N.W. Pammenter[2]. **Development of treatment protocols towards eliminating seed-borne fungi of the recalcitrant-seeded species of *Trichilia dregeana* Sond**. Submitted to lab.

Contributions: AP Makhathini carried out experimental work, recorded data and writing of the manuscript.

Prof Pammenter and the late Prof Berjak (1939-2015) were the supervisors, assisted in the experimental design and editing of the manuscript.

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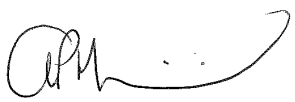

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AP MAKHATHINI

ACKNOWLEDGEMENTS

I am grateful to my supervisors, the late Professor Patricia Berjak (1939 – 2015) and Professor Norman Pammenter for their enthusiastic guidance, support and encouragement in my learning experience. May you be blessed in abundance!

I need to also extend my gratitude to the following:

- To God, the Father, the Son and the Holy Spirit for the opportunity to be part of a brilliant research team, for good health and wisdom, the glory is yours, forever, AMEN.
- To past and present members of Plant Germplasm Conservation Research Group for their understanding and flexibility and most importantly for just being there for me when I needed them, I am truly indebted.
- To DUT, Department of Biotechnology & Food Technology staff members for encouragement and support through to the end of this journey, especially Prof S. Singh, Prof K. Permaul, Prof H. Baijnath, Dr K. Maduray, Prof F. Swalaha, Dr J.J. Mellem, Dr N.P. Mchunu, Ms S. Juglal, I greatly appreciate.
- To my friends: Noks, Hle, Kuhle, Kammy, Fiks, Masego, Jency, Babu Elliot and Agnes for your kindness, support and encouragement, I will not forget and forever thankful.
- To Mr Thembela Mathews Mshengu, my driver, seed collecting and cleaning partner, for when my brain could not function anymore you took over, I cannot thank you enough!
- To my husband, Nhlahla Lawrence Zincume, for encouragement and understanding, Thank you!
- To my children, Khwezi and Khanya, from now on we shall sing and play soccer on your terms!
- To my brothers, Mphiwe and Mxolisi Makhathini your prayers, encouragement and support means a lot, Thank you!
- To my beloved parents, Ntombi Doris and Themba Patrick Makhatini† who laid the foundation of my education, Thank you for your prayers, support, love and always believing in me! “Shuku and Gxabhashe, I made it!”
- I would like to finally acknowledge and express my gratitude to National Research Foundation and Durban University of Technology, for financial support and time off.

DEDICATION

Mary, Tabernacle of the Most High:

The angel went to her and said, "Greetings, you who are highly favoured! The Lord is with you." - Luke1:28

ABSTRACT

Recalcitrant seeds cannot be stored under normal storage conditions and are characterized by not undergoing dehydration, sensitivity to desiccation, high susceptibility to desiccation injury, and the fact that they are shed at relatively high moisture content leading to early germination. The storage of these seeds for seed banks and conservation programmes is an important aspect of seed conservation strategies. One of the major problems restricting storage lifespan of these seeds is the presence and proliferation of microorganisms. The purpose of this study was to develop treatment protocols, evaluate the impact of fungicides by performing seed vigour and germination tests, isolation and identification of fungi in recalcitrant seeds proliferating in tissue culture stage and lastly to assess developed protocols under pot trials conditions.

Plant germplasm selected for seed harvest was that of *Trichilia dregeana*, *Protorhus longifolia* and *Garcinia livingstonei*. Isolates were obtained by performing a series of microbiological tests and plating out on Potato Dextrose Agar (PDA) to obtain pure cultures. Thereafter, the pure cultures were characterized using different media *viz*. Czapek Dox medium (CDM), Sabourad Dextrose Agar (SDA), Water Agar (WA), Malt Extract Agar (MEA) and PDA, microscopy was used to observe and capture fungal fruiting bodies and these were confirmed using 18S DNA identification. A total of 6 pure fungal isolates were prevalent to *T. dregeana*; namely *Paecilomyceslilacinus*, *Fusarium* sp. *Aureobasidium pullulans*, *Penicillium brevicompactum* and *Trichoderma asperellum.* The most prone to fungi was *P. longifolia* as a total of 18 fungal isolates were identified to be *Trichophyton rubrum*, *Phialemonium* sp., *Penicillium chrysogenum*, *Penicillium polonicum*, *Penicillium olsani*, *Trichoderma asperellum*, *Fusarium* sp., *Hypocrea atroviridis*, *Colletotrichum gloeosporiodes*, *Penicillium* sp., *Irpex* sp., *Cladosporium cladosporiodes*, *Aspergillus* sp., *Acremonium* sp., *Penicillium brevicompactum*, *Alternaria* sp., *Cytospora* sp. and *Penicillium adametzioides*. Only one isolate *Fomitopsis meliae* was isolated and identified from *G. livingstonei*. According to the text all of the isolates were of plant (seed) origin.

The identified isolates were then subjected to bio control agents of *Trichoderma harzianum* strain B77 (Eco77) and *Trichoderma harzianum* strain Kd (EcoT) [2 X 10^9 spores/gram], and *Bacillus subtilis* [1 X 10^6 propagules/ml] and chemical agents using the well diffusion method [10 mg ml^{-1}, 20 mg ml^{-1}, 50 mg ml^{-1}, 80 mg ml^{-1} and 100 mg ml^{-1}] to compare the most and least effective control agents. Thereafter the zones of inhibition diameters were measured. The effectiveness of these fungicides was then compared from 3 to 7 days of incubation at 25 °C. Eco77 gave the widest effectiveness across all the isolated fungal species, however, there were instances where EcoT produced a significantly higher inhibition than Eco77 (e.g. for *Aspergillus* sp., *Cytospora* sp., *P. lilacinus* and *P. chrysogenum*). With regards to chemical controls, Orius exhibited highest effectiveness and ripenit showed the least effectiveness. For purposes of this study, in promoting environmental safety and awareness as per ISO 14001 a decision was taken to develop treatment protocols using Eco77, Nipastat® and Biotaine™.

Developed selected protocols were surface decontamination alone however seeds were dusted with Benomyl 500 WP when stored in hydrated storage (DBe) or followed by treatment with a strain of *Trichoderma harzianum*, Eco77 and Biotaine™ (active ingredient chlorhexidine gluconate) (DEBBe) or with (DEnN) or without (DEn) Nipastat® (a mixture of parabens) (as a powder applied to seeds, or encapsulation in alginate gel, incorporating Nipastat. Under *in vitro* conditions DEBBe and DEn were the best treatments in terms of controlling contamination levels and a higher germination percentage of explants at 30 days of culture. Pot trials which lasted for 6 months after germination, outside the greenhouse, showed good results when assessing biomass allocation of leaves, stems and roots with treatments DEBBe, DEn and DEnN. However, storage time and type of treatments that the seeds were initially exposed to had an impact on the physiology of seed development.

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UNITS OF MEASUREMENTS

%	percent
^{o}C	degree Celcius
cfu	colony forming unit
conc.	concentration
d	day(s)
g	gram(s)
g g^{-1}	g H_2O per g
g g^{-1}dmb	g H_2O per g of dry matter, dry mass basis
g kg^{-1}	gram per kilogram
h	hours(s)
L	liter(s)
min	minute(s)
mL	milliliter(s)
mm	millimeter(s)
mm^2	square millimeter(s)
µL	microliter
µm	micrometer
s	second(s)
M	molarity
µM	micromolar
mV	millivolts
mg L^{-1}	millligrams per litre
PIRG	Percent Inhibition of Radial Growth
ppm	parts per million

RH	Relative Humidity
v/v	volume per volume
w/v	weight per volume

ABBREVIATIONS AND SYMBOLS

α-L-	Alpha Lyase
β	beta
β-D-	beta deoxy
ABC	ATP- binding cassette
ANOVA	analyses of variance
ATSDR	Agency for Toxic Substances and Disease Registry
BASF	Baden Aniline and Soda Factory
BCA	Bio-control Agent
Bt	*Bacillus thuringiensis*
c.	circa
d	day(s)
DMSO	dimethyl sulphoxide
dmb	dry mass basis
DW	dry weight
Eco77	*Trichoderma harzianum* strain B77
EcoT	*Trichoderma harzianum* strain Kd
EFSA	Europian Food Safety Authority
EO	Eloctrolyzed oxidizing
EPA	Environmental Protection Agency
fmb	fresh mass basis
FW	fresh weight
GA_3	Gibberellic acid
IAA	Indole-3-acetic acid
IAAld	Indole-3-acetaldehyde

IEt	Indole-3-ethanol
ISR	Induced systemic resistance
JA	Jasmonic acid
LSF	liquid state fermentation
MBC	methyl 2-benzimidazole carbamate
MEA	Malt Extract Agar
MS	Murashige Skoog
n	number
NADCC	Sodium dichloro-isocyanurate
NCP	New Chemicals Programme
REDOX	oxidation-reduction potential
PCNB	Pentachloronitrobenzene
PDA	Potato Dextroxe Agar
PI%	Percentage of growth inhibition
PIRG	Percentage Inhibition of Radial Growth
PR	pathogenesis-related proteins
R	Radial growth
RPD	Report Plant Disease
SA	salicyclic acid
SABS	South African Bureau of Standards
SANS	South African National Standards
SAR	systemic acquired resistance
SASRI	South African Sugar Research Institute
SDA	Sabourad Dextrose Agar
spp.	species
T	*Trichoderma*

™	Trademark
T22T-22, DRHCl, T77	*Trichoderma* strains
UV	Ultraviolet
WA	Water Agar

1. INTRODUCTION

1.1. Seed types

Plant germplasm conservation is of global concern as plant genetic resources are diminishing through humans utilizing natural resources indiscriminately, and because of land clearing for agriculture and silviculture and urbanization; adding to these losses are the effects of climate change and natural disasters (Rao, 2004, Jackson and Kennedy, 2009, Berjak and Pammenter, 2014): considering these factors, one cannot argue or disagree about the necessity for *ex situ* germplasm preservation as a complementary practice to *in situ* conservation (FAO, 2013).

Among the various *ex situ* plant germplasm conservation methods, the most efficient, convenient and economical approach to conserve plant genetic diversity over extended periods is through seed storage (Paunescu, 2009, Engelmann, 2011, Berjak and Pammenter, 2013). Longevity of seeds in storage is mainly determined by storage temperature and water content, the latter being governed by relative humidity (RH). Good quality seeds are storable for decades and more, but only when generally kept at -18ºC and 15% RH (Ford-Lloyd and Jackson, 1991, FAO, 2013). This strategy, however, cannot be applied to all plant species because of the post-harvest behaviour of seeds, which determines the most suitable method of conservation.

There are three distinct categories of seeds types *viz*. orthodox, intermediate and recalcitrant. Seeds tolerating extreme desiccation and surviving in the dehydrated state for periods that are predictable depending on storage conditions are said to show orthodox storage behaviour (Roberts, 1973, Chin, 1980, Berjak and Pammenter, 1997, Berjak and Pammenter, 2004a, Sun, 1999, Pammenter and Berjak, 1999, Kermonde and Finch-Savage, 2002, Berjak and Pammenter, 2014), whereas, seeds categorized as showing intermediate postharvest behaviour are relatively desiccation-tolerant, but will not withstand removal of water to levels as low as orthodox seeds. Such seeds, may also be chilling-sensitive, even in the dehydrated state, particularly if they are of tropical origin (Ellis et al., 1990, Ellis et al., 1991, Pammenter and Berjak, 1999).

Recalcitrant seeds are those that either lack, or do not express, the various processes and mechanisms embodying the essential characteristics of desiccation tolerance as occurs in orthodox seeds (e.g. work done on recalcitrant seeds of *Castanospermum australe*, revealed the absence of certain late embryogenesis accumulating proteins (LEAs) which have been shown to be critical for tolerance (Delahaie et al., 2013).

1.2. Recalcitrant seed physiology

Recalcitrant seeds are classified as those that are highly metabolic when shed from the parent plant and, depending on the developmental stage at which this takes place and the species, some seeds could undergo final stages of development after being shed while other begins to germinate immediately (Farrant et al., 1989, Calistru et al., 2000, Berjak and Pammenter, 2008). In contrast, orthodox seeds are metabolically inert and water uptake is a necessity for them to initiate germination (Côme and Corbineau, 1989).

To clearly differentiate between orthodox and recalcitrant seed types, understanding development is of vital importance. Three phases that define orthodox seed development are histodifferentiation, reserve deposition and maturation drying (Kermonde and Finch-Savage, 2002). Histodifferentiation (i.e. phase one) is a stage in which undifferentiated cells divide and specialize, thus leading to development of tissues with specific functions. These events occur in all seed types (Wang and Hedley, 1991, Olsen et al., 1992). In phase two, various forms of reserves are deposited in the endosperm and these will later provide nutrients to a germinating seedling. Phases one and two, thus far contribute towards increasing dry and fresh mass of the seeds. Maximum dry mass is ultimately reached and this marks the beginning of the final stage of seed development. This stage, maturation drying, is marked by rapid loss of water, and thus fresh mass, accompanied by preparation for germination, thus replacing events intrinsic to seed development. Only then the seeds can be categorized as physiological mature. Completion of these three sequential phases leads to seeds being shed and remaining quiescent until water is available to induce germination (Kermonde and Finch-Savage, 2002, Goveia et al., 2004). In contrast, in recalcitrant seeds, the physiological maturity and drying occurring in phase three of the development of orthodox seeds does not take place (Berjak et al., 1989, Berjak et al., 1990, Farnsworth, 2000, Connor and Sowa, 2002,

Kermonde and Finch-Savage, 2002), resulting in seeds being shed at high water contents (0.3 – 4.0 g g^{-1} dry mass basis [dmb] depending on the species [(Berjak and Pammenter, 2001, Berjak and Pammenter, 2004b)].

Water plays a vital role in facilitating intracellular order and structural stability (Berjak, 2006), and moreover, is involved in every dynamic process in living cells as it serves as a universal solvent or medium through which movement of solutes and biochemical reactions take place (Sun, 2002). Non-orthodox seeds continue to sustain metabolic activities even after being shed, these being facilitated by appropriate water contents. Assessments of various characteristics of the relationship between water status and seed development have been carried out on seeds of *Podocarpus henkelii* (Dodd et al., 1989), *Quercus robur* (Grange and Finch-Savage, 1992, Finch-Savage, 1992a, Finch-Savage et al., 1992) and *Avicennia marina* (Farrant et al., 1993a, Farrant et al., 1997) showing the latter stages of seed development of non-orthodox seeds to differ from that in orthodox types.

1.3. Desiccation sensitivity

Responses to desiccation play a major role in differentiating recalcitrant from orthodox seeds. Mechanisms for desiccation tolerance in orthodox seeds have evolved over many millennia allowing the seeds to survive during and after the maturation drying phase. Recalcitrant seeds lack the ability for metabolic switch-off as it occurs in orthodox seeds, or only partially express these mechanisms (Berjak and Pammenter, 2001, Berjak and Pammenter, 2008, Berjak and Pammenter, 2013, Delahaie et al., 2013), thus limiting them from completing the final phase facilitating the ability to tolerate desiccation.

Intensive work that has been done on seeds, particularly seed storage for germplasm conservation, has demonstrated that tolerance to dehydration is not just the ability of the species to tolerate desiccation; but there is a time component to tolerance in the dry state (Berjak and Pammenter, 2013). Desiccation sensitivity implies that seeds of the species concerned lack or do not express genes that confer tolerance, but this has not been proven or identified. It is likely though that a number of putative mechanisms are missing or fail, rather than one individual process (Berjak and Pammenter, 2013).

1.3.1. Desiccation tolerance mechanisms

With the exception of orthodox seeds, desiccation tolerance, may not be common among species, but occurs across a range of life-forms, and some commonalities seem to appear in the mechanisms employed by the various life-forms having such tolerance (Berjak, 2006, Bharuth et al., 2007, Berjak and Pammenter, 2013). Several mechanisms have been proposed that in synergy confer the ability of specific tissues or life cycle stages of a species to be desiccation tolerant:

a) Intracellular physical characteristics

The major features which become apparent in embryo cells, the underlying mechanisms and processes involved and their interactions in developing seeds upon acquisition of desiccation tolerance, have been extensively reviewed (Vertucci and Farrant, 1995, Pammenter and Berjak, 1999, Kermonde and Finch-Savage, 2002, Bewley et al., 2006, Bharuth et al., 2007, Berjak and Pammenter, 2008). These features are briefly discussed in this section, to provide comparison with the features of desiccation-sensitive seed physiology. One of the major requirements for plant cells to tolerate dehydration is their ability to withstand reduction of fluid volume within the vacuoles. This is associated with their breakdown of large vacuoles into considerably smaller ones and/or intravascular deposition of insoluble material (generally protein), thus giving the cell mechanical resilience against collapsing (Pammenter and Berjak, 1999, Berjak and Pammenter, 2001, Berjak and Pammenter, 2004b, Berjak and Pammenter, 2013). Carbohydrate and lipid reserve accumulations further provide solid material contributing to buffering capacity of plant cells (Vertucci and Farrant, 1995, Berjak and Pammenter, 2013), thus a assisting in increasing the cell resilience to the mechanical stress accompanying extreme dehydration. Studies carried out on seeds of various species described as highly or moderately recalcitrant or orthodox showed that the extent of vacuolation in embryonic axis cells at maturity was related to the degree of desiccation sensitivity of the seeds (Berjak et al., 1984, Farrant et al., 1989, Farrant et al., 1997, Goveia et al., 2004).

The major components of the cytoskeleton are microfilaments and microtubules, which impose spatial organization on the cytoplasm and nucleus and also provide internal support to cells. In the case of orthodox seeds, the cytoskeleton is dismantled in an orderly fashion upon dehydration and reassembles

upon rehydration (Mycock et al., 2000, Berjak and Pammenter, 2013). Research on the embryonic axes of non-orthodox *Quercus robur* (Mycock et al., 2000), *Inga vera* (Faria et al., 2004) and *Bridelia micrantha* (Merhar et al., 2004) revealed that dismantling of the cytoskeleton occurred upon injurious levels of dehydration, and that complete reassembly upon rehydration was not re-established. Furthermore, progressive loss of the ability of microfilaments to become re-orientated during rehydration was observed in *Trichilia dregeana* seeds (Gumede et al., 2003). In contrast, work done by (Faria et al., 2005) showed that the cytoskeleton was rapidly re-assembled during rehydration of orthodox seeds of *Medicago truncutula*. The intolerance of the cytoskeleton to desiccation, therefore, has physiological and structural consequence in recalcitrant seeds (Pammenter and Berjak, 1999).

The skeletal cytomatrix is not the only structural/organizational component of the cell affected by dehydration but also the integrity of the nucleoskeleton and the genetic material is compromised. Maintenance of the nuclear integrity during dehydration and in the dry state, as well as its re-establishment on rehydration is a vital requisite for desiccation tolerance. Even prior to dehydration of desiccation tolerant tissues, DNA replication stops and the chromatin becomes highly condensed (Sargent et al., 1981, Calistru, 2004), with replication resuming upon imbibition, as the chromatin re-disperses. Stability within the DNA structure during dehydration of cells of desiccation tolerant seeds is highly maintained, whereas within desiccation sensitive types, it becomes degraded (Osborne and Boubriak, 1994, Osborne et al., 2000).

b) Intracellular organelles dedifferentiation and metabolic 'switch-off'

Metabolic 'switch off', dedifferentiation of organelles and reduction of the elements of endomembrane system interact, thus limiting unregulated metabolism of the consequences of generation of free radicals (Berjak and Pammenter, 2013). Observations by Farrant et al. (1997) showed that at the phase corresponding to maturation drying in orthodox seeds, (*i.e.* prior to shedding), seeds of *Avicennia marina*, which are highly desiccation sensitive, had a large number of well-developed mitochondria per unit cell volume, compared with those of *Phaseolus vulgaris* seeds where the mitochondria had become substantially dedifferentiated. In addition, endomembranes such as the rough endoplasmic reticulum

become reduced and cisternae of Golgi apparatus become dissociated (Pammenter and Berjak, 1999, Berjak and Pammenter, 2001, Berjak and Pammenter, 2004b, Berjak and Pammenter, 2013). The dedifferentiation of organelles observed in orthodox seeds suggests their tolerance of desiccation is partly because membrane surface area is minimized prior to water loss. In the case of recalcitrant seeds, the retention of well-developed, differentiated organelles is thought to contribute significantly to the desiccation sensitivity of this seed type (Pammenter and Berjak, 1999, Faria et al., 2004). Additionally, as water is lost, damage occurs as a result of the ongoing metabolism, which becomes unbalanced (Pammenter and Berjak, 1999, Walters et al., 2001).

c) Anti-oxidant mechanisms and free radicals

Strong oxidizing agents produced during normal metabolism (for example, during the reduction of mitochondrial cytochromes [Leprince et al., 1994, Leprince et al., 2000a]) are free radicals and reactive oxygen species (ROS). Anti-oxidant systems control ROS levels within hydrated cells, thus preventing injurious consequences of escaped free radicals (Pammenter and Berjak, 1999, Bailly et al., 2001, Walters et al., 2001, Berjak and Pammenter, 2004b, Buitink et al., 2002, Hasleas et al., 2003, Mayaba and Beckett, 2003, Kranner and Britic, 2005).

Lipid peroxidation is the most commonly studied consequence of free radical/ROS overproduction, which ultimately leads to leaking seed membranes during hydration as shown by Varghese and Naithani (2000) for neem, by Leprince et al. (2000b) for cucumber and pea, Sinha et al. (2005) for water lettuce. When metabolism is at distress, as in the case of dehydration occurrence, unregulated free radicals may have a potential to be produced (Leprince and Hoekstra, 1998, Leprince et al., 2000b, Farrant et al., 2004). To avoid cellular damage, anti-oxidant system control and operation must be effective (Pammenter and Berjak, 1999, Berjak and Pammenter, 2001, Oliver et al., 2001, Berjak and Pammenter, 2004b, Kranner et al., 2005). During maturation drying of orthodox seeds, their metabolism is 'switched-off' but components of their anti-oxidant systems remain active, thus facilitating safe dehydration and rehydration. Desiccation sensitive seeds were also suggested to have anti-oxidant and free radical scavenging systems (Chaitanya et al., 2000), as has now been demonstrated for, e.g. *Trichilia dregeana*

(Varghese et al., 2011). However, these become impaired under conditions of water stress and consequently cannot effectively quench the ROS generated (Hendry et al., 1992, Leprince et al., 1999) especially under slow drying conditions (Pammenter et al., 1998, Varghese et al., 2011).

d) Role of protective molecules

Presence of sucrose and certain raffinose series oligosaccharides

High concentrations of sucrose with certain raffinose series oligosaccharides (or galactosyl cyclitols) are known to accumulate in orthodox seeds as they mature (Leprince et al., 1993, Horowicz and Obendorf, 1994, Blackman et al., 1995, Steadman et al., 1996, Obendorf, 1997, Black et al., 1999, Berjak and Pammenter, 2008, Berjak and Pammenter, 2013). These two carbohydrate-types, together with LEAs, are major constituents contributing to the intracellular vitrified (glassy-like) state in seeds once the water content is low (i.e. ≤ 0.3 g g^{-1} dry mass). The intracellular vitrified state curtails molecular diffusion and thus minimizes unregulated metabolism (Pammenter and Berjak, 1999, Berjak and Pammenter, 2001, Hoekstra et al., 2001, Kermonde and Finch-Savage, 2002, Alpert, 2006, Berjak and Pammenter, 2004b, Berjak and Pammenter, 2008, Berjak and Pammenter, 2013). In addition, the intracellular vitrified state may also contribute to the prolonged life-span of desiccation tolerant seeds in the dry state (Leapold et al., 1994) and the ultimate breakdown of the glassy matrix may underlie seed deterioration during storage. However, it is important to note that, these carbohydrates do not work in isolation, as many other cytomatrical component molecules must be incorporated in the vitrified state. The vitrified state imposes a highly viscous intracellular situation, and therefore limits residual reactivity and the migration of free radicals. Moreover, to ensure seed survival in the desiccated condition, maintenance of intracellular vitrification is vital; however, the glassy state is metastable and therefore does not guarantee infinite viability retention even under sub-zero storage conditions (Berjak, 2006, Berjak and Pammenter, 2013).

Late embryonic accumulating/ Abundant Proteins (LEAs)

Development of many seed species is accompanied by an accumulation of LEAs. These proteins play a role in the acquisition and maintenance of desiccation tolerance in orthodox seeds. Their amphipathic nature is thought to allow them to interact with a wide range of macromolecules, thus preventing them from denaturation under desiccated conditions (Blackman et al., 1995, Oliver et al., 2001, Stupnikova et al., 2006, Berjak and Pammenter, 2008). However, the situation regarding the occurrence of LEAs in recalcitrant seeds is equivocal, as they have been found to occur in a range of species from different habitats (e.g. *Avicennia marina* [(Farrant et al., 1993a)]). Ultimately, the LEAs and possibly other proteins, together with sucrose and certain oligosaccharides act to confer desiccation tolerance in orthodox seeds (Fu et al., 1995, Walters et al., 1997). A review by Berjak (2006) on the glassy state in desiccation-tolerant organisms emphasizes that the vitrified state in seeds cannot be considered to contain only sucrose and certain oligosaccharides, but must also include an array of other cytomatrical constituents. LEAs, are principally considered as vital components of the vitrified state, thought to add tensile strength and stability to the matrix (Berjak, 2006).

1.3.2. Operation of repair mechanisms upon rehydration

Protective mechanisms acquired by orthodox seeds before and after drying are not the only mechanisms enabling survival of desiccation as also vital is the ability to repair desiccation damage upon rehydration. Orthodox seeds have been shown to have the ability to repair damage accumulated in the dry state, soon after hydration has been initiated. Recovery takes place in the lag phase of water intake before radicle emerges (Osborne and Weber, 1987, Berjak and Pammenter, 2008). This however, requires highly efficient repair mechanisms and restitution of normal cell structure and function. In addition to repair mechanisms, the presence of an efficient operational antioxidant system is of vital importance reviewed by Pammenter and Berjak (1999), and Berjak and Pammenter (2008), and this aspect had been demonstrated in the DNA repair mechanisms during early rehydration by Boubriak et al. (1997). However, Boubriak et al. (2000) also found that when DNA fragmentation was induced by radiation of newly harvested highly recalcitrant *A. marina* seeds, embryos were able to repair, but that ability was compromised if the embryos had first been dehydrated, indicating that repair mechanisms are also

sensitive to water loss. Studies by Connor and Sowa (2003) also showed that after initial dehydration of recalcitrant *Quercus alba* acorns, on rehydration a reversible shift occurred between the gel and the liquid crystalline phases of membranes, but this ability declined in line with declining seed viability. Those authors also showed that the secondary structure of proteins was irreversibly affected by dehydration.

1.3.3. Damage of seeds in relation to desiccation

Damage due to desiccation occurs when water that is critical for survival is withdrawn from cells leading to changes in physical and physiological properties (Walters et al., 2002a). These changes due to desiccation in orthodox seeds are reversible once cells accumulate sufficient water to re-initiate cell activities and therefore, the effects of dehydration are manifested not only by the differences between the hydrated and dry states, but by the ability of cells to regain full functionality upon imbibition (Walters et al., 2002a).

In non-orthodox/recalcitrant seeds, water removal from the tissues can lead to two types of cellular damage (Vertucci and Farrant, 1995, Pammenter and Berjak, 1999, Walters et al., 2001) *viz*. metabolism-linked damage and desiccation damage *sensu stricto*. Damage *sensu stricto* has a negative impact on mechanical activities and structural properties of the cells, resulting in failure to regulate coordinated metabolic functions within the cells. Unbalanced metabolism leads to aqueous-based degradative processes that are probably mediated by free radicals (Pammenter et al., 1998, Pammenter and Berjak, 1999, Walters et al., 2001, Walters et al., 2002a), whereas, desiccation damage *sensu stricto* takes place at low water contents (Walters et al., 2001). Reduced water contents may lead to ineffective or inadequate protective antioxidant systems, resulting in damage that is detrimental to cells (Finch-Savage et al., 1994). Inadequate operation of antioxidant systems during slow water loss from recalcitrant zygotic axes of *Trichilia dregeana*, has been shown to be a major component contributing to metabolism-linked damage (Varghese et al., 2011). In recalcitrant seeds desiccation damage *sensu stricto* describes the damage that occurs when the water required to maintain integrity of the intracellular structures is removed (Pammenter et al., 1991, Walters et al., 2001). Such damage was observed to have taken place at water contents below

0.5 g g^{-1} dmb in rapidly dehydrated recalcitrant axes of *Camellia silences* and was correlated with the viability loss (Walters et al., 2001).

1.3.4. Desiccation and drying rate

The acquisition of desiccation tolerance in orthodox seeds involves, among other factors, reduction of vacuolar volume, which is often achieved by filling these compartments with insoluble material during the maturation drying phase (Vertucci and Farrant, 1995, Berjak, 2006, Bharuth et al., 2007, Berjak and Pammenter, 2013). However, this is not the case with mature recalcitrant seeds, where vacuoles are prominent in cells of the embryonic axes, and seemingly, the more desiccation sensitive the seed the greater is the degree of vacuolation (Farrant et al., 1997, Berjak and Pammenter, 2013). In addition, recalcitrant seeds remain metabolically active after being shed and do not lose water readily, but naturally would dry slowly (Pammenter and Berjak, 1999, Berjak and Pammenter, 2002, Berjak and Pammenter, 2004b, Berjak and Pammenter, 2013). The rate at which dehydration take place influences the extent of water loss that the axes in non-orthodox seeds can withstand without any significant damage. Dehydration occurring at slow rates allows more time for unbalanced metabolic activities to occur and, without any or having only limited protective mechanisms, the consequences are detrimental. In contrast, rapid drying limits the time during which metabolism-linked damage can occur. Because most recalcitrant seeds are far too large to dry rapidly, embryonic axes are conventionally excised for the purposes of germplasm cryopreservation. These explants should survive cryopreservation and yield recovery levels comparable with their potential to survive transiently under ambient conditions after rapid drying (Berjak et al., 1993, Pammenter and Berjak, 1999, Makeen et al., 2005), or ultra-rapid drying (Berjak et al., 1990, Vertucci et al., 1991, Berjak et al., 1999, Walters et al., 2001, Walters et al., 2002a, Pammenter et al., 2002).

Therefore, to preserve viability despite water loss, research done on embryonic axes of recalcitrant seeds utilizes a rapid drying technique, usually a laminar air-flow (Corredoira et al., 2004, Makeen et al., 2005) or a flash-drier (Walters et al., 2002a, Peran et al., 2006). If recalcitrant embryonic axes are dried rapidly, freezable (solution) water can be removed with minimal perturbation of metabolic processes (Pammenter

et al., 1991, Thammasiri, 1999, Kioko et al., 2006). However, removal of non-freezable (structure-associated) water results in loss of integrity of cellular and structural organization, and is referred to as desiccation damage *sensu stricto* (Pammenter and Berjak, 1999, Pammenter et al., 2000, Walters et al., 2001, Kim et al., 2005). As initially reported by (Berjak et al., 1990), flash-drying technique is thus by so far the most efficient of these methods and involves exposing naked explants to a stream of dry air. However, the morphological and histological attributes of explants still play a significant role, irrespective of the technique used in governing the actual rate at which water is lost.

Damage associated with desiccation sensitivity in non-orthodox seeds is a consequence of the lack or inadequate operation of protective mechanisms necessary to withstand loss of freezable or non-freezable water. In this regard, non-freezable water cannot be removed from the cells of recalcitrant seeds/axes (irrespective of drying rate) without lethal consequences. However, with sufficiently rapid drying, removal of freezable water can be tolerated to relatively low water contents simply because insufficient time is available for metabolism-linked damage to accumulate (Pammenter et al., 1998, Pammenter and Berjak, 1999, Walters et al., 2001, Berjak and Pammenter, 2008). These techniques will not be further discussed as they are not relevant to the current study.

1.4. Potential for recalcitrant seed storage

Storage of recalcitrant seeds requires maintenance of water content at, or slightly below, that of the newly shed state. However, viability retention in such hydrated storage generally ranges from only days to months, depending on the species (Roberts and King, 1980, Pammenter et al., 1984, Farrant et al., 1988, Berjak et al., 1989, Calistru et al., 2000, Berjak and Pammenter, 2004a, Berjak and Pammenter, 2008). Such seeds may also be sensitive to low temperatures, being damaged by chilling injury at temperatures of 10 - 15 ˚C or lower, depending on the species (Roberts and King, 1980). Species that are temperate, such as *Aesculus hippocastanum* (Dickie et al., 1991), *Acer pseudoplatanus* (Tompsett and Pritchard, 1993) and *Quercus robur* (Finch-Savage, 1992a) are chilling tolerant, although they never become desiccation tolerant despite losing a portion of tissue water during their development. Some temperate species (e.g. *Q. robur*), are recorded as surviving in hydrated storage for more than a year (Suszka and

Tylkowski, 1980). In contrast, with seeds of tropical species (exemplified by *Avicennia marina* which does not lose water during their development) may be highly sensitive to desiccation prior to, and after they are shed (Farrant et al., 1993a).

Plants producing non-orthodox seeds that are cultivated for economic purposes (e.g. cocoa [*Theobroma cacao*), tea [*Camellia sinensis*] and rubber [*Hevea brasiliensis*]) must be stored for a short while in order to make their transportation between laboratories, seed repository or cultivation centres possible (Chin, 1980, Berjak et al., 1989, Berjak and Pammenter, 2004a). This necessitates optimization of the means to prevent water loss and ensuring that the seeds are not exposed to damaging low – or high – temperatures.

