

# **THE EFFECT OF PROVENANCE ON THE RESPONSE OF THE RECALCITRANT SEEDS OF *TRICHILIA DREGEANA* TO DRYING AND CHILLING**

By

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**INYUVESI  
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## **PREFACE**

The experimental work described in this Masters thesis was carried out in the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, from May 2009 to September 2010, under the supervision of Professor P. Berjak and Professor N. Pammenter.

This study represents original work by the author and has 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 is duly acknowledged in the text.

Rebecca O. Oyerinde

December 2011

**DECLARATION 1 – PLAGIARISM**

I, ....., 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.
3. This thesis does not contain other persons’ data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed

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## ABSTRACT

One of the factors that affect the post-harvest behavior of seeds is provenance. Different geographical locations are characterised by different environmental conditions, hence, plant materials harvested from different locations may respond differently to laboratory-induced stresses.

The aim of the present study was to assess the role that provenance plays on the degree of recalcitrance of a single species – a factor that needs to be taken into consideration when choices are to be made for plant germplasm conservation. In this study, seeds of *Trichilia dregeana*, which have been shown to display recalcitrant post-harvest behavior, were harvested from four locations that were slightly different in climatic conditions: Mtunzini (MTZN), Durban (DBN), Pietermaritzburg (PMB) and Port Edward (P.ED), all within the KwaZulu-Natal province of South Africa. Clean seeds, whose aril and seed coat have been removed, were subjected to different drying and chilling stresses and their responses to the stresses are examined. Excised embryonic axes of the seeds were also subjected to flash (very rapid) drying and their responses to the stress are shown.

Although the vegetation of these locations can be categorized as being sub-tropical, the present study suggests that there may be ‘degrees’ in the sub-tropical nature based on the rainfall and maximum temperature data. The ‘degree of sub-tropicality’ is suggested to be in the order MTZN > DBN > P.ED > PMB. Mean seed size assessed as seed length, width and fresh mass varied significantly across the four regions, with seeds from MTZN being the smallest and those from P.ED being the largest. The shedding water content of the embryonic axes of the seeds ranged from 1.28 - 2.35 g g<sup>-1</sup> dry weight (DW) but it varied significantly across the four provenances in the order MTZN < DBN < P.ED < PMB.

The first 20 min of flash drying the embryonic axes resulted in rapid loss of water, with axes of seeds from the four regions losing more than 50% of the shedding water contents. Mean percentage germination for each provenance was still relatively high (70% and above) after flash drying the embryonic axes of the seeds for 150 min. However, shoot

production, root and shoot length and dry mass accumulation showed that axes of seeds from MTZN were the most adversely affected by rapid drying while those from PMB were the least sensitive. When clean whole seeds were dried slowly by burying in silica gel for 36 h, all seeds of the four provenances still retained more than 50% of their shedding water. MTZN seeds lost viability completely from the 24 h of slow drying while seeds from the other three locations retained some viability after 36 h. The order of survival of slow drying was MTZN < DBN < P.ED = PMB.

Storage of cleaned whole seeds at chilling temperatures (3°C, 6°C and 16°C) caused increase in the water content of the embryonic axes of the seeds across the four locations. All seeds of the four provenances had lost more than 50% survival after they had been stored at 3°C for 10 weeks. Survival was completely lost from the 12<sup>th</sup> week for MTZN seeds, from the 14<sup>th</sup> week for DBN and P.ED seeds and from the 16<sup>th</sup> week for PMB seeds. There was no survival recorded for seeds across the four regions at the 18<sup>th</sup> week. The seeds were able to tolerate storage at 6°C for a little longer than those stored at 3°C. Only MTZN seeds completely lost viability at the 18<sup>th</sup> week; seeds from the other three locations retained some viability throughout the 18 weeks of storage. Storage at 16°C was the most tolerable, as all seeds from the four provenances maintained some viability until the 18<sup>th</sup> week in storage, with MTZN having the least survival and those from P.ED having the highest survival.

Phylogenetic analysis of the internal transcribed spacer (ITS) region of the DBN and PMB samples showed intraspecific levels of genetic variation, and were separated by a genetic distance of 0.9%, an indicator that differences in storage behavior, chilling and desiccation sensitivity between seeds obtained from different collection localities may be genetically based.

This study showed that MTZN seeds were the most sensitive, while seeds from PMB and P.ED were the most tolerant, to drying and chilling treatments, respectively. The prevailing climatic conditions may have a bearing on the responses observed in this study.

## TABLE OF CONTENTS

<b>TITLE PAGE.....</b>	<b>i</b>
<b>PREFACE.....</b>	<b>ii</b>
<b>DECLARATION 1 – PLAGIARISM.....</b>	<b>iii</b>
<b>ACKNOWLEDGMENT.....</b>	<b>iv</b>
<b>ABSTRACT.....</b>	<b>v</b>
<b>TABLE OF CONTENTS.....</b>	<b>vii</b>
<b>LIST OF FIGURES.....</b>	<b>xi</b>
<b>LIST OF TABLES.....</b>	<b>xv</b>
<b>CHAPTER ONE: INTRODUCTION .....</b>	<b>1</b>
1.1 Biodiversity and plant genetic resources .....	1
1.1.1 The need for plant germplasm conservation.....	1
1.1.2 Conservation of plant germplasm .....	5
1.1.2.1 Methods of plant germplasm conservation.....	5
1.2 Seeds and seed development.....	6
1.2.1 Post-harvest behaviour of seeds.....	7
1.2.1.1 Orthodox seeds.....	8
1.2.1.2 Non-orthodox seeds .....	9
1.2.2 Why conserve recalcitrant seeds?.....	10
1.3 Seed storage and <i>in vitro</i> storage of germplasms.....	12
1.3.1 Seed storage .....	12
1.3.1.1 Conventional methods of seed storage .....	12
1.3.1.2 Storage at ultra low moisture content .....	13
1.3.1.3 Moist or imbibed storage .....	14
1.3.2.2 Normal growth.....	16
1.4 Desiccation sensitivity and damage .....	17
1.5 Chilling sensitivity and injury.....	18
1.6 Drying and drying rates .....	22
1.7 Cryopreservation.....	25

1.8	Effect of provenance .....	25
1.9	Phylogeny .....	27
1.10	The present study .....	29
1.10.1	Provenances investigated in this study .....	29
1.10.2	Species under investigation in this study .....	33
1.11	Aim and objectives of the present study .....	38
<b>CHAPTER TWO: MATERIALS AND METHODS .....</b>		<b>39</b>
2.1	Seed collection and cleaning.....	39
2.2	Measurement of seed physical characteristics .....	39
2.3	Seed decontamination .....	39
2.4	Seed storage .....	40
2.5	Determination of water content .....	40
2.6	Dehydration.....	41
2.6.1	Flash drying .....	41
2.6.2	<i>In vitro</i> germination medium and germination of flash dried embryonic axes .....	42
2.6.3	Silica-gel drying.....	42
2.6.4	<i>In vitro</i> germination medium and germination of silica-gel dried seeds ..	43
2.7	Chilling .....	43
2.8	Assessment of germinability and growth parameters .....	43
2.9	Temperature and rainfall data .....	43
2.10	Phylogeny .....	45
2.10.1	DNA extraction.....	45
2.10.2	Polymerase Chain Reaction (PCR) amplification.....	46
2.10.3	DNA purification, quantification and analysis .....	47
2.11	Statistical analysis.....	47
<b>CHAPTER 3: RESULTS .....</b>		<b>48</b>
3.1	2008 and 2009 temperature and rainfall data for the locations from which collections were made.....	48
3.2	Morphological differences of <i>T. dregeana</i> seeds from the four provenances ..	50

3.3	Initial water contents and germination of <i>T. dregeana</i> seeds from the four provenances.....	52
3.4	Responses of <i>T. dregeana</i> embryonic axes and seeds from the provenances to drying.....	53
3.4.1	Rapid (flash) drying of embryonic axes.....	53
3.4.1.1	Water content and germination.....	53
3.4.1.2	Percentage root and shoot production after flash drying .....	56
3.4.1.3	Root and shoot length of seedlings from previously flash-dried embryonic axes .....	57
3.4.1.4	Seedling dry mass .....	60
3.4.2	Slow (Silica-Gel) drying.....	60
3.4.3	Comparison between axes water content and germination of embryonic axes of <i>T. dregeana</i> excised and dried rapidly and slowly dried whole seeds.....	63
3.5	Responses of seeds of <i>T. dregeana</i> from the four provenances to storage at sub-ambient temperatures.....	67
3.5.1	Response to storage at 3°C.....	67
3.5.2	Response to storage at 6°C.....	69
3.5.3	Response to storage at 16°C.....	71
3.6	Comparison between <i>T. dregeana</i> seeds harvested in 2009 and 2010 from Mtunzini.....	73
3.6.1	Flowering to fruit shedding conditions of <i>T. dregeana</i> from Mtunzini for 2009 and 2010.....	74
3.6.2	Differences in response to drying of 2009 and 2010 <i>T. dregeana</i> seeds from MTZN .....	74
3.6.2.1	Flash drying of embryonic axes of <i>T. dregeana</i> seeds .....	74
3.6.2.2	Silica-gel drying.....	76
3.6.3	Differences in responses of 2009 and 2010 <i>T. dregeana</i> seeds from Mtunzini to storage at different cold temperatures .....	78
3.6.3.1	Storage at 3°C .....	78
3.6.3.2	Storage at 6°C .....	80

3.6.3.3	Storage at 16°C .....	82
3.7	Phylogeny .....	84
<b>CHAPTER FOUR: DISCUSSION.....</b>		<b>86</b>
4.1	Prevailing climatic conditions and morphological differences of seeds harvested from the different provenances.....	86
4.2	Initial water contents and germination of <i>T. dregeana</i> seeds from the four provenances.....	87
4.3	Response of <i>T. dregeana</i> seeds from the four provenances to drying .....	88
4.3.1	Flash drying .....	88
4.3.2	Slow drying.....	922
4.3.3	Comparison between the two drying rates across provenances.....	94
4.4	Responses of <i>T. dregeana</i> from each provenance to storage under chilling conditions.....	95
4.5	Comparison between <i>T. dregeana</i> seeds harvested from Mtunzini in 2009 and 2010.....	98
4.6	Correlation between the provenances studied and their responses to dehydration and chilling stresses .....	99
4.7	Phylogeny .....	101
4.8	Concluding remarks .....	103
<b>REFERENCES.....</b>		<b>105</b>

## LIST OF FIGURES

- Figure 1.1: Map of South Africa showing KwaZulu-Natal province (a); Map of KwaZulu-Natal indicating (red arrows) the provenances studied (b)..... 32
- Figure 1.2: *Trichilia dregeana* tree. .... 37
- Figure 1.3: *Trichilia dregeana* open fruits with seeds. Photo taken by Prof. Berjak, P..  
..... 37
- Figure 1.4: *Trichilia dregeana* seeds from the four provenances. Photo taken by Prof. Berjak, P..... 38
- Figure 3.1: Mean seed fresh mass of *T. dregeana* seeds of different provenances. Mean (n = 20) with different letters are significantly different (one-way ANOVA, Duncan and Scheffe post hoc tests  $p \leq 0.05$ ). Bars represent one standard deviation on either of the mean ..... 51
- Figure 3.2: Mean length and width of *T. dregeana* seeds of the four provenances. Mean (n = 20) with different letters are significantly different (one-way ANOVA Duncan and Scheffe post hoc tests  $p \leq 0.05$ ). Bars represent one standard deviation on either side of the mean..... 52
- Figure 3.3: Water content of flash dried embryonic axes of *T. dregeana* from different provenances (n = 5), bars represent standard deviation..... 54
- Figure 3.4: Percentage germination of flash dried embryonic axes of *T. dregeana* from different provenances (n = 25)..... 56
- Figure 3.5: Seedlings of *T. dregeana* after 12 weeks in culture, from previously flash dried axes for each provenance (I: MTZN; II: DBN; III: PMB; IV: P.ED)

	and flash drying time (a: 0; b: 20; c: 40; d: 60; e: 90; f: 120; g: 150 FD minutes).....	58
Figure 3.6:	Water contents of embryonic axes from silica-gel dried whole seeds <i>T. dregeana</i> from different provenances.....	62
Figure 3.7:	Percentage germination of silica-gel dried whole seeds of <i>T. dregeana</i> from different provenances.....	62
Figure 3.8:	The relationship between the water content of excised axes that have been flash dried and axes dried slowly in whole seeds, and germination of axes that have been flash dried and slowly dried whole seeds of <i>T. dregeana</i> from MTZN .....	64
Figure 3.9:	The relationship between the water content of excised axes that have been flash dried and axes dried slowly in whole seeds, and germination of axes that have been flash dried and slowly dried whole seeds of <i>T. dregeana</i> from DBN .....	64
Figure 3.10:	The relationship between the water content of excised axes that have been flash dried and axes dried slowly in whole seeds, and germination of axes that have been flash dried and slowly dried whole seeds of <i>T. dregeana</i> from PMB .....	66
Figure 3.11:	The relationship between the water content of excised axes that have been flash dried and axes dried slowly in whole seeds, and germination of axes that have been flash dried and slowly dried whole seeds of <i>T. dregeana</i> from P.ED .....	66
Figure 3.12:	Axes water content of <i>T. dregeana</i> seeds from the four provenances stored at 3°C (n = 5), bars represented standard deviation .....	68

Figure 3.13:	Percentage germination of <i>T. dregeana</i> seeds from the four provenances stored at 3°C (n = 25).....	69
Figure 3.14:	Axes water content of <i>T. dregeana</i> seeds from the four provenances stored at 6°C (n = 5), bars represented standard deviation .....	70
Figure 3.15:	Percentage germination of <i>T. dregeana</i> seeds from the four provenances stored at 6°C, (n = 25).....	71
Figure 3.16:	Axes water content of <i>T. dregeana</i> seeds from the four provenances stored at 16°C, (n = 5), bars represented standard deviation. ....	72
Figure 3.17:	Percentage germination of <i>T. dregeana</i> seeds from the four provenances stored at 16°C, (n = 25).....	73
Figure 3.18:	Axes water content of flash dried embryonic axes of 2009 and 2010 <i>T. dregeana</i> seeds from MTZN (n = 5), bars represented standard deviation .....	75
Figure 3.19:	Percentage germination of flash dried embryonic axes of <i>T. dregeana</i> seeds from MTZN in 2009 and 2010 (n = 25).....	75
Figure 3.20:	Axes water content of silica-gel dried seeds of 2009 and 2010 <i>T. dregeana</i> from MTZN (n = 5), bars represented standard deviation .....	77
Figure 3.21:	Percentage germination of silica-gel dried seeds of 2009 and 2010 <i>T. dregeana</i> from MTZN (n = 5). ....	77
Figure 3.22:	Axes water content of 2009 and 2010 <i>T. dregeana</i> seeds from MTZN, stored at 3°C (n = 5), bars represented standard deviation .....	79

Figure 3.23: Percentage germination of 2009 and 2010 <i>T. dregeana</i> seeds from MTZN, stored at 3°C (n = 25).....	79
Figure 3.24: Axes water content of 2009 and 2010 <i>T. dregeana</i> seeds from MTZN, stored at 6°C (n = 5), bars represented standard deviation .....	81
Figure 3.25: Percentage germination of 2009 and 2010 <i>T. dregeana</i> seeds from MTZN, stored at 6°C (n = 25).....	81
Figure 3.26: Axes water content of 2009 and 2010 <i>T. dregeana</i> seeds from MTZN, stored at 16°C (n = 5), bars represented standard deviation .....	83
Figure 3.27: Percentage germination of 2009 and 2010 <i>T. dregeana</i> seeds from MTZN, stored at 16°C (n = 25).....	83
Figure 3.28: Phylogenetic tree based on analysis of 337 nucleotides of the nuclear ribosomal ITS1 region of <i>T. dregeana</i> , with <i>T. emetica</i> as the outgroup. Nodal support values were derived from neighbour joining (nj), maximum parsimony (mp) and maximum likelihood (ml) values (nj/mp/ml). .....	84

## LIST OF TABLES

Table 1. 1:	Straight line or air distance (km) between the locations studied .....	29
Table 2. 1:	Sequences of primers used in amplification of the nuclear ribosomal ITS1 region of <i>T. dregeana</i> DNA .....	46
Table 2. 2:	Thermal cycling protocol used in PCR amplification of the ITS region ..	46
Table 3.1:	2008-2009 rainfall and temperature data for the collection locations as derived from the data supplied by South African Weather Service.....	48
Table 3.2:	Comparison of geographical characteristics and accumulated rainfall and temperature from flowering to fruit shedding period across the provenances studied .....	49
Table 3.3:	Initial water contents ( $\pm$ one standard deviation) and viability of embryonic axes and whole seeds of <i>T. dregeana</i> . Means with different letters are significantly different, one-way ANOVA, ( $p \leq 0.05$ ). .....	53
Table 3. 4:	Percentage water loss relative to the initial axes water contents after flash-drying the embryonic axes of <i>T. dregeana</i> from different provenances...	54
Table 3.5:	Percentage shooting of <i>T. dregeana</i> axes from different provenances after each flash drying time. Means with different letters are significantly different (Two-way NOVA, Tukey's Post Hoc test, $p \leq 0.05$ ).....	57
Table 3.6:	Mean root length of seedlings obtained from <i>T. dregeana</i> axes that have been previously dried rapidly, for each and provenance and for each flash drying time. Mean with different letters are significantly different, Two-way ANOVA, ( $p \leq 0.05$ ).....	58

Table 3.7: Mean shoot length of seedlings obtained from *T. dregeana* axes that have been previously dried rapidly, for each and provenance and for each flash drying time. Mean with different letters are significantly different, Two-way ANOVA, ( $p \leq 0.05$ )..... 59

Table 3.8: Average dry mass of seedlings produced by rapidly dried axes of *T. dregeana* seeds of all four provenances. Mean with different letters are significantly different, Two-way ANOVA, ( $p \leq 0.05$ ) ..... 60

Table 3. 9: HKY genetic distances (%) between *T. dregeana* and *T. emetica* samples.. ..... 85

# CHAPTER ONE

## INTRODUCTION

### 1.1 Biodiversity and plant genetic resources

Biodiversity refers to the variety of all living forms: microorganisms, plant and animal species in a given region, including their genetic materials and the ecosystems of which they are part (Tamayo *et al.*, 1997). Plant Genetic Resources (PGRs) are the sum of all combinations of genes resulting from the evolution of plant species. It includes wild or domesticated plant species, as well as products of classical breeding, biotechnology and genetic engineering (Hidalgo *et al.*, 2007).

#### 1.1.1 The need for plant germplasm conservation

Almost all life forms are directly or indirectly dependent on plants for survival and the need to conserve plant genetic resources is of greater importance than ever before. Plants provide a wide range of ecosystem services, from basic human needs like food, shelter and clothing, to fuels, medicines, fibres, rubber and other products for the use of humans. In addition, they produce oxygen and remove carbon dioxide from the atmosphere (Secretariat of the Convention on Biological Diversity [SCBD], 2009). However, many plant species are vulnerable to extinction due to anthropogenic activities and natural factors which have collectively contributed to the loss of PGRs in the wild through extinction events. Some of these factors include: deforestation for cultivation and settlement, industrialisation, bush encroachment, invasion of alien species, unsustainable harvesting of plants for traditional medicines, population growth, climate change, unsustainable agricultural and forestry practices, urbanisation, pollution, land use changes, among many others (SCBD, 2002; Department of Environmental Affairs and Tourism [DEAT], 2010).

Use of plants for traditional medicinal practices is one of the major factors contributing to the threat of extinction of wild plant species. Traditional herbal medicine is regarded by the World Health Organisation (WHO), as one of the surest means to achieve a total health care coverage of the world population. Approximately 80% of the world's populations (more than 4 billion people) presently use herbal medicine for their health needs (WHO, The International Union for Conservation of Nature and Natural Resources [IUCN] and World Wide Fund for Nature [WWF], 1993); with about 20,000 plants species being used for the purpose. China and India are the two largest users of herbal medicine with an estimate of over 5,000 and 7,000 plant species being used within these countries, respectively. India for example consumes about 2,000 tons of herbal medicine annually. Arab physicians use between 200 and 250 plant species for the same purpose (SCBD, 2002; Saad, *et al.*, 2005; Verma and Singh, 2008). About 4,000 taxa are used across Africa while over 700 plant species are reported as being traded for medicinal purpose on the continent (Mulholland and Drewes, 2004). About 4,000 tons of medicinal plants or plant parts are traded in Durban, a city within the province of KwaZulu-Natal South Africa, alone, yielding about R270 million (*c.* \$36 million) annually (von Ahlefeldt *et al.*, 2003; Berjak, 2005). Herbal medicines yield more than \$60 billion in the global market annually and this is expected to increase by an average rate of 6.4% per annum (Sharma *et al.*, 2008).

Plant parts that are used for medication concoctions include roots, leaves, bark, rhizomes, fruits, seeds, stems and flowers but sometimes, whole plants are utilised (Verma and Singh, 2008; Sharma *et al.*, 2008). Harvesting of these plants or parts thereof for medicinal use leads to scarcity of plants and in turn, loss of biodiversity. The demand for herbal remedies is increasing worldwide, leading to an unsustainable, indiscriminate harvesting and overexploitation of the plants, especially the wild species (which are harvested mostly for medicinal purposes [Alves and Rosa, 2007]).

Another potent factor that is contributing to biodiversity loss and receiving urgent worldwide attention is climate change. Climate change is a consequence of increasing atmospheric concentrations of greenhouse gases (water vapour, CO<sub>2</sub>, CH<sub>4</sub>, N<sub>2</sub>O and

Ozone) – largely a result of increasing emissions resulting from human activities. Climate change is evidenced by increasing temperature, altered precipitation patterns, length of growing season and extreme weather events such as storms, fires, drought, etc. (Hannah *et al.*, 2002). In the past century, there was average temperature rise of about 0.6°C, but it is projected to increase by 0.1°C to 0.4°C per decade i.e. 1°C to 4°C over the next century. The SCBD report (2009) reported that a rise in global temperature will increase the rate of extinction of plant species. Plant adaptation or migration and changes in distribution are consequent upon climate change. In most cases, however, plants may not be able to keep up with the rapid rate at which the climate is changing (Jump and Peñuelas, 2005); their ability to reach a new climatically suitable environment may be prevented by habitat loss and fragmentation, or their persistence in a new climatic environment may be hampered by invasive alien species (Thomas *et al.*, 2004). Scenarios such as these can lead to extinction events.

The physiological functions, the phenological timing (i.e. the timing of seasonal activities of plants and animals like earlier shooting and flowering in plants [Fitter and Fitter, 2002; Walther *et al.*, 2002; Parmesan and Yohe, 2003]) and the successful completion of plant's life cycle are dependent on, among other things, specific environmental conditions. Changes in climatic conditions can, and are, impacting these functions significantly. Vulnerable plant species (such as plants with narrow climatic and geographic ranges or limited habitat requirements) and ecosystems (such as mangroves and montane grasslands) are more likely to be significantly impacted and threatened with extinction (SCBD, 2003). Willis *et al.* (2008) are of the opinion that high extinction risk may be due, and proportional, to climate change. Thomas *et al.* (2004) sampled about 20% of the Earth's terrestrial surface and predicted that, by 2050, 15-37% of the species found in the region sampled may be extinct. However, Michael *et al.* (2009) felt that the projected impacts of climate change may likely be occurring 30–50 years earlier than predicted due to the pace of climate change.

South Africa, a country classified as semi-arid, with a generally temperate climate but characterised by a great variety of climatic zones and habitat types, is not excluded from

the factors that are contributing to diversity loss. The country has one of the richest flora (and fauna) globally, with seven major terrestrial biomes, and is ranked as the country with the fifth highest number of plant species on the planet (DEAT, 2010), a large proportion of which are endemic (see Spenceley, 2001). Sadly, about 91% of the country is comprised of dry lands, making the country vulnerable to desertification, which is expected to increase with climate change (Von Maltitz and Scholes, 2006). Spenceley (2001) reported that South Africa has the highest concentration of threatened plant groups on the planet, constituting about 36% of the threatened plant groups. By 2050, about 27% of South African Proteaceae are projected to be extinct due to land use changes while climate change will drive *c.* 21-27% to extinction. That projection is even with maximum dispersal, which is unlikely for the plants in that family (Thomas *et al.*, 2004). The country is also plagued by the problem of soil degradation due to, among other factors, wind and water erosion; and vegetation degradation due to grazing, bush encroachment and deforestation (DEAT, 2010).

The activities and factors mentioned above contribute, either directly or indirectly, to decreased food production, poor human health, increased poverty, famine, water scarcity, increased prevalence of tropical diseases and worst of all, the loss of biodiversity and extinctions (DEAT, 2010). The effects and consequences of biodiversity loss are most severe on the poor who can not access or afford substitutes or alternative livelihood practices or resources. This is especially true for rural communities which are largely directly dependent on plants and plant allies for their livelihood.

It is necessary to conserve plants and plant genetic resources to mitigate the effects and consequences of biodiversity loss which will affect present and future generations. Kundu *et al.* (2009) summarily gave two main reasons for why biodiversity should be conserved: 1) to protect against unforeseen and unpredictable disasters and dangers, especially extinction and 2) to provide availability for the future.

### **1.1.2 Conservation of plant germplasm**

Conservation of plant genetic resources refers to the maintenance of plant populations in their natural habitat, or samples of these populations in germplasm banks, with the presumption that these materials are useful or potentially useful, and seeks to maintain and manage them for both current and future benefits (Pineda and Mejía, 2007). On the other hand, plant germplasm refers to plant structures that are able to give rise to a new generation of a given plant species either naturally or via *in vitro* manipulations. These structures include seeds, propagules or DNA/gene fragments (Hidalgo *et al.*, 2007).

#### **1.1.2.1 Methods of plant germplasm conservation**

There are two methods for the conservation of plant germplasm: *ex situ* and *in situ* conservation (Kundu *et al.*, 2009). *In situ* conservation is the conservation of plant germplasms in the natural habitats in which they have developed. *Ex situ* conservation is the conservation of plant germplasm outside the natural environments in which they have evolved (Hidalgo *et al.*, 2007).

According to Hayward and Hamilton (1997), *in situ* conservation does not just refer to the conservation of plant species in their natural habitats, but also includes other normal habitats like man-made gardens and farms. It also includes national parks and forest reserves. According to those authors, *in situ* conservation aims at conserving possible maximum diverse genotypes while allowing continuous evolution. This helps plant species adapt genetically to pests and diseases (i.e. select for genes that favour resistance) and changing environmental conditions. It also helps in conserving a wide range of biodiversity rather than just the targeted species. However, *in situ* conservation requires land space to be carried out; this may be a major problem with the trend of drastic loss of land to other uses (Engelman, 1991). It also requires trained personnel (Engelman, 1991), as it does not ensure the survival of targeted species unless there is efficient management of individual species (Heywood and Iriondo, 2003). Also, species conserved in this way may be threatened considerably by unforeseen natural and anthropogenic disasters like

fires, pest and pathogen, socio-economic developments and land-use changes (Engelman, 1991; Chin, 1996; Li and Pritchard, 2009).

*Ex situ* conservation is complementary to *in situ* conservation and it involves the collection of selected germplasm or representative samples of desired species and storage of these in facilities like seed banks, field gene banks, cryobanks, culture libraries and botanical gardens (Heywood and Iriondo, 2003). It provides material for researchers to investigate the physiology, morphology and biochemistry of the stored germplasm, and also serve as material for education, breeding and re-introduction programmes. Most important is its use in the conservation of genetic varieties that are of great value and whose existence in nature may be threatened (Heywood and Iriondo, 2003; Hidalgo *et al.*, 2007).

## **1.2 Seeds and seed development**

Seeds constitute the germplasm that is preferentially used for *ex situ* germplasm conservation (Berjak, 2006). They are easily accessible as many plants species produce seeds, are relatively easy to collect compared to other germplasm and they can be stored in relatively small places. Seed storage is also economical in terms of infrastructure, manpower, technology and cost. In addition, the risk of genetic damage while in storage is minimal (Heywood and Iriondo, 2003; Li and Pritchard, 2009). Seeds are essential for many breeding programmes and conservation of genetic resources. They are also indispensable for present and future human needs and survival (Berjak *et al.*, 1996; Chin, 1997; Berjak and Pammenter, 2004).

A seed is the propagating unit which has naturally accumulated all the necessary materials needed for survival and subsequent seedling establishment. It encloses, in itself, a whole plant embryo. Generally, there are, at least, three phases of seed development for the vast majority of plant species, especially those producing orthodox seeds (to be discussed later). The first is embryogenesis which is characterised by rapid cell division and histodifferentiation after fertilization. It leads to the formation of the embryonic axis

and associated nutritive tissues, it also results in the rapid gain of seed fresh weight. The second phase (also referred to as seed maturation) is characterised by synthesis, deposition and mass accumulation of storage reserves (which comprise proteins, carbohydrates and lipids), leading to a rapid increase in dry weight, as well as cell expansion. The third developmental phase is maturation drying (recently referred to as the desiccation phase (Angelovici *et al.*, 2010)) and it is specific to orthodox seeds. In this phase, there is rapid loss in the seed water content as the maximum dry weight is, or nearly, reached. The seed may be shed at this stage and it is quiescent and ready for germination under favourable conditions such as the availability of water (Welbaum and Bradford, 1989; Bewley and Black, 1994; Waterworth and Bray, 2007; Angelovici *et al.*, 2010).

### **1.2.1 Post-harvest behaviour of seeds**

Seeds are broadly grouped into two categories based on their post-harvest behaviour and storability: these are the orthodox and recalcitrant seed categories (Roberts, 1973). According to that author, 'orthodox' seeds are those that are shed dry at low water content and can still be further dried to lower water content (0.02 to 0.05 g g<sup>-1</sup> [dry mass basis]). The longevity of such seeds can be predicted in storage under low temperature and at low water content. He referred to those seeds that can not withstand water content lower than a relatively high value (around or > 0.45 g g<sup>-1</sup>) as 'recalcitrant'. There are those seeds whose storage behaviour does not conform to either of the two categories described above, but rather they possess partly the features of orthodox as well as of recalcitrant seeds. These have been referred to as 'intermediate' seeds (Ellis *et al.*, 1990; Hong and Ellis, 1996). However, there are no boundaries to delineate distinctly these seed categories as variations in the post-harvest behaviour of the seeds abound within each, and across the categories, thus, it is better to consider seed behaviour as constituting a continuum from extreme orthodoxy to extreme recalcitrance with varying gradations between and among the two extremes (Berjak and Pammenter, 1997, 2001, 2004).

### 1.2.1.1 Orthodox seeds

Orthodox seeds are those that can withstand extreme dehydration and are amenable to long-term storage for predictable periods, under low (can be sub-zero) temperature and low water content, the latter maintained by low relative humidity, without loss of viability (Roberts, 1973; Roberts and Ellis, 1977, 1989). Orthodox seeds undergo maturation drying as the final phase of their development, which is consequent upon a suite of mechanisms or factors that have been entrained during the reserve deposition phase of their development (Pammenter and Berjak, 1999; Vertucci and Farrant, 2005). These seeds are generally shed dry from the parent plants in a quiescent state, following a genetically-controlled shut down of all metabolic processes and activities.

The mechanisms entrained during maturation drying confer the ability to withstand desiccation in orthodox seeds (Bewley and Black, 1994; Vertucci and Farrant, 1995; Farrant *et al.*, 1997; Pammenter and Berjak, 1999; Berjak and Pammenter, 2004). Before entering the desiccated state, there is volume reduction of vacuoles, which is achieved by vacuolar filling with insoluble materials or by vacuole sub-division, and the orderly dissociation of the cytoskeleton with a controlled modification of the nucleoskeleton (Berjak and Pammenter, 2004). There are other vital factors and mechanisms that enable cells to tolerate desiccation. These include intracellular de-differentiation (which decreases membrane surface area), presence and efficient operation of anti-oxidants, accumulation and roles of protective molecules e.g. late embryogenic abundant/accumulating proteins (LEAs), certain oligosaccharides/galactosyl cyclitols and sucrose; deployment of certain amphipathic molecules, presence of an effective oleosin layer around lipid bodies and presence and operation of repair mechanisms during rehydration. (Berjak and Pammenter, 1997, 2001, 2003, 2004, 2008; Pammenter and Berjak, 1999).

