Investigating pollination success between *Pinus radiata* and selected pine species

by

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Dedication

Ouma Hanna, Cori, Mia, Philip, Cara, Heidi and Vicky

One for all and all for one!

Declaration of originality

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation includes two papers published in two different peer-reviewed journals, one paper approved for publication with final correction in a third journal, and one paper accepted for publication in a fourth journal. I was principally responsible for the development and writing of these papers (published and unpublished). Declarations are included at the back of the dissertation indicating the nature and extent of the contributions of co-authors.

Date: March 2018

Abstract

Pinus radiata is not only an important commercial plantation species in the Southern Hemisphere, but it is highly susceptible to *Fusarium circinatum*. Interspecific hybridisation attempts between *P. radiata* and some pine species is an ongoing process to improve *F. circinatum* tolerance. To date, limited pollination success between *P. radiata* and some pine species (*P. tecunumanii, P. oocarpa, P. pringlei* and *P. patula*) might be a result of biological or reproductive barriers that limit gene flow between species. Seven *Pinus* species (*P. tecunumanii, P. oocarpa, P. pringlei, P. maximinoi, P. elliottii, P. taeda* and *P. patula*) and one interspecific hybrid (*P. patula*×*tecunumanii*) with *F. circinatum* tolerance were selected as possible hybridisation partners for *P. radiata*.

Previous studies indicated that the eight pure pine species are grouped in three distinctly different subsections with no difference in the karyotype. A generic *Pinus* cross-pollination protocol was developed and tested. Pollination success was evaluated with pathology diagnostic tests and DNA fingerprinting. Not only did the pathology tests indicate that all the screened seedlings were indeed infected and susceptible to *F. circinatum* (as would be expected from *P. radiata* pure species seedlings), but DNA fingerprinting assigned seedlings to the *P. radiata* population. Low pollen germination and pollen contamination from external sources might have contributed to the limited pollination success. However, Computed Tomography (CT) scans were successfully conducted to determine seed set of intact cones at 104 weeks after pollination. The generic protocol was adapted (green woven cloth bags to be replaced regularly, seal openings created by bulb applicators daily, sterilise breeding equipment, needles and female strobili before bagging and pollination events) and tested to determine whether pollination success could be improved.

Studies on the climatic conditions inside (micro) and outside (macro) the pollination bags indicated that placement of the bags in the top northern side of *P. radiata* trees at the Karatara seed orchard, might increase temperatures closer to Sabie temperatures where the eight pine taxa (eight pure species and one hybrid) yield viable seed. Temperature fluctuations between day and night time are more severe at Karatara than Sabie and might be a constraint to pollination success. Three circadian models were developed to simulate climatic conditions at Karatara (micro and macro) and Sabie (average between Tweefontein, Witklip and Spitskop seed orchards). Biplots (discriminant analysis and principal component analysis), resembling the natural provenance climate data, grouped *P. elliottii, P. taeda* and *P. radiata* with Karatara, indicating good site species matching. When altitude was ignored as a vector, *P. patula* was grouped with the trio as well.

The limited success of previous interspecific hybrid attempts with *P. radiata* warranted detailed studies of pollen germination and growth. Climatic regimes approximating the above mentioned micro- and macro-climates affected pollen grain size (re-hydration), pollen tube size and pollen tube growth rates (PTGR). Furthermore, to simulate the re-hydration effect of the pollen droplet on pollen grains, de-hydrated and re-hydrated pollen grain size (length and width) were compared between the between the nine pine taxa (eight species and one hybrid). Although there were significant differences in PTGR, pollen grain size and pollen tube size among species, none of the selected pine species had a size or PTGR comparable to *P. radiata*. It was concluded that the *in vitro* experiments, despite the significant differences observed, did not provide comprehensive answers to the limited pollination success.

In vivo pollination success between *P. radiata, P. tecunumanii, P. oocarpa* and *P. maximinoi* were determined by counting the number of visible ovules, pollen grains inside the ovules, pollen grains outside the ovules and pollen tubes. Data was collected with a standard fixation-dehydration-embedding histology sequence over the seven-week period, indicating that *P. radiata×oocarpa, P. radiata×tecunumanii* and *P. radiata×maximinoi,* differed significantly from *P. radiata×radiata* for all four factors investigated. The paraffin wax method was not suitable to study conelets older than eight weeks. MicroCT scans were used to confirm if fertilisation occurred between 15 to 16 months after pollination. Seed were visible from week 68 after pollination and viability of seed could be determined with quantitative porosity and defect analyses.

Pearson correlation with biplots between natural provenance data (altitude, precipitation and temperature), PTGR (length and width) and hydrated pollen grain size (length and width) at micro- and macro-temperature regimes for the eight pure species, identified three distinct climatic response groups. These are (1) *P. oocarpa* and *P. tecunumanii*; (2) *P. maximinoi* and *P. patula*; and (3) *P. elliottii* and *P. taeda*. Although *P. pringlei* and *P. radiata* were not included in the three distinct climatic response groups, *P. pringlei* grouped with *P. patula* because of altitude, while *P. radiata* grouped with *P. elliottii* and *P. taeda* due to precipitation and minimum temperature.

Data collected at Karatara during this study, correlated with previous studies indicating that *P. radiata* is better adapted to drier summers and wetter winters than the more tropical species. Therefore, pollination success at Karatara is not likely to be constrained by site factors, but if the pollen partner is not comparable with the temperature regimes at Karatara, it might result in limited pollination success. Therefore, data collected indicated that *P. elliottii* and *P. taeda* might be potential hybridisation partners for *P. radiata*. During this study valuable information was collected in terms of pollen grain size, PTGR, climatic data, three circadian models, *in vivo* pollination success and microCT scans. As this study focused on climate and pollen aspects, future studies need to concentrate on the female strobili, such as the hormonal and/or chemical interaction and whether ovule abortions are affecting pollination success.

Opsomming

Pinus radiata is nie net ‘n belangrike kommersiële plantasie spesie in die Suidelike Halfrond nie, maar ook hoogs vatbaar vir *Fusarium circinatum*. Interspesifieke hibridisasie pogings tussen *P. radiata* en party dennespesies word voortdurend gedoen om weerstand teen *F. circinatum* te verhoog. Beperkte bestuiwing sukses tussen *P. radiata* en sommige dennespesies (*P. tecunumanii, P. oocarpa, P. pringlei* en *P. patula*) tot op hede mag die gevolg van biologiese of reproduktiewe versperrings wees wat gene vloei tussen spesies beperk. Sewe *Pinus* spesies (*P. tecunumanii, P. oocarpa, P. pringlei, P. maximinoi, P. elliottii, P. taeda* en *P. patula*) en een interspesifieke hibried (*P. patula*×*tecunumanii*) met *F. circinatum* weerstand is gekies as moontlike hibridisasie pasmaats vir *P. radiata*.

Vorige studies het getoon dat die agt suiwer dennespesies gegroepeer is in drie duidelik verskillende subseksies met geen verskil in kariotipe nie. ’n Generiese *Pinus* kruisbestuiwingsprotokol is ontwikkel en getoets. Bestuiwing sukses is geëvalueer met patologie ontledingstoetse en DNS vingerafdrukke. Die patologie toetse het aangedui dat al die getoetste saailinge tog met *F. circinatum* geïnfekteer is en vatbaar is daarvoor, soos verwag sou word van suiwer *P. radiata* saailinge. DNS vingerafdrukke het saailinge toegeken aan die *P. radiata* populasie. Lae stuifmeel ontkieming en stuifmeel kontaminasie van eksterne bronne mag bygedra het tot die beperkte bestuiwing sukses. Rekenaar tomografie (RT) skanderings is suksesvol uitgevoer om saad vorming in geslote keëls op 104 weke na bestuiwing te bepaal. Die generiese protokol is aangepas (groen geweefde lapsakkies wat gereeld vervang word, seël openinge daagliks wat veroorsaak is deur bolglasstafies, steriliseer teelgereedskap, naalde en vroulike strobili voor sakplasing en bestuiwings geleenthede) en getoets om te bepaal of hierdie aanpassings wel kanse op bestuiwings sukses verhoog.

Studies op die klimaatstoestande binne (mikro) en buite (makro) die bestuiwingsakkies het getoon dat plasing van die sakkies in die top noordelike kant van *P. radiata* bome in die Karatara saad boord, mag die temperatuur nader aan Sabie se temperature bring waar die agt dennespesies lewensvatbare saad produseer. Temperatuur wisselinge tussen dag en nag is groter by Karatara as Sabie en mag ’n moontlike reproduktiewe versperring wees. Drie sirkadiese modelle is ontwikkel om klimaatstoestande by Karatara (mikro en makro) en Sabie (gemiddeld tussen Tweefontein, Witklip en Spitskop saad boorde) te simuleer. Alhoewel die spesifieke posisionering van die bestuiwingsakkies in die boomtoppe en aan die noordelike kant van die bome die gaping in dagtemperature tussen Karatara en Sabie mag verklein het, het die nagtemperature grootliks verskil. Wisseling in die nagtemperature was meer opmerklik by Karatara as by Sabie. Bi-stipping (diskriminantanalise en hoofkomponentanalise), wat die natuurlike herkoms se klimaatsdata

weerspieël, het *P. elliottii, P. taeda* en *P. radiata* by Karatara gegroepeer, wat goeie area spesie passing aandui. Wanneer hoogte bo seevlak as vektor geïgnoreer word, kan *P. patula* ook by die drie gegroepeer word.

Die beperkte sukses van vorige interspesifieke hibridisasie pogings met *P. radiata* het gedetailleerde studies op stuifmeel ontkieming en groei gewaarborg. Klimaatstelsels wat amper dieselfde as die bogenoemde mikro- en makro- klimate is, het stuifmeelkorrel grootte (hidrasie), stuifmeelbuis grootte en stuifmeelbuis groeitempo's (SBGT) affekteer. Verder, om die hidrasie-effek van die stuifmeeldruppel op stuifmeelkorrels te simuleer, is gedehidreerde en gehidreerde stuifmeelkorrelgroottes van die nege denne taksa (ag *Pinus* spesies en een hibried) met mekaar vergelyk. Alhoewel daar betekenisvolle verskille was in die SBGT, stuifmeelkorrel grootte en stuifmeelbuis grootte van die verskillende spesies, het geen van die dennespesies 'n grootte of SBGT gehad wat vergelykbaar is met *P. radiata* nie. Daar is tot die gevolgtrekking gekom dat die *in vitro* eksperimente, ten spyte van die verskille waargeneem, nie voldoende antwoorde tot die beperkte bestuiwing sukses verskaf het nie.

In vivo bestuiwing sukses tussen *P. radiata, P. tecunumanii, P. oocarpa* en *P. maximinoi* is bepaal deur die aantal sigbare saadknoppe, stuifmeelkorrels binne die saadknoppe, stuifmeelkorrels buite die saadknoppe en stuifmeelbuise te tel. Data is versamel met 'n standaard fiksasie-dehidrasie-inbedding histologie volgorde oor die sewe-week tydperk. Dit het aangewys dat *P. radiata*×*oocarpa, P. radiata*×*tecunumanii* en *P. radiata*×*maximinoi* aansienlik verskil het van *P. radiata*×*radiata* vir al vier faktore wat ondersoek is. Die paraffienwas metode was nie geskik om keëls ouer as agt weke te bestudeer nie. MikroRT skanderings is gebruik om te bepaal of bevrugting plaasgevind het tussen 15 tot 16 maande na bestuiwing. Saad was sigbaar vanaf week 68 na bestuiwing en lewensvatbaarheid van saad kon bepaal word met kwantitatiewe porositeit en defekte analise.

Pearson korrelasie met bi-stipping tussen natuurlike herkoms data (hoogte bo seevlak, neerslag en temperatuur), SBGT (lengte en breedte) en gehidreerde stuifmeelbuis grootte (lengte en breedte) by mikro- en makro-temperatuur stelsels vir die agt suiwer spesies, het drie duidelike klimaat reaksie groepe geïdentifiseer. Dit is (1) *P. oocarpa* en *P. tecunumanii,* (2) *P. maximinoi* en *P. patula,* en (3) *P. elliottii* en *P. taeda. Pinus pringlei* en *P. radiata* is nie ingesluit by die drie duidelike klimaat reaksie groepe nie, maar *P. pringlei* kan by *P. patula* gegroepeer word as gevolg van hoogte bo seevlak, terwyl *P. radiata* by *P. elliottii* en *P. taeda* gegroepeer kan word weens neerslag en minimum temperatuur.

Data versamel by Karatara deur die loop van hierdie studie, het ooreengestem met vorige studies wat bewys dat *P. radiata* beter aangepas is by droër somers en natter winters as die meer tropiese spesies. Daarom is bestuiwing sukses by Karatara waarskynlik nie beperk deur area faktore nie, maar as die stuifmeel pasmaat nie vergelykbaar is met die temperatuurstelsels by Karatara nie, mag dit beperkte bestuiwing sukses tot

gevolg hê. Dus het data wat versamel is, aangedui dat *P. elliottii* en *P. taeda* potensiële hibridisasie pasmaats vir *P. radiata* kan wees. Gedurende hierdie studie is waardevolle inligting versamel in terme van stuifmeelkorrel grootte, SBGT, klimaatsdata, drie sirkadiese modelle, *in vivo* bestuiwing sukses en mikroRT skanderings. Omdat hierdie studie gefokus het op klimaat en stuifmeel aspekte, moet toekomstige studies fokus op die vroulike strobili, soos die hormonale en/of chemiese interaksie en of saadknop aborsies bestuiwing suksesse affekteer.

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Scientific outputs derived from this project till date

1. Scientific publications

Ham H, Botha A-M, Kanzler A, du Toit B. 2017. Climate as possible reproductive barrier in *Pinus radiata* (D. Don) interspecific hybridisation. *Annals of Forest Research* 60(1): 1-16.

Ham H, Botha A-M, Kanzler A, du Toit B. 2017. *Pinus radiata* hybridisation: Pollen tube elongation and pollen grain size as possible reproductive barriers. Accepted subject to final corrections: *iForest*, May 10th.

Ham H, Botha A-M, Kanzler A, du Toit B. 2017. *In vivo* interspecific pollination success between *P. radiata*, *P. maximinoi, P. oocarpa* and *P. tecunumanii*. Accepted by *Journal of Forestry Research*, July 19th.

Ham H, Du Plessis A, Le Roux SG. 2017. Microcomputed tomography (microCT) as a tool in *Pinus* tree breeding: pilot studies. *New Zealand Journal of Forestry Science* 47(1): 2-8.

2. Papers presented at symposia

Ham, H., Dvorak, W., Kanzler, A., and du Toit, B. 2013. *P. radiata* hybridisation: the quest for success. Breeding and Genetics Resources of Southern US and Mexican Pines, IUFRO Working Group 2.02.20, International Symposium, Jacksonville - Florida, USA, 5 February.

Ham, H., Dvorak, W., Kanzler, A., and du Toit, B. 2013. *Pinus radiata* hybridisation: progress to date. International CAMCORE Conference, Durban, October 13.

Ham, H., Dvorak, W., and Kanzler, A. 2014. Possible effects of karyotype, palynology and reproductive morphology on *Pinus* interspecific hybridisation success. Research to support a transformed, sustainable and competitive Southern African forest sector: ICFR symposium, July 22, Durban.

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Chapter 1

Project rationale

1. Introduction

Pinus section *Trifoliae* (*Pinus* subgenus *Pinus*) consist of medium to large trees native to North and Central America and the Caribbean Islands, including several of the world's most ecologically and economically important tree species (Hagman 1975; Hernández-León et al. 2013). Taxonomists have divided the genus *Pinus* into three subgenera, five sections and up to 16 sub-sections, depending on the classifier (Critchfield and Little 1966; Price et al. 2000; Grotkopp et al. 2004). Within the genus *Pinus,* about 95 species with numerous varieties and hybrids can be found (Krugman and Jenkinson 1974; Critchfield 1975; Gernandt et al. 2005; Plomion et al. 2007). Four of the 95 species (*P. radiata, P. patula, P. elliottii* and *P. taeda*) form the backbone of the South African softwood resources of which *P. radiata* is the most important species in the Cape pine-growing region (DAFF 2014).

The extant populations of *P. radiata* occur naturally over a small range of land (latitudes 28 to 37 °N; Hocking 1930; Dillon et al. 2013) with five disjunctive populations at Año Nuevo, Monterey and Cambria (on the Californian coast), Guadalupe and Cedros Islands off the coast of Baja California (Ledig 2000; Lavery and Mead 2000; Gapare et al. 2012; Dillon et al. 2013). The five provenances grow under the same climatic and edaphic conditions (Gapare et al. 2012), but previous studies in Australia and New Zealand indicated that Monterey and Cambria provenances have similar growth potential, while Año Nuevo and Monterey provenances have the best overall attributes (Gapare et al. 2011). *Pinus radiata* is one of the most highly bred and widely planted pines in the Southern Hemisphere (Balocchi 1997; Burdon 2001; Burdon et al. 2008) and was introduced into Southern Hemisphere countries at different times: South Africa (1850), Australia (1857), Chile (1975) and New Zealand (unknown) (Lavery and Mead 2000; Richardson and Higgings 2000).

The amount of DNA in pines varies greatly among species, which can favour recombination (Ledig 2000; Morse et al. 2009), but the large number of species and intraspecific taxa indicate a high degree of variation (population with two or more clear phenotypes) (Pravdin 1985). Although the genetic diversity in *Pinus radiata* is low compared to other *Pinus* species (Wu et al. 1998), the karyotype and phylogenetic tree can shed light on the evolutionary relationships between *P. radiata* and other *Pinus* species before embarking on pollination success.