There are three ways in which recalcitrant seed post-harvest life span can be extended, to optimize short-, medium- and long-term storage. For short-to medium-term storage, recalcitrant seeds are maintained at lowest temperature that they can tolerate without adverse effects, under conditions not permitting water loss and limited, or ideally, no proliferation of mycoflora associated with seeds (Calistru et al., 2000, Schmidt, 2000, Sutherland et al., 2002, Berjak and Pammenter, 2008). In contrast, for long-term storage of the genetic resources of recalcitrant-seeded species, cryopreservation (storage in liquid nitrogen) of components with the potential to produce seedlings/plantlets (e.g. embryonic axes, axillary buds, shoot apices) is currently the best option (Chin and Roberts, 1980, Wesley-Smith et al., 1992, Berjak and Pammenter, 1997, Dumet et al., 1997, Berjak and Pammenter, 2001, Kioko et al., 2006, Berjak and Pammenter, 2008). However, with this method one needs to incorporate many precautions and consider a variety of factors to ensure a successful cryopreservation (e.g. elimination of all contaminants, explant size, water content, use (or not) of cryoprotectants, and cooling and thawing rates). Furthermore, irrespective of the explant type, recovery and ongoing development after cryostorage necessitates the use of *in vitro* technology (FAO, 2013). Long-term cryostorage will not be further discussed in this chapter, as investigations in this study are towards developing and optimizing short-term and medium-term storage conditions for whole seeds. Such investigations necessitate preliminary trials on a species-specific basis. Nevertheless, whole seed storage is strictly a short-to medium-term option, basically because the seeds are metabolically active, and will progress from development to germination at water

content not much reduced from what it was at shedding (Farrant et al., 1988, Berjak et al., 1989, Drew et al., 2000, Eggers et al., 2007, Berjak and Pammenter, 2008).

Recalcitrant seeds produced by some species (e.g. *Trichilia dregeana*), are shed considerably before the embryonic axes are fully developed and can be stored in the hydrated state for several months at approximately 16 ºC (but not much lower, as they are chilling sensitive [Kioko, 2003]) before visible germination in storage is observed (Goveia et al., 2004, Berjak and Pammenter, 2008). In the case of recalcitrant seeds that are not chilling sensitive, storage shelf life may be further extended by refrigeration. At the other extreme, are seeds of *Theobroma cacao*, are known not to survive below 10 ºC (Chin and Roberts, 1980, Berjak and Pammenter, 2008), whilst *Trichilia emetica* seeds have been found to be lethally damaged at 6 ºC (Kioko et al., 2006, Berjak and Pammenter, 2008) as those of *Telfaria occidentalis* (Ajayi et al., 2006). Furthermore, genetic differences among populations of purportedly the same species from different provenances have been found to play a role in the relative chilling sensitivity of the seeds (Bharuth et al., 2007).

It had originally been suggested that lowering water contents of recalcitrant seeds to levels allowing basal metabolism but precluding the onset of germination in storage might be a means to prolong their life span (Chin and Roberts, 1980). However, this, has been proven to be deleterious to both shelf life and quality. This has been shown to be the case for seeds of a range of species (Corbineau and Côme, 1986a, Corbineau and Côme, 1986b, Drew et al., 2000, Eggers et al., 2007). In the cases of *Trichilia dregeana* (Drew et al., 2000, Eggers et al., 2007), *T. emetica*, *Syzygium cordatum* and the gymnosperm, *Podocarpus henkelii* (Eggers et al., 2007), not only did seed storage life span decline in the sub-imbibed condition relative to that of seeds stored at the shedding water contents, but fungal proliferation was exacerbated.

In attempting to prolong seed life span in storage, therefore, various manipulations and techniques have been developed. Among these, pericarp removal from freshly harvested *Avicennia marina* seeds and coating the exposed tissues with a crude alginate gel extended storage life span four-fold (Motete et al., 1997, Pammenter et al., 1997). Those authors suggested that this may have been a result of slowing post-

shedding germinative development, and more importantly, the alginate coating reduced the rate at which the root primodia lost water and suffered dehydration damage. Furthermore, as there was no measurable change in a range of metabolic parameters, it was suggested that the extended storage life span was due, at least partly, to the alginate gel inhibiting fungal proliferation (Motete et al., 1997, Pammenter et al., 1997).

However, it is important to note, manipulations using alginate gel encapsulation or storage in hydrated storage to extend storage life span of recalcitrant seeds may be successful, but cannot be recommended for longer-term germplasm conservation purposes, as the stored seeds sooner or later lose their viability in storage (Pammenter et al., 1994).

Another approach to extending life span of recalcitrant seeds is manipulation of the storage atmosphere. Nitrous oxide (anaesthetic gas which reduces respiration rate) treatment of *Litchi chinensis* and *Dimocarpus logan* seeds, was shown to facilitate increased life spans (Sowa et al., 1991). However, oxygen remains essential, as it is required to maintain viability of the metabolically-active seeds whilst in storage (Willan, 1985).

Studies have also demonstrated that darkness inhibits germination of seeds of particular species (Teketay, 1998), although this is not universal. Nevertheless, light needs to be controlled during storage of seeds as not to promote germination. However, all manipulations mentioned will fail if problems imposed by the seed associated microflora are not addressed. This problem is exacerbated since the best option for storing whole recalcitrant seeds is under conditions not permitting seed water loss and at moderate temperatures (Berjak and Pammenter, 2014), which are precisely the conditions that favour the proliferation of microflora, principally the seed-associated mycoflora (Berjak and Pammenter, 2014).
Short- to medium-term maintenance of recalcitrant seeds is carried out under hydrated storage condition (HS), which promote proliferation, particularly of associated fungi which compromise life span and also virtually preclude other efforts to preserve the germplasm by cryopreservation. Therefore, it is of vital importance to ensure that plant material to be used for storage or culture after cryostorage is free from

microbial contaminants, but this is difficult and even impossible when plant material (i.e. whole fruits or seeds) is collected from the ground (Sutherland et al., 2002, Berjak and Pammenter, 2013). As a guaranteed strategy to eliminate or minimize contaminants, the most effective and efficient decontamination procedures which are non-injurious to the seeds/explants must be employed.

1.5. Fungal status of seeds

Recalcitrant seeds can be stored for a few days or months and this is dependent on type of plant germplasm and the species (Chin and Roberts, 1980, Berjak et al., 1989, Berjak, 1996). Their survival in storage is diminished at low relative humidity and, depending on the species, low temperature as well: generally, therefore, temperatures of up to 25 °C and a saturated atmosphere are required (Côme and Corbineau, 1989). However, the high relative humidity, seed water content and temperature that tropical recalcitrant seeds generally require, also favour proliferation of microorganisms (Berjak, 1996, Mittal et al., 1998, Calistru et al., 2000). A diverse population of mycological propagules generally inhabits recalcitrant seeds as both external and internal contaminants (Berjak et al., 1990, Calistru et al., 2000), often composed of the genera *Alternaria, Cladosporium* and *Fusarium* (Berjak et al., 1990, Calistru et al., 2000, Mittal and Mathur, 2002). Presence of this mycoflora as either external or internal contaminants reduces germination ability of both recalcitrant and intermediate seeds (Mittal et al., 1998, Calistru et al., 2000).

Hydrated-storage of recalcitrant seeds is accompanied by progressive debilitation, loss of vigour and eventually viability, the time taken depending on the species. This is also the time at which fungus becomes more prominent. It is probable that seed deterioration offers fungus the conditions to proliferate and/or the fungi themselves cause the seed debilitation (Calistru et al., 2000). Furthermore, seeds stored at high water content often germinate during storage at favourable temperatures (Suszka and Tylkowski, 1980, Pammenter et al., 1994, Chien and Lin, 1997). Whilst germinating recalcitrant seeds have specialized mechanisms that counteract the proliferation of fungi, these eventually fail when the seeds become debilitated (Berjak, 1996, Merhar et al., 2003). Fungi infection and proliferation thus remain a serious problem for the conventional short- to medium-term storage of recalcitrant seeds. Moreover,

when embryonic axes are excised from the seeds and used for long-term storage by cryopreservation, fungal infection has been identified as one of the root causes of failure (Mycock and Berjak, 1990a, Calistru et al., 2000, Berjak and Pammenter, 2014).

1.6. Seed associated fungi and classification

Seed germination requirements are diverse and differ from one species to another but share a common factor with regard to water availability to induce development and growth. Orthodox seed fungal pathogens have been the subject of extensive research in comparison with those of recalcitrant seeds where fungi particularly proliferate in hydrated storage and as well when explants are cultured. Toxigenic fungi in orthodox-seeded crops have been historically divided into two distinct categories. The first includes those that invade and produce toxins before harvest, which are often referred to as 'field fungi.' The second category becomes a problem after harvest, hence the name 'storage fungi' (Roberts, 1972, Christensen and Sauer, 1982, Miller, 1995).

Fungi invasion before harvest is governed primarily by plant host–fungus and other biological interactions (e.g. with insects), while growth of fungi postharvest is governed by the nature of seed nutrients, physical (temperature and moisture) and biotic factors (i.e. insects). However, the original source of the fungi in both categories is the field (McLean and Berjak, 1987), although storage fungi will also invade seeds from sources of inoculum in the store. There are four types of toxigenic fungi identifiable (Miller, 1994):

a) Plant pathogens such as *Fusarium graminearum* and other related species,
b) Fungi that grow and produce mycotoxins on/in senescent or stressed plants tissues such as *Fusarium moniliforme* and sometimes *Aspergillus flavus*,
c) Fungi that initially colonize the plant, and are transmitted to the developing seeds; these predispose the seeds to mycotoxin contamination after harvest (e.g. *Aspegillus flavus*); and,
d) Fungi that are found in the soil or associated with decaying plant material, and which occur on the developing seeds in the field and later proliferate in storage if conditions promote this (e.g. *Penicillium verrucosum* and *Aspergillus ochraceus*).

1.6.1. Field fungi

These plant myco-pathogens, consisting of members of the genera *Alternaria, Cladosporium, Culvalaria, Epicoccum, Fusarium* and *Verticillium*, are recognized field fungi that have been reported to invade seeds before harvest or after cutting and swathing, but before the harvest is threshed (Foley, 1962, Christensen and Kaufmann, 1969, Christensen and Kaufmann, 1974, Murray, 1974, Delatour et al., 1980, Mittal and Sharma, 1982, Wicklow and Wilson, 1986, Bacon and Williamson, 1992, Payne, 1992, Wicklow, 1994, Miller, 1995). These fungi require high relative humidities (RH) and seed moisture content (Christensen and Kaufmann, 1969, Christensen and Kaufmann, 1974, Roberts, 1972, Christensen and Sauer, 1982, Calistru et al., 2000, Berjak and Pammenter, 2004a). Spores and mycelia fragments present in and on soil, plant debris, seeds, or standing crops, are transported by wind, rain or insects to initiate infection in the developing host plants, particularly in immature seeds (Agarwal and Sinclair, 1987, Mills, 1989, Miller, 1995, Calistru et al., 2000). Myco-pathogenes of the genus *Fusarium* are a persistent mycotoxin producing fungi often found contaminating tissues from a range of non-orthodox seeds such as *Avicennia marina, Castanospermum australe, Litchi chinensis, Podocarpus henkelii, Landolpia kirkii, Scadoxus membranaceus* and *Camellia sinensis.* In addition to *Fusarium* ssp. for *L. kirkii*, genera of *Alternaria, Cladosporium, Aspergillus* and *Penicillium* are common fungal contaminants (Murray, 1974, Delatour et al., 1980, Mittal and Sharma, 1982, Mycock and Berjak, 1990a, Pongpanich, 1990, Singh and Singh, 1990, Abdelmonem and Rasmy, 1996, Kehr and Schroeder, 1996, Calistru et al., 2000, Sutherland et al., 2003). Growth of some field fungi may continue even under storage environment if the seeds are not adequately dried, but usually when seed moisture content becomes the limiting factor as a result of drying, the activity of the field fungi declines and they are no longer able to proliferate (Christensen and Kaufmann, 1974, Miller, 1995, Calistru et al., 2000, Sutherland et al., 2002). It is only at this stage that the storage fungi will emerge and become established (McLean and Berjak, 1987, Mycock and Berjak, 1992a, Calistru et al., 2000).

1.6.2. Storage fungi

The storage fungal contaminants, are described as saprophytes or opportunistic invaders of dried orthodox seeds and dead organic matter (Dube, 1990, Hudson, 1986, Erdey et al., 1997) or alternatively described as pathogens (Mycock and Berjak, 1990a, Mycock and Berjak, 1992a, Erdey et al., 1997). These are dominated by xerotolerant mycobiota of the genera *Aspergillus* and *Penicillium*. These fungi are metabolically active in seeds stored at 70% RH or higher, under which conditions starchy grains will equilibrate to moisture contents of 13% (wet mass basis) or more (Roberts, 1972, Christensen and Kaufmann, 1969, Christensen and Kaufmann, 1974). *Aspergillus* species have been isolated from seeds of many species, including maize, (Payne, 1992), cotton (Khalid et al., 2001) and different types of nuts (Diener, 1989, Doster and Michilides, 1994, Waliyar et al., 1994). Moreover, the occurrence of these fungal species has also been reported in seed-derived spices (black, white and red pepper) (Ito et al., 1994). Noting that storage fungi have been isolated from developing seeds in the field (McLean and Berjak, 1987), nevertheless much inoculum is introduced during seed processing for storage (Christensen and Kaufmann, 1969, Christensen and Kaufmann, 1974, McLean and Berjak, 1987). Under poor storage conditions (elevated temperature and/or high RH), a small amount of inoculum can proliferate rapidly, spreading from seed to seed leading to significant problems including poor seed quality, lower seedling yield and ultimately, decay of the stored seeds (Agarwal and Sinclair, 1987). Particularly, the significant incidence of *Aspergillus flavus* and *Aspergillus parasiticus* is a cause of major concern in most parts of the world as they are producers of aflatoxins (Groopman et al., 1988, Newberne, 1993).

Fungal spores may also be transported to, and dispersed by pests (e.g. insects and mites) during storage. Insects generally further worsen the physical state of seeds as well as increasing the seed moisture content via the production of metabolic water, to levels that allow fungal to proliferate or allow fungal proliferation (Agarwal and Sinclair, 1987, Williams, 1991b). Moreover, fungal propagules may also gain access to the interior of the seeds via discontinuities in the pericarp such as cracks (Mycock et al., 1988) or the micropyle (Mycock et al., 1988, Mycock and Berjak, 1992a), as well as having been systemically transmitted from the parent plant (Mycock and Berjak, 1992a, Mycock and Berjak, 1992b). Another route

by which fungi gain access to seeds is through the stigma-style continuum during flowering (Marsh and Payne, 1984a).

The progressive deterioration of stored seeds is characterized by a succession of species, starting with *Aspergillus versicolor* and continuing through *Aspergillus ochraceus*, *Aspergillus candidus*, *Aspergillus flavus* and finally *Penicillum* spp. (Christensen and Kaufmann, 1974, McLean and Berjak, 1987). Each member of this cascade characteristically invades and degrades specific seed tissues, but depends on both the moisture content of seeds (Christensen and Kaufmann, 1974, Christensen and Sauer, 1982) and the extracellular enzyme capabilities of each species (Mycock and Berjak, 1992a, Mycock and Berjak, 1992b). As a result, these fungi cause gradual decay which is initially characterized by seed vigour loss and then death (Christensen and Sauer, 1982, Jha, 1995, Eggers et al., 2007).

1.7. The aspects of associated fungi on recalcitrant seeds

Seeds are seldom entirely free of microbial contamination, and the seed-associated fungi may be on the external surface, located internally or both. It has become evident over the years that to categorize seed-associated fungi as either field or storage mycoflora lacks consistency, as research by many scientists have shown the persistence of various field fungi, in particular the *Fusarium* species, during relatively long cold storage of orthodox seeds (Russel and Berjak, 1983, Agarwal and Sinclair, 1987, McLean and Berjak, 1987, Sayer, 1991, Berjak et al., 1992). Thus, in addition to their role as plant pathogens these fungi may also be considered as facultative storage fungi (Mycock and Berjak, 1992a). As these fungi may be systematically transmitted from the germinating seed into the developing plant (Daniels, 1983, Agarwal and Sinclair, 1987, Mycock and Berjak, 1992a, Mycock and Berjak, 1992b), the survival of seed-borne fungi during storage has important implications in control measures against these plant pathogens.

When fungal contamination occurs by systemic transmission, the mycelium is likely to become established deep in the seed tissues, making the eradication process almost impossible (Berjak, 1996). Post-harvest recalcitrant seeds also remain very prone to fungal contamination and proliferation as they

are shed at relatively high water contents, offering an immediately available moisture source and substrates for opportunistic fungi.

There is no doubt that at harvest, recalcitrant and intermediate seeds (i.e. the non-orthodox seed category) harbour a spectrum of fungal species (Sutherland et al., 2002). Species of *Alternaria*, *Cladosporium* and *Fusarium* as well as *Aspergillus* and *Penicillium* have been isolated from the surface and internal seed tissues of seven unrelated recalcitrant species from a variety of provenances in South Africa, ranging from sub-tropical estuaries to warm-temperate montane areas (Mycock and Berjak, 1990a, Berjak and Pammenter, 2014). *Hevea brasiliensis* seeds of Malaysian provenance were found to habour 23 fungal species (Singh and Singh, 1990). The prevalence of fungal infection may well be related to the fact that most of the seeds tested in those studies had tropical/subtropical provenances, thus possibly being more prone to fungal infection. However, it was found that seeds of *Podocarpus henkelii*, an African gymnospermous temperate species, also harboured fungi, as did those of *Camellia sinensis* (Calistru, 2004). In addition, a spectrum of fungi has been found to be associated with seeds of species of cool temperate origin, such as *Quercus robur* (Murray, 1974). The fungus that commonly infects the acorns after shedding, *Sclerotinia batschiana* (Kehr and Schroeder, 1996), was also found in the seeds of *Castanea* species (Delatour et al., 1980).

Sutherland et al. (2002), stated that the spectrum of fungal species could differ with seed provenance, where geographical areas are widely separated. Recalcitrant seeds assessed in South Africa appear to harbour an essential different spectrum of fungi (Mycock and Berjak, 1990b) when compared with those originating in the Asia-Pacific area (Pongapanich, 1990). However, there are some exceptions to this limited distribution: e.g., *Phomopsis* sp. has been isolated from oak seeds (Kehr and Schroeder, 1996), *Trichilia dregeana* (Sutherland et al., 2003) and *Hevea brasiliensis* (Singh and Singh, 1990). Although this fungal genus is common to seeds from all these trees, it appears that the species were different, as *Phytophthora heveae* (not a fungus species) was isolated only from seeds of rubber trees, *Hevea brasiliensis*, while *Phytophthora azadirachtae* were found to have contaminated only neem seeds, whereas fungi such as *Cladosporium*, *Mucor*, *Phoma*, *Phomopsis*, *Alternaria* and *Botryodiplodia* were

most frequent contaminants of recalcitrant seeds from a wide range of provenances (Sutherland et al., 2003).

Fusarium species have been isolated from a variety of recalcitrant seeds, appearing to become the dominant fungal contaminant during storage (Mycock and Berjak, 1990b, Calistru et al., 2000). It appears that few representatives of species of *Aspergillus* and *Penicillium* are associated with recalcitrant seeds (Mittal and Sharma, 1982, Pongapanich, 1990, Singh and Singh, 1990), none of which was categorized as a storage fungus (Sutherland et al., 2002).

The apparent absence of the storage fungal species from the fungal spectrum of desiccation sensitive seeds is not surprising. The xerotolerant species of *Aspergillus* and *Penicillium* predominate in air-dried orthodox seeds during storage, when the low seed water activity and the osmotic conditions became unfavourable to the field fungi. However, in intermediate seeds that are hydrated to relatively low water contents, xerotolerant fungi could become active if their propagules are present intra-seminally or in the storage containers. In contrast, recalcitrant seeds offer ideal conditions and substrates for field fungi, such as *Fusarium*, as these pathogens need high seed water contents for ongoing activity. All desiccation sensitive seeds tested to date have been shown to habour a variety of fungal species as well as bacteria, yeasts and viruses, and fungal inoculum was found in fresh seeds even when newly hand harvested (Mycock and Berjak, 1990a, Calistru et al., 2000).

1.8. Impact of secondary metabolites produced by fungi on seeds

In addition to degradation of stored reserves of seeds, their quality is affected by secondary fungal metabolites which are collectively referred to as mycotoxins. Mycotoxins are produced as secondary metabolites by seed-associated mycobiota. Production of these toxins, which are frequently specific to particular fungal genera, is influenced by seed moisture, mechanical damage, temperature, nutrients and duration of storage (Bennett and Keller, 1997). Knowledge about mycotoxins dates back to 1100 AD (Gupta and Sharma, 1995), where the first report on human mycotoxicoses was reported. These toxins, particularly, those produced by species of *Fusarium* and *Aspergillus*, have been associated with a wide

variety of human and animal stock pathologies, including carcinomas (Erdey et al., 1997). Exposure to mycotoxins can occur directly or indirectly via contact and consumption of contaminated seeds or their products (Figure 1). The nature, type, distribution, toxicology and ecology of toxins produced by *Fusarium* (Joffe, 1986, Mills, 1989, Marasas, 1991, Shotwell, 1991, Norred, 1993, Erdey et al., 1997, IARC, 2000, Abdel-Wahhab and Kholif, 2008) and *Aspergillus* (Abramson, 1991, Shotwell, 1991, Payne, 1992, Luchese and Harrigan, 1993, Cotty et al., 1994, Ramos and Hernandez, 1996, Abbel-Wahhab and Kholif, 2008) species have been extensively reviewed. In prolonging shelf life of stored recalcitrant seeds it is necessary to eliminate or minimize the incidence of fungal contaminants (Calistru et al., 2000). This would also prevent production of mycotoxins which may be phytotoxic (Sinha and Kumari, 1989, Van Asch et al., 1992, MacLean et al., 1992a, Lamprecht et al., 1994).

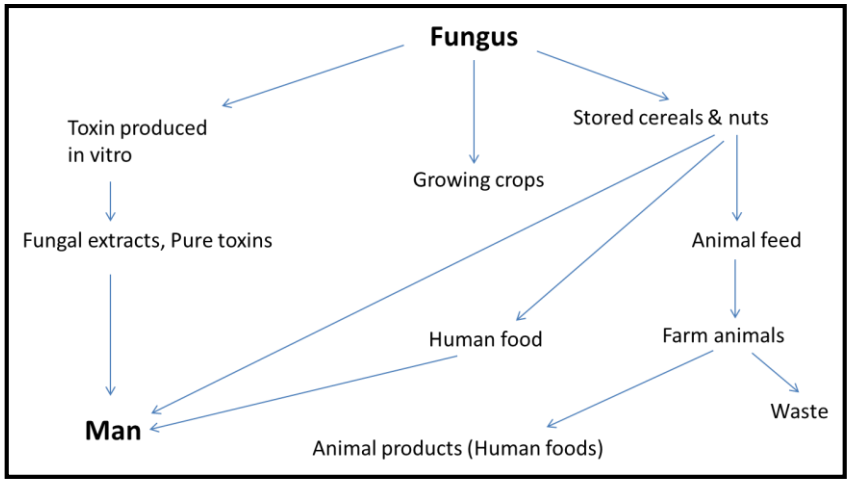

Figure 1: Exposure of human and animals to mycotoxins (Abdel-Wahhab and Kholif, 2008).

1.9. Treatment methods to eradicate contaminants

The prevention of pathogenic seed-borne inoculum is a task that is almost impossible to achieve, more especially if the inoculum is deeper-seated within the internal structures of the seed i.e. embryonic axes/embryos (Calistru et al., 2000, Sutherland et al., 2002, Berjak and Pammenter, 2014, Berjak et al., 2014). Control of these micro-organisms maybe attained through integrated disease management programmes, usually involving both methods of prevention of seed infection in the field and the eradication of pathogens already within the seeds (Agarwal and Sinclair, 1987, Erdey et al., 1997, Calistru

et al., 2000, Sutherland et al., 2002, Abdel-Wahhab and Kholif, 2008, Berjak et al., 2014). Preventative measures in the field involve the selection of seed production areas where the pathogens of major concern are unable to establish or maintain themselves at critical levels during the period of seed development, and use of foliar fungicidal sprays, amongst others (Agarwal and Sinclair, 1987, Sutherland et al., 2002, Abdel-Wahhab and Kholif, 2008). While these practices are viable at the commercial level, the costs involved in disease management in the field are considerable. In contrast, eradicative seed treatments are usually easy to apply, have minimal or no effect on the environment, may involve only small monetary outlay, if at all, and may give virtual freedom from disease in crops (Maude, 1983, Agarwal and Sinclair, 1987). Such treatments are most useful where the seed is the main or only repository of disease (Maude, 1983). The types of eradicative seed treatments are shown in Figure 2 and include biological, chemical, mechanical and physical methods.

1.9.1. Biological control methods of plant pathogens

Biological control is the deliberate use of one or more non-pathogenic antagonists that regulate/inhibit or reduce the population of seed-borne pathogens without adversely affecting the viability status or quality of the plant material; this approach typically involves an active human role (Cook, 1985, Agarwal and Sinclair, 1987, Tuite, 1988, Emmert and Handelsman, 1999, Pal and Gardner, 2006), an approach first described by Smith (1919). The approach was used in the entomology and plant pathology fields, where live killer insects, entomopathogenic nematodes or microbial pathogens were used to control different pathogenic insects. The microbial inocula that inhibit or control plant pathogens are called biological control agents (BCA), and are usually referred to as antagonists since their mode of action is based on competing with a disease causing agent/microbe.

A wide variety of biological agents are used in controlling pathogenic organism in plants. These include, bacteria, fungi, nematodes, yeasts, plant-based extracts and protozoan cultures as live attenuated agents, or their cellular components or secondary metabolites. Examples include *Calotropis gigantea* leaf extracts, *Arbuscular mycorrhuzae*, *Azotobacter* spp., *Bacillus* spp., *Pseudomonas fluorescenes*, *Pasteuria penetrans*, *Trichoderma* spp., *Pichia pastoris*, *Rhizobacteria*, non-pathogenic strains of *Fusarium* spp.

(Duijff et al., 1998, Benhamou et al., 2002, Viji et al., 2003, Compant et al., 2005a) and cultures of *Penicillium oxalicum* Corrie and Thom, *Penicillium decumbens* Thom and *Trichoderma harzianum* (Santamarina et al., 2002). These bio-controls may be used in isolation or as a cocktail with low impact chemicals to obtain appropriate control of pathogens (Monte, 2001). Strains of *Trichoderma* as used by Calistru et al. (1995) were found to have significant effect against orthodox-seed-associated fungal contaminants in maize. Those investigations later prompted application of non-pathogenic fungal antagonists in recalcitrant seed storage as demonstrated by Finch-Savage et al. (2003), where the use of a broad spectrum *Trichoderma virens* as a bio-control agent, resulted in protection against fungal infection and in reduced fungal proliferation during storage of *Quercus robur* seeds.

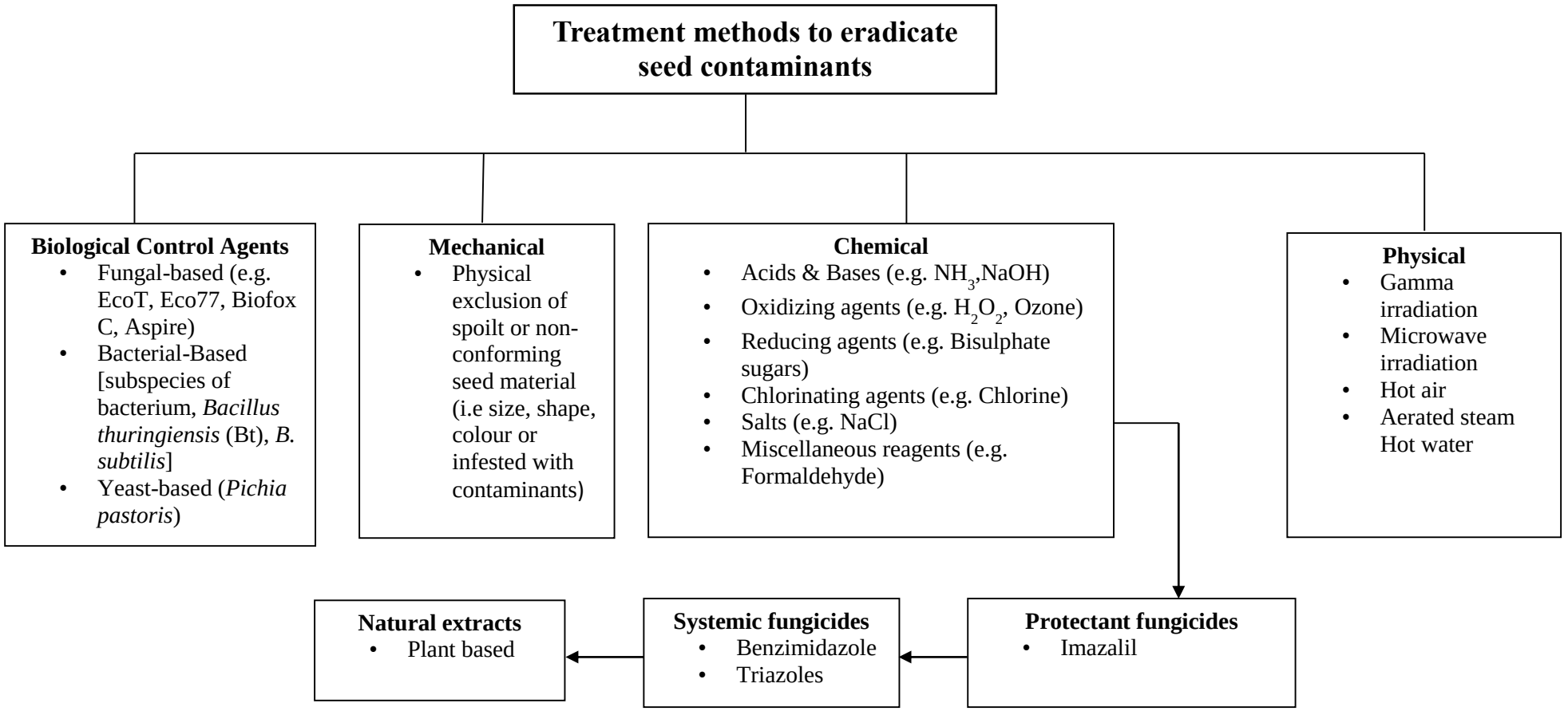

Figure 2: Brief summary of some treatment methods to eradicate seed contaminants (Agarwal and Sinclair, 1987, Erdey et al., 1997, Butt and Copping, 2000, Calistru et al., 2000, Sutherland et al., 2002, Abdel-Wahhab and Kholif, 2008, Viji et al., 2013, Berjak and Pammenter, 2014, Berjak et al., 2014).

1.9.1.1. Biological control method using *Trichoderma harzianum*

The genus *Trichoderma*, (Ascomycetes) comprising filamentous fungi have been known since the first application as a bio-control agent in the 1930s (Ha, 2010). *Trichoderma* species occur as teleomorphs (sexual stage) and anamorphs (asexual stage) belonging to the family Hypocrea and class Sordariomycetes (Dube, 1990, Harman et al., 2012). The genus occurs widely in nature as a dominant component of the microflora in all types of soils with a pH ranging from 2.5 to 9.5, but preferably from slightly to moderate acidic environments (Hagedorn, 2004, Shakeri and Foster, 2007). These fungi are saprophytic in nature and are known for their ability to colonize and improve the balance of the soil microflora and as well as colonizing the roots of plants (Harman et al., 2004b, Shakeri and Foster, 2007). Most strains of *Trichoderma* have bio-control properties manifested by competing with soil phytopathogenic fungi and production of toxins (Schirmbock et al., 1994). Commercial production of *T. harzianum* is by fermentation as well as by cultures grown on solid substrate (Schirmbock et al., 1994). Colonies grown on potato dextrose agar (PDA) become green due to the formation of conidia (Figure 3, A and B).

The mycelium of *Trichoderma* spp. growing on PDA has a woolly appearance (see Figure 3, A and B). Maturity occurs within 5 to 7 days but the timing entirely depends on type of medium used for culture (De Hoog et al., 2000). Morphological characteristics tend to differ with different species of *Trichoderma*. These differences can be noted visually by inspecting mycelium growing on medium, growth rate in culture and pigmentation (varies from dull yellow to dark green, or even reddish – but some produce no colour). Arrangement of conidiophores and shape of conidia (Figure 4) can also be easily distinguished by using a compound microscope. The shape of conidia ranges from globose to ellipsoidal, obvoidal or short cylindrical with different size of conidia up to 3 µm in diameter. The conidia are smooth to rough-walled and are located at the tip of flask-shaped phialides (Dube, 1990, Sutton et al., 1998, Harman and Kubicek, 1998, Shakeri and Foster, 2007). The attachment of conidia at the tips of phialides is often disrupted while preparing for microscopical observations and therefore most usually the conidia are viewed as scattered shapes on a prepared microscopic slide (Sutton et al., 1998, Harman and Kubicek, 1998).

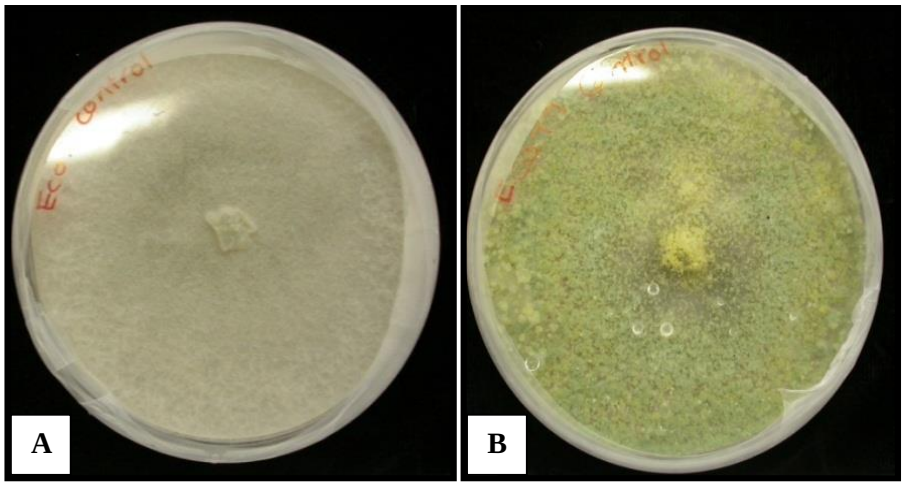

Figure 3: A - *T. harzianum* EcoT strain and B - *T. harzianum* Eco77 strain growth on potato dextrose agar (PDA). B - also shows a green pigment due to the formation of conidia

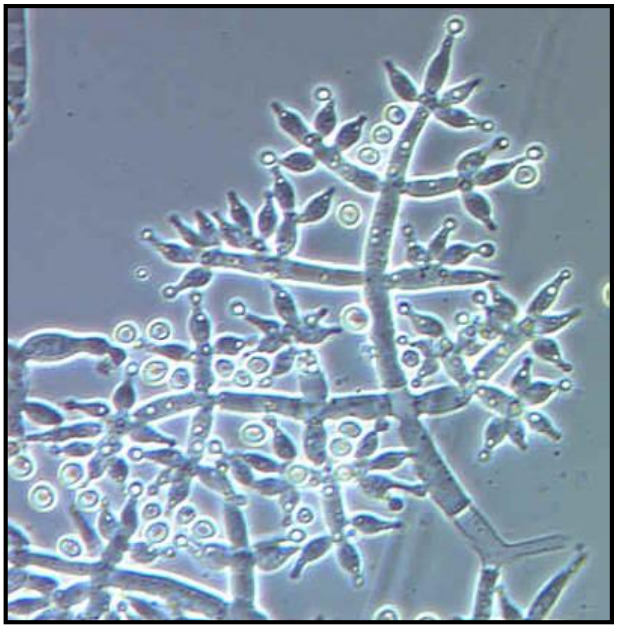

Figure 4: Microscopic appearance of part of mycellium of *T. harzianum* with conidia at the tip of phialides from each hyphal branch (Source: http://www.ars.usda.gov, accessed, 06 August 2014).