On rehydration, dehydration-associated changes in orthodox seed cells are reversible (Pammenter *et al.*, 1991) as the cells resume cell cycling (Berjak and Pammenter, 1997). Initially, there is the operation of repair mechanisms and then resumption of full metabolic activities leading to germination and seedling establishment (Berjak and

Pammenter, 2004). It should be noted, however, that all the mechanisms and processes that confer desiccation tolerance in orthodox seeds function together, and not in isolation, to bring about the tolerance of orthodox seeds to physiological stresses (Berjak and Pammenter, 2004). Most annual and biennial agricultural and horticultural crop species show orthodox seed behaviour (Roberts and King, 1980) as do many tree species (Chin 1996).

### **1.2.1.2 Non-orthodox seeds**

#### ***Recalcitrant seeds***

As originally categorised by Roberts (1973), recalcitrant seeds were described as those that cannot withstand water content below a relatively high level, and do not undergo the final phase of development known as maturation drying. They are sensitive to dehydration/desiccation and some are also chilling-sensitive (Roberts, 1973; Chin and Roberts, 1980; Berjak *et al.*, 1989) (desiccation and chilling sensitivity are discussed later). These seeds are not only shed wet from the parent plant (Roberts and King, 1980; Berjak, 2006), but are metabolically active to the point that cell cycling may not be curtailed before, or at shedding (Berjak and Pammenter, 1997, 2003, 2008; Boubriak *et al.*, 2000). Also, the metabolism of such seed grades from development into germination, with the transition being difficult to identify (Berjak, 2006). At least one, and probably more, of the mechanisms and processes that confer desiccation tolerance in orthodox seeds are either absent, incomplete or non-functional in recalcitrant seeds (Berjak and Pammenter, 1997; 2004). Their lifespan, after being shed from the parent plants, is therefore relatively short, from only days to months depending on the species (Roberts and King, 1980). This is primarily because viability is lost if the seeds lose water or are not provided with additional water, or the seeds may initiate germination immediately, or very soon, after being shed.

Plant species that show recalcitrant seed behaviour are mostly tropical, perennial trees including plantation crops, fruits and timber species (Chin, 1996); well-known examples include cocoa, coffee, oil-palm, coconut, rubber, litchi, mango, mangosteen, durian,

rambutan and jackfruit (Roberts and King, 1980; Song *et al.*, 2003; Berjak, 2006). Some temperate species such as horse chestnut, sycamore, sugar maple, silver maple, English oak, red oak and tea also produce recalcitrant seeds (Berjak and Pammenter, 2001). Recalcitrant seeds are, on average, larger than orthodox seeds (King and Roberts, 1980a; Berjak and Pammenter, 2003; Daws *et al.*, 2005), containing considerable amounts of reserve material that facilitate rapid seedling establishment (Berjak and Pammenter, 2004). Daws *et al.* (2005) also showed that desiccation-sensitive seeds have less physical defenses (in terms of endocarp and testa) than orthodox seeds; this trait also enhances rapid germination in recalcitrant seeds. Recently, however, with the focus on hitherto unresearched, primarily tropical species, many forest trees (Sacandé *et al.*, 2004) and even herbaceous species (e.g. many members of the Amaryllidaceae [Sershen *et al.*, 2008]) have been shown to produce recalcitrant, or otherwise non-orthodox, seeds.

### ***Intermediate seeds***

Non-orthodox, but not ‘strictly’ recalcitrant, seeds are those originally categorised by Ellis *et al.* (1990), as intermediate. They may withstand dehydration (to around 0.14 g g<sup>-1</sup> [Berjak and Pammenter, 1996]) more than recalcitrant types but not to the extent of those that show orthodox seed behaviour (Ellis *et al.*, 1990). Seeds of *Coffea arabica*, *C. canephora*, *Elaeis guineensis* (oil palm), *Carica papaya* and several *Citrus* species have been described as showing intermediate seed behaviour (Ellis and Hong, 1996). Intermediate seeds of a number of tropical species appear to be chilling-sensitive therefore cannot be stored at low temperatures without damage (Hong and Ellis, 1996). Chilling-sensitivity is also characteristic of recalcitrant seeds of some species as originally noted by Chin and Roberts (1980).

### **1.2.2 Why conserve recalcitrant seeds?**

By nature, recalcitrant seeds should not pose any problem of conservation or species regeneration *per se* in the habitat where they usually occur. These are generally in the tropics and sub-tropics, and sometimes in the temperate regions, but necessarily providing conditions counteracting dehydration. In most cases, the moist tropical

environment favours seedling establishment soon after the seeds are shed from the parent plants (Roberts and King, 1980; Berjak *et al.*, 1992a; Pammenter and Berjak, 2000a, 2000b) and as such, the acquisition of seed desiccation tolerance would have no, or little, selective advantage (Berjak and Pammenter, 2004). Hence, recalcitrant plant species persist as a seedling bank rather than seed bank and, as seedlings, they have the advantage of being less susceptible to predation and other threats than seeds (Pammenter and Berjak, 2000a, 2000b). For temperate species that show recalcitrant seed behaviour e.g. chestnut and oak (Chin, 1996; Song *et al.*, 2003), while the environment may not favour immediate seedling establishment, the seeds usually remain in the dormant state until favourable conditions for germination arise (Berjak *et al.*, 1992a; Pammenter and Berjak, 2000a, 2000b; Berjak and Pammenter, 2004). However, such seeds must have relative impermeable outer coverings that will prevent or curtail water loss. This trait is also essential for the few species from relatively dry areas that produce recalcitrant seeds, although some amaryllids that are fleshly seeded with no impermeable coat have been found in very dry habitats (Sershen *et al.*, 2008).

However, as explained earlier in this chapter, the ever-increasing demand by humans for plants of medicinal value is faster than the rate at which nature replenishes these species. In South Africa and especially in the KwaZulu-Natal province, many plants, that show recalcitrant seed behaviour, are traded in medicinal or *muthi* markets. These vulnerable plants cover a wide range of families such as the Zamiaceae (e.g. *Encephalartos natalensis*), Lecythidaceae (e.g. *Barringtonia racemosa*), Anacardiaceae (e.g. *Prothorus longifolia*), Canellaceae (e.g. *Warburgia salutaris*), Euphorbiaceae (e.g. *Croton sylvaticus*), Meliaceae (e.g. *Ekebergia capensis*, and *Trichilia emetica*), Amaryllidaceae (e.g. *Boophane distcha*, *Crinum moorei*, *Haemanthus albiflos*, *H. deformis* and *Scadoxus puniceus*), among many others (von Ahlefeldt *et al.*, 2003). Sadly, not only is the bark harvested recklessly but other plant parts, e.g. bulbs, roots, fruits, seeds, stems and leaves and even whole plants are harvested. Some of these species are vulnerable, endangered/critically endangered, regionally extinct and extinct in the wild already, while some are near extinction altogether (Hilton-Taylor, 1996; von Ahlefeldt *et al.*, 2003;

Dines *et al.*, 2005). Additionally, the biology of these seeds has prevented their long-term storage and this has impacted on their conservation.

### **1.3 Seed storage and *in vitro* storage of germplasms**

#### **1.3.1 Seed storage**

Storage of seeds remains the most effective, convenient and relatively inexpensive way of conserving plant genetic resources (Hong and Ellis, 1996; Chin, 1997; Berjak, 2006). The needs and the conditions of seed storage are dependent on seed post-harvest behaviour (Berjak *et al.*, 1996; Chin, 1997). Low moisture content and low temperature are the two major pre-requisites for long term storage of seeds. However, the best storage conditions can only maintain, and not improve, the quality of seed stored; hence, seeds of high quality and vigour must be selected for storage (Hill, 1999; Berjak and Pammenter, 2003).

##### **1.3.1.1 Conventional methods of seed storage**

Conventionally, seeds are stored at low temperature and low relative humidity (RH), with which the low water content of the seeds is in equilibrium. According to Roberts and King (1980), no seeds (orthodox or recalcitrant) will remain viable for extended periods, under such conditions, if they are sufficiently wet. However, orthodox seeds undergo maturation drying during their development and are shed at water contents of *c.* 0.15 g g<sup>-1</sup> (H<sub>2</sub>O per g dry mass) or less (Berjak and Pammenter, 2001). Such seeds can withstand further drying to about 0.05 g g<sup>-1</sup> or lower (Roberts and King, 1980; Chin, 1997; Berjak and Pammenter, 2001). Storage at -18°C or lower allows such seeds to remain viable for years – decades or longer (Roberts and Ellis, 1977). The general rule (known as Harrington's rule of thumb) is that for orthodox seeds, a 1% reduction in seed moisture contents doubles the storage life of the seeds, likewise, a 5°C reduction in storage temperature increases seed storage life by twofold (Harrington, 1960; Chin, 1997; Hill, 1999).

### 1.3.1.2 Storage at ultra low moisture content

This method of storage involves drying seeds to very low moisture content, i.e. *c.* 0.01 to 0.02 g g<sup>-1</sup>, with little or no viability loss. However, this is suitable only for orthodox seeds. Such ultra-dry seeds can be stored at ambient temperature (*c.* 20°C), thus removing the necessity of having to store at low temperatures (e.g. -20°C). It is efficient and economical especially in situations where there is no constant supply of power to support refrigeration. For example, ultra-dried lettuce seeds with water content of 0.025 g g<sup>-1</sup> had better survival rate than those at 0.053 – 0.064 g g<sup>-1</sup> (Chin, 1997, Engels and Engelmann, 1997).

However, it has been reported that there is an optimum water content level to which seeds can be dried to maximize their storage longevity (Walters and Engels, 1998). Those authors reported that over-drying seeds to extremely low water content, depending on the species, can be counter-productive. An earlier review showed that ultra-dry conditions accelerate ageing processes in seeds (Vertucci and Farrant, 1995). Walters and Engels (1998) also reported that even if seeds are dried to optimum moisture level, their longevity decrease as their storage temperature increase.

The methods of storage highlighted above, however, cannot be used for seeds that display recalcitrant behaviour because low temperature and RH are rapidly lethal to such seeds (Chin, 1991, 1997). In storing recalcitrant seeds, the understanding of their desiccation sensitivity and ongoing metabolism is highly important. Unlike orthodox seeds, recalcitrant seeds are shed at water contents of as high as 4.0 g g<sup>-1</sup> dry mass or more and slow water loss to about 0.8 g g<sup>-1</sup> causes lethal damage (Berjak and Pammenter, 1997, 2001; Berjak, 2006). In light of this, recalcitrant seeds can be stored only in the short- to medium-term. Various short- to medium-term storage methods that have been, and are being tried, are highlighted below:

### **1.3.1.3 Moist or imbibed storage**

This method of storage entails storing seeds in moist media such as sawdust, sand, charcoal or different combinations, moistened with water, or submerging the seeds in water. This method is usually plagued with the problems of micro-organisms and germination during storage. The seeds are usually stored at low temperature *c.* 7°C to prevent germination or the use of germination inhibitors. This method of storage lasts for few weeks to few months. Cocoa seeds were stored using this method for three months with 60% survival; also rubber seeds stored this way for a month had 60% germination (Chin, 1996, 1997).

### **1.3.1.4 Partial dehydration/desiccation method**

It is also referred to as sub-imbibed storage (King and Roberts, 1980b, Eggers, *et al.*, 2007). In this method, recalcitrant or desiccation sensitive seeds are slightly dried to lower moisture content than in fully hydrated seeds, to levels that precludes injury and viability loss but inhibit germination in storage. This method is usually coupled with fungicidal treatment; then the seeds are stored in aerated environment like perforated bags. *Hevea* seeds that were partially dried to 20% (0.25 g g<sup>-1</sup>) moisture content and stored at 20°C had 50% germination after one year. In like manner, cocoa seeds slightly dried to 35 - 53% (0.54 - 1.13 g g<sup>-1</sup>) moisture content and stored at 22°C had more than 50% survival after six months. Seeds of *Clausena lansium* partially dried to 49.4% (0.98 g g<sup>-1</sup>) moisture content, treated with fungicide and stored at 15°C maintained 100% and 90% survival after 400 and 600 days, respectively (Chin, 1996, 1997).

### **1.3.1.5 Hydrated or wet storage**

This is achieved by storing the seeds at the water content at which they are shed, at ambient or slightly lower temperatures (King and Roberts, 1980a; Berjak *et al.*, 1989). While this has been successful in prolonging the lifespan for relatively short times, from weeks to months, it has inherent disadvantages like germination in storage; but in particularly the proliferation of micro-organisms, especially fungi, which will destroy the seeds if their activity is not curtailed (Mycock and Berjak, 1995; Berjak, 1996).

### **1.3.1.6 Controlled atmosphere storage**

Seeds are stored in an atmosphere that is different from normal air in terms of carbon dioxide and oxygen level or in sealed containers (Chin, 1996). Durian, cocoa and *Shorea talura* seeds that were stored under different controlled sealed containers and atmosphere (CO<sub>2</sub>) remained viable for 32 days, 45 days and six months, respectively (Chin, 1996). This is an indication that this method of storage may not be appropriate for storing recalcitrant seeds.

### **1.3.1.7 Cryogenic storage**

In terms of germplasm conservation, short- to medium-term storage of seeds showing recalcitrant post-harvest behaviour is not promising (Chin, 1997), the most promising method being cryogenic storage. This method entails storing seeds at ultra low temperature usually in liquid Nitrogen (-196°C) or in the vapour phase (*c.* -150°C). At these temperatures, there is minimal metabolic activities and cell division, and deterioration is negligible (Chin, 1997).

### **1.3.2 *In vitro* storage of germplasm**

Conservation of whole non-orthodox seeds is not very promising even with cryogenic storage (Chin, 1996), hence the use of biotechnological *in vitro* techniques (discussed below) are used to conserve their alternative germplasm in the short-, medium- or long-term (Berjak *et al.*, 1996). These techniques are useful as well for plant species that do not produce seeds and for those that are vegetatively propagated e.g. banana and cassava (Engels and Engelmann, 1997). *In vitro* techniques have the advantages of high multiplication rates; provide aseptic systems that reduce the risk of microorganisms, pests and pathogens; reduction of space, and risk of genetic erosion is reduced (Engelman, 1991). These techniques provide ample opportunity for various germplasm to be conserved in the long-term; these may include explants, embryonic axes, cells, buds, meristems and somatic embryoids (Berjak *et al.*, 1996). However, some of the problems and disadvantages associated with these techniques include the need for regular subculturing and risk of genetic variations (Engelman, 1991).

### **1.3.2.1 Minimal growth storage**

In this method of *in vitro* storage, cultures are kept under conditions that limit growth. This is achieved by exposing the cultures to chemical and physical factors like low temperatures (below 10°C), low pO<sub>2</sub>, light reduction, reduction in gas pressure, mineral oil overlay, reduction in nutrient level and the addition of growth retardant to culture medium. These factors may be applied singly or in different combinations (Engelman, 1991; Berjak *et al.*, 1996; Chandel and Pandey, 1996). This method was used to conserve the shoot cultures of *Eucalyptus grandis*, by culturing on low-nutrient medium and storing at 4 °C under very low light, for six months without any loss of viability (Berjak *et al.*, 1996). This method is ideal for short-term conservation as listed in Chandel and Pandey (1996), and it is appropriate for slow growing species (Engelman, 1991).

### **1.3.2.2 Normal growth**

Germplasms can be conserve indefinitely under normal conditions provided necessary cautions are taken and there is constant supply of nutrients (Chandel and Pandey, 1996). The method avoids stress induction on cultures as is the case in minimal growth method, and it does not require a facility for low temperature maintenance. However, there is the danger of genetic alteration, loss through contamination and human errors. This method also requires high maintenance.

### **1.3.2.3 Cryopreservation**

Cryopreservation is the only promising method of long-term conservation of non-orthodox germplasms other than seeds (discussed in section 1.7). The germplasm is stored at ultra low temperature i.e. in liquid nitrogen or in its vapour phase, where all biochemical activities are arrested, and they can be stored indefinitely without subculturing, thus making it economical.

For any success to be achieved in storing recalcitrant seeds, the need to eliminate, or at least reduce to the barest minimum, any microbial inoculum present on, or in the seeds is imperative (Berjak and Pammenter, 2001). Where the fungi are present on the seed

surface, the use of surface treatment with fungicides should eliminate the inoculum (Berjak, 2006). If they are present in the seeds, then other treatment is required, e.g. removal of seed coat and use of systemic fungicides. Occasionally for recalcitrant seeds, application of thermotherapy, by the controlled use of heated water, may be successful (Berjak, 2006). Even if the problem of fungal proliferation can be ameliorated (Calistru *et al.*, 2000) by appropriate treatment, seeds in hydrated storage will still die within a short time because of non-cessation of metabolism. The ongoing metabolism gradually grades into germination which requires an exogenous water supply, without which the seeds are subjected to water stress ultimately leading to death (Pammenter *et al.*, 1994).

#### **1.4 Desiccation sensitivity and damage**

Water plays a vital role in supporting all living systems. At a cellular level, its roles are multiple: from structural, molecular, biochemical to metabolic as highlighted by Walters *et al.* (2002). It can serve as a solute, solvent, reactant, product, protectant and stabilizer for most biological reactions (Vertucci, 1989, Vertucci and Farrant; 1995). It is the driving force for all cellular organisation (Hoekstra *et al.*, 2001). Studies have shown that there are five levels of water/hydration within a cell, with each level having different water potentials, controlling different metabolic activities and having differing properties as depicted in the binding sites and states (see Walters *et al.*, 2002). Level V is the dilute, solution-like, non-binding water (usually, in the case of fully hydrated seeds). Level IV is the concentrated or pore solution, bound to the capillary site. Level III is the bridge over hydrophobic moieties on macromolecules; it is bound to the hydrophobic site. Level II is glassy in characteristics and it is associated with polar surfaces or hydroxyl groups, in essence, it is bound to the hydrophilic site. Lastly, Level I is considered as a structural components, typified by the non-freezable, intracellularly-, and ionic-bound water (see Vertucci and Farrant, 1995; Walters *et al.*, 2002). Thus, when water is removed from the cell at any level, some levels of damage are expected. However, this damage may be reversible as in the case of desiccation-tolerant species because of their acquisition of desiccation-tolerance mechanisms. When the damage is irreversible, however, the species is said to be desiccation-sensitive.

All recalcitrant seeds are sensitive to desiccation. As explained earlier, there is no cessation of metabolic activities in recalcitrant seeds; this is facilitated because they are usually shed hydrated (unlike orthodox seeds that are metabolically quiescent because they are dry), extensively vacuolated and are characterised by a high degree of subcellular development (Farrant *et al.*, 1997). Metabolism still continues when the seeds are dehydrated, in fact, slight dehydration stimulates germinative metabolism, leading to root/shoot growth, as it was in *Ekebergia capensis* (Pammenter *et al.*, 1998a), *Quercus kelloggii*, *Q. shumardii*, *Q. velutina* and *Q. virginiana* (Chmielarz and Walters, 2007). However, this germinative metabolism process induced by slight dehydration will need an exogenous supply of water shortly thereafter.

When recalcitrant seeds are dehydrated, three types of damage are possible: mechanical, metabolism-induced and desiccation damage *sensu stricto*. Mechanical damage occurs when there is reduction in cell volume. This results in the loss of turgor pressure in the cell and loss of membrane materials. These lead to increased chance of membrane fusion and molecular crowding and interaction that can bring about protein denaturation, as well as alteration of membrane properties (Vertucci and Farrant, 1995; Côme and Corbineau, 1996; Hoekstra *et al.*, 2001, 2003). The ongoing metabolism under water deficiency brings about intracellular damage which maybe lethal to the seeds. Metabolism is unbalanced, unregulated and deranged – this is metabolism-induced damage. It is also a consequence of uncontrolled oxidative reactions. (Pammenter and Berjak, 1999; Berjak, 2005). Desiccation damage *sensu stricto* happens if non-freezable water that is intimately bound to the surfaces of macromolecules is removed, leading to conformational damage and the denaturation of the macromolecules (Pammenter and Berjak, 1999; Pammenter *et al.*, 2000).

### **1.5 Chilling sensitivity and injury**

The seeds of some plant species that show recalcitrant seed behaviour are also sensitive to low temperature i.e. chilling (Chin and Roberts, 1980). Low, chilling temperatures limit the geographical distribution and most importantly, the productivity of sensitive

species (Gai *et al.*, 2008). Most biological reactions using enzymes as catalysts are temperature-dependent. Thus, when there is alteration in the temperature (in this case, reduction to chilling temperature), enzymatic activities and function may be disturbed, consequently agitating cell function and balance. It has been suggested that some other temperature-dependent, rate-limiting reactions may cease altogether because of low temperature (King and Roberts, 1980a, 1980b). Other adverse effect of chilling may include reduction in the ionization of enzymes, buffers and substrate, increased in hydrogen bonding and Van der Waal's forces. The consequences of the latter are: firstly, it may results in enzyme molecules reacting together to form a larger unit that is less active; secondly, water clusters may be formed around the enzyme active sites reducing their efficiency. In addition, reduction in temperature increases the viscosity of cellular fluid. All these generally reduce enzyme functions and metabolic activities within the cell (King and Roberts, 1980a).

Membrane phase transition from the liquid crystalline to gel phase is a consequence of chilling sensitivity in chilling-sensitive species (Raison and Chapman, 1976). In addition, because of chilling, membrane components are separated out laterally in which the components are sorted out according to their molecular type (Platt-Aloia and Thomson, 1987). There seems to be a threshold temperature, referred to as phase transition temperature ( $T_m$ ), below, or at which membrane phase changes occur in cell membranes (Bryant *et al.*, 2001; Sacandé *et al.*, 2001).  $T_m$  depends on various factors within the cell like water content, presence of impurities in the phospholipid bilayer and presence of solutes in the suspending solution (Bryant *et al.*, 2001). This temperature is usually below 10 – 15°C (but not sub-zero) for chilling sensitive species (Sacandé *et al.*, 2001). When the  $T_m$  of membrane is high, as it may be in a fresh seed, the storage of such seed at temperature lower than  $T_m$  leads to the rigid gel phase formation and lateral phase separation of the membrane components. The integrity of the membranes is also compromised because they are disrupted, thus causing increased permeability and solute leakage. This results in chilling injury as is the case in neem seed stored at chilling temperatures (Sacandé *et al.*, 2001). Membrane phase changes may impact the enzymes

and other activities that are associated with membrane and membrane-bound organelles e.g. mitochondria (Berjak *et al.*, 1996).

Cold-labile enzymes like those in the TCA cycle and some glycolytic enzymes, under chilling condition, may affect the cell cytoskeleton with which they are associated, thus disorienting and dismantling the cytoskeleton (Berjak *et al.*, 1996). Protein denaturation can also be consequent upon chilling (King and Roberts, 1980b), thus every protein-derived molecule will be impacted adversely. It has been reported earlier that during chilling, there is decrease in lipid fluidity within membranes. This may lead to changes and alterations in the membrane thickness and permeability, electric field and cation concentrations, and consequently, is the alteration in the conformation and activities of membrane-bound enzymes. There is likelihood also that alteration in membrane permeability may result in the liberation of toxic materials which may be damaging and lethal to the cells (King and Roberts, 1980b). Irreversible chilling injuries like damage to cellular components and metabolism are reflected in lack of germination or low seedling vigour (Chang *et al.*, 2010).

Seeds of some tropical species cannot tolerate temperatures below 10°C without loss of viability (Berjak and Pammenter, 1996); the value may be lower or higher, depending on the species but can not be sub-zero because of their hydrated state. Chilling injury has been recorded in recalcitrant seeds of cocoa, rambutan and Borneo camphor. Seed storage protein (globulin) and photosynthetic protein (Rubisco large subunit) were degraded and destroyed when cotton (*Gossypium hirsutum* L.) seedlings were subjected to chilling treatment (Gai *et al.*, 2008). Thus seeds stored hydrated should be maintained at the lowest temperature that precludes chilling injury (Berjak, *et al.*, 2004; Berjak, 2006).

### ***Reactive Oxygen Species (ROS) and Oxidative Stress***

Reactive Oxygen Species (ROS) are produced normally in every metabolic plant cell as byproducts of activities like photosynthesis and respiration (Wang *et al.*, 2010). The major sources of ROS production in plant cells are all active organelles like chloroplasts, mitochondria and peroxisomes. These organelles are involved in highly oxidizing

metabolic activities as well as high rate of electron flow. ROS comprise free radicals such as superoxide radicals ( $O_2^{*-}$ ), hydroxyl radicals ( $OH^*$ ), perhydroxy radicals ( $HO_2^*$ ) and alkoxy radicals ( $RO^*$ ); and non-radical, molecular forms of ROS such as hydrogen peroxide ( $H_2O_2$ ) and singlet oxygen ( $^1O_2$ ) (Gill and Tuteja, 2010; Møller and Sweetlove, 2010). ROS play dual roles of benefit and damage. ROS are beneficial in that they can stimulate the expression of new genes, they are involved in signaling the pathway for the synthesis of antioxidants and they are important components of signal transduction pathways that control essential metabolic processes like growth (Roach *et al.*, 2008; Gill and Tuteja, 2010; Gonzalez-Aguilar *et al.*, 2010). On the other hand, they are harmful, toxic and can cause damage to plant cell. However, plant cells have developed efficient antioxidant systems to counteract the damaging effect of ROS.

There are enzymatic and non-enzymatic antioxidants that scavenge ROS that are produced in the cell. Enzymatic antioxidants include superoxide dismutase (SOD), catalases (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione S-transferases (GST) and glutathione peroxidase (GPX). Non-enzymatic, low molecular antioxidants are ascorbic acid (Vitamin C), glutathione (GSH), proline (Pro),  $\alpha$ -tocopherols (Vitamin E), flavonoids and carotenoids (Car) (Pukacka and Ratajczak, 2005; Gill and Tuteja, 2010).

Usually, there is equilibrium and balance between ROS production and their scavenging/consumption by antioxidants. However, when plant tissues are subjected to biotic or abiotic stress e.g. water stress, extreme temperatures (as is the case in the present study), radiation, air pollution etc. this equilibrium is disturbed, resulting in excessive production of ROS, and consequently, oxidative stress (Roach *et al.*, 2008). In addition, the stress may overwhelm the antioxidant systems and can cause them to be dysfunctional and inefficient in scavenging the ROS sufficiently (Møller and Sweetlove, 2010).

The adverse effect of excessive production of ROS include: peroxidation of lipids of cell membrane, cell metabolic disorder, damaged cellular components and structures, damage to lipid, protein, carbohydrate and DNA (Pukacka and Ratajczak, 2005; Roach *et al.*, 2008; Møller and Sweetlove, 2010; Wang *et al.*, 2010; Gill and Tuteja, 2010). Over production of ROS as a result of environmentally-induced stress is the main cause of (i) loss of crop productivity worldwide (Gill and Tuteja, 2010), (ii) seed deterioration (Pukacka and Ratajczak, 2005), (iii) reduction in yield and quality of seed (Wang *et al.*, 2010) and ultimately cell death (Roach *et al.*, 2008; Møller and Sweetlove, 2010; Gill and Tuteja, 2010). Pukacka and Ratajczak (2005) reported 40% loss in viability of beech seeds stored at 4°C for six weeks. The viability loss, according to those authors, was due to excessive production of ROS, resulting in oxidative stress and the inefficiency of the antioxidant machinery to counteract them.

In addition to the factors highlighted above, there are other important factors that contribute to the desiccation-sensitivity and the storage behaviour of recalcitrant seeds. These include developmental status, seed size, drying rate, conditions under which they are dried and rehydration conditions. Additionally, provenance, intra- and inter-seasonal variation and the inherent nature/characteristics of the seeds affect their post-harvest responses (Farrant *et al.*, 1985; Berjak, 1988; Berjak *et al.*, 1992a, 1992b; Song *et al.*, 2003; Daws *et al.*, 2004, 2005; Berjak and Pammenter, 2008). Consequently, recalcitrant seeds do not respond predictably to physiological stresses, suggesting that there are varying degrees of recalcitrance within and across species (Berjak and Pammenter, 1994).

## **1.6 Drying and drying rates**

Low water content is one of the pre-requisites for the successful storage of seeds, thus posing a great necessity for seeds to be dried before they can be stored (Hill, 1999). Drying is a natural phenomenon in orthodox seeds as explained earlier. Orthodox seeds that are not shed dry naturally can be dried sufficiently to be amenable for successful storage. For recalcitrant seeds, however, the reverse is the case. Apart from the fact that

they are usually shed with high water contents, most recalcitrant seeds are large (Section 1.2.1.2), and some have thick, impermeable seed coats (Chin *et al.*, 1989). Because of the large size, their ratio of surface area to volume is usually less than the ratio for smaller seeds; hence their rate of natural or induced water loss is usually slow. In addition, the impermeable seed coat in some seeds minimises and/or reduces the rate of water loss.

As the dehydration progresses slowly in recalcitrant seeds, metabolism still continues, and in fact, can stimulate and enhance the onset of germinative metabolism (as in the case of *Avicennia marina* (Farrant *et al.*, 1985), *Aesculus hippocastanum* (Tompsett and Pritchard, 1998) and *Ekebergia capensis* (Pammenter *et al.*, 1998a)); but in time, it leads to unbalanced, deranged metabolism. And because some or all the mechanisms that confer tolerance to desiccation in orthodox seeds are missing in recalcitrant seeds, lethal damage occurs (Berjak, *et al.*, 1992a). Emphasis is laid on time or duration here (Pammenter *et al.*, 1994, 1998a, 1998b, 2000), the rate at which drying occurs is a critical factor for consideration. It has been observed in a number of experiments that, the slower the water loss, the greater the intracellular damage. This is because slow drying gives enough time for lethal aqueous-based deleterious damage to take place (Berjak and Pammenter, 1997; Varghese *et al.*, 2008) and worse still, the water content may still be relatively high. On the other hand, if water can be rapidly removed (as in the case of flash dried embryonic axes), it provides insufficient time for metabolism-linked damage to accumulate (Berjak and Pammenter, 2008; Varghese *et al.*, 2008). Thus, lower water content can be reached, while viability is still retained (Berjak *et al.*, 1992b; Pammenter *et al.*, 1998b), than when dried slowly (Pammenter and Berjak, 1999).

However, whole, recalcitrant seeds cannot be dried sufficiently rapidly without loss of viability, because of their large size (as in the case of *Araucaria* species (Tompsett, 1984); hence the use of their embryonic axes to achieve rapid water loss. In most recalcitrant seeds, the embryonic axes are very small (*c.* 1 -3 mm), constituting a small fraction of the whole seeds (Berjak *et al.*, 1996; Berjak and Pammenter, 1996, 2008; Berjak, 2006); for example, the mass of the embryonic axis of *Trichillia dregeana* and *Euterpe edulis* is 0.40% and 0.54% of the mass of the whole seeds respectively (Kioko,

2002, Panza *et al.*, 2007). Embryonic axes are the future young plants no matter how small they are; that means they can survive and grow into whole plants if isolated and handled appropriately. Thus, they are perfect specimen for any suitable drying manipulations. Because of their small size, the surface area to volume ratio is usually large, hence they can lose water more rapidly. The invention of the flash drying technique (Berjak *et al.*, 1990) helps to dry embryonic axes very rapidly.