2. Taxonomic classification

Because the genetic distance (evolutionary similarity) between pine species influences the degree of their reproductive isolation, taxonomic grouping based on morphology and molecular studies have relevance to this study. For the pine species investigated in this study and using the subsection grouping of Price et al. (2000), *P. elliottii* and *P. taeda* belong to subsection *Australes, P. oocarpa, P. patula, P. pringlei* and *P. tecunumanii* are found in the *Oocarpae*, *P. radiata* is part of subsection *Attenuatae* and *P. maximinoi* is a member of the

subsection *Ponderosae* (Figure 1.1). The species included in these subsections are also generally supported by Millar (1986), Perry (1991), Farjon & Styles (1997) and others. However some believe that the *Australes* and *Oocarpae* are monospecific (Gernandt et al. 2005; Hernàndez-Leòn et al. 2013), or at the least, have co-evolved with each other, and in some cases, crossed together naturally (Dvorak et al. 2001). Most importantly, the classification of Price et al. (2000) moved the California closed-cones pines (including *P. radiata*) out of the *Oocarpae* subsection favoured by Critchfield and Little (1969), as well as, others and placed them in their own subsection, *Attenuatae*. Subsequent molecular markers studies by Wu et al. (1998) and Dvorak et al. (2001) confirmed that the genetic distances between the more tropical pines in *Oocarpae* and *Australes,* and the winter rainfall species in the *Attenuatae* were large. This might partially explain why it is difficult to make successful cross combinations between the *Attenuatae* and the *Oocarpae* or *Australes* and why levels of resistance to the same disease can be so different between subsections. In this study, *P. oocarpa*, *P. pringlei*, and *P. tecunumanii* in the *Oocarpae,* are very resistant to *Fusarium circinatum* while *P. radiata* of the *Attenuatae* is highly susceptible (Hodge and Dvorak 2000; Roux et al. 2007; Kanzler et al. 2012; Mitchell et al. 2011, 2012, 2013;). Generally, increasing taxonomic distance between pine species limits hybridisation success due to interspecific incompatibility (pre-zygotic barrier affecting pollen tube development), inviability (post-zygotic barrier reducing the ability to produce mature seed) (Hagman 1975; Potts and Dungey 2004) or possibly environment. Although interspecific incompatibility is genetically determined (McWilliam 1959), Potts and Dungey (2004) suggested that interspecific incompatibility is more likely to happen than inviability. Hagman (1975) stated that incompatibility occurred in the interspecific hybrid of *Betula verrucosa*×*pubescens* (silver birch x white birch) due to the retarded growth of the pollen tubes and this might also happen in *Pinus* species as well. Although interspecific hybrids between pines in the same subsection generally pose no problem and are successfully made, crosses between pines in different subsections show a high degree of incompatibility especially between the pollen tube growth and nucellus (Hagman 1975). Therefore, possible reasons for limited pollination success of *P. radiata* attempts could be related to karyotype, palynology and/or reproductive morphology. Reference to *P. tecunumanii* throughout the manuscript refers to the low elevation, more tropical populations of the species in Belize (northern Guatemala), Honduras and Nicaragua (Dvorak 1985; Dvorak et al. 2009).

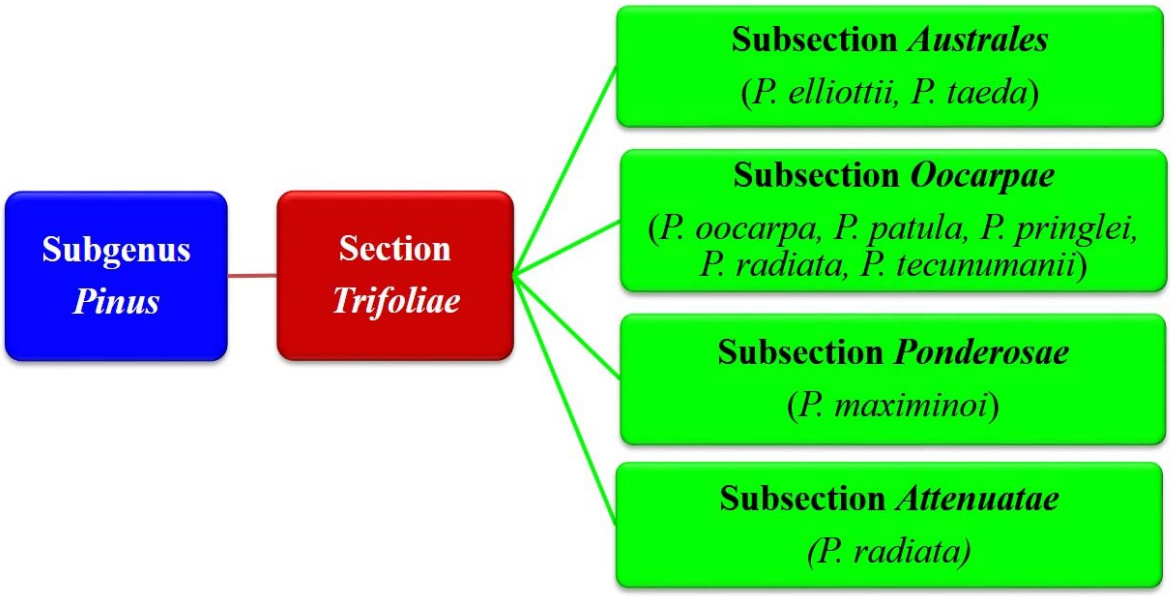

Figure 1.1: Taxonomic grouping of the pine used in the study (Price et al. 2000)

3. Karyotype

Karyotype refers to chromosome numbers, morphology, the size and shape and can be used to define species character and help solve problems related to variation and affinity between species (Pederick 1970; van der Walt and van Zyl 1988; Steyn and Robbertse 1992). Although conifers have among the most complex and largest genomes of higher plants, early studies showed distinct karyological features for certain subsections, groups of related species and occasionally for individual species. The karyotype of all pine species is remarkably similar in chromosome number, length and centromere location; comprising of a haploid complement of 12 chromosomes with little difference in length. Eleven chromosomes are isobrachial (median centromere), while the smallest chromosome is slightly heterobrachial (submedian centromere location) (Sax and Sax 1933; Bowden 1945; Mehra and Khoshoo 1956; Khoshoo 1960; Pederick 1967, 1970; Saylor 1972; Ohri and Khoshoo 1986; Karvonen et al. 1993; Hall et al. 2000; Ledig 2000; Joyner et al. 2001; Williams et al. 2002; Grotkopp et al. 2004; Cai et al. 2006; Plomion et al. 2007; Shepherd and Williams 2008; Morse et al. 2009; Williams 2009; Nkongolo and Mehes-Smith 2012). Although the karyotype (genome size) is uniform, the amount of DNA varies greatly between species. Little is known about the exact genome size of most *Pinus* species, but closely related pines have very similar chromosome structure with little variation in genome size (Sax and Sax 1933; Pederick 1970; Hall et al. 2000; Ledig 2000; Williams et al. 2002; Cai et al. 2006; Shepherd and Williams 2008; Williams 2009). However, it has been suggested that tropical pines might have a smaller genome sizes than temperate pines (Hall et al. 2000).

Karotypic orthoselection (evolutionary selection) does not fully account for genome size variation in pines. However, genomic shock (two related but homologous genomes are combined into a single cell causing chromosome elimination or even polyploidy) within interspecific hybridisation, can be studied to explain the result of meiotic abnormalities and consequently pollen abortion (Williams et al. 2002). Despite karyotypic constancy and the notable absence of genomic shock, it is not known whether hybrid genome content is similar to the individual parental species. Interspecific hybridisation may alter the genome size without changing the chromosome number, but changes on the chromosomes might be slight (Williams et al. 2002). Hall et al. (2000) supports this theory, as nuclear genome sizes are unusually variable within pine species in regions of Mexico where natural interspecific hybridisation does occur between related species within a subsection (Plomion et al. 2007). Even though there are contrary views on the effect interspecific hybridisation (e.g. Williams 2002; Morse et al. 2009) has on genome size, reproductive barriers may exist at the chromosome level that inhibit the formation and survival of embryos. As the karyotype of the species is consistent, the phylogeny, palynology (study of pollen grains, pollen tube development and elongation), and reproductive morphology of *Pinus* species might be the single most important factor contributing to the limited success of interspecific hybrids between different *Pinus* subsections as it is genetically determined (Little and Critchfield 1969; Dungey et al. 2003; Price et al. 2000; Hodge and Dvorak 2014; Fernando 2014).

4. Phylogenetic tree

The evolutionary relationships among various *Pinus* species were investigated and classified into the *Oocarpae* and *Australes* subsections. This was followed with the phylogenetic tree constructed from RAPD marker data (Figure 1.2; Dvorak et al. 2000). Results indicated that the *Attenuatae* group (*P. radiata, P. attenuata* and *P. muricata*) is very distinct from the *Oocarpae* and *Australes* subsections (containing *P. oocarpa*, *P. tecunumanii, P. taeda* and *P. elliottii*); suggesting pollination success between these two groups would be more difficult than within the *Attenuatae* group. Unfortunately, *P. maximinoi* was not part of the Dvorak et al. (2000) study, but previous studies indicated it belongs to the *Ponderosae* group (Gernandt et al. 2009). Regarding *F. circinatum,* it seems that some species in the *Oocarpae* group are more tolerant than species in the *Attenuatae* group (Shelbourne 1974; Hodge and Dvorak 2000; Dungey et al. 2003; Mitchell 2011, 2012, 2013), making interspecific hybridisation between these groups a worthwhile endeavour. A phylogenetic tree, which illustrates possible relationships between species, can also assist with inferences such as how the environment affects a species, and to predict distribution areas (Seong and Offner 2013).

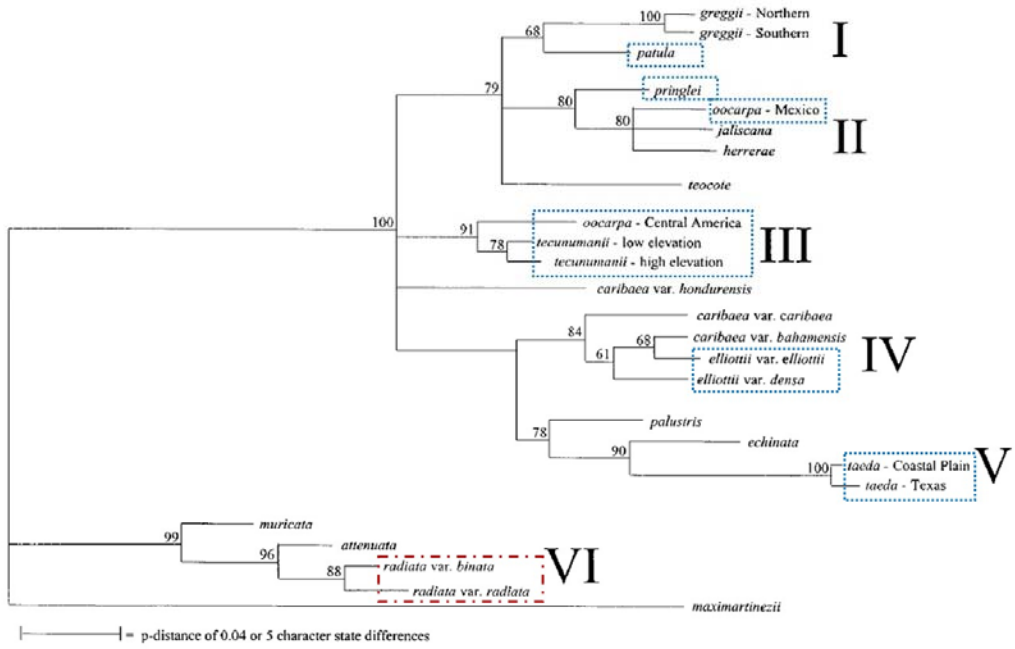

Figure 1.2: Phylogenetic tree indicating the evolutionary relationship between the eight *Pinus* species investigated in this study. Clades I to V represents the *Oocarpae* and *Australes* (some degree of *Fusarium circinatum* tolerance), while Clade VI revers to *Attenuatae* (no *Fusarium circinatum* tolerance). *Pinus maximinoi* (*Ponderosae*) was not part of this study (adapted from Dvorak et al. 2000).

5. Pathogen tolerance

Pinus radiata is susceptible to *F. circinatum* (Pitch canker), creating major problems in nurseries and commercial plantations in the Cape region of South Africa since 2005 (Mitchell et al. 2011; Kanzler et al. 2014; Iturritxa et al. 2014, 2017). The economic impact it has on *P. radiata* nurseries, field losses, re-establishing and products, is difficult to estimate as data reporting these (published and unpublished) are insufficient to quantify damage and losses (Mitchell et al. 2011, 2012). However, it is estimated that 40% annual re-planting and 20% die-back in mature *P. radiata* plantations are due to infection by *F. circinatum* in South Africa (D. Carstens, Pers. Comm.). Disease control measures (preventative chemical sprays) are costly, environmentally unfriendly and technically difficult to apply in commercial plantations (Iturritxa et al. 2014). Therefore, preventative treatments are limited to good silvicultural management, hygiene in pine nurseries (Iturritxa et al. 2017), and interspecific hybridisation of *P. radiata* with tolerant *Pinus* species (Lavery and Mead 2000; Rogers et al. 2006; Apiolaza 2014). Although *F. circinatum* tolerance varies considerably between *Pinus* species, taxa from sub-section *Oocarpae* (*P. oocarpa, P. tecunumanii*, and *P. pringlei*) demonstrated the greatest level of resistance to *F. circinatum* infection (Hodge and Dvorak 2000; Mitchell et al. 2011, 2012, 2013). Interspecific hybrids of *P. radiata* with *P. tecunumanii* and *P. oocarpa* might thus be a

viable option (Hodge and Dvorak 2000) as indicated by *P. patula*×*oocarpa,* and *P. patula*×*tecunumanii* (Mitchell et al. 2012, 2013; Kanzler et al. 2014) to reduce susceptibility to *F. circinatum*. *Pinus radiata* breeding material have been screened for *F. circinatum* tolerance in parallel studies since 2011. *Pinus taeda* and *P. elliottii* have been included as tolerant parents during these trials, as tolerant levels compare well to *P. tecunumanii*, *P. oocarpa* and *P. maximinoi,* as indicated by Mitchell et al. (2011, 2012, 2013). Interspecific hybrids can also potentially extend the geographic planting range of *P. radiata* (Hodge and Dvorak 2000; Gapare et al. 2012; Ellstrand 2014; Gapare 2014; Hodge and Dvorak 2014; White et al. 2014). Limited success has however been achieved to date with *P. radiata* hybrids (Hodge and Dvorak 2014) and might be a result of reproductive barriers limiting gene flow between species (Ellstrand 2014).

6. Reproductive barriers

Reproductive barriers between pines can be caused by a myriad of different pre-zygotic and post-zygotic factors that include genetic differences between parent species and environmental clines in which they naturally evolved. If these barriers are pronounced, hybrid seed production will be limited to non-existent (Sax and Sax 1933; McWilliam 1959; Potts and Dungey 2004; Plomion et al. 2007; Ellstrand 2014). Pre-zygotic barriers can include chromosome instability, lack of fertility, sterility, different taxonomic groups, different stages of development (from failure of pollen germination to inadequate embryogenesis [lack of fertilisation]) and pollen management (Sweet and Thulin 1969; Sundberg et al. 1987; Slee and Abbott 1990; Ledig 2000; Harushima et al. 2001; Nel and van Staden 2005; Kormutak et al. 2007; Shepherd and Williams 2008). Post-zygotic barriers include incompatibility between the ovule tissue and foreign pollen tubes (Hardin et al. 1972; Ottaviano et al. 1980; Harushima et al. 2002; Dafni et al. 2005; Williams 2009).

Reproductive barriers can be genetic (hybrid sterility, hybrid weakness, and gametophytic competition), making the study of gene flow extremely important (Ledig 2000; Harushima et al. 2002; Ellstrand 2014). Although DNA markers can be used to analyse the quantitative trait loci (QTL) that seem to be responsible for reproductive barriers, it cannot determine whether the barrier is on the male (pollen) or female (flower) parent, whether it induces abortion or involves gametophyte competition (Ledig 2000; Harushima et al. 2001, 2002; Isik 2014).

Three major reproductive (two pre-zygotic and one post-zygotic) barriers have been identified in *Eucalyptus* interspecific hybrids: structural barrier (length of the pollen tube is too short for fertilisation); physiological barrier (pollen tube abnormalities and arrest in the pistil); and physiological barrier (slow and inadequate development of the embryo after fertilisation) (Potts and Dungey 2004). *Pinus* studies also indicated that pollen can influence pollination success: high viability pollen increases the number of surviving cones and fertile seeds (Doyle et al. 2002; Dungey et al. 2003; Nel and van Staden 2003, 2005); the morphology of pine ovulate cones are very specific in accepting pollen, resulting in filter-out pollen of non-conspecific taxa, thus

limiting pine species hybridising with one another (Dungey 2001; Dungey et al. 2003; Nel and van Staden 2005; Schwendemann et al. 2007); incompatibility between genetically distant species (phenotypic tree) and poor pollen germination (Doyle et al. 2002; Dungey et al. 2003); variation in pollen tube growth rate (Varis et al. 2008); and pollen grain germination ability affected by temperature regimes and day length (Varis et al. 2008).