Trichoderma spp. have over 70 years history as successful bio-control agents (Harman et al., 2008). This genus tends to be antagonistic to other fungi and is resistant to many chemical fungicides (Harman et al., 2004*b*). It has been suggested that growth of plant pathogens is inhibited by five mechanisms viz. (a) mycoparasitism (the antagonist uses all or some of the nutrients from the host [(Chet et al., 1981)], (b) competition (competes for space and nutrients [(Elad, 1996)], (c) antibiosis (the production of inhibitory secondary metabolites [(Tronsmo, 1996)], (d) Solubilization and sequestration of inorganic plant nutrients (reducing soil pH by releasing organic acids which promote solubility of phosphates, thus making them readily available to plants) [(Vinale et al., 2008*b*)] and lastly (e) induction of plant resistance mechanisms (induces the plant to respond to attack by pathogens [Kuc, 2001, Oostendorp et al., 2001, Pal and Gardner, 2006]). Studies by (Elad, 1996) demonstrated that *Trichoderma harzianum* as a bio-control agent for *Rhizoctonia solani* was capable of mycelium lysis and concluded that it also improved plant growth compared with chemical treatments, while investigations by Yedidia et al., (1999) showed that treated plants were more developed than untreated plants.

1.9.1.2. *Bacillus subtilis* as a bio-control agent

Originally named *Vibrio subtilis* in 1835, it was renamed as *Bacillus subtilis* in 1872 (Perez et al., 2000). It belongs to the genus *Bacillus* and is a Gram-positive, catalase-positive bacterium commonly found in soil and vegetation (Figures 5 and 6). It is rod-shaped, and has the ability to form a tough, protective endospore, allowing the organism to endure extreme environmental conditions (Perez et al., 2000, Schaechter et al., 2006).

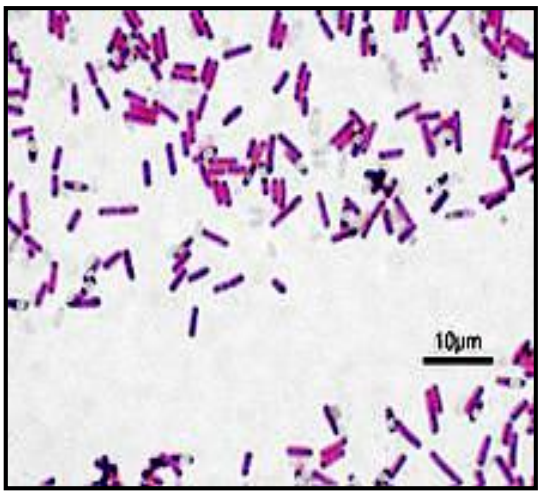

Figure 5: Microscopic appearance of Gram-positive rods of *Bacillus subtilis* (Source: Http://en.citizendium.org, accessed, 13 August 2014).

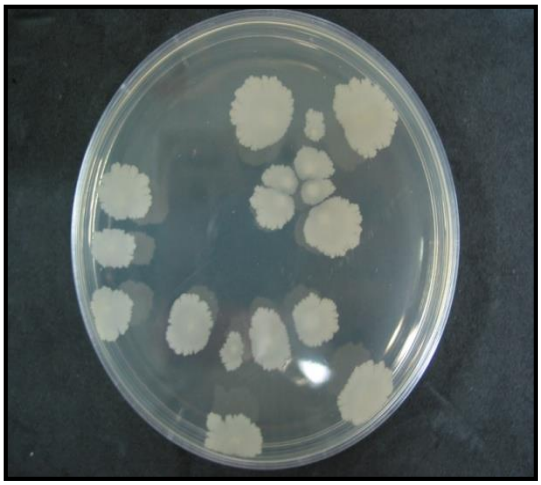

Figure 6: Colonies of *Bacillus subtilis* growing on a nutrient agar (NA).

Bacillus strains produce a broad spectrum of bioactive peptides. One of these, the lipopeptide surfactins, is a well-known class of compounds. Many strains of *B. subtilis* produce cyclic lipopeptides belonging to the family iturin. There are many other compounds in this family that are powerful antifungal agents such as bacillomycin L, bacillomycin D, bacillomycin F and mycosubtilins (Siddiqui, 2006). According to that author the surfactans produced by *B. subtilis* show surfactant activity and antagonistic activity against *Rhizoctonia solani.* In previous studies *Bacillus subtilis* displayed *in vitro* antagonism against a wide

range of phytopathogenic fungi, as a consequence of the production of antifungal metabolites (Grover et al., 2009).

Bacillus subtilis contains antifungal peptidolipids and antifungal phosphono-oligopeptides, e.g. rhizocticin A (Besson et al., 2006, Gong et al., 2006, Kugler et al., 1989). Another lipopeptide called fengycin is known to have antifungal activity against filamentous fungi (Deleu et al., 2008, Siddiqui, 2006). It has also shown antifungal activity against the growth of *Fusarium oxysporum* and the antibiotics, iturin A and bacillomycin L have been shown to inhibit growth of *Saccharomyces cerevisiae* (Besson et al., 2006).

When using bacteria in conjunction with fungal test isolates, the antifungal substances from bacteria will have to be isolated before testing for their action on the fungus, as the bacteria and the fungi require different conditions for growth. Also, these secondary metabolites would have to be isolated to determine their optimal antifungal activities. In previous studies the agar well diffusion method was used to test antifungal activity of *Bacillus subtilis* against test fungi *Microsporum fulvum* and *Trichophyton* species (Kumar et al., 2008). The effects of different broth media on the growth and production of antifungal substances by the bacteria were determined on nutrient broth, sucrose broth, trypticase dextrose broth and trypticase soya broth. Maximum antibiotic production was observed with trypticase soya broth (TSB). Also, growth was optimum under shaking conditions at 48 h as compared to stationary conditions. After centrifugation the extracts / secondary metabolites from the supernatant were then used to inoculate the wells (Kumar et al., 2008).

In another study, application of *B. subtilis* spore suspension on yam surface completely replaced the postharvest rot-causing fungi, such as *Botryodiplodia theobromae, Fusarium moniliforme* and *Penicillium sclerotigenum* (Swain and Ray, 2009).

1.9.1.3. *Pichia pastoris* as a bio-control agent

Amongst soil microorganisms, yeasts have drawn less attention as bio-control agents of fungal plant pathogens compared with bacterial and filamentous fungal antagonists. The ability of yeasts to induce resistance of host tissues offers good potential for their use as bio-control agents. Many genera of yeasts have been used to control post-harvest diseases, mainly of fruits. Inhibition of fungal pathogens associated with fruits that are similar to those associated with soil-borne fungal root pathogens, strongly suggests that yeasts also have potential for biological control (Khaled and Sivasithmparam, 2005). Numerous species within the genus *Pichia* have strong antifungal properties mediated through the production of lytic enzymes, toxic fatty acids, toxic proteins, and ethyl acetate (Fleet, 2007). Commercial preparations of some species are available for the pre- and post-harvest control of fruit, grain and vegetable spoilage fungi (Fleet, 2007). The yeast strain, *Pichia anomala* DBVPG 3003, secretes a killer toxin that has antifungal activity. In previous studies *Pichia anomala* was shown to inhibit growth of *Penicillium roqueforti* in high-moisture winter wheat, barley, and oats (Peterson and Schnurer, 1998).

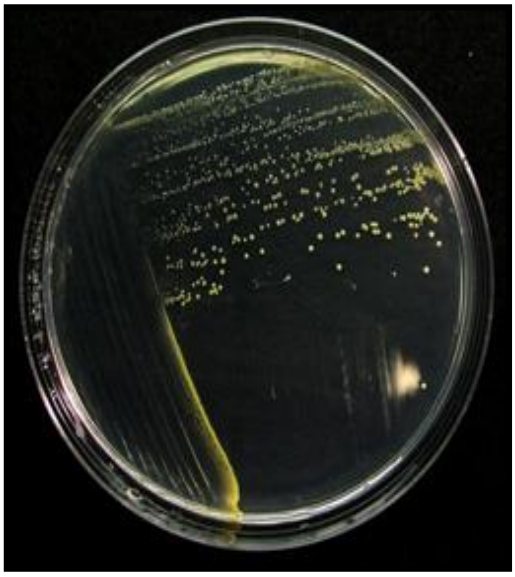

Figure 7: Colonies of *Pichia pastoris* growing on Yeast peptone dextrose agar (YPDA)

1.9.2. Chemical control methods and their applicability on plant material

Application of synthetic chemicals on seeds is reported to be a highly effective method for controlling seed-associated mycoflora, relatively economical and user friendly (Agarwal and Sinclair, 1987). These chemicals include broad-spectrum protectant fungicides, such as captan and thiram, and systemic fungicides, such as acibenzolar-S-methyl, benomyl, carbendazim, imazalil and thiabendazole. However, efficacy reports on these protectant fungicides as treatments against seed-borne pathogens are variable, being reported as ineffectual in some cases (Halfon-Meiri and Solel, 1989), and only partially effective in others (Kannaiyan, 1992). Their activity appears to be limited to seed surfaces and the tissues of the seed coat (Agarwal and Sinclair, 1987) which may account for the inability of these chemicals to eradicate seed-borne pathogens completely.

Systemic fungicides, on the other hand (exemplified by Celest, Heritage and Orius), have been reported to be effective in reducing or eliminating seed-borne inoculum, even when applied to seed surfaces as a spray, slurry or dust (Moreno-Martinez et al., 1994). However, these can be phytotoxic (Kelly, 1993, Petit et al., 2008, EFSA, 2009) and their usage is limited by their selectivity for specific fungi (Agarwal and Sinclair, 1987). Furthermore, the current trends in research are guided by a number of standard practice bodies that encourage minimization or even elimination of chemicals from the environment (e.g. ISO 14001:2004) and this opinion is increasingly recognized worldwide.

Selecting an ideal fungicide must mainly be guided by a plant material response towards that type of fungicide and, most importantly, the level of impact the fungicide will have on the environment on either or both a short-term or long-term basis. With relevance to the seed and farmer (or the user) such factors include: economics (i.e. how costly is the fungicide), how stable it is, user friendliness, wide means of application (e.g. either applied as a spray or as a coating or both) and last but not least, it should be registered and endorsed by relevant laws world-wide (Copeland and McDonald, 2001).

1.9.2.1 Fungicides

Fungicides treat or control diseases caused by fungal pathogens, especially seed-associated fungal contaminants, while insecticides control insect pests (Zaman, 1995, Gullino et al., 2000, Vincelli and Williams, 2011). Fungicides play an important role in the agricultural industry as they ensure that seeds and seedlings are free from soil-borne fungal pathogens that usually cause seed rots, damping-off, seedling blights and root rots. In addition, they control fungal pathogens that tend to infest surfaces of plant material (e.g. leaves), causing smuts of barley and oats. Moreover, certain fungicides inhibit fungal pathogens that are located in deeper tissues of the seed material (McMullen and Lamey, 2000), thus ensuring better yields at the end of harvest season. Fungicidal seed treatments are therefore important for controlling soil-borne fungal pathogens, surface-borne pathogens on the seeds and internally seed-borne fungal pathogens (Figure 8).

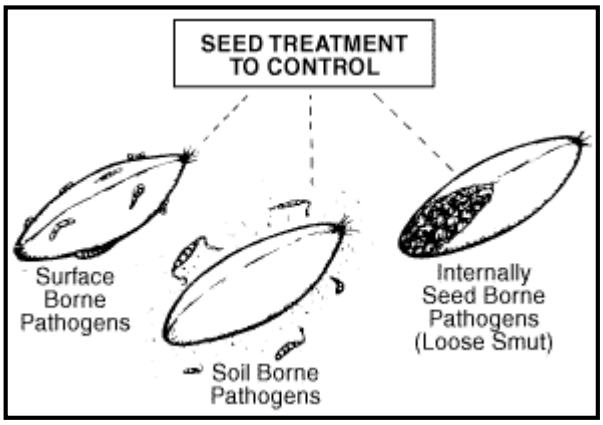

Figure 8: Reasons for seed treatment (McMullen and Lamey, 2000).

1.9.2.2 Classification of fungicides

Fungicides can be sourced from chemicals or biological materials and are divided into two main types, viz. contact and systemic (Vincelli and Williams, 2011). Contact fungicides are also known as protectant fungicides or non-systemic fungicides, and are categorized by remaining on the plant or seed surface after application. This type of fungicide is applied before the host tissues are invaded by a fungal contaminant and their mode of action against the contaminant is by killing or inhibition (Yuste and Gostinear, 1999).

However, application of contact fungicides should be complemented by the use of systemic fungicides, which penetrate into the tissues to ensure eradication of deeper seated inoculum within the seed, as is the case with recalcitrant seeds (Berjak and Pammenter, 2004b, Berjak et al., 2014). Systemic fungicides are further categorized depending on their depth of penetration within the plant or seed:

(a) **Localized penetrants** - form a protective barrier on the plant surfaces and will permeate leaves in the localised areas of application. These fungicides have some curative activity, but do not move upward or downward in the plant.

(b) **Acropetal penetrants** - form a protective barrier on the plant, permeate into the plant, and move upward in the xylem. These fungicides have protective and good curative activity.

(c) **Systemic penetrants** - form a protective barrier on the plant, permeate into the plant, move upwards in the xylem, and downwards in the phloem. These fungicides have protective activity and good curative activity.

An additional advantage of systematic fungicides is that they can suppress the fungus after it has infected the plant, whereas contact fungicides need to be present on the surface before infection for them to be effective. However, systemic fungicide mode of action may be specific for the disease causing agent (plant mycopathogen) which has been associated with the production of resistant fungal strains and therefore their usage and efficacy need to be monitored appropriately (Dias, 2012, Berjak et al., 2014).

Systemic fungicide application prolongs storage longevity of recalcitrant seeds by minimizing microbial contaminants, especially fungi (Kelly, 1993, Petit et al., 2008, EFSA, 2009). Both contact and systemic antifungal treatments are used extensively in our laboratory to ensure that recalcitrant seeds to be used for further experimentation are as free of contaminants as possible, or that, if inoculum persists, it will not tend to proliferate in hydrated storage (HS) or under *in vitro* conditions. However, their application is carefully considered and monitored as we are aware of possible phytotoxic effects on resultant seedlings (Kelly, 1993, Petit et al., 2008, EFSA, 2009). Adverse effects of the application of systemic fungicides include compromising GA_3 synthesis in wheat seedlings (Gao et al., 1988), interference with photosynthesis, as shown by Saladin et al. (2003) for *Vitis vinifera* and seed and seedling mortality, as

reported for onion by Fullerton et al. (1995). A summary of examples of contact and systemic fungicides is listed in Table 1.

The UKZN–Westville plant germplasm laboratory has adopted a standard operating procedure,for all post-harvest seeds to be used either for storage or to yield explants for *in vitro* purposes (Berjak and Pammenter, 2004b, Cheruiyot et al., 2007, Berjak et al., 2014). This procedure involves usage of contact based decontaminants (Sutherland et al., 2002). For example, 1-1.5% m/v solutions of sodium hypochlorites (NaOCl) and calcium hypochloride ($Ca(OCl)_2$); sodium dichloroisocyanurate (NaDCC) as a 0.5 or even 0.3% m/v solution; and 0.1% m/v mercuric chloride ($HgCl_2$). However, if the contaminant is located below the testa, with no evidence of penetrating into the underlying tissues, a similar approach is used following removal of the seed coverings, as long as such treatments have no detrimental effects on deeper tissues of the seed. To obviate phytotoxic effects (Schmidt, 2000, Berjak et al., 2014), duration of exposure and concentrations of decontaminants to be used on seed or any explant-type need to be investigated and verified in advance of large-scale application. To date, chemical control by fungicides still remains the best means of treating seeds, soil, foliar and post- harvest material. Benzimidazole (used for almost all our post-harvest seeds in storage) and other fungicides are briefly discussed below:

1.9.2.2(a) Benzimidazole

Benzimidazole fungicides were introduced for plant disease control in the 1960s. They were later used as foliar fungicides in the early 1970s, to treat seeds and for use in post-harvest applications. They gained popularity because of unique properties not seen before with the other protectants. These included low use rates, broad spectrum and systemic activities with post-infection action that allowed for extended spray interval. All these qualities made them very popular, however, but led to misuse such as poor spray coverage and curative spraying by farmers (Smith, 1988). The first case of resistance to benzimidazoles was shown by powdery mildew in greenhouses in 1969. By 1984, resistance had been reported by many of the pathogens against which benzimidazoles should to be active (Smith, 1988, Morton and Staub, 2008). The reason for the rapid development of resistance was that these fungicides are single site inhibitors of fungal microtubule assembly during mitosis, via tubulin-benzimidazole-interactions. The primary patent holders of this class were DuPont (Benlate), Merck, Sharp & Dohme (Mertec) and Nippon

Soda (Topsin M). The current ranking of global sales is benomyl, carbendazim, thiophanate and thiabendazole (Morton and Staub, 2008). Among this class of systemic fungicides, benomyl is the most used and effective chemical against a wide range of plant diseases, and in crop protection (García et al., 2003). It also acts as an effective contact fungicide for stored recalcitrant seeds such as those of *T. dregeana* [dusting of the seed surfaces (Kioko, 2003)] as well as by coating seeds using a suspension: e.g. a 0.3% suspension of benomyl was highly effective in increasing the storage period of *Hevea brasiliensis* (Chin, 1988).

1.9.2.2(b) Morpholine

These types of fungicides are best known for their excellent control of cereal diseases, powdery mildew on vegetables, grapes and leaf-spot disease (called sigatoka) of banana. During the 1980s fenpropidin and fenpropimorph were key fungicides in the European cereal market, while tridemorph was used extensively for sigatoka. This class of chemical, although having seen shifts in sensitivity by some pathogens (sigatoka in Central and South America), is still in use (Morton and Staub, 2008). Key patents were held by BASF (Calixin and Corbel) and Dr. R. Maag (Corbel and Tern). Dimethomorph, though a morpholine, is quite distinct from the morpholines mentioned above with its activity being against Oomycetes via the inhibition of cell wall formation (Morton and Staub, 2008). The current ranking of global sales is: dimethomorph, fenpropidin, fenpropimorph, sprioxamine. Morpholine fungicides belong to a broad group of fungicides that is often referred to as sterol biosynthesis inhibitors (SBI). Other SBIs include the next four groups of fungicides (Morton and Staub, 2008):

(i) ***Piperazines***: the major player in this group was triforine, which was used extensively as a home and garden product (especially on roses). Key to the acceptance of triforine was its efficacy and safety to a wide range of plants. The key producer was CelaMerck (Saprol).

(ii) ***Imidazoles:*** include a small number of compounds in this class that are active against plant pathogens. The most important are imazalil (Janssen Pharmaceutica) and prochloraz (Boots Company Limited). The primary uses for imazalil were as a seed treatment and post-harvest

treatment, while prochloraz (trade name: Sportak) was used on cereals, being especially active on *Pseudocercosporella* eyespot.

(iii) ***Pyrimidines*** are a class of fungicides that were extensively explored by Eli Lilly giving rise to nuarimol, fenarimol and triadimenol. The major player of these was fenarimol (Rubigan®) on pome fruit, grapes and turf.

(iv) ***Triazoles*** are the largest class of fungicides. Bayer was the first to launch a triazole, namely triadimefon (Bayleton) in 1973. This was soon followed by triadimenol (Baytan) and bitertanol (Baycor). Janssen Pharmaceuticals sold the agricultural use rights to Ciba-Geigy for propiconazole (Tilt) which was launched in 1979. Numerous other triazoles have been launched since, with Bayer's most recent entrée being prothiaconazole (Proline) in 2004. The reason for the longevity of this class of fungicides is that while being highly efficient broad spectrum products, resistance has occurred over time as a slow shift resulting in a decreased sensitivity to their mode of action as de-methylation inhibitors (DMI). The newer triazoles, being intrinsically more active, push the sensitivity curves back to their original ED 50 values. The current ranking of global sales is: tebuconazole, epoxiconazole, propiconazole, difenoconazole, flusilazole, tetraconazole, fluquinconazole, flutriafol (Smith, 1988, Morton and Staub, 2008).

1.9.2.2(c) Anilides

Anilides are a diverse group of fungicides. The earliest introduction was anilazine (Dyrene), primarily as a leaf spot fungicide from Bayer and Nissan, followed by the seed treatment carboxin (Vitavax), which is highly effective on bunts, smuts and assorted Basidiomycetes such as *Rhizoctonia* spp. This was followed by the dicarboximides, iprodione (Rovral) from Rhone-Poulenc, vinclozolin (Ronilan) from BASF and procymidone (Sumisclex) from Sumitomo. These fungicides all had exceptional protectant activity against the genera *Botrytis, Monilinia* and *Sclerotinia.* Combating resistance became an issue with the wide-scale use of these fungicides.

The best of this group of anilides were the phenylamide fungicides metalaxyl (Apron/ Ridomil) from Ciba–Geigy and benalaxyl (Galben) from Isagro. These, along with phosphonate fosetyl-Al (Aliette) from Rhone-Poulenc, which was also introduced in 1977, brought a completely new level of control of the Oomycetes through their systemic properties by offering protection to the plants as seed treatments, and soil or foliar applications. Oxadixyl (Sandofan) from Sandoz was a later member of the phenylamides. The limited use of the phenylamide fungicides has once again been the development of resistance, even though the manufacturers tried introducing combinations with protectant fungicides such as mancozeb and chlorothalonil. The latest anilide to be registered in 2003 was boscalid (Emerald, Endura and Pristine) from BASF. Boscalid is registered for foliar use on a wide range of vegetables, fruits and nut crops, either alone or in a mixture with pyraclostrobin as Pristine (Smith, 1988, Morton and Staub, 2008).

1.9.2.2(d) Strobilurins

Strobilurins were launched in 1996. They are the second largest chemical group of fungicides and are widely used on cereals and, more recently, on soybeans. Companies have recently also promoted the plant health attributes of this group of fungicides on soybeans and corn. The strobilurin fungicides have a broad spectrum, are highly efficacious, and are suitable for a wide range of crops. Some resistance against microbes has led to companies adjusting the recommendations to be used by developing mixtures and other uses, including seed treatments (Smith, 1988, Morton and Staub, 2008).

Table 1: List of some fungicide chemical classes and their mode of action (Dicklow, 2006, Vincelli and Powell, 2007, Jung et al., 2010)

Class	Mode of action:	Chemical common name	Trade names	Pathogen controlled .
Acylalanine	**C**	Mefenoxam	Quell, Subdue Maxx	*Pythium, Phytophthora* and downy mildews (WM)
Aniline	**AP**	Boscalid	Emerald 70EG (WDG)	Broad spectrum
Aromatichydro-carbon	**C**	Etridiazol (ethazole)	Koban 30WP, Terrazole 35WP	Broad spectrum
Aromatic hydro-carbon	**C**	PCNB	Andersons FFII 15.4G, Defend 4F, Engage 75W, Fluid Fungicide II, Parflo 4F	Broad spectrum
Benzamide	**C**	Flutolanil	Contrast	**Basidiomycetes:** rusts, smuts, *Rhizoctonia, Sclerotium rolfsii.*
Benzimidazole	**AP**	Thiophanate-methyl	Cleary's 3336, Domain, Fungo Flo, Banrot (mixture with etridiazole), Zyban (mixture with mancozeb)	Broad spectrum systemic but **excludes**WM.
Benzonitrile	**C**	Chlorothalonil	Daconil Ultrex, Concorde ConSyst (mixed with thriophanate-menthyl) Spectro90 (mixed with thiophanate-methyl	Broad spectrum kills spores on surface.
Carbamate	**C**	Propamocarb	Banol	WM
Carbamate	**C**	Maneb	Maneb Plus Zinc F4, Maneb 75 DF, Pentathlon 4F (75 DG)	WM
Carboximide	**AP**	Flutolanil	Prostar 70WP, Moncut 70-DF	Broad spectrum
Copper, complexes	**AP**	Copper hydroxide	Kocide, Champ, Junction (mixed with mancozeb)	Broad spectrum including some bacteria, residues & phytotoxicity may be a problem
Dicarboximide	**LP**	Iprodione vinclozolin	Chipco26019 Ornalin, Volan	**Not** for WM. Broad spectrum, esp. *Rhizoctonia, Botrytis.*
Dithiocarbamate	**C**	Mancozeb manganese+zinc	Dithane, Fore, Manzate, Stature (mixed with dimethomorph) Cleary's ProtectTO	Broad spectrum protectant kills spores on surfaces.
Dicarboximide	**LP**	Vinclozolin	Curan 4F, LescoTouche EG, Vorlan 500F	Broad spectrum
Demethylation Inhibitor	**AP**	Propiconazole	ArmorTECH PPZ 143, Banner GL 3.6WSP	Broad spectrum
Demethylation Inhibitor	**AP**	Tebuconazole	Torque	Broad spectrum
Imidazole	**AP**	Triflumizole	Terragard	Broad spectrum systemic but not WM
Phenylamide	**AP**	Mefenoxam	Subdue, Ridomil, Quell, Mefanoxam	Broad spectrum
Phosphate	**SP**	Phosphite (salts)	Apron (seed treatment), Alude 5.2F, Biophos, Fosphite SP	Broad spectrum
Strobilurin	**AP**	Fluoxastrobin	Disarm 480 SC	Broad spectrum
Strobilurin	**LP**	Trifloxystrobin	Compass 50WDG	Broad spectrum

Notes: "**Water moulds (WM)**" are *Pythium, Phytophthora* and downy mildews.

"Broad spectrum" means the fungicide controls most groups of fungi; exceptions are noted.

Acropetal Penetrant (AC); Contact (C); Localized Penetrant (LP) and Systematic Penetrant (SP)

1.9.2.2(e) Preservatives applied as fungicides/fungistats

A preservative is any agent, natural or synthetic, with the capability of inhibiting the proliferation of microorganisms either by preventing their growth or by completely killing the microbes (Todar, 2001, Baumann et al., 2005). The former are known as static agents, due to their action of hindering microbial growth. The latter are known as cidal agents as they bring about the death of the organism. The act of preservation does not only involve the use of chemicals to prevent microbial invasion which subsequently ends in the degradation of the seed or plant material quality. It also includes the use of factors extrinsic to the seed formulation such as the use of low temperature, which possibly inactivates microbial enzymes which may be present in the seed that may lead to the degradation of its quality. Therefore preservation can be termed as any act which intends to increase the shelf life by preventing degradation of quality (Baumann et al., 2005).

In consideration of the vast number of recalcitrant seeds requiring extended post-harvest storage, the characteristics of an ideal antimicrobial to meet the needs of these seeds representative of many species is equally vast, making it virtually impossible for a single antimicrobial agent to satisfy these requirements (Gardiner, 2008). Therefore to generalize the characteristics of an ideal antimicrobial agent, a few properties have been narrowed down to accommodate the needs of various plant materials (e.g. fruits, seeds, explants):

(i) **A broad spectrum of activity** thus making most if not all types, species, genes and strains of microorganisms susceptible to the antimicrobial.

(ii) **Efficacy in antimicrobial activity** is essential to ensure that the least amount is effective. This is because high concentrations risk irritation and toxicity to the consumer and may also affect the consistency of the product (Gardiner, 2008).

(iii) **Stability of antimicrobial**. This refers to heat labile compounds and their stability in respect to storage and temperatures with which the product may come into contact. This also includes the pH sensitive compounds (refer to Table 2) and the stability thereof (Willey et al., 2008).

(iv) **Solubility**. The antimicrobial needs to be in homogenous solution with the sample being treated to ensure good distribution for effective action.

(v) **Compatibility**. The interactions between the preservative and the sample being treated should not have a negative effect on the workings of each formulation but rather a synergistic relationship.

Noting the above, one cannot eliminate or control, post-harvest diseases by using a preservative but can only minimize prevalence of degradation on plant based material. Some examples of these preservatives and their pH of optimum activity are presented in Table 2 (Elder and Crowley, 2012).

A preservative blend of interest in the current study is Nipastat™. It is within a class of aminobenzoate esters (see Table 2), a mixture of parabens (i.e. methyl p-hydroxybenzoate, ethyl p-hydroxybenzoate, butyl p-hydroxybenzoate, isobutyl p-hydroxybenzoate and propyl p-hydroxybenzoate)[1]. This blended mixture is generally used as a preservative of a wide range of cosmetics and toiletries[2]. It works adequately either as rinse-off or a leave-on formulation. This product is effective against bacteria, moulds and yeasts, and is recommended to be used at about 0.05-0.3% of the total weight of the finished products[3].

Nipastat™ solubility varies and depends on a solvent being used. Our personal experience is that it is insoluble in water and almost 100% soluble in absolute alcohol, however, Clariant, Industrial and consumer specialties, (2014) advise an ~ 65% in methanol; ~ 60% in ethanol and acetone, ~ 35% in propylene glycol; ~ 40% in hexylene glycol; with less than 1% solubility in pure glycerine; in mixture of glycerine and water (1:1); in liquid paraffin and in water[2]. It is significant to note that even though the solubility of this paraben is lowest in water compared with other solvents, its microbicidal activity is not compromised. However, this insoluble nature of Nipastat™ makes it difficult to blend it directly with any product to be preserved, but the solubility improves markedly when added to water in the temperature range of 60–100 ^{o}C[2], although the recommended handling temperature is ~ 80 ^{o}C[1].

Nipastat™ affords broad spectrum control of various pathogens, including genera of both Gram positive and Gram negative bacteria (namely, *Aerobacter aerogenes, Alkaligenes faecalis , Bacillus cereus,*

Escherichia coli, *Micrococcus flavus*, *Staphylococcus aureus*, *Streptococcus haemolyticus*, *Serratia marcescens* and *Proteus vulgaris*)[2]. In addition to its antibacterial activities, are anti-mould and anti-yeast properties against *Aspergillus niger*, *Mucor racemosus*, *Pityrosporium ovale*, *Saccharomyces cerevisiae* and *Candida albicans*[2].

Table 2: Some examples of preservatives and their pH of optimum activity (Elder and Crowley, 2012).

Preservative(s)	pH of optimum activity	Reference(s)
Aminobenzoate esters e.g. Parabens, Nipastat™	pH 4 - 8	(Johnson and Steer, 2006)
Quarternary ammonium compounds e.g. Benzalkonium Chloride, Benzathonium chloride	pH 4-10	(Kibbe, 2006a, Kibbe, 2006b)
Aryl acid e.g. Benzoic acid/salts	<pH 4.5	(Weller, 2006a)
Aryl alcohol e.g. Benzyl alcohol	<pH 5.0	(Cahill, 2006)
Quarternary ammonium compounds e.g. Cetrimide	pH 7-9	(Owen, 2006a)
Binguanides e.g. Chlorhexidine	pH 5-7	(Owen, 2006b)
Chlorocresol	pH 4-9	(Nema, 2006)
Chloroxylenol	Little pH effect	(McIndoe, 2006)
Formaldehyde donators e.g. Imidurea	pH 3-9	(Guest, 2006a)
Formaldehyde donators e.g. Bronopol	pH 5-8	(Denyer and Hodges, 2006)
Alkyl acids e.g. Propionic acids	pH 3-9	(Amidon, 2006)
Alkyl acids e.g. Sorbic acids /salts	pH 4.5	(Cook, 2006)
Phenolic compounds e.g Phenol, m-cresol	pH 4.9	(Galichet, 2006, Guest, 2006*b*)
Phenylmecuric salts e.g. acetate, borate, nitrate	pH 5.8	(Hepburn, 2006a, Hepburn, 2006b, Hepburn, 2006c)
Thiomersal	Acidic pH	(Weller, 2006b)

[1]Anonymous. 2002. Nipa esters the original parabens, preservatives for cosmetics, toiletries and pharmaceuticals, Clariant. http://www.innovadex.com/Personalcare/Detail/1022/42535/Nipastat™

[2]Nipastat. 2010.Clariant, Industrial and consumer specialities.Preservatives for the cosmetic industry, pp. 1-http://www.essentialingredients.com/spec/Nipastat.pdf

[3] Nipastat by Clariant, Personal care, innovadex: http://www.innovadex.com

Having come across this brilliant preservative, with such a wide range of antimicrobial properties, we were interested to evaluate its efficiency against test isolates sourced from contaminated *in vitro* seedlings of *Garcinia livingstonei*, *Protorhus longifolia* and *Trichilia dregeana* with a view to formulating an application procedure using Nipastat™ for seed treatment of these plant species.

1.9.3. Mechanical treatment methods

Seed samples are generally not uniformly sound, possibly including seeds that are discoloured due to infections, distorted and small or enlarged. The removal of all inert material by processing, seed screening and sieving, and visibly selecting the healthy looking seeds may assist in reducing usage of infected seed material, but does not totally eliminate the problem and thus it is not a long term solution in ensuring storage of healthy seeds of any plant species (Agarwal and Sinclair, 1987).