The effect of drying rate was shown by the work of Pammenter and others (1998b, 2000). Those authors' work on *Trichilia dregeana*, *Castanospermum australe*, *Camellia sinensis* and *Ekebergia capensis* showed that rapid rate of drying resulted in lower water contents before viability was lost, compared to slower rate of drying. In *Landolphia kirkii*, rapid (flash) drying reduced the water contents of the embryonic axes from 1.50 g g<sup>-1</sup> to 0.32 g g<sup>-1</sup> within 30 minutes, while it took 3 days of slow drying whole seeds for the axes to reach the same water contents (Pammenter *et al.*, 1991). Similar observations were made in *Avicennia marina* (Farrant *et al.*, 1985) and jackfruit, *Artocarpus heterophyllus* (Wesley-Smith *et al.*, 2001)

It should be noted however, that flash drying does not confer desiccation tolerance, as in orthodox seeds. Flash-dried embryonic axes will lose viability within a short time if they are left at ambient temperature and are not appropriately handled and used within short time (Pammenter and Berjak, 1999; Walters *et al.*, 2002). It should be emphasised also that, no matter how rapidly recalcitrant explants are dried, there is a water content limit, below which, they will die. Moreover, this water content is higher than the water content to which orthodox or intermediate seeds can be dried, and has been suggested to be at, or near, but not be lower than the level of structure-associated, non-freezable water (Berjak *et al.*, 1992a; Pammenter and Berjak, 1999; Berjak and Pammenter, 2008; Varghese *et al.*, 2008). The major advantage of flash drying embryonic axes is that it provides suitable explants, at suitable water contents, for cryopreservation – a process that offers the only hope for long-term storage of recalcitrant seeds (Berjak and Pammenter, 2008).

## 1.7 Cryopreservation

Because of the characteristics of recalcitrant seeds and the various threats posed, in which short- and medium-term storage will not suffice to conserve them, a method of long-term storage – cryopreservation, is being attempted and explored for its applicability to the germplasm. However, this approach is rarely appropriate for large, wet, whole seeds because at freezing temperatures, ice crystal will be formed in the hydrated living tissues, and this is lethal to the cell structures (Towill, 1991; Berjak, 2005; Daws and Pritchard, 2008). Thus, excised embryonic axes, which are usually small, (or alternative explants where the embryonic axes are too big) are used (Berjak, 1996; Berjak, 2006; Berjak and Pammenter; 2008). In the process of cryopreservation, the explants must be dried to an appropriate water content very rapidly to bypass any desiccation damage and must be immediately plunged into ultra-low, cryogenic temperature, usually in liquid nitrogen (LN) at  $-196^{\circ}\text{C}$  (Berjak, 1996). Ultimately, the explants used should be able to retain viability after retrieval from LN and thawing (Towill, 1991). However, a number of processes and procedures are involved, and most importantly, optimal protocols have to be defined and designed for each species for successful cryopreservation (see Engelman, 1997).

Thus far, protocol optimization for successful cryopreservation has been at inter-species level. However, intra-species differences have to be considered in cryopreservation, as variable success has been achieved in the cryopreservation of a single species (Berjak, 1996). Variations abound within a species with respect to size, water content, quality, adaptability, developmental status and biochemical variations (Berjak, 1996). These variations, among other things, are due to the different provenances where such species have developed. Basic protocols, as in the present study, are necessary for choices of best, suitable explant to be made for successful cryopreservation at intra-species level.

## 1.8 Effect of provenance

There is controversy about categorising the post-shedding behaviours of the seeds of some species e.g. *Zizania palustris*, *Coffea arabica*, *Camellia sinensis*, *Citrus limon*,

*Azadirachta indica*, etc. (Berjak *et al.*, 1995; Walters *et al.*, 2002; Daws *et al.*, 2004) which have been classified as recalcitrant, intermediate or even orthodox, which, among other things, could be related to the provenance i.e. the region where such seeds originate (Berjak *et al.*, 1995; Daws *et al.*, 2006). Berjak and Pammenter (1994) have commented that the natural habitat of species is an important factor to consider when handling recalcitrant seeds. The environment in which a plant grows contributes to the performance of its seeds and their responses to physiological stress (Weiner *et al.*, 1997). This is because different habitats are characterised by different environmental conditions like rainfall, humidity, sunlight, length of day/night, temperature and altitude.

While recalcitrance is recorded as most commonly found in the humid tropical or subtropical habitats, recalcitrant seeds are not confined to these habitats. Recalcitrant species can be found to a lesser extent, in temperate zones and in seasonal and stressful habitats as well (Roberts and King, 1980; Pammenter and Berjak, 2000a, 2000b). As the habitat becomes drier and probably cooler, the proportion of seeds showing recalcitrant seed behaviour declines (Tweddle *et al.*, 2003). That is, the warmer the natural habitat, the more abundant the recalcitrant-seeded species and the shorter their lifespan as seeds, because they will naturally germinate at, or very soon after, shedding (Pammenter *et al.*, 1994). Seeds that are found in warmer regions accumulate a greater heat sum during their development, enabling them to be further developed when shed, compared with those found in cooler regions (Daws *et al.*, 2004). As it has been described by Berjak *et al.* (1992b) and Goveia *et al.* (2004), developmental status affects the responses of seeds to desiccation. In many instances, this may be linked directly to provenance.

Negash (2003) recorded significant differences in the germination ability of the seeds of *Podocarpus falcatus* from four provenances in Ethiopia. That author suggested that the differences observed could be attributed to, among other factors, the previous and prevailing climatic conditions at the time of the seed development. Altitudinal variations of different provenances had significant impact on the seedling growth of *Pinus oocarpa* in México (Sáenz-Romero *et al.*, 2006) and *Eucalyptus urophylla* in Indonesia (Wright and Osorio, 1996). Chilling sensitivity of *Ekebergia capensis* seeds was dependent on

provenance as reported by Bharuth *et al.* (2007, 2008), with seeds from the northerly habitat being more sensitive than those from the south. Different degrees of recalcitrance were observed in *Aesculus hippocastanum* seeds across Europe (Daws *et al.*, 2004). *Acer pseudoplatanus* has been categorised as showing intermediate and recalcitrant seed behaviour based on provenance (Daws *et al.*, 2006). Nine species of *Coffea* studied by Dussert *et al.* (2000) showed significant difference in seed development duration. This difference was directly correlated to the duration of wet season in the native environments of the nine species. Marked differences have also been observed, due to provenance, in seed behaviour, seedling growth and growth parameters of some recalcitrant species such as *Eucalyptus tereticornis* (Ginwal, 2009), *Cichorium intybus*, white cedar, *Hypericum perforatum*, (Keller and Kollmann, 1999); some orthodox species such as *Chamaecyparis thyoides* (Mylecraine *et al.*, 2005), *Cordia africana* (Khalil and Abdelgadir, 2003), *Acacia senegal* (Khalil and Siam, 2003), *Bombacopsis quinata* (Hodge *et al.*, 2002); and others such as *Centaurea cyanus*, *Papaver rhoeas*, *Daucus carota*, *Leucanthemum vulgare* and *Silene alba* along West-East in Europe (Keller and Kollmann, 1999); *Ribes cereum* and *Symphoricarpos oreophilus* (Rosner *et al.*, 2001).

Consideration of provenance as an important factor in seed physiological responses has only recently become a focus. It is becoming apparent that this aspect needs to be explored in depth, in order to contribute to the choice of seeds for storage, for any physiological manipulation, and especially in the context of climate change (in which mitigation measures are indispensable) and habitat restoration.

## **1.9 Phylogeny**

Fluctuations and random changes characterize environmental conditions (Bradshaw, 1956). Various plants habitats are likewise characterized with a wide range of climatic and edaphic factors (Hufford and Mazer, 2003). Plants are able to adapt to these changes (which occur both in time and space) because of their plasticity (Bradshaw, 1956). Local environmental conditions affect plants at the organizational level such as the root, leaves,

meristems etc. Interactions between various organs, in addition to the response and plasticity of each organ to environmental variations, may result in different behaviours observed in a whole plant species and/or genetically distinct individuals (Hufford and Mazer, 2003; de Kroon *et al.*, 2005).

Seeds of a plant species obtained from different populations may respond differently in terms of germination; and the variations in the germination responses may be as a result of the seeds' genotypes (epitypes) or maybe environmentally induced (ecotypes) (Quinn, 1977; Hufford and Mazer, 2003). According to Quinn (1977), variable environmental conditions may, with time, alter the genotypic compositions of seeds. The genotypic variation among seeds may arise as a result of different factors such as different source of pollen during fertilization, changes in mode of pollination (self- or cross-pollination), differential rate of pollen tube growth, etc. (Quinn, 1977).

A putatively single plant species from different populations may diversify morphologically as observed in the number and size of leaves of *Sapium sebiferum* (Zou *et al.*, 2007) and *Theobroma cacao* (Bertolde *et al.*, 2009), in the flowers and fruits of *Aglaia elaeagnoidea* (Muellner *et al.*, 2009), or physiologically as in the germination of *Bromus tectorum* (Kao *et al.*, 2008), net CO<sub>2</sub> assimilation of *Sapium sebiferum* (Zou *et al.*, 2007), photosynthetic rates and stomatal conductance of *Theobroma cacao* (Bertolde *et al.*, 2009) etc. However, morphological or physiological similarities or differences are not sufficient to show species relatedness or the variation of one species to the other. Morphological and phenotypic characters in particular have limitations and are unreliable because they are often influenced by environmental conditions (Singh *et al.*, 2010; Domyati *et al.*, 2011)

Beyond morphological tools, various techniques like biochemical and molecular tools are being employed to detect variations between and within species (Mondini *et al.*, 2009; Domyati *et al.*, 2011). Molecular tools are being employed more often because they can detect genetic relatedness among species or variations within species at the lowest hierarchy – the DNA (Monsen-Collar and Dolcemascolo, 2010). The non-functional,

non-coding, fast-evolving internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA) is one of the most sequenced and analysed regions of the nrDNA (Schlötterer *et al.*, 1994; Álvarez and Wendel, 2003). This is because its degree of variation is higher than that of other genic coding regions of the DNA, whereas it is flanked by highly conserved sequences and can easily be amplified with universal primers. In addition, it is not difficult to amplify the ITS region from DNA samples that are small, degraded or diluted because of the high copy number of rDNA genes (Gardes and Bruns, 1993; Schlötterer *et al.*, 1994). Phylogenetic analysis of the ITS regions is very useful in assessing the extent of genetic variation or similarity, at the species level, between populations of the same species (Muellner *et al.*, 2009).

## 1.10 The present study

### 1.10.1 Provenances investigated in this study

In this study, four provenances within the KwaZulu-Natal (KZN) province of South Africa were investigated for their effects on the physiological responses of *Trichilia dregeana* to stresses induced within the laboratory environment. These were Mtunzini, Durban, Pietermaritzburg and Port Edward (Figure 1.1). The distance between the locations are shown in Table 1.1 as calculated from South Africa Distance Calculator ([http://distancecalculator.globefeed.com/South\\_Africa\\_Distance\\_Calculator.asp?state=02](http://distancecalculator.globefeed.com/South_Africa_Distance_Calculator.asp?state=02))

**Table1. 1:** Straight line or air distance (km) between the locations studied

	DBN	PMB	MTZN	P.ED
DBN	-			
PMB	66.42	-		
MTZN	122.72	151.86	-	
P.ED	153.89	160.17	276.25	-

Mtunzini (meaning ‘place in the shade’) is a small coastal town situated on a hill, almost halfway along the coastline of the KZN province. It is about 140 km north of Durban. The geographical coordinates is 28° 57' 0" S, 31° 45' 0" E. It is sub-tropical with an

average, annual rainfall of over 1000 mm. Most rainfall occurs in the summer (mean temperature: 17°C min. - 40°C max.) while average winter temperature is about 17°C minimum. The location is characterised by pristine dune forest, an estuary and mangrove swamp forest. It has many indigenous trees with about 250 species and a variety of fauna, including South Africa's rarest bird of prey, the palm nut vulture; and about 1000 ha (9 km<sup>2</sup>) of nature reserve. (<http://www.zululandbirdingroute.co.za/sz/mtunzini.html>; <http://www.zululandzigzag.co.za/zululand-mainmenu-37/mtunzini.html>).

Durban is a city on the eastern coast/seaboard of KZN/South Africa, facing the India Ocean with geographical coordinates of 29° 58' S, 30° 57' E. It is characterised by a mild sub-tropical climate with warm, wet summer (average temperature: 21°C min. - 33°C max.) and mild moist/dry winter (average temperature: 11°C min. - 25°C max.). Generally, it is warm all year round, with an average temperature of 21°C. It has an average, annual rainfall of 1,009 mm and humidity of 79%; it encompasses about 2,292 km<sup>2</sup> land area. Topographically, most places are hilly, with few flat areas, thus the city is marked with large altitudinal variations as well as local heterogeneity of climate. The vegetation is classified as coastal forest or thornveld. There are about 2,000 plant species in the city, 82 terrestrial mammal species and 380 species of birds. There are also 69 species of reptiles, 25 endemic invertebrates and 37 frog species. It is a biodiversity hotspot i.e. having both the rich and the endangered plant (and animal) species. Unfortunately, the city has lost about 70% of its original land area to urbanisation and other uses. Consequently, the species that are found in the city are under threat because of habitat loss (Cadman and Dames, 1993;

<http://www.sa-venues.com/weather/kwazulunatal.htm>;

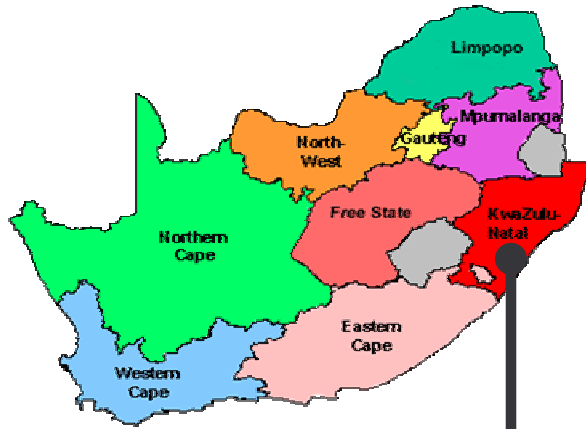
<http://old.weathersa.co.za/Climat/Climstats/DurbanStats.jsp>;

[http://wiki.ulwazi.org/index.php5?title=Biodiversity\\_of\\_Durban%2C\\_kwaZulu-Natal](http://wiki.ulwazi.org/index.php5?title=Biodiversity_of_Durban%2C_kwaZulu-Natal))

Pietermaritzburg is situated in the KZN midlands with coordinates of 29°37' S, 30°23' E, land area of 649 km<sup>2</sup> and 596 m elevation. It is about 80 km from Durban. The rainfall ranges from about 700 mm to over 1,300 mm depending on location, averaging about 844 mm per annum. It has a 46 ha botanic garden, various nature reserves and parks which

host different indigenous fauna and flora. The city has a wide range of soil types as well as environmental conditions, thus providing conditions for an extensive variety of plant species to thrive. Unfortunately, the area is terribly plagued with various types of weeds and invasive alien species, blocking the water courses and displacing the indigenous forests of the city. It is thus referred to as the weed capital of South Africa (<http://www.africanexplore.com/citypages/kzn/pietermaritzburg.html>; <http://www.ferncliffeconservancy.co.za/article7/>).

Port Edward is the southernmost town in the KZN province with coordinates of 31°03'00" S, 30°13'00" E. It is about 170 km South of Durban. The town claims to have the smallest desert, called the 'red desert', in the world. The desert covers just about 11 ha, surrounded by luxuriant, sub-tropical vegetation. The Umtamvuna Nature Reserve in the town is a dense forest that houses many rare and endemic trees and plants species, as well as various species of birds. Port Edward has an extensive, unspoilt coastline. The extensive beaches are also backed by coastal forests. In summer, the average maximum temperature is usually above 30°C while it is about 20°C in winter (<http://www.coastingafrica.com/book/13SouthCoast.pdf>; [http://www.sa-venues.com/game-reserves/kzn\\_umtamvuna.htm](http://www.sa-venues.com/game-reserves/kzn_umtamvuna.htm); [http://www.southafrica-travel.net/kwazulu/port\\_edward.html](http://www.southafrica-travel.net/kwazulu/port_edward.html)).



(a)



(b)

**Figure 1.1:** Map of South Africa showing KwaZulu-Natal (a); Map of KwaZulu-Natal indicating (red arrows) the provenances studied (b).

### 1.10.2 Species under investigation in this study

The plant species investigated in this study was *Trichilia dregeana* Sond. from the Meliaceae family (Figure 1.2). The English common names are forest mahogany, Natal forest mahogany, cape mahogany, thunder tree, Christmas bells or red ash; and umKhulu and uMathunzini in the Zulu language (Pooley, 1993; Grace *et al.*, 2003; Maroyi, 2007). There are about 12 scientific names that are synonyms to *T. dregeana* (e.g. *T. splendida* A.Chev.; *T. tomentosa* A.Chev.; *T. redacta* Burt Davy; *T. vestita* C.DC.; *T. dregei* E.Mev. ex C.DC.; etc.).

Generally, *T. dregeana* is a tall evergreen tree with a height of 10 m to 40 m maximum; and can be buttressed. The cylindrical bole is about 100 – 200 cm in diameter, and can reach a height of 12 m before branching to form a shady, dense, spreading, rounded crown (Pooley, 1993; Maroyi, 2007). Unlike the related species *T. emetica* Vahl. (with which it is often confused), *T. dregeana* has very shiny, dark green leaves with paler, almost hairless abaxial surface. Leaflets tips are pointed with narrower base, and have 8 – 12 side veins. Leaves of *T. emetica* are shorter than, and not as dark as, those of *T. dregeana*; with rounded tips, brownish hair on the abaxial surface and 13 – 16 side veins (Pooley, 1993).

Flowering to fruiting/fruit maturity seasons take about eight months. The flowers are very fragrant, sweet-scented, whitish-green, with velvety petals up to 24 mm long in dense bunches. Flowering takes place between October and December (September in Zimbabwe (Maroyi, 2007)). Fruits are found from March to May. The fruit capsules are round (about 30 mm diameter), pale pinkish green turning darker on ripening.

Characteristically, the fruits have indented tips with no neck (*T. emetica* fruits have pointed/beaked tips with distinct necks). On ripening, the fruit of *T. dregeana* split into three segments to reveal six black seeds, each covered by bright red to scarlet, waxy aril (Palgrave, 1977; Pooley, 1993). Flowering and fruiting occurs earlier in *T. emetica* (September – November and January – April, respectively).

In terms of distribution, there are about 200 – 300 species of *Trichilia*, native to, and concentrated in America with few in Africa (about 14 – 18 species) and Madagascar (about six species) (Philips, 1926; Dyer, 1975; Grundy and Campbell, 1993; Maroyi, 2007). *T. dregeana* is found in the high rainfall evergreen coastal forests of KwaZulu-Natal and Eastern Cape; Mpumalanga and Limpopo provinces of South Africa. It also occurs in Swaziland, Zimbabwe, and northward to tropical Africa (Angola, Democratic Republic of Congo, Congo, Cameroon, Côte d'Ivoire, Guinea and Ethiopia (Pooley, 1993; Maroyi, 2007)). It may however be found in many countries, outside its natural habitat, where it is planted for ornamental purposes.

Altitudinal variations typify the distribution of *T. dregeana* across Africa. It is found in similar vegetation (i.e. transitional vegetation zone between savanna-mosaic forest and moist, evergreen forest) in West Africa and DR Congo, but at altitudes 800 – 1,600 m in West Africa and below 500 m in the DR Congo. It is found in Ethiopia where the average annual rainfall is 1,500 – 2,500 mm and at altitude of 1,350 – 2,000 m, whereas it is found at lower altitude in Uganda and Tanzania. The altitude gradually reduces toward southern Africa, and it is found close to sea-level near Durban (Maroyi, 2007).

Virtually every part of *T. dregeana* is useful for humans and animals. It has similar usefulness to *T. emetica*. Butterflies and bees feed on the flowers of these plant species while the fruits are eaten by human and birds (Pooley, 1993). The aril serves as food for baboons. Although the seed coat is poisonous, boiled seeds are edible after the removal of the seed coat. The aril is also edible; can be eaten or made into a drink or sauce. Poultices made from the leaves can be used to treat bruises and eczema. The leaves are also believed to induce sleep and hot infusions from the leaves can be soothing when applied to bruises. In addition, the leaves can also be used in the treatment of lumbago, rectal ulcer and dysentery. In Nigeria, leaves are used for the treatment of syphilis and can be eaten for the treatment/relief of gonorrhoea. Decoctions made from the roots are consumed as tonic and can be used against fever. It is also useful as purgative. Daily consumption of the bark decoctions and hot infusions can be used to treat diarrhea. The bark decoction is also useful in the treatment of back pain that results from kidney problems. Other

medicinal uses are the treatment of stomachache, fever, scabies as well as a purgative in form of enema and as a blood purifier. The bark can also be used to make fish poison (Palgrave, 1977; Grace *et al.*, 2003; Eldeen *et al.*, 2005; Krief *et al.*, 2005 Maroyi, 2007). Medicinal use of the tree especially in Africa also includes treatment of inflammatory problems such as kidney pain, sore back/back ache and inflammation of the bronchus (Eldeen *et al.*, 2007).

The wood is used for furniture, carving, household equipments, shelving and construction. The wood is also used to repair ships and canoes, as well as for firewood and charcoal. The tree is planted for ornamental purposes in cities, parks and gardens. It is also grown as a shade tree for coffee in Ethiopia. The trees serve as breeding sites for some butterfly species (Pooley, 1993; Maroyi, 2007).

One of the most important components of the seeds that contribute to their usefulness is the seed oil. *T. dregeana* seeds are rich in oil; comprising 55% - 65% of the seeds and they can produce as much oil as many other oil-rich dicotyledonous seeds. The approximate composition of the fatty acid in the oil is: palmitic acid 34%; stearic acid 3%; oleic acid 51% and 11% of linoleic acid. One kilogram of fresh seeds can produce 308 ml of oil. Thus it can serve as potential source of income to the rural people (Grundy and Campbell, 1993; Maroyi, 2007). The oil is used to make candles, soap and cosmetics and can be used for cooking. The residue after the extraction of oil is used as fertilizer as well as animal feed. It can be used to hasten the healing of a fractured limb when rubbed into a cut made in the skin; used as massage oil or consumed internally for the treatment/relief of rheumatism and also useful as general body ointment (Palgrave, 1977; Pooley, 1993; Maroyi, 2007). The oil provides good finish on wooden surfaces (Grundy and Campbell, 1993).

Various phytochemical compounds have been extracted from the different parts of *T. dregeana* and these extracts have been shown to be biologically active; having anti-microbial and anti-inflammatory activities. Examples are the extractions of cycloart-23-ene-3,25-diol from the leaves by Eldeen *et al.*, (2007) and limonoids (which include

evodulone and prierianin derivatives, dregeanin, dregeana 1-5 and rohituka 7 in *T. dregeana*) from the seeds and the seed coats (Maroyi, 2007). Apart from having antimicrobial and anti-inflammatory properties, limonoids (which are commonly found in the Meliaceae) are also known as antifeedants and growth regulators of insects, thus can serve as potent insecticides (Maroyi, 2007; Wheeler *et al.*, 2001). Limonoids also show cell-adhesion inhibitory properties. Extracts from the bark (as well as leaves and roots) have anti-bacterial properties. They also inhibit prostaglandin synthesis which is useful in the suppression of pain and inflammation (Eldeen *et al.*, 2005; Maroyi, 2007). It is therefore convenient to suggest that *T. dregeana* can be a potential source for the production of pharmaceutical products.

In terms of yield, one or two years of abundant seed yield may be followed by year(s) of low yield. The species is easily propagated by the seeds, especially fresh seeds. *T. dregeana* is frost-sensitive. The wood is susceptible to borer attack, thus must be treated against insects. The seeds have also been categorised as showing recalcitrant post-harvest behaviour (Maroyi, 2007). *T. dregeana* is not considered as being threatened because of its abundant existence and wide distribution. Although there is no record of evidence to substantiate this, it is assumed that wild populations of the species may be compromised locally, because common trees found are those planted in cities, parks and gardens for ornamental purposes (Pooley, 1993; Kioko, J.I; 2002; Pammenter *et al.*, 2003; Maroyi, 2007).



**Figure 1.2:** *Trichilia dregeana* tree.

Source: <http://www.plantzafrica.com/planttuv/trichildreg.htm>



**Figure 1.3:** *Trichilia dregeana* open fruits with seeds. Photo taken by Prof. Berjak, P.



**Figure 1. 4:** *Trichilia dregeana* seeds from the four provenances. Photo taken by Prof. Berjak, P.

### 1.11 Aim and objectives of the present study

The aim of the present study is to evaluate how provenance may affect the physiological responses of the seeds of a single species to biological stresses.

The objectives are to assess: (1) the response to desiccation and drying rates by the seeds collected from the four provenances; (2) the impacts of storage at different chilling temperatures; and (3) if the seeds from the four provenances were genetically similar enough to represent the same species.

The significance of the present study is that it will lay the foundation upon which further research should be built. Cryopreservation remains the only way by which the germplasms of non-orthodox species can be conserved in the long-term. However, it is characterised with a lot of considerations and optimization of protocols for each species. The present study highlighted the basic considerations at intra-species level, such that even within a species, better choices can be made for cryopreservation. It also gives room for selection, within species, for ecological restoration. This necessitates collection from wide range of locations such that as much genetic diversity as possible can be conserved.

## CHAPTER TWO

### MATERIALS AND METHODS

#### 2.1 Seed collection and cleaning

*Trichilia dregeana* seeds were obtained from three coastal areas, Mtunzini (MTZN), Durban (DBN) and Port Edward (P.ED) and one inland area, Pietermaritzburg (PMB); all of which are within the KwaZulu-Natal province of South Africa. While open fruits were harvested directly from *T. dregeana* trees from MTZN, P.ED and PMB, newly shed *T. dregeana* seeds were collected daily off the ground in DBN. Harvesting within all the four provenances was carried out in 2009 but at different times of the fruiting season. Seeds from MTZN were again collected in 2010. Seeds were collected in MTZN in late May (2009) and early May (2010); in DBN (between June and July, 2009); in P.ED (early June, 2009) and PMB (between July and August, 2009). Upon arrival in the laboratory, the fruits and seeds were first dusted with Benomyl 500 WP (active ingredient, benzimidazole [500 g kg<sup>-1</sup>]; Villa Crop Protection, S. Africa) to curtail the proliferation of fungi during the period (a few days) required for cleaning and preparing the seeds for storage. This involved extracting the seeds from open fruits and removing the fatty, waxy aril and seed coat to reveal the pale-green dicotyledonous seeds.

#### 2.2 Measurement of seed physical characteristics

Twenty seeds (with the aril and seed coat intact) from each provenance were measured for length and breadth using a vernier caliper. The fresh weights of the seeds were then determined using a DE series, Model 400 2-place weighing balance.

#### 2.3 Seed decontamination

Cleaned seeds from all the four provenances (2009) were surface-decontaminated by soaking in 1% (v/v) sodium hypochlorite (NaOCl) for 30 minutes and thereafter rinsed

three times with sterile, distilled water. MTZN seeds harvested in 2010 were further soaked in an anti-fungal ‘cocktail’ for 4 h, after which they were rinsed with sterile distilled water three times. The ‘cocktail’ comprised 0.5 ml L<sup>-1</sup> Early Impact (active ingredients, triazole [94 g L<sup>-1</sup>] and benzimidazole [150 g L<sup>-1</sup>]; Zeneca Agrochemicals, S. Africa) and 2.5 ml L<sup>-1</sup> Previcur N (active ingredient, propamocarb hydrochloride [722 g L<sup>-1</sup>]; AgrEvo, S. Africa). Seeds were then placed in a monolayer between paper towels on laboratory bench overnight to allow them to dry back to the shedding water content. Thereafter, some seeds were immediately directed towards experiments whilst the remaining seeds were stored hydrated (details below).

#### **2.4 Seed storage**

In order to ascertain seeds response to chilling, they were stored hydrated at a range of temperatures for four and half months. Decontaminated seeds that were to be stored at different temperatures were placed in 297 mm × 150 mm brown paper bags (300 seeds per bag) and dusted with Benomyl 500 WP. Each paper bag was thereafter sealed in a 330 mm × 420 mm GLAD<sup>®</sup> plastic bag. The sealed plastic bags were then stored at 3°C, 6°C or 16°C for 18 weeks. Two bags (i.e. 600 seeds from each provenance) were stored at each temperature treatment.

Decontaminated seeds that were to be used for all subsequent laboratory experiments, (essentially, the drying experiments), were placed in a monolayer on plastic mesh, suspended 200 mm above 1% NaOCl-moistened paper towel that lined the base of a 5L plastic bucket, sealed with lids. The lids were lined with paper towel in order to absorb condensed water from the seeds while in storage. The plastic mesh and buckets were decontaminated with 1% (v/v) NaOCl before use. The sealed buckets were then stored at 16°C for a very short period and were quickly used for the drying experiments.

#### **2.5 Determination of water content**

Water content was determined gravimetrically for individual embryonic axes after drying the axes to constant mass in an oven at 80°C for 48 hours. The embryonic axes of the

seeds were carefully removed using a sharp scalpel blade. Each axis was put in a small, aluminium-foil weighing boat and the fresh mass (FM) before, and dry mass (DM) after, drying was determined using a Mettler AE 240 5-place balance. The difference between FM and DM is the total water and it is expressed on dry mass basis as g water per g dry mass ( $\text{g g}^{-1}$ ).

## **2.6 Dehydration**

In order to assess the response of *T. dregeana* seeds to dehydration, they were subjected to different drying rates as well as a range of drying times. Two types of drying were used: rapid (flash) drying and slow (silica-gel) drying.

### **2.6.1 Flash drying**

Flash drying involves rapidly drying explants (embryonic axes in this study) on a mesh above a fan, which circulates silica-gel dried air over the explants, in an enclosed container e.g. glass jar (Pammenter *et al.*, 2002). The embryonic axes used in this study were excised with small (about 2 mm) cotyledonary attachments; except for those that were used for water content determination, which were excised with no cotyledonary attachment. The cotyledonary attachment was to enhance shooting, as it has been established by Goveia (2007) that excised embryonic axes of *Trichilia sp.* without the cotyledonary attachment produced little or no shooting at all. Thirty axes were used for each drying time, with five embryonic axes sampled for water content and 25 axes used for *in vitro* germination.

#### ***Determination of percentage water loss after flash drying***

For the purpose of comparison among the provenances, water loss after each flash drying time relative to the initial water content for each provenance was determined and expressed as percentage as follows:

$$\text{Percentage loss of water originally present} = (\text{WC}_{t_0} - \text{WC}_t) / \text{WC}_{t_0} * 100$$

Where  $\text{WC}_{t_0}$  is water content at time zero

$\text{WC}_t$  is water content at each flash drying time

### **2.6.2 *In vitro* germination medium and germination of flash dried embryonic axes**

*In vitro* germination medium for embryonic axes was made up of full strength Murashige and Skoog (1962) medium (MS, 4.42 g L<sup>-1</sup>) to which 30 g L<sup>-1</sup> sucrose and 8 g L<sup>-1</sup> agar were added, at pH 5.6 – 5.8; and was kept sterile.