The reproductive structures (flowers) of forest trees and their development can be severely influenced by environmental factors (photoperiod, temperature, soil moisture, water availability, photosynthesis rates and nutrition) and can pose reproductive barriers (Alzoti et al. 2010). Although silviculture practices (irrigation, fertilisation and thinning) can also affect female flowering, pollen quantity, pollen shedding, and seed production; reproductive barriers will determine whether gene flow is successful or not. Even though genetic incompatibility will remain a constraint on the production of viable offspring (Dungey et al. 2003), reproductive barriers may be overcome to some extent with new techniques or adaptation in breeding strategies and pollination protocols.

7. *Pinus radiata* breeding

Pinus, Eucalyptus and *Populus* species have been subjected to advanced breeding techniques for more than 50 years (Hyun 1976; Shelbourne et al. 1989; Kuang et al. 1999; Gapare 2014) as they contain an enormous amount of variability that breeders can utilise to produce trees with desired characteristics in the shortest possible time (Zobel and Talbert 1984; Sorensson 2002; Suzuki and Suzuki 2014).

In comparison to other pine species, *P. radiata* has a relative small and fragmented natural range that exhibits historic genetic bottlenecks that often lead to inbreeding depression, reduced seed production, low seed viability, depressed growth and decreased adult fertility (Wilcox 1983; Shelbourne et al. 1989; Wu et al. 1998; Kuang et al. 1999; Vogl et al. 2002). Tree improvement of *P. radiata* is mainly done within populations of pure species. The oldest known interspecific *P. radiata* hybrid (*P. attenuata*×*radiata)* was made in 1929 (Critchfield 1975; Dungey 2001), while various pine breeding programmes in New Zealand reported limited success with hybrids thereafter (Khurana and Kohsla 1998; Dungey et al. 2003). Early studies revealed that *P. radiata* can be crossed readily with only two, very closely related species (*P. attenuata* and *P. muricata)* but several pine species show potential (Critchfield 1967; Dungey 2001; Burdon et al. 2008). Interspecific crosses between closely related species within the *Attenuatae* group (*P. radiata* and *P. attenuata*) were more successful than crosses between *P. radiata* and *P. muricata*. Furthermore, interspecific crosses between *P. radiata* and species from the *Oocarpae* group (*P. greggii, P. patula, P. oocarpa* and *P. tecunumanii)* had limited success (Dungey et al. 2003). Success was measured with average crude seed yield per cone that ranged from 0 to 23.1 for *Atttenuatae* and 0 to 0.91 for the *Oocarpae* groups respectively (Dungey et al. 2003). However, unpublished CAMCORE reports indicated limited *P. radiata* interspecific successes of *P.*

radiata×*tecunumanii* and *P. radiata*×*oocarpa*, using DNA analysis of seedling needle tissue (Hodge and Dvorak 2000).

8. Study objectives and structure of thesis

The reproductive process in plants represents a critical phase in their life-cycle (Varshney and Varshney 1981) and adequate knowledge of the reproductive cycle and possible limitations or reproductive barriers need to be investigated and understood (Hagman 1975; Fernando et al. 1997; Taylor and Hepler 1997; Fernando 2014). The role that these barriers might play in limiting the pollination success of *P. radiata* with some pine species to increase *F. circinatum* tolerance are not well understood. Therefore, the focus of the dissertation is to determine if barriers preventing pollination success in *P. radiata* can be identified and mitigated to some extent. This study will also determine why past pollination attempts with *P. radiata* have failed, and to establish a new protocol for successful pollination with *P. radiata*.

Although *P. radiata* can be crossed readily with only two, very closely related species (*P. attenuata* and *P. muricata),* several pine species need to be investigated as hybrid partners to improve *F. circinatum* tolerance. Due to limited success with *P. radiata* interspecific hybridisation (<1%; W. Dvorak Pers. Comm.), this study investigated possible reasons that could limit pollination success between *P. radiata* and seven pine species (*P. maximinoi, P. oocarpa, P. tecunumanii, P. elliottii, P. taeda, P. pringlei* and *P. patula*). One interspecific hybrid (*P. patula*×*tecunumannii*), currently employed for *F. circinatum* tolerance in South Africa, were also included as a possible interspecific hybrid partner for *P. radiata*. These species were chosen due to their *F. circinatum* resistance, ease of propagation, wood properties and geographic adaptation. Although *P. patula* is considered susceptible to *F. circinatum*, it was included in the study as the female parent of *P. patula*×*tecunumannii*.

Objectives addressed in this study, are:

a) Determine and evaluate the current pine pollination protocol used by pine breeders
b) Investigate and compare climate conditions inside pollination bags to natural provenance data
c) Compare *in vitro* pollen viability, pollen tube elongation and pollen grain size between the nine pine taxa (eight pure species and one interspecific hybrid)
d) Determine *in vivo* pollination success
e) Investigate if Computed and MicroComputed Tomography can be employed in pine breeding programmes to generate 3D images to determine seed set and viability

This dissertation consists of six chapters (Figure 1.3): project rationale (Chapter 1); evaluate pollination protocol (Chapter 2); investigate the climate inside pollination bags (Chapter 3); determine *in vitro* pollination

success (Chapter 4); determine *in vivo* pollination success (Chapter 5); and summary of project findings (Chapter 6).

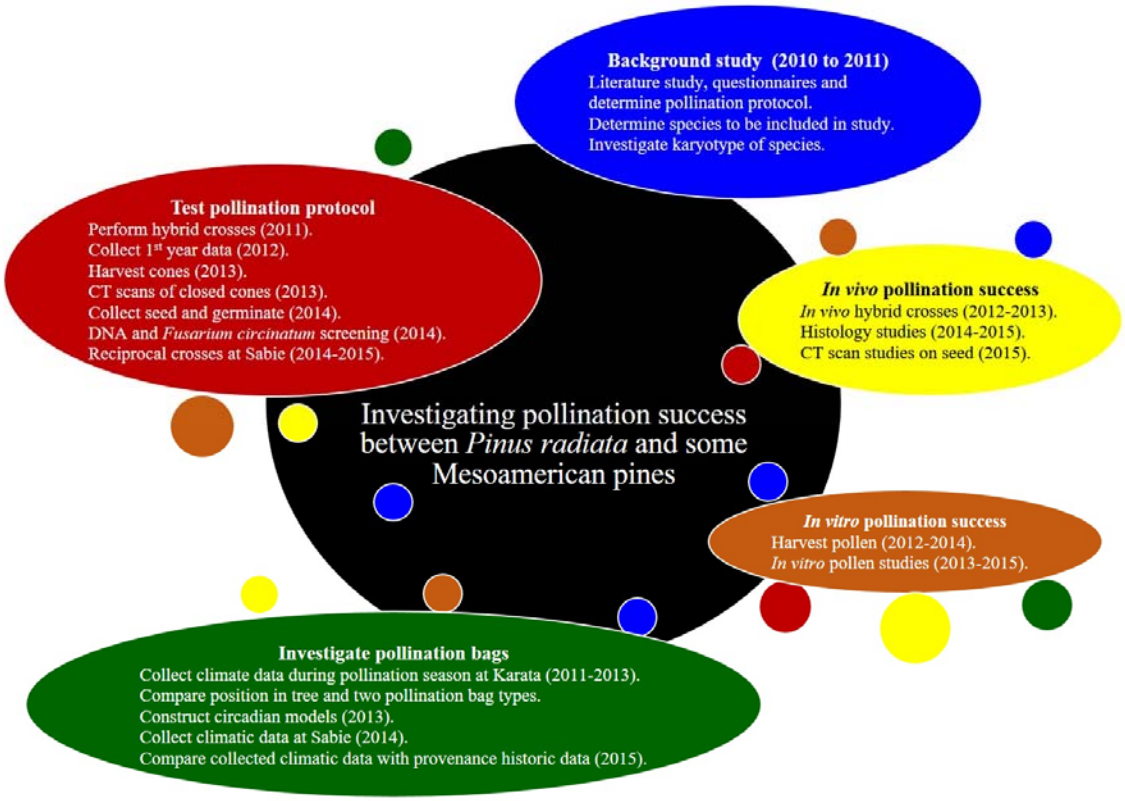

Figure 1.3: Diagram illustrating the flow of the project investigating the pollination success between *Pinus radiata* and selected pine species.

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Chapter 2

Determine and evaluate generic pollination protocol

1. Introduction

Plant breeding (tree improvement) is a sequential process, starting with the evaluation and the selection of the best candidates to improve genetic gain. The genetic improvement of forest tree species is based on recurrent selection to progressively increase the frequency of desirable genes in the breeding population through cycles of cross-pollination (Shelbourne et al. 1989; Codesido and Fernández-López 2014). Not only is there significant variation among clones in terms of male and female flowering processes, but there is strong genetic control. Therefore, synchronisation of pollen shedding and flower receptivity is important to limit pollen contamination (background pollen) during cross-pollination activities, especially in seed orchards with large number of parents (Codesido and Fernández-López 2014).

In any breeding programme, genetic gain, avoidance of inbreeding depression and genetic diversity are essential, and these may be affected, negatively or positively, by factors such as self-fertilisation, inbreeding, relatedness among parents, pollen contamination, spatial distribution, flowering phenology and synchrony as well as male and female fertility. It can be limited by: phenotypic selection, breeding (tree improvement cycle), seed and seed production. Therefore, systematic monitoring of these steps is thus extremely important. The ideal situation in any breeding programme is the absence of pollen contamination, self-pollination, linkage disequilibrium, inbred or random mating (Codesido and Fernández-López 2014). Background pollination (pollen contamination) from unselected sources outside the seed orchard is always present and is relative high (45 to 60 %) when orchards are fully mature (Pulkkinen et al. 1995). This needs to be controlled to lower selfing and inbreeding depression.

In general, cross-pollination has been performed on conifer species since 1937 (Bramlett and O'Gwynn 1981; Nel and van Staden 2003), resulting in the formation of new genotypes through segregation of the parental genes and their recombination (Hagman 1975; Plomion et al. 2007). Hybridisation may explain complex patterns of intra- and interspecific morphological and genetic variation in pines (Hernández-León et al. 2013) and gene-flow, but incompatibility or reproductive barriers can prevent success (Barbour et al. 2006). These barriers are present in both open- and cross-pollination (Hagedorn 2000), but may vary between species (McWilliam 1959; Hagman 1975). Previous studies have indicated that *P. radiata* exhibits a high degree of reproductive incompatibility with the commercially important closed-cone pines of Mexico and Central-America (Moran and Griffins 1985).

While interspecific hybridisation is a viable option to improve *F. circinatum* tolerance (Kanzler et al. 2012, 2014), the high levels of reproductive incompatibility observed in *P. radiata* could complicate the hybridisation process. Interspecific hybrids of *P. radiata* with *P. tecunumanii* and *P. oocarpa* need to be investigated (Hodge and Dvorak 2000) as *P. patula*×*oocarpa* and *P. patula*×*tecunumanii* shows promise for improved *F. circinatum* resistance compared to pure *P. patula* (Roux et al. 2007; Kim et al. 2008; Kanzler et al. 2014). Since traditional selection and breeding within *P. radiata* for improved resistance might provide

little to no improvement, artificial interspecific hybridisation of *P. radiata* with other tolerant *Pinus* species that are more resistant to *F. circinatum* seems to be the next best and only option (Hodge and Dvorak 2000).

This study aimed to determine and assess the pollination protocol employed by various pine breeding programmes in the international tree breeding organisation (CAMCORE) network. A questionnaire was sent to pine breeders to determine a generic protocol for assessment in a *P. radiata* seed orchard in South Africa (Southern Cape), the main *P. radiata* commercial plantation area. What makes testing the protocols in the Southern Cape different than in orchards in other countries where *P. radiata* is grown, is that the Southern Cape does not experience sub-freezing temperatures, and pollen tubes of tropical pines presumably would survive the winter. Therefore, it would not be one of the causes for pre or post zygotic abortions in the interspecific crosses made.

2. Materials and methods

2.1 Determine generic pollination protocol

The focus of the study was to gain insight into the current breeding strategies of different organisations involved in pine breeding and to develop a generic pollination protocol for testing. The study can be classified as a reputability study conducted amongst key informants (Bless and Higson-Smith 1995). A total of 13 key informants were purposefully selected (Babbie and Mouton 2001) as typical pine breeders in the CAMCORE network. A reputability study focusses more on a deeper understanding of a topic than on making statistical inferences about a larger population (de Vaus 2002), allowing for a smaller sample of respondents.

While the key informants at the respective companies were the unit of analysis for the study (Bless and Higson-Smith 1995) it was also considered that although these individuals work for companies, their views and opinions might not represent the views and opinions of the companies. It was, therefore, necessary to critically assess responses and information to ensure that the interest and concerns of those involved in the study were safeguarded (Robson 2002).

The companies involved in pine breeding are located throughout the southern hemisphere, making direct observations and personal face-to-face interviews impossible. Survey research (Babbie and Mouton 2001) was therefore selected as the best method to gather original data. Questionnaire surveys are an important part of social research and clear guidelines on the construction of questionnaires and execution of surveys are presented by authors such as De Vaus (2002), Bless and Higson-Smith (1995) and Babbie & Mouton (2001).

When constructing a questionnaire it is very important to clearly define the different research topics and key fields for which information is required (Bless and Higson-Smith 1995). The following topics were identified

as key to the study: pollination protocol employed by company, hybridisation success to date and future projects. Questions were formulated around these issues, starting with easier questions that led the respondent to more difficult questions towards the end of the questionnaire (de Vaus 2002). Care was taken to limit the length of the questionnaire as long questionnaires tend to reduce the response rate (Babbie and Mouton 2001). Questions were carefully phrased to prevent leading or negative questions and to ensure that responses were exhaustive and mutually exclusive (de Vaus 2002). The questionnaire was also pre-tested amongst peers to check on clarity, meaning, redundancy and non-responses (Babbie and Mouton 2001).

The questionnaires were sent to respondents via e-mail as an attached document with a cover letter, explaining the aim of the study and guaranteeing confidentiality (Robson 2002). Respondents were encouraged to contact the researcher directly for further questions or clarifications. In any survey, non-responses could be a serious problem, especially where a small number of experts were selected to participate in the study. Regular follow-ups were made to prompt and encourage respondents (de Vaus 2002), but after one month it was recognised that the longer responses are delayed the less likely they will be returned (Babbie and Mouton 2001). This study made use of open-ended questions to allow more in depth discussion. The analysis of open-ended questions is, however, not as simple as for closed questions. Open-ended questions require coding and a combination of information into a number of categories for simple descriptive statistical analysis. Procedures described by Robson (2002), Babbie & Mouton (2001) and De Vaus (2002) were followed in the analysis of the questionnaire.

2.2 Assess generic pollination protocol

Hybrid crosses

Controlled crosses were performed on *P. radiata* trees at the Cape Pine Karatara seed orchard (33 ° 54 ' 0 " South; 22 ° 50 ' 0 " East), during the winter (July and August) of 2011. Female strobili of ten *P. radiata* ramets of clone AR366 were pollinated with *P. oocarpa* and *P. tecunumanii* pollen lots (bulked from five trees per lot). The pollen was collected by CAMCORE in natural stands in the El Castaño and San Jeronimo (Guatemala) populations, respectively during January 2011. A standard pollen management protocol for *Pinus* species was followed as determined by CAMCORE: catkins were harvested from mature trees in natural stands and dried in paper bags for approximately 48 hours at 25 ºC. Pollen lots were sifted with a 100 µm sieve to discard any unwanted particles and pollen lots were placed in glass Petri dishes on silica gel in sealed containers (desiccators) until the water moisture content was below 10 %. The pollen was then couriered from Guatemala to North Carolina, where moisture content (MC) and germination percent were rechecked at North Carolina State University. *Pinus oocarpa* and *P. tecunumanii* had a MC of 7.8 % & 6.2 % and a germination percent of 25 % and 55 %, respectively. The relatively low germination percent of the pollen lots were thought to be a function of the long travel time to return from isolated natural stands in Guatemala after pollen collection and the rustic storage conditions in Guatemala City. The two bulk pollen lots (200 ml each) were then expressed couriered to MTO in South Africa. Upon arriving at the Karatara nursery in the Southern Cape,

the pollen was placed in a desiccator with silica gel and kept at 4 to 5 °C prior to being applied to female strobili of *P. radiata*, six weeks after reception.

The generic *Pinus* cross-pollination protocol based on an analysis of the questionnaire data and described in Section 3.1, was followed. Female strobili were bagged on 19 July 2011 and pollinated approximately two weeks later. Three pollination events were performed at three separate dates (4, 10 and 16 August 2011), approximately six days apart. Pollination bags were removed on 30 August, two weeks after the last pollination event. The *P. radiata* trees were approximately 40 m tall, therefore, interspecific hybrid crosses were performed at random throughout the trees and within easy access of a cherry picker to optimise number of female strobili pollinated. The following data was collected: date of bagging, date of cross-pollination events, removal of pollination bags, first year cone survival (June 2012) and number of cones harvested (June 2013) (see Critchfield and Kinloch 1986).