1.9.4. Physical treatment methods

This is one of the oldest methods practiced to date, and is used to control seed-borne pathogens via physical processing of the plant material to ensure selection and usage of 'clean' material. There are a various ways of which this could be done as illustrated in Figure 1.

1.9.5. Future prospects for biological eradicative methods

There are many advantages as well as disadvantages to the use of biological control methods as part of an overall plant management programme and there is need to collect data over a long-term as to ensure safety of humans and livestock especially when the seeds/products enter the food chain.

1.9.5.1 Advantages of using biological methods:

Probably the most important advantage to the use of biological control is that it typically offers longer term management than the more traditional technology routes. Longer term control is achieved because biocontrol agents act as a host-specific control method, continually present and impacting the invading pathogen. Advantages include longer term control relative to other technologies, lower overall costs, as well as pathogen-specific control that leads to enhanced environmental compatibility.

1.9.5.2 Disadvantages of using biological methods:

There are several disadvantages to consider, including effect or control times of years instead of weeks, agents available for only a limited number of target plant germplasm, and relatively strict environmental conditions for success. Therefore, it is important to consider all aspects of the problem before deciding on the use of one or more management strategies.

1.10. Hypthesis, Aim and objectives of this study

1.10.1. Hypothesis

Assessment of the chemical and biochemical activities may assist in the development of protocols for medium or long-term storage of some recalcitrant seeded plant germplasm.

1.10.2. Aim

The purpose of this study was to isolate and identify fungi in recalcitrant seeds that proliferate at the culture stage, and to assess the efficacy of biological and chemical treatments on identified fungal isolates. This information was then used to develop the best treatments to control pathogens (particularly fungi) on recalcitrant seeds when the control agents were applied prior to short-term storage at 16 ^{o}C. Finally,

the effect of pathogen control treatments on the subsequent seed germination, and seedling growth and development was assessed over a six month growth period. Plant species selected for seed harvest were *Protorhus longifolia* (collected at the University of KwaZulu-Natal, Westville campus, KwaZulu-Natal- fruits only in November), *Trichilia dregeana* (collected all around Durban, KZN KwaZulu-Natal - Fruits from May to July) and *Garcinia livingstonei* (collected in Durban Golf course and at uMthunzini farm, KwaZulu-Natal- Fruits only in December). The three plant species are indigenous to South Africa, are important in traditional practices and all produce recalcitrant seeds. In summary, the aim of the study was to assess physiology, viability, longevity and storage under experimental storage protocols in order to evaluate and improve the storage capability.

1.10.2. Objectives

Objective 1: Screen freshly-harvested recalcitrant seeds of *Trichilia dregeana*, *Garcinia livingstonei* and *Protorhus longifolia* for internal contaminants, i.e. to: detect, localize, isolate and identify common seed-associated fungi across the selected plant species.

Objective 2: Assess efficacy of biological and chemical treatments in curtailing/eliminating proliferation of the selected fungal isolates using the Agar-Plate Technique.

Objective 3: Assess and develop treatment protocols from Objective 2 for seeds prior to hydrated storage.

Objective 4: Assess the efficacy of developed protocols in terms of survival of inherent fungi, effects on storage longevity of the seeds in hydrated storage.

Objective 5: Given that many of the chemicals (and biological) control agents used involved toxic chemicals or secondary metabolites it was deemed necessary to assess the effect of these chemicals on the post-treatment germination and subsequent growth.

1.11. Plant species investigated: importance of forest trees

The conservation of forests is of vital importance for us and future generations. The three plant species chosen for investigation in this study are widely used in traditional practices and have the common characteristic of being trees or bushes producing recalcitrant seeds:

1.11.1. *Trichilia dregeana*

Trchilia dregeana (Pooley, 1993), common name Natal or Forest mahogany, a woody tree belonging to the family of Meliaceae., is found in Asia, America and Africa. However, *T. dregeana* and *T. emetica* are the only species of the genus *Trichilia* found in South Africa and grow well in places of heavy rainfall in coastal and montane evergreen forest. *Trichilia dregeana* can easily be confused with *T. emetica* which is very similar in appearance, but there are minor differences such as the seed and fruit size, colour and the size of leaves. The species produces large trees which attain impressive heights of up to 35m and a diameter of up to 1.8 m as they mature. It has strikingly uniform, very dense, deep green rounded canopy with few internal branches visible (Moll, 1992, Kioko et al., 2006). They produce creamy-white flowers, from October to December and fruiting occurs mainly between January and May. When ripe the fruits burst open on the tree to show red and black seeds (Figure 9). The leaves of the tree are glossy dark green in colour and have pair of side vein (Figure 10). New leaves are shiny, red brown and turn lime green before darkening (Schmidt et al., 2002, Whitaker et al., 2009).

Figure 9: *T. dregeana* capsules revealing seeds.

Figure 10: *T. dregeana* tree growing along Ritson road of Durban, KwaZulu-Natal

The trees provide excellent shade and have a non-aggressive root system. Plants can be grown easily from the seeds or cutting, grow quickly when watered and can be planted in either shade or sun light. The wood is suitable for carving, is used for furniture, fishing floats, musical instruments and household implements. The seed arils are cooked as a vegetable or crushed to yield a milky juice taken as a drink or with side dishes. The seeds are rich in fats, which are used to produce soaps, as body ointment and hair oil as well as for cooking (Schmidt et al., 2002). The species is also an important medicinal plant with the seed, oil, leaves, roots and bark being used for many purposes depending on different cultures. The oil has properties of hastening healing particularly of fractures (Thomas and Grant, 2004).

1.11.2. *Protorhus longifolia*

This is a medium sized evergreen indigenous tree, up to 15 m tall. It grows in forests, forest margins, on river banks and riparian vegetation. The tree belongs to the family of Anacardiaceae, which occur in the Eastern Cape, KwaZulu-Natal coast and the Drakensberg Escarpment in Mpumalanga (Moll, 1992, Schmidt et al., 2002).

The main stem of the tree is tall and straight, reaching a diameter of 1 m; it is brown in colour but becomes darker with age. The stem produces a sticky exudate when its bark is injured. The leaves are scattered, simple, glossy elliptic, dark green above and paler below. The lateral veins on the leaves are prominently parallel and forking near margins. Flowers from July to October and are borne in leaf axils or terminally, greenish white to red in colour. The fruit is a drupe, single seeded, and pale purple (Figure 11) when mature (Schmidt et al., 2002). The leaves are very similar to those of a mango tree and its powdered bark is poisonous. The species grows readily from seed and growth is relatively quick. It tolerates slight frost and is very drought-resistant. *Protorhus longifolia* is used in medicine and has been used as a specimen tree (focus of interest in a garden) and screening plant.

Figure 11: Branch of *Protorhus longifolia* showing ripe and unripe fruits

1.11.3. *Garcinia livingstonei*

Garcinia livingstonei is commonly called African mangosteen and belongs to the family Clusiaceae. The African mangosteen is widespread in the warmer parts of Africa, from just north of Durban as far as Somalia and Guinea. In southern Africa it spreads far up to the Limpopo and Zambezi valleys. The tree reaching 18 m and is pyramidal shape when young but spreads later, with thick, woody young branches and yellow to red resin (Figure 12). The fruits are single-seeded and are known for their delicious orange-

red berry fruits (Figures 12 and 13), sticky yellow juice and delicious acid-sweet taste that can be used in fermenting alcoholic beverage. They produce flowers which cluster in leaves or on knobby side spurs, are cream to greenish yellow in colour and sweet scented (Figure 14).These trees are sensitive to cold, while quite hardy to both drought and heavy rain (Van Wyk and Van Wyk, 1997, Mabberley, 2002).

Figure 12: Tree showing ripe and unripe fruits of *Garcinia livingstonei* in Mtunzini farm, KZN.

Figure 13: Ripe and unripe fruits of *G. livingstonei*

Figure 14: Flowers of *G. livingstonei*

2. METHODOLOGY

2.1. Fungal location, isolation and identification of common test isolates

A sample of ~100 g of seeds was immersed in a ¼- strength Ringer's solution (i.e. 1 tablet per 500 ml of sterile distilled water) in 1 l beaker and placed onto an orbital shaker for 20 minutes at 150 rpm. This gave rise to sample number 1: *whole seed (WS)*, followed by removal of seed coats under sterile conditions to serve as sample number 2: *seed coat (SC)* and lastly sample number 3: *naked seed (NS)*. Sample numbers 2 and 3 were also immersed into a Ringer's solution as per sample number 1 above. All these (samples 1, 2 and 3 immersed in ¼-strength Ringer's solution) served as 10^{o} dilutions. Serial dilutions were performed aseptically and up to 10^{-6} dilution for each sample. After this 1 ml of each sample was transferred into Petri dishes prepared using molten potato dextrose agar (PDA) (39 $g.l^{-1}$, adjusted to pH: 5.6 and autoclaved at 121 ^{o}C for 20 minutes) and incubated at room temperature for 10 d. The plates were assessed at 2 days intervals. The highest dilutions gave rise to a single growing mycelium. The single growing mycelium was isolated and sub-cultured three times onto sterile PDA to ensure an axenic culture. Common isolates from the samples (WS, SC and NS) were purified and identified. These isolates were used in the preliminary studies. The procedure was replicated three times for consistency.

2.2. Isolation, purification and identification of fungal inoculum associated with the inner surface of the testa

For isolation of the inoculum of fungi associated with the inner surface of the testa, seed coverings were removed from newly-harvested randomly selected seeds which underwent a series of surface-decontamination protocols developed for the study . Once seeds were surface decontaminated, they were then placed in Hydrated storage (HS) according to treatment protocols. Thereafter, any fungal proliferation emerging in HS and in culture were isolated and immersed in ¼-strength Ringer's solution

in 250 ml and agitated for 10 min at 150 rpm. Three 10 ml aliquots of this solution were then serially diluted (10^{-1} – 10^{-6}), from which 1 ml of each was mixed with ~ 30 ml of Potato Dextrose Agar before setting. The cultures were incubated at 25-30 °C for 5-10 d, and monitored daily. As mycelia developed, individual plugs (10x10 mm) were sub-cultured into fresh PDA and incubated. This was repeated three times to ensure axenic cultures. Petri dishes were also examined under stereomicroscope and the number of seeds infested and the fungal colonies developed were recorded as follows:

$$\% \text{ Frequency of genus isolated} = \frac{\text{No. of same genus isolated}}{\text{Total no. of genus isolated}} \times 100$$

The frequency of each fungus was determined in the percentage from the colonies of all fungi developed.

2.3. Characterisation of fungal isolates

For identification of the isolates, plugs of axenic cultures were introduced into 9 ml of Ringer's solution and vortexed to disperse the spores. A sterile needle was immersed in the Ringer's suspension attracting a minute droplet (minimizing the number of spores transferred), then plated on each of five different media, *viz.* malt extract agar (MEA), Sabourad agar (SDA), PDA, water agar and Czapek Dox agar (CDA) to facilitate fungal identification microscopically by inspection of colony margins, surface and underside textures, pigmentation and growth rates over 7 d period (Cappucino and Sherman, 1992, Jha, 1995, Cappucino and Sherman, 2014).

2.4. Slide preparation for fungal identification

For microscopical identification according to Raper and Fennel (1965), Ellis (1971), Domsch et al. (1980) and Nelson et al. (1983) teased-out mycelium on microscope slides were stained with Latco-phenol Cotton Blue, then covered using Menzel Glaser 18 x 18 mm cover slips.

2.5. Microscopical identification

The prepared slides were viewed and characterized at low and high power with a Nikon Eclipse 80*i* microscope equipped with apochromatic objective lenses and images of fruiting body structures were captured.

2.6. Molecular identification

Molecular identification of fungi was done as conformance test at Inqaba Biotec, South Africa using an ITS PCR with ITS-1 and ITS-4.

2.7. Biological control products

Four products were used for the purposes of this investigation: *Trichoderma harzianum* strain Kd (EcoT) and *Trichoderma harzianum* strain B77 (Eco77); Plant Health Products (PTY) Ltd, South Africa), *Bacillus subtilis* and *Pichia pastoris* (stock culture provided by Durban University of Technology, South Africa) with sterile distilled water used as the control. The main bio mechanism of control for the selected bio agents are induced resistance and via antimicrobials, competition and via antimicrobials, induced resistance alone, (Xu et al., 2010), respectively. The number of spores gram^{-1} of spore suspension was 2 x 10^9 for the *Trichoderma* strains and 1 x 10^6 propagules ml^{-1} for *Bacillus subtilis* and *Pichia pastoris*.

2.8. Control of identified fungal isolates

2.8.1. Biological control

a) Plug Agar diffusion method

For the bio-control agents EcoT, Eco77, *P. pastoris* and *B. subtilis* plugs of 6 mm wide were aseptically cut out using a sterile surgical blade. Pre-test test isolates were suspended into ¼-strength Ringer's solution, mixed with a vortex and 0.1 ml was spread evenly with a sterile hockey stick onto PDA plates to which the plugs of bio-control agents were placed on the centre of the plate. The zone of inhibition

was measured (i.e. the diameter of the clearing zone). This was done in triplicates per test isolate. The control made use of the same experimental procedure in 2.8.1 (*a)* but PDA plates contained only test isolate addition and no bio-control agent plug.

b) Disc-Assay method

A 2 ml of 24 h *B. subtilis* pre-culture was used to inoculate 200 ml TSB (Tryptic soy broth) medium. This was grown in a shaking incubator at 150 rpm, and at 37 °C ±2 °C for 48 h (Kumar et al., 2008).

A 2 ml of 48 h *P. pastoris* pre-culture was used to inoculate 200 ml yeast peptone dextrose broth (YPD). This was grown in a shaking incubator at 160 rpm, and at 30 °C for 48 h. After incubation both the cultures were centrifuged at 7000 rpm for 10 min. The pellet was discarded and the supernatants used for the disc assay.

Paper discs were prepared using a paper punch and contained in a sealable bottle for autoclaving. The sterile paper discs were submerged in the *B.subtilis* and *P. pastoris* bio-control supernatant, as well as in *Trichoderma* EcoT and Eco77 (1 $g.l^{-1}$) for 30 minutes. Test isolate was suspended into ¼-strength Ringer's solution, mixed with a vortex and 0.1 ml was spread evenly with a sterile hockey stick onto PDA plates. The bio-control agent impregnated discs, were placed on the centre of the 90 mm PDA plates containing test isolates. This was also carried out in triplicates per test isolate.

Control 2.8.1 *(b)* was carried out using paper discs which were submerged into sterile distilled water instead of bio-control supernatants, and placed onto PDA plates containing 0.1 ml of spread test isolate.

c) Agar well diffusion method

Test isolate was suspended into Ringers solution, mixed with a vortex and 0.1 ml was spread evenly with a sterile hockey stick onto PDA plates. From each of these plates a 10 mm plug was cut out aseptically. To each of these wells, 1 ml of bio-control agents (1 g l^{-1}) *T. harzianum* EcoT, and *T. harzianum* Eco77

were poured. Supernatants of *P. pastoris* and *B. subtilis* were prepared as in 2.8.1 *(b)* and 1 ml was poured into the wells. This was done in triplicates per test isolate.

The control in 2.8.1 *(c)* made use of the same experimental procedure in 2.8.1 *(c)* but the wells contained 1ml of sterile distilled water instead of bio-control agents.

2.8.2. Chemical control

Table 3: Seven chemical fungicides, each with a different active ingredient, used in the study.

Fungicides	Active ingredient	Company
Afugan	pyrazophos (organophosphate)	Hoechst AG, West Germany
Biotaine™	chlorhexidine gluconate	Dismed Pharma (PTY) LTD
Celest	fludioxonil	Syngenta AG
Orius 200 EW	triazole	Makhteshim-Agan South Africa (PTY) LTD
Odeon 720 DC	chlorothalonil	Makhteshim-Agan, South Africa (PTY) LTD
Sporekill	didecyl dimethyl ammonium chloride	HydroTech Properties (EDMS) BPK/ Seed (PTY) LTD
Ripenit	ethephon	R.T. Chemicals
Nipastat®	mixed parabenzoates (parabens)	Clariant chemicals, South Africa

An agar well diffusion method was used to assess the efficacy of each of the chemical control agents investigated in this study. A 100 µl sample of a test isolate was spread on a PDA plate using a sterile swab and 50 µl of a chemical control was transferred in an inoculated plate into a well and a sterile water was used as a control. The plates were incubated at 25 ^{o}C for 7 d. Observations were made from the third day and final results recorded on the seventh day. The experiment was carried out in triplicate.

2.9. Seed collection and preparation

Seeds of *Trichilia dregeana* and *Protorhus longifolia* were collected at University of KwaZulu-Natal, Durban (Westville and Howard College campuses), central Durban and surrounding areas. Seeds of *Garcinia livingstonei* were collected from Mthunzini farm, North of Durban, some at Durban Golf course and in Hillcrest, Pinetown. Most fruits collected from trees were mature and had already opened exposing the seeds. The seeds were then processed manually by removing the aril and seed coat, revealing the green cotyledonary surfaces. These were collected into clean (2 l) plastic beakers loosely covered with paper towel which was frequently moistened to prevent seed from drying until the required numbers were attained. The seeds were then subjected to a range of treatments as detailed in Table 4, and placed into hydrated storage.

2.10. Development of seed treatment protocols for *Trichilia dregeana, Garcinia livingstonei* and *Protorhus longifolia* before hydrated storage

Table 4: Summary of developed seed treatment protocols

Treatment	Eco77 (Bio-control) 1 g.l^{-1}	Biotaine™ 2% (v/v)	Benomyl 500 WP (Seed- dusting)	Nipastat® 1 g.l^{-1}	Encapsulation 2% (m/v) low viscosity alginic acid (sodium salt)
DBe (Control)	-	-	√	-	-
DEBBe	√	√	√	-	-
DEBN	√	√	-	√ seed dusting	-
DEn	-	-	-	-	√
DEnN	-	-	-	√ incorporated into alginate capsule	√

D = decontamination; **E** = Eco77; **B** = Biotaine; **Be** = Benomyl 500 WP, **N** = Nipastat; **En** = encapsulation

2.10.1. Treatment DBe

Cleaned **s**eeds were surface decontaminated by treatment with a 1% (v/v) NaOCl solution containing a few drops of the wetting agent, Tween 20/80® , for 10 min, respectively. Seeds were subsequently rinsed three times in sterile distilled water. They were then left to dry between sheets of towel paper overnight at a room temperature. Once they were dried, hydrated storage conditions were employed as explained below (section 2.11).

2.10.2. Treatment DEBBe

Cleaned **s**eeds were surface decontaminated by treatment with a 1% (v/v) NaOCl solution containing a few drops of the wetting agent, Tween 20/80®, for 10 min, respectively (as per treatment DBe, above), then soaked in 1 $g.l^{-1}$ suspension of Eco 77 spores (with shaking at 150 rpm) for 4 h, followed by rinsing three times in sterile distilled water. The seeds were then immersed in 2% (v/v) Biotaine™ for 10 min and thereafter rinsed three times into sterile distilled water. They were then left to dry between sheets of towel paper overnight at room temperature, after which they were placed within sieves in sterile buckets, after which hydrated storage conditions were carried as explained below (section 2.11).

2.10.3. Treatment DEBN

Cleaned **s**eeds were surface decontaminated by treatment with a 1% (v/v) NaOCl solution containing a few drops of the wetting agent, Tween 20/80®, for 10 min, respectively (as per treatments DBe and DEBBe, above), then soaked in 1 $g.l^{-1}$ suspension of Eco 77 spores (with shaking at 150 rpm) for 4 h, followed by rinsing three times in sterile distilled water. The seeds were then immersed in 2% (v/v) Biotaine™ for 10 min and thereafter rinsed three times into sterile distilled water. They were then left to dry between sheets of towel paper overnight, after which they were placed within sieves in sterile buckets, then layered with Nipastat® (powder), the buckets sealed and stored at 16 ^{o}C in hydrated storage condition (see section 2.11)

2.10.4. Treatment DEn

Cleaned **s**eeds were surface decontaminated by treatment with a 1% (v/v) NaOCl solution containing a few drops of the wetting agent, Tween 20/80® , for 10 min, respectively (as per treatments DBe, DEBBe and DEBN, above), then left to dry between sheets of towel paper overnight. Once seeds were surface-dry, they were immersed in a solution of 2% w/v (low viscosity) alginic acid (sodium salt) for 15 min under aseptic conditions. The alginic acid was then polymerised in 0.1 M $CaCl_2$ for 15 min, completely encapsulating the seeds. The capsule was then dried in a laminar air-flow for 4 h. The encapsulated seeds were then placed within sieves in sterile buckets which were sealed and then maintained at 16 ^oC in hydrated storage (see section 2.11).

2.10.5. Treatment DEnN

Cleaned **s**eeds were surface decontaminated by treatment with a 1% (v/v) NaOCl solution containing a few drops of the wetting agent, Tween 20/80®, for 10 min, respectively (as per treatments DBe, DEBBe, DEBN and DEn, above), then left to dry between sheets of towel paper overnight, after which they were immersed for 15 min in a solution of alginic acid (as in DEn, above), into which Nipastat® was incorporated at 1 $g.l^{-1}$. The alginate was polymerised in 0.1 M $CaCl_2$ for 15 min, completely encapsulating of the seeds. The capsule was then dried in a laminar air-flow for 2 h. The encapsulated seeds were then placed within sieves in sterile buckets, which were sealed and stored at 16 ^oC in hydrated storage (see section 2.11).

2.11. Hydrated storage (HS) conditions and Gravimetric determination of water content

Surface decontaminated seeds for treatments DBe (control) and DEBBe were lightly dusted with Benomyl 500 WP (active ingredient, benzimidizole; Villa Protection, South Africa), while cleaned seeds for treatment DEBN were dusted with Nipastat, whilst seeds from treatments DEn and DEnN were encapsulated in alginate gel, the latter incorporating Nipastat (Figure 15). All the treated seeds were placed as a monolayer on a plastic mesh suspended 200 mm above paper towel saturated with sterile water to which a few drops of sodium hypochlorite (NaOCl) as commercial household bleach had been added, in white, translucent 5 l plastic buckets. Bucket lids were lined with paper towel (as a precaution to prevent condensate from dripping back onto seeds) before the buckets were sealed and stored in 16 ºC constant temperature room. Plastic sieve, bucket lids and the buckets had been previously washed using domestic dishwashing liquid soap (active ingredient, anionic detergents, Unilever, South Africa), rinsed using hot tap water, decontaminated by soaking in a 1% m/v NaOCl solution overnight, subsequently dried using a paper towel and lastly wiped with 70% v/v ethanol prior to use.

Figure 15: *Trichilia dregeana* seeds coated with alginate gel contained in a sieve within a 5 l plastic bucket

Water content of freshly harvested and treated whole seeds was determined gravimetrically and expressed on a dry mass basis, i.e. g H_2O g^{-1} dry mass ($g.g^{-1}$). Using a five-place micro balance (Mettler MT5; Germany), whole seeds (n = 5) were weighed individually in aluminium foil boats, after which they were dried to constant weight (at 80 ^oC for 48 h). Dried seeds were then brought to ambient temperature in the weighing boats over activated silica gel in closed glass Petri dishes, and reweighed to obtain the dry mass.

2.12. Seed culture conditions after hydrated storage, and seedling maintenance

A sample of 30 seeds was randomly selected for removal from hydrated storage using forceps. Of these five seeds were used for water content evaluation as per the gravimetric determination below. The other 25 seeds were further surface decontaminated by exposure to 1% (m/v) NaOCl solution containing a few drops of the wetting agent, Tween 20/80®, for 10 min, then rinsed three times in sterile distilled water. The seeds were then immersed in 0.1% (m/v) mercuric chloride ($HgCl_2$) for 15 min and thereafter rinsed 3 times with sterile distilled water. Seeds were then further decontaminated with 0.01% (m/v) of Cicatrin (active ingredient, neomycin sulphate) for 10 min, and finally rinsed three times with sterile distilled water.

The decontaminated seeds were cultured on water agar (WA) for 30 d. Five seeds of *T. dregeana* and *P. longifolia* were plated per 90 mm Petri dish, stored in a dark cardboard at ambient temperature until germinated to the stage of shoot emergence, after which the plates were transferred to a growth room with a 16 h photoperiod (66 µmol m-$2s^{-1}$ PPFD) provided by Biolux tubes [Osram L58W] and maintained at 27 ^oC day/21 ^oC night (standard culture conditions). Plates were checked daily for any incidence of contamination and (if there was any), uncontaminated seeds were transferred to a fresh sterile WA plate to prevent them from being cross contaminated (contamination was recorded accordingly). A similar procedure was followed for *G. livingstonei* with an exception of using plates; instead Magenta boxes were used to accommodate the size of seeds as they are bigger as compared to *T. dregeana* and *P.*

longifolia. After 30 d, seedlings were assessed for average root length, root, and shoot development and total number of seedlings excluded due contamination.

2.13. Seedling maintenance outside the greenhouse

Ten seeds were sampled from hydrated storage at time intervals of 0, 1, 2, 4, 6, 16, 32 and 64 days. Therafter, one seed was planted per 500 ml potting bags filled with commercial potting soil mix (Grovida Potting Mix, Grovida Horticultural Products CC, South Africa) contained within trays of 450 x 350 mm which were placed in a shade-house. The young plants were watered daily and weeds were removed every second week. Plants were grown for six months, after which they were harvested, and stem diameter, leaf area and number of leaves per plant recorded, and biomass allocation to roots and stems were assessed.

2.14. Biomass allocation

After 6 months of growth outside the greenhouse, saplings were harvested. On the day of harvest, they were watered heavily to ensure easy separation from the potting soil which prevented the roots from breaking. The plants were then put on mesh tray under soft running tap water to rinse off soil particles, snails and earthworms, after which they were transferred to paper towel to remove excess water. The saplings were then subdivided into leaves, stem and roots. The number of leaves was counted, leaf area measured using an area meter (C1-202, CID, Inc., USA), and stem diameter and root length measured using a calibrated ruler. Once data were collected, the plant parts were wrapped in heavy-duty aluminium foil and dried to constant weight (at 80 ^{o}C for 48 h). Ten samples of each of the leaves, stems and roots were used for all five treatments (i.e. DBe, DEBBe, DEBN, DEn & DEnN) from plants grown from seeds after 0, 1, 2, 4, 6, 16, 32 and 64 d of storage.

2.15. Seed vigour trials

Using forceps, 25 randomly selected seeds were removed from hydrated storage after 0, 1, 2, 4, 6, 16, 32 and 64 days. These were then placed on moistened filter paper in 65 mm Petri dishes (one seed per Petri dish to give 25 replicates) for seeds of *T. dregeana* and *P. longifolia*. Petri dishes were closed, but not sealed (i.e. not air tight) and kept at ambient temperature for 31 d. The seeds were watered with distilled water every second day, when they were checked for initiation of germination and root length (if any) was measured. However, for *G. livingstonei* Magenta boxes were used to accommodate the size of the seed but the same procedure was followed as per *Trichilia* and *Protorhus* species (See Figures 18A, B and C).

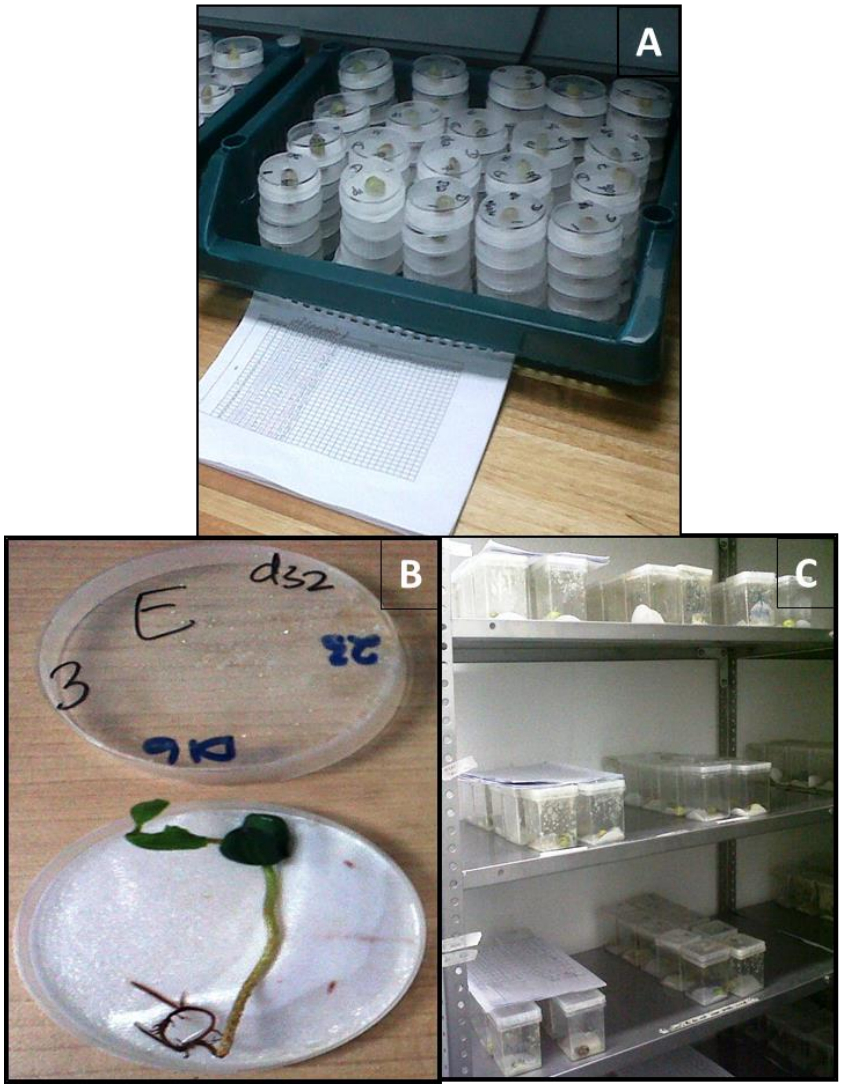

Figure 16: Shows seeds of *T. dregeana* (A), *P. longifolia* (B) and *G. livingstonei* (C) contained within Petri dishes (A and B) and Magenta boxes (C)

2.16. Statistical Analysis

Data obtained were analyzed using SPSS 23 for Windows 7 and GenStat 17th Edition. The data that were not normally distributed were log transformed before analysis, but the original (untransformed) data are presented here. One-way and two- way analyses of variance (ANOVA) were used where applicable to analyze the data and the means compared using Tukey HSD[a,b] post Hoc Test. Chi square test was used where necessary to test differences between two samples.

3. RESULTS

3.1. Isolation, identification and frequency of fungi from *T. dregeana*, *P. longifolia* and *G. livingstonei* seeds

Preliminary experiments involved sampling major parts of the reproductive structures, namely embryonic axis (EA), fruit (F), seed coat (SC) and whole seed (WS) of each plant species to determine common and dominant fungi among selected plant species. This investigation led to a sum total of approximately 150 fungal isolates. The most common and dominant isolates were *A. flavus*, *A. niger*, *Fusarium* spp., *Trichoderma* spp., *Aspergillus* spp., *Penicillium* spp., *Cephalosporium* sp. and *Acremonium* sp. Ultimately, it was decided to concentrate more on isolates that proliferated from decontaminated plant material in culture and also in HS as these were more difficult and showed more resistance.

The most prevalent fungal isolates identified thereafter (Table 5) were species of the genus *Penicillum* in both plant species *T. dregeana* and *P. longifolia* with a frequency of 100%, and *Fomitopsis meliae* was the only isolate from *G. livingstonei*. The rest of the identified isolates from plant species of *T. dregeana* and *P. longifolia* were *Trichophyton rubrum*, *Phialemoniun* sp., *Trichoderma asperellum*, *Fusarium* sp., *Hypocrea atroviridis*, *Colletotrichum gloeosporioides*, *Irpex* sp., *Cladosporium* sp., *Aspergillus* sp., *Acremonium* sp., *Alternaria* sp., *Cytospora* sp., *Hypocrea atroviridis* and *Aureobasidium pollulans*.

Among the plant species investigated *P. longifolia* (18 fungal isolates) was more prone to contaminants compared with *T. dregeana* (6 fungal isolates) and *G. livingstonei* (1 isolate). Fungal proliferation from whole seeds in culture was visible from day 5 on the surface of the seeds and escalated vigorously as it spread on the surface of water agar. Fungal growth in hydrated storage (HS) was only visible after 16 days in both *T. dregeana* and *G. livingstonei* whereas with *P. longifolia* as soon as the 7th day of storage.

Table 5: Outcomes for isolation, identification and frequency of fungi from *T. dregeana*, *P. longifolia* and *G. livingstonei*

Plant species	Fungi isolated	No. of isolates	% Frequency
Trichilia dregeana[6]	*Paecilomyces lilacinus*	6	55
	Aureobasidium pullullans	1	9
	Penicillium brevicompactum	1	9
	Fusarium sp.	1	9
	Trichoderma asperellum	1	9
	Penicilllium sp.	1	9
Protorhus longifolia[18]	*Trichophyton rubrum*	1	3.6
	Phialemonium sp.	1	3.6
	Penicillium chrysogenum	3	10.7
	Penicillium polonicum	3	10.7
	Penicillium olsonii	2	7
	Trichoderma asperellum	2	7
	Fusarium sp.	2	7
	Hypocrea atroviridis	2	7
	Colletotrichum gloeosporioides	1	3.6
	Penicillium sp.	1	3.6
	Irpex sp.	1	3.6
	Cladosporium cladosporioides	1	3.6
	Aspergillus sp.	1	3.6
	Acremonium sp.	1	3.6
	Penicillium brevicompactum	3	10.7
	Alternaria sp.	1	3.6
	Cytospora sp.	1	3.6
	Penicillium adametzioides	1	3.6
Garcinia livingstonei[1]	*Fomitopsis meliae*	1	100

Trichilia dreageana[6] = sum total of 11 fungal isolates; *Protorhus longifolia*[18] = sum total of 28 fungal isolates and *Garcinia livingstonei*[1]= sum total of 1 fungal isolate

3.2. Effect of different antimicrobial agents on the micro-organisms associated with the seeds of *T. dregeana*, *P. longifolia* and *G. livingstonei*

3.2.1. Bio control agents: effects and efficacy against fungal isolates

We must note that for *B. subtilis,* only agar well diffusion method produced significant effects on fungal isolates and therefore only results from this method are discussed whereas for both *Trichoderma* strains EcoT and Eco77, plug agar diffusion method was the most efficient method thus results obtained using this method are discussed accordingly:

Interaction amongst biological treatments and fungal isolates of *T. dregeana* (Table 6) showed that *B. subtilis* agar well diffusion method had the smallest average zone of inhibition zones when compared with both strains of *Trichoderma* EcoT and Eco77. The fungal isolate demonstrating the most resistance against *B. subtilis* was *T. asperellum* (12 mm) and the most susceptible fungal isolate was *A. pullulans* (18 mm). However, overall response of the fungal isolates to *B. subtilis* as biocontrol did not show much differences amongst isolates; as noticed, both *P. lilacinus* (17 mm) and *A. pullulans* (18 mm) were the most susceptible fungi belonging to the same category/subunit whereas the other fungal isolates were at the same level of susceptibility. *P. lilacinus*, *A. pullulans* and *P. brevicompactum* were not different from each other in the way they responded against Eco77 however responded differently when compared to *Fusarium* sp., *T. asperellum* and *Penicillium* species.