Flash drying was carried out for 20, 40, 60, 90, 120 and 150 min. At each drying interval, five embryonic axes were used to determine water content gravimetrically (as described in section 2.5 above), while 25 embryonic axes were rehydrated straight away after dehydration by direct immersion in sterile Calcium-Magnesium (CaMg) solution for 30 minutes in the dark. The CaMg solution was comprised of 1.0 µM CaCl<sub>2</sub>.2H<sub>2</sub>O and 1.0 mM MgCl<sub>2</sub>.6H<sub>2</sub>O (after Mycock, 1999; Berjak *et al.* 2000). Immediately after rehydration, the embryonic axes were first decontaminated with 1% (v/v) NaOCl for 10 min in the laminar flow and rinsed three times with sterile distilled water. Then, they were soaked in 0.01 % (w/v) Cicatrin<sup>®</sup> (components per gram: Neomycin sulphate, 3300 units; Bacitracin Zinc, 250 units; L-cysteine, 2.0 mg; Glycine, 10 mg and dl-Threonine, 1.0 mg; Active ingredient: Neomycin and Bacitracin; Pfizer, S. Africa) for 5 min and were washed again thrice with sterile, distilled water. Subsequently, the embryonic axes were plated on full strength MS medium in 65 mm Petri dishes, with five axes per Petri dish and sealed with parafilm. The cultures were maintained in the growth room under a 14/10 h light (65 µmol m<sup>-2</sup> sec<sup>-2</sup>)/dark photoperiod at 23 - 25°C. Freshly excised embryonic axes subjected to no flash drying served as the control, in which five embryonic axes were used for initial (0 min) water content determination and 25 embryonic axes were decontaminated and plated as described above.

### **2.6.3 Silica-gel drying**

Whole seeds were buried in activated silica gel, sealed in 330 mm × 420 mm plastic bags, for 6, 12, 18, 24, 30 and 36 h. Thirty seeds, for each drying time, were buried in each plastic bag. For each time interval, five seeds were randomly sampled for axes water content determination (see section 2.5 above) while 25 seeds were assessed for germinability *in vitro*.

#### **2.6.4 *In vitro* germination medium and germination of silica-gel dried seeds**

The *in vitro* culture medium comprised quarter-strength MS, that is, 1.11 g L<sup>-1</sup> MS, 30 g L<sup>-1</sup> sucrose; and 8 g L<sup>-1</sup> agar (solidifying medium) brought to pH range of 5.6 – 5.8.

For *in vitro* culturing, silica-gel dried seeds were decontaminated within a laminar flow, by first soaking them in 0.1 % (w/v) mercuric chloride (HgCl<sub>2</sub>) for 15 min followed by three rinses with sterile, distilled water. The seeds were subsequently soaked in 0.01 % (w/v) cicatrin® for 10 min and rinsed thrice with sterile, distilled water. The seeds were then plated on the quarter-strength MS medium in 70 mm × 70 mm × 95 mm Magenta boxes, with five seeds per box. The boxes were sealed with lids and parafilm, and the cultures were maintained in the growth room under 14/10 h light (65 μmol m<sup>-2</sup> sec<sup>-1</sup>)/dark photoperiod at 23 – 25°C.

#### **2.7 Chilling**

Cleaned seeds that were stored hydrated at 3°C, 6°C and 16°C for 18 weeks (described in section 2.4) represented those that were subjected to different chilling temperatures. At each storage temperature, seeds were assessed for water content and germinability fortnightly, from 2 to 18 weeks. The germination medium and the method of germination for seeds stored at different chilling temperatures were the same as those described in 2.6.4 above.

#### **2.8 Assessment of germinability and growth parameters**

Germinability of embryonic axes and whole seeds was scored as positive when radicle elongation was approximately 2 mm. Furthermore, measurement of root and shoot length, percentage roots and shoots and total seedling dry mass from previously flash dried axes were also assessed.

#### **2.9 Temperature and rainfall data**

Monthly minimum and maximum temperature and rainfall data from 2008 to 2010, for each provenance, were supplied by the South African Weather Service

([www.weathersa.co.za](http://www.weathersa.co.za); Private Bag X097, Pretoria, 0001; Tel: +27(0)123676000). The following parameters were given: average of the daily maximum temperature (in °C) by month, average of the daily minimum temperature (in °C) by month and total of the rainfall (in mm) by month.

To characterise the different locations in terms of heat and rainfall, the following parameters were derived from the given weather data:

**I. Data for the whole of 2008 and 2009:**

1. Annual accumulated maximum temperature for each year =

$$\sum [(average\ of\ daily\ max.\ temperature\ for\ month\ 1 \times number\ of\ days\ in\ month\ 1) + \dots + (average\ of\ daily\ max.\ temperature\ for\ month\ 12 \times number\ of\ days\ in\ month\ 12)]$$

2. Annual accumulated minimum Temperature for each year =

$$\sum [(average\ of\ daily\ min.\ temperature\ for\ month\ 1 \times number\ of\ days\ in\ month\ 1) + \dots + (average\ of\ daily\ min.\ temperature\ for\ month\ 12 \times number\ of\ days\ in\ month\ 12)]$$

3. Annual rainfall for each year =

$$\sum [(total\ of\ the\ rainfall\ for\ month\ 1) + \dots + (total\ of\ the\ rainfall\ for\ month\ 12)]$$

Where 1 to 12 represent January to December.

4. Average daily maximum temperature for each year =

$$Annual\ accumulated\ maximum\ temperature\ for\ each\ year / number\ of\ days\ in\ each\ year$$

5. Average daily minimum temperature year =

$$Annual\ accumulated\ minimum\ temperature\ for\ each\ year / number\ of\ days\ in\ each\ year$$

(366 days in 2008 and 365 days in 2009)

**II. Data from flowering to fruit shedding period:**

1. Accumulated maximum temperature from flowering to fruit shed =  
 $\sum [(average\ of\ daily\ max.\ temperature\ for\ month\ 1 \times number\ of\ days\ in\ month\ 1) + \dots + (average\ of\ daily\ max.\ temperature\ for\ month\ 9 \times number\ of\ days\ in\ month\ 9)]$

2. Monthly accumulated maximum temperature from flowering to fruit shed =  
Accumulated maximum temperature from flowering to fruit shed/9

3. Accumulated minimum temperature from flowering to fruit shed =  
 $\sum [(average\ of\ daily\ min.\ temperature\ for\ month\ 1 \times number\ of\ days\ in\ month\ 1) + \dots + (average\ of\ daily\ min.\ temperature\ for\ month\ 9 \times number\ of\ days\ in\ month\ 9)]$

4. Monthly accumulated minimum temperature from flowering to fruit shed =  
Accumulated minimum temperature from flowering to fruit shed/9

5. Accumulated rainfall from flowering to fruit shed =  
 $\sum [(total\ of\ the\ rainfall\ for\ month\ 1) + \dots + (total\ of\ the\ rainfall\ for\ month\ 9)]$

6. Monthly average rainfall from flowering to fruit shed =  
Accumulated rainfall from flowering to fruit shed/9

Where 1 to 9 represent Sept – May, Nov – July, Dec – Aug and Oct – June for MTZN, DBN, PMB and P.ED locations respectively.

9 is the number of months that elapsed from flowering to fruit shedding period.

## **2.10 Phylogeny**

### **2.10.1 DNA extraction**

DNA was extracted from 30 mg (dry mass) leaf of *Trichilia dregaeana* from DBN and PMB. The dry leaves were first ground to a fine powder in liquid nitrogen (LN) before DNA was extracted using the DNeasy® Plant Mini Kit (Qiagen) according to manufacturer's instructions. DNA was quantified using a Nanodrop Spectrophotometer

and the quality (occurrence as high molecular weight DNA) was determined by electrophoresing the DNA isolates in a 1% w/v agarose gel that contained 0.05mg ml<sup>-1</sup> ethidium bromide (EtBr) in 1X TBE (Tris- Borate-EDTA [Ethylene Diamine Tetraacetic Acid]) for 1.5 h at 120V. Molecular weight of the bands was determined by comparison of their mobility with that of selected bands of a standard molecular weight marker.

### 2.10.2 Polymerase Chain Reaction (PCR) amplification

The non-coding nuclear ribosomal internal transcribed spacer 1 (ITS1) was amplified as follows: Polymerase Chain reactions (PCR) were run in a final volume of 25 µl; this comprised 30 ng of extracted DNA in a final volume of 9µl of and 16 µl of mastermix. The mastermix contained 0.8 µl deionised water, 2.5 µl buffer (10X), 4 µl MgCl<sub>2</sub> (25 mM), 4 µl forward primer and 4 µl reverse primer (6.25 µM) (Table 2.1), 0.5 µl dNTPs (10mM) and 0.2 µl *Taq* (5 units/µl) (Supertherm). Primers 1 and 2 were used to amplify the ITS1 region (Table 2.1).

**Table 2. 1:** Sequences of primers used in amplification of the nuclear ribosomal ITS1 region of *T. dregeana* DNA

DNA markers	Primers	Target sequences (5'-3')
ITS 1	1 (forward)	TTCCGTAGGTGAACCTGCG
	2 (reverse)	GCTGCGTTCTTCATCGATGC

**Table 2. 2:** Thermal cycling protocol used in PCR amplification of the ITS1 region

Stage	Purpose	Temperature (°C)	Time (min)
1	Initial denaturation	94.0	2
*2	Denaturation	94.0	1
	Annealing	42.0	2
	Extension	72.0	2
3	Final extension	72.0	7
4	Storage	15.0	∞

\* Stage 2 repeated 35 times

The thermal cycling profile used in the PCR amplification is presented in (Table 2.2). The PCR products (DNA fragments) were separated using gel electrophoresis as described previously. Bands were excised from the gel and the PCR products purified using a Qiagen Gel Extraction kit according to manufacturer's instructions.

### **2.10.3 DNA purification, quantification and analysis**

The purified PCR products were sequenced at Inqaba Biotec (Pretoria, South Africa). A *Trichilia emetica* ITS sequence (Accession number EF136577.1) was selected for use as an outgroup as a BLAST (Basic Local Alignment Search Tool) search of the NCBI (National Centre for Biotechnology Information) Genbank revealed that this was the sequence most similar to *T. dregeana*. Sequences were aligned using the CLUSTAL W option (Thompson *et al.*, 1994) of the BioEdit program (Version 5.0.9 for Windows 95/98/NT) and by visual inspection. Alignments were analysed phenetically by the neighbour-joining (nj) method and cladistically using the maximum parsimony (mp) and maximum likelihood (ml) methods in PAUP version 4.0-beta version (Swofford *et al.*, 2001). In all analyses, nodal support was assessed by bootstrap resampling analysis (1000 replicates). The most appropriate model for use in neighbour joining and maximum likelihood analyses (HKY) was determined using jModeltest (Posada, 2008). For parsimony analyses, starting trees were obtained by stepwise addition. The addition sequence was random, with 1 tree held at each step and with 10 replicates.

### **2.11 Stastical analysis**

Statistical analyses of all the data obtained were carried out using SPSS 15.0 for Windows. One-way analysis of variance (ANOVA) was used to test for inter-provenance differences in seed weight, seed length and width. One-sample, Kolmogorov-Smirnov test was used to test whether germination data were normally distributed. Chi square test and One-way and Two-way ANOVA were used where necessary to test the differences in germination, water contents and growth parameters across the provenances, respectively.

## CHAPTER THREE

### RESULTS

#### 3.1 2008 and 2009 temperature and rainfall data for the locations from which collections were made

*Trichilia dregeana* seed collections from the four locations studied were carried out between May and August, 2009. However, flowering started in the previous year (2008) since the duration from flowering to fruit shedding is usually about eight months or more. Hence, Table 3.1 gives the idea of the heat energy (temperature) and the amount of rainfall that was prevalent in these provenances, from January to December, for both years, as well as daily maximum and minimum temperature averages for each year.

**Table 3.1:** 2008-2009 rainfall and temperature data for the collection locations as derived from the data supplied by South African Weather Service.

	Annual rainfall (mm)	Accumulated annual maximum Temperature (°C)	Accumulated annual minimum Temperature (°C)	Average daily maximum Temperature (°C)	Average daily minimum Temperature (°C)
<b>MTUNZINI (MTZN)</b>					
2008	1231.6	9898.3	5243.6	27.0	14.3
2009	908.6	9764.8	5942.5	26.7	16.3
<b>Average</b>	<b>1070.1</b>	<b>9831.5</b>	<b>5593.1</b>	<b>26.9</b>	<b>15.3</b>
<b>DURBAN (DBN)</b>					
2008	1182.0	9150.4	6074.7	25.0	16.6
2009	924.4	9051.2	5985.7	24.8	16.4
<b>Average</b>	<b>1053.2</b>	<b>9100.8</b>	<b>6029.9</b>	<b>24.9</b>	<b>16.5</b>
<b>PIETERMARITZBURG (PMB)</b>					
2008	780.4	9694.7	4948.6	26.5	13.5
2009	792.8	9141.1	4795.1	25.0	13.1
<b>Average</b>	<b>786.6</b>	<b>9417.9</b>	<b>4871.8</b>	<b>25.8</b>	<b>13.3</b>
<b>PORT EDWARD (P.ED)</b>					
2008	1387.8	8601.6	6325.7	23.5	17.3
2009	1180.2	8517.3	6314.2	23.3	17.3
<b>Average</b>	<b>1284.0</b>	<b>8559.4</b>	<b>6319.9</b>	<b>23.4</b>	<b>17.3</b>

**Table 3.2:** Comparison of geographical characteristics and accumulated rainfall and temperature from flowering to fruit shedding period across the provenances studied

FEATURES	PROVENANCE			
	MTZN	DBN	PMB	P.ED
Geographical Coordinates	28°57' S, 31°45' E	29°51' S, 30°59' E	29°37' S, 30°23' E	31°2' S, 30°13' E
Location within KwaZulu-Natal (KZN) province	KZN North Coast*	KZN Central Coast*	KZN Inland	KZN South Coast*
Flowering to fruit shedding period for 2008 – 2009	September – May	November – July	December – August	October – June
Accumulated rainfall (mm) from flowering to fruit shedding	756.20	697.20	632.40	843.20
Monthly average rainfall (mm) from flowering to fruit shedding	84.02	77.47	70.27	93.69
Accumulated max. temperature (°C) from flowering to fruit shedding	7602.50	6972.00	6928.40	6603.20
Accumulated min. temperature (°C) from flowering to fruit shedding	4625.30	4690.70	3575.60	5030.00
<i>T. dregeana</i> harvest	May (Late)	June – July	July – August	June (Early)

\* The term KZN north coast and KZN south coast are local terms for the coast north and south of Durban, respectively. All the coastal provenances in this study are on the east coast but Durban is located centrally.

Rainfall for 2008 was higher than 2009 across all the provenances, except Pietermaritzburg. Also, the annual maximum temperature for 2008 was higher than 2009 across the four provenances. For MTZN and P.ED, accumulated minimum temperature was lower in 2008 than it was in 2009. The reverse was the case for DBN and PMB where the accumulated minimum temperature was higher in 2008 than 2009 (Table 3.1).

Flowering to fruit shed was recorded to take nine months in this study and the flowering season for each provenance was derived by counting nine months backward from the harvest time. The accumulated rainfall, maximum and minimum temperature from flowering to fruit shed as well as the monthly average rainfall for this period are shown in Table 3.2. The rainfall was in the order P.ED > MTZN > DBN > PMB, with the south and north coastally provenances having the highest values and the inland provenance having the lowest. Accumulated maximum temperature and minimum temperature during flowering to fruit shed were in the order MTZN > PMB > DBN > P.ED and P.ED > DBN > MTZN > PMB, respectively. That means Port Edward had the highest rainfall as well as the highest minimum temperature. MTZN was the warmest provenance, having the highest accumulated maximum temperature. Also, maximum temperature decreased from the north coast down to the south coast, while the inland is relatively hotter than the coastal areas with the exception of the north coast (Table 3.2).

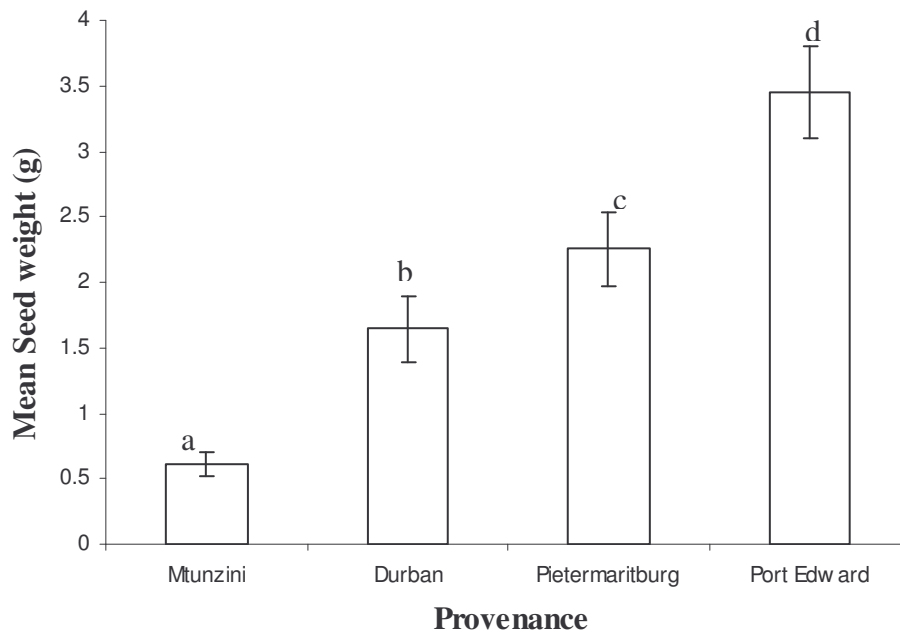
### **3.2 Morphological differences of *T. dregeana* seeds from the four provenances**

There were variations in the harvest time of *T. dregeana* seeds among the provenances. While fruit shedding was earliest at the North coast provenance (MTZN) being over by late May, fruit shedding and harvesting continued until July at the East coast provenance (DBN). Harvesting was carried out in the South coast (P.ED) in early June, while it was not possible to harvest from the inland provenance (PMB) until July when the seeds had matured (Table 3.2).

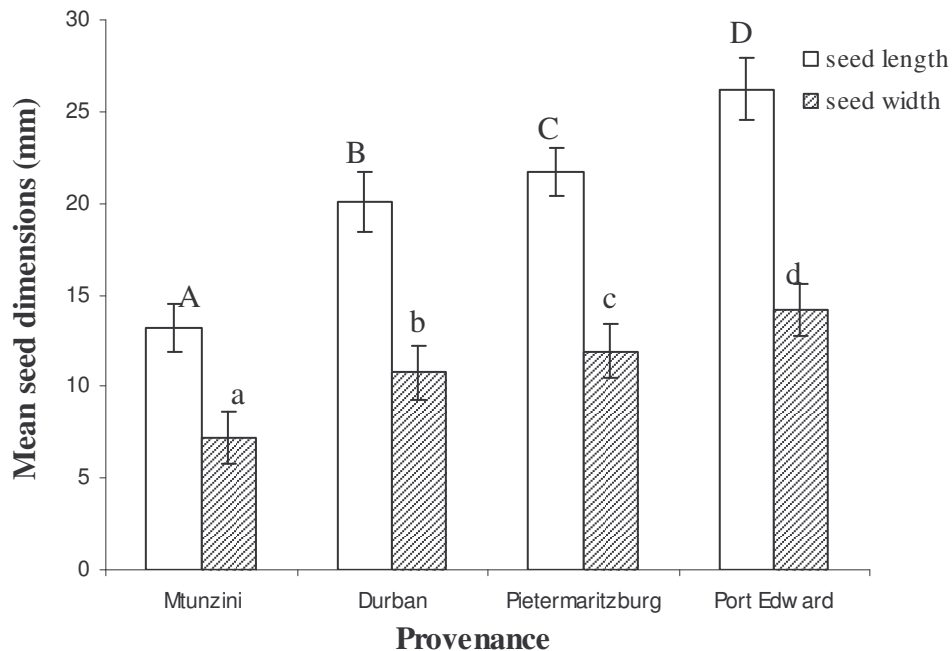
The seeds increased in fresh mass (Figure 3.1), length and width (Figure 3.2) from the North coast (MTZN), through the East coast (DBN) to the inland (PMB) and the South coast (P.ED) provenances, i.e. MTZN < DBN < PMB < P.ED. Means in seed weight,

length and breadth were significantly different across the provenances (one-way ANOVA,  $p \leq 0.05$ ).

Additionally, variability in seed sizes across the provenances also increased with increase in seed mean weight, length and breadth. This suggests that seeds from MTZN which were the smallest of the four provenances were more consistent in size than those from the other provenances. This is evident in the standard deviation bars on the mean graphs.



**Figure 3.1:** Mean seed fresh mass of *T. dregeana* seeds of different provenances. Mean ( $n = 20$ ) with different letters are significantly different (one-way ANOVA, Duncan and Scheffe post hoc tests  $p \leq 0.05$ ). Bars represent one standard deviation on either side of the mean.



**Figure 3.2:** Mean length and width of *T. dregeana* seeds of the four provenances. Mean (n = 20) with different letters are significantly different (one-way ANOVA Duncan and Scheffe post hoc tests  $p \leq 0.05$ ). Bars represent one standard deviation on either side of the mean.

### 3.3 Initial water contents and germination of *T. dregeana* seeds from the four provenances

The water contents of embryonic axes excised from newly harvested seeds from the provenances ranged from 1.28 to 2.35 g g<sup>-1</sup>, in the order MTZN < DBN < PMB < P.ED (Table 3.3). The initial water contents seemed to have inverse relationships with average maximum temperature along the coast line, i.e. the warmer the provenance, the lower the axis water content of the seed obtained from that provenance.

Development of radicle length of about 2 mm was taken as germination of the embryonic axes while protrusion of about 2 mm long radicle from between the cotyledons of whole seeds was taken as germination. Whole seeds, the aril and seed coat of which had been removed showed 100% germination across the four provenances. Freshly excised embryonic axes showed high percentage of survival as well, but statistical analysis showed that the germination of axes from seeds from PMB and P.ED were lower than those axes MTZN and DBN seeds (Table 3.3).

**Table 3.3:** Initial water contents ( $\pm$  one standard deviation) and viability of embryonic axes and whole seeds of *T. dregeana*. Means with different letters are significantly different, one-way ANOVA, ( $p \leq 0.05$ ).

Provenance	Embryonic axes water content ( $\text{g g}^{-1}$ )	Embryonic axes germination (%)	Whole seed germination (%)
MTZN	$1.28 \pm 0.38^a$	100 <sup>b</sup>	100
DBN	$1.51 \pm 0.20^{ab}$	96 <sup>ab</sup>	100
PMB	$1.92 \pm 0.41^{bc}$	80 <sup>a</sup>	100
P.ED	$2.35 \pm 0.25^c$	80 <sup>a</sup>	100

### 3.4 Responses of *T. dregeana* embryonic axes and seeds from the provenances to drying

Results for the two drying rates that were employed in this study i.e. rapid (flash) and slow (silica-gel) drying, are reported below.

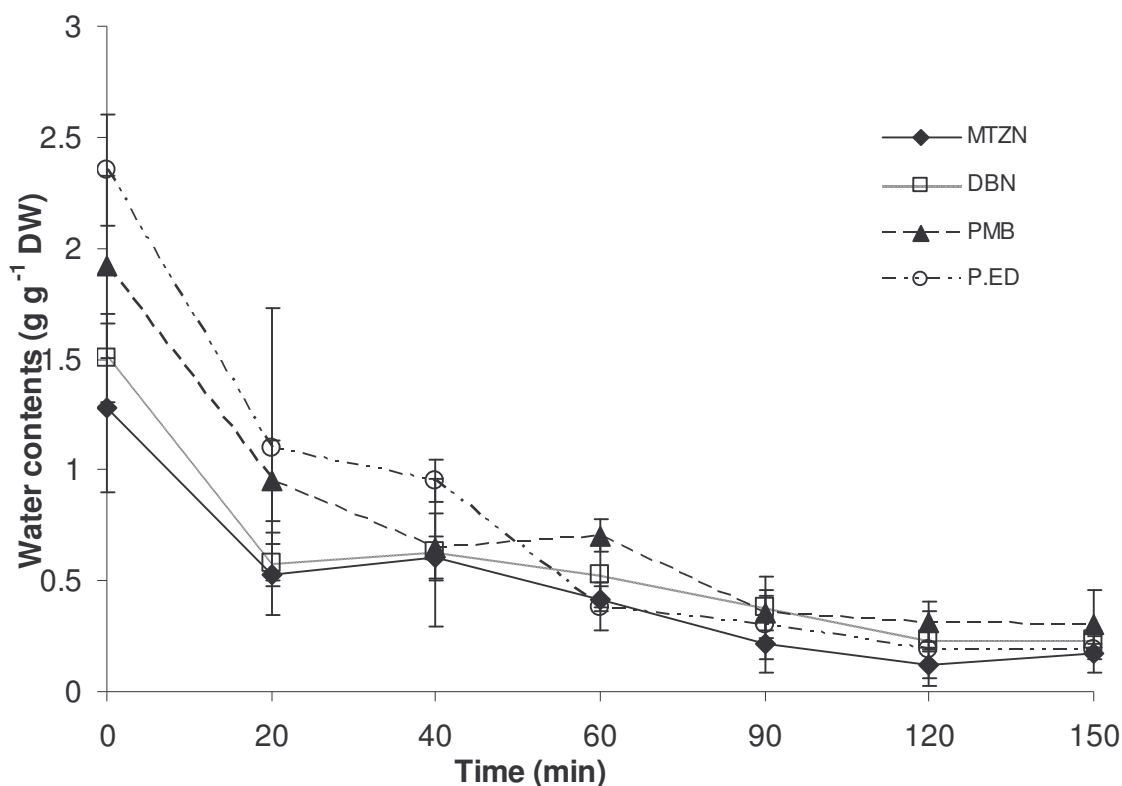
#### 3.4.1 Rapid (flash) drying of embryonic axes

Rapid drying of the embryonic axes of *T. dregeana* was achieved by flashing drying for 0, 20, 40, 60, 90, 120 and 150 minutes as described earlier.

##### 3.4.1.1 Water content and germination

As would be expected, water contents decreased as drying time increased (Figure 3.3). The decrease was rapid initially and after the first 20 minutes of drying, axes excised from the seeds of all provenances had lost more than 50% of the water content they possessed at shedding (MTZN:  $1.28 \pm 0.38$  to  $0.53 \pm 0.18$ ; DBN:  $1.51 \pm 0.20$  to  $0.58 \pm 0.08$ ; PMB:  $1.92 \pm 0.41$  to  $0.95 \pm 0.18$  and P.ED:  $2.35 \pm 0.25$  to  $1.10 \pm 0.63$ ). Thereafter, the water contents declined gradually until the end of the drying course (Figure 3.3).

Embryonic axes (henceforth referred to as axes) excised from seeds belonging to the four provenances fell into two groups in term of their responses to flash drying (Figure 3.3).



**Figure 3.3:** Water content of flash dried embryonic axes of *T. dregeana* from different provenances (n = 5), bars represent standard deviation.

**Table 3. 4:** Percentage water loss relative to the initial axes water contents after flash-drying the embryonic axes of *T. dregeana* from different provenances

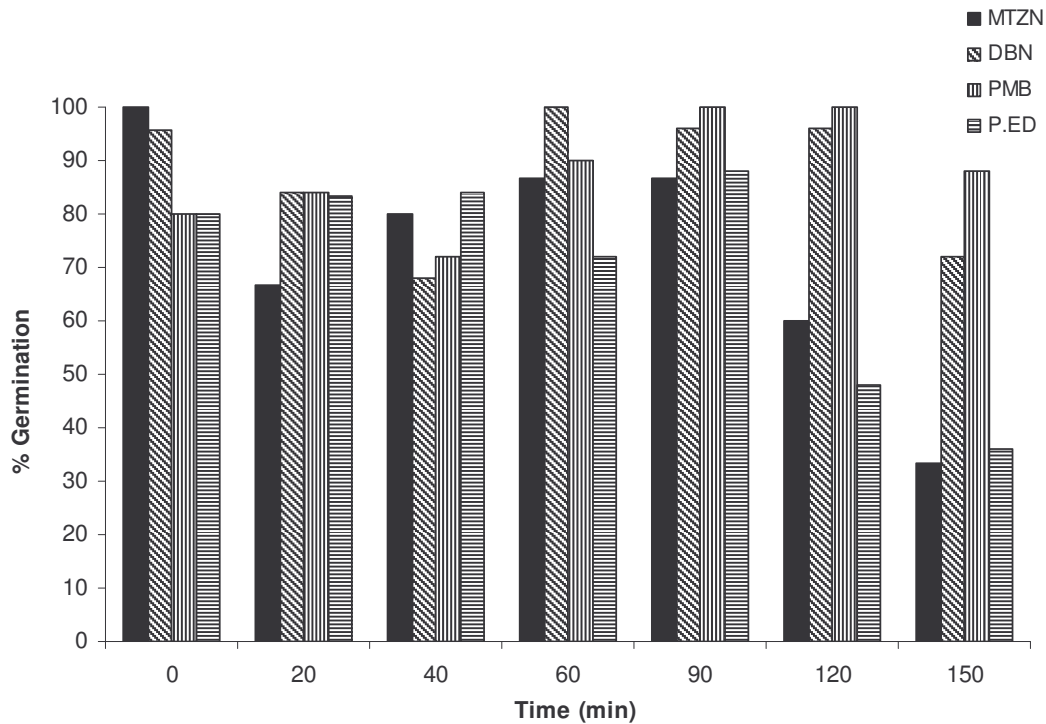
	MTZN	DBN	PMB	P.ED
<b>FD TIME</b>				
0	-	-	-	-
20	58.49%	61.31%	50.41%	53.08%
40	52.87%	58.30%	66.14%	59.59%
60	86.20%	64.83%	63.29%	83.69%
90	83.25%	74.62%	81.37%	87.18%
120	90.23%	84.32%	83.58%	91.85%
150	86.59%	84.71%	84.16%	92.01%
<b>Mean</b>	76.27%	71.35%	71.49%	77.90%

Considering the mean water content for all flash drying times for each provenance, axes from MTZN and DBN responded alike to flash drying as their mean water contents, for all flash drying times, were similar. In the same way, mean water contents of axes from

PMB and P.ED were not significantly different. However, the former group (MTZN and DBN) was significantly different from the latter (PMB and P.ED) (Two-way ANOVA,  $p \leq 0.05$ ). Conversely, to ascertain the axes of which location dried most, the percentage water loss at each flash drying time, relative to the initial axes water content was determined for each location (Table 3.4). Embryonic axes from P.ED dried the most, having the highest mean percentage water loss, while those from DBN seeds had the least mean percentage water loss. The order of response to flash drying was DBN > PMB > MTZN > P.ED (Table 3.4).

The percentage germination for all flash drying times was summed up for each provenance and the mean taken to represent the provenance response to flash drying. Viability of the axes after flash drying was in the order P.ED < MTZN < DBN < PMB. Axes from P.ED provenance had the lowest viability, having a mean of 70% germination. Those from PMB had the highest viability with mean of 88%. MTZN and DBN had means of 73% and 87% germination, respectively. The percentage germinations of axes from P.ED and MTZN were statistically the same, so also were those from DBN and PMB. However, these two groups (MTZN/P.ED and DBN/PMB) were significantly different from one another ( $p \leq 0.05$ ) (Figure 3.4).

Adding up the percentage germinations of the four provenances for a particular time, the mean of the percentage germinations represented the viability at that time. Axes flash dried for 90 min had the highest percentage germination with a mean of 93% while those flash dried for 150 min had the least viability, having a mean of 60% (Figure 3.4). It is noteworthy, however, that *T. dregeana* embryonic axes still had more than 50% survival after flash drying for 150 min at relatively low water contents of  $0.17 - 0.30 \text{ g g}^{-1}$  ( $0.17 \pm 0.08$ ,  $0.23 \pm 0.03$ ,  $0.30 \pm 0.15$  and  $0.19 \pm 0.02 \text{ g g}^{-1}$  for MTZN, DBN, PMB and P.ED, respectively).



**Figure 3.4:** Percentage germination of flash dried embryonic axes of *T. dregeana* from different provenances (n = 25).

### 3.4.1.2 Percentage root and shoot production after flash drying

Root production was the criterion used for percentage germination. Hence the result obtained for germination after flash drying was the same for root production. The shoot productions by embryonic axes from *T. dregeana* seeds for the provenances, at each flash drying time, were shown in Table 3.5.