Computed Tomography scans

Fertilisation success and seed set can be investigated by Computed Tomography (CT). CT scans originated in 1971 as a medical diagnostic tool and uses non-lethal X-ray energies (~ 75 kV) (Stuppy et al. 2003; Staedler et al. 2013). It is a non-invasive approach to 3D visualisation and quantification of biological structures (Stuppy et al. 2003; van der Niet et al. 2010) with complete 360 ^{o} sampling (Kalathingal et al. 2007). The data, based on differential X-ray attenuation, are analogous to those otherwise obtainable only by serial sectioning (Stuppy et al. 2003; van der Niet et al. 2010). However, CT scans remain strongly underused in plant sciences despite its high potential (Staedler et al. 2013), non-destructive sampling and its ability to highlight small objects (van der Niet et al. 2010). The qualitative and quantitative investigation of the internal morphology and histology of plants can be a potential application of CT scans (Stuppy et al. 2003; du Plessis et al. 2016; Guelpa et al. 2016).

CT scans were used as an alternative method to determine seed set and confirm number of seeds obtained from cross-pollination accurately and measurable (Kalathingal et al. 2007). As CT scans is non-destructive (Kriebel 1972), more samples can be studied at one time (Stuppy et al. 2003), while 3D geometric representation is a reliable representation, but sample size might present statistical problems (Perrin et al. 2010; van der Niet et al. 2010; Staedler et al. 2013). However, the repeatable examination of live samples is not feasible due to the intensity of the X-rays (Stuppy et al. 2003). Studies with CT scans are very scarce in plant sciences due to plant tissues that are mostly constituted of light elements which display low X-ray absorptions. This can be compensated with very long scanning times that can decrease quality due to the probability of motion artefacts. A large number of pictures of X-ray shadows of an object (around 1 000) are carried out at a different rotation angle. These pictures are then processed by algorithms to reconstruct a volume model of the sample, including whatever is contained in the field of view around the sample (Staedler et al. 2013). Previous studies were able to distinguish between pith, xylem, cortex, vascular bundles, leaf bases, seeds and ovuliferous scales (Pika-Biolzi et al. 2000). This is due to contrasting plant tissues (soft versus hard) that absorbs the X-ray differently

thickness as the consistency of cell walls and cell contents differs (Stuppy et al. 2003; du Plessis et al. 2016; Guelpa et al. 2016).

Mature cones (22 cones per hybrid cross combination) were harvested approximately 22 months after the third pollination event, tagged, weighed (fresh weight), measured (length and width) and prepared for CT scans. CT scanner (General Electric Phoenix V|Tome|X L240/NF180) with X-ray settings of 100 kV and 200 µA, acquired 1 700 images in a full rotation at image acquisition time of 500 ms per image, with no averaging and no skipping of images. Detector shift was activated to minimize ring artefacts. Background calibration was performed and the scan time was approximately 60 minutes per scan. Reconstruction was done with system-supplied Datos reconstruction software. Analysis was performed with Volume Graphics VGStudio Max 2.1 or Visualization Sciences Group Avizo Fire 8.0 commercial 3D analysis software packages.

During the CT scans of mature cones, two dimensional X-rays were used to observe mature cones (after harvesting before drying) non-destructively at a high resolution and contrast. The mature cones were exposed to collimated (parallel) X-rays and the absorbed radiation was measured at repeated angles to allow 3D image reconstructions. Although this technique can be classified as non-destructive sampling, the sample size is too small for significant statistical analysis. However, reconstructed 3D images create a permanent record and life-like image for future analysis, comparison or prediction studies.

Confirmation of hybrid status

Hybrid status was confirmed in three phases as determined by the questionnaire survey: (1) compare seed of hybrid crosses with pure species; (2) *F. circinatum* screening of seedlings; and (3) DNA fingerprinting of needles obtained from seedlings.

Seed size comparison

After the CT scans, the mature cones were dried at 48 °C for eight hours in a commercial oven. Dried cones were weighed (dry weight), seed were extracted and the wings removed. Seeds lots were counted, weighed and photographed. Seed from three pure *Pinus* species (*P. radiata, P. oocarpa* and *P. tecunumanii*) were also obtained from CAMCORE members in South Africa and included as controls. A total of 25 seeds per interspecific cross combination and controls were measured (length and width) with a Leica EZ4 HD stereomicroscope (Critchfield and Kinloch 1986). The microscope was calibrated before measurements were taken to optimise accuracy of measurements. Seed size (length and width) measurements employed a completely randomised design with 25 treatments, of which three control species (*P. radiata, P. oocarpa* and *P. tecunumanii*) and 22 hybrid combinations (11 each of *P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii*). A total of 1 250 measurements were recorded with 25 measurements per treatment (hybrid combinations and pure species) for both seed length and width. The data were subjected to analysis of variance (ANOVA; Appendix B) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.2; SAS Institute Inc, Cary, USA). Shapiro-Wilk test was performed on the standardised residuals from the model to

verify normality (Shapiro and Wilk 1965). Fisher least significant difference was calculated at the 5 % level to compare treatment means (Ott and Longnecker 2001). A probability level of 5 % was considered significant for all significance tests.

The viability of seeds was tested by placing all the seeds (22 cones harvested and three control *Pinus* species) in a glass beaker filled with distilled water for 24 hours at room temperature (24 °C). Empty seeds or seeds with underdeveloped or partially formed embryos are lighter and will float on the water surface, indicating lower viability and germination percentage. The number of seeds, both afloat and submerged, was documented. Seeds (both floating and submerged) were sown in black seedling trays to germinate and were watered twice daily (ten minutes each) in a glasshouse at Stellenbosch University. After two months, standard commercial fertiliser was applied weekly. The germination percentage and number of seedling survival were documented monthly.

***Fusarium circinatum* screening of seedlings**

After six months, seedlings showed possible symptoms of *F. circinatum*. Seedlings were brown with wilted needles and white spores visible at the growth-point (bud). After eight months, only the control seedlings (*P. oocarpa* and *P. tecunumanii*) and some *P. radiata*×*tecunumanii* hybrid combinations appeared healthy. Forty seedlings were randomly selected from both the healthy and unhealthy interspecific hybrid combinations (20 each for *P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii*) and sent for diagnostic tests at the Department of Plant Pathology, Stellenbosch University. Seedlings were surface-sterilised with a 70 % ethanol solution and plated out onto PDA (a general growth medium for the isolation of fungi and bacteria) plates. Isolations were made from the tip, crown and roots of all the seedlings. Various *Fusarium* species colonies were selected and genomic DNA was extracted from aerial fresh mycelia using the Wizard SV Genomic DNA Purification System Kit (Promega, South Africa). *Fusarium circinatum* can be identified with a PCR assay and species-specific primers CIRC4A and CIRC1A (Schweigkofler et al. 2004). The *F. circinatum* isolate FCC 3577 (obtained from the Forestry and Agriculture Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa) was used as the positive control.

DNA fingerprinting of needles obtained from seedlings

Fifty of the remaining visibly healthy seedlings (25 each for *P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii*) were randomly selected and sent for DNA fingerprinting at the Forestry and Agriculture Biotechnology Institute (FABI, University of Pretoria), to confirm hybrid status. DNA reference samples of *P. tecunumanii* were provided by FABI, while reference samples of *P. radiata* were collected in the field from the CAMCORE conservation park in Jonkershoek, outside Stellenbosch. Needles from 40 *P. radiata* trees in the conservation park were randomly selected, 10 trees per provenance (Cambria, Año Nuevo, Monterey and Guadalupe Island).

DNA fingerprinting was performed with SSR markers. All the *P. radiata* needle samples were successfully fingerprinted and represented a unique genotype. A STRUCTURE analysis was first conducted using only

the *P. radiata* reference samples and the pre-existing *P. tecunumanii* reference set that was previously developed by FABI. Unfortunately, at the time of the DNA analyses FABI was still working on a *P. oocarpa* reference set and *P. radiata*×*oocarpa* hybrid combinations was only compared to the *P. radiata* reference samples.

3. Results

3.1 Determine generic pollination protocol

Thirteen questionnaires were send out to 11 CAMCORE pine breeding partners and two *P. radiata* breeding programmes in Australia and New Zealand. Eleven (all CAMCORE partners) questionnaires were returned, resulting in a success rate of 85 %. The following generic pollination protocol was compiled from these questionnaires:

- Identify and isolate clusters of female strobili at the second stage (female strobili buds enlarged and enclosed within bud scales) of the six stage development system (also supported by Bramlett and O'Gwynn 1981; Williams 2009).
- Place a pollination bag (white densely-woven cloth with clear window) over the female strobili clusters (bagging) and pollinate with a bulb applicator, approximately 1 ml of pollen per pollination event when ovulate cones are receptive at stage five (female strobili completely extended, opening between scales is at maximum) (also supported by Bramlett and O'Gwynn 1981; Williams 2009).
- Perform controlled crosses daily between 10:00 and 16:00 as the female strobili and pollination bags might be covered with night-time dew and deemed too wet to work with before 10:00.
- These wet conditions could contribute to sticky or over hydrated pollen grains, hampering pollination success.
- Pollinate female strobili three times, six days apart, to increase pollination success (also supported by Huyn 1972). Take care that the pollen remains fresh and dry to improve pollination success (also supported by Slee and Abbott 1990).

3.2 Assess generic pollination protocol

Hybrid crosses

Twenty-two cones were harvested (11 each for both *P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii*) on 17 July 2013, approximately 104 weeks after pollination. *Pinus radiata*×*oocarpa* had a female strobili survival of 79 % in July 2012 and a 79 % cone survival at harvesting (July 2013), compared to 70 % female strobili and 55 % cone survival for *P. radiata*×*tecunumanii*. The morphology of the harvested cones of both hybrids resembled commercial *P. radiata* pure species cones. The average fresh weight of *P. radiata*×*oocarpa* cones was 110 g compared to 109 g for *P. radiata*×*tecunumanii* cones. The mature cone dry weight was on average

106 g and 105 g for *P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii*. The average moisture content loss of cones was 3.4 % for *P. radiata*×*oocarpa* and 3.7 % for *P. radiata*×*tecunumanii*.

Computed Tomography scans

Defect analysis of CT scans indicated different densities (Figure 2.1). Blue had the lowest density and therefore, provides a good indication of non-viable seed or air pockets. Red had a higher density, indicating the cone axis which is woodier than the blue herbaceous areas. Viable seed were indicated by a greyish to white colour with a black pit representing the embryo. Of the 22 cones, the following had the most prominent results:

- Cone 205.2 (*P. radiata*×*tecunumanii*) had the highest seed weight, number of seed, number of submerged seed and number of seedlings at six months.
- Cone 226.1 (*P. radiata*×*oocarpa*) had the lowest number of seed submerged and number of seedlings at six months with more blue areas visible than cone 205.2.
- Cone 226.2 (*P. radiata*×*oocarpa*) had the lowest seed weight.
- Cone 226.3 (*P. radiata*×*oocarpa*) had the lowest cone weight after drying. The blue areas are much smaller in cone 226.3 than 226.2, indicating a lower number of air-pockets.
- Cone 204.3 (*P. radiata*×*tecunumanii*) had the heaviest cone after drying.
- Cone 227.4 (*P. radiata*×*oocarpa*) had three different density shades.

Number of filled (viable) seed was counted to compare with the emerged seed. However, the number of seed was on average 200 times higher than the number of emerged seed as it is difficult to count seed in the rotating 3D image. Indicating that this is not a reliable test to predict the number of seed set. However, scanning of enclosed cones can help to predict whether viable seed are present.

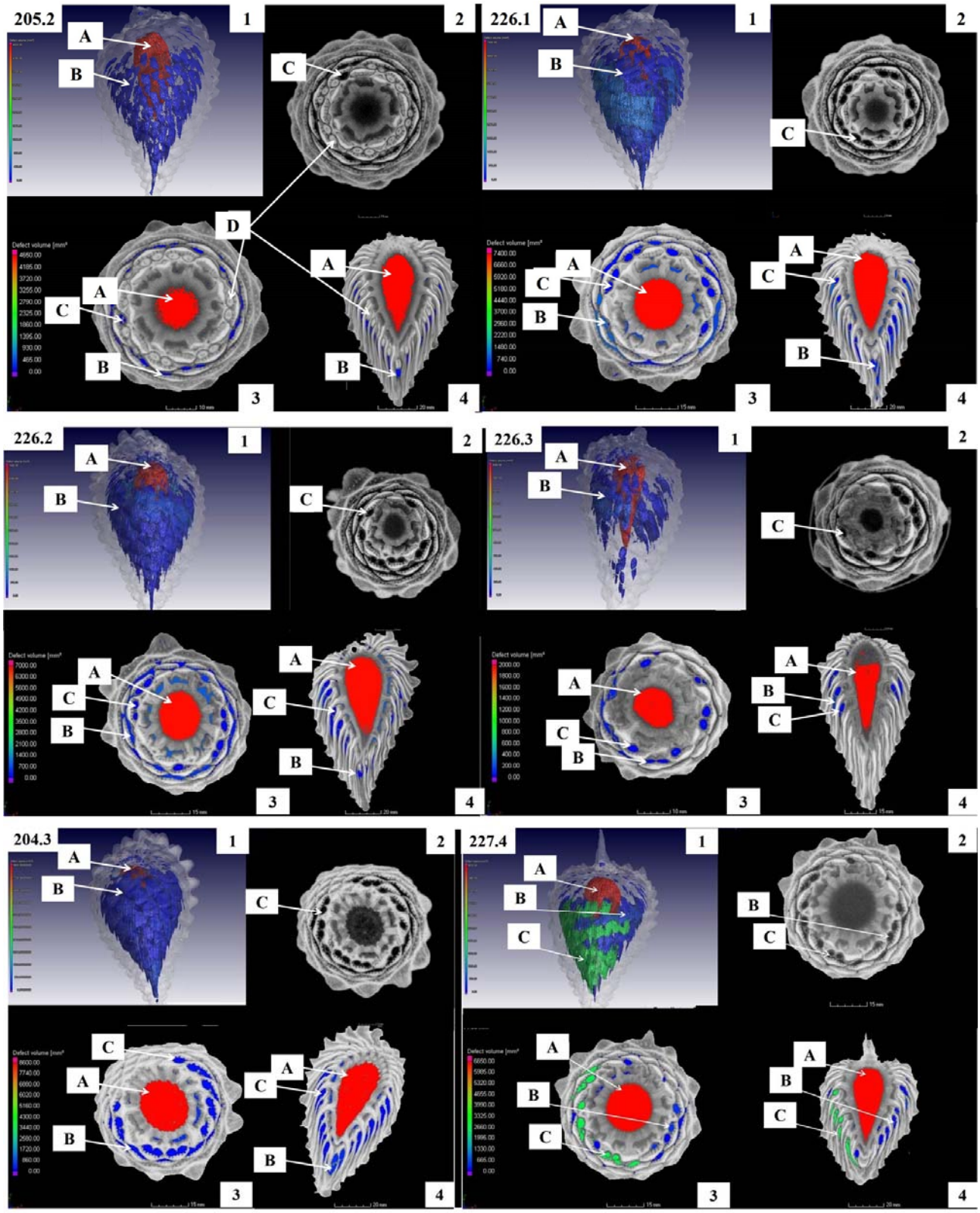

Figure 2.1: Defect analysis of cone 205.2 (*P. radiata*×*tecunumanii*) with the highest seed weight, number of seed, submerged seed and germination of seed at six months; cone 226.1 (*P. radiata*×*oocarpa*) with the lowest number of seed submerged and germinated at six months; cone 226.2 (*P. radiata*×*oocarpa*) lowest seed weigh; cone 226.3 (*P. radiata*×*oocarpa*) lowest cone weight after drying; cone 204.3 (*P. radiata*×*tecunumanii*) heaviest cone after drying; and cone 227.4 (*P.*

radiata×*oocarpa*) with three different colours and densities (1: 3D image; 2: cross section of cone without volume differences; 3: cross section of cone; 4: longitudinal section of cone; A: red indicating higher volume with no air pockets; B: blue indicating lower volume with air pockets; C: blue and green indicating seed filled with air and non-viable; D: viable seed with no air pockets)

Confirmation of hybrid status

Seed size comparison

A total of 722 and 1 214 seeds were extracted from *P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii* cones, respectively. Only three seeds (0.4 %) from the *P. radiata*×*oocarpa* and 425 (35 %) from the *P. radiata*×*tecunumanii* cones were filled (viable) seed according to the water test (Figure 2.2). However, number of filled seed was higher for *P. oocarpa* (29), *P. radiata* (40) and *P. tecunumanii* (39).