Comparison amongst *Fusarium* sp., *T. asperellum* and *Penicillium* sp. showed the responses to be significantly different. Eco77 was the most effective against *Penicillium* sp. (45 mm) and *T. asperellum* (36 mm) was the second most susceptible isolate. Eco77 effects against the two fungal isolates was significantly different when compared with other isolates however different when they were also compared. Furthermore, EcoT was most effective against *T. asperellum* (35 mm) and *A. pullulans* (31 mm). However, with EcoT differences were observed with fungal isolates of *A. pullulans* and *T.*

asperellum, even though they were not different when compared to each other. *Fusarium* sp. and *Penicillium* sp. were not different when compared to each other and also to *P. brevicompactum* however different when further compared to *P. lilacinus*.

Table 6: Effects of biological control agents on fungal isolates from *T. dregeana*. Mean inhibition zone (mm) (p = 0.001). Different superscripts upper-case letters in each row indicate significant differences (subsets).

Treatment	Fungal isolate(s)					
	Mean inhibition zones (mm)					
	P. lilacinus	*Fusarium* sp.	*A. pullulans*	*Penicillium* sp.	*P. brevicompactum*	*T. asperellum*
EcoT	21 ^{A}	26^{B}	31^{C}	26^{B}	25^{AB}	35^{C}
Eco77	19^{A}	26^{B}	19^{A}	45^{D}	20^{A}	36^{C}
B. subtilis	17^{B}	13^{AB}	18^{B}	16^{AB}	15^{AB}	12^{A}

LSD of means at 5% = 4.087

Moreover, *Trichoderma* strain Eco77 continued to be an effective biological fungicide against the *G. livingstonei* isolate (*F. meliae*) which was the only fungal isolate from this plant species (Tables 7). A total average zone of inhibition of 18 mm was obtained (i.e. 20% overall inhibition). *Trichoderma* strain EcoT and *B. subtilis* exhibited a total average zone of inhibition of 15 mm and 14 mm respectively (Table 7). Individual comparisons however showed differences when both *B. subtilis* and *Trichoderma* strain EcoT were compared to *Trichoderma* strain Eco77 within this plant species. In addition, no differences were observed when comparing *B. subtilis* and EcoT throughout all fungal isolates. In summary, within this plant species *B. subtilis* and EcoT were not as effective as Eco77.

Table 7: Effects of biological control agents on fungal isolates from *G. livingstonei*. Mean inhibition zone (mm) (p = 0.001). Different superscripts upper-case letters in each row indicate significant differences (subsets).

Treatment	Fungal isolate
	Mean inhibition zones (mm)
	F. meliae
EcoT	15^{A}
Eco77	18^{B}
B. subtilis	14^{A}

LSD of means at 5% = 0.94

For *P. longifolia* fungal isolates (Table 8), EcoT was the most effective biological fungicide. The highest inhibition zone of 45 mm was achieved against *T. asperellum* and the smallest inhibition zone was 10 mm against *P. lilacinus*. In addition, the range of inhibition for EcoT against the other fungal isolates was from 10 to 45 mm. This was the second widest range achieved when comparing to *B. subtilis* (10 – 30 mm) and Eco77 (6 - 45 mm). Similarily to EcoT, *Trichoderma* strain Eco77 was able to produce an inhibition zone of 45 mm against *T. asperellum*. Both strains of *Trichoderma* were very effective against this isolate whereas no inhibition was observed with *B. subtilis* (0 mm). The highest zones of inhibition for *B. subtilis* was achieved against *Aspergillus* sp., *Cytospora* sp. and *H. atroviridis* (30 mm) and the range of inhibition achieved against *Aspergillus* sp. and *Cytospora* sp. by *Trichoderma* strains was 12 – 27 mm, thus *B. subtilis* was quite effective and efficient against these two fungal isolates. However, *Trichoderma* strains were very effective against *H. atroviridis* exhibiting 45 mm zone of inhibition with Eco77 and 32 mm zone of inhibition with EcoT.

Table 8: Effects of biological control agents on fungal isolates from *P. longifolia*. Mean inhibition zone (mm) (p = 0.001). Different superscripts upper-case letterin each column indicate significant differences (subsets).

Treatment	Fungal isolate(s)																
	Mean inhibition zones (mm)																
	Acremonium sp.	*Alternaria* sp.	*Aspergillus* sp.	*C. cladosporioides*	*C. gloeosporioides*	*Cytospora* sp.	*Fusarium* sp.	*H. atroviridis*	*Irpex* sp.	*P. adametzioides*	*P. brevicompactum*	*P. chrysogenum*	*P. lilacinus*	*P. olsonii*	*P. polonicum*	*Penicillium* sp.	*Phialemonium* sp.	*T. asperellum*
B. subtilis	20^{C}	23^{C}	30^{D}	17^{C}	14^{B}	30^{D}	10^{B}	30^{D}	20^{C}	26^{D}	22^{CD}	20^{C}	0^{A}	13^{B}	16^{BC}	15^{B}	0^{A}	0^{A}
Eco77	10^{AB}	13^{B}	13^{B}	15^{BC}	21^{C}	15^{BC}	18^{C}	45^{D}	22^{C}	11^{AB}	12^{BC}	21^{C}	18^{BC}	15^{BC}	6^{A}	17^{BC}	18^{C}	45^{D}
EcoT	11^{A}	14^{AB}	12^{A}	17^{B}	27^{CD}	27^{D}	24^{CD}	32^{D}	26^{CD}	12^{A}	13^{AB}	31^{D}	10^{A}	15^{A}	16^{B}	15^{A}	20^{BC}	45^{E}

L.S.D. of means at 5% =5.585

Comparisons within these plant species in determining the most effective biological control agent leads to the conclusion that although large inhibition zones (> 50%) were not produced, *Trichodermal* strains were more effective in minimizing contamination levels than *B. subtilis*, with Eco77 being the most effective antimicrobial agent. Therefore Eco77 was the only biological antimicrobial agent considered for further development of seed decontamination protocols.

3.2.2. Chemical control agents: effects and efficacy against fungal isolates

A comparison of the effects of the various chemical control agents on fungal isolates obtained from *T. dregeana* (Table 9) showed that Ripenit was the weakest antimicrobial agent as it had the smallest inhibition zones (averaging at 2 mm with *P. lilacinus*, *A. pullullans*, *P. brevicompactum*, *T. asperellum* and *Penicillium sp*.). However, it was observed to be more effective against *Fusarium* sp. (20 mm). Orius was least effective against *P. brevicompactum* and *Fusarium* sp. (i.e. both averaged at 2 mm of inhibition zone), however it was highly effective against the fungal isolates of *P. lilacinus* and *T. asperellum* (i.e. 90 mm zone of inhibition). Afugan, Odeon and Sporekill had inhibition zones that ranged from 7 to 12 mm when tested against species of *Fusarium* sp., *P. brevicompactum* and *T. asperellum*. Similarly, Celest was also not very efficient in inhibiting *P. lilacinus* and *A. pullulans* with inhibition zones of 2 mm, however achieving an inhibition zone ranging from 17 to 23 mm with *P. brevicompactum*, *Fusarium* sp., *T asperellum* and *Penicillium* sp.

Nipastat performed consistently in inhibiting *P. lilacinus*, Fusarium sp. *Penicillium* sp. (i.e. inhibition zones ranging from 44 to 60 mm were obtained) but *A. pullulans* and *P. brevicompactum* were more resistant with inhibition zones from 16 to 19 mm. Biotaine maintained inhibition zones above 20 mm for four fungal isolates (i.e. *A. pullulans, P. brevicompactum, T. asperellum* and *Penicillium* sp.) but was less effective against *P. lillacinus* and *Fusarium* sp.

The results indicated that Nipastast, Orius and Biotaine were the most effective chemical control agents with Ripenit being the least effective.

Table 9: Effects of chemical control agents on fungal isolates from *T. dregeana*. Mean inhibition zone (mm) (p = 0.001). Different superscripts upper-case letters in each raw indicate significant differences (subsets).

Treatment	Fungal isolate(s)					
	Mean inhibition zones (mm)					
	P. lilacinus	*A. pullullans*	*P. brevicompactum*	*Fusarium* sp.	*T. asperellum*	*Penicillium* sp.
Afugan	20^{C}	21^{C}	22^{C}	9^{B}	2^{A}	12^{C}
Biotaine	18^{B}	21^{C}	22^{C}	14^{A}	22^{C}	23^{C}
Celest	2^{A}	2^{A}	17^{B}	17^{B}	23^{C}	18^{B}
Orius	90^{D}	17^{B}	2^{A}	2^{A}	90^{D}	22^{C}
Odeon	21^{D}	2^{A}	7^{B}	12^{C}	23^{D}	2^{A}
Sporekill	17^{B}	18^{B}	31^{C}	18^{B}	8^{A}	34^{C}
Ripenit	2^{A}	2^{A}	2^{A}	20^{B}	2^{A}	2^{A}
Nipastat	44^{C}	16^{A}	19^{B}	60^{D}	22^{B}	60^{D}

LSD of means at 5% = 2.937

Only one isolate (*F. meliae)* was obtained from *G. livingstonei* (Table 10). Celest, Odeon and Ripenit were not effective against *F. meliae* (i.e. 100% fungal growth was obtained). Afugan and Orius gave inhibition zones of 12 mm whilst Nipastat and Sporekill gave an average of 14 mm and 15 mm. Biotane, however, exhibited an average inhibition zone that was the largest produced by the chemical agents (20 mm; i.e. ~ 23 % inhibition). Summarizing from these results Biotaine was the most effective chemical control. This chemical control was then followed by Sporekill and Nipastat which when compared to each other showed no differences.

Table 10: Effects of biological control agents on fungal isolates from G. *livingstonei*. Mean inhibition zone (mm) (p = 0.001). Different superscripts upper-case letters in each column indicate significant differences (subsets).

Treatment	Fungal isolate
	Mean inhibition zones (mm)
	F. meliae
Afugan	12^{B}
Biotaine	20^{D}
Celest	0^{A}
Nipastat	14^{C}
Odeon	0^{A}
Orius	12^{B}
Ripenit	0^{A}
sporekill	15^{C}

LSD of means at 5% = 0.61

For the isolates from *P. longifolia* (Table 11) Ripenit, Celest and Odeon were poor antimicrobial chemical agents. However, the ranking of efficacy was not the same as observed in isolates from *Garcinia livingstonei* (Table 10). Significant differences were observed when all the antimicrobial chemicals selected were compared against each other except for Biotane and Sporekill which both had an overall average inhibition of 24 mm (~ 27% overall inhibition). However, Orius was the most effective antimicrobial chemical as it exhibited a total overall zone of inhibition of 27 mm (i.e. 30% inhibition) disregarding fungal type. Orius was also able to achieve a 100% inhibition against *Aspergillus* sp. and *T. asperellum*. A 100% inhibition was also observed for Celest against *T. asperellum* eventhough it was observed to be the least effective against the other fungal fungal isolates tested. Moreover, Afugan was able to exhibit a 100% inhibition zone against *C. cladosporioides*. Chemicals that achieved a consistant performance against the fungal isolates were Biotaine, Nipastat, Orius and Sporekill.

Table 11: Effects of biological control agents on fungal isolates from *Protorhus longifolia*. Mean inhibition zone (mm) (p=0.001). Different superscripts upper-case letters in each column indicate significant differences (subsets)

Fungal isolates	Treatment							
	Afugan	**Biotaine**	**Celest**	**Nipastat**	**Odeon**	**Orius**	**Repenit**	**Sporekill**
Acremonium sp.	0^{A}	31^{F}	0^{A}	20^{D}	11^{C}	23^{DE}	0^{A}	35^{G}
Alternaria sp.	9^{B}	35^{G}	0^{A}	16^{CD}	11^{C}	0^{A}	0^{A}	32^{G}
Aspergillus sp.	0^{A}	27^{E}	0^{A}	18^{D}	27	90^{G}	0^{A}	32^{G}
C. cladosporioides	90^{E}	35^{G}	0^{A}	17^{CD}	20^{D}	24^{DE}	0^{A}	33^{G}
C. gloeosporioides	0^{A}	22^{C}	0^{A}	12^{B}	10^{C}	18^{C}	0^{A}	25^{E}
Cytospora sp.	14^{CD}	15^{B}	0^{A}	8^{A}	0^{A}	10^{B}	17^{B}	16^{B}
Fusarium sp.	9^{B}	22^{C}	0^{A}	14^{BC}	0^{A}	2^{A}	0^{A}	28^{F}
H. atroviridis	9^{B}	23^{D}	0^{A}	11^{A}	0^{A}	13^{B}	0^{A}	11^{A}
Irpex sp.	16^{D}	35^{E}	0^{A}	15^{C}	0^{A}	30^{F}	0^{A}	25^{E}
P. adametzioides	0^{A}	28^{E}	0^{A}	20^{D}	22^{DE}	24^{DE}	0^{A}	25^{E}
P. brevicompactum	0^{A}	17^{B}	0^{A}	9^{A}	26^{F}	25^{E}	0^{A}	21^{D}
P. chrysogenum	11^{BC}	2^{A}	0^{A}	13^{BC}	0^{A}	22^{D}	0^{A}	18^{C}
P. lilacinus	11^{BC}	27^{E}	0^{A}	20^{D}	23^{E}	16^{C}	0^{A}	28^{F}
P. olsonii	12^{C}	2^{A}	0^{A}	10^{A}	0^{A}	16^{C}	15^{B}	14^{B}
P. polonicum	19^{D}	28^{E}	0^{A}	12^{B}	1^{B}	27^{E}	0^{A}	25^{E}
Penicillium sp.	0^{A}	20^{C}	0^{A}	12^{B}	28^{F}	23^{DE}	0^{A}	20^{CD}
Phialemonium sp.	19^{D}	28^{E}	0^{A}	12^{B}	1^{B}	27^{E}	0^{A}	25^{E}
T. asperellum	0^{A}	28^{E}	90^{B}	17^{CD}	11^{C}	90^{G}	0^{A}	23^{DE}

LSD of means at 5% = 2.9

The data indicate that the most effective chemical control agents could have been Orius, Sporekill, Biotane and Nipastat. However, both Biotaine (surface decontaminant) and Nipastat (preservative) had the most consistent activity against the isolates and therefore these two were considered for further assessment in developing seed decontamination protocols (refer to Section 2.10).

3.3. The effects of developed protocols on the quality and internal fungal status of *T. dregeana*, *P. longifolia* and *G. livingstonei* seeds

3.3.1. (a) Seed vigour assessments on moistened paper towel in a Petri dish

It was important to assess seed vigour as this gave insight into the impact of protocols on seed germination, and whether there were differences amongst treatments in influencing germination levels. Figures 17 and 18 show seed growth and growth trend whereas Table 12 shows effect of treatments on the external morphology of the seed. However, for purposes of this study only results for seeds that were stored for 64 days for *T. dregeana* and *G. livingstonei* and for 16 days for *P. longifolia* are discussed as they showed some differences.

Figure 18A – All the seeds of *T. dregeana* started germinating around day 3. Germination levels for treatments DBe, DEBBe, DEBN and DEn started rising as from day 7 to day 15 and then entered short stationary growth phase. With most treatments root growth ceased at day 21, except for DEnN which had a slower root growth rate but continued growing past day 21. From these results one can conclude that treatment DEnN seedlings had more potential when compared to the other treatments.

Figure 18B - A slight differentiation on root emergence was observed with *P. longifolia* seeds and this was more noticeable with treatments DEBBe and DEBN. Seeds appeared brown and dead with no emergence of root growth (Figure 17). However, treatments DBe, DEn and DEnN had a positive response. Seeds exposed to these treatments showed a good growth curve, whereby first root emergence was noticeable from day 3 and thereafter, an exponential growth phase of roots was observed for both treatments DBe and DEnN up to day 13. Treatment DEn resulted in similar root growth but greater than that of DBe and DEnN treated seeds. It was also noted that root emergence of encapsualated seeds

delayed by at least 2 days, which is possibly a consequence of the alginate gel holding the cotyledons together tightly.

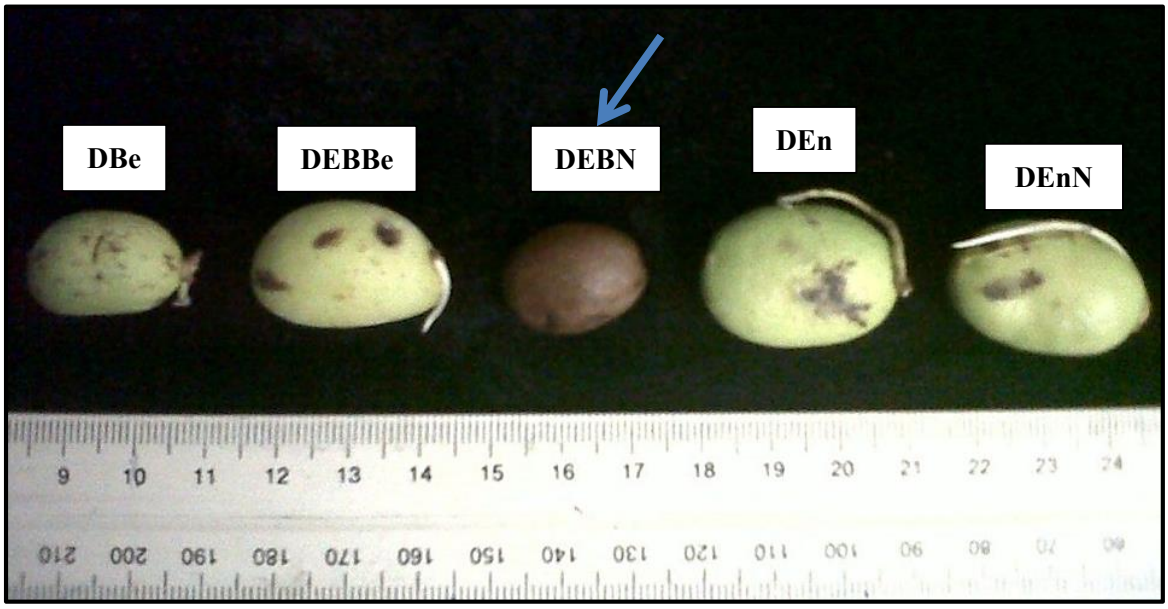

Figure 17: Seeds of *G. livingstonei* after seed vigour assessment for 30 d

Figure 18C – A longer lag phase was observed with *G. livingstonei* seeds when compared with those of *T. dregeana* and *P. longifolia*. Treatment DEnN treated seedsshowed first emergence of root around day 9 which thereafter showed a progressive exponential phase up until day 30. A similar pattern was observed with treatment DEn, however first root emergence was around day 13. Treatments DBe and DEBBe showed root emergence only around day 25, whereas treatment DEBN had a negative impact on seeds.

In summary, treatment DEBN was harmful to seeds of *P. longifolia* and *G. livingstonei* which led to no root emergence. None of the developed treatment protocols had a detrimental impact on *T. dregeana* seeds. Treatment DEnN was the best for both *T. dregeana* and *G. livingstonei* whereas for seeds of *P. longifolia*, treatment DEn was the best. Order of best treatments: DEnN, DEn, DBe, DEBBe and DEBN.

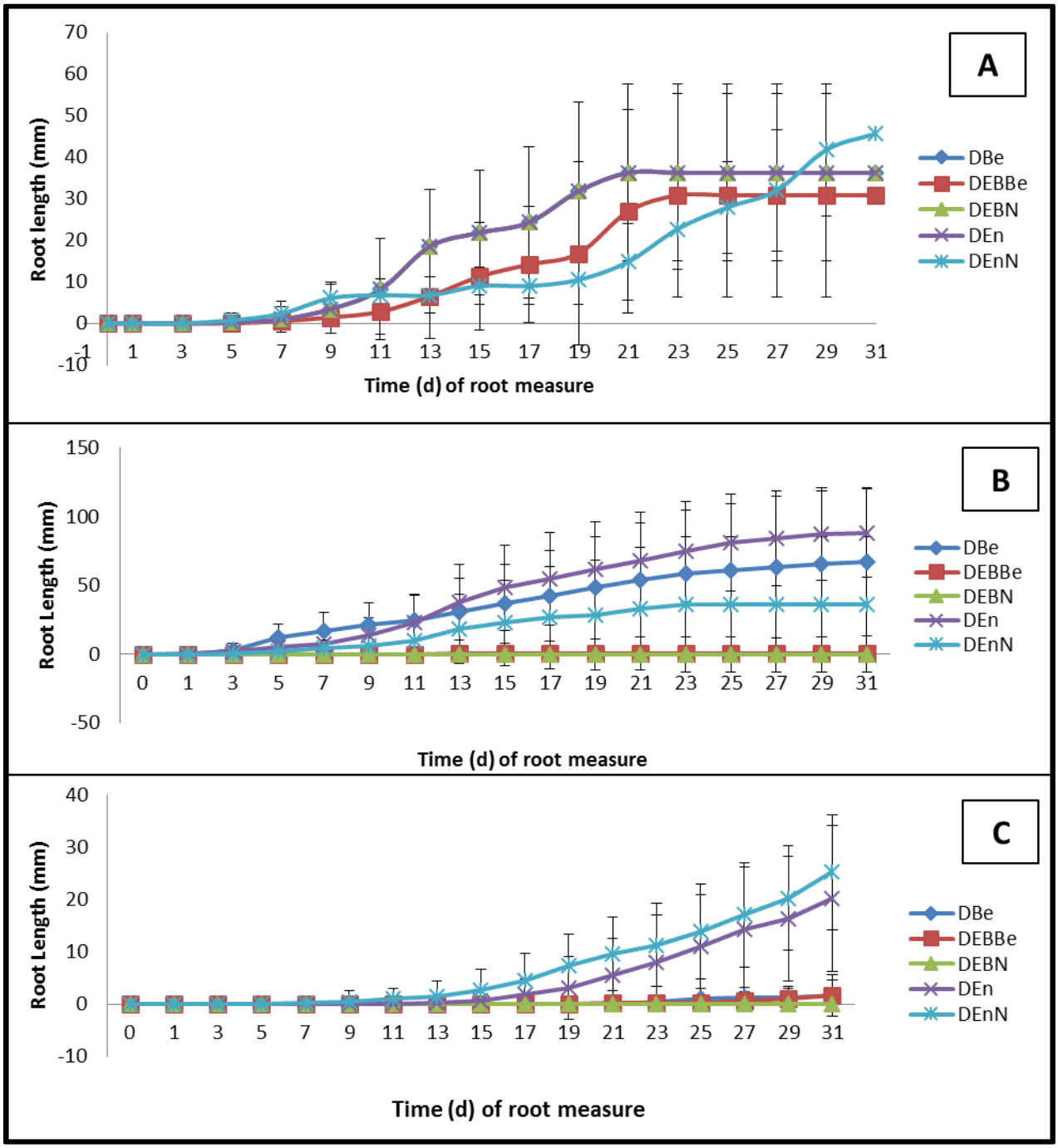

Figure 18: Germination of seedlings produced by seeds after being stored hydrated for 64 d (A & C); for 16d (B). Root growth was assessed for 30 d on moistened filter paper (n = 25).

A *- T. dregeana;* ***B*** *- P. longifolia;* ***C*** *- G. livingstonei*

3.3.1. (b) Comparisons amongst treatments and hydrated storage on seed vigour

Comparison amongst treatments and hydrated storage effects on seed vigour (Table 12) assisted in highlighting the major differences within plant species between treatments. For *T. dregeana* trials treatments DBe did not show much difference with hydrated storage time as observed from day 0 to day 16, however, days 32 and 64 were significantly different from days 0, 1, 4 and 16. Storage days 32 and 64 were not different when compared to each other. However seeds that had been stored for 64 days produced seedlings with the longest roots (on average 54 mm). Treatment DBe served as a control against all other treatments. It was observed that the control seedlings had shortest roots at day 0, however neither treatment nor storage time seemed to have affected the seed vigour as hydrated storage days increased.

Comparing average root length for treatment DEBBe at day 0 and day 64 no detrimental effects were observed and it was noted that roots were longest at day 16 (50 mm). Similarily to treatment DBe, seeds subjected treatment DEBN had the longest roots at day 64 (54 mm) and was different when compared to storage days 0, 1, 2, 4 and 6. No differences were noted when further compared to days 16 and 32. A similar trend was noted for treatment DEn, a longer average root was obtained at 64 d of HS (56 mm). This was the longest root length recorded amongst all assessed treatments whereas treatment DEnN was the most consistent and no significant differences were observed at days 0, 1, 2, 4, 6, 16, 32 and 64. Seeds that were initially stored for 32 days produced the longest root length (49 mm) for this treatment.

G. livingstonei trials (Table 12) showed high sensitivity towards treatment and storage times. The lowest roots were for DEBN treated seeds at storage days 16, 32 and 64 (0 mm). DEn treated seeds that had been stored for 32 days produced the longest roots (42 mm). These seeds were significantly different when compared with storage days 0, 1, 2, 4, 6, 16 and 64. Seeds treated using DEnN also had moderately long rootsregardless of storage times. The produced roots lengths were ranging from 16 – 32 mm and the longest achieved for those stored for 4 days (33 mm). It was also noted that for this particular plant species, those seeds that were treated using treatments DEn and DEnN and stored for 32 and 64 days had

produced roots the length of which did not differ when compared to those that were not exposed to storage (day 0). Therefore these treatments and storage times had no detrimental impacts as they produced results similar to the control treatment DBe.

P. longifolia results were similar to that of *Garcinia* sp. as a similar sensitivity was observed for seeds exposed to both treatment and hydrated storage. When comparing those seeds initially treated with DBe and had not been exposed to storage at (i.e. day 0), their roots ranged from 46 – 106 mm and those exposed to other treatments (i.e. DEBBe, DEBN, DEn and DEnN) and hydrated storage from days 16 – 64 ranged from 0 – 67 mm. The longest rootswere achieved by those seeds that were treated with treatment DBe (141 mm), stored for 4 days. Seeds stored for 4 days regarless of treatment produced the longest roots throughout except for those treated using DEBN. Treatment DEBN affected the seeds the most as the last germinating seeds were observed for those stored up to 1 day only.

In summary *P. longifolia* plant seeds were most affected by treatment and storage time followed by *Garcinia livingstonei*. *Trichilia dregeana* was the least affected and the impact was positive (i.e. roots were longer for longer stored seeds and also when comparing to those that were treated using the control treatment [DBe]). Overall, treatments DEn and DEnN were best performing treatments (see Figure 20 A, B, C and Table 12).

Table 12: Comparisons amongst treatments and hydrated storage effect on seed vigour. Root length was measured at 2 day interval for 31 days (in mm), however, for statistical analysis only results at 31 days were considered.

Plant species	Treatment	Hydrated storage (*d*)							
		Average root length (mm)							
		0	**1**	**2**	**4**	**6**	**16**	**32**	**64**
T. dregeana	**DBe**	40^{A}	42^{A}	45^{A}	40^{A}	42^{A}	42^{A}	52^{B}	54^{B}
	DEBBe	45^{AB}	41^{AB}	38^{A}	40^{AB}	39^{AB}	50^{B}	49^{B}	46^{B}
	DEBN	48^{AB}	48^{AB}	46^{AB}	44^{AB}	41^{A}	50^{BC}	52^{C}	54^{C}
	DEn	42^{AB}	44^{AB}	45^{AB}	42^{A}	45^{AB}	49^{BC}	49^{AB}	56^{C}
	DEnN	47^{A}	47^{A}	45^{A}	43^{A}	48^{A}	46^{AB}	49^{A}	46^{A}
G. livingstonei	**DBe**	17^{B}	17^{B}	15^{B}	14^{B}	24^{C}	21^{C}	9^{AB}	2^{A}
	DEBBe	15^{A}	20^{A}	12^{A}	10^{A}	10^{A}	10^{A}	10^{A}	10^{A}
	DEBN	12^{B}	16^{B}	8^{AB}	11^{B}	7^{AB}	0^{A}	0^{A}	0^{A}
	DEn	23^{BC}	12^{AB}	17^{AB}	22^{BC}	9^{A}	31^{C}	42^{D}	20^{B}
	DEnN	26^{AB}	19^{A}	21^{A}	33^{B}	16^{A}	16^{A}	32^{B}	25^{AB}
P. longifolia	**DBe**	106^{C}	107^{C}	89^{BC}	141^{D}	112^{CD}	67^{B}	4^{A}	0^{A}
	DEBBe	50^{B}	23^{AB}	0^{A}	55^{B}	30^{B}	0^{A}	0^{A}	0^{A}
	DEBN	46^{B}	41^{B}	0^{A}	0^{A}	0^{A}	0^{A}	19^{AB}	0^{A}
	DEn	106^{B}	88^{B}	104^{B}	115^{B}	90^{B}	88^{B}	0^{A}	0^{A}
	DEnN	84^{C}	68^{C}	54^{BC}	115^{D}	88^{CD}	36^{B}	19^{AB}	0^{A}

LSD of means at 5% = 7.205 for *T. dregeana*; LSD of means at 5% = 10.11 for *G. livingstonei*; LSD of means at 5% = 29.4 for *P. longifolia.* $p \leq 0.05$ = significant =* Different superscripts upper-case letters in each row indicate significant differences.

3.3.1 In culture assessments of contamination levels, root and shoot development and average root length

The preliminary investigations led to the development of seed treatment protocols as outlined in methodology, Chapter 2. These were applied to seeds as treatments, the seeds were placed into HS and sampled periodically and set out to germinate. Seeds were inspected to assess contamination, root and shoot development and average root length (assessed at the end of 30 days in culture). Results discussed are focusing at 64 days of HS for both *T. dregeana* and *G. livingstonei* and at 16 days HS for *P. longifolia*.

Table 13 shows results after 64 d in HS for *T. dregeana* seeds, placed in culture for 30 d, with overall contamination being maintained below 6 explants for all five treatments. Treatments DEBBe, DEBN and DEnN were not statistical different, but were different to treatments DBe and DEn. Treatments DEBBe, DEBN and DEnN were 100% efficient in eliminating contamination since no explant showed signs of infection. However treatments DBe (4 explants were infected) and DEn (with 5 explants showing infection) were not efficacious. Treatment DEnN had a significantly higher yield of roots and shoots developed (100%), whereas the other treatments (DBe, DEBBe and DEn) had a range from 5 to 20 explants. Root length was longest with treatment DEn (56 mm) followed by treatment DEnN seedlings (46 mm) although the difference was not significant from the other treatments (root lengths raging from 31 – 56 mm). Concluding for *T. dregeana*, treatment DEnN was the best treatment considered for short to medium term storage, followed by DEn, DEBN, DEBBe and lastly DBe.

Similarily, the protocols also showed good results for *G. livingstonei* seeds (Table 13) regarding elimination of contamination. Treatment DEnN completely prevented growth of fungi, with 100 % of seeds being unblemished and germinable even after 6 months in HS (results not shown). In contrast, most of treatment DBe seeds (untreated) had developed discoloured spots and showed signs of infection. Highest contamination levels were obtained if DBe and DEn treatments were applied (7 and 5 explants showing infection respectively). On average 1 explant was contaminated after DEBBe and DEBN

treatments. Treatment DEnN resulted in with highest root development (24 explants) and treatment DEBN resulted in to have the least (only 7 explants). Highest shoot development was however obtained with treatment DEBBe (24 explants), followed by treatments DBe and DEnN (23 explants). The least shoots developed were obtained with treatment DEBN (only 9 explants) and also had the shortest roots development which averaged at 15 mm. Treatment DEnN again gave rise to longest roots which averaged at 63 mm; this was not different from treatments DBe and DEn but was different from DEBBe and DEBN. It was therefore concluded that for this plant species treatment DEnN was the best, followed by DEBBe, DBe, DEn and lastly DEBN.

In contrast to *P. longifolia* seeds (results not shown), treatments DEBBe and DEBN were the only treatments where seeds appeared to have survived for 64 d in HS, with no visible contamination. However, after 30 d on WA, seeds had not yet germinated. Moreover, after 32 d of HS treatment DEBBe, emerged as a better treatment, achieving 40% and greater than 60% of seeds having developed roots and shoots, respectively. Of the 20% of seeds showing contamination, none germinated. Therefore, it was decided to focus more on day 16 of HS to give us a better understanding of all treatments effects on seeds. Results on day 16, however, were similar to those at days 32 and 64 in terms of overall treatments effects on seeds. Treatments DEBBe and DEBN, maintained low levels of contamination (1 and 4 explants infected) when compared to treatments DBe, DEn and DEnN (which each had 5 explants being infected). Percentage roots developed was also higher with treatments DEBBe and DEBN (i.e. 10 and 12 explants developing roots). Similarly, for shoot developed, 21 and 17 were obtained for DEBBe and DEBN treatments, respectively. However, average root length was different with values of 9 and 11 mm. Treatment DEn gave the most undesirable results with no root development and only 3 explant produced shoots; followed by treatment DBe which had 5 explants developing root and also 5 explants developing shoots. Treatment DEnN performed better when compared to treatment DEn and DBe, achieving root and shoot development with 9 explants. Even though treatment DEBBe had the lowest contamination levels when compared to treatment DEBN, DEn and DBe, treatment DEBN was chosen as the best treatment as it had produced seedlings with higher yields of shoots and roots being obtained with this

treatment. Therefore we finally concluded that DEBBe, DEnN, DBe and lastly DEn were the least performing.