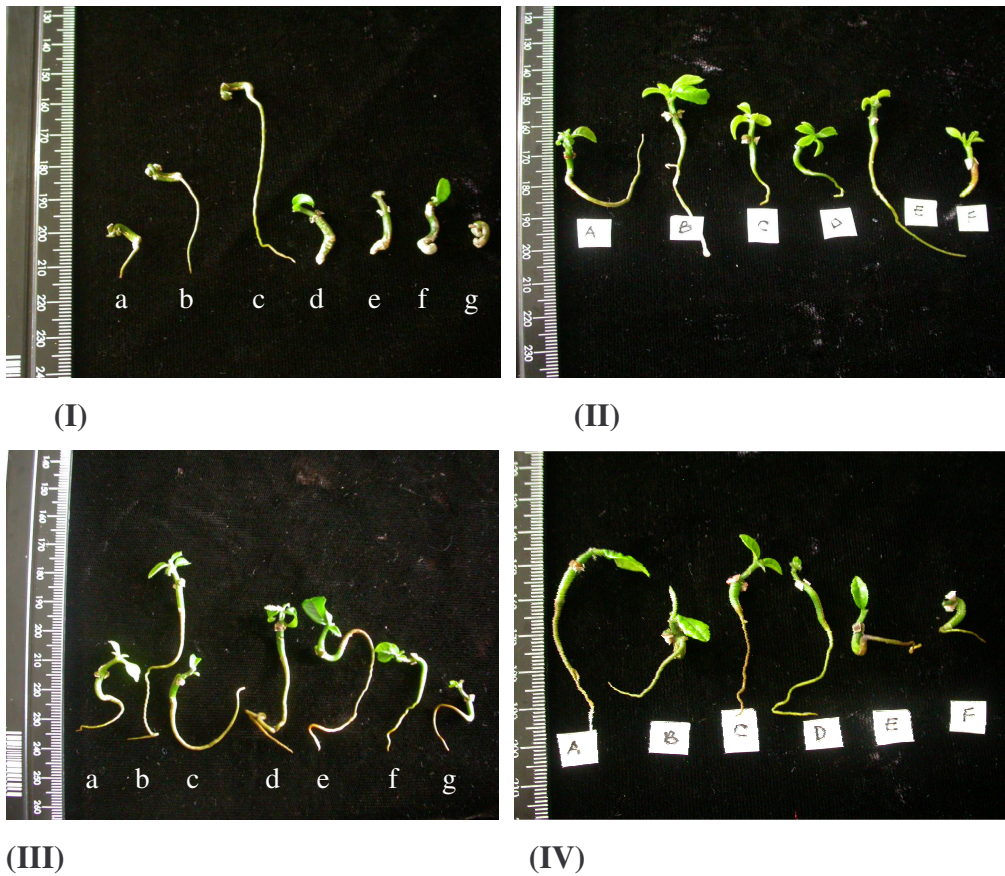
Axes obtained from PMB seeds had the highest shoot production, having a mean of 74%, whilst those axes from P.ED had the lowest shoot production with a mean of 38%. It should be noted that axes from only PMB seeds produced more than 50% shoot at every flash drying time. Axes flash dried for 90 min had the highest shoot production (71%) while those axes flash dried for 150 min had the lowest with 39% (Table 3.5).

**Table 3.5:** Percentage shooting of *T. dregeana* axes from different provenances after each flash drying time. Means with different letters are significantly different (Two-way ANOVA, Tukey's Post Hoc test,  $p \leq 0.05$ )

FD Time (min)	Percentage shoot production				
	MTZN	DBN	PMB	P.ED	Mean
0	40	70	60	52	56 <sup>ab</sup>
20	53	28	72	34	46 <sup>a</sup>
40	47	48	64	56	54 <sup>ab</sup>
60	53	58	75	32	54 <sup>ab</sup>
90	67	76	90	52	71 <sup>b</sup>
120	27	48	84	12	44 <sup>a</sup>
150	0	40	72	28	39 <sup>a</sup>
Mean	41 <sup>a</sup>	53 <sup>ab</sup>	74 <sup>b</sup>	38 <sup>a</sup>	

#### 3.4.1.3 Root and shoot length of seedlings from previously flash-dried embryonic axes

Seedlings obtained from axes that had been previously dried rapidly were assessed for their vigour, in terms of the root and shoot lengths, for each flash drying time and for each provenance, after 12 weeks in culture (Figure 3.6).



**Figure 3.5:** Seedlings of *T. dregeana* after 12 weeks in culture, from previously flash dried axes for each provenance (I: MTZN; II: DBN; III: PMB; IV: P.ED) and flash drying time (a: 0; b: 20; c: 40; d: 60; e: 90; f: 120; g: 150 FD min).

**Table 3.6:** Mean root length of seedlings obtained from *T. dregeana* axes that have been previously dried rapidly, for each provenance and for each flash drying time. Mean with different letters are significantly different, Two-way ANOVA, ( $p \leq 0.05$ )

FD time (min)	Average root length (mm)				Mean
	MTZN	DBN	PMB	P.ED	
0	6.27	14.09	19.00	16.70	13.85 <sup>ab</sup>
20	8.20	9.50	39.19	10.15	18.28 <sup>b</sup>
40	13.75	7.82	13.11	17.90	13.38 <sup>ab</sup>
60	7.77	11.54	18.44	16.06	13.65 <sup>ab</sup>
90	6.08	15.58	29.30	14.05	17.06 <sup>b</sup>
120	6.67	7.46	24.40	10.33	13.90 <sup>ab</sup>
150	4.40	5.33	11.50	7.43	8.98 <sup>a</sup>
Mean	7.83 <sup>a</sup>	10.93 <sup>ab</sup>	22.61 <sup>c</sup>	14.04 <sup>b</sup>	

**Table 3.7:** Mean shoot length of seedlings obtained from *T. dregeana* axes that have been previously dried rapidly, for each provenance and for each flash drying time. Mean with different letters are significantly different, Two-way ANOVA, ( $p \leq 0.05$ )

FD time (min)	Average shoot length (mm)				
	MTZN	DBN	PMB	P.ED	Mean
0	7.33	12.60	17.22	13.23	13.02 <sup>b</sup>
20	8.38	9.86	17.83	9.00	12.90 <sup>b</sup>
40	8.71	10.50	14.31	12.00	11.92 <sup>b</sup>
60	10.25	12.71	15.27	12.50	13.09 <sup>b</sup>
90	7.20	9.95	14.44	11.00	11.07 <sup>ab</sup>
120	10.00	9.75	14.52	9.00	12.23 <sup>b</sup>
150	0.00	8.25	12.22	5.40	9.33 <sup>a</sup>
Mean	7.96 <sup>a</sup>	10.86 <sup>b</sup>	14.95 <sup>c</sup>	11.08 <sup>b</sup>	

*T. dregeana* seedlings obtained from previously flash dried embryonic axes of PMB seeds were more vigorous than others as seedlings from that provenance had the highest root and shoot lengths. On the hand, seedlings from MTZN provenance were the least vigorous of all the four provenances, having the lowest root and shoot lengths (Table 3.6 and 3.7). The order of vigour of seedling growth for all the four provenances was MTZN < DBN < P.ED < PMB. Tukey's post hoc test of Two-way ANOVA showed that root lengths from MTZN and DBN were significantly the same, also DBN and P.ED were significantly the same (Table 3.6). The shoot lengths of seedlings from DBN and P.ED were not significantly different, although the value for the latter was higher than the former; otherwise they were significantly different from other provenances (Table 3.7).

Root and shoot lengths were not consistent with the flash drying times. Seedlings obtained from 20 min flash drying time had the highest root length; influenced by the exceptional value of seedlings from 20 min flash dried PMB axes. Root length was in the order 20 > 90 > 120 > 0 > 60 > 40 > 150 min and the significant differences are shown in Table 3.6. Order of shoot length at different flash drying time is shown in Table 3.7 and was in the order 60 > 0 > 20 > 40 > 120 > 90 > 150 min.

### 3.4.1.4 Seedling dry mass

**Table 3.8:** Average dry mass of seedlings produced by rapidly dried axes of *T. dregeana* seeds of all four provenances. Mean with different letters are significantly different, Two-way ANOVA, ( $p \leq 0.05$ )

FD time (min)	Average seedling dry mass accumulation (mg)				Mean
	MTZN	DBN	PMB	P.ED	
0	12.62	12.57	13.53	15.82	13.70 <sup>de</sup>
20	14.31	15.12	17.91	12.30	14.91 <sup>e</sup>
40	13.72	16.69	14.21	14.34	14.75 <sup>e</sup>
60	8.66	13.97	15.78	10.19	12.53 <sup>cd</sup>
90	8.10	10.21	12.11	12.48	10.98 <sup>bc</sup>
120	7.29	9.76	9.41	9.76	9.32 <sup>ab</sup>
150	4.69	9.02	9.41	8.97	8.69 <sup>a</sup>
Mean	10.44 <sup>a</sup>	12.61 <sup>b</sup>	12.82 <sup>b</sup>	12.53 <sup>b</sup>	

Seedlings from MTZN accumulated the least dry matter during the growth period (Two way ANOVA;  $p \leq 0.05$ ). Seeds of the remaining three provenances were not significantly different from one another (Table 3.8).

Dry mass accumulation decreased with drying time from 60 to 150 min, the differences being significant (Table 3.8). Seedling dry mass accumulation progressed in the order  $150 < 120 < 90 < 60 < 0 < 40 < 20$  min and the significance of differences are shown in Table 3.7.

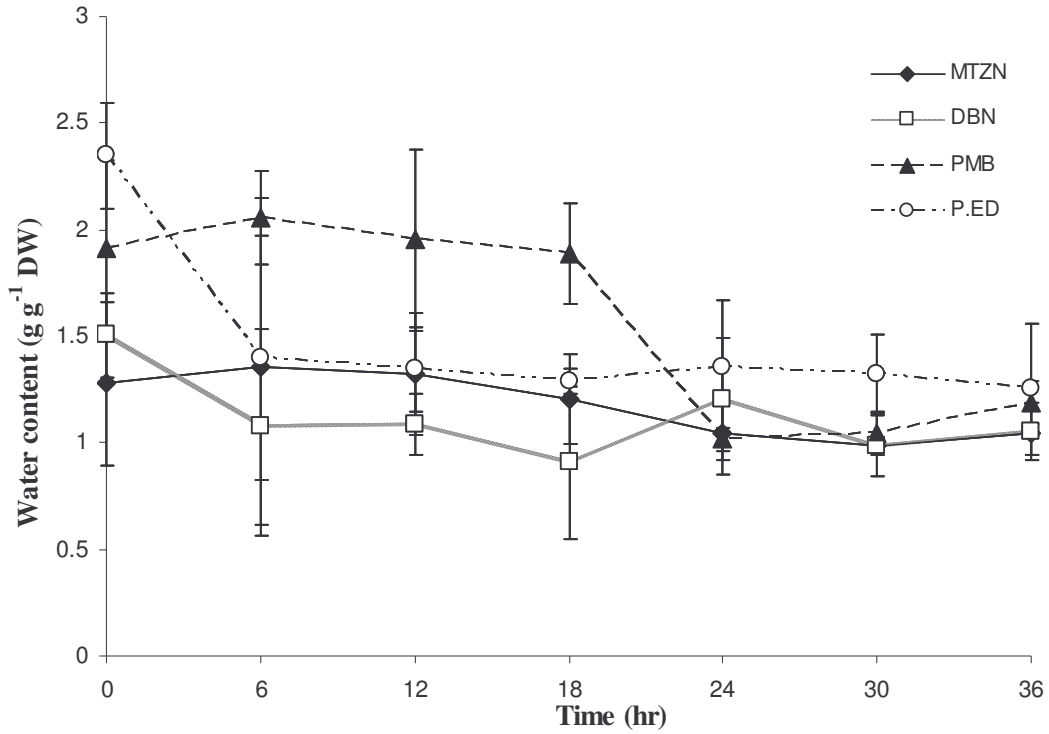
### 3.4.2 Slow (Silica-Gel) drying

Whole seeds of *T. dregeana* were dried slowly by burying them in silica gel at different times up to 36 h. Embryonic axes excised from whole seeds that were buried in silica gel lost water very slowly (Figure 3.6). All the axes of seeds from the four provenances had more than 50% of the initial water content at the end of the slow drying time, i.e. after being buried in silica gel for 36 h. The water contents ( $\pm$  standard error) at 36 h of being buried in silica gel were MTZN,  $1.05 \pm 0.06$ ; DBN,  $1.06 \pm 0.06$ ; PMB,  $1.19 \pm 0.05$  and

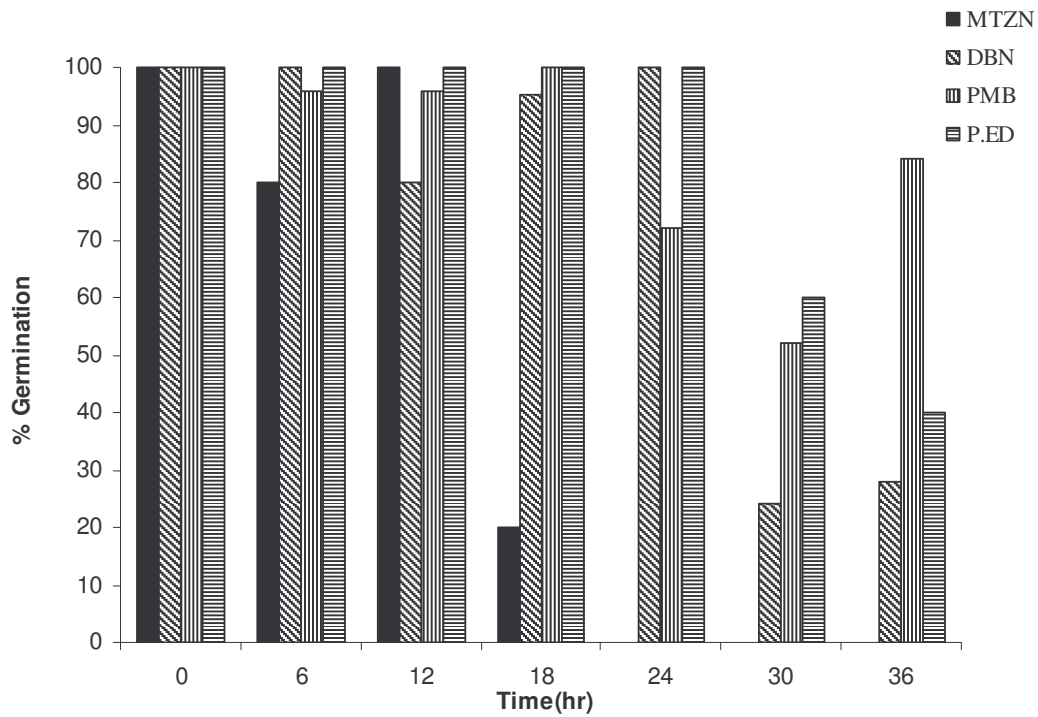
P.ED,  $1.25 \pm 0.14 \text{ g g}^{-1} \text{ DW}$ ; compared to the initial water contents that were  $1.28 \pm 0.17$ ;  $1.51 \pm 0.09$ ;  $1.92 \pm 0.18$  and  $2.35 \pm 0.11 \text{ g g}^{-1} \text{ DW}$  for MTZN, DBN, PMB and P.ED, respectively.

Tukey's post hoc test of Two way ANOVA ( $p \leq 0.05$ ) showed that the initial mean water content (mean for the four provenances combined) was significantly different from all other slow drying times. The mean water contents at 6, 12 and 18 h of slow drying time were not significantly different; so also were the mean water contents at 18, 24 and 36 h times. Another group with non-significant water content differences comprised those that were slow dried for 24, 36 and 30 h. However, each of these groups was significantly different from the others (Figure 3.6). The order of water content reduction is  $0 > 6 > 12 > 18 > 24 > 36 > 30$  h of slow drying.

Mean axes water content (mean for all the drying times combined) of slow dried seeds from DBN was the least, followed by those from MTZ. However, they were not significantly different from one another. The mean axes water content of PMB seeds was the highest, followed by those from P.ED, but these two provenances were not significantly different one from another (Figure 3.6). However, the two groups were significantly different from one another. The descending order of axes water content for the provenances after slow drying the whole seeds was  $\text{PMB} > \text{P.ED} > \text{MTZN} > \text{DBN}$ .



**Figure 3.6:** Water contents of embryonic axes from silica-gel dried whole seeds *T. dregeana* from different provenances.



**Figure 3.7:** Percentage germination of silica-gel dried whole seeds of *T. dregeana* from different provenances.

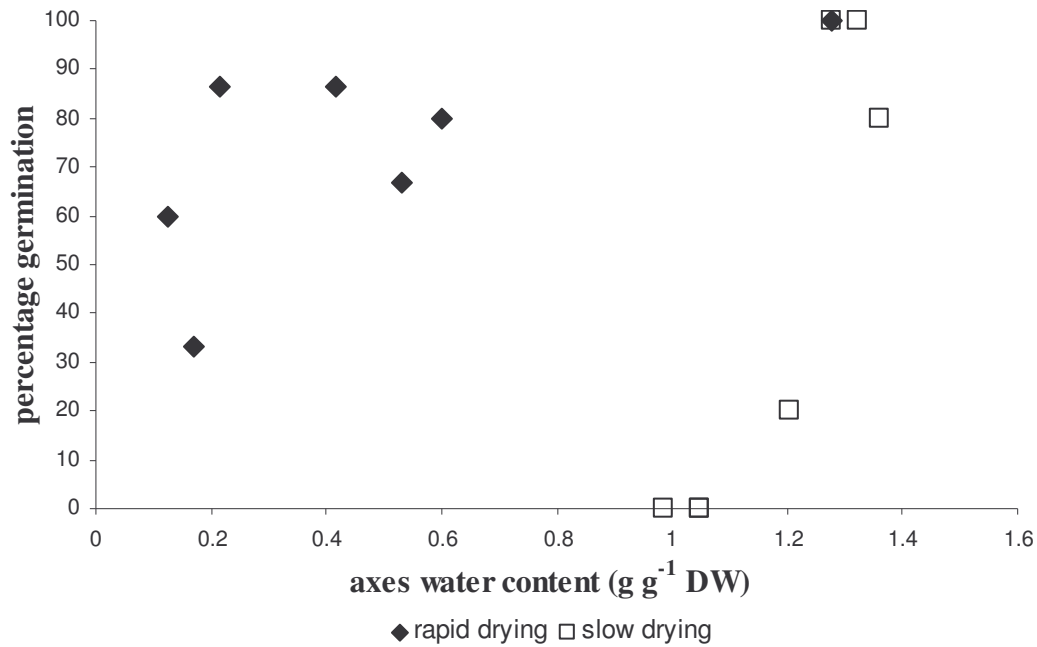
The germination of silica gel dried seeds was assessed initially on daily basis, up to 6 days. It was observed, however, that viability was almost, or totally lost, across the provenances, after the seeds were buried in silica gel for just three days. Seeds from DBN had 4% germination while those from P.ED had 8% germination after this time (result not shown). Hence, the drying time was reduced to a maximum of 36 h.

Figure 3.7 showed the germination of the seeds after they had been slowly dried in silica gel for different times up to 36 h. MTZN seeds had lost more than 50% viability at 18 h slow drying and it was completely lost by 24 h. DBN seeds lost more than 50% viability from 30 h, while seeds from P.ED lost more than 50% only by the last drying time, i.e. 36 h. PMB seeds retained more than 50% viability throughout the drying course. MTZN showed the lowest viability with a mean of 43% germination over all the drying times. DBN seeds had a mean of 75% germination, while PMB and P.ED both had 86% mean germination. The germinations of the seeds from the four provenances were significantly different from each other after slow drying, except for those from PMB and P.ED ( $p \leq 0.05$ ).

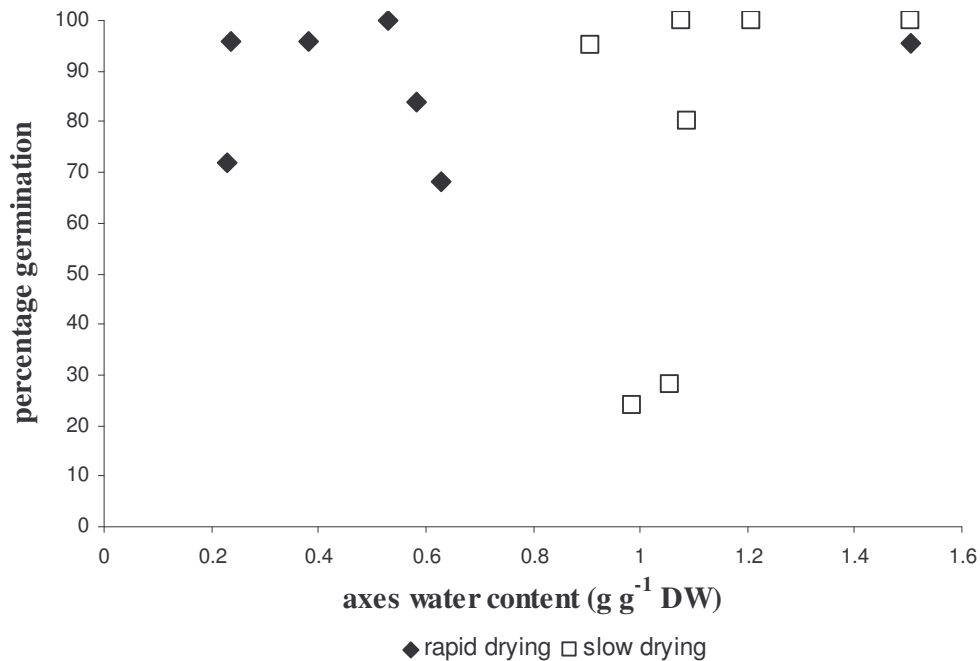
The percentage germination of dried whole seeds (seeds of all four provenances combined) fell into three homogenous groups in terms of drying times. The germination after 0, 6 and 12 h slow drying was one group and differences were statistically non-significant. Another homogenous group comprised those that were dried for 18 and 24 h, while those that were slowly dried for 36 and 30 h made up the last group. However, these three groups were significantly different one from another (Figure 3.7). The percentage germination of the control was the highest (100%) while that of 30 h slow drying was the lowest (35%).

### **3.4.3 Comparison between axes water content and germination of embryonic axes of *T. dregeana* excised and dried rapidly and slowly dried whole seeds.**

After 120 min flash drying, the water content of embryonic axes of seeds harvested from MTZN had been reduced from the initial water content of 1.28 g g<sup>-1</sup> to 0.12 g g<sup>-1</sup> DW,



**Figure 3.8:** The relationship between the water content of excised axes that have been flash dried and axes dried slowly in whole seeds, and germination of axes that have been flash dried and slowly dried whole seeds of *T. dregeana* from MTZN.



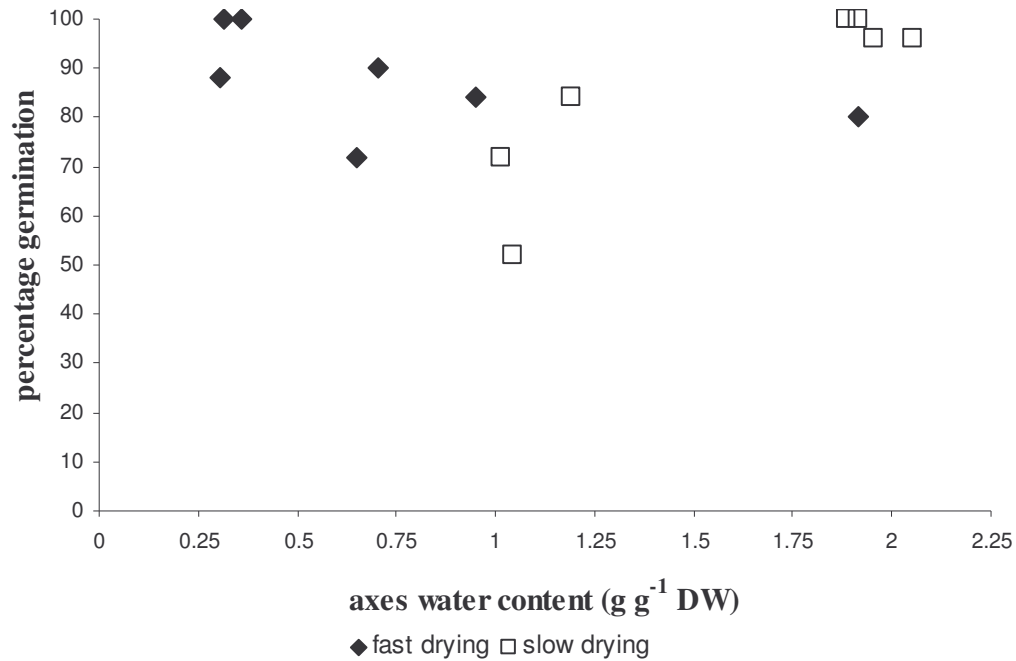
**Figure 3.9:** The relationship between the water content of excised axes that have been flash dried and axes dried slowly in whole seeds, and germination of axes that have been flash dried and slowly dried whole seeds of *T. dregeana* from DBN.

with 60% survival at that water content. In slowly, silica-gel dried seeds, axes water content was barely reduced after being buried in silica gel for 36 h, the water content being  $1.05 \text{ g g}^{-1}$  DW at this last time (36 h). As high as the water content remained at each drying time, viability of whole seeds was still rapidly lost. For instance, from 24 to 36 h in silica gel, the axes water content was still relatively high at  $0.99$  to  $1.05 \text{ g g}^{-1}$  DW, but viability was 0% (Figure 3.8) at each of those six-hourly times.

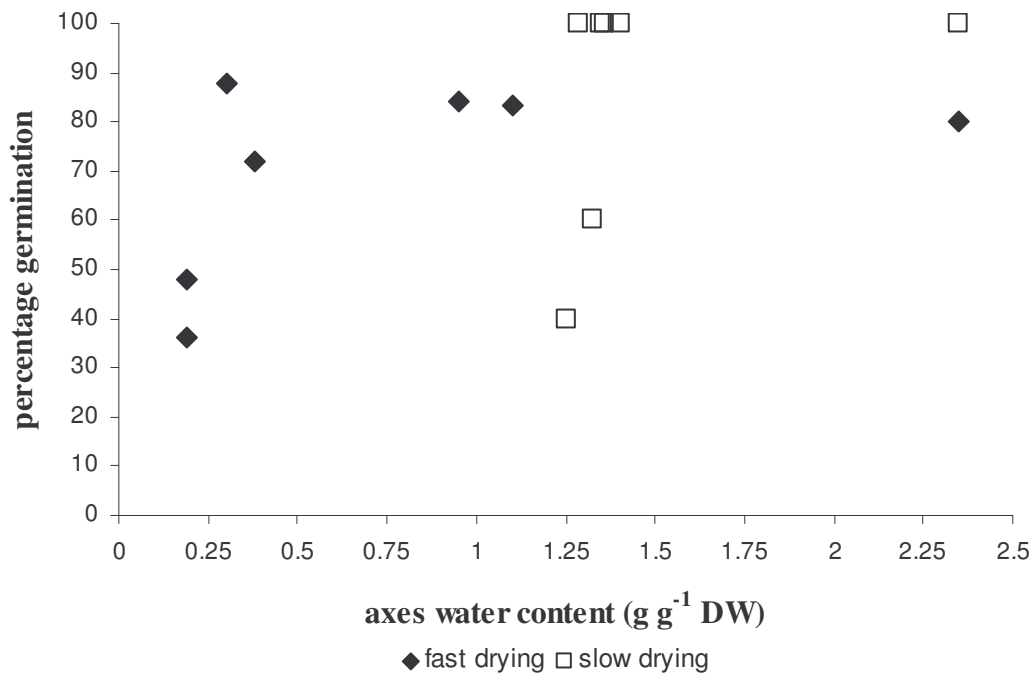
From the initial water content of  $1.51 \text{ g g}^{-1}$  DW, embryonic axes of seeds from DBN had been reduced to  $0.23 \text{ g g}^{-1}$  DW after 150 min flash drying and the survival at this water content was 72%. Survival was not less than 50% at any water content to which the embryonic axes were flash dried to (Figure 3.9).

Silica gel drying did not reduce the axes water content of DBN seeds to any level achieved by flash drying. After 30 and 36 h in silica gel, the axes water contents of DBN seeds were  $0.99$  and  $1.06 \text{ g g}^{-1}$  DW and the survival of whole seeds at these water contents was 24 and 28%, respectively (Figure 3.9).

Figure 3.10 shows the relationship between the water contents of excised axes that have been dried rapidly and axes that were dried slowly in the whole seeds, and germination of axes that have been flash-dried and whole seeds of *T. dregeana* that have been dried slowly in silica-gel, harvested from PMB. In the first 20 min of flash drying, the embryonic axes reduced from an initial water content of  $1.92 \text{ g g}^{-1}$  DW to  $0.95 \text{ g g}^{-1}$  DW, and after 150 min flash drying, the water content was  $0.30 \text{ g g}^{-1}$  DW. There was 72% viability and above, for every water content reached at each flash drying time. After 36 h in silica gel, the axes water content of PMB seeds was  $1.19 \text{ g g}^{-1}$  DW (greater than half the initial water content). However, PMB seeds maintained good viability after slow drying, in having more than 50% viability at each slow drying time (Figure 3.10).



**Figure 3.10:** The relationship between the water content of excised axes that have been flash dried and axes dried slowly in whole seeds, and germination of axes that have been flash dried and slowly dried whole seeds of *T. dregeana* from PMB.



**Figure 3.11:** The relationship between the water content of excised axes that have been flash dried and axes dried slowly in whole seeds, and germination of axes that have been flash dried and slowly dried whole seeds of *T. dregeana* from P.ED.

After flash drying the embryonic axes of *T. dregeana* seeds obtained from P.ED for 120 and 150 min, the water content had been reduced from 2.35 g g<sup>-1</sup> DW to 0.19 g g<sup>-1</sup> DW, but the viability at this water content was 48% and 36% for those flash drying times, respectively. However, at 0.30 g g<sup>-1</sup> DW, viability was as high as 88%. Silica gel drying reduced the initial axis water content to 1.25 g g<sup>-1</sup> DW after 36 h and the viability at this water content was 40%. For higher water contents obtained at other drying times before 36 h, survival was 60% and above. P.ED seeds, like those obtained from PMB, apparently maintained good viability after slow drying (Figure 3.11).