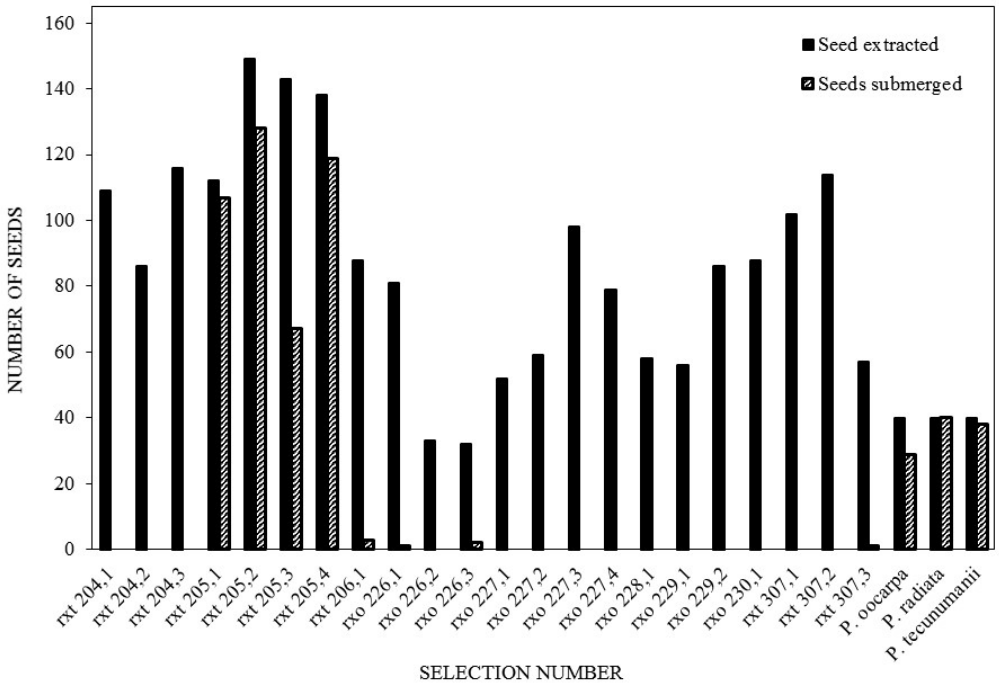

Figure 2.2: Number of seed harvested and submerged during viability testing (rxt: *P. radiata*×*tecunumanii*, rxo: *P. radiata*×*oocarpa*)

There were no significant differences in seed length among the putative hybrid crosses and the control species (*P. radiata, P. oocarpa* and *P. tecunumanii*). For seed width, the *P. radiata* control was significantly larger than the *P. tecunumanii* and *P. oocarpa* controls as expected, but it was difficult to discern differences among the putative hybrids.

In total, 2 043 seeds were sown but only 373 seedlings (18.3 %) were alive after six months (Figure 2.3). Only 0.6 % of *P. radiata*×*oocarpa*, 26 % of *P. radiata*×*tecunumanii*, 38 % of *P. radiata*, 52 % of *P. oocarpa* and

74 % of *P. tecunumanii* seedlings were alive after six months. The survival percentages of *P. radiata* and *P. oocarpa* were extremely low and did not compare well with *P. tecunumanii.* Ten *P. radiata* seedlings died between two and six months while the number of seedlings of *P. oocarpa* stayed constant. The number of seedlings of *P. tecunumanii* increased to three and *P. radiata*×*tecunumanii* to 30 seedlings between two to six months, indicating that *P. tecunumanii* in this study germinated slightly slower than *P. radiata* and *P. oocarpa*.

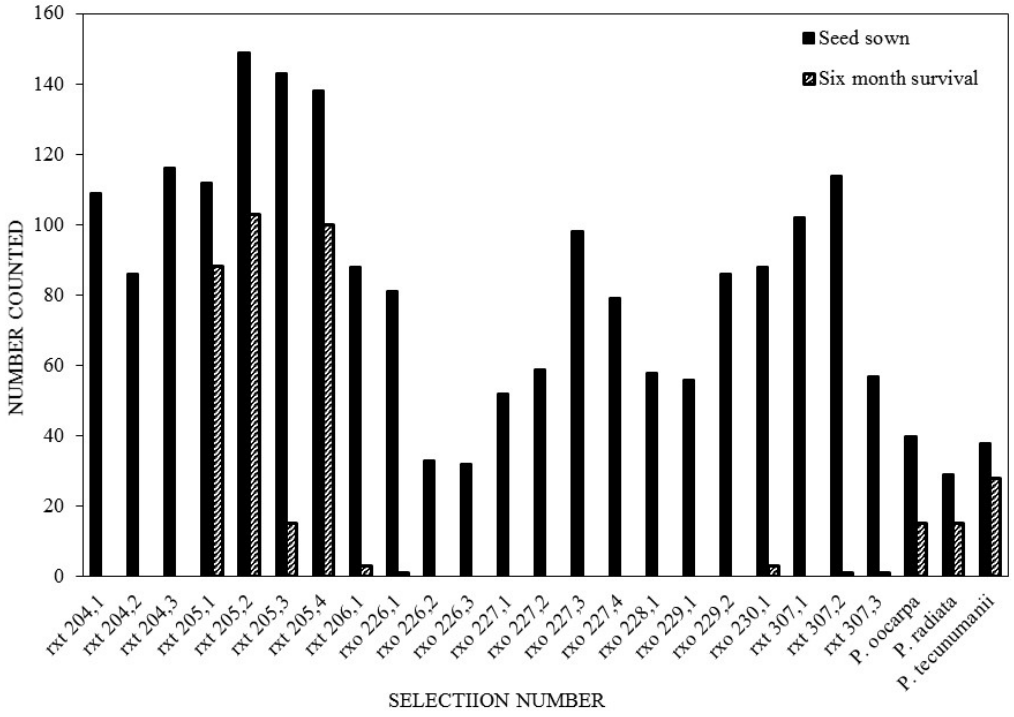

Figure 2.3: Number of seed sown compared to six month seedling survival (rxt: *P. radiata*×*tecunumanii*, rxo: *P. radiata*×*oocarpa*)

***Fusarium circinatum* and DNA screening of six month old seedlings**

Of the 40 seedling selected for disease testing, *F. circinatum* was isolated from the crown area of twelve seedlings (30 %), while 20 seedlings (50 %) had *F. oxysporum* in the crown and roots. No *Pythium* species were isolated from any of the 40 seedlings sent for diagnostic tests, indicating healthy growing conditions. The marker (> 90 % and K = 2) to *P. radiata* analysis indicated that all *P. radiata*×*tecunumanii* and *P. radiata*×*oocarpa* putative hybrids showed strong assignment (> 90 % and K = 2) to *P. radiata*. Therefore, it appears that the putative hybrids were most likely pollinated by *P. radiata* pollen, indicated pollen contamination.

4. Discussion

During a questionnaire survey, a generic pine pollination protocol was determined and assessed in a *P. radiata* seed orchard at Karatara. *Pinus radiata* female strobili were pollinated with *P. oocarpa* and *P. tecunumanii* pollen lots collected in the natural stands in El Castaño and San Jeronimo (Guatemala) by a CAMCORE delegation. Pollen lots were dried to MC of 7.8 % & 6.2 % and a germination percent of 25 and 55 % for *P. oocarpa* and *P. tecunumanii* respectively before couriered to MTO (South Africa) via North Carolina (USA). Unfortunately, pollen germination tests were not repeated when the pollen shipment arrived at Karatara.

Initial pollination success was assessed by the number of mature seed cones harvested 22 months after pollination, number of seed per cone and number of six month old seedlings. Seed set can be influenced by abortion of conelets and female strobili throughout the reproductive cycle of 22 months and might not be a good indicator of pollination success. Previous studies indicated that abortion of pine seed cones varies between 40 and 90 % (Hagedorn 2000), while on average 69 % for *P. patula* were observed (Bester et al. 2000; Nel and van Staden 2003). The survival of female strobili (one year after pollination) and seed cones (two year after pollination) were acceptable during this study; 70 and 55 % for *P. radiata*×*tecunumanii*; and 79 % for *P. radiata*×*oocarpa*. Mature cones harvested during this study reflected the morphology of *P. radiata* cones and could not be used as an indication whether interspecific hybridisation was successful. No significant difference were evident in seed length and width between the putative hybrids. Therefore, seed size also cannot be used as a preliminary indicator of hybridisation success.

CT scanning proved to be a useful tool to follow the development of seeds in the intact cone. Defect analyses showed that there are mainly three types of densities (red, green and blue). Seed were clearly visible, while blue and green coloured seed indicated a high percentage of dull (non-viable or empty) seed. When mature seed cones are harvested in huge quantities, this can be a useful technique to distinguish between cones with high numbers of empty or viable seed. However, the technique is very expensive and personnel need good computer and analytical skills. But, bigger volumes of mature cones can be scanned at one time and cones with too little viable seed can be rejected. This can speed up the process of extracting seed in commercial nurseries working with millions of cones per season.

The number and morphology of mature seed cones could not predict whether interspecific hybridisation was successful. Number of filled seeds would be a good indicator, as a wide cross should have only three to 10 viable seeds. Therefore, the number and size of seed per cone and cross-combination were determined. In *P. wallichiana,* 42 to 91 and 42 to 67 g for 1 000 seed were observed (Aslam et al. 2010). However, the number of seed harvested from this study was on average 66 and 110 per cone and the weight per 1 000 seed was 6.3 to 8.2 g and 8.8 to 22 g for *P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii* respectively. All the seed were subjected to the water test, which indicated that only an average of 0.3 and 39 seed per cone were viable for *P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii* respectively. Water soaking test was not deemed reliabile

enough to establish seed viability. Therefore, all the seed (total of 1 936) were sown to determine seed germination percentage. Unfortunately, seedling survival at six month mirrored the water test.

Fusarium circinatum and DNA screening confirmed no interspecific hybrids were obtained during the experiment. Two *Fusarium* species (*circinatum* and *oxysporum*) were identified on the six month old seedlings. *Fusarium* species may be a secondary and endophytic disease when plants are stressed while *F. oxysporum* are associated with Top Blight on conifer seedlings. As *P. radiata* is highly susceptible to *F. circinatum*, it raises concern that the newly bred seedlings were infected with *F. circinatum* after propagated in a *F. circinatum* free glasshouse. Previous studies indicated that spores can infect seeds, female strobili, needles and mature cones. It can thus be present on the seed surface and internal seed tissue, but seed surface treatments might not be sufficient to limit infection. Seed from trees that are grown in an infested area will never be free from the contamination risk as the spores are in the air and on needles. Furthermore, infected seed can germinate and produce asymptomatic seedlings from which the fungus can be isolated. The fungus can also switch from a latent to an active form of infection, if conditions are favourable. Preventative measures in a seed orchard might include regular spore counts and sterilisation of breeding equipment (Nel 2002; Wingfield et al. 2008; Ioos et al. 2009). In future, the pollination protocol should be amended and include thorough sterilisation of breeding equipment before, during and after the pollination events with associated spore counts throughout the year.

During DNA screening two seedlings appeared to be selfed, while the presence of non-maternal alleles in the other seedlings confirmed that they were outcrossed by different *P. radiata* genotypes. In the future, all hybrid seedlings need to be verified by genotyping and the identity of all ramets in the current *P. radiata* orchard need checked to make sure their pedigree assignments are correct. This will assist with accurate parental confirmation.

The impact of the X-rays during CT scanning on seed viability was not investigated as the penetration of the X-rays intensity could not be measured without damaging the cones. However, previous studies indicated that X-rays are absorbed to varying degrees by different parts of the seed. This helps to distinguish between different tissues and injuries but physiologically changes are not revealed (Simak 1957; Kriebel 1972). Very mild X-ray dosses (~ 100 kV) does not injure physiologically sound (freshly collected) seed and the germination ability should remain high (Simak 1957; Gustafsson and Simak 1958) although this might differ between species (Gustafsson and Simak 1958). Low radiation of 600 to 1 200 r causes a slight increase in germinability, rate of germination, both hypocotyl and root length (Gustafsson and Simak 1958; Ohba and Simak 1961). Whereas radiation of 1 200 to 4 800 r reduced germination percentage, rate of germination and seedling development (Gustafsson and Simak 1958; Ohba and Simak 1961). Furthermore, mature seed is less affected by X-rays than immature seed (Gustafsson and Simak 1958; Johnson 1976).

In conclusion, the general protocol that is currently used with interspecific hybridisation of *Pinus* species was tested to determine *P. radiata* hybridisation success with both pathology diagnostic tests and DNA fingerprinting. Pollen germination was very low and might have played a critical role in the failed attempts to make *P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii* hybrid crosses at the Karatara seed orchard. There might be a number of reasons, but subfreezing temperatures affecting the pollen tube development of the tropical pines was not one of these. Pollination bags needs to exclude unwanted pollen (pollen contamination) and allow the movement of gasses to prevent the build-up of heat, moisture and carbon dioxide (Sedgley and Griffin 1989; Nel and van Staden 2003; Plomion et al. 2007). If pollination bags are not placed on cones at the right time, unwanted pollen (self or wild pollen) could contaminate the cones. Foreign pollen contamination was a major problem in the experiment, which not only causes concern for the hybrid program, but also suggests contamination in the local *P. radiata* breeding program where advanced generation progeny are produced by controlled pollination crossing of the best *P. radiata* selections. It might also suggest that *P. radiata* pollen might outcompete foreign pine pollen. All *P. radiata* reference samples were successfully fingerprinted and represented unique genotypes. STRUCTURE was successfully able to discriminate between *P. radiata* and *P. tecunumanii* reference samples, with strong assignment of all individuals to their respective species. All seedlings were assigned to the *P. radiata* population, suggesting that there was most likely pollen contamination from surrounding *P. radiata* pollen and that the controlled pollinations using the *P. tecunumanii* polymix was unsuccessful. CT scans can provide additional information to pine breeding programmes. Literature indicated that mature seed is not affected by an exposure of 100 kV X-ray radiation for a duration of 60 minutes.

As interspecific hybridisation in this study was not successful with *P. radiata,* the generic pollination protocol that derived from the questionnaire survey needs to be adapted. The following recommendations are made for follow-up studies and will be addressed in the next chapters:

- Investigate different bagging options and sterilisation of breeding equipment before, during and after pollination events.
- Placement of pollination bags inside the trees to manipulate number of sunshine hours.
- The effect of environmental conditions (temperature, relative humidity, dew point) inside the pollination bag on pollination success.
- Compare Karatara climatic conditions to the natural provenances of the pine species chosen for this study.
- The effect of temperature on the pollen grain germination, pollen tube size and pollen tube growth rate (PTGR). These studies should include both *in vitro* and *in vivo* conditions, compared to natural growth conditions.
- The effect of pollination success on conelet development. These studies should determine whether the pollen grain size, pollen tube length and width are sufficient to increase fertilisation success. The number of pollen grains inside the ovule should also be investigated as an indication of pollination success.

Therefore, new guidelines that addresses the environmental conditions inside pollination bags, pollen quality, pollen quantity, pollen tube growth, pollination and fertilisation success could contribute towards better hybridisation success.

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Chapter 3

Investigate climate conditions inside the pollination bags

1. Introduction

Pinus is considered the most important commercial forestry genus within the Pinaceae family, consisting of various species and varieties (Critchfield 1975; Gernandt et al. 2005; Plomion et al. 2007). Some pines have developed geographical races that are morphologically and physiologically distinct, limiting broad adaptability and hybridisation potential (Krugman and Jenkinson 1974). Although interspecific hybridisation can occur naturally in various pines, it is mostly limited to species within a subsection (Plomion et al. 2007) and as the genetic distance between species increases, one would expect to see an increase in hybridisation barriers (Ellstrand 2014) and less pollination success. Where possible, interspecific hybridisation can be used in pine breeding programmes to increase genetic variation through new combinations that would not happen naturally (Zobel and Talbert 1984). There have been various interspecific hybridisation successes that address the following breeding objectives: *Fusarium circinatum* and low levels of frost tolerance *(P. patula×tecunumanii*) (Kanzler et al. 2014); *Cronartium ribicola* tolerance and cold hardiness (*P. strobus×wallichiana*) (Lu and Derbowka 2012); drought tolerance and productivity *(P. elliotti×taeda*) (Dungey 2001); hardiness, rapid growth rate and timber quality (*P. rigida×taeda*) (Huyn 1976; Barnes and Mullin 1978); and tree production and pulp properties (*P. elliottii×caribaea*) (Van der Sijde and Roelofsen 1986; Wright et al. 1991; Cappa et al. 2013).

Despite the above mentioned examples, interspecific hybridisation successes, especially of wide crosses between taxonomic subsections, are difficult and often result in pollination failure (Dungey et al. 2003; Ellstrand 2014). Environmental conditions, biological barriers (for example pollen availability, flowering times, geographic distribution) and physical (climate, wind) limitations (Burdon 1977; Boyer 1981; Greenwood and Schmidtling 1981; Dickson 1995; Dungey 2001; Alzoti et al. 2010) have to be considered in hybridisation programmes to increase pollination success. Reproductive structures and their development (months before pollination season) can be severely influenced by environmental factors (Alzoti et al. 2010) as these factors are not necessarily similar during male (catkins) and female (strobili or conelets) development (Burdon 1977; Boyer 1981; Greenwood and Schmidtling 1981; Dickson 1995). The length of the receptive period (female strobili) and availability of pollen (male strobili) are also affected by weather conditions during the pollination season (Dickson 1995; Alzoti et al. 2010).

Pine breeding programmes make use of fairly simple equipment and protocols to address the above mentioned pollination constraints (Chapter 2). One of the key tools is the pollination bag (micro-fibre with clear window in South Africa) used in controlled pollination to limit contamination from foreign pollen (Bramlett and O'Gwynn 1981; Nel and van Staden 2005). During controlled-crosses, the environment inside the pollination bag can create unfavourable climatic (temperature and humidity) conditions which could affect pine pollen germination (McWilliam 1959a; Bester et al. 2000). Various studies in different geographic regions, have tested different pollination bag materials that allows for improved air exchange between the inside (micro) and outside (macro) of the bag, while still restricting foreign pollen contamination (Ferrand 1988; Sweet et al.

1992; Hagedorn and Raubenheimer 1996; Hagedorn et al. 1997; Hagedorn 2000; Nel and van Staden 2003; Neal and Anderson 2004). However, limited information is available on the micro (inside pollination bag) compared to the macro-climate (outside the pollination bag) as a possible barrier to pollination success.