Table 13: In culture trials after 64 days storage of *T. dregeana* and *G. livingstonei* seeds and 16 days storage of *P. longifolia* seeds.

$p \leq 0.05$ = significant =* Different superscripts upper-case letters in each row indicate significant differences.

Plant Species	Sample no.	Observation @ 30d in culture	Statistical analysis used	Treatment(s) DBe	DEBBe	DEBN	DEn	DEnN
T. dregeana	25	No. of Cont. explants	χ^2(4d.f. cont vs non. cont) = 14.847; *p=0.005*	4^{b}	0^{a}	0^{a}	5^{b}	0^{a}
		No. of Roots developed	χ^2(4d.f. roots dev. vs none) = 14.098; *p =0.007*	16^{a}	20^{b}	15^{a}	20^{b}	25^{c}
		No. of Shoots developed	χ^2(4d.f. shoots dev. vs none) = 38.37; *p=0.0005*	16^{b}	5^{a}	15^{b}	20^{c}	25^{d}
		Avg. length of roots (mm)	One way ANOVA; *p = 0.0005*; F=7.691; *df* =4	36^{ab}	31^{a}	36^{ab}	56^{c}	46^{bc}
G. livingstonei	25	No. of Cont. explants	χ^2(4d.f. cont vs non. cont) = 14.847; *p=0.005*	7^{b}	1^{a}	1^{a}	5^{b}	0^{a}
		No. of Roots developed	χ^2(4d.f. roots dev. vs none) = 40.494; *p =0.005*	23^{c}	19^{b}	7^{a}	21^{bc}	24^{c}
		No. of Shoots developed	χ^2(4d.f. shoots dev. vs none) = 40.945; *p=0.0005*	23^{b}	24^{b}	9^{a}	22^{b}	23^{b}
		Avg. length of roots (mm)	One way ANOVA; *p = 0.0005;* F=10.942; *df* = 4	43^{bc}	38^{b}	15^{a}	54^{bc}	63^{c}
P. longifolia	25	No. of Cont. explants	χ^2(4d.f. cont vs non. cont) = 3.571; *p=0.467*	5^{b}	1^{a}	4^{b}	5^{b}	5^{b}
		No. of Roots developed	χ^2(4d.f. roots dev. vs none) = 17.712; *p =0.001*	5^{b}	10^{c}	12^{c}	0^{a}	9^{c}
		No. of Shoots developed	χ^2(4d.f. shoots dev. vs none) = 38.961; *p=0.0005*	5^{a}	21^{d}	17^{c}	3^{a}	9^{b}
		Avg. length of roots (mm)	One way ANOVA; *p = 0.003; F=4.241; df =4*	1^{a}	9^{ab}	11^{b}	0^{a}	6^{ab}

3.4. Effects of treatment and hydrated storage on developmental aspects of seedlings grown *ex vitro* for six months after germination

Biomass allocation of seedlings grown in pot trials was one of the important physiological aspects to monitor and assess. This served as an additional measure to ascertain whether the physiology of seedlings was affected by treatment protocols as conditions outside the greenhouse in pot trials are much more diverse as compared to culture conditions in the laboratory. Biomass allocation of plantlets (Figures 19, 20 and 21) therefore involved assessments of total leaf area per plant, dry biomass of leaves, roots and stem, stem diameter and leaf lengths.

Figure 19: *G. livingstonei* plantlets harvested after six months

Figure 20: *P. longifolia* plantlets harvested after six months

Figure 21: *T. dregeana* plantlets harvested after six months

3.4.1. Effects of treatment and storage on mean leaf area

Trials on *T. dregeana* (Tables 14 and 22) showed that both seed treatment and storage time had a significant impact on leaf area (p=0.001) with mean leaf area values ranging from 291 to 609 mm^2. In particular the longer the storage period the greater the impact. In this regard the leaf area of the control (0 days) of treatment DBe was 609 mm^2 but for plants obtained from seeds that had been stored for 64 days it had decreased to 166 mm^2.

Similarily *Garcinia* sp. trials (Tables 14 and 22) showed a similar trend where the longer seeds were stored produced plants with smaller leaf area, irrespective of the treatment that the seeds were initially exposed to. In addition, it was also observed that the impact caused by both treatment and storage time was significant and had a negative impact on the leaf area (p= 0.001). Leaf area obtained for *Garcinia* sp. was smaller when compared to those of *Trichilia* plant species.

Observations on *P. longifolia* trial (Table 14 and 22) were similar to *Garcinia* sp. regarding smaller leaf area and the trend observed with both *Trichilia* sp. and *Garcinia* sp. plants where the longer stored seeds tended to produce plants with smaller leaf area disregarding the treatment that it was initially exposed to. Leaf area of *P. longifolia* ranged from (0 to 59 mm^2). *P. longifolia* was affected the most eventhough it range was wider when compared to those of *Garcinia* sp. trials. Both treatment and storage time had a significant impact on leaf area ($p = 0.002$).

In summary, leaf area of these three plant species was negatively affected by both treatment and storage time. However, treatment DEn and DEnN were the best for all *Trichilia* sp. storage periods. However, for *Garcinia* sp. and *Protorhus* sp., seed that had been initially treated with DEn and DEnN exposed to storage for 6 days produced the largest leaf area when compared to control treated seeds that were not exposed to storage.

Table 14: Treatment and storage impact on leaf area (mm^2) *(T. dregeana: p = 0.001; G. livingstonei: p = 0.0005; P. longifolia: p = 0.0005).*

Plant species	Treatment	Hydrated Storage (*d*)							
		Leaf Area (mm^2)							
		0	**1**	**2**	**4**	**6**	**16**	**32**	**64**
T. dregeana	DBe	609^{C}	376^{B}	371^{B}	389^{B}	379^{B}	320^{B}	315^{B}	166^{A}
	DEBBe	453^{BC}	539^{C}	498^{BC}	357^{ABC}	333^{AB}	313^{A}	417^{ABC}	359^{AB}
	DEBN	414^{AB}	466^{B}	396^{AB}	418^{AB}	324^{A}	389^{AB}	370^{AB}	316^{A}
	DEn	474^{BC}	517^{C}	461^{ABC}	381^{AB}	439^{ABC}	354^{AB}	340^{A}	454^{ABC}
	DEnN	291^{A}	368^{A}	371^{A}	350^{A}	348^{A}	301^{A}	317^{A}	355^{A}
G. livingstonei	DBe	6^{A}	11^{B}	16^{C}	4^{A}	5^{A}	5^{A}	4^{A}	2^{A}
	DEBBe	12^{C}	10^{C}	9^{BC}	7^{B}	8^{BC}	5^{B}	6^{B}	1^{A}
	DEBN	10^{C}	3^{AB}	5^{B}	3^{AB}	1^{AB}	0^{A}	0^{A}	0^{A}
	DEn	7^{B}	4^{AB}	10^{B}	7^{B}	10^{B}	7^{B}	2^{A}	3^{A}
	DEnN	15^{D}	11^{C}	10^{C}	8^{BC}	8^{BC}	6^{B}	7^{BC}	2^{A}
P. longifolia	DBe	6^{A}	43^{BC}	0^{A}	8^{AB}	59^{C}	24^{B}	0^{A}	0^{A}
	DEBBe	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	2^{A}	0^{A}	0^{A}
	DEBN	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEn	2^{A}	6^{A}	10^{A}	21^{A}	49^{B}	13^{A}	8^{A}	0^{A}
	DEnN	6^{A}	9^{A}	0^{A}	0^{A}	45	16^{A}	0^{A}	0^{A}

LSD of means at 5% = 129.03 for *T.dregeana*, LSD of means at 5% = 3.8 for *G. livingstonei and* LSD of means at 5% = 21.7 for *P. longifolia.* $p \leq 0.05$ = significant =* Different superscripts upper-case letters in each row indicate significant differences.

3.4.2. Effects of treatment and storage on mean leaf dry mass

Assessments of total leaf dry mass of *T. dregeana* (Table 15 and 22) irrespective of storage time ($p = 0.47$) showed no differences regardless of treatment ($p = 0.39$). After six months growth period the average leaf biomass of the control plants was 0.49 g (i.e. non-stored seeds) whereas the biomass of other plants ranged from 0.26 to 0.68 g. Control treatment DBe at storage day 64 resulted in the lowest leaf biomass of (0.15 g) whereas treatment DEBN resulted in the largest leaf biomass of 0.34 g when compared with the control treatment (DBe) and the other treatments (DBBe, DEn and DEnN). Therefore, it was summarized that total leaf biomass was neither negatively nor positively influenced by these factors.

Contrarily, *G. livingstonei* trials (Tables 15 and 22) showed that DEBN treated seeds produced plantlets that had the smallest number of leaves thus giving the smallest leaf biomass ranging from 0.0 to 0.211 g. However, it was evident from the results (Tables 15 and 22) that treatment, storage and treatment*storage had a *p* value of 0.001 thus showing significance throughout all treatments. It was further observed that day 0 resulted in biomasses that were higher when compared to those of days 16, 32 and 64. A similar trend was observed for those stored for 1, 2, 4 and 6 days, however not consistent. It was also noted that at day 64 better leaf yields were obtained with seeds initially exposed to treatments DEn and DEnN (i.e weighing at 0.04 and 0.03 g). However, all the factors assessed for this plant species showed a negative influence on leaf mass.

P. longifolia trials (Tables 15 and 22) showed that this plant species was most sensitive when compared to *T. dregeana* and *G. livingstonei* with regard to leaf biomass. Both treatment and storage had a significant impact on leaf biomass ($p = 0.001$), and also differences were observed on where the two factors interact ($p = 0.008$). The highest biomass of 1 g was obtained for those seeds initially treated using treatment DBe and that had been stored for 6 days.

Table 15: Treatment and storage impact on leaf dry mass (g). (*T. dregeana*: $p = 0.386$; *G. livingstonei: p = 0.0005; P. longifolia: p = 0.0005*).

Plant species	Treatment	Hydrated Storage (*d*)							
		Leaf Dry Biomass (g)							
		0	**1**	**2**	**4**	**6**	**16**	**32**	**64**
T. dregeana	DBe	0.49^{A}	0.33^{A}	0.28^{A}	0.40^{A}	0.29^{A}	0.24^{A}	0.28^{A}	0.15^{A}
	DEBBe	0.40^{A}	0.44^{A}	0.40^{A}	0.30^{A}	0.28^{A}	0.32^{A}	0.42^{A}	0.29^{A}
	DEBN	0.32^{A}	0.37^{A}	0.32^{A}	0.36^{A}	0.27^{A}	0.33^{A}	0.25^{A}	0.34^{A}
	DEn	0.37^{A}	0.68^{A}	0.40^{A}	0.37^{A}	0.38^{A}	0.31^{A}	0.25^{A}	0.30^{A}
	DEnN	0.26^{A}	0.26^{A}	0.28^{A}	0.28^{A}	0.27^{A}	0.24^{A}	0.26^{A}	0.24^{A}
G. livingstonei	DBe	0.090^{B}	0.155^{BC}	0.190^{C}	0.040^{AB}	0.055^{AB}	0.061^{AB}	0.051^{AB}	0.02^{A}
	DEBBe	0.149^{BC}	0.160^{BC}	0.103^{B}	0.078^{AB}	0.191^{C}	0.062^{AB}	0.071^{AB}	0.018^{A}
	DEBN	0.133^{B}	0.033^{A}	0.055^{A}	0.038^{A}	0.211^{C}	0^{A}	0^{A}	0^{A}
	DEn	0.098^{B}	0.068^{AB}	0.101^{B}	0.091^{B}	0.120^{B}	0.009^{A}	0.037^{A}	0.039^{A}
	DEnN	0.234^{C}	0.133^{B}	0.127^{B}	0.093^{B}	0.091^{AB}	0.074^{AB}	0.076^{AB}	0.026^{A}
P. longifolia	DBe	0^{A}	0^{A}	0^{A}	0^{A}	1^{B}	0^{A}	0^{A}	0^{A}
	DEBBe	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEBN	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEn	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEnN	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}

LSD of means at 5% =1.745 for *T.dregeana*, LSD of means at 5% = 0.066 for *G. livingstonei and* LSD of means at 5% =0.2 for *P. longifolia.* $p \leq 0.05$ = significant =* Different superscripts upper-case letters in each row indicate significant differences.

3.4.3. Effects of treatment and storage on mean root biomass

In the *T. dregeana* trials (Tables 16 and 22), both treatment ($p = 0.04$) and storage ($p = 0.001$) produced plants with a root biomass that showed significant impact , however, where the two factors interact ($p = 0.277$), no effects on root biomass were observed. Seeds treated with DBe at 0 day of storage had a root biomass that averaged at 0.61 g with the biggest biomass being obtained from plants that emerged from seeds that were in storage for 2 days and 4 days with treatments DEBBe and DEBN which both averaged

0.67 g. Seeds that had been initially exposed to 64 days storage produced plantlets that had the smallest root biomass of 0.25 g for control treatment DBe whereas the other treatment ranged from 0.36 g to 0.55 g. Treatment DEBN had a positive impact on root biomass when considering time of storage at 64 days even though the seed appearance after treatment was not good (i.e. seeds had brown patches). Impact observed for seeds treated with treatment DEBBe was the same for days 1, 2, 4, 6, 16 and 32.

In the *G. livingstonei* trials (Table 16 and 22) seeds that were not stored and treated using DBe produced on an average of 0.291 g root biomass, which was much less than that produced by *T. dregeana*. However, it was noted that at day 4 regardless of storage showed a negative impact on root biomass produced by *Garcinia* sp. and was significantly different when compared to that of day 0 for *Trichilia* sp. trials. Seeds treated using treatment DEBN did not produce any roots after 16 days HS. Whereas, treatment DEn was a better performing treatment and was consistant.

Protorhus sp. plantlets responded in a similar manner when compared to those of *Garcinia* sp. (Tables 16 and 22), however these seeds were most sensitive hence no root biomass obtained was 0 g. Root biomass obtained with this plant species was very small irrespective of treatment or storage time (i.e. root biomass was rounded of to zero). Since they were also no significant differences observed regarding effects on root biomass we could not summarize which was the better performing treatment or storage time for this plant species.

Table 16: Treatment and storage impact on root dry mass (g) *(T. dregeana: p = 0.042; G. livingstonei: p = 0.0005; P. longifolia: p = 0.0005).*

Plant species	Treatment	Hydrated Storage (*d*)							
		Root Dry Biomass (g)							
		0	**1**	**2**	**4**	**6**	**16**	**32**	**64**
T. dregeana	DBe	0.61^{BC}	0.64^{BC}	0.56^{BC}	0.65^{C}	0.58^{BC}	0.43^{AB}	0.41^{AB}	0.25^{A}
	DEBBe	0.54^{AB}	0.67^{B}	0.67^{B}	0.59^{AB}	0.66^{B}	0.56^{AB}	0.66^{B}	0.42^{A}
	DEBN	0.51^{AB}	0.45^{A}	0.47^{AB}	0.67^{B}	0.50^{AB}	0.56^{AB}	0.47^{AB}	0.55^{AB}
	DEn	0.57^{A}	0.60^{A}	0.61^{A}	0.57^{A}	0.53^{A}	0.60^{A}	0.42^{A}	0.43^{A}
	DEnN	0.38^{AB}	0.50^{AB}	0.56^{AB}	0.54^{AB}	0.55^{AB}	0.58^{B}	0.40^{AB}	0.36^{A}
G. livingstonei	DBe	0.291^{B}	0.315^{B}	0.460^{C}	0.144^{AB}	0.221^{B}	0.127^{AB}	0.151^{AB}	0.028^{A}
	DEBBe	0.416^{C}	0.343^{C}	0.266^{BC}	0.264^{BC}	0.317^{BC}	0.210^{B}	0.178^{AB}	0.063^{A}
	DEBN	0.286^{C}	0.131^{B}	0.167^{BC}	0.097^{AB}	0.063^{AB}	0^{A}	0^{A}	0^{A}
	DEn	0.210^{AB}	0.219^{AB}	0.248^{B}	0.227^{B}	0.279^{B}	0.179^{AB}	0.108^{AB}	0.094^{A}
	DEnN	0.459^{C}	0.422^{C}	0.375^{C}	0.337^{BC}	0.340^{BC}	0.241^{BC}	0.295^{BC}	0.067^{A}
P. longifolia	DBe	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEBBe	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEBN	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEn	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEnN	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}

LSD of means at 5% = 0.206 for *T.dregeana*, LSD of means at 5% = 0.129 for *G. livingstonei and* LSD of means at 5% = 0.1 for *P. longifolia*. $p \leq 0.05$ = significant =* Different superscripts upper-case letters in each row indicate significant differences.

3.4.4. Effects of treatment and storage on mean stem biomass

T. dregeana trials (Tables 17 and 22) showed that stem biomass was significantly affected by seed treatment and storage. After six months growth the control stem biomass averaged 0.50 g whereas for other plants, obtained fromDEBBe, DEBN, DEn and DEnN treated seeds, ranged from 0.22 to 0.47 g. It appeared that increasing storage period had a positive impact on stem biomass with the largest stem biomass of (0.64 g)

being obtained from DEBBe treated seeds stored for 32 d followed by treatment DEn treated seed stored for 64 days (0.51 g).

G. livingstonei trials (Tables 17 and 22) showed that treatment DEBN resulted in the smallest stem dry biomass ranging from 0 to 0.11 g and showed differences when compared with the other treatments. Treatment DEnN was a better performing treatment when compared to other treatments and seeds initially treated using this treatment produced stems, which weight ranged from 0.04 – 0.14 g. Treatment DEBBe treated seeds produced two stems per embryonic axis (Figure 25), especially those stored for 1 day, however these were thin in diameter and therefore did not make a noticeable difference in the final stem dry biomass but certainly ensured a better chance of survival. It was further noted that day 0 and day 1 of DEBBe treated seeds were not different when compared, however day 1 was significantly different when compared to days 2, 4, 6, 16, 32 and 64.

P. longifolia plantlets responded in a similar way when compared to those of *Garcinia* sp. (Tables 16 and 22), in summary the treatment or storage time or both, caused (or resulted in) death of seeds irrespectively. Since they were also no significant differences observed regarding effects on stem biomass we could not distinguish from a better or worst performing treatment or storage time for this plant species.

Table 17: Treatment and storage impact on stem dry mass (g). (*T. dregeana*: *p = 0.008; G. livingstonei: p = 0.0005; P. longifolia: p = 0.0005).*

Plant species	Treatment	Hydrated Storage (*d*)							
		Stem Biomass (mm)							
		0	**1**	**2**	**4**	**6**	**16**	**32**	**64**
T. dregeana	DBe	0.50^{BC}	0.35^{AB}	0.27^{AB}	0.57^{C}	0.33^{AB}	0.33^{AB}	0.49^{BC}	0.22^{A}
	DEBBe	0.47^{AB}	0.44^{A}	0.44^{A}	0.38^{A}	0.36^{A}	0.44^{A}	0.64^{B}	0.44^{A}
	DEBN	0.35^{A}	0.36^{A}	0.37^{AB}	0.36^{A}	0.37^{A}	0.29^{A}	0.55^{B}	0.44^{AB}
	DEn	0.35^{AB}	0.64^{C}	0.42^{AB}	0.42^{AB}	0.41^{AB}	0.35^{AB}	0.29^{A}	0.51^{BC}
	DEnN	0.22^{A}	0.26^{A}	0.27^{AB}	0.33^{AB}	0.31^{AB}	0.29^{AB}	0.56^{C}	0.45^{BC}
G. livingstonei	DBe	0.058^{B}	0.118^{C}	0.161^{D}	0.057^{A}	0.079^{B}	0.073^{B}	0.057^{A}	0.017^{A}
	DEBBe	0.114^{BC}	0.135^{C}	0.077^{B}	0.089^{B}	0.1^{B}	0.076^{B}	0.054^{AB}	0.023^{A}
	DEBN	0.111^{C}	0.039^{AB}	0.06^{B}	0.042^{B}	0.027^{AB}	0^{A}	0^{A}	0^{A}
	DEn	0.091^{B}	0.089^{B}	0.112^{BC}	0.107^{BC}	0.137^{C}	0.081^{A}	0.049^{A}	0.046^{A}
	DEnN	0.128^{B}	0.115^{B}	0.122^{B}	0.104^{B}	0.137^{B}	0.104^{B}	0.097^{B}	0.041^{A}
P. longifolia	DBe	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEBBe	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEBN	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEn	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEnN	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}

LSD of means at 5% = 0.185 for *T.dregeana*, LSD of means at 5% = 0.04 for *G. livingstonei* and LSD of means at 5% = 0.1 for *P. longifolia.* $p \leq 0.05$ = significant =* Different superscripts upper-case letters in each row indicate significant differences.

Figure 22: *G. livingstonei* plantlet after six months growing in the field showing two stems that emerged from one embryonic axis observed only for treatment DEBBe

3.4.5. Effects of treatment and storage on mean leaf length

T. dregeana trials showed that regardless of storage time, treatment had a significant impact on leaf length (Tables 18 and 22, $p = 0.001$) . Leaf length of control plants, obtained from non-stored DBe treated seeds, reached 81 mm after six months growth, but for other plants ranged from 50 to 66 mm. This then showed that the treatments had a negative impact on leaf length with an exception of day 1 for all treatments excluding treatment DBe. With regard to storage irrespective of treatment and where the two factors interact also showed that the longer stored seeds the shorter the leaf length ($p = 0.001$). Plantlets yielded from seeds stored for 64 d had the shortest leaf lengths (28 mm) for treatment DBe and the longest leaf lengths (56

mm) for DEn treated seeds. Comparing days 0 and 64 for treatment DEnN, leaf length was longer at day 64 (53 mm).

G. livingstonei trials (Tables 18 and 22) showed that treatment and storage regardless of each other had a significant impact on leaf length ($p = 0.001$). Further assessing to where the two factors interact significant interaction was also observed ($p = 0.001$). DBe treated seeds at day 0 produced seedlings that had leaf lengths that averaged at 21 mm and when comparing to those that had been stored for 64 days, their leaf lengths were shorter (9 mm). These were also found to be significantly different when compared. The longest leaves were produced by plants obtained from those seeds that had been stored for 2 days (42 mm). Plants obtained from DEBN treated seeds were affected the most, and their leaf lengths ranged from 0 – 31 mm. When comparing days 0 and 64 for DEBBe and DEnN treated seeds, a significant difference was observed that was showing a negative impact on leaf length. However, DEn treated seeds showed no differences and resulted in longest leaf lengths (19 mm) when compared to other treatments producing a range from 0 to13 mm.

Similarily, *P. longifolia* trials (Tables 18 and 22) showed significant differences caused by treatments, storage and where the two factors interact ($p = 0.001$). However, a different trend was observed for this particular plant species where leaf lengths were shorter for those plants obtained from seeds that had not been stored. Plants that hat produced moderately longer leaves were those emerged from DBe, DEn and DEnN treated seeds, stored for 6 days. Treatments DEBBe and DEBN were the worst performing treatments. DEn treated seeds produced plantlets with longest leaves (58 mm) for those seeds that had been stored for 6 days. In summary, DEn treated seeds survived up to 32 days of storage whereas the other seeds did not suvive thus making it the best treatment with regard to leaf length.

Table 18: Treatment and storage impact on leaf length (mm). (*T. dregeana*: *p = 0.0005; G. livingstonei: p = 0.0005; P. longifolia: p = 0.0005).*

Plant species	Treatment	Hydrated Storage (*d*)							
		Leaf Length (mm)							
		0	**1**	**2**	**4**	**6**	**16**	**32**	**64**
T. dregeana	DBe	81^{D}	56^{C}	54^{BC}	51^{BC}	48^{BC}	46^{B}	50^{B}	28^{A}
	DEBBe	63^{C}	63^{C}	59^{BC}	52^{AB}	47^{A}	46^{A}	53^{AB}	51^{AB}
	DEBN	56^{BCD}	63^{CD}	60^{CD}	51^{AB}	49^{AB}	57^{BCD}	45^{A}	52^{ABC}
	DEn	66^{CD}	66^{CD}	63^{BCD}	49^{AB}	55^{AB}	50^{AB}	48^{A}	56^{ABC}
	DEnN	50^{A}	51^{A}	54^{A}	50^{A}	46^{A}	48^{A}	47^{A}	53^{A}
G. livingstonei	DBe	21^{B}	28^{B}	42^{C}	16^{AB}	23^{B}	20^{B}	18^{AB}	9^{A}
	DEBBe	36^{C}	29^{BC}	24^{B}	24^{B}	26^{BC}	24^{B}	20^{B}	8^{A}
	DEBN	31^{C}	10^{AB}	16^{B}	14^{B}	8^{A}	0^{A}	0^{A}	0^{A}
	DEn	24^{AB}	24^{AB}	34^{B}	29^{B}	31^{B}	29^{B}	16^{A}	19^{A}
	DEnN	46^{C}	32^{B}	31^{B}	30^{B}	30^{B}	27^{B}	27^{B}	13^{A}
P. longifolia	DBe	13	57	0^{A}	8^{A}	36^{B}	20^{AB}	0^{A}	0^{A}
	DEBBe	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	6^{A}	0^{A}	0^{A}
	DEBN	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEn	5^{A}	9^{A}	10^{A}	20^{A}	58^{B}	20^{A}	9^{A}	0^{A}
	DEnN	17^{A}	10^{A}	0^{A}	0^{A}	53^{B}	15^{A}	0^{A}	0^{A}

LSD of means at 5% = 8.807 for *T.dregeana*, LSD of means at 5% = 10.6 for *G. livingstonei and* LSD of means at 5% = 20.3 for *P. longifolia*. $p \leq 0.05$ = significant =* Different superscripts upper-case letters in each row indicate significant differences.

3.4.6. Effects of treatment and storage on mean root length

T. dregeana (Tables 19 and 22) showed that treatment irrespective of storage had an influence on root length (p = 0.001), storage days regardless of treatment also had an impact (p = 0.001) and furthermore where the two factors interact (p = 0.001). Control treatment DBe at day 0 produced plantlets that had an average root length of 215 mm which for other treatments ranged from 188 to 244 mm. Moreover, storage time regardless of treatment showed a negative impact on root growth of plants obtained from seeds stored for 64 d (94 mm) for DBe and longest for seeds stored for 6 days (300 mm) for treatment DEBBe. When

comparing day 0 for treatments DEBN, DEn and DEnN to day 64, these treatments resulted in growth of plants with longer roots when their seeds were stored for 64 days thus having a positive impact when compared to the control treatment DBe.

G. livingstonei trials (Tables 19 and 22) showed that both treatment and storage time had an influence on root length ($p = 0.001$). When DBe, DEn and DEnN treated seeds at day 0 were compared to those that had been stored for 64 days, no differences were observed. However, plants emerged from DEn treated seeds stored for 64 days produced longer roots (150 mm) as compared to plants obtained from non-stored seeds (138 mm). DEBBe treated seeds at day 0 produced plants with the longest roots (313 mm) whereas the others ranged from 138 -214 mm. Furthermore, when DEBBe treated seeds were stored for 64 days, plants with longer roots were produced (183 mm) when compared to other treated seeds only producing plants with a root length ranged from 0 to 160 mm. However, it was noted that DEn treated seeds did not produce the plants with the longest roots but obtained values were neither affected by storage time nor treatment.

P. longifolia trials (Tables 19 and 22) showed that treatment, storage time and where the factors interact had a significant impact on seeds ($p = 0.001$). DEBBe and DEBN treated seeds were affected negatively as most seeds did not germinate. Plants emerged from DEn treated seeds produced plants with the most consistent root lengths and the seeds could be stored for longer (i.e stored up to 32 days).

Table 19: Treatment and storage impact on root lengths (*T. dregeana*: *p = 0.002; G. livingstonei: p = 0.0005; P. longifolia: p = 0.002)*.

Plant species	Treatment	Hydrated Storage (*d*)							
		Root Length (mm)							
		0	**1**	**2**	**4**	**6**	**16**	**32**	**64**
T. dregeana	DBe	215^{BC}	264^{CD}	208^{BC}	242^{BCD}	281^{D}	249^{BCD}	198^{B}	94^{A}
	DEBBe	244^{ABC}	257^{BC}	238^{AB}	258^{BC}	300^{C}	266^{BC}	221^{AB}	194^{A}
	DEBN	188^{AB}	213^{AB}	177^{A}	274^{C}	253^{BC}	257^{BC}	238^{BC}	197^{AB}
	DEn	199^{AB}	160^{AB}	173^{AB}	192^{AB}	218^{ABC}	228^{BC}	270^{C}	216^{ABC}
	DEnN	216^{A}	192^{A}	208^{A}	249^{A}	201^{A}	229^{A}	196^{A}	219^{A}
G. livingstonei	DBe	152^{AB}	228^{B}	262^{B}	128^{AB}	167^{AB}	142^{AB}	118^{A}	84^{A}
	DEBBe	313^{B}	266^{AB}	228^{AB}	225^{AB}	256^{AB}	189^{A}	212^{A}	183^{A}
	DEBN	192^{B}	130^{B}	148^{B}	98^{AB}	110^{B}	0^{A}	0^{A}	0^{A}
	DEn	138^{A}	184^{A}	209^{A}	186^{A}	182^{A}	181^{A}	127^{A}	150^{A}
	DEnN	214^{AB}	320^{B}	317^{B}	259^{AB}	230^{AB}	237^{A}	337^{B}	160^{A}
P. longifolia	DBe	36^{A}	115^{B}	0^{A}	16^{A}	76^{B}	48^{AB}	0^{A}	0^{A}
	DEBBe	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	37^{A}	0^{A}	0^{A}
	DEBN	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEn	28^{AB}	17^{A}	17^{A}	32^{AB}	141^{C}	60^{B}	15^{A}	0^{A}
	DEnN	65^{B}	17^{A}	0^{A}	0^{A}	127^{C}	30^{AB}	0^{A}	0^{A}

LSD of means at 5% = 58.8 for *T.dregeana*, LSD of means at 5% = 107.1 for *G. livingstonei and* LSD of means at 5% = 51.5 for *P. longifolia*. $p \leq 0.05$ = significant =* Different superscripts upper-case letters in each row indicate significant differences.

3.4.7. Effects of treatment and storage time on mean stem diameter

T. dregeana trials (Tables 20 and 22) showed that treatment regardless of storage ($p = 0.001$) time and storage time ($p = 0.001$) regardless of treatment had a significant effect on stem diameter, but there were no significant intractions. Control treatment DBe at day 0 resulted in production of plantlets with a diameter averaging at 14 mm whereas with other treatments diameters ranged from 12 to 14 mm. Stem diameters

were largest with DEBBe treated seeds (35 mm) after seeds had been stored for 64 days, bigger stem diameters were also observed for this treatment at days 6, 16 and 32.

G. livingstonei trials (Tables 20 and 22) showed that all the factors assessed (i.e. both treatment and storage regardless of each other and where the two interact) had a significant influence on stem diameter (p = 0.001). At the end of the growth period, after seeds were not stored (i.e. day 0) regardless of treatment were compared to those seeds stored for 64 days plants with lower stem diameter were observed thus a negative impact on seeds was observed as storage days increased. Stem diameter of plants obtained from non-stored seeds ranged from 3 to 6 mm, whereas stem diameter of plants emerged from seeds stored for 64 days ranged from 0 to 3 mm. Plants with widest stem diameters were produced by seeds treated using treatment DBe and that had been stored for 4 days (10 mm). Seeds treated using treatment DEBN were negatively affected the most whereas treatments DEBBe, DEn and DEnN treated seeds resulted in the most consistent results.

Similarily to *G. livingstonei*, results obtained for *P. longifolia* trials (Tables 20 and 22) showed a similar trend of interaction caused by treatment, storage time and where the factors interact had a significant impact on seeds (p = 0.001). It was observed that DEBBe and DEBN treatments had the most detrimental effects on seeds. Treaments DBe, DEn and DEnN treated seeds which were stored for 16 d produced stems, however, it was also noted that seeds stored for 2 days and had been treated using DBe and DEnN did not produced stems. Furthermore, those treated using DEnN and had been stored for 4 days also did not produce stems.

Table 20: Treatment and storage impact on stem diameter (mm). (*T. dregeana*: *p = 0.01; G. livingstonei: p = 0.0005; P. longifolia: p = 0.0005)***.**

Plant species	Treatment	Hydrated Storage (*d*)							
		Stem Diameter (mm)							
		0	**1**	**2**	**4**	**6**	**16**	**32**	**64**
T. dregeana	DBe	14^{AB}	16^{AB}	11^{AB}	19^{B}	16^{AB}	16^{AB}	15^{AB}	9^{A}
	DEBBe	14^{A}	12^{A}	12^{A}	18^{A}	18^{A}	19^{A}	20^{A}	35^{B}
	DEBN	13^{A}	12^{A}	13^{A}	17^{A}	12^{A}	17^{A}	16^{A}	11^{A}
	DEn	12^{A}	12^{A}	13^{A}	17^{A}	12^{A}	16^{A}	19^{A}	13^{A}
	DEnN	14^{A}	11^{A}	11^{A}	19^{A}	11^{A}	16^{A}	17^{A}	15^{A}
G. livingstonei	DBe	10^{B}	14^{B}	19^{C}	7^{AB}	13^{B}	8^{AB}	10^{B}	4^{A}
	DEBBe	14^{B}	17^{B}	13^{B}	12^{AB}	13^{B}	13^{B}	13^{B}	8^{A}
	DEBN	13^{B}	5^{B}	9^{BC}	7^{B}	5^{B}	0^{A}	0^{A}	0^{A}
	DEn	12^{B}	15^{BC}	20^{C}	17^{C}	15^{BC}	14^{BC}	7^{A}	12^{B}
	DEnN	18^{B}	13^{AB}	17^{B}	15^{B}	14^{B}	12^{AB}	15^{B}	9^{A}
P. longifolia	DBe	4^{AB}	14^{C}	0^{A}	3^{A}	8^{B}	4^{AB}	0^{A}	0^{A}
	DEBBe	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	2^{A}	0^{A}	0^{A}
	DEBN	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEn	2^{A}	2^{A}	2^{A}	4^{AB}	15^{C}	5^{B}	2^{A}	0^{A}
	DEnN	5^{B}	2^{A}	0^{A}	0^{A}	13^{C}	4^{A}	0^{A}	0^{A}

LSD of means at 5% = 8.3 for *T.dregeana*, LSD of means at 5% = 4.7 for *G. livingstonei* and LSD of means at 5% = 4.8 for *P. longifolia***.** $p \leq 0.05$ = significant =* Different superscripts upper-case letters in each row indicate significant differences.