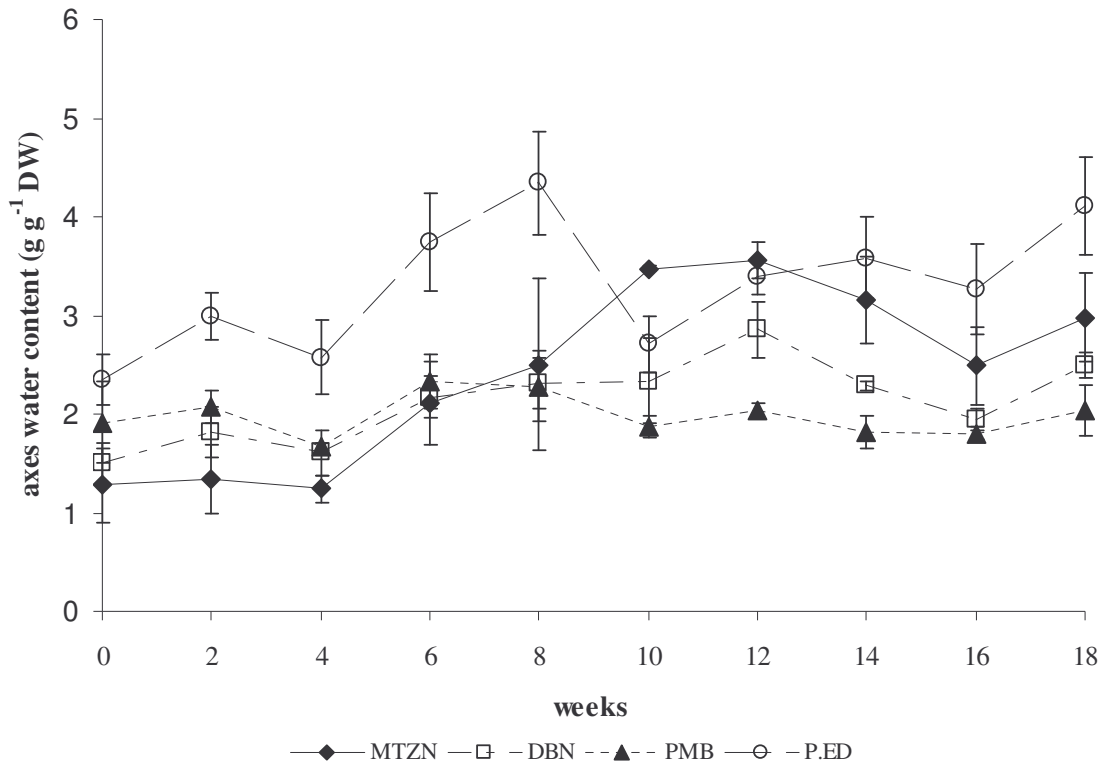
### **3.5 Responses of seeds of *T. dregeana* from the four provenances to storage at sub-ambient temperatures**

Seeds from each provenance were stored at 3°C, 6°C and 16°C for 18 weeks and were assessed fortnightly for water contents and germination.

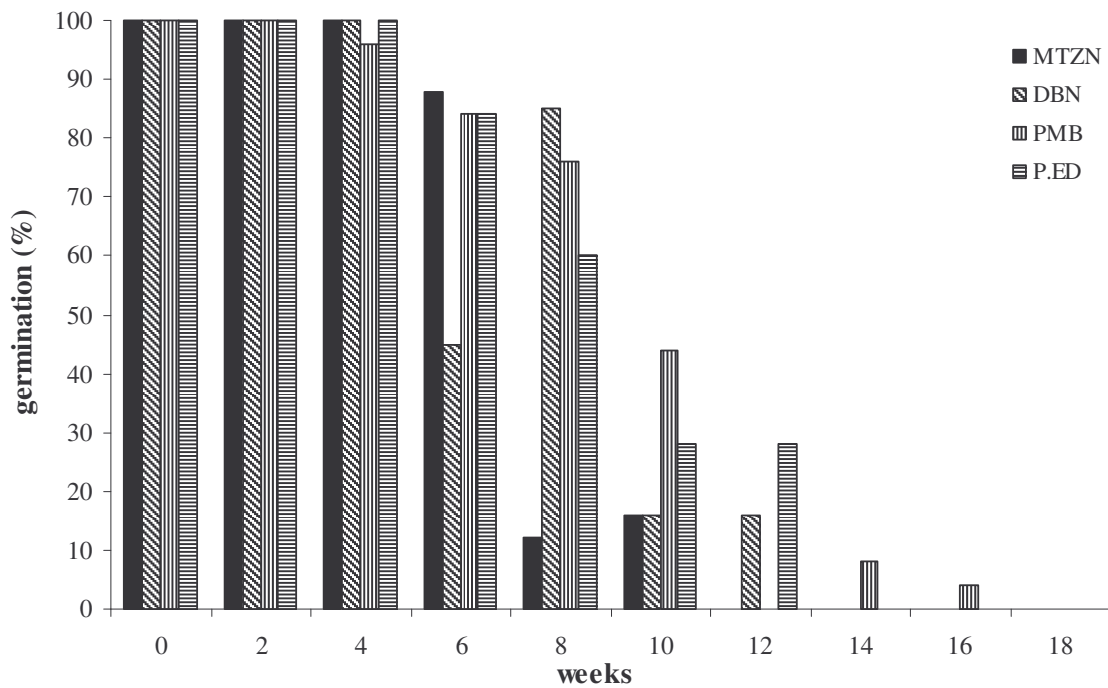
#### **3.5.1 Response to storage at 3°C**

Axes of *T. dregeana* seeds from PMB and P.ED increased in water contents up to the eighth week in storage, while those seeds from MTZN and DBN increased in water content until the 12<sup>th</sup> week in storage (Figure 3.12). The water content of axes from PMB seeds declined slightly from the 10<sup>th</sup> week and remained almost constant from then until the 18<sup>th</sup> week in storage. Axes water content of seeds from MTZN and DBN also declined from the 14<sup>th</sup> week, while those from P.ED increased from the 12<sup>th</sup> week, after a sharp drop in the 10<sup>th</sup> week, up to the 18<sup>th</sup> week in storage. Axes of seeds from P.ED had the highest mean water content throughout the 18 weeks of storage at 3.41 g g<sup>-1</sup> DW, while those from PMB had the lowest at 1.99 g g<sup>-1</sup> DW. Axis water contents of MTZN and DBN seeds were 2.60 and 2.20 g g<sup>-1</sup> DW, respectively. Axis water contents of seeds from DBN and PMB were not significantly different; otherwise, the water contents of the other provenances were significantly different from each other (Two-way ANOVA,  $p \leq 0.05$ ) (Figure 3.12).

After 10 weeks in storage at 3°C, seeds from all the four provenances had lost more than 50% viability (Figure 3.13). Survival was completely lost by the 14<sup>th</sup> week in storage, for seeds from MTZN, DBN and P.ED. MTZN seeds were the most vulnerable to the chilling treatment as their survival was less than 50% from the eighth week, and was lost completely from the 12<sup>th</sup> week. On the other hand, PMB seeds seemed to be the most tolerant of this storage temperature as survival was still maintained (although very little) till the 16<sup>th</sup> week in storage. For each provenance, the percentage germinations for all the samplings times were combined and the mean taken to represent the response of each provenance to storage at 3°C. Mean percentage germination was 42%, 46%, 51% and 50% for MTZN, DBN, PMB and P.ED seeds, respectively. However, the germination percentages were not significantly different (chi square,  $p \leq 0.05$ ) (Figure 3.13).



**Figure 3.12:** Axes water content of *T. dregeana* seeds from the four provenances stored at 3°C (n = 5), bars represented standard deviation.



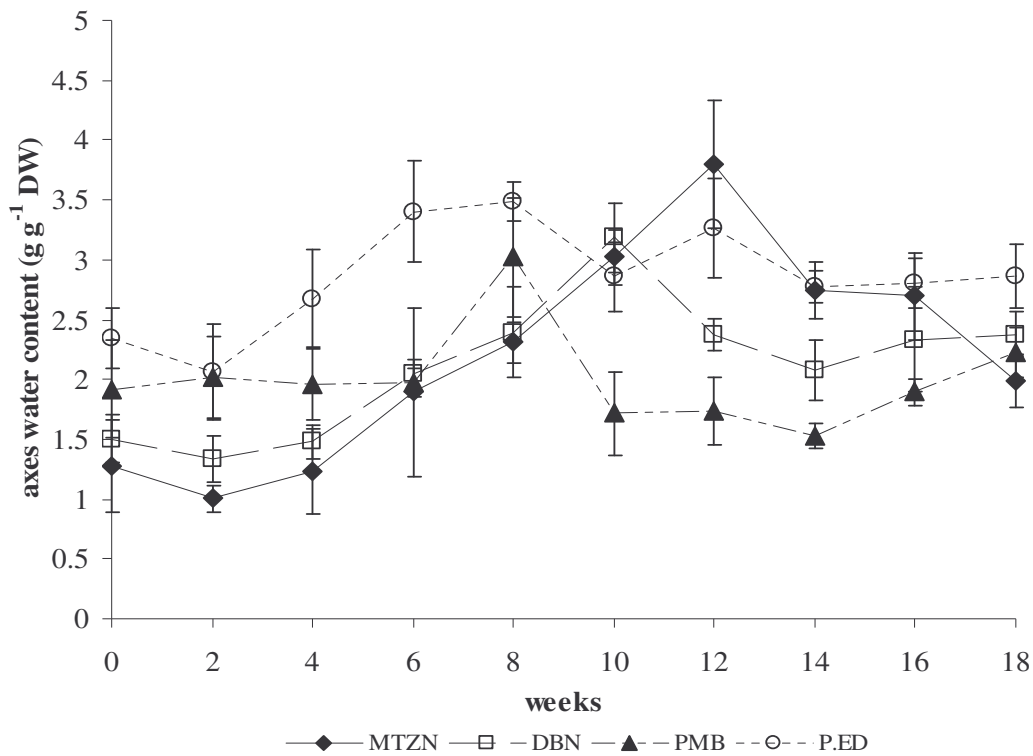
**Figure 3.13:** Percentage germination of *T. dregeana* seeds from the four provenances stored at 3°C (n = 25)

### 3.5.2 Response to storage at 6°C

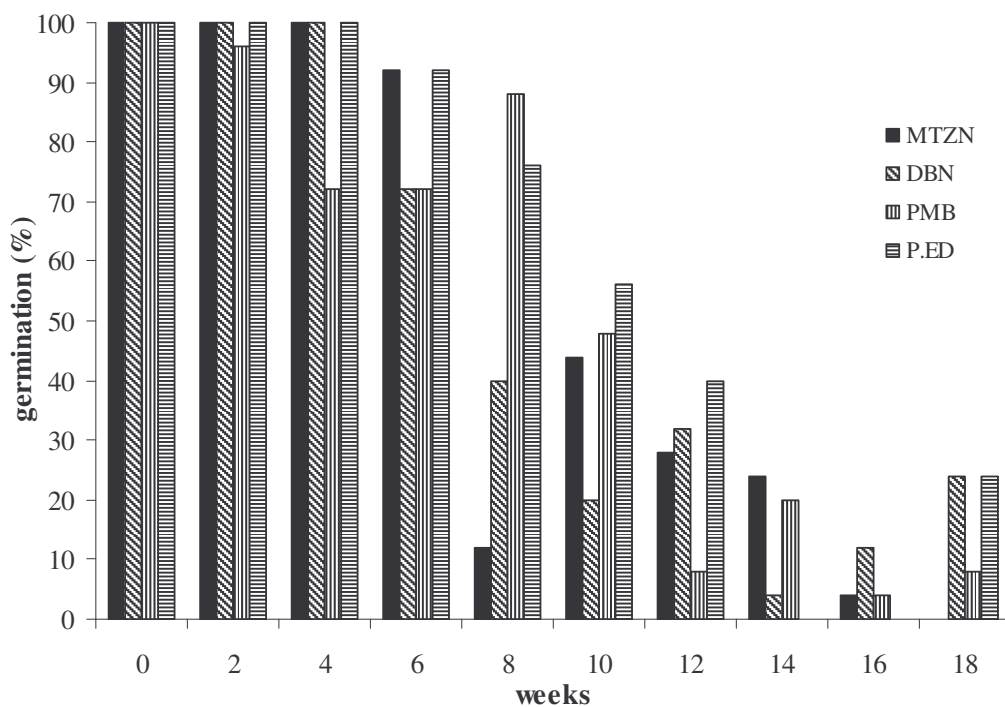
Water content of axes from seeds from P.ED and PMB increased till the eighth week in storage at 6°C. On the other hand, it increased till the 10<sup>th</sup> and 12<sup>th</sup> week for seeds from DBN and MTZN respectively, after which it declined until the end of the storage period. Axes water content of seeds from P.ED declined slightly from the 12<sup>th</sup> week, but was still relatively high at the 18<sup>th</sup> week in storage. The water content of axes of seeds from PMB remained relatively constant, except for a peak at the eighth week and a slight increase from the 14<sup>th</sup> week until the 18<sup>th</sup> week (Figure 3.14). Mean axes water contents, throughout the storage period, were 2.00, 2.18, 2.32 and 2.91 g g<sup>-1</sup> DW for seeds from PMB, DBN, MTZN and P.ED, respectively. These values were significantly different from each other (Two-way ANOVA,  $p \leq 0.05$ ).

Seeds were able to tolerate storage at 6°C a little longer than those stored at 3°C. While MTZN and DBN seeds had less than 50% viability from the eighth week in storage at 6°C, seeds from PMB and P.ED had more than 50% survival till the 8<sup>th</sup> and 10<sup>th</sup> week in

storage respectively (Figure 3.15). Survival was lost completely only in the 18<sup>th</sup> week of storage for MTZN seeds. P.ED seeds also showed no survival in the 14<sup>th</sup> and 16<sup>th</sup> week but had 24% viability in the 18<sup>th</sup> week of storage. Seeds from DBN and PMB maintained some viability throughout the 18 weeks storage period. MTZN, DBN, PMB and P.ED seeds had mean percentage germination (all storage times combined) of 45%, 45%, 46% and 54%, respectively, but these values were not significantly different (chi square,  $p \leq 0.05$ ).



**Figure 3.14:** Axes water content of *T. dregeana* seeds from the four provenances stored at 6°C (n = 5), bars represented standard deviation.



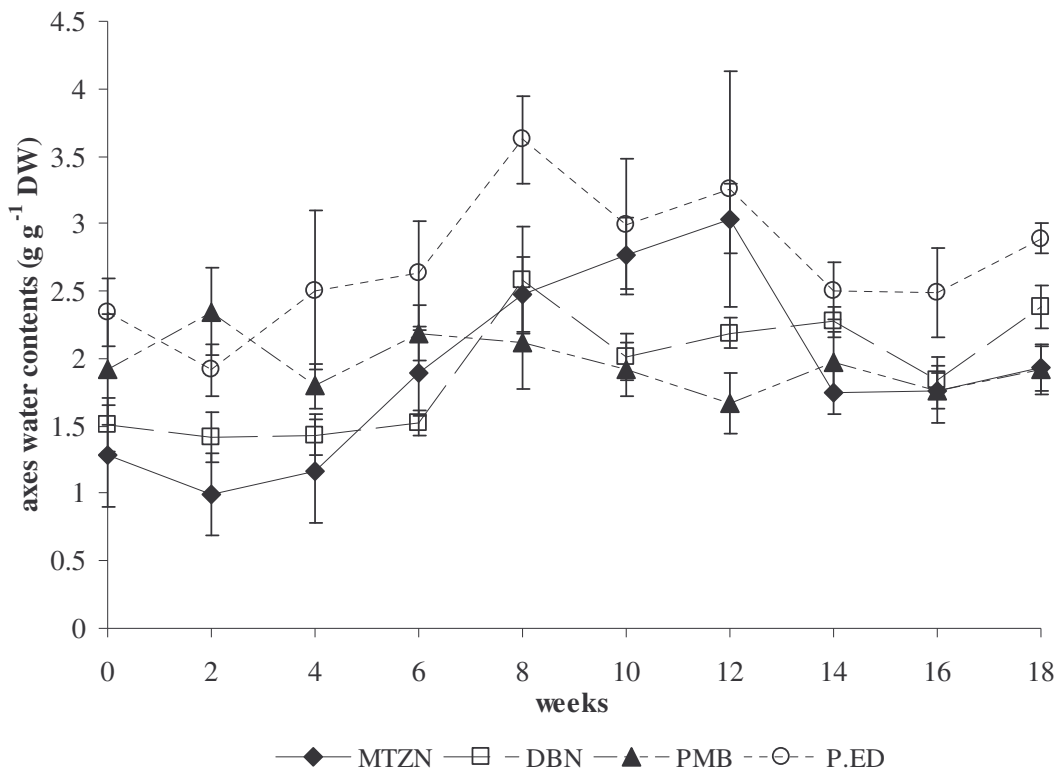
**Figure 3.15:** Percentage germination of *T. dregeana* seeds from the four provenances stored at 6°C, (n = 25).

### 3.5.3 Response to storage at 16°C

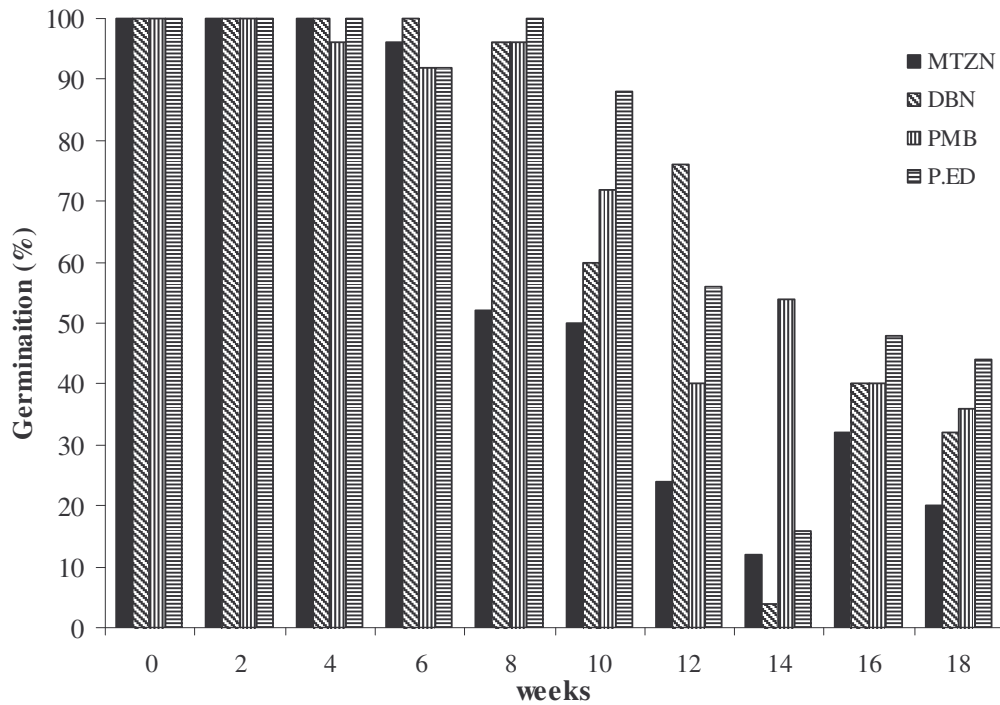
Water content of axes of seeds from DBN and P.ED increased till the eighth week in storage at 16°C, while those from MTZN increased steadily till the 12<sup>th</sup> week in storage. Axes of seeds from PMB were different in that they tended to lose water in storage, especially from the sixth to the 12<sup>th</sup> week; however, the axes water content of PMB seeds did not change much during storage, compared to those from the other three provenances (Figure 3.16). At 16°C, seeds from MTZN had the lowest mean axis water content, the value being 1.72 g g<sup>-1</sup> DW; those from P.ED had the highest value of 2.76 g g<sup>-1</sup> DW. Mean axis water contents of seeds from DBN and PMB were the same, having 1.96 g g<sup>-1</sup> DW. The three values were significantly different from each other (Two way ANOVA,  $p \leq 0.05$ ).

Seeds from all the four provenances, stored at 16°C, maintained some viability throughout the 18 weeks in storage; an indication that 16°C is the most tolerable of all the storage temperature tested. Across all provenances, survival was  $\geq 50\%$  up till the 10<sup>th</sup>

week in storage (Figure 3.17). Seeds from DBN and P.ED still had more than 50% survival in the 12<sup>th</sup> week, while seeds from PMB had 54% viability in the 14<sup>th</sup> week. The lowest survival after 18 weeks in storage was 20%, recorded for seeds from MTZN. Mean percentage germination (for all storage time combined) for each provenance was 54%, 68%, 70% and 72% for MTZN, DBN, PMB and P.ED, respectively, but these values were not statistically significant (chi square,  $\leq 0.05$ )



**Figure 3.16:** Axes water content of *T. dregeana* seeds from the four provenances stored at 16°C, (n = 5), bars represented standard deviation.



**Figure 3.17:** Percentage germination of *T. dregeana* seeds from the four provenances stored at 16°C, (n = 25).

### 3.6 Comparison between *T. dregeana* seeds harvested in 2009 and 2010 from Mtunzini

In the results obtained earlier in this study, it was shown that *T. dregeana* seeds collected from MTZN apparently were different from seeds collected from the other three provenances, in that they were the smallest in size and in most cases, the most sensitive to drying as well as to chilling treatment. It was presumed that late harvest of those seeds, i.e. in late May 2009 (Table 3.2) contributed to their high level of sensitivity. To establish the sensitivity to chilling and dehydration of seeds from MTZN relative to seeds from the other three provenances, seeds were collected from trees in MTZN shortly after their maturity, in April, 2010 (a month earlier than the previous year). The seeds were subjected to drying and chilling treatments as those of the previous year.

### 3.6.1 Flowering to fruit shedding conditions of *T. dregeana* from Mtunzini for 2009 and 2010

For the nine months that covered the flowering to fruit shedding period of *T. dregeana* at MTZN, the accumulated rainfall was higher in 2009 than in 2010. On the other hand, maximum and minimum temperatures were higher in 2010 than in 2009. That means at MTZN, 2010 was hotter with less rain than 2009 (Table 3.8). However, the differences between rainfall and maximum and minimum temperatures, for 2009 and 2010 were not significantly different.

**Table 3.8:** Comparison between 2009 and 2010 conditions during the period from flowering to fruit maturity of *T. dregeana* at MTZN provenance

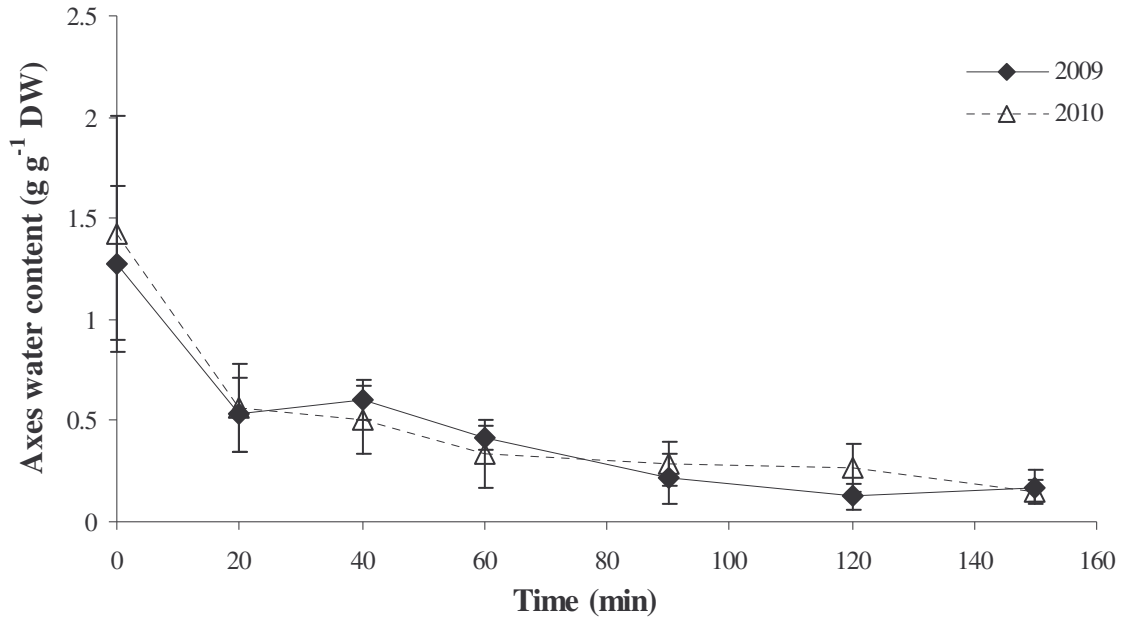
FEATURES	2009	2010
Geographic coordinates	28°57' S, 31°45' E	
Location within KZN province	KZN North coast	
Flowering to fruit maturity period	September (2008) – May (2009)	September (2009) – May (2010)
Accumulated rainfall (mm) from flowering to fruit maturity	756.20	692.00
Monthly average rainfall (mm) from flowering to fruit maturity	84.02	76.89
Daily average max. temperature(°C) from flowering to fruit maturity	27.85	28.19
Daily average min. temperature(°C) from flowering to fruit maturity	16.94	19.00
Harvest of <i>T. dregeana</i> seeds	May (Late)	April (Late)

### 3.6.2 Differences in response to drying of 2009 and 2010 *T. dregeana* seeds from MTZN

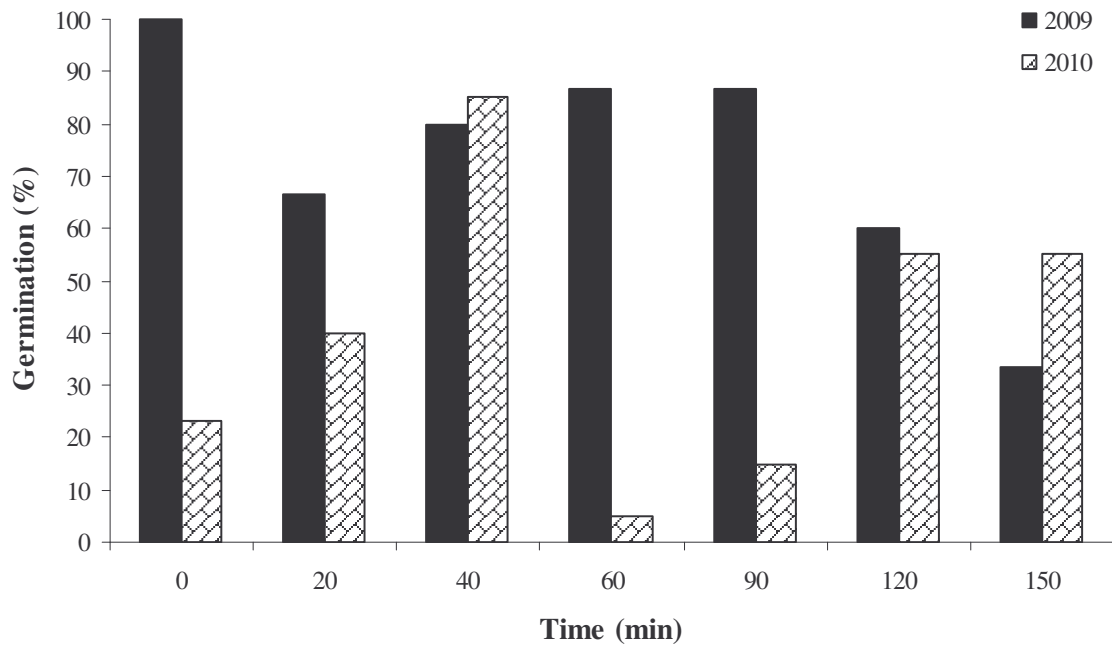
Like those in the previous year, seeds harvested in 2010 were subjected to two types of drying: flash (rapid) drying and silica-gel (slow) drying, for the same lengths of time (see section 2.6).

#### 3.6.2.1 Flash drying of embryonic axes of *T. dregeana* seeds

The initial axes water content was 1.28 and 1.42 g g<sup>-1</sup> DW for 2009 and 2010 seeds, respectively. At each flash drying time, the water contents for the two years were very similar, for example, after flash-drying for 20 min, the water contents of the embryonic axes were 0.53 and 0.57 g g<sup>-1</sup> DW.



**Figure 3.18:** Axes water content of flash dried embryonic axes of 2009 and 2010 *T. dregeana* seeds from MTZN (n = 5), bars represented standard deviation.



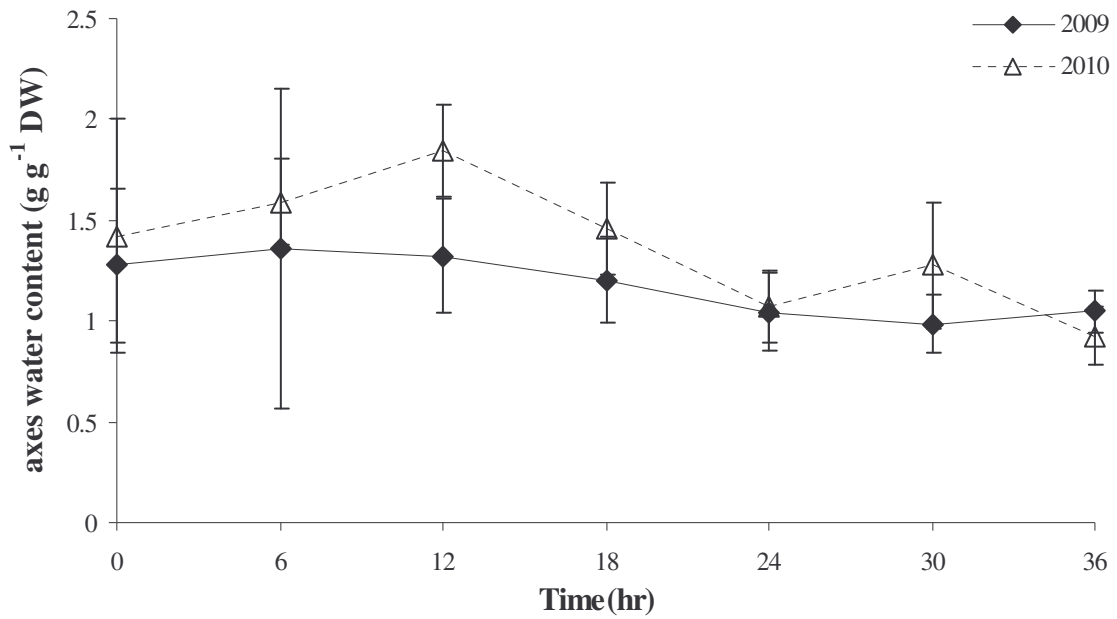
**Figure 3.19:** Percentage germination of flash dried embryonic axes of *T. dregeana* seeds from MTZN in 2009 and 2010 (n = 25).

After 40 min flash-drying, the corresponding values were 0.21 and 0.28 g g<sup>-1</sup> DW, for 2009 and 2010, respectively. At the end of the flash drying time course i.e. 150 min, the water content of embryonic axes of *T. dregeana* seeds, for 2009 and 2010, were 0.17 and 0.15 g g<sup>-1</sup> DW, respectively (Figure 3.18). Despite the similarity in the water content after flash drying times, germination was far from being similar (Figure 3.19). Except at 40 and 150 min drying times where embryonic axes of *T. dregeana* in 2010 had higher percentage germination than those in 2009. Embryonic axes from seeds harvested in 2009 had higher survival, after flash drying, than those harvested in 2010. For instance, in 2009, the control had 100% germination while it was 23% in 2010. Also, after 60 and 90 min flash-drying times, 2009 embryonic axes showed 87% germination for both times; while for those of 2010, the values were 5% and 15%, respectively. Combining the germination of all flash drying times and taking the mean for each year, 2009 seeds had mean germination of 73% while 2010 seeds had 40% and these values were significantly different (chi square,  $p \leq 0.05$ ).