To understand the effect that pollination bags might have on pollination success, it is important to understand the reproductive biology of pines. The female strobilus receptivity consists of six stages and can be summarised as follows: Stage 1 - female strobili buds appear on vegetative shoots but are tightly enclosed in bud scales (too early for isolation bagging); stage 2 – female strobili buds are still enclosed inside bud scales (ideal for isolation bagging); stage 3 – each strobilus emerges through the top of scales (too late to bag); stage 4 – strobilus elongates but ovuliferous scales have not opened fully (pollen application possible although early); stage 5 – ovuliferous scales of the female strobilus are now at right angles to the cone axis (optimum for injecting pollen into isolation bag); stage 6 – female strobilus is not receptive because ovuliferous scales have swollen completely (too late for pollination). Depending on climate and species, it takes approximately seven days from stage 2 when strobili can be bagged to stage 5 when pollination can be applied (Bramlett and O'Gwynn 1981; Williams 2009).

The objective of this study was to investigate if the environmental conditions (temperature, relative humidity (RH) and dew point (DP)) during isolation of female strobili within pollination bags and their position on the tree (top, middle, north and south) can affect pollination success. The climatic conditions of seed orchards where the pine species under consideration are being grown in South Africa were, therefore, investigated. Micro- and macro-climate variables were collected at Karatara (Southern Cape) for three consecutive pollination seasons and complimented with macro-climate data from three seed orchards around Sabie, Mpumalanga Province (Tweefontein, Witklip and Spitskop). This data together with natural provenance climatic data (altitude, precipitation and temperature (maximum, minimum and mean)) were used to assess the possible effects of temperature on pollination success.

2. Materials and methods

The generic pollination protocol employed in Chapter 2 (Section 2.2.1) were amended during this study as follows: to limit pollen contamination two types of pollination bags were investigated (green and white cloth); and placement of pollination bags inside the trees to manipulate number of sunshine hours. These experiments were only performed to collect actual climatic data inside (micro) and outside (macro) the pollination bags. No pollinations were performed during the collection of the data.

2.1 Environmental conditions of pollination bags

The primary study was performed in a *P. radiata* pine seed orchard at Karatara (33 ° 54 ' 0 " South; 22 ° 50 ' 0 " East) owned by MTO Forests. The orchard is situated at 239 m.a.s.l. and receives an annual rainfall of 650 mm, mainly in winter. It differs from many other *P. radiata* pine seed production areas as the minimum temperatures never fall below freezing even during the coolest months of the year.

Environmental conditions between the top and middle of tree, as well as north and south facing branches, were investigated during the pollination season (July and August) over three consecutive years (2011, 2012 and 2013). For consistency, one clone (AR 366) and 10 ramets per experiment (year interval) were used. Clusters of female strobili were identified and isolated at the second stage of the six-stage development system as described by Bramlett and O'Gwynn (1981). Micro-fibre pollination bags (white and green cloth with a clear window, Figure 3.1) were placed over female cone clusters and tied to the branch (13 July). White cloth bags are generally used at Karatara. However, the green cloth bags (used by Sappi) are closer interweave with fewer openings to limit pollination contamination.

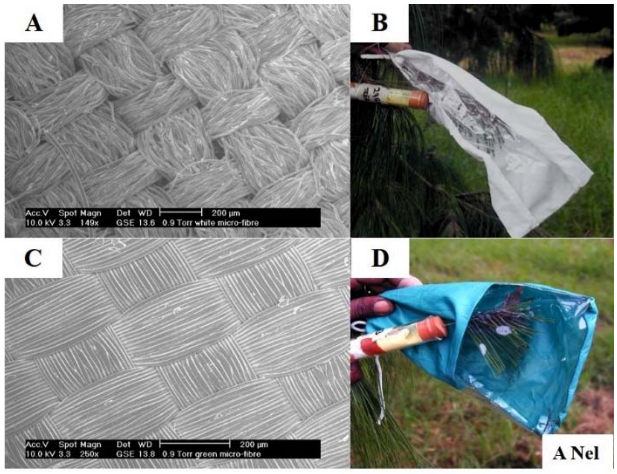

Figure 3.1: White (A and B) and green (C and D) woven cloth pollination bags investigated during this study

EL USB 2 data loggers (water resistant) were attached to the inside (micro) and outside (macro) of pollination bags to register changes in micro- and macro-climate (Figure 3.2). Two data loggers were used per pollination bag and will be referred to as a logger-bag set. Hourly temperature (˚C), RH (%) and DP (˚C) measurements were logged from time of bagging until six weeks after pollination (total of 50 days). Other data collected included: the date when data loggers were started, date of bagging, location in the ramet (top, middle, north or south), tree identification code, date of cross pollination, date of de-bagging and date when data loggers were removed. This experiment aimed to collect only climatic data and no pollinations were performed, but the impact of the sterilisation on the equipment were tested for feasibility purposes only.

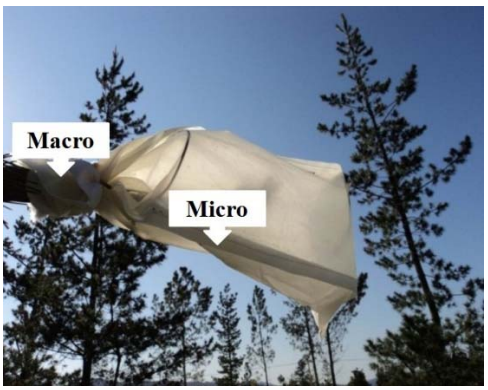

Figure 3.2: Photo showing the logger-bag set with the position of the micro (inside pollination bag) and macro (outside pollination bag) data loggers recording hourly temperature (˚C), RH (%) and DP (˚C) measurements

Both micro and macro data were collected annually as follows (Figure 3.3):

- 2011: Ten *P. radiata* trees were selected and a logger-bag set was placed in the top of each tree for optimum sunlight exposure (total of 10 logger-bag sets with minimal tree shade effect). Half were white and half were green cloth bags.
- 2012: Ten *P. radiata* trees were selected and a logger-bag set was placed in the middle of each tree on either the northern or southern side (five logger-bag sets on northern side and five on southern side, all shaded by branches in the middle of the tree). Care was taken to place the logger-bag sets on branches that were oriented towards either north or south while branches that faced more east or west were ignored. Half were white and half were green cloth bags.
- 2013: Fourteen *P. radiata* trees were selected and each logger-bag set was placed on the northern side towards the top of trees to simulate optimum sunlight exposure (14 logger-bag sets with minimal tree shading effect). Half were white and half were green cloth bags.

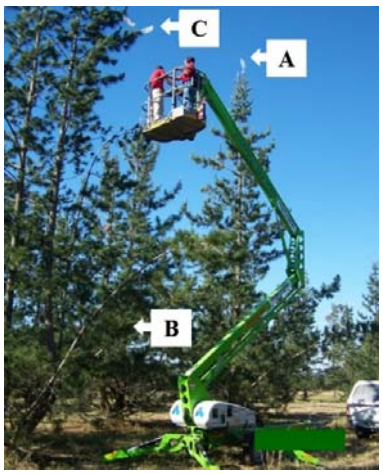

Figure 3.3: Photo indicating the different positions of the pollination bags in the trees (A: top of tree, B: middle of tree, C: top of tree north) during three consecutive pollination seasons

Measurements were downloaded from the data loggers with EasyLog USB version 5.51 and exported to MS Excel 2010. Descriptive statistics, graphs and tables were generated in MS Excel and presented in the results section. To determine the effect of temperature and RH on pollination success, data from the day before pollination, day of pollination and two days after pollination were isolated. During the study period (13 July to 31 August 2011, 2012 and 2013), sunrise was approximately at 07:30 and sunset around 17:30. Therefore, day period was taken as 08:00 to 17:00 and night from 17:01 to 07:59. Temperature, RH and DP data were not subjected to statistical analysis, but recorded values and daily averages were plotted to show differences (similar to analysis by Nel and van Staden 2003). Theron (2000) emphasises that the use of that averages are a reliable way to illustrate climatic trends. The time of hand pollination and pollen droplet (emergence after pollination) are indicated on the graphs. The pollination drop (pollen droplet) is defined as an aqueous, protein-rich substance secreted at the micropyle of the ovule during pollination which retracts after capturing the pollen grain. It only appears between 02:00 and 04:00 in *P. taeda* (Williams 2009). It can also be rich in sugars (Nepi et al. 2009).

A secondary study was performed in three seed orchards (Tweefontein 25 ° 0 ' 38 " South, 30 ° 45 ' 28 " East; Witklip 25 ° 12 ' 54 " South, 30 ° 51 ' 41 " East; and Spitskop 25 ° 07 ' 34 " South, 30 ° 47 ' 08 " East) around Sabie (Mpumalanga, South Africa) where *P. oocarpa*, *P. tecunumanii*, *P. pringlei*, *P. maximinoi*, *P. elliottii*, *P. taeda* and *P. patula* are planted as potential reciprocal hybrid partners for *P. radiata* during the pollination season (May to June) in 2015. Hourly temperature, RH and DP data were collected and analysed over a 50-day period as described earlier (10 logger-bag sets per locality). As the pine species flowers at different times than *P. radiata*, data were collected in May (Month 1) and June (Month 2) 2015 and compared to Karatara (July as Month 1 and August as Month 2). To mimic natural conditions, logger-bag sets were placed randomly in trees (north, south, middle and top) as determined in the initial pollination protocol (Chapter 2). The data were averaged per day and hour, for example 50 (days) temperature readings at 08:00 were averaged to give one value for 08:00. This was done to determine whether reciprocal crosses might be feasible with *P. radiata* pollen at these three seed orchards. Only macro-climate data were collected to compare with the micro- and macro-climate data collected at Karatara.

Data collected at Karatara and Sabie were used to develop three circadian climatic models, simulating temperature, RH and DP fluctuations, for Sabie (average of Tweefontein, Spitskop and Witklip), Karatara micro- (Kmic) and Karatara macro-climate (Kmac). These circadian models will be used in follow-up studies to compare pollen tube germination and elongation of the eight species investigated in this study (Chapter 4).

2.2 Natural climatic conditions versus Karatara

Provenance data (state, country longitude and latitude) per species were obtained from CAMCORE (Woodbridge personal communication 2015). Data were filtered to extract provenance data for *P. radiata*

(Karatara and USA), *P. tecunumanii*, *P. oocarpa*, *P. maximinoi*, *P. pringlei*, *P. elliottii*, *P. taeda*, *P. patula*, Tweefontein, Witklip and Spitskop (Figure 3.4). Although Tweefontein, Witklip and Spitskop are seed orchards and not species, they are representative of a sample of South African provenances for the seven pine species and are thus compared to Karatara (South African provenance for *P. radiata*).

BIOCLIM (2016) historical data (interpolations of observed data, representative of 1950 to 2000) for temperatures (maximum, minimum and mean), altitude and precipitation were extracted from map tiles 11, 22, 23, 36, 37 and 46. Unfortunately, the BIOCLIM data base does not have any RH and DP data for all the provenances. Although various databases do have RH information, it is only available for limited cities which is sometimes at a lower altitude or a considerable distance from the actual provenance where CAMCORE collects pollen and plant material. As CAMCORE uses the same GPS data points when collecting plant material as the BIOCLIM database, it was decided to use the more accurate provenance climatic data of the BIOCLIM database. Data were imported into QGIS 28 Wien as raster images and an overlay was conducted with delimited text layering. Data was extracted for January (as Month 1) and February (as Month 2) from the natural provenances of the eight species in the Northern Hemisphere, for comparison with the pollination season at Karatara, Tweefontein, Witklip and Spitskop (July as Month 1 and August as Month 2).

The experimental design was a completely randomised design. The treatment design was a factorial with two factors namely species with 14 levels (*P. radiata*, *P. oocarpa*, *P. tecunumanii*, *P. maximinoi*, *P. patula*, *P. elliottii*, *P. taeda*, *P. pringlei*, Karatara, Tweefontein, Witklip, Spitskop, Kmic and Kmac) and time with two levels (Month 1 and Month 2). Altitude was only compared between provenances as it remained the same for both months. Precipitation and temperature (mean, maximum and minimum) were compared as two separate months and the combined effect. The data were subjected to analysis of variance (ANOVA; Appendix B) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.2; SAS Institute Inc, Cary, USA). Shapiro-Wilk test was performed on the standardised residuals from the model to verify normality (Shapiro and Wilk 1965). Fisher's least significant difference was calculated at the 5 % level to compare treatment means (Ott and Longnecker 2001). A probability level of 5 % was considered significant for all significance tests.

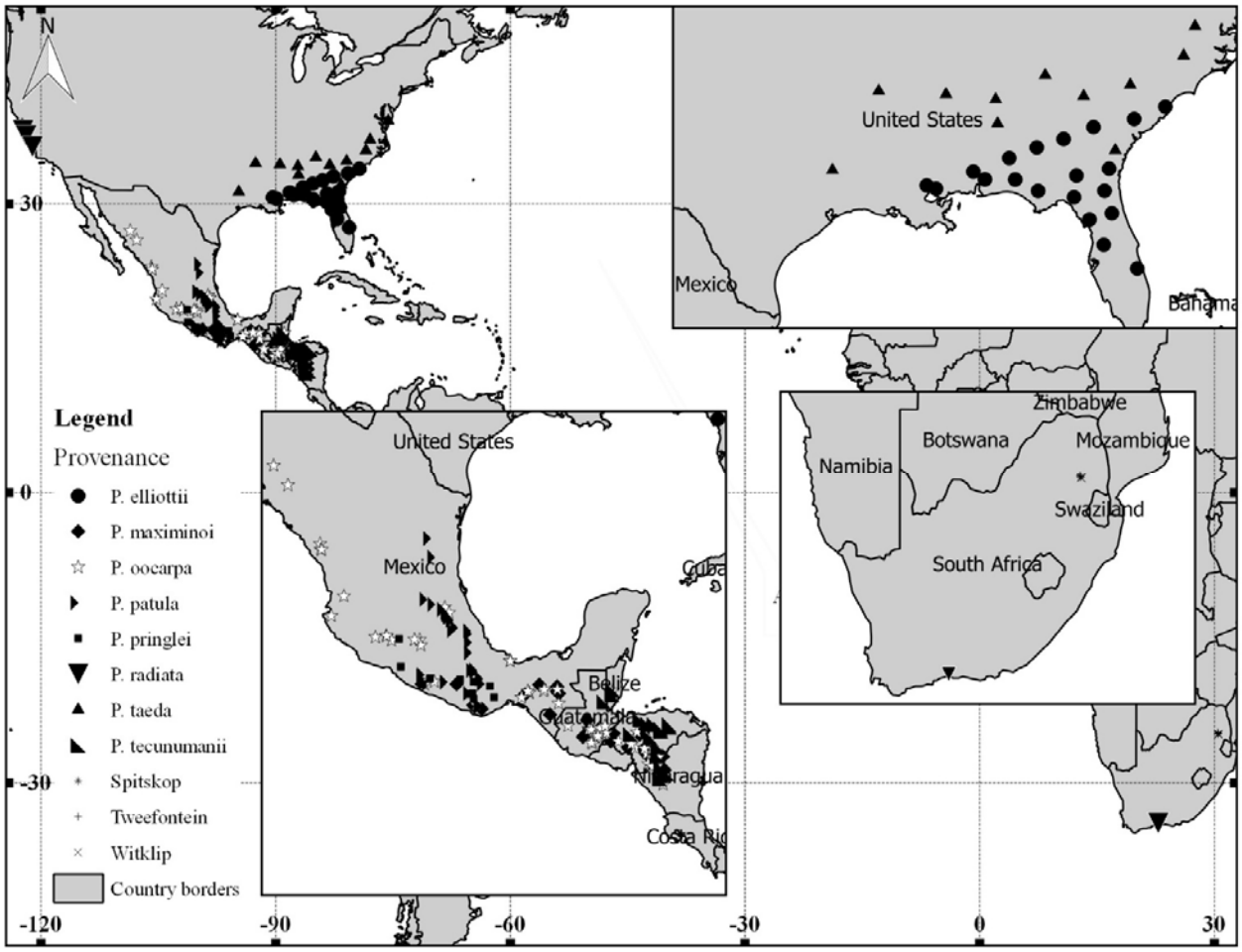

Figure 3.4: Map of North America and South Africa indicating the natural provenances of *P. elliottii, P. maximinoi, P. oocarpa, P. patula, P. pringlei, P. radiata, P. taeda, P. tecunumanii,* Karatara and Sabie (Spitskop, Tweefontein and Witklip)

A Discriminant Analysis (DA) and Principal Component Analysis (PCA) were performed on the interaction between provenance and species with all five variables (Rencher 2002). Biplots were constructed to distinguish between natural and distinctive groups (DA), while quantitative variables were determined for correlations between multidimensional datasets (PCA) (Kohler and Luniak 2005; Erasmus et al. 2016).

3. Results

3.1 Environmental conditions inside pollination bags at Karatara

There was no significant difference in the temperature, RH and DP measurements between the green and white cloth bags. To limit pollination contamination the following are recommended for future pollination seasons at Karatara: better interwoven green cloth bag; seal openings created by bulb applicator regularly; daily sterilisation of breeding equipment; and replace bags every three years.