3.4.8. Effects of treatment and storage on mean number of leaves

For *T. dregeana* trials (Tables 21 and 22) a similar response of root biomass, stem diameter and stem biomass was observed in the number of leaves; however, there was no interaction between the response to treatment or storage time. DBe treated seeds at day 0 gave rise to plantlets with an average of 5 leaves per plantlet and seeds treated with other treatments produced plantlets with 4 – 6 leaves. DEBBe treated seeds produced a better yield of 8 leaves per explant at day 1 however decreased to an average of 6 leaves at day

64. DEnN treated seeds gave a better yield of leaves with an increase of storage time. Control treatment DBe resulted in the lowest number of 2 leaves per explant when compared to the other treatments at day 64.

G. livingstonei trials (Tables 21 and 22) showed that all the factors assessed (i.e. both treatment and storage regardless of each other and where the two interact) had a significant influence on the number of leaves ($p = 0.001$). Comparisons between non-stored seeds and those stored for 64 days showed that values obtained for seeds treated using DBe and DEn were not significantly different and produced plantlets with 2 to 4 leaves. However, number of leaves per plantlet obtained from non-stored, DEBBe, DEBN and DEnN treated seeds, was significantly smaller from those obtained from seeds stored for 64 days. Most detrimental treatment on seeds was DEBBe as no leaves were produced as from 16 days of storage. The most consistent results were obtained with DEnN treated seeds (i.e. number of leaves per plantlets ranged from 3 to 6). However, we also noted that the highest number of leaves was achieved with treatment DBe for those seeds only stored for 4 days (an average of 10 leaves per explant was obtained).

Similarily to *Garcinia* trials, it was noted for *Protorhus* species (Tables 21 and 22) that all the factors assessed (i.e. both treatment and storage regardless of each other and where the two interact) had a significant influence on the number of leaves produced ($p \geq 0.05$). Comparing the non-stored seeds to those stored for 64 days a descrease in number of leaves was observed however not significantly different for all treatements. It was further noted that DBe, DEn and DEnN treated seeds stored for 6 days produced plantlets with reasonably high number of leaves [i.e. DBe (7); DEn (8) and DEnN (10)]. Results obtained for treatments DEn and DEnN were significantly different when compared to other storage times (*viz*. 0, 1, 2, 6, 16, 32 and 64). Furthermore, seeds treated using DEBBe and DEBN were affected the most regardless of storage time.

Table 21: Treatment and storage impact on number of leaves (*T. dregeana*: *p = 0.0005; G. livingstonei: p = 0.0005; P. longifolia: p = 0.0005).*

Plant species	Treatment	Hydrated Storage (*d*)							
		No. of Leaves							
		0	**1**	**2**	**4**	**6**	**16**	**32**	**64**
T. dregeana	DBe	5^{B}	5^{B}	5^{B}	5^{B}	6^{B}	5^{B}	5^{B}	2^{A}
	DEBBe	5^{A}	8^{C}	7^{BC}	6^{AB}	6^{AB}	6^{AB}	7^{BC}	6^{AB}
	DEBN	6^{A}	6^{A}	6^{A}	6^{A}	6^{A}	6^{A}	6^{A}	5^{A}
	DEn	5^{A}	5^{A}	5^{A}	5^{A}	5^{A}	5^{A}	6^{A}	5^{A}
	DEnN	4^{A}	5^{AB}	5^{AB}	6^{B}	5^{AB}	4^{A}	5^{AB}	6^{B}
G. livingstonei	DBe	3^{AB}	5^{B}	5^{B}	10^{C}	3^{AB}	2^{A}	2^{A}	2^{A}
	DEBBe	5^{B}	6^{B}	2^{A}	3^{AB}	3^{AB}	3^{AB}	2^{A}	2^{A}
	DEBN	4^{B}	1^{A}	4^{B}	2^{A}	1^{A}	0^{A}	0^{A}	0^{A}
	DEn	4^{A}	4^{A}	4^{A}	4^{A}	3^{A}	3^{A}	3^{A}	3^{A}
	DEnN	6^{B}	5^{AB}	5^{AB}	5^{AB}	4^{A}	4^{A}	6^{B}	3^{A}
P. longifolia	DBe	3^{AB}	7^{B}	0^{A}	2^{AB}	7^{B}	4^{B}	0^{A}	0^{A}
	DEBBe	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEBN	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}
	DEn	1^{A}	1^{A}	1^{A}	3^{A}	8^{B}	3^{A}	2^{A}	0^{A}
	DEnN	2^{AB}	1^{A}	0^{A}	0^{A}	10^{C}	4^{B}	0^{A}	0^{A}

LSD of means at 5% = 1.7 for *T.dregeana*, LSD of means at 5% = 2 for *G. livingstonei and* LSD of means at 5% = 3.7 for *P. longifolia*. $p \leq 0.05$ = significant =* Different superscripts upper-case letters in each row indicate significant differences.

Table 22: The significance of effects of treatment, storage time and treatment * storage time on each dependent variable of selected plant species.

Plant species	Variable	Treatment			Storage days			Treatment X Storage days		
		df	F	Sig.	df	F	Sig.	df	F	Sig.
Trichilia dregeana	Leaf Area (mm^2)	4	4.65	0.00*	7	5.59	0.00*	28	1.97	0.003*
	Leaf length (mm)	4	5.58	0.00*	7	17.83	0.00*	28	4.44	0.000*
	Leaf Biomass (g)	4	1.04	0.39	7	0.95	0.47	28	1.00	0.462
	Root length (mm)	4	4.24	0.00*	7	6.15	0.00*	28	2.50	0.000*
	Root biomass (g)	4	2.50	0.04*	7	3.93	0.00*	28	1.15	0.277
	Stem diameter (mm)	4	3.35	0.01*	7	3.01	0.00*	28	1.66	0.021
	Stem biomass (g)	4	3.49	0.01*	7	3.18	0.00*	28	2.18	0.010*
	No. of leaves	4	2.50	0.04*	7	3.93	0.00*	28	1.15	0.277
Garcinia livingstonei	Leaf Area (mm^2)	4	18.878	0.000*	7	22.230	0.000*	28	3.341	0.000*
	Leaf length (mm)	4	31.276	0.000*	7	16.997	0.000*	28	2.534	0.000*
	Leaf Biomass (g)	4	11.663	0.000*	7	14.054	0.000*	28	2.444	0.000*
	Root length (mm)	4	25.290	0.000*	7	5.261	0.000*	28	1.294	0.149
	Root biomass (g)	4	25.555	0.000*	7	20.977	0.000*	28	1.641	0.023
	Stem diameter (mm)	4	43.586	0.000*	7	14.409	0.000*	28	2.927	0.000*
	Stem biomass (g)	4	25.691	0.000*	7	18.628	0.000*	28	2.701	0.000*
	No. of leaves	4	21.869	0.000*	7	10.455	0.000*	28	3.937	0.000*
Protorhus longifolia	Leaf Area (mm^2)	4	8.042	0.000*	7	8.151	0.000*	28	1.995	0.002*
	Leaf length (mm)	4	10.170	0.000*	7	8.923	0.000*	28	2.850	0.000*
	Leaf Biomass (g)	4	8.061	0.000*	7	6.837	0.000*	28	1.813	0.008
	Root length (mm)	4	7.820	0.000*	7	7.876	0.000*	28	2.410	0.000*
	Root biomass (g)	4	8.109	0.000*	7	11.676	0.000*	28	3.521	0.000*
	Stem diameter (mm)	4	10.630	0.000*	7	9.484	0.000*	28	3.267	0.000*
	Stem biomass (g)	4	5.528	0.000*	7	6.037	0.000*	28	1.351	0.113
	No. of leaves	4	8.968	0.000*	7	7.481	0.000*	28	2.000	0.002*

$p \leq 0.05$ = significant =* Different superscripts upper-case letters in each row indicate significant differences

4. DISCUSSION

Extending the storage lifespan of recalcitrant seeds demands urgent attention to help conserve the genetic resources of many important indigenous recalcitrant seeded plant species. Postharvest infection which hinders prolonged storage of this type of seeds has not received the attention that the magnitude of the problem poses. There are three types of contamination in plant tissue culture (i) acute contamination which occurs almost immediately after establishment of cultures and is associated with an ineffective surface decontamination; (ii) contamination that becomes evident after culture establishment and is associated with endogenous micro-organisms; and (iii) chronic contamination which is manifested after a long period in what was deemed a sterile stock culture (George, 1996).

Seed surfaces harbour a wide variety of microbial communities more especially fungal species, the inoculum of which is frequently internally located (Mycock and Berjak, 1990, Sutherland et al., 2002, Berjak and Pammenter, 2014, Berjak et al., 2014). Moreover, the internally located fruit structures (i.e. cotyledons or embryonic axes) could be exposed to a variety of cross contaminants during the different procedural stages when they being processed for culture purposes. Therefore, fungi or other seed associated contaminants need to be effectively eliminated, and necessary steps taken as to ensure subsequent decontamination of explants. However, present study has shown that with some species this task is almost impossible (Table 5).

To establish an optimal decontamination procedure requires an appropriate combinations of sterilant types, concentrations and exposure times. Previous studies have shown that when fungi ares eliminated, bacterial contaminations predominate, which then requires administration of a mixture of fungicides and antibiotics (Berjak and Pammenter, 2004b, Berjak et al., 2014). Screening for a potential antimicrobial agent remains an important task in ensuring an efficient procedure. Depending on the type and position of contaminants,

the decontaminant of choice can either be administered by surface-decontamination (for superficially-located contaminants) or by addition to the culture medium/ encapsulation of plant material with the antimicrobial agent (for endogenous micro-organisms).

In order to optimize a decontamination procedure successfully, for selected plant germplasm of *T. dregeana, P. longifolia* and *G. livingstonei* (or any other species), it is vital that an antimicrobial agent, no matter how efficient it may be in eliminating contaminants, should have minimal adverse effects on the viability of seeds. Hypochlorites [i.e. $Ca(OCl)_2$ used at 1% m/v or NaOCl used between 2% and 5% m/v] are generally good surface decontaminants, effective against a wide range of microorganisms and even at lower concentrations but at longer exposure (e.g. Abdel-Mallek et al., 1995). In this study, sodium hypochlorite was chosen for surface decontamination of whole seeds as it eliminated most of the superficial fungal and bacterial contaminants, and did not have a negative impact on whole seed vigour or viability. Therefore, application of 1% v/v NaOCl served as the basis of all decontamination protocols developed before HS and seed *in vitro* culture. This is in agreement with the report by Berjak et al. (2014) that 1% v/v NaOCl was effective in eliminating all surface contaminants in embryonic axes with no detrimental effects. Therefore, treatment with DBe served as a control against treatments: DEBBe, DEBN, DEn and DEnN. The above mentioned treatments were the best performing treatments with respect to seed vigour, viability of seeds, and elimination or control of seed contamination in HS and subsequent growth.

The types of pathogens reported for recalcitrant seeded species are listed by Mycock and Berjak (1990) and Calistru et al., (2000). During the present investigation, seed infections were observed and hence some of the selected plant species pathogens were isolated, purified into pure axenic cultures and identified. Subsequent pathogen testing showed the presence of pathogens in all parts of the selected plant germplasm *viz*. whole fruits, seed coats, seed, pericarp, cotyledon and embryonic axis. Therefore different fungal species were associated with recalcitrant seeds of *T. dregeana, P. longifolia* and *G. livingstonei* and there

were specificities in the association of these species with the investigated seeds. From the seeds of *T. dregeana, P. longifolia* and *G. livingstonei*, 6, 18 and 1 fungi species were isolated. Only 3 out of 6 associated with *T. dregeana* were among the 18 associated with *P. longifolia* and the only 1 from *G. livingstonei* was not included in either 6 or 18 lists of *Trichilia* or *Protorhus* species.

Of the fungal genera isolated, *Fusarium*, *Alternaria* and *Cladosporium* particularly, have been singled out as field fungus in the context of seed pathology (e.g. Christensen and Kaufmann, 1974, Mycock and Berjak, 1990). This classification implies that the fungi concerned gain access to the seed prior to, or immediately after harvest when the moisture content of the seed is still relatively high. While certain species of *Aspergillus* and *Penicillium* (known as the storage fungi) might initially be concealed prior to, or at harvest, they are dried down to moisture contents suitable for storage (McLean and Berjak, 1987). Therefore the list of fungi isolated in the current study is comparable to a spectrum of contaminants obtained by Mycock and Berjak (1990). During HS and in culture conditions, there was narrowing in the range of fungal species involved (i.e. from a sum total of 150 fungal species to a total of only 25 isolates when only HS and culture isolates were considered). *Paecilomyces, Penicillium, and Fusarium* species emerged as the major isolates for *T. dregeana* and *P. longifolia*. Thus like the situation in orthodox, air-dry, stored seeds, a fungal succession does occur during storage of recalcitrant seeds. However, the succession does not include any of the species traditionally classified as storage fungi, e.g. *Aspergillus glaucus* group spp. and *A. flavus* group spp. for both plant species of *Trichilia* and *Garcinia*. The absence of any species of *Fusarium* and *Penicillium* from HS and culture explants for *G. livingstonei* (Table 5) is interesting since *Fusarium* spp. are widely present in soil and plant debris in South Africa (Marasas et al, 1988, Mycock and Berjak, 1990) and these are implicated in a spectrum of plant diseases, many of which are seed-borne (Argawal and Sinclair, 1987). *Garcinia* species seeds are well protected by a seed coat and pericarp which produces a sticky yellow juice and delicious acid-sweet taste. Moreover, the seeds are well cushioned within a watery pulp. It was observed that the seeds within fruits are more prone to fruit worms rather than fungi. The

seeds resilience to fungal growth and proliferation may be attributed to chemical composition of seed coat and pericarp and warrants further study.

Screening of potential antimicrobial agents against isolated fungi played a vital role in selecting the best suitable bio-control and chemical agents. Therefore a variety of both biological and chemical agents were assessed in order to develop and optimize decontamination procedures for further studies on seeds (Figures 18 – 22; Tables 6, 7 and 8). There were specifities in the effectiveness of biological and chemical agents against fungal isolates from the three tree species. However, none of the agents had an acceptable level of effectiveness across the experimental units. Although Eco77 (*Trichoderma harzianum* strain B77) gave the widest effectiveness against all isolated fungal species, there were instances where EcoT (*Trichoderma harzianum* strain Kd) produced a significantly higher inhibition than Eco77 (e.g. for *Aspergillus* sp., *Cytospora* sp., *P. lilacinus* and *P. chrysogenum*). This varying degree of inhibition is held to be related to the different antagonistic potential of different strains and species of *Trichoderma* (Roiger and Jeffers, 1991).

T. harzianum showed antagonism (Tables 6, 7 and 8) against the isolated mycoflora. This is in accordance with investigation by Gajera and Vakharia (2010) which showed that *Trichoderma* spp. produce chitinases and β-1, 3-glucanase, enzymes responsible for degradation of cell walls of fungi , which lead to lysis of hyphae. The fact that glucans and chitin are the main structural components of fungal cell walls, suggests that these hydrolases are involved in mycoparasitic activity De la Cruz et al. (1992). However, other mechanisms can also be used by *Trichoderma* spp. *viz* parasitism. Shalini et al. (2006) states that the *Trichoderma* isolates inhibit a pathogenic fungus by sticking their mycelial tips on the large hyphae of *Rhizoctonia solani*. This process is rapidly followed by excessive coiling on the target fungus. It is speculated that the process of sticking with the mycelial tips is further advanced by production of enzymes

(Gveroska and Ziberoski, 2011). Competition may be displayed, such that the antagonist will use all the nutrients from the medium or can produce an inhibitory metabolite.

Bio-control treatment with *Bacillus subtilis* showed a lower antimicrobial activity when compared to that of *Trichoderma* strains. It is important to note that an indirect approach was preferred since the direct application of bio-control treatment assay (i.e. using 6 mm plug of bio-control agent placed at the centre of an agar plate containing fungal [isolate] contaminant evenly inoculated as slurry) and *Bacillus subtilis* could not display any anti-fungal properties. Reasons for this were concluded to be due to the incubation temperature (25^{o}C) favouring fungal growth and not bacterial (*B. subtilis*). However, changes in incubation temperature would not have been suitable for consistency purposes. Moreover, the reason that may have contributed to low activity of *B. subtilis* is that the medium used to assess the assay (PDA) provides nutritional requirements better suited for fungal organisms. The use of *B. subtilis* supernatant was therefore more ideal for purposes of this study as it contained the secondary metabolites which may have been produced within 48 h of culture. Culture conditions were in soluble (liquid) form and the metabolites therefore were able to diffuse into solid medium and readily elicit antifungal effects.

Mycosubtilins, bacillomycins and peptidolipids are some of the compounds that may have been involved in inhibiting fungal growth. When compared with other studies, *B. subtilis* was also effective against *R. solani* due to surfactants it produced. Concluding from the results of biocontrol agents used in this study, the next logical step was to develop and optimise decontamination methods using *Trichoderma* strains (Eco 77 and EcoT).

The chemical fungicides used (*viz*. Afugan, Biotane, Celest, Orius, Odeon, Sporekill, Ripenit and Nipastat) were selected as they are already used as potential antimicrobial agents as single application or used in

combination as decontaminants of whole seeds, axes or any plant material including those maintained in the greenhouse or hedges. They are known to effectively control a wide range of fungi in plant tissue culture or in the field. However, it is important to note that it is easier to use these chemicals as aerosols in the field as the plant material is more mature than the ones grown in culture. Plant material in culture is usually immature and sensitive (e.g. embryonic axes or explants of plants etc.). Therefore, the way chemical mixtures are prepared, application and time of exposure to plant material is critical. Hence all this was carefully considered and analyzed before finalizing the chemical list chosen for investigation.

Nipastat was the most consistent in inhibiting fungal isolates when compared against all chosen chemical agents (Tables 9, 10 and 11). Nipastat is a preservative by nature and is recommended for usage at concentrations of 0.05 to 0.3%. Previous studies have indicated that some of the common moulds and yeasts are inhibited by Nipastat at a concentration of 0.13% as are bacteria using 0.1% and 0.03% or higher in the fungal toxicity trials. Nipastat has been proven to be a good fungicide at higher concentrations, but for the current study it was found to be most effective at 0.1% with all 3 plant species chosen for the study.

In *T. dregeana* trials the minimum diameter of inhibition was 19 mm and 60 mm being the highest zone followed by Sporekill and Biotaine with respect to consistency. However, Berjak et al. (2014) noted toxic effects to *T. dregeana* axes after being exposed on a 0.03% Nipastat-enriched half strength MS medium, but killed all the fungi and bacteria associated with the embryonic axes. Moreover, studies by Motete et al. (1997) showed that when *Avicennia marina* seeds were encapsulated in Nipastat-containing alginate gel, fungal proliferation was inhibited.

Isolates from *G. livingstonei* showed F. *maliae* as the only dominating fungus (Table 10) and showed resistance against all chemical agents used (i.e. exhibited inhibition zones below 25 mm). *F. maliae* was not susceptible to Celest, Odeon and Ripenit as a 100% fungal growth was obtained. However, it is

important to note that Sporekill and Nipastat gave a higher inhibition diameter of 15 mm which was about 2 mm bigger than that of Afugan and Orius. Biotaine proved to be the best antimicrobial agent against *F. maliae* when compared with other chemical agents (20 mm; ~ 23% inhibition).

This was however expected of Biotaine as it is a widely used surface decontaminant (active ingredient: chlorhexidine gluconate). Chlorhexidine is a broad-spectrum biocide effective against Gram-positive bacteria, Gram-negative bacteria and fungi. The mechanism of action depends on the rapid uptake of chlorhexidine thus impairing the integrity of the cell wall and the plasma membrane resulting in leakage of cell contents and cell death (McDonnell and Denver, 1999). For purposes of this study, biotaine was introduced after seeds had been exposed to Eco77 spore suspension for 4 hours to surface decontaminate the seeds and also to remove excess fungal *Trichoderma* spores on the seed surface. This treatment proved not to be detrimental to seed vigour but effective in decreasing fungal growth in HS.

Biotaine continued to meet the expectation as the best antimicrobial agent when looking at total inhibition against *P. longifolia* isolates (~ 26% inhibition) whereas Nipastat remained to show a better consistency against all isolates. The present results indicate the efficacy of Nipastat and Biotane in eliminating or decreasing proliferation of fungi, and their non-toxic nature, provide a good argument for their use rather than the other chosen chemical agents.

Generally, biological agents gave a comparatively higher average inhibitory effects across a spectrum of the isolated fungal species than the chemical agents.

A graphic representation of seed vigour for all three plant species of *T. dregeana*, *P. longifolia* and *G. livingstonei* (Figures 18A, B and C) clearly showed that seeds may malfunction or perish due to infections or type of treatment it has been exposed to prior to HS or culture. In general, for all plant species investigated in this study, not too many differences amongst treatments were observed but some significance differences occurred towards the end of 30 days assessment period. Treatment DEnN (i.e. seeds encapsulated with alginate gel with or without Nipastat), had the least effects on seeds and reasonably controlled contamination levels throughout the duration of vigour assessment. Nipastat as a powder sprinkled over seeds had a drying effect on seeds but was able to contain fungi at the site of proliferation thus preventing it from spreading. This phenomenon was observed with all seeds of the three plant species in HS or culture. Detrimental impacts caused by treatment type were more distinguishable with the *P. longifolia* seeds. These seeds were more sensitive towards treatments DEBBe and DEBN. The graph indicating viability/vigour continued to drag along the x-axis until the assessment duration was over, basically depicting seed death. The most resilient seeds were those of *G. livingstonei.* It was also noted that the cotyledons of this plant species remained green and healthy looking for longer periods, both in culture and field trials. In this study, all seeds that were subjected to minimal surface decontamination (treatment DBe, Table 13) exhibited higher levels of seed contamination (ranged from 4, 7 and 5 seeds; n = 25), indicating that surface decontamination was necessary to precede culturing. In this regard, NaOCl solution containing drops of Tween 20/80 (a wetting agent) and later dusted with Benomyl in HS or culture on their own, did not provide sufficient protection from fungal contaminants. These results suggest the presence of acute and possibly endogenous contaminants (George, 1996) on and in internal structures of the seeds of *T. dregeana*, *P. longifolia* and *G. livingstonei*. The other treatments (DEBBe, DEBN, DEn and DEnN); which included a combination of both biological and chemical treatments, eliminated the visible contamination from seeds.

Therefore, developed treatment protocols included a control (DBe), combination of both bio and chemical agents (DEBBe and DEBN) and a chemical agent used in a capsule of alginate gel or sprinkled on seeds (DEn and DEnN). These treatment protocols were developed with an understanding that controlling plant diseases using chemicals is a challenge because of the potential toxic effects. Therefore the urgent need for an eco-friendly approach for crop protection is crucial, and the use of biological antimicrobial agents could serve as an alternative. In this current research, observations were made with regards to how both biological and chemical agents could serve as excellent eliminators of contaminants with minimal impacts on seeds in HS, in culture or in the field.

It must be noted that seeds assessed in culture were exposed to a double dose of surface decontamination since the concern was about cross contamination through manipulation from HS to culture. Under aseptic conditions the 25 seed sample from HS were re-surface decontaminated with 1% v/v NaOCl, 0.01% w/v cicatrin and 0.1% w/v $HgCl_2$ and time exposure (5, 10 and 15 min) which included 3 times rinse with sterile distilled water before each decontaminant. Even though mercury is highly toxic (ATSDR, 2001), none of the deleterious effects occurred as per findings on barley seeds (Jonathan et al., 2002) and also when applied to the axes of *Andrographis paniculata* (Talei et al., 2011). Moreover, the toxic effects of $HgCl_2$ depend on two factors namely, exposure time and concentration used. In the present study a very low concentration (0.1% w/v) was used, although the exposure time was longer (15 min). The treatment nevertheless compromised seed vigour, assessed as root emergence and shoot production. Similar results were obtained when mercuric chloride was used on embryonic axes of *T. dregeana* (Berjak et al., 2014).

Table 6 (Chapter 3) shows results after 64 days of HS for both *Trichilia* and *Garcinia* species and only at 16 days for *P. longifolia*. Treatment DEn and DEnN involving the encapsulation of seeds with alginate gel (with or without Nipastat) produced best seedlings, which maintained a 0% contamination, with 100% of seeds being unblemished and germinable (even up to 6 months in HS – data not shown) for species of

Trichilia and *Garcinia*. Treatments DEBBe and DEBN, which incorporated bio-control agents of *Trichoderma* strain Eco77 were second best regarding contamination levels, root and shoot development. However, *P longifolia* seeds were rather sensitive to treatments, but the major problems were seed germinating too early in HS and also contamination prevalence. Treatments incorporating *Trichoderma* strain Eco77 for treatment of *P. longifolia* seeds resulted in good batch of seed material, even up to 64 days of HS. These seeds were not too prone to contamination problems encountered with other treatments and early germination. However less than 5% survived due to problems outlined. This is the reason for discussing results of seeds stored for 16 days. A study by Varghese (not published) also showed that *P. longifolia* seeds were very prone to fungal contamination and once the seed coat is removed germination is prompt. However, pursuing further with the investigations it was decided to try all the developed protocols in the field as to note any differences when comparing with the culture trials.

(a) Trichilia dregeana

Looking at the above-ground biomass and total yield for field allocation for seedlings that originated from seeds had been stored in HS for 64 days, a significantly larger leaf area was observed with treatment DEn and this may be due to seeds responding positively to treatment and thus producing healthy seedlings. This is confirmed when considering seeds from treatment DEBN, whereby, they looked unhealthy and had brown patches. This also corresponds with reported findings by Araus et al. (2001), Zelitch (1982), Ashley and Boerma (1989). These authors reported that in several species yield is closely related to total canopy photosynthesis during growth and may be increased by faster approach to full cover (i.e. early vigour) and higher leaf extension by minimizing stress. Moreover, when plants, are under stress, the assimilation rate is generally more limiting to yield than it is under optimal conditions as indicated by higher association commonly observed between yield and above ground biomass at maturity (Araus et al., 2001). However, this pattern was not observed at storage day 64 with regard to leaf dry biomass but prevalent at days 1 and 6 as a higher leaf biomass was observed for the same treatment. A higher stem biomass and leaf length was

also observed for the same treatment (DEn) for seedling material that came from the seeds stored for 64 days in HS which also corresponds to literature. However, the number of leaves was more with *Trichoderma* treated seeds (DEBBe) at day 64 when compared to all other treatments and also underground biomass allocation (root biomass) was more with *Trichoderma* treated seeds. The concept of bio-control treatments of seedlings or plant material is an effective and economical method to provide a more vigorous transplant with disease protection when it is in culture *in vitro* or planted to the field (Nemec et al., 1996). Adding to bio-control activity, *Trichoderma* strains have been reported to promote plant growth (Chang et al., 1986, Inbar et al., 1994, Ozbay et al., 2004), and therefore, this serves as possible explanation to understanding how *T. harzianum* strains control minor pathogens thus leading to stronger growth and nutrient uptake.

(b) Protohus longifolia

This plant species continued to differ even under field conditions when being compared to the other two plant species (i.e. *T. dregeana* and *G. livingstonei*). The above ground biomass allocation was consistent with results obtained under culture conditions. It was observed that DBe treatment had no detrimental effects on seeds (seeds looked green, with no black patches and looked healthy with prompt germination), however contamination was a problem. Treatments DEn and DEnN were second best treatments regarding side effects on seeds and with controlled contamination prevalence. It was observed that for seeds that came from HS which had showed contamination and then recovered did not perform well in the field (e.g. they would germinate and quickly die off). This was the very reason why most of the DBe treated seeds that had survived HS at 6, 16 and 32 days never developed into seedlings. Moreover, *Trichoderma* treated seeds did not perform well in the field; however, it was noted that seed processing for this treatment was harsh (i.e. prolonged exposure to *Trichoderma* spore suspension for 4 hours then to Biotaine and then dried

overnight). This was done to try to standardize protocols for all three plant species, but future work should assess whether time of exposure can be decreased. However, seedlings produced from this treatment had greener leaves with no black spots on the leaves but with no production of two stems as noticed with *Trichilia* and *Garcinia* species.

(c) Garcinia livingstonei

G. *livingstonei* seeds were not susceptible to a wide range of fungal contaminants; only one type of fungal species (i.e. *F. meliae*) was isolated. Looking at the fruit morphology, it is well protected by a seed coat and pericarp which produces a sticky yellow juice and delicious acid-sweet taste. Moreover, the seeds are well cushioned within a watery pulp. It was observed that the seeds within fruits are more prone to fruit worms other than fungi. The seeds resilience to contamination with fungi may be attributed to chemical composition of seed coat and pericarp and the properties of seeds of this species warrants further investigation.

In summary, a protocol for the elimination of fungal agents associated with recalcitrant seeds of *T. dregeana*, *P. longifolia* and *G. livingstonei*, was successfully developed including either the encapsulation of seeds in calcium alginate or seed treatment a combination of chemical (Nipastat, Biotaine) and biological (Eco-77) agents without any apparent effect on the viability and vigour of seeds or saplings.

5. CONCLUSIONS

The research reported here has not only shown the detrimental impacts of seed-associated fungal contaminants on maintaining seed viability, but has also significantly demonstrated the efficacy of Nipastat, Biotaine, and Eco77 as a decontaminant for these, and potentially other types of explants, for subsequent short-to-medium term storage and other purposes. To conclude:

(1) Different fungal species were associated with recalcitrant seeds of *Trichilia dregeana*, *Protorhus longifolia* and *Garcinia livingstonei* and they related uniquely to the investigated seeds. This is a further justification for the study suggesting that an effective storage protocol necessitate an in depth study of seeds of the individual tree species.

(2) Exposure of fungal isolates from the three species to both biological and chemical agents also demonstrated specifities regarding efficiency. However, none of the agents on its own had an acceptable level of effectiveness across the experimental units. Although Eco77 (*Trichoderma harzianum* strain B77) gave the widest effectiveness across all the isolated fungal species, there were instances where EcoT (*Trichoderma harzianum* strain Kd) produced a significantly higher inhibition than Eco77 (e.g. for *Aspergillus* sp., *Cytospora* sp., *P. lilacinus* and *P. chrysegenum*).

(3) Generally, biological agents gave a comparatively higher average inhibitory effects across a broader spectrum of the isolated fungal species than chemical agents.

(4) A protocol for the elimination of fungal agents associated with recalcitrant seeds of *T. dregeana*, *P. longifolia* and *G. livingstonei*, was successfully developed including either the encapsulation of seeds in calcium alginate or seed treatment with a combination of chemical (Nipastat, Biotaine) and biological (Eco77). Seeds subjected to different pre HS treatments showed different responses in all seed quality parameters. However, the most important quality parameter was seed vigour which was least affected by *Trichoderma* Eco77. Germination was found to be higher in seeds subjected to either *Trichoderm*a treatment compared with seeds treated with chemical antimicrobial agents. Various growth parameters of seedlings derived from post HS, including, root length, dry

weight, number of shoots and leaves were also adversely affected by chemical treatments. In conclusion, decontamination protocols for recalcitrant seeds need to be developed on a species specific basis, and treatment with chemical anti-fungal agents can have adverse effects on seed germination and subsequent seedling growth.

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7. APPENDICES

7.1 Publication 1

Development of treatment protocols towards eliminating seed-borne fungi of recalcitrant-seeded species of *Trichilia dregeana* Sond.

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Summary

Recalcitrant seeds survive in storage in the short- to medium-term only under hydrated conditions and at temperatures above zero. A major problem restricting storage lifespan is the presence and proliferation of fungi. The present study was designed to improve the hydrated storage life span of *Trichilia dregeana* seeds by curtailing/eliminating fungal contaminants, and to assess the impact of the treatments on seed viability and subsequent performance of seedlings. Six fungal species prevalent to *T. dregeana* seeds in hydrated storage and *in vitro* were isolated and identified from axenic cultures and 18S DNA analysis. After *in vitro* testing of the efficacy of chosen biocontrol agents and chemical compounds in inhibiting the growth of axenic cultures of the test isolates, the selected treatments of the seeds were: surface decontamination alone or followed by treatment with a strain of *Trichoderma harzianum*, Eco77®, and Biotaine™ (active ingredient chlorhexidine gluconate) with or without Nipastat® (a mixture of parabens) as a powder applied to seeds, and encapsulation in alginate gel, incorporating Nipastat or not. Under *in vitro* conditions

treatments with Eco77, Biotaine and Nipastat encapsulated in an alginate gel were best for controlling contamination levels and affording higher germination of seeds after 30 days in culture. Six months after removal from storage and planting in the pot trials, significant changes in biomass allocation to leaf area, leaf length, stem biomass and root length were recorded.

Introduction

Recalcitrant seeds do not undergo maturation drying (Roberts, 1973) and are desiccation sensitive and as such are unable to be stored under conditions suitable for orthodox seeds (Pammenter and Berjak, 1999; Berjak and Pammenter, 2008, 2013 *viz*. low relative humidity (RH) and below-zero temperatures.

Many recalcitrant seeds especially those of tropical origins are also sensitive to chilling and cannot be stored at temperatures below 10-15°C (Roberts, 1973; Hong and Ellis, 1996). Even when maintained at the water content at which they were shed, storage lifespan of recalcitrant seeds is extremely short compared with that of orthodox seeds, varying from less than two weeks to a few months (Han *et al*., 1997; FAO, 2013; Berjak and Pammenter, 2014). Hydrated storage at moderate temperatures of approximately 16 °C is a requirement in maintaining seed vigour and viability for all tropical species (Berjak and Pammenter, 2014) and this is adopted as a standard operating procedure in our laboratory; however, these very conditions also promote fungal growth of any seed-associated inoculum. In the case of *Trichilia dregeana* used in this study, the removal of the waxy aril assists in eliminating surface contaminants (Berjak *et al*., 2014); however, if the inoculum is deeper-seated, then the procedure will delay, but not eliminate, fungal proliferation (Berjak, 2005; Myeza, 2005; Berjak *et al*., 2014).