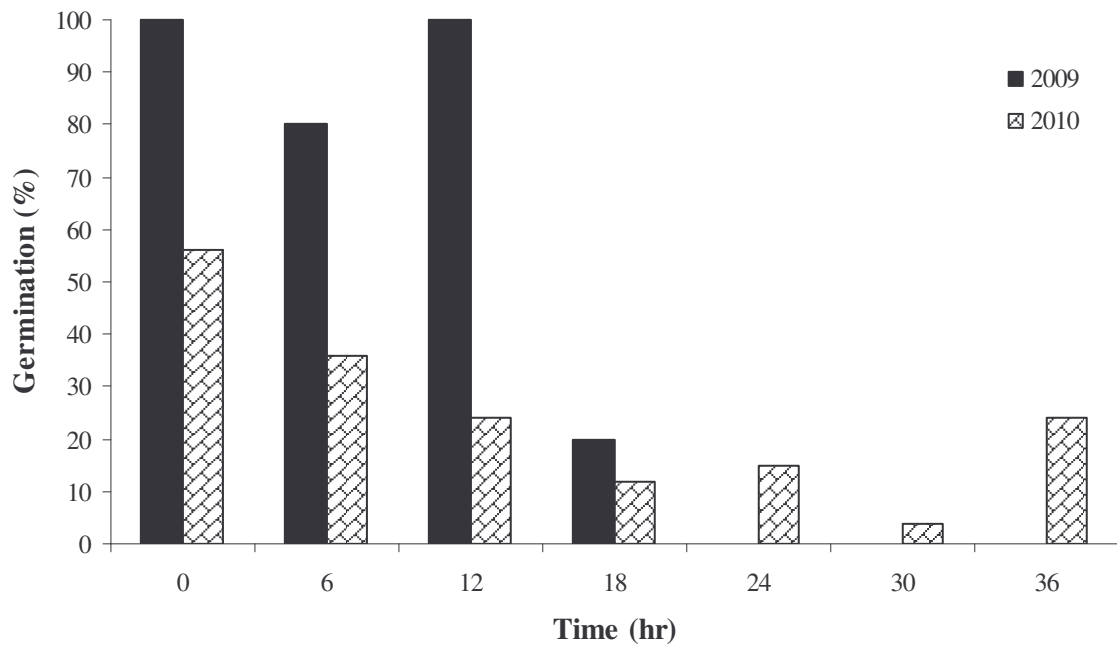
### 3.6.2.2 Silica-gel drying

From the sixth hour to the 36<sup>th</sup> of burying whole seeds in silica gel, the embryonic axes of seeds harvested in 2009 lost water very slowly, with the initial axes water content and after 36 h of drying being 1.28 and 1.05 g g<sup>-1</sup> DW, respectively. For the seeds harvested in 2010, the initial axis water content was 1.42 g g<sup>-1</sup> DW. Reduction in axis water content was only noticeable from the 24<sup>th</sup> to the 36<sup>th</sup> h of drying, and at the end of 36 h the axis water content was 0.92 g g<sup>-1</sup> DW (Figure 3.20).

Figure 3.21 showed the percentage germination of whole seeds that were slow-dried by burying in silica-gel. Seeds harvested in 2009 lost viability completely after 18 h while viability was sustained (although little) at each drying time, throughout the 36 h for those seeds harvested in 2010. However, for those times where viability was sustained in 2009 seeds, their viability was higher than those of 2010 seeds. Mean percentage germination for 2009 seeds was 43% and was significantly different from 2010 seeds, which had a mean percentage germination of 24% (chi square,  $p \leq 0.05$ ).



**Figure 3.20:** Axes water content of silica-gel dried seeds of 2009 and 2010 *T. dregeana* from MTZN (n = 5), bars represented standard deviation.



**Figure 3.21:** Percentage germination of silica-gel dried seeds of 2009 and 2010 *T. dregeana* from MTZN (n = 25).

### 3.6.3 Differences in responses of 2009 and 2010 *T. dregeana* seeds from Mtunzini to storage at different cold temperatures

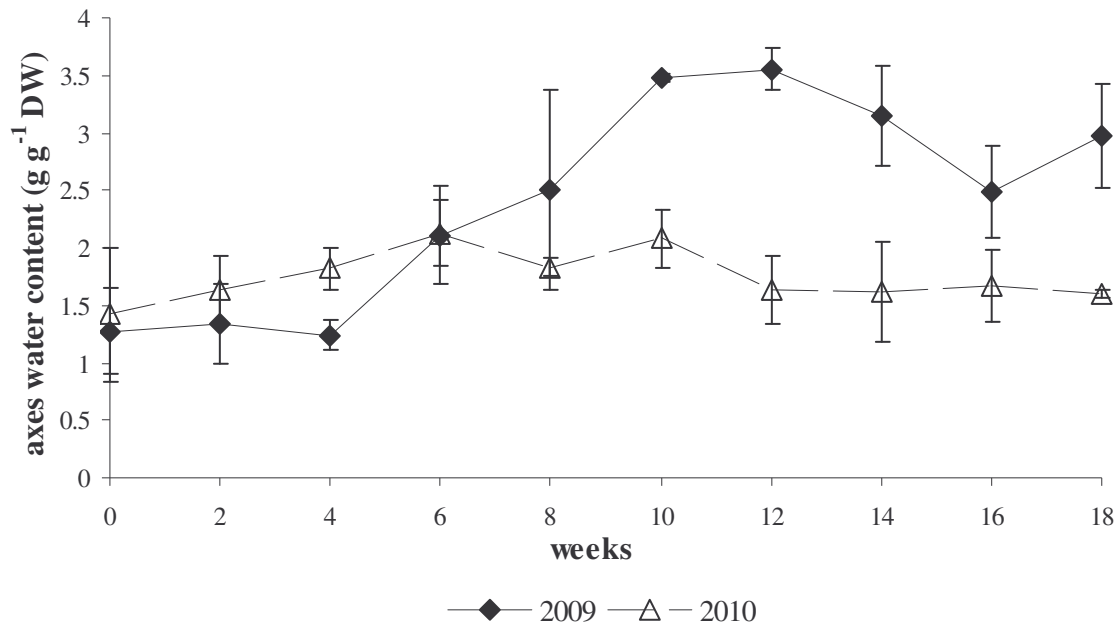
Seeds harvested in 2010 were stored at 3°C, 6°C and 16°C for 18 weeks and were sampled fortnightly for axes water content and germination, as with the seeds that were harvested in 2009.

#### 3.6.3.1 Storage at 3°C

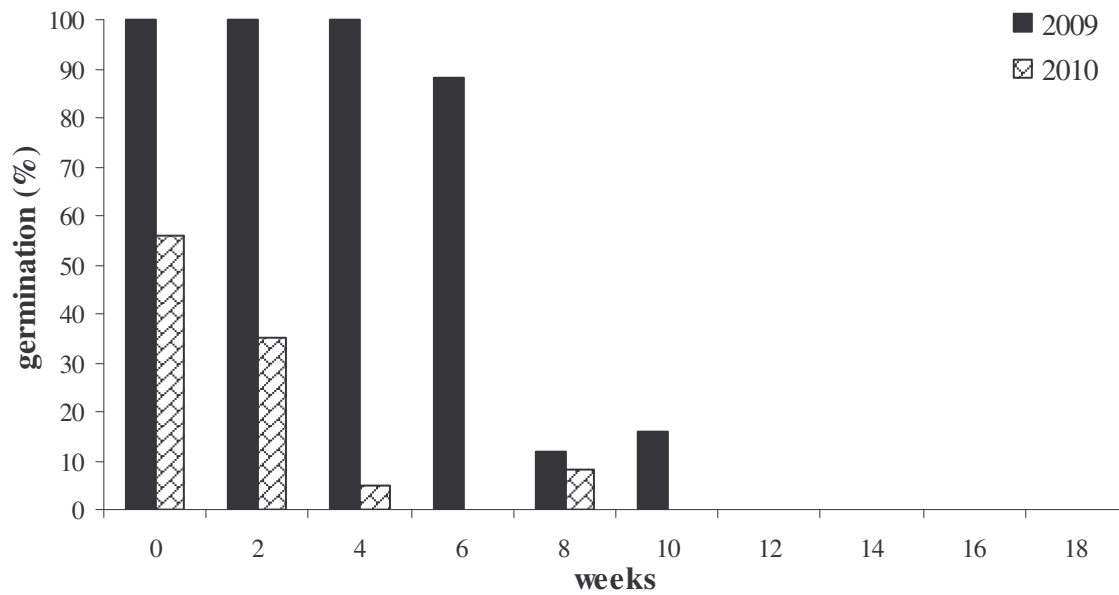
Water content of embryonic axes of seeds harvested in 2009 increased steadily and rapidly from two weeks to 12 weeks in storage. The axes water content at two weeks was 1.34 g g<sup>-1</sup> DW and it was as high as 3.55 g g<sup>-1</sup> DW at 12 weeks in storage. The water content tended to decline from the 14<sup>th</sup> week until the 18<sup>th</sup> weeks, however, the values were still relatively high when compared to the water content at two weeks in storage (Figure 3.22). At two weeks, the water content of seeds harvested in 2010 was 1.64 g g<sup>-1</sup> DW, and this appeared to increase until the 10<sup>th</sup> week in storage. From the 12<sup>th</sup> to the 18<sup>th</sup> week in storage, water content remained constant at around 1.60 to 1.67 g g<sup>-1</sup> DW. The water content was similar for both years at six weeks in storage; and except for the first four weeks where axes of 2010 seeds had higher water content than those of 2009, the axes water content of 2009 seeds was much higher than those of 2010 from the eighth to 18<sup>th</sup> week in storage (Figure 3.22).

Seeds harvested in 2009 retained some survival until the 10<sup>th</sup> week in storage, while survival only lasted until the 8<sup>th</sup> week for 2010 seeds, with no survival at 6 weeks (Figure 3.23). For the three times that 2010 seeds survived, survival was less than 50% at those times, while 2009 seeds showed 100% survival at the first four weeks, 88% at six weeks, and only thereafter was survival less than 50% for 10 and 12 weeks in storage.

However, seeds from both years were very sensitive to 3°C, in that survival was completely lost at 12 and 10 weeks in storage for 2009 and 2010 seeds, respectively. The mean percentage germination for each year was 35% and 5% for 2009 and 2010, respectively, and they were statistically different (chi square,  $p \leq 0.05$ ).



**Figure 3.22:** Axes water content of 2009 and 2010 *T. dregeana* seeds from MTZN, stored at 3°C (n = 5), bars represented standard deviation.

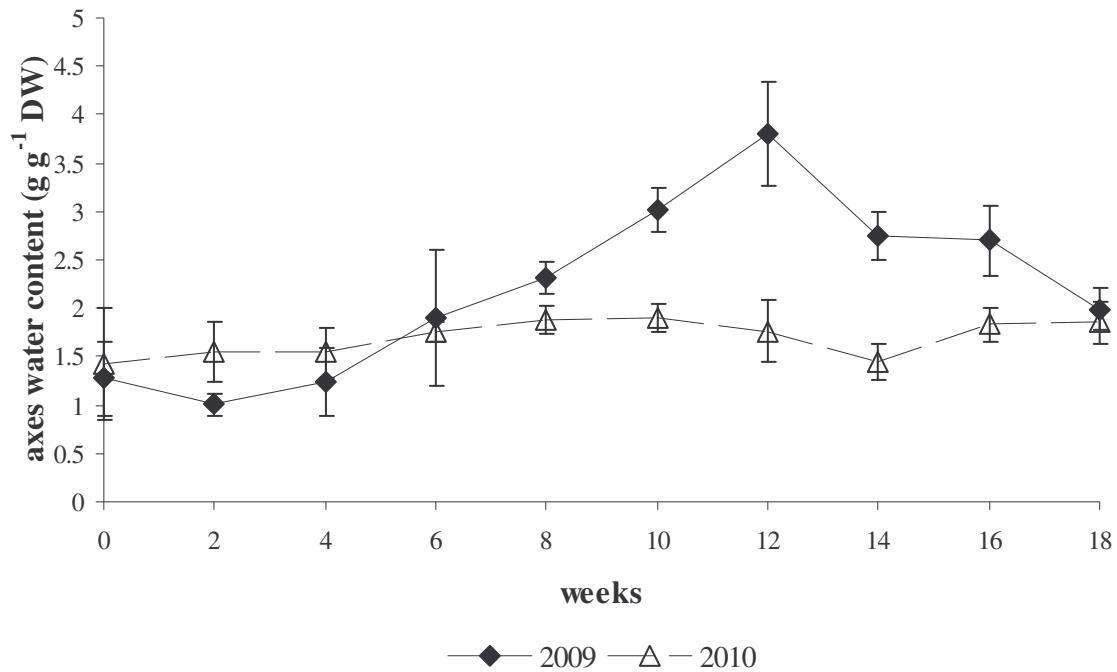


**Figure 3.23:** Percentage germination of 2009 and 2010 *T. dregeana* seeds from MTZN, stored at 3°C (n = 25).

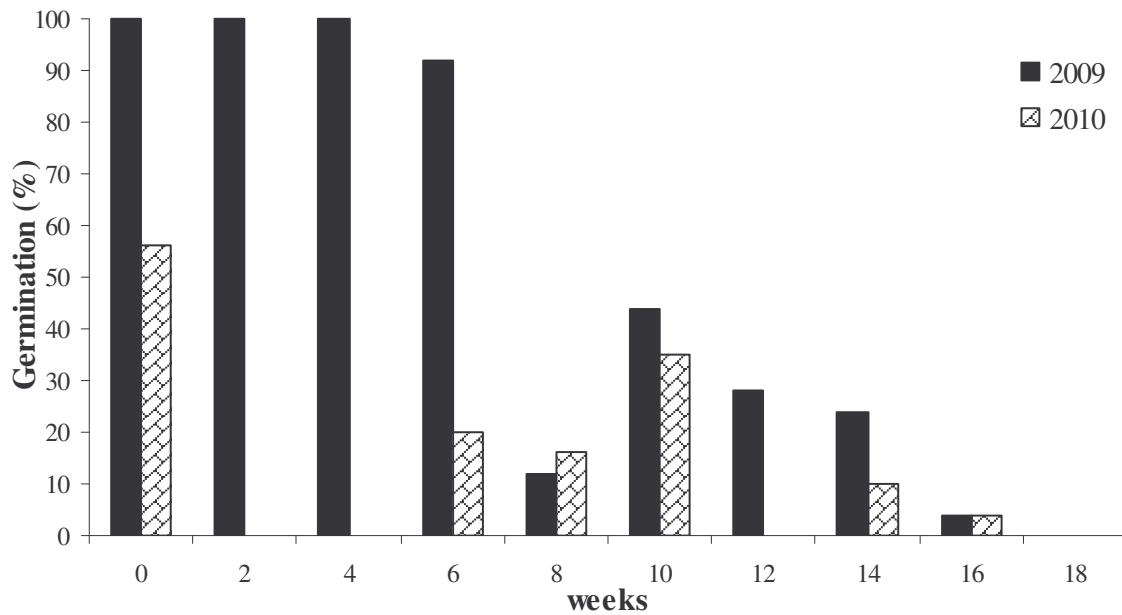
### 3.6.3.2 Storage at 6°C

The axis water contents of the seeds harvested in 2009 and 2010, stored at 6°C are shown in Figure 3.24. Water content of axes of seeds harvested in 2009 increased steadily from 1.00 g g<sup>-1</sup> DW at two weeks to 3.80 g g<sup>-1</sup> DW at 12 weeks in storage, after which the water content declined to 1.99 g g<sup>-1</sup> DW at 18 weeks. For seeds harvested in 2010, their axes water contents were generally lower than those harvested in 2009, except for the first four weeks where 2010 seeds had higher water content than 2009. Unlike 2009 seeds, there was not such a marked increase in axis water content of 2010 seeds in storage. At two weeks, the axes water content of 2010 seeds was 1.55 g g<sup>-1</sup> DW and the highest was 1.90 g g<sup>-1</sup> DW at 10 weeks in storage. At the end of the storage period, the axes water content of 2010 seeds was 1.85 g g<sup>-1</sup> DW.

Seeds harvested in 2009 survived from the second week until the 16<sup>th</sup> week in storage. On the other hand, seed harvested in 2010 survived on five occasions only: 6, 8, 10, 14 and 16 weeks in storage. Seeds harvested in 2009 showed 92% and above survival at the first six weeks in storage, thereafter, survival was less than 50%. Survival was less than 50% for the storage times that 2010 seeds survived (Figure 3.25). Mean percentage germination for seeds collected in 2009 and 2010 was 45% and 9%, respectively, and these values were significantly different (chi square,  $p \leq 0.05$ ).



**Figure 3.24:** Axes water content of 2009 and 2010 *T. dregeana* seeds from MTZN, stored at 6°C (n = 5), bars represented standard deviation.

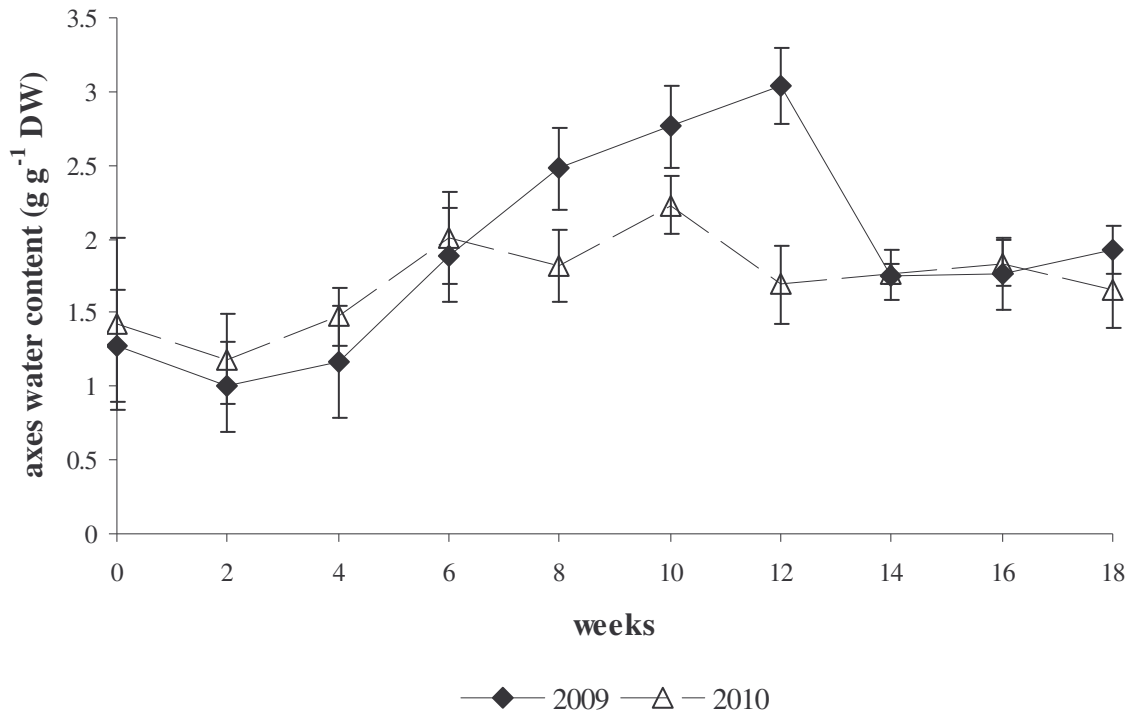


**Figure 3.25:** Percentage germination of 2009 and 2010 *T. dregeana* seeds from MTZN, stored at 6°C (n = 25).

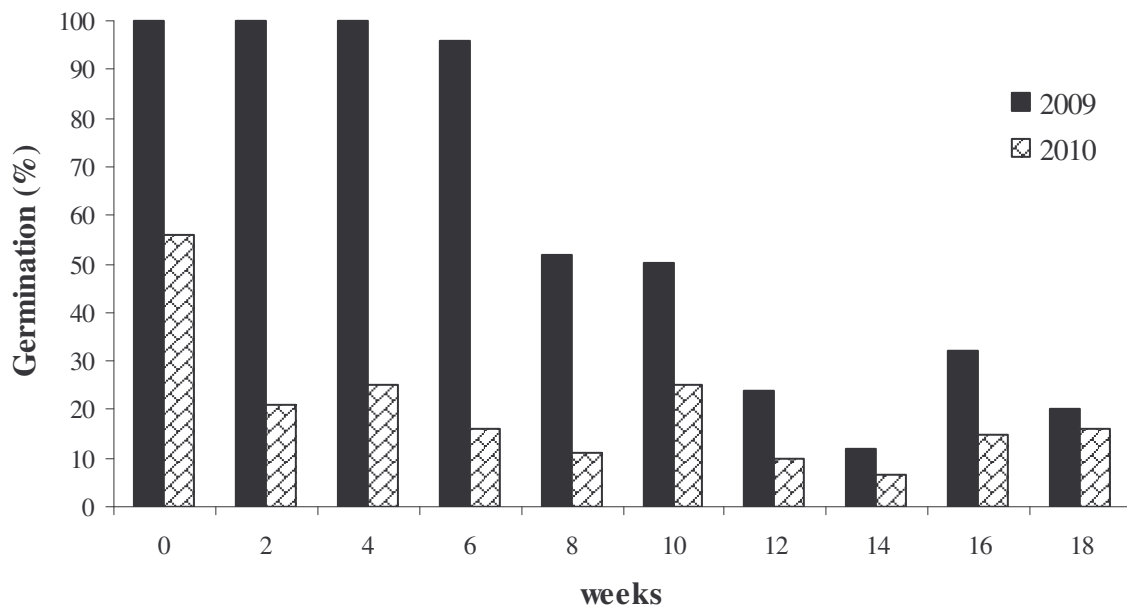
### 3.6.3.3 Storage at 16°C

At two weeks, the water contents of embryonic axes of 2009 seeds, stored at 16°C was 1.00 g g<sup>-1</sup> DW, increased to highest value of 3.04 g g<sup>-1</sup> DW at 12 weeks in storage. After 12 weeks in storage, the water content declined, but was still higher than the water content at two weeks in storage. At 18 weeks in storage, the water content was 1.93 g g<sup>-1</sup> DW. For the first six weeks in storage, 2010 seeds had higher axes water content than 2009 seeds. At two weeks, the axes water content of 2010 seeds was 1.19 g g<sup>-1</sup> DW, the highest value was 2.23 g g<sup>-1</sup> DW at 10 weeks in storage and at 18 weeks, the water content was 1.66 g g<sup>-1</sup> DW. At 14 weeks in storage, the water contents for the two years were similar with 1.75 and 1.76 g g<sup>-1</sup> DW for 2009 and 2010, respectively (Figure 3.26).

Some seeds from both 2009 and 2010 survived throughout the 18 weeks in storage (Figure 3.27). However, seeds harvested in 2009 had higher percentage germination than those of 2010 at every storage time, throughout the 18 weeks in storage. 2009 seeds had 50 – 100% survival from the second to the 10<sup>th</sup> week in storage. Survival was only less than 50% in 2009 seeds from the 12<sup>th</sup> to the 18<sup>th</sup> week in storage. On the other hand, seeds harvested in 2010 had less than 50% survival at every time, throughout the 18 weeks storage period. The highest survival was 25% at 4 weeks in storage. The mean percentage germination for 2009 and 2010 seeds was 54% and 16%, respectively, and these values were statistically different ( $p \leq 0.05$ ).



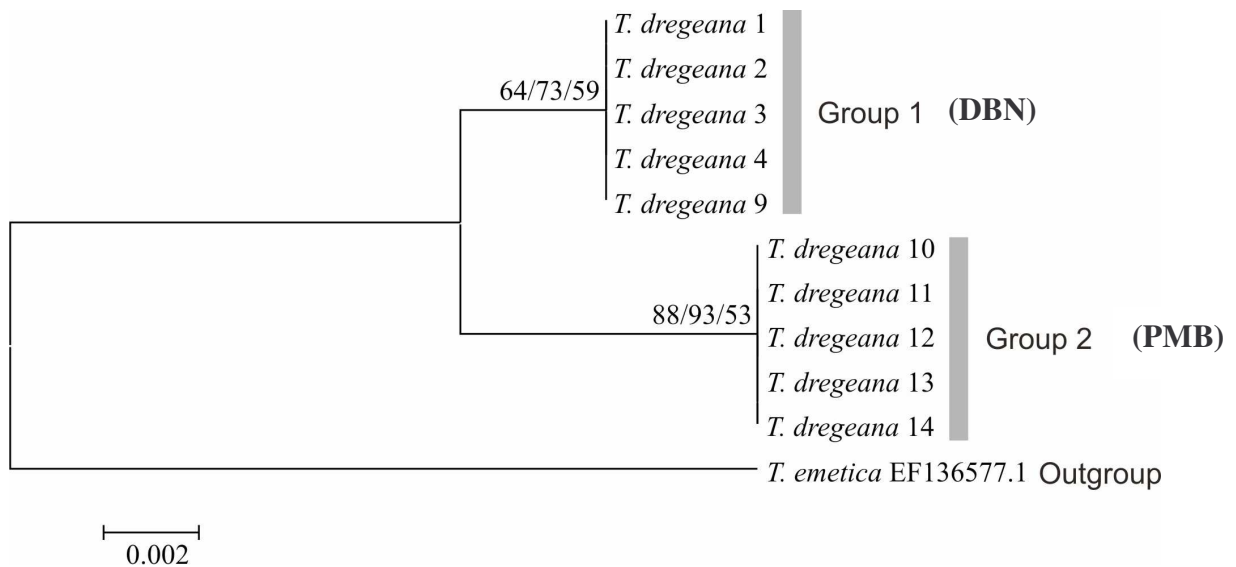
**Figure 3.26:** Axes water content of 2009 and 2010 *T. dregeana* seeds from MTZN, stored at 16°C (n = 5), bars represented standard deviation



**Figure 3.27:** Percentage germination of 2009 and 2010 *T. dregeana* seeds from MTZN, stored at 16°C (n = 25)

### 3.7 Phylogeny

The trimmed ITS1 alignment comprised 337 characters. Neighbour-joining, maximum parsimony and maximum likelihood analyses yielded congruent trees, which are summarized in Figure 3.28. *Trichilia dregeana* samples formed a monophyletic clade with respect to the outgroup, *T. emetica*, from which they were separated by a mean HKY genetic distance of 3.0% (Table 3.9).



**Figure 3. 28:** Phylogenetic tree based on analysis of 337 nucleotides of the nuclear ribosomal ITS1 region of *T. dregeana*, with *T. emetica* as outgroup. Nodal support values are derived from neighbour joining (nj), maximum parsimony (mp) and maximum likelihood (ml) values (nj/mp/ml).

**Table 3. 9:** HKY genetic distances (%) between *T. dregeana* and *T. emetica* samples

	1	2	3	4	9	10	11	12	13	14	
<i>T. dregeana</i> 1	-										
<i>T. dregeana</i> 2	0.0	-									
<i>T. dregeana</i> 3	0.0	0.0	-								
<i>T. dregeana</i> 4	0.0	0.0	0.0	-							
<i>T. dregeana</i> 9	0.0	0.0	0.0	0.0	-						
<i>T. dregeana</i> 10	0.9	0.9	0.9	0.9	0.9	-					
<i>T. dregeana</i> 11	0.9	0.9	0.9	0.9	0.9	0.0	-				
<i>T. dregeana</i> 12	0.9	0.9	0.9	0.9	0.9	0.0	0.0	-			
<i>T. dregeana</i> 13	0.9	0.9	0.9	0.9	0.9	0.0	0.0	0.0	-		
<i>T. dregeana</i> 14	0.9	0.9	0.9	0.9	0.9	0.0	0.0	0.0	0.0	-	
<i>T. emetica</i>	2.8	2.8	2.8	2.8	2.8	3.1	3.1	3.1	3.1	3.1	-

The ingroup *T. dregeana* samples formed two clades, Group 1 and Group 2, corresponding to DBN and PMB samples, respectively. Group 1 was poorly-supported (bootstrap values 64% (nj), 73% (mp), 59% (ml)), whilst Group 2 was moderately supported (bootstrap values 88% (nj), 93% (mp), 53% (ml)) (Fig. 3.28). Groups 1 and 2 were separated by a genetic distance of 0.9% (Table 3.9).

## CHAPTER FOUR

### DISCUSSION

#### 4.1 Prevailing climatic conditions and morphological differences of seeds harvested from the different provenances

The environment in which seeds and fruits are formed affects the development of such seeds and fruits (Negash, 2003). This is usually evident by variations in seed size and weight. The environmental conditions such as rainfall, temperature light as well as soil fertility and moisture influence the quality of seeds that are produced (Copeland and McDonald, 2001). In the present study, the four locations assessed (Mtunzini, Durban, Pietermaritzburg and Port Edward) were characterised by different climatic conditions viz: rainfall, and minimum and maximum temperatures. *Trichilia dregeana* seeds from these locations varied significantly in length, width and fresh mass. Also, the maturity of the seeds, and consequently, harvesting occurred at different times of the year. In evening primrose crops (*Oenothera spp.*), Fieldsend and Morison (2000) showed that the prevailing climatic conditions during seed growth had strong influence on seed quality and consequently, on the fatty acid and oil content of the species.

In this study, seed size was inversely proportional to accumulated maximum temperature during development. MTZN had the highest accumulated maximum temperature from flowering to fruit shedding, but the seeds from that location were the smallest in size. On the other hand, P.ED had the lowest accumulated maximum temperature from flowering to fruit shedding but the seeds harvested from there were the biggest (Table 3.2, Figure 1.3 and 1.4). This observation was unlike the findings of Daws *et al.* (2004) who showed that *Aesculus hippocastanum* seeds that developed under warmer conditions were significantly larger than those that developed under cooler climatic conditions (although those authors worked across a spatial scale and, consequently a wider range of

temperatures). Also, in the present investigation seed mass increased from North (MTZN) to South (P.ED), in the southern hemisphere away from the equator. This observation is opposite to the findings of Daws *et al.* (2004) who showed that the seed mass of *Aesculus hippocastanum* increased in a North-South gradient, in the Northern hemisphere towards the equator. Temperature may be responsible for different sizes observed in this study. There seems to be an optimum temperature required during flowering or maturation, below or above which, seed size decreases (Abdullah *et al.*, 2001). Hence, the observed variation in seed size may be an indication of how far or close, the prevailing environmental temperatures were, to the optimum temperature, during seed development.

#### **4.2 Initial water contents and germination of *T. dregeana* seeds from the four provenances**

Recalcitrant seeds show a wide variation in their shedding water content (Pammenter and Berjak, 2000c); this is usually high from 0.30 to ~ 4.00 g g<sup>-1</sup> (Berjak and Pammenter, 2003). The initial water contents for *T. dregeana* seeds from the provenances were significantly different from each other, with MTZN, DBN, PMB, and P.ED having 1.28, 1.51, 1.92 and 2.35 g g<sup>-1</sup> DW, respectively. Previous work on *T. dregeana* showed a wide range of initial water contents e.g. c. 1.70 g g<sup>-1</sup> DW (Kioko, 2002), 2.04 g g<sup>-1</sup> (Goveia, 2007) and <1 g g<sup>-1</sup> (Pammenter *et al.*, 2000). Other recalcitrant seeds like *Ekebergia capensis* had initial water content of 1.85 g g<sup>-1</sup> (Pammenter *et al.*, 1998a); it could be as high as those found in the recalcitrant amaryllid species, e.g. *Nerine filamentosa* had 7.08 g g<sup>-1</sup>, *N. angulata* had 4.20 g g<sup>-1</sup>, etc. (see Von Fintel, 2006), or as moderate as 0.68 g g<sup>-1</sup> of *Azadirachta indica* (Berjak *et al.*, 1995). The high shedding water content of *T. dregeana* and other recalcitrant seeds is because, unlike orthodox seeds, there is no, or little, maturation drying phase in their development stages and most importantly, there is no cessation of metabolism in recalcitrant seeds and they are shed wet (Berjak and Pammenter, 2003, 2008). It should be noted, however, that not only recalcitrant seeds, but some orthodox seeds are shed at high water content, relative to other orthodox seeds. For example, the shedding water content of muskmelon is 0.54 – 0.72 g g<sup>-1</sup> DW (Welbaum and Bradford, 1989). Therefore, high shedding water content alone, in itself,

does not suffice to categorise a seed as recalcitrant, it is the ability/inability of the seeds to withstand dehydration without the consequent lethal impact (Berjak, *et al.*, 1995).

A germination test is the most reliable physiological indicator of the seed lot quality of non-orthodox seeds (Pritchard, 1996). Germination of fresh whole seeds from all four provenances and which had had the seed coat and aril removed was 100%. This showed that the seeds from all the four provenances were viable and were physiologically capable of developing into seedlings. The aril and the seed coat of the seeds were removed before germination because previous work on *T. dregeana* by Kioko (2002) showed that the presence of the seed coat alone, or with the aril on the seeds, drastically reduced the germination of the seeds. Goveia (2007) also recorded 100% germination for *Strychnos gerrardii* after the fruit pulp and seed coat were removed.