Temperature

Data loggers in the top of trees that were exposed to maximum sunlight during the pollination season of 2011 indicated that there were larger temperature differences within the micro than the macro environment (Figure 3.5). The minimum and maximum temperatures in the micro environment ranged between 10 and 32 °C, while temperatures ranged between 11 to 22 °C in the macro environment. The micro temperature was on average 10 °C higher than the macro during the day and on average 1 °C lower during the night. Micro temperatures increased and decreased more rapidly than the macro temperatures close to sunrise and sunset and the micro temperatures peaked approximately at 12:00 while the macro temperatures peaked at about 15:00 each day. During pollination events, the micro temperature was on average 8 °C higher than the macro temperature, but during the pollen droplet period it was only 1 °C lower.

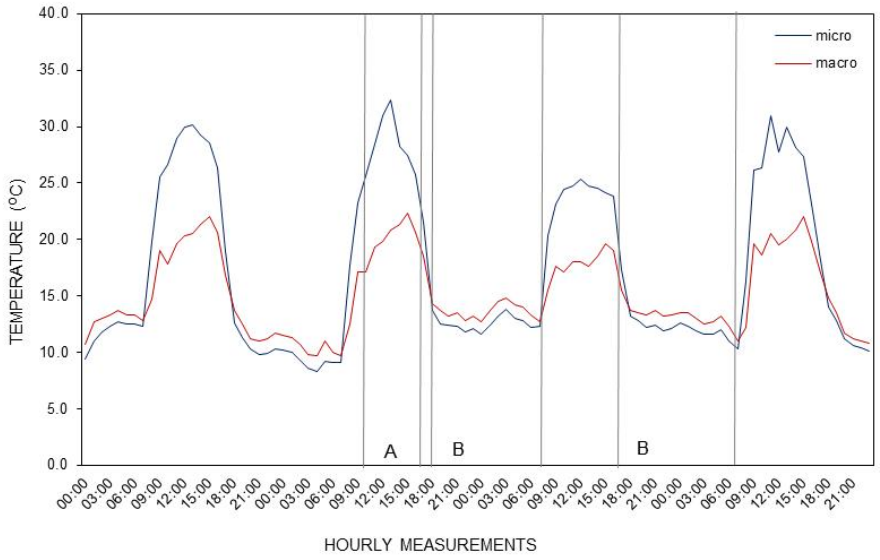

Figure 3.5: Average micro and macro temperature for 2011 (A: pollination period; B: pollen droplet)

During the pollination season of 2012, data logger-bag sets were placed approximately in the middle of the trees and as close as possible to the northern (less shaded) or southern (shaded) side of the trees. This was done to compare aspect (north and south) and position in tree (middle in 2012 and top of tree 2011). The temperature differences between micro and macro were not as evident as in 2011. Northern micro and macro temperatures differed less than 1 °C during the night and less than 2 °C during the day (Figure 3.6) and ranged from 4 to 31 °C. Southern micro temperatures ranged from 4 to 25 °C while macro temperatures ranged from 5 to 25 °C. Micro and macro temperatures differed by approximately 1 °C for both the pollination and pollen droplet periods (Figure 3.7).

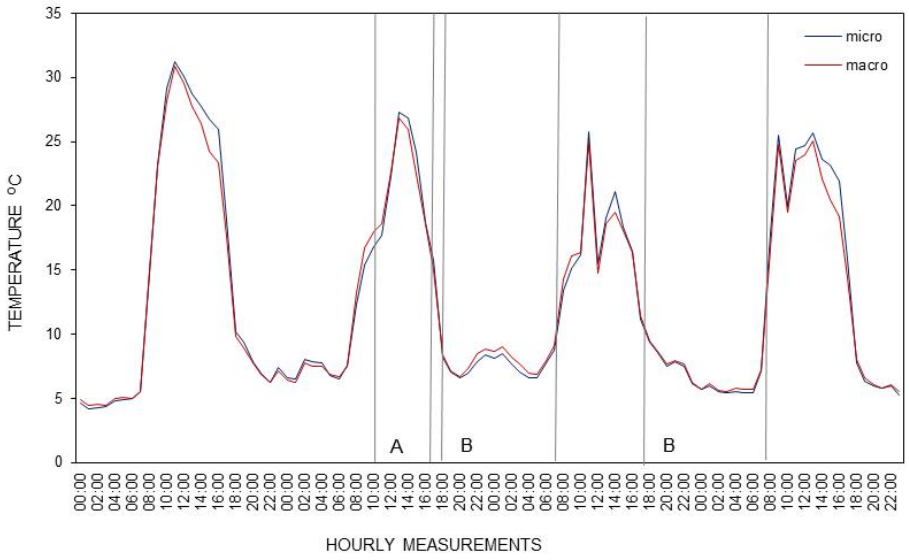

Figure 3.6: North micro and macro temperature for 2012 (A: pollination period; B: pollen droplet)

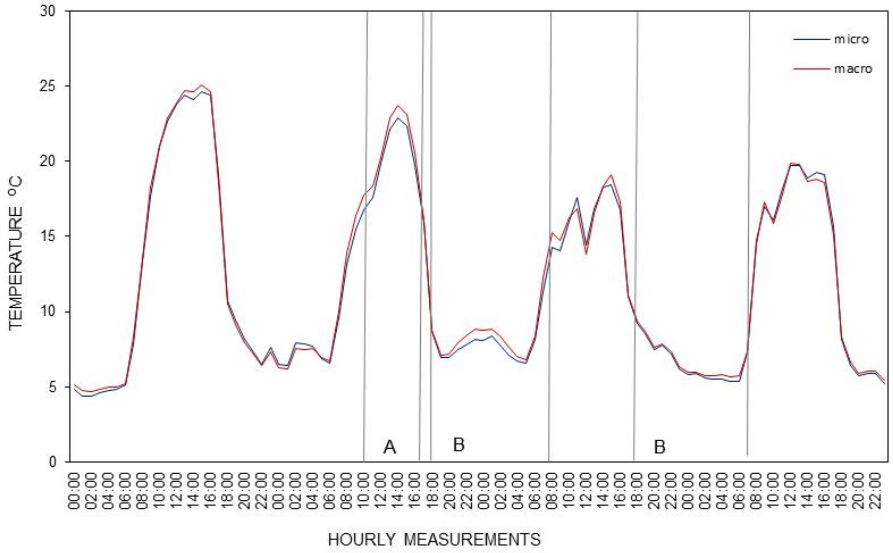

Figure 3.7: South micro and macro temperature for 2012 (A: pollination period; B: pollen droplet)

There was less of a temperature difference between micro and macro temperatures than observed during the 2011 season for both northern and southern logger-bag sets. However, when the temperatures of northern and southern logger-bag sets were compared, there was a clear difference (Figure 3.8). North was on average 5 °C warmer during the day but less than 1 °C cooler than south. However, the increases and decrease in temperatures around sunrise and sunset were more rapid than in 2011. While the micro and macro temperatures for both north and south peaked at the same time (Figures 3.6 and 3.7) there were differences in peak temperature time in both micro and macro temperatures between north and south. Temperatures on the northern side peaked at around 12:00 while temperatures on the southern side peaked at about 15:00 (Figure 3.8 and 3.9). This difference was quite pronounced in the micro temperatures. For both north and south

aspects the micro temperatures were higher during pollination and lower during pollen droplet than the macro temperatures.

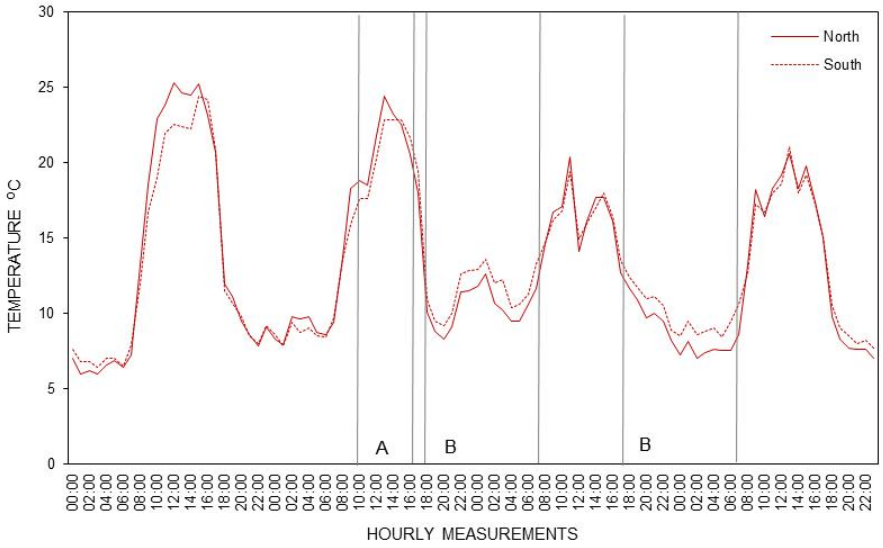

Figure 3.8: North and south macro temperature for 2012 (A: pollination period; B: pollen droplet)

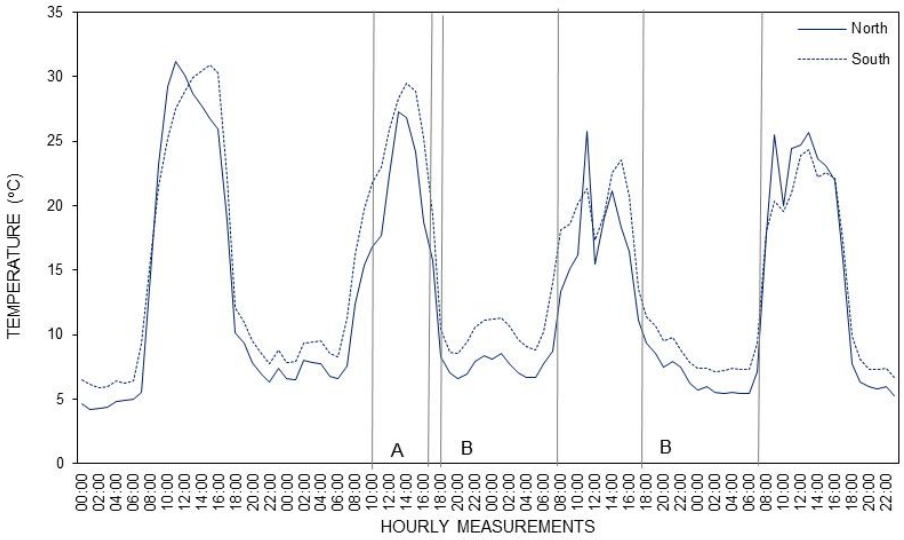

Figure 3.9: North and south micro temperature for 2012 (A: pollination period; B: pollen droplet)

Data loggers were placed in the northern top part of trees during the pollination season of 2013 for maximum exposure to direct sunlight. The average micro and macro temperatures showed the same trend as for the 2011 and 2012 season. During daytime the micro temperatures were on average 5 °C higher and less than 1 °C lower at night than the macro temperatures (Figure 3.10). The micro temperatures ranged from 7 to 32 °C while the macro ranged from 7 to 26 °C. Peaks in micro and macro temperature were however slightly closer together at about 12:00. During pollination micro temperature was much higher but lower during pollination

drop than the macro temperatures. On average, the 2013 temperatures correlated better with the 2011 than the 2012 pollination season.

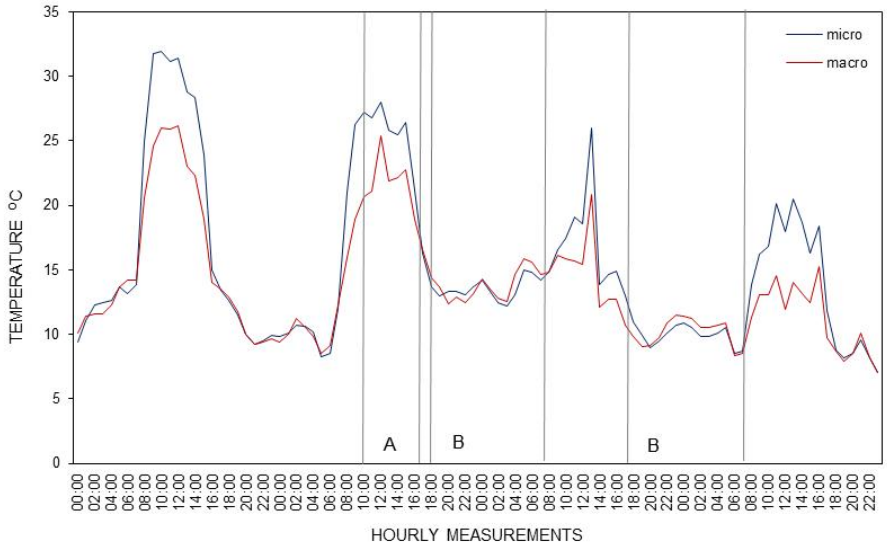

Figure 3.10: Average micro and macro temperature for 2013 (A: pollination period; B: pollen droplet)

Dew point

DP is the temperature at which water vapour condenses into liquid water (dew) at the same rate at which it evaporates. It is expressed in degrees Celsius and indicates the degree of dryness in the air. For example, 10 to 16 °C is comfortable for humans but less than 10 °C is considered dry air. DP will always be lower than actual temperature. At the equator, the DP will be much higher than in a desert region.

Micro DP ranged from 3 to 14 °C while macro ranged from 2 to 11 °C during the 2011 pollination season (Figure 3.11). The micro DP is as much as 6 °C higher during the day and only 1 °C higher during the night than the macro DP. There was a sharper increase and decrease in the micro DP during the day than the macro DP. During pollination and pollen droplet the micro DP tended to be higher than the macro DP. The average DP for 2011 showed the same trend as temperature for 2011 pollination season.

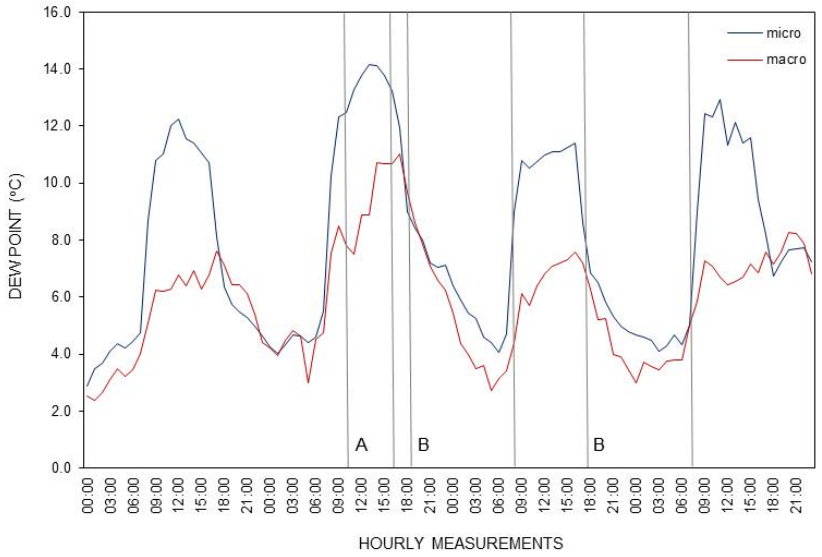

Figure 3.11: Average micro and macro DP for 2011 (A: pollination period; B: pollen droplet)

Northern micro DP ranged from 2 to 20 °C while macro ranged from -3 to 11 °C (Figure 3.12). Furthermore, micro DP was as much as 8 °C higher during the day but only on average 1 °C higher during the night than macro DP. However, during the first pollen droplet, macro DP was up to 8 °C lower than micro DP. Northern micro DP also increased and decreased more rapidly than macro DP. The negative macro DP indicates that the air was dry and that dew could have formed on needles. Southern micro and macro DP showed the same pattern as northern (Figure 3.13). The micro DP ranged from 0 to 18 °C while the macro ranged from -2 to 11 °C. This was expected as the data loggers on the southern side of the tree received more shade than the northern side. The lowest southern macro DP corresponds with the lowest northern macro DP. The difference between south micro and macro DP was up to 8 °C higher at pollination during daytime, while it was on average 1 °C higher during the night except during the first pollination drop.