Seed pathogens have been shown to affect the health of recalcitrant seeds and cause germination failure (Sutherland *et al*., 2002). Some research has been done on fungal contamination of recalcitrant seeds (e.g. Calistru *et al*., 2000; Sutherland *et al*., 2002; Berjak *et al*., 2004; Berjak *et al*., 2014) but no successful approach to counteract the fungal attack has been published. As it is important to be able to maintain

recalcitrant seeds under conditions that will best maintain their vigour and viability, it is imperative that storage conditions be optimised, which includes the vital aspect of minimising – or ideally, eliminating – the seed-associated fungi. The fungi associated with recalcitrant seeds not only affect germination adversely, but also can cause diseases in the resultant plants (Gure, 2004). Moreover, the seeds themselves provide favourable conditions for fungal proliferation, by supplying an ideal nutrient source (Sutherland, *et al*., 2002; Calistru, 2004; Berjak and Pammenter, 2014). Therefore, to curtail seed-associated fungi, treatment prior to storage or planting using chemical or biochemical agents is required. These antimicrobial treatments are divided into two types, *viz*. contact and systemic fungicides (Sutherland *et al*., 2002; Vincelli and Williams, 2011).

All these problems associated with seed borne fungi prompted the current investigations using whole seeds of the tropical recalcitrant seeded species, *Trichilia dregeana* Sond. (Meliaceae). Seeds were collected, surface decontaminated and subjected to several treatments which were assessed in terms of their effects on seed vigour, viability and efficacy against identified fungal species persistent in hydrated storage (HS) and also in seed culture *in vitro*. Treatments found to be satisfactory were used to developed seed treatment protocols. The efficacy of these protocols was optimized and then assessed under culture and pot trial conditions.

Materials and Methods

Fungal isolation and purification

Seed coverings were removed from newly-harvested seeds which underwent a series of surface-decontamination protocols and then placed in HS. Thereafter, any fungal proliferations emerging in HS and in culture were isolated and immersed in ¼-strength Ringer's solution and agitated for 10 min at 150

rpm. Three 10 *ml* aliquots of this solution were then serially diluted (10^{-1} – 10^{-6}), from which 1 *ml* of each was mixed with ~ 30 *ml* of Potato Dextrose Agar before setting. The cultures were incubated at 25-30°C for 5-10 d, and monitored daily. As mycelia developed, individual plugs (10x10 mm) were sub-cultured into fresh PDA and incubated. This was repeated three times to ensure axenic cultures. The cultures were examined with a stereomicroscope and the frequency with which a genus occurred was expressed as the number of isolates of the same genus divided by the total number of genera isolated per plant species.

Characterisation of fungal isolates

Plugs of axenic cultures were introduced into 9 ml of Ringer's solution and vortexed to disperse the spores. A sterile needle was used to collect an inoculum then plated on each of five different media, *viz.* malt extract agar (MEA), Sabourad agar (SDA), potato dextrose agar (PDA), water agar (WA) and Czapek Dox agar (CDA). Fungal identification was carried out microscopically by inspection of colony margins, surface and underside textures, pigmentation and growth over a 7 d period (Cappuccino and Sherman, 1992; 2014; Jha, 1995). Teased-out mycelium on microscope slides were stained with Latco-phenol Cotton Blue. The prepared slides were viewed and characterized at low and high power with a Nikon Eclipse 80*i* equipped with apochromatic objective lenses. Images of fruiting body structures were captured and identified according to Raper and Fennel (1965); Ellis (1971); Domsch *et al.* 1980 and Nelson *et al.* (1983). Molecular identification of fungi was done to confirm or validate results at InqabaBiotec, South Africa using an ITS PCR with ITS-1 and ITS-4.

Biological control agents

Three agents were used: *Trichoderma harzianum* (two strains: EcoT® and Eco77®; Plant Health Products (PTY) Ltd, South Africa) and *Bacillus subtilis* (stock culture provided by Durban University of Technology, Department of Biotechnology and Food Technology, South Africa).Sterile distilled water acted as the

control. The spore innocula were g^{-1} was 2 x 10^9 for the *Trichoderma* strains and 1 x 10^6 propagules ml^{-1} for *Bacillus subtilis.*

Control of identified fungal isolates using biological treatment agents

Bacillus subtilis was grown in a 10 ml nutrient broth for 24h at 25˚C, and then centrifuged at 300*g* (ThermoScientic, *Pico*21 centrifuge, Germany) for 5 min. From the supernatant: 50µl of *B. subtilis* was transferred into a well in a PDA plate where the surrounding surface of the well had been inoculated with 100µl of a test fungal spore suspension. The plates were incubated at 25˚C for 7 d, with observations being made daily of the diameter of inhibition. The experiment was carried out in triplicate.

For EcoT and Eco77 dual cultures were set up by placing plugs of the fungal isolate and the biological control agent opposite each other at the edges of a 90 mm Petri dish plate. The plates were incubated at 25˚C and results were recorded on day 7. The experiment was carried out in triplicate.

Control of identified fungal isolates using chemical treatment agents

Eight chemical treatments were employed; namely Afugan (pyrazophos, Hoechst AG, Germany), BiotaineTM (chlorhexidine gluconate, Dismed Pharma, PTY, LTD), Celest (fludioxonil, Syngenta AG), Orius 200 EW (triazole, Makhteshim-Agan, South Africa, PTY, LTD), Odeon 720 DC (chlorothalonil, Makhteshim-Agan, South Africa, PTY, LTD), Sporekill (dodecyl dimethyl ammonium chloride, HydroTech Properties [EDMS] BPK/Seed (PTY) LTD), Ripenit (ethephon, R.T. chemicals) and Nipastat® (mixed parabenzoates [parabens], Clariant chemicals, South Africa). Concentrations investigated were 10, 20, 50, 80 and 100 µl/mg but results discussed here were those at 50 µl/mg. The same method was used to assess the efficacy of each of the chemical treatment agents investigated in this study as described above for the biocontrol agents, using 100µl sample of fungal spore suspension and 50 µl/mg of a chemical control

in the wells. The plates were incubated at 25˚C and results recorded on the seventh day. The experiment was carried out in triplicate.

Seed collection preparation and hydrated storage

Seeds of *Trichilia dregeana* were collected at the University of KwaZulu-Natal, Durban, South Africa and surrounding urban areas between April and July, 2008-2012. Most fruits were mature and had already opened exposing the seeds. The aril and seed coat were removed revealing the green cotyledonary surfaces. These were collected into clean (2 *l*) plastic beakers loosely covered with moist paper towel. The seeds were then surface decontaminated in 1% (v/v) NaOCl solution containing a few drops of the wetting agent, Tween 20/80®, for 10 min then rinsed three times in sterile distilled water. They were then left to dry between sheets of towel paper overnight at room temperature. Thereafter, they were subjected to a range of treatments (Table 1) and placed into hydrated storage. All the treated seeds were placed as a monolayer on a plastic mesh suspended 200 mm above paper towel saturated with sterile water to which a few drops of 12 % (v/v) sodium hypochlorite had been added, in white, translucent 5 *l* plastic buckets. Bucket lids were lined with paper towel (as a precaution to prevent condensate from dripping back onto seeds) before the buckets were sealed and stored at constant 16ºC . Plastic sieve, bucket lids and the buckets had been previously washed using domestic liquid soap (active ingredient, anionic detergents), rinsed using hot tap water, decontaminated by soaking in 1% w/v NaOCl overnight, subsequently dried using a paper towel and lastly wiped with 70 % v/v ethanol prior to use.

Seed culture conditions after hydrated storage, and seedling maintenance

A sample of 30 seeds was randomly selected for removal from hydrated storage using sterile forceps. Of these, five seeds were used for gravimetric (X temp) water content measurement on individual seeds,

expressed on a dry mass basis (g H_2O g^{-1} dry mass). The other 25 seeds were further surface decontaminated in 1% (w/v) NaOCl containing a few drops of the wetting agent, Tween 20/80®, for 10 min, then rinsed three times in sterile distilled water. The seeds were then immersed in 0.1 % (w/v) mercuric chloride ($HgCl_2$) for 15 min and thereafter rinsed 3 times with sterile distilled water. Seeds were then further decontaminated with 0.01% (m/v) of Cicatrin (active ingredient, neomycin sulphate) for 10 min, and finally rinsed three times with sterile distilled water.

The decontaminated seeds were cultured on water agar for 30 d. Five seeds of *T. dregeana* were plated per 90 mm Petri dish, stored in a dark cardboard at ambient temperature until germinated to the stage of shoot emergence, after which the plates were transferred to a growth room with a 16 h photoperiod (66 µmol m-2s-1 PPFD provided by Biolux tubes [Osram L58W] and maintained at 27ºC day/21ºC night (standard culture conditions). Plates were checked daily for any incidence of contamination and (if there was any), uncontaminated seeds were transferred to a fresh sterile WA plate to prevent them from being cross contaminated (contamination was recorded accordingly).

Seed vigour was assessed by rate of germination of seeds (n=25) placed on filter paper in 65 mm Petri dishes (one seed per Petri dish). Seeds were watered every second day and germination assessed daily.

Seedling establishment maintenance in the pot trials

Seeds were sampled from hydrated storage after 0, 1, 2, 4, 6, 16, 32 and 64 days. They were then planted into 500 m*l* potting bags filled with commercial potting soil mix placed in the pot trials. The young plants were watered daily and weeds removed every second week for six months, after which plants were harvested. On the day of harvest, they were watered heavily to ensure easy separation from the potting soil. The roots were gently washed under running water and transferred to paper towel to remove excess water. The seedlings were then subdivided into leaves, stem and roots. The number of leaves was counted,

leaf area measured using an area meter (C1-202, CID, Inc., USA), and stem diameter and root length measured using a calibrated ruler. Separated parts were then dried to constant weight. Ten samples were used for each of the five treatments from plants grown from seeds after 0, 1, 2, 4, 6, 16, 32 and 64 d of storage.

Statistical Analysis

Data obtained were analyzed using SPSS 23 for Windows 7 and GenStat 17th Edition. The data that was not normally distributed were log transformed before analysis, but the original (untransformed) data are presented here. One-way and two- way analyses of variance (ANOVA) were used where applicable to analyze the data and the means compared using Tukey HSD^{a,b} post Hoc Test. Chi square test was used where necessary to test differences between two samples.

Results

Identified fungal species from Trichilia dregeana

Six fungal species were identified from the seeds of *T. dregeana viz*., *Paecilomyces lilacinus*, *Fusarium* sp., *Areobasidium pollulans, Penicillium* sp., *Penicillium brevicomputum* and *Trichoderma asperellum* . *Paecilomyces lilacinus* was the most prevalent fungus (frequency 0.55) on PDA followed by the other identified fungal species (each at 0.09).

Biological antimicrobial agents

Interaction amongst biological treatments and fungal isolates of *T. dregeana* (Table 2) showed that *B. subtilis* had the smallest average inhibition zones when compared with both strains of Trichoderma EcoT and Eco77. The fungal isolate demonstrating the most resistance against *B. subtilis* was *T. asperellum* (12 mm) and the most susceptible fungal isolate was *A. pullulans* (18 mm). However, overall response of the fungal isolates to *B. subtilis* as a biocontrol did not show much differences amongst isolates; as noticed, both P. *lilacinus* (16.7 mm) and *A. pullulans* (18 mm) were the most susceptible fungi belonging to the same category/subunit whereas the other fungal isolates were at the same level of susceptibility. *P. lilacinus, A. pullulans* and *P. brevicompactum* were not different from each other in the way they responded against Eco77 however responded differently when compared to *Fusarium sp*, *T. asperellum* and *Penicillium* species. Comparison amongst *Fusarium*, *T. asperellum* and *Penicillium* species were also significantly different. Eco77 was the most effective against *Penicillium* sp. (45.33 mm) and *T. asperellum* (36 mm) was the second most susceptible isolate. Eco 77 effects against the two fungal isolates was significantly different when compared with other isolates however different when they were also compared. Furthermore, EcoT was most effective against *T asperellum* (34.67 mm) and *A. pollulans* (30.67 mm). However, with EcoT differences were observed with fungal isolates of *A. pullulans* and *T. asperellum*, even though they were not different when compared to each other. *Fusarium* sp. and *Penicillium* sp. were not

different when compared to each other and also to *P. brevicompactum* however different when further compared to *P. lilacinus*.

In summary *B. subtilis* was the least effective antimicrobial agent as compared to Trichodermal strains of EcoT and Eco77, however when comparing the two trichodermal strains no differences were noted but had a slight difference when the actual inhibition zones where compared with Eco77 exhibiting slightly bigger zones of inhibition. In addition, the most problematic fungal genera in storage and in culture are Penicillium and *Fusarium* and therefore Eco77 achieved the highest inhibition zones [i.e. with *Penicillium sp*. (45 mm) and with Fusarium (26 mm)] was achieved. Although Eco77 was more effective than the other tested biological agents both strains of Trichoderma were also considered for further development of seed treatment protocols.

Chemical antimicrobial agents

A comparison of the effects of the various tested chemicals on fungal isolates (Table 3) showed that Ripenit was the weakest antimicrobial agent as it had the smallest inhibition zones (averaging at 2 mm with *P. lilacinus, A. pullullans, P. brevicompactum, T. asperellum* and *Penicillium sp*.). However, it was observed to be more effective against *Fusarium* sp. (19.67 mm). Orius was least effective against *P. brevicompactum* and *Fusarium* sp. (i.e. both averaged at 2 mm of inhibition zone), however it was highly effective against the fungal isolates of *P. lilacinus* and *T. asperellum* (i.e. 90 mm zone of inhibition). Afugan, Odeon and Sporekill had inhibition zones that averaged from 7 to 12 mm when tested against species of *Fusarium* sp., *P. brevicompactum* and *T. asperellum*. Similarly, Celest was also not very efficient in inhibiting *P. lilacinus* and *A. pullulans* with inhibition zones of 2 mm, however achieving an inhibition zone ranging from 17 to 23 mm with *P. brevicompactum, Fusarium* sp., *T asperellum* and *Penicillium* sp.

Nipastat performed consistently in inhibiting *P. lilacinus*, Fusarium sp. *Penicillium* sp. (i.e. inhibition zones ranging from 44 to 60 mm were obtained) but *A. pullulans* and *P. brevicompactum* were more resistant with

inhibition zones from 15 to 19 mm. Biotane maintained inhibition zones above 20 mm for four fungal isolates (i.e. *A. pullulans*, *P. brevicompactum*, *T. asperellum* and *Penicillium* sp.) but was less effective against *P. lillacinus* and *Fusarium* sp.

The results indicated that Nipastast, Orius and Biotaine were the most effective chemical control agents with Ripenit being the least effective. On the basis of the data presented in Tables 2 and 3 seed treatment protocols based on Eco77, EcoT, Nipastat and Biotaine were established. These were treatments DBe, DEBBe, DEBN, DEn and DEnN (See Table 1 for definitions of the abbreviations).

In culture: preliminary trials on treated and non-treated T. dregeana seeds after 64 d of hydrated storage

Table 4 shows the results from material that had been in hydrated storage 64 days and then assessed for 30 days in culture. During this period treatments DEBBe, DEBBe and DEnN showed no contamination. It was also noted that these three treatments were not significantly different from treatments DBe which had 4 contaminated explants and DEn which had 5 contaminated explants. Treatment DEnN had a significantly high roots and shoots development (i.e. 100 % of explants produced shoots and roots), whereas 5 - 20 explants in the other treatments (DBe, DEBBe and DEn rooted and shooted. Average root length was the highest with treatment DEn (56 mm) and this was statistically different when compared with treatments DBe, DEBBe and DEBN but not different from DEnN. DEnN treated seeds produced an average root length of 46 mm which was not statistically different from treatments DBe and DEBN which averaged at 34 mm however, it was different from DEBBe. DEnN was therefore considered the best treatment to maintain or improve seed life span in hydrated storage.

Pot trials: effects of developed protocols on the quality of T. dregeana seeds after 64 d of hydrated storage

Preliminary results in culture led to field trials investigations and seeds were treated using the developed protocols and subjected to hydrated storage at times 0, 1, 2, 4, 6, 16, 32 and 64 *d*. Noting that the difference came about when sampling these seeds and were then grown in 500 *ml* potting bags outside the greenhouse to insinuate the natural growth conditions.

Leaf area (Tables 5 and 6): Both seed treatment and storage time had a significant impact on leaf area ($p = 0.001$) with mean leaf area values ranging from (291 – 609 mm^2). In particular the longer the storage period the greater the impact. In this regard the leaf area of the control (0 days) of treatment DBe was 609 mm^2 but for material that had been stored for 64 days it had decreased to 166.0 mm^2.

Leaf biomass (Tables 5 and 7): Seed treatment ($p = 0.39$) and storage ($p = 0.47$) had no significant impact on leaf biomass. The average leaf biomass of the control was 0.49 g at day 0 whereas the other treatments ranged from 0.26 to 0.68 g. Control treatment DBe at storage day 64 had the lowest leaf biomass of (0.15 g) whereas treatment DEBN had the largest leaf biomass of 0.34 g when compared with the control treatment (DBe) and the other treatments (DEBBe, Den and DEnN) Therefore it was concluded that leaf biomass was neither negatively influenced nor positively influenced by these factors.

Root biomass (Tables 5and 8): Both treatment ($p = 0.04$) and storage ($p = 0.001$) regardless of each other had a significant impact on root biomass however where the two factors interact ($p = 0.277$), no effects on root biomass were observed. Seeds treated with DBe at 0 day had a root biomass that averaged at 0.61 g with the biggest biomass being obtained with seeds that were in storage for 2 days and 4 days with

treatments DEBBe and DEBN which both averaged at (0.67 g). Seeds that had been initially exposed to 64d storage produced plantlet that had the smallest root biomass of 0.25 g for control treatment DBe whereas the other treatment had a range from 0.36 g to 0.55 g. Treatment DEBN had a positive impact on root biomass when considering time of storage at 64 days even though the seed appearance after treatment was not good (i.e. seeds had brown patches). Impact observed for seeds treated with treatment DEBBe was the same for days 1, 2, 4, 6, 16 and 32.

Stem biomass (Tables 5 and 9): Stem biomass was significantly affected by seed treatment and storage. At the start of the experiment the control stem biomass averaged 0.50 g whereas the other treatments (DEBBe, DEBN, DEn and DEnN) ranged from 0.22 – 0.47 g. It appeared that increasing storage period had a positive impact on stem biomass with the largest stem biomass of (0.64 g) being obtained from DEBBe treated seeds stored for 32 d followed by treatment DEn treated seed stored for 64 days (0.51 g).

Discussion

As report by Sutherland *et al*., 2002 and Berjak and Pammenter, 2014 the risk of surface contaminants penetrating the inner tissues of *T. dregeana* when placed into storage was reduced by collecting the material directly from the tree and immediately performing surface decontamination treatments. The fungal contaminants isolated from *T. dregeana* seeds included *Paecilomyces lilacinus, Fusarium sp., Areobasidium pollulans, Penicillium sp., Penicillium brevicomputum* and *Trichoderma asperellum. Paecilomyces lilacinus* was the predominant isolate. This spectrum of contaminants is similar to Berjak *et al*. (2014) working with other tropical and sub-tropical recalcitrant seed species.

Eco77 was effective against all of the *T. dregeana* seed fungal isolates especially the *Penicilium* sp., however, it was noted that both strains (EcoT and Eco77) of *T. harzianum* were equally good antagonists. This varying degree of inhibition is possibly related to the previously reported differences in antagonistic potential of different strains and species of *Trichoderma* (Roiger and Jeffers, 1991).

Both Biotaine (surface decontaminant) and Nipastat (preservative) demonstrated consistent activity against the fungal isolates of *T. dregeana* and showed no detrimental effects against seed viability, similar to the observations of Motete *et al*. (1997) working with *Avicennia marina* seeds. Biotaine is a widely used surface decontaminant (active ingredient: chlorhexidine gluconate) whereas Chlorhexidine is a broad-spectrum biocide effective against Gram-positive bacteria, Gram-negative bacteria and fungi. The mechanism of action of chlorhexidine is related to cell wall and plasma membrane integrity resulting in leakage of cell contents and cell death (MacDoneld *et al*., 1999). For purposes of this study, biotaine was introduced after seeds had been exposed to Eco77 spore suspension for 4 hours. This treatment proved not to be detrimental to seed vigour and viability but effective in decreasing fungal growth in HS.

Application of 1% v/v NaOCl served as the basis of all developed decontamination protocols which was done before HS and seed *in vitro* culture, it is noteworthy that use of this surface decontaminant only eliminated the contaminants arising from cross contamination and not the seed-borne fungi. This is in

agreement with the report by Berjak *et al*, (2014) who noted that 1% v/v NaOCl was effective in eliminating all surface contaminants in embryonic axes with no detrimental effects on seed growth. In this regard the present *in vitro* seed culture trials revealed that treatment DEnN was significantly different from the other treatments with regards to shoot and root development (which was 100%) and in eliminating contamination (100% was obtained). This protocol (DEnN) was formulated from synseed technology which was first developed in the 1980s as reviewed by Reddy *et al*. (2012); however, it is ideally suited for embryonic axes because of explant size. Despite the size of the whole seed used for the current study, this method proved to be suitable as it was possible to incorporate the Nipastat into the alginate and form an nipastat-alginate bead that could be stored under HS for a period of 64 days with no contamination or in storage germination. Similar observations were also obtained for *A. marina by* Motete *et al*. (1997). Although the Nipastat is a powder and when applied to the seed had a drying effect it was able to contain fungi at the site of proliferation thus preventing from spreading from one seed to another.

A significantly larger leaf area was obtained from material treated with treatment DEn and this may be due to seeds responding positively to treatment and thus producing healthy seedlings. This was confirmed by treatment DEBN, where the plant material was unhealthy and had brown patches on the leaves. This also corresponds with reported findings by Araus *et al*. (2001); Zelitch, 1982 and Ashley and Boerma, 1989. Those authors reported in several species that total canopy photosynthesis during growth is closely related to yield and may be increased by faster approach to full cover (i.e. early vigour) and higher leaf extension by minimising stress. When plants are under stress the assimilation rate is generally more limiting to yield than it is under optimal conditions as indicated by higher association commonly observed between yield and above ground biomass maturity Araus *et al*. (2001). However, this pattern was not observed at day 64 with regard to leaf dry biomass but was noted at days1 and 6 as a higher leaf biomass was observed for the same treatment. A higher stem biomass and leaf length was also observed for the same treatment (DEn) at day 64 which thus corresponds to literature. However, the number of leaves and underground biomass allocation (root biomass) were more with *Trichoderma* treated seeds (DEBBe) at day 64 when compared

with all of the other treatments. Adding to biocontrol activity, *Trichoderma* strains have been reported to promote plant growth (Chang *et al*., 1986; Inbar *et al*., 1994; Ozbay *et al*., 2004), and therefore, this serves as possible explanation to understanding how *T. harzianum* strains control minor pathogens thus leading to stronger growth and nutrient uptake.

Concluding comments

The current research has not only shown the detrimental impacts of seed-associated mycoflora contaminants on maintaining seed viability, but has also significantly demonstrated the efficacy of Nipastat, Biotane, and Eco-77 as a decontaminant for these, and potentially other types of explants, for subsequent short-to-medium term (temporal) storage and other purposes. Exposure to Nipastat, Biotane and Eco-77 has the potential for a complete elimination of seed-associated field and storage fungi with no adverse effects on seeds or explants. The current study also demonstrated that alginate encapsulation (with or without Nipastat), has significant potential in the eradication of field, storage fungi or opportunistic contaminants via cross contamination and for the formation of synseeds. This has a marked a practical impact for both short and medium term storage of *T. dregeana* seeds.

Acknowledgements

Late Professor Patricia Berjak (1939 – 2015) mentorship, support and technical input is appreciated wholeheartedly. Ongoing financial assistance from the National Research Foundation (NRF) of South Africa is appreciated and acknowledged.

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Table 1: Summary of developed seed treatment protocols

Treatment	Eco77 (Biocontrol) 1 *g/l*	Biotaine™ 2% (v/v)	Benomyl 500 WP (Seed- dusting)	Nipastat® 1 *g/l*	Encapsulation 2% (w/v) low viscosity alginic acid (sodium salt)
DBe (Control)	-	-	√	-	-
DEBBe	√	√	√	-	-
DEBN	√	√	-	√ seed dusting	-
DEn	-	-	-	-	√
DEnN	-	-	-	√ incorporated into alginate capsule	√

D = surface decontaminated; **E** = Eco77; **B** = Biotaine; **Be** = Benomyl 500 WP, **N** = Nipastat;

En = encapsulation

Table 2: Inhibition of the growth of fungal isolates from the seed of *T. dregeana* by *B. subtilis, EcoT* and *Eco77*. Mean inhibition zone (mm) (p = 0.001). Different superscript upper-case letters in each row indicate significant differences.

Treatment	Fungal Isolate(s)					
	Mean inhibition zones (mm)					
	P. lilacinus	*Fusarium sp.*	*A. pullulans*	*Penicillium sp.*	*P. brevicompactum*	*T.asperellum*
EcoT	21.33 ^{A}	26^{B}	30.67^{C}	26.33^{B}	25.33^{AB}	34.67^{C}
Eco77	19.33^{A}	26^{B}	18.67^{A}	45.33^{D}	20.33^{A}	36^{C}
B. subtilis	16.67^{B}	13^{AB}	18^{B}	15.67^{AB}	15^{AB}	12^{A}

L.S.D of means at 5% = 4.087

Table 3: Inhibition of the growth of fungal isolates from the seed of *T. dregeana* by a number of fungicidal agents: Afugan, Biotaine, Celest, Orius, Odeon, Sporekill, Ripenit and Nipastat. Mean inhibition zone (mm) (p = 0.001). Different superscript upper-case letters in each row indicate significant differences (subsets).

Treatment	Fungal Isolate(s)					
	Mean inhibition zones (mm)					
	P. lilacinus	*A. pullullans*	*P. brevicompactum*	*Fusarium sp.*	*T. asperellum*	*Penicillium sp.*
Afugan	19.67^{C}	21^{C}	22^{C}	9^{B}	2^{A}	12.33^{C}
Biotaine	17.67^{B}	21^{C}	21.67^{C}	14^{A}	22.33^{C}	22.67^{C}
Celest	2^{A}	2^{A}	17^{B}	17^{B}	23^{C}	18^{B}
Orius	90^{D}	17.33^{B}	2^{A}	2^{A}	90^{D}	21.67^{C}
Odeon	21^{D}	2^{A}	7.33^{B}	12^{C}	22.67^{D}	2^{A}
Sporekill	17.33^{B}	18^{B}	31.33^{C}	17.67^{B}	8.33^{A}	34^{C}
Ripenit	2^{A}	2^{A}	2^{A}	19.67^{B}	2^{A}	2^{A}
Nipastat	44^{C}	15.67^{A}	19^{B}	60^{D}	21.67^{B}	60^{D}

LSD of means at 5% = 2.937

Table 4: *T. dregeana* in culture trials assessed for 30 days in culture after a pre storage of 64 days of hydrated storage of seeds. No. of contaminated explants [χ^2(4d.f. cont vs non. cont) = 14.847; *p=0.*05]; No. of seeds producing roots [χ^2(4d.f. roots dev. vs none) = 14.098; *p =0.007*]; No. of seeds producing shoots [χ^2(4d.f. shoots dev.vs none)] and Avg. length of roots (mm) [one way ANOVA; *p* =0.05]. Different superscript lower-case letters in each row indicate significant differences. **D** = surface decontaminated; **E** = Eco77; **B** = Biotaine; **Be** = Benomyl 500 WP, **N** = Nipastat; **En** = encapsulation

Plant Species	N	Observation @ 30d in culture	Treatment(s)				
			DBe	DEBBe	DEBN	DEn	DEnN
T. dregeana	25	No. of cont. explants	4^{B}	0^{A}	0^{A}	5^{B}	0^{A}
		No. of seeds producing roots	16^{A}	20^{B}	15^{A}	20^{B}	25^{C}
		No. of seeds producing shoots	16^{B}	5^{A}	15^{B}	20^{C}	25^{D}
		Avg. length of roots (mm)	36^{AB}	31^{A}	36^{AB}	56^{C}	46^{BC}

Table 5: Significant effects of treatment, storage time and their interactions on leaf area, leaf biomass, root biomass and stem biomass.

Plant species	Variable	Treatment			Storage days			Treatment X Storage days		
		df	F	Sig.	df	F	Sig.	df	F	Sig.
T. dregeana	Leaf Area (mm^2)	4	4.65	0.00*	7	5.59	0.00*	28	1.97	0.003*
	Leaf Biomass (g)	4	1.04	0.39	7	0.95	0.47	28	1	0.462
	Root biomass (g)	4	2.5	0.04*	7	3.93	0.00*	28	1.15	0.277
	Stem biomass (g)	4	3.49	0.01*	7	3.18	0.00*	28	2.18	0.01*

Table 6: Effects of treatment and storage on leaf area variable of *T. dregeana*. $p \leq 0.05$ = significant =* Different superscripts upper-case letters in each row indicate significant differences (subsets). **D** = surface decontaminated; **E** = Eco77; **B** = Biotaine; **Be** = Benomyl 500 WP, **N** = Nipastat; **En** = encapsulation

Treatment	Hydrated Storage (d)							
	Leaf area (mm^2)							
	0	**1**	**2**	**4**	**6**	**16**	**32**	**64**
DBe	609^{C}	375^{B}	370^{B}	389^{B}	379^{B}	319^{B}	314^{B}	166^{A}
DEBBe	453^{BC}	538^{C}	498^{BC}	357^{ABC}	332^{AB}	313^{A}	417^{ABC}	359^{AB}
DEBN	414^{AB}	466^{B}	396^{AB}	418^{AB}	323^{A}	388^{AB}	370^{AB}	316^{A}
DEn	474^{BC}	517^{C}	460^{ABC}	381^{AB}	439^{ABC}	354^{AB}	340^{A}	453^{ABC}
DEnN	291^{A}	368^{A}	370^{A}	350^{A}	348^{A}	301^{A}	316^{A}	355^{A}

LSD of means at 5% = 129.03

Table7: Effects of treatment and storage on leaf biomass variable of *T. dregeana*. $p \leq 0.05$ = significant =* Different superscripts upper-case letters in each row indicate significant differences (subsets). **D** = surface decontaminated; **E** = Eco77; **B** = Biotaine; **Be** = Benomyl 500 WP, **N** = Nipastat; **En** = encapsulation

Treatment	Hydrated Storage (d)							
	Leaf biomass (g)							
	0	**1**	**2**	**4**	**6**	**16**	**32**	**64**
DBe	0.49^{A}	0.33^{A}	0.28^{A}	0.40^{A}	0.29^{A}	0.24^{A}	0.28^{A}	0.15^{A}
DEBBe	0.40^{A}	0.44^{A}	0.40^{A}	0.30^{A}	0.28^{A}	0.32^{A}	0.42^{A}	0.29^{A}
DEBN	0.32^{A}	0.37^{A}	0.32^{A}	0.36^{A}	0.27^{A}	0.33^{A}	0.25^{A}	0.34^{A}
DEn	0.37^{A}	0.68^{A}	0.40^{A}	0.37^{A}	0.38^{A}	0.31^{A}	0.25^{A}	0.30^{A}
DEnN	0.26^{A}	0.26^{A}	0.28^{A}	0.28^{A}	0.27^{A}	0.24^{A}	0.26^{A}	0.24^{A}

LSD of means at 5% = 1.745

Table 8: Effects of treatment and storage on root biomass variable of *T. dregeana*. $p \leq 0.05$ = significant =* Different superscripts upper-case letters in each row indicate significant differences (subsets). **D** = surface decontaminated; **E** = Eco77; **B** = Biotaine; **Be** = Benomyl 500 WP, **N** = Nipastat; **En** = encapsulation

Treatment	Hydrated Storage (d)							
	Mean Root biomass (g)							
	0	**1**	**2**	**4**	**6**	**16**	**32**	**64**
DBe	0.61^{BC}	0.64^{BC}	0.56^{BC}	0.65^{C}	0.58^{BC}	0.43^{AB}	0.41^{AB}	0.25^{A}
DEBBe	0.54^{AB}	0.67^{B}	0.67^{B}	0.59^{AB}	0.66^{B}	0.56^{AB}	0.66^{B}	0.42^{A}
DEBN	0.51^{AB}	0.45^{A}	0.47^{AB}	0.67^{B}	0.50^{AB}	0.56^{AB}	0.47^{AB}	0.55^{AB}
DEn	0.57^{A}	0.60^{A}	0.61^{A}	0.57^{A}	0.53^{A}	0.60^{A}	0.42^{A}	0.43^{A}
DEnN	0.38^{AB}	0.50^{AB}	0.56^{AB}	0.54^{AB}	0.55^{AB}	0.58^{B}	0.40^{AB}	0.36^{A}

LSD of means at 5% = 0.206

Table 9: Effects of treatment and storage on stem biomass variable of *T. dregeana*. $p \leq 0.05$ = significant =* Different superscripts upper-case letters in each row indicate significant differences (subsets). **D** = surface decontaminated; **E** = Eco77; **B** = Biotaine; **Be** = Benomyl 500 WP, **N** = Nipastat; **En** = encapsulation

Treatment	**Hydrated Storage (d)**							
	Mean Stem biomass (g)							
	0	**1**	**2**	**4**	**6**	**16**	**32**	**64**
DBe	0.50^{BC}	0.35^{AB}	0.27^{AB}	0.57^{C}	0.33^{AB}	0.33^{AB}	0.49^{BC}	0.22^{A}
DEBBe	0.47^{AB}	0.44^{A}	0.44^{A}	0.38^{A}	0.36^{A}	0.44^{A}	0.64^{B}	0.44^{A}
DEBN	0.35^{A}	0.36^{A}	0.37^{AB}	0.36^{A}	0.37^{A}	0.29^{A}	0.55^{B}	0.44^{AB}
DEn	0.35^{AB}	0.64^{C}	0.42^{AB}	0.42^{AB}	0.41^{AB}	0.35^{AB}	0.29^{A}	0.51^{BC}
DEnN	0.22^{A}	0.26^{A}	0.27^{AB}	0.33^{AB}	0.31^{AB}	0.29^{AB}	0.56^{C}	0.45^{BC}

LSD of means at 5% = 0.185