### **4.3 Response *T. dregeana* seeds from the four provenances to drying**

#### **4.3.1 Flash drying**

Usually, the flash drying of embryonic axes of recalcitrant seeds allows them to reach lower water content, more rapidly than whole seeds, while high viability is still retained. Flash drying had been observed to result in three stages of water loss over a period of drying time. The first few minutes of flash drying usually results in a very rapid rate of water loss from the embryonic axes. Flash drying for further minutes results in a slow rate of water loss. At the last stage of flash drying, water content will be almost constant because the embryonic axes will be losing water very slowly. This trend of water loss has been observed by Pammenter *et al.* (1998a, 1998b), Kioko (2002) and Ajayi *et al.* (2006). For all the four locations assessed in the present study, the first 20 min of flash drying resulted in initial rapid rate of water loss but from 20 to 90 min flash drying, the rate of water loss was slow. Flash drying for 90 to 150 min, resulted in slower rate of water loss, almost becoming constant (Figure 3.3).

Contrary to what was observed by Kioko (2002) who showed that it took about 210 min for the flash-dried embryonic axes of *T. dregeana* to reach constant water content, constant water content was reached, in this study, from 120 min of flash drying. This

observation is similar to that shown by Goveia (2007), where flash dried embryonic axes of *T. dregeana* (with basal or 2 mm segment) lost little or no water, from 120 min flash drying time.

Flash-dried embryonic axes of *T. dregeana* seemed not to dehydrate to the extent to which some other recalcitrant explants do. In this study, the water content reached after 150 min flash drying was 0.17, 0.23, 0.30 and 0.19 g g<sup>-1</sup> DW for MTZN, DBN, PMB and P.ED respectively. Similarly, a water content of 0.30 g g<sup>-1</sup> DW was recorded by Goveia (2007) after the embryonic axes of the same species were dried for 150 min and 0.16 g g<sup>-1</sup> DW by Kioko (2002) after 210 min. On the other hand, *Warburgia salutaris* embryonic axes dehydrated rapidly from around 2.4 g g<sup>-1</sup> to 0.1 g g<sup>-1</sup> in 45 min of flash drying (Kioko, 2002). Flash-dried embryonic axes of *Camellia sinensis* reduced in water content from 3.50 g g<sup>-1</sup> to 0.07 g g<sup>-1</sup> within 120 min (Berjak, *et al.*, 1993) and embryonic axes of *Artocarpus heterophyllus* that were dried rapidly reduced in water content from 3.54 g g<sup>-1</sup> to 0.19 g g<sup>-1</sup> in 90 min (Wesley-Smith *et al.*, 2001).

It is characteristic of cuticle tissues to prevent water loss. Therefore, the thick cuticle of the embryonic axes may prevent the natural loss of water of *T. dregeana* and may be responsible for the seemingly resistance to induced dehydration (Kioko, 2002). That author also suggested the possibility of the role of the amorphous masses in the vacuoles of the cells of *T. dregeana* embryonic axes. The amorphous mass-filled cells (which are absent in *T. emetica*) might have positively influenced the water retention ability of the embryonic axes.

Initial slight dehydration, depending on the species, has been reported to result in an initial stimulation and increase of germination rate. Examples include the first eight days of drying the propagules of *Avicennia marina* (Farrant *et al.*, 1985), *Aesculus hippocastanum* (Tompsett and Pritchard, 1998), *Ekebergia capensis* seeds that had been rapidly dried for 6 h (Pammenter *et al.*, 1998a), *T. dregeana* and *Warburgia salutaris* (Kioko, 2002), and the first 15 – 45 min of flash drying the axes of *Telfairia occidentalis* (Ajayi *et al.*, 2006). Tompsett and Pritchard (1998) suggested that since most recalcitrant

seeds (e.g. *Aesculus hippocastanum*) may not have completed their developmental growth at the time of harvest or seed fall, the initial drying may be an extension of the developmental stage of maturation drying that should have taken place should the seeds still be on their parent plants. However, in the present study, the viability after rapidly drying for 20 min (in which the water contents were reduced to the range of 0.53 to 1.10 g g<sup>-1</sup> across the four provenances) was less than those obtained in the control, in axes of seeds from MTZN and DBN, and slightly higher than the control in PMB and P.ED. There was no increase in germination after flash drying for 40 min as well, except for axes of P.ED seeds. However, viability of 87% and above was recorded for embryonic axes that have been flash-dried for 90 min, across the four locations studied with water content being in the range of 0.21 to 0.38 g g<sup>-1</sup>. Similarly, 100% germination was recorded, at every interval, for jackfruit embryonic axes that have been rapidly dehydrated from initial water content of 3.54 g g<sup>-1</sup> to 0.40 g g<sup>-1</sup> (Wesley-Smith *et al.*, 2001). If the germination obtained after flash drying for 90 min in this study was combined for all the provenances, the total germination was higher than the control germination. Being the only drying time that produced the highest viability, it may be that there is an optimum flash drying time for *T. dregeana* axes that produces optimum viability. At the water content obtained after 150 min flash drying, embryonic axes of *T. dregeana* from DBN and PMB still had 72 and 88% germination, respectively.

Further dehydration of embryonic axes has a damaging effect and usually leads to reduced rate and total germination loss thereof. Kioko (2002) observed that at water content below 0.2 g g<sup>-1</sup>, the viability of flash-dried embryonic axes of *T. dregeana* declined sharply, while Wesley-Smith *et al.* (2001) reported a decrease in viability from 100% at 0.4 g g<sup>-1</sup> to 60% at 0.19 g g<sup>-1</sup>. In the present study, water content of flash-dried embryonic axes of MTZN and P.ED was below 0.2 g g<sup>-1</sup> from 120 min and viability declined drastically to 60% and below from that time. After flash-drying for 4 h to 0.16 g g<sup>-1</sup>, Kioko (2002) observed that the root meristematic cells still maintained general cellular organization, with discernable organelles and normal nuclear morphology, however, dismantling of the cytoskeleton appeared to be taking place. This may explain why there

was no total loss of, but reduced, viability, when embryonic axes of *T. dregeana* were flash dried to water content below 0.2 g g<sup>-1</sup> DW (Kioko, 2002).

Although viability was not totally lost after the flash-drying time course in this study across the four provenances, percentage shooting, root and shoot length and seedling dry mass had the least values after 150 min flash drying (Figure 5, Table 4-6). It may be that the deleterious effect of dehydration was having impact from this point, but flash drying was not carried out beyond 150 min. The sensitivity to desiccation was more visible in the shoot production in this study. In fact embryonic axes from MTZN did not produce any shoots at 150 min. Goveia (2007) also recorded reduced shoot production for *T. dregeana* that have been flash-dried for 150 min. No shoot development was noted in any of the drying treatments used for jackfruit embryonic axes, only the control, un-dried embryonic axes developed shoot (Wesley-Smith *et al.*, 2001). Similarly, Kioko (2002) reported that flash-dried embryonic axes of *T. dregeana* lost the capacity for shoot production. According to that author, the inability for shoot production was because the shoot pole of the embryonic axes dehydrated more rapidly than the root pole, upon flash drying. It was suggested that the rapid dehydration for a prolonged time imposed more severe stress on the shoot meristems than the root. Wesley-Smith *et al.* (2001) suggested this view earlier and in addition, those authors added that increased sensitivity to desiccation, rehydration or combination of all these factors could be responsible for failure to produce shoot, or the delay thereof.

Percentage water loss relative to the initial water content after flash drying showed that axes of P.ED seeds lost water the most after flash during, followed by MTZN, axes of DBN seeds showed the least water loss (Table 3.4). The trend of the water loss, across the four locations, was not consistent with seed size. However, P.ED and MTZN had the highest rainfall in 2008 and 2009 and, consequently, during flowering to fruit shedding period. This may contribute to the trend in water loss, but it can not be proven beyond this.

### 4.3.2 Slow drying

The slower the rate of water loss, the more the damage that accumulates within the cells. Unlike rapid drying, slow drying gives enough time for deleterious aqueous-based damage to occur within the cells (Section 1.6). A number of experiments have shown that materials that are dried slowly do not usually decline to the lower water content achieved by rapidly dried materials (e.g. *Ekebergia capensis*, Pammenter *et al.* 1998a). Worse still, viability of slow-dried materials is usually very low, at relatively high water content. Slow-dried seeds of *T. dregeana* and *T. emetica* lost viability totally at  $0.55 \text{ g g}^{-1}$  and  $0.42 \text{ g g}^{-1}$ , achieved after six and three days, respectively (Kioko, 2002). Ajayi *et al.* (2006) reported that at any water content, rapidly-dried embryonic axes of fluted pumpkin had 25% more viability than slow-dried embryonic axes. The propagules of *Avicennia marina* declined both in rate and total germination after slow-drying for prolonged period, this occurred at a water content, higher than the water content at which rapidly-dried materials retained maximum rate and germination (Farrant *et al.*, 1985). Wesley-Smith *et al.* (2001) reported embryonic axes of jackfruit reached  $0.70 \text{ g g}^{-1}$  after rapid drying for 30 min, while a similar water content of  $0.67 \text{ g g}^{-1}$  was reached after slow drying for three days. Those authors also reported 100% germination at  $0.37 \text{ g g}^{-1}$  after rapid drying while slow drying to the same water content resulted in 0% survival.

Slow drying gives more time for the water content to remain at an intermediate level, between fully hydration and the lower limit of survival. Prolonged period at this intermediate water content gives time for metabolism-induced degradative activities to take place leading to reduced, or loss of, viability (Wesley-Smith *et al.*, 2001). Additionally, since slow drying takes a longer time than rapid drying, it gives more room for germination-associated changes to take place and proceed further within the cell. It is suggested, that as these changes proceed, the tissues becomes increasingly sensitive to desiccation, and therefore, less water loss can be tolerated (Farrant *et al.*, 1985).

In the present study, embryonic axis water content of seeds dried slowly by burying in silica gel for 36 h did not in any way come close to the water content of embryonic axes that were rapidly dried in 20 min. Water content was still greater than  $1.00 \text{ g g}^{-1}$  ( $1.05 -$

1.25 g g<sup>-1</sup>) across the four provenances after 36 h in silica gel. Similarly, it took over 5 d for axes water content of slow-dried seeds of *Ekebergia capensis* to decline to 1.00 g g<sup>-1</sup> (Pammenter *et al.*, 1998a).

In the present study, slow drying was carried out initially on daily basis but after three days, the highest survival recorded was 8% for P.ED seeds while it was completely lost in seeds from MTZN. No survival was recorded beyond three days. Similarly, Kioko (2002) did not record any survival for *T. dregeana* and *T. emetica* seeds that were dried slowly for six and three days, respectively.

Although the water contents reached after 36 h in silica gel were somewhat similar in the seeds obtained from the four locations in this study, the recorded survival was in no way similar. While survival declined drastically in MTZN seeds from 100% at 12 h (1.32 g g<sup>-1</sup>) to 20% at 18 h (1.20 g g<sup>-1</sup>) and totally lost thereafter, DBN seeds maintained 80 – 100% from the start to 24 h (1.21 g g<sup>-1</sup>) before declining sharply to 28% and below afterward. Survival of PMB seeds was 52 – 100% across the drying time. P.ED seeds maintained 100% up to 24 h (1.36 g g<sup>-1</sup>), 60% at 30 h (1.33 g g<sup>-1</sup>), then 40% at 36 h (1.25 g g<sup>-1</sup>). The survival of these seeds after slow-drying showed a positive relation with seed size. Probably, the sizes of the seed affected the drying rate relatively, and the extents to which the embryonic axes were adversely affected within the cotyledons while drying.

Rapid drying does not allow water to be evenly distributed within the axes. During drying, the water at the peripheral cells dries first, with the sensitive interior tissues sufficiently hydrated to retain survival and germination upon rehydration (Pammenter *et al.*, 1998a). Wesley-Smith *et al.* (2001) reported that rate of drying determined the distribution of water among the tissues of embryonic axes. Those authors stated that slow drying allows water to be more evenly distributed throughout the entire embryonic axes, with the implication that germinative cells from the interior suffered greater dehydration. Putting this into perspective, it is suggested, in this study, that the extent of water distribution as a result of dehydration is consequent upon sizes of the embryonic axes and the seeds. For example, MTZN seeds were the smallest of the four provenances and

because of that, the seeds dried faster apparently and remained in that state for longer period than seeds from other provenances. This gives enough time for water and dehydration to be more evenly distributed within the axes with the resultant adverse effect. P.ED seeds were the largest and the big cotyledons may serve as temporary barrier, protecting the embryonic axes. In addition, because the embryonic axes of the P.ED seeds were bigger themselves, dehydration may only result in the removal of water from the outermost tissue layers, with the germinative cells from the core being less affected. It is assumed, therefore, that it is not the water content reached, but the relative time taken for embryonic axes to be evenly dehydrated, within the cotyledons, for a prolonged period, that determined the survival of the seeds that were dried slowly in silica gel, across the four locations.

#### **4.3.3 Comparison between the two drying rates across provenances**

Most researchers who have worked on the effects of the two drying rates unequivocally concluded that rapid drying (usually of embryonic axes) is faster, with less damage, than slow drying. Much lower water content is reached within a very short time (from minutes to few hours) while viability is still retained when rapid drying is employed. On the other hand it will take days for slow-drying of either embryonic axes or whole seeds to reach the same water content and some times, the water content does not reduce to the extent of rapid drying, and viability is usually lost drastically (Farrant, *et al.*, 1985; Pammenter *et al.*, 1991; Pammenter *et al.*, 1998a, 2000; Wesley-Smith *et al.*, 2001).

Similarly, in the present study, the rates of drying have marked difference on the water content reached after each drying method, across the four locations (reported earlier). However, seeds from the four provenances did not respond in the same way to viability loss or retention after slow-drying. Only seeds from MTZN and DBN seemed to follow the trend observed by past researchers. Embryonic axes of seeds from MTZN had overall mean of 73% viability after flash drying while it was drastically reduced to an overall mean of 43% after the seeds were dried slowly. Seeds from DBN responded in the same way but the differences in viability loss/retention were not as distinctive as it were in seeds from MTZN. Flash drying of the embryonic axes yielded overall mean of 87%

viability while it was 75% after seeds were slow dried. In seeds obtained from PMB, there was very little difference between rapid and slow drying, with overall mean of 88% and 86%, respectively. Seeds from P.ED showed complete deviation from expectation, with slow drying yielding higher mean viability of 86% than rapid drying of 70%. The reason for this observation is not known, however, it seems that seed size played some role in the rate at which whole seeds dried slowly and how water was distributed evenly within the cells (discussed above). Also, excision injury to the embryonic axes of seeds from P.ED may account for the reduced viability.

#### **4.4 Responses of *T. dregeana* from each provenance to storage under chilling conditions.**

Recalcitrant seeds are not only desiccation sensitive, some are also sensitive to chilling, especially those from tropical/subtropical origin (Nishida and Murata, 1996). Some recalcitrant species, such as mango, cocoa and Borneo camphor, are sensitive to moderate chilling temperature of 10°C to 15°C (Roberts and King, 1980), while some others, such as fluted pumpkin, will lose viability within two weeks of storage at 6°C (Ajayi *et al.*, 2006). *T. emetica* also lost viability steadily with storage time at 6°C (Kioko *et al.*, 2006). In the present study, *T. dregeana* seeds from the four locations were stored at 3°C, 6°C and 16°C. One consistent observation was that the water content of the seeds' embryonic axes increased with storage time. Additionally, the lower the storage temperature, the higher the increase in water content of the seeds stored at that temperature. One possible cause of this observation may be the storage method. The seeds were packed in brown paper bags and sealed in plastic bags. This storage method did not provide any medium for water that is given off during respiration to be absorbed; possibly, the water was reabsorbed by the seed, leading to an increase in water content. The seeds were also dusted with benomyl, which became moistened (by the water that was given off during respiration) with storage time, and in turn, kept the seeds moistened throughout the storage period. The effect of this may also contribute to the observed increase in water content. Walker *et al.* (1991) also reported a significant increase in water content when 4-weeks old plants of chilling-sensitive cultivated tomato were stored under chilling conditions.

The lower the storage temperature, the higher the sensitivity to that temperature and the least the survival observed in each provenance. Gualanduzzi *et al.* (2009) suggested that, post-harvest storage at different chilling temperatures impose different degree of chilling stress. Pammenter *et al.* (1994) suggested that the accumulation of damage is positively related to the duration of applied stress (in this case, chilling). Also, it was reported by Whitaker (1995) that the effect of chilling can be primary or secondary. The primary injury is a physical injury that occurs instantaneously at short-term exposure to threshold chilling temperature but it is reversible (Engelman, 1991). On the other hand the irreversible, secondary injury is at biochemical/metabolic level, which likely takes place when tissues are stored for longer period under chilling conditions. In this study, seeds stored at 3°C had the lowest viability while those stored at 16°C had the highest viability, across the four provenances. Also, for each of the storage temperatures across the four provenances, it was observed that the longer the storage period, the lower the viability. However, the survival of the seeds from each provenance, at each storage temperature, was different. At 3°C, seeds from MTZN were the most sensitive, losing viability drastically at eight weeks and total loss from the 12<sup>th</sup> week. On the other hand, PMB seeds were the most tolerant, as they were still able to maintain some viability until the 16<sup>th</sup> week. The survival at this temperature was in the order MTZN < DBN < P.ED < PMB. At 6°C, only seeds from MTZN and P.ED totally lost viability at some weeks, i.e. MTZN at 18<sup>th</sup> week and P.ED at 14<sup>th</sup> and 16<sup>th</sup> week. DBN and PMB seeds maintained some viability, howbeit little, throughout the 18 weeks in storage. However, mean viability, at this storage temperature, was in the order MTZN = DBN < PMB < P.ED. Viability was not totally lost for seeds stored at 16°C throughout the 18-week storage treatment, across the four locations. However, seeds from P.ED were the most tolerant while MTZN were the most sensitive to this storage temperature. The order of survival at this temperature was MTZN < DBN < PMB < P.ED.

The underlying causes of chilling sensitivity in recalcitrant seeds are not very clear, neither is this topic as extensively researched as desiccation sensitivity. However, past studies suggest some main effects of chilling. Chilling temperature affects the function of enzymes within the cells. Reduction in temperature below a threshold can cause

enzymatic activities and functions to be disturbed, consequently, agitating cell functions (King and Roberts, 1980a, 1980b). The effect of chilling injury on TCA cycle has been suggested by Berjak *et al.* (1996). Recently, decrease in the synthesis of citrate in the TCA cycle, was observed by Tsuchida *et al.* (2010), when cucumber fruits were stored under chilling conditions.

Adverse effects of chilling have also been reported at the ultrastructural level. Niki *et al.* (1978) reported disruptions in cell organelles like the nuclei, mitochondria, ribosomes and tonoplast. When the callus tissues of *Cornus stolonifera* were kept at 0°C, those authors also observed ultrastructural changes in proplastids and rough endoplasmic reticulum (RER). The RER was reported to release ribosomes/microvesicles and thus were turned to smooth endoplasmic reticulum. The microvesicles in turn, developed into large vesicles and occupied a large portion of the cytoplasm. These changes were believed to be consequences of degradative processes taking place within the cells as a result of cold stress on the callus tissues. Hydrated storage of *Azadirachta indica* seeds under chilling conditions resulted in reduced viability which was accompanied by the degeneration of the organelles such as mitochondria, plastids, vacuoles and the plasmalemma (Berjak *et al.*, 1995). Similarly, organelles within the cells of *T. emetica* seeds were seriously degraded and the plasmalemma had receded from the cell wall after 20 d at 6°C (Kioko *et al.*, 2006).

Another damage associated with chilling sensitivity is membrane phase change (from liquid crystalline to gel state) and phase separation/redistribution of membrane components. Associated with temperature induced phase changes of membrane lipids are also changes in their permeability, fluidity and the viscosity (Yoshida and Niki, 1979; Platt-Aloia and Thomson, 1992; Nishida and Murata, 1996; Berjak *et al.*, 1996; Sacandé *et al.*, 2001; Campos *et al.*, 2003; Gualanduzzi *et al.*, 2009). Production of reactive oxygen species (ROS) which consequently lead to lipid peroxidation and oxidative stress is associated with chilling injury as well, as it was observed in zucchini squash fruits stored under chilling conditions (Gualanduzzi *et al.*, 2009).

Deleterious effects of chilling sensitivity are not marked in seeds only but also in fruits like in zucchini (Gualanduzzi *et al.*, 2009), mango (Dea *et al.*, 2010), cucumber (Tsuchida *et al.*, 2010); photosynthetic activities as in coffee seedlings (Oliveira *et al.*, 2002) and in leaves (Campos *et al.*, 2003). Storage of 4-weeks old plants of *Lycopersicon esculentum* and rooted cutting of *L. hirsutum* at 2°C for 3 d reduced the uptake of photosynthetic CO<sub>2</sub> and stomatal conductance (Walker *et al.*, 1991). All the phenological stages of reproduction was reduced in chickpea, as well as abscission of young buds and flowers and abortion of pods at < 10°C in the field. Pollen viability was also suppressed under this condition. Chilling also caused chlorosis, necrosis and curling of the leaves of chickpea (Kumar *et al.*, 2010)

The effects of chilling highlighted above are impacting cells either independently or collectively; but the ultimate effect is cell death, leading to lack of germination or low seedling vigour. These effects increase with increased storage period and with lower chilling temperatures.

#### **4.5 Comparison between *T. dregeana* seeds harvested from Mtunzini in 2009 and 2010**

The comparison between seeds that were harvested in 2009 and 2010 from MTZN was done because it was assumed that late harvest of MTZN seeds in 2009 probably influenced the lowest tolerance, shown by those seeds, to chilling and drying. Therefore, the seeds were collected a month earlier from that location in 2010.

The accumulated temperature during seed development of *T. dregeana* at MTZN was higher in 2010 than in 2009 and vice versa for rainfall. For all the treatment carried out in this study, (rapid and slow drying, storage at 3°C, 6°C and 16°C), seeds harvested from MTZN in 2010 had significant poorer performance in their responses to all the treatments than those harvested in 2009 from this location. Apparently, irrespective of the time of harvest, seeds from MTZN were the most sensitive of the four provenances sampled in this study.

Variability abounds in characteristics of recalcitrant seeds such as seed size, shedding water content, and response to stresses (Berjak and Pammenter, 1996, 2008). According to those authors, the variations are not inter-specific alone, but these differences occur within the same species intra- or inter-seasonally, within or across provenances and from seed-to-seed within the same harvest. The intra-species variations were discernible in this study, between two seasons of MTZN (2009 and 2010), and across the four provenances.

#### **4.6 Correlation between the provenances studied and their responses to dehydration and chilling stresses**

Temperature and rainfall were the two environmental factors that were considered in this study. Muasya *et al.* (2008) believe that temperature during seed development had a more decisive effect on seed quality than rainfall. Bergin *et al.* (2008) also did not find any correlation between rainfall of the locations studied and the growth of *Podocarpus totara* plants. Reports on the correlation between prevalent temperature during seed development and percentage viability are not consistent. A study on common bean (*Phaseolus vulgaris*) from two locations in Kenya associated higher temperature during seed development with low percentages of seed viability (Muasya *et al.*, 2008). On the other hand, Daws *et al.* (2004) associated high temperature during seed development of *Aesculus hippocastanum* with high viability.

Along the coastal locations (MTZN, DBN and P.ED) in this study, *T. dregeana* seeds from MTZN, being the location with the highest maximum temperature during development, were the smallest in size and the most sensitive to dehydration and chilling stresses. On the other hand P.ED had the lowest maximum temperature during development and the seeds from this location were the biggest and one of the most tolerant to chilling and dehydration. This observation is similar to the observation of Muasya *et al.* (2008) but contrary to that of Daws *et al.* (2004). This implies that the role of temperature during seed development is species- and provenance-dependent. Of the four provenances studied, accumulated rainfall during seed development was highest in P.ED, but lowest in PMB. Yet, PMB seeds and embryonic axes had the highest germination in some of the treatments applied. Irrespective of the wide difference in their

rainfall data, P.ED and PMB seeds were the least sensitive to dehydration and chilling (except for flash drying, where PMB was the least sensitive and P.ED was the most sensitive). This may corroborate Muasya *et al.* (2008) that rainfall does not play as decisive a role as temperature. In a North-South gradient along the coastal region, seeds from the northerly provenance (MTZN) were the most sensitive while those from down south were the least sensitive to chilling and dehydration. Similar observation was made in N-S gradient by Bharuth *et al.* (2008).

Of all the three coastal areas, DBN is the closest to the inland site (PMB). The accumulated maximum temperatures during seed development for these two locations were very similar, with PMB being slightly higher. However, unlike the coastal areas where the location with the lowest accumulated maximum temperature was the least sensitive, PMB, with a similar accumulated temperature, was less sensitive than DBN. This may be because PMB had the lowest rainfall of these two (and of all the four) locations.

Usually large seed size is associated with recalcitrant seed behaviour (King and Roberts, 1980a; Chin, 1996). Probably, large seed size has a role to play on the degree of recalcitrance as it was reported by Daws *et al.* (2005) that larger seed size will reduce rate of drying. It was reported by those authors that seed size has a significant positive relationship with the time of germination. This may be the case when recalcitrant behaviours are compared among different species. However, within the same species, as in this study, it may be said that, large seed size could be an indication of good quality. Seed size in maize was positively correlated with seed vigour and stronger seedling establishment (Enayatgholizadeh *et al.*, 2011). As in this study, the correlation between seed size and recalcitrant behaviour was negative, being within the same species. Viability was positively correlated with seed size after the imposition of chilling and dehydration stresses.

The natural habitat of species producing recalcitrant seeds is related to lifespan in storage of the seeds and their susceptibility to chilling. Generally, species from warmer and

wetter habitat will have shorter lifespan and be sensitive to chilling (Berjak, *et al.*, 1992a; Pammenter *et al.*, 1994). Although all the locations in this study have been categorized to be sub-tropical (Chapter one, section 1.9.1) there may be degrees within this category based on the maximum temperature and rainfall data for these locations, especially during seed development (Chapter 3, Table 3.2). P.ED could be said to be very wet (highest rainfall) but cool (lowest maximum temperature), MTZN was wet but very hot (highest maximum temperature), DBN was moderately wet but hot while PMB was dry (least rainfall) and hot. The ‘degree’ of the sub-tropical nature of these habitats could be suggested to be in the order MTZN > DBN > P.ED > PMB. The different combinations of temperature and rainfall, among other factors, could affect the degree of sensitivity of seeds from each provenance, this causing MTZN seeds to be the most sensitive while seeds from PMB and P.ED were the least sensitive to dehydration and chilling.

#### 4.7 Phylogeny

The ITS regions have been successfully used to study species relatedness or variations within and between species such as in fungi e.g. yeast (Chen *et al.*, 2001), animals e.g. *Drosophila* (Schlötterer *et al.*, 1994) and plants e.g. *Aglaia elaeagnoidea* (Muellner *et al.*, 2009). Phylogenetic study, in addition to morphological and physiological differences helped to identify a new endophyte species of the fungus *Neotyphodium* in the grass *Bromus auleticus* (Iannone *et al.*, 2009).

Phylogenetic analysis of the ITS1 region of nrDNA from *T. dregeana* leaves from coastal (DBN) and inland (PMB) populations within the KZN province showed that the two groups were genetically different, forming poorly- to moderately-supported monophyletic clades. The two populations were separated by genetic distance of 0.9% (Table 3.9); which is consistent with intraspecific level differences when compared with the interspecific genetic distances of 2.8% and 3.1% between these two clades and the outgroup, *T. emetica*. Their growth in different geographical environments characterised by different climatic and other factors (e.g. edaphic) may be responsible for the observed genetic differences between the two populations as the characteristics of the habitats of a

plant species may influence the genetic diversity of the plant species (Odat *et al.*, 2004). *Cassia occidentalis* has considerable morphological variations when collected from different regions in India, due to different climatic, soil and other conditions (Arya *et al.*, 2011). Their study showed that *C. occidentalis* was genetically diversified, which was believed to play direct or indirect role in the phytochemical variations within the species.

The genetic differences between the two populations might contribute to their morphological differences as the seeds from the two populations varied in size, length and breadth (Chapter 3, Figure 3.1 and 3.2). Notably, their responses to laboratory-induced stresses such as desiccation and chilling showed that the embryonic axes and seeds *T. dregeana* from PMB were more tolerant of dry and cold conditions than those obtained from DBN. This observation may be a direct reflection of the prevailing climatic conditions of the two locations as climatic data in Table 3.2 (Chapter 3) suggested that DBN is more humid or more 'sub-tropical' than PMB. The growth and development of the plants under different environmental conditions might have resulted in selection, adaptation and consequently, the different responses of the seeds from different locations to similar stresses. Samples of a species that develop under different environmental conditions are known to sometimes vary morphologically and physiologically (Groot and Boschhuizen, 1970). Those authors showed that *Plantago major* L. plants from different habitats were significantly different in their morphological features. They also concluded that the plants varied genetically. The observed differences between DBN and PMB populations may be as a result of combined effects of genetic and environmental variations.

DBN and PMB are the closest locations, with respect to distance, of the four locations studied (Chapter one, Table 1.1). In addition, the climatic data for these two locations and morphological features of their seeds were not so divergent when put side by side with those from MTZN and P.ED (chapter 3, Table 3.2, Figure 3.1 and 3.2). As in the distance, climatic data and morphological features, the responses of the embryonic axes and seeds from DBN and PMB to chilling and desiccation were not so far apart when compared with the north (MTZN) and south (P.ED) coastal locations. Nonetheless,

despite their closeness, the phylogenetic study still showed some degree of genetic variation (0.9% genetic distance in ITS sequences) between samples from DBN and PMB. The study of *Ammopiptanthus mongolicus* by Ge *et al.*, (2005) showed a positive correlation between geographical distance and genetic distance in *A. mongolicus*, while in *Ranunculus acris* there was no significant correlation between genetic distances and geographical distances (Odat *et al.*, 2004). Thus, there is the probability that higher genetic distance and hence, higher degree of genetic divergence would have been detected if phylogenetic analysis was carried out on samples from locations (i.e. MTZN and P.ED) that showed more distinct variations in geographic distance, climatic factors, morphological features and stress tolerance. Unfortunately, this was not possible due to technical problems.

#### **4.8 Concluding remark**

The present study covered provenances within a narrow range, that is, within one province in South Africa. Hence the differences in response of *T. dregeana* seeds from the four provenances in this study may not be distinctive as those of Daws *et al.* (2004) who studied the effect of provenances on *Aesculus hippocastanum* across the continent of Europe or Dussert *et al.* (2000) who studied *Coffea* seeds in six countries within Africa. However, some conclusion can be drawn from this study.

High temperature during seed development seemed to have negative relationship with seed size of *T. dregeana*. Consequently, seed size also had negative correlation with dehydration and chilling sensitivity. Seeds from PMB were the most tolerant of flash drying and to storage at 3°C while seeds from P.ED were the most tolerant to storage at 6°C and 16°C. Seeds from these two locations responded in the same way to slow drying. Also, seeds from these two locations were larger. Therefore, when selection of seeds have to be made, for applications such as cryopreservation or for ecological restoration, the environmental conditions, especially in terms of temperature, during seed development of *T. dregeana*, and seed size should be considered. Seeds from MTZN showed the most recalcitrant behaviour while those from PMB and P.ED showed the least.

This study further confirmed that *T. dregeana* is desiccation sensitive and, in particular, sensitive to chilling, as the seeds could not be stored at 3°C and 6°C without significant loss of viability. However, the degree of sensitivity is influenced by, among other things, the prevailing environmental conditions under which the seeds develop as it was shown in this study. The seeds can still be stored at 16°C for some months before viability is significantly lost.

The phylogenetic analysis showed that *T. dregeana* from PMB and DBN populations are genetically different; however, the study was preliminary. Before concrete conclusions can be made on the genetic variations in *T. dregeana*, further research has to be done with samples from more populations.

The biochemical aspect of the effects of provenance needs to be extensively researched. Since this study is a primary study towards the long-term conservation of the species, it is suggested that cryopreservation trials should be attempted on the seeds from the locations studied.

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