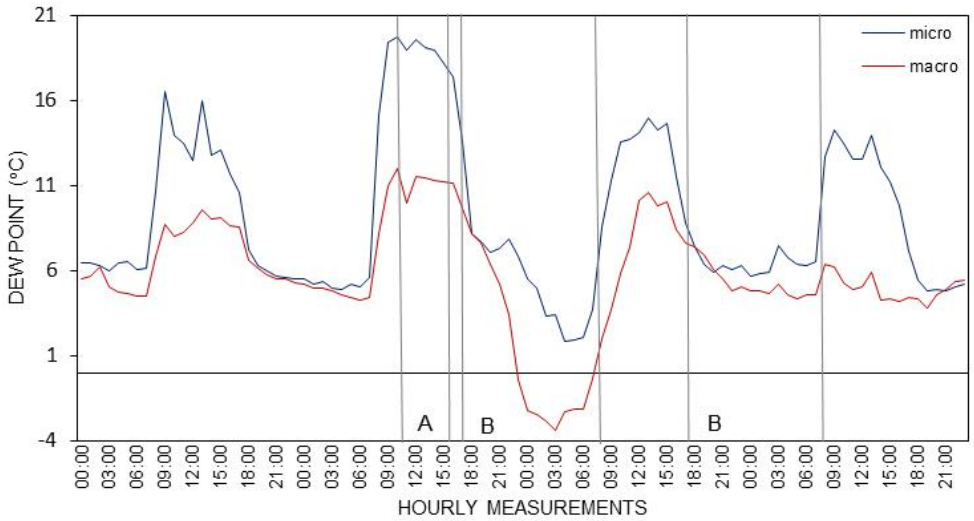

Figure 3.12: North micro and macro DP for 2012 (A: pollination period; B: pollen droplet)

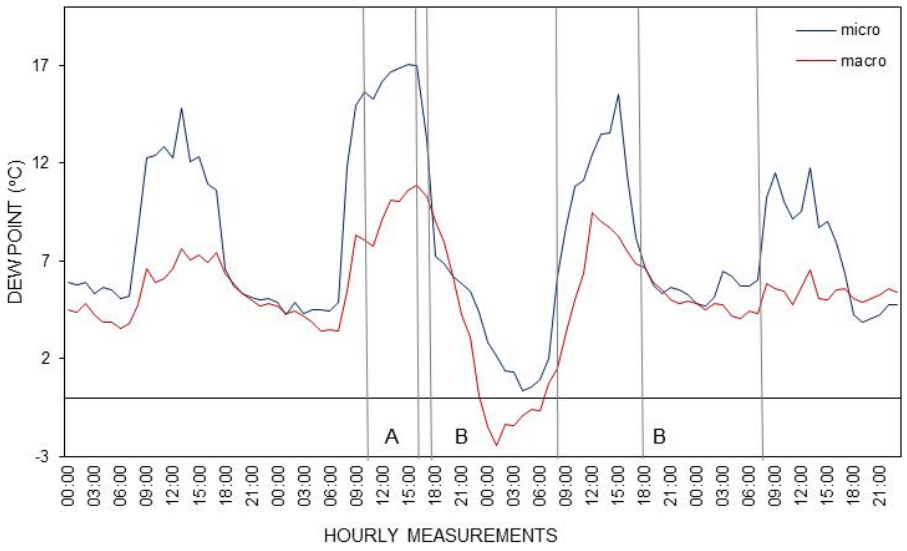

Figure 3.13: South micro and macro DP for 2012 (A: pollination period; B: pollen droplet)

Northern macro DP was generally higher than southern macro DP except during the night of the first pollen droplet (Figure 3.14). The north macro DP was up to 4 °C higher during the day and up to 2 °C higher in the night than the south macro DP. The lowest macro DP was on the northern side during the first pollen droplet. There is a sharper increase and decrease in temperatures on the northern than the southern side.

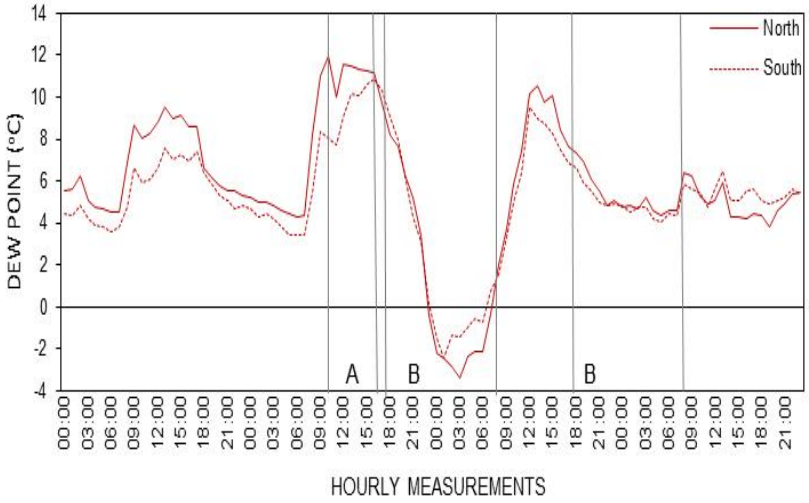

Figure 3.14: North and south macro DP for 2012 (A: pollination period; B: pollen droplet)

The northern micro DP was generally higher than the southern one (Figure 3.15). The north micro DP was up to 4 °C higher during the day and up to 2 °C higher during the night than the south micro DP. Furthermore, the north had sharper fluctuations in DP temperature than the south.

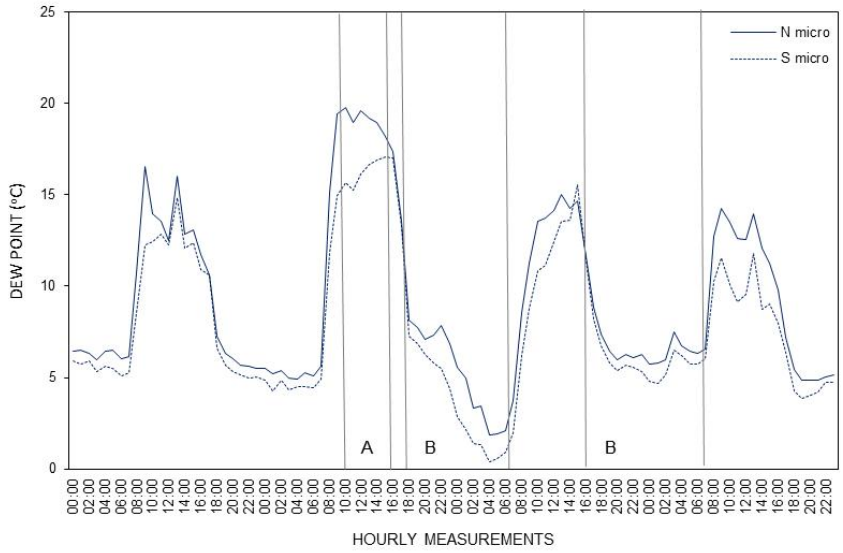

Figure 3.15: North and south micro DP for 2012 (A: pollination period; B: pollen droplet)

The macro DP for the 2013 pollination season ranged from -3 to 11 °C while the micro ranged from 1 to 15 °C (Figure 3.16). As previous figures indicated, the micro increased and decreased more rapidly than the macro DP temperatures. The micro DP was up to 8 °C higher during the day and up to 4 °C higher during the night than the macro DP. The macro DP for 2013 corresponds with the 2011 pollination season and was not as low as during the 2012 pollination season.

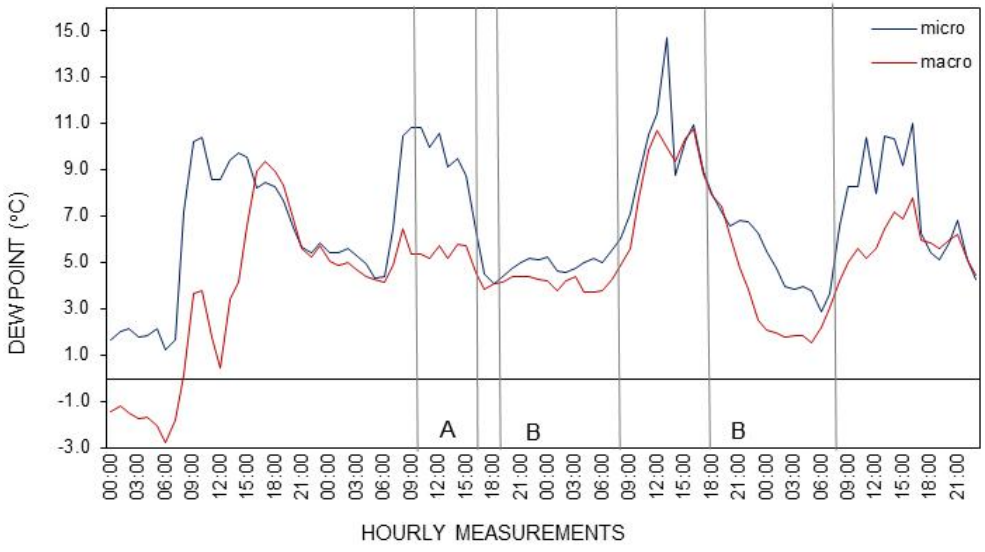

Figure 3.16: Average micro and macro DP for 2013 (A: pollination period; B: pollen droplet)

Relative Humidity

Humidity is the amount of water vapour in the air. RH measures the current absolute humidity relative to the maximum for that temperature and is expressed as a percentage. It is a function of both water content and

temperature. In general, when the RH is lower than 25 %, the air feels dry. When RH is higher than 60 %, the air feels wet and higher than 70 % water condenses on cold surfaces. At Karatara pollinations are performed when there is no condensation visible in the pollination bags.

During the 2011 pollination season macro RH ranged from 43 to 82 % while micro RH ranged from 34 to 84 %. The average difference between micro and macro was 7 % during the night and up to 14 % during the day. Micro RH was lower during pollination than macro RH but higher during pollen droplet (Figure 3.17).

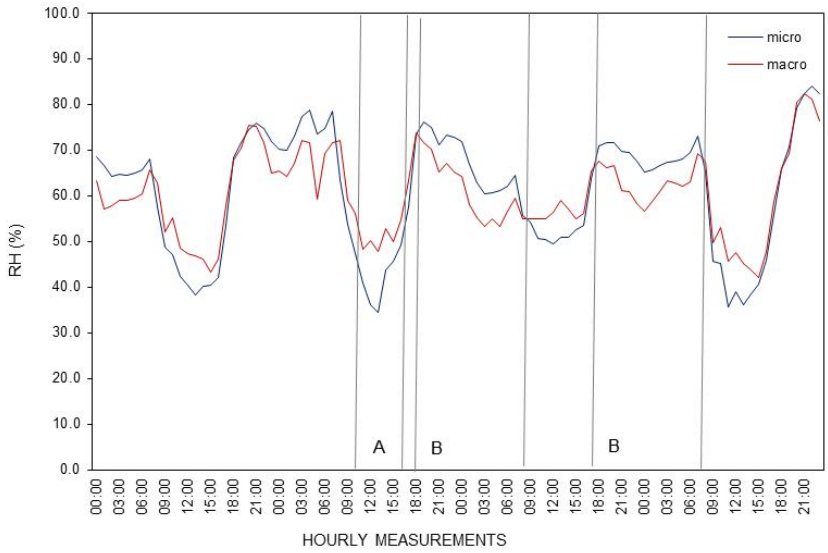

Figure 3.17: Average micro and macro RH for 2011 (A: pollination period; B: pollen droplet)

Northern macro RH ranged from 18 to 88 % while micro RH ranged from 38 to 90 % (Figure 3.18) during the 2012 pollination season. Thus, micro RH was as much as 26 % higher than macro. During pollination, macro RH was slightly lower than micro RH, but similar to 2011. The micro RH was higher during pollen droplet than macro RH. Southern macro RH ranged from 33 to 86 % while micro ranged from 33 to 87 % (Figure 3.19). During pollination, micro RH was slightly lower than macro RH but there was not a large difference in micro and macro RH during pollination drop.

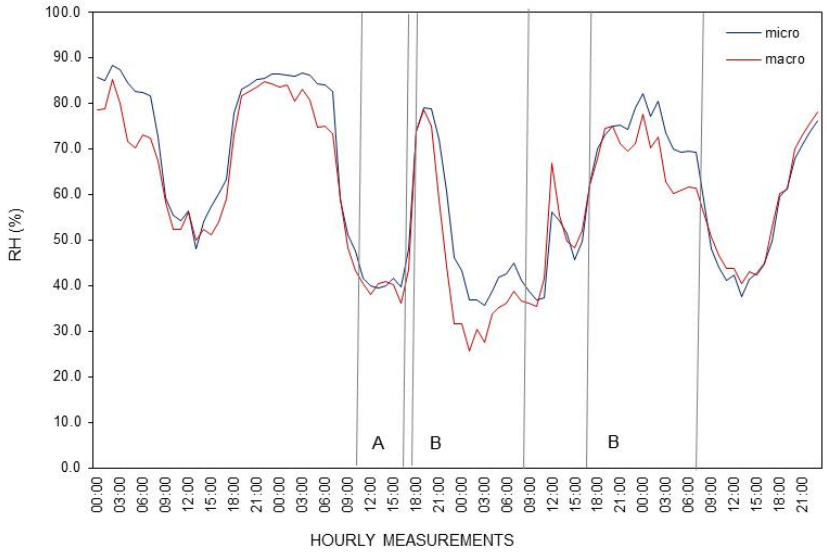

Figure 3.18: North micro and macro RH for 2012 (A: pollination period; B: pollen droplet)

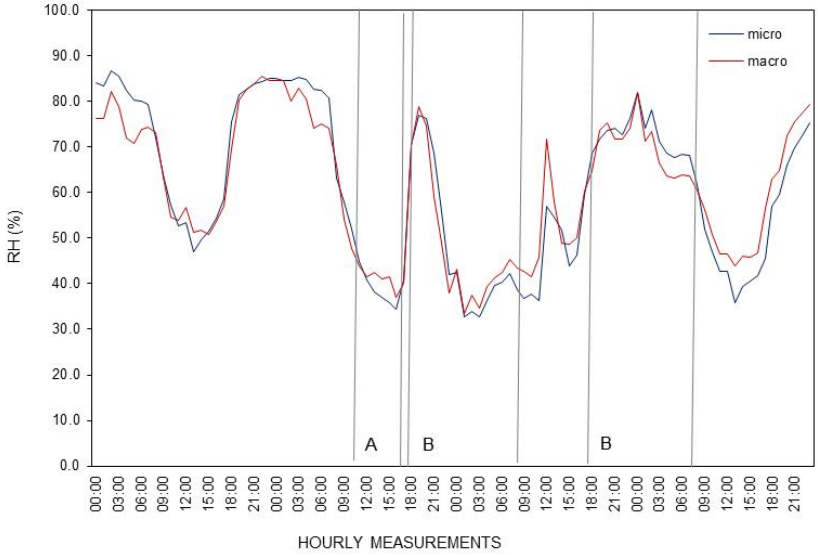

Figure 3.19: South micro and macro RH for 2012 (A: pollination period; B: pollen droplet)

Northern macro RH was often lower than southern RH with more pronounced differences during mid-day (Figure 3.20), while northern and southern micro RH appeared closer together than macro RH (Figure 3.21). However, northern micro was higher during the day at time of pollination, as well as during the night during pollen droplet. Micro RH ranged from 29 to 86 % while macro RH ranged from 19 to 90 % during the 2013 pollination season (Figure 3.22). Macro was lower during pollination and pollen droplet than micro RH.

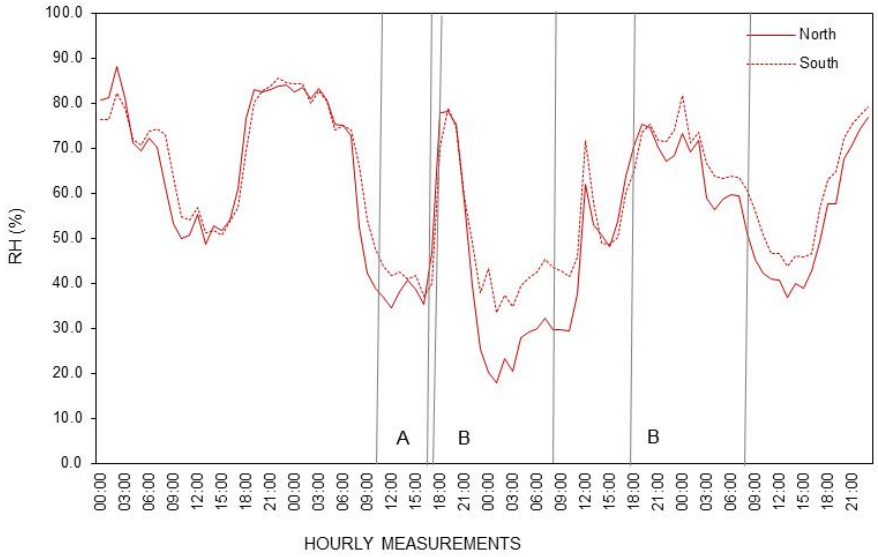

Figure 3.20: North and south macro RH for 2012 (A: pollination period; B: pollen droplet)

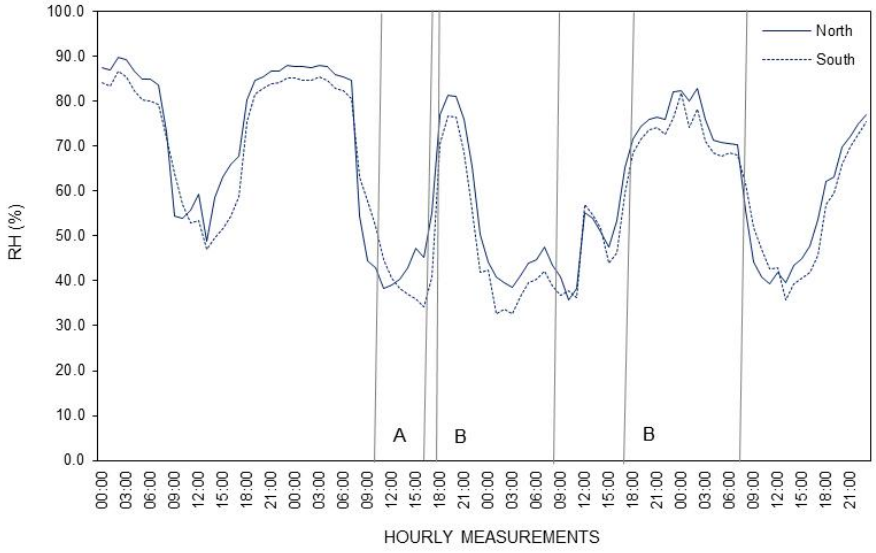

Figure 3.21: North and south micro RH for 2012 (A: pollination period; B: pollen droplet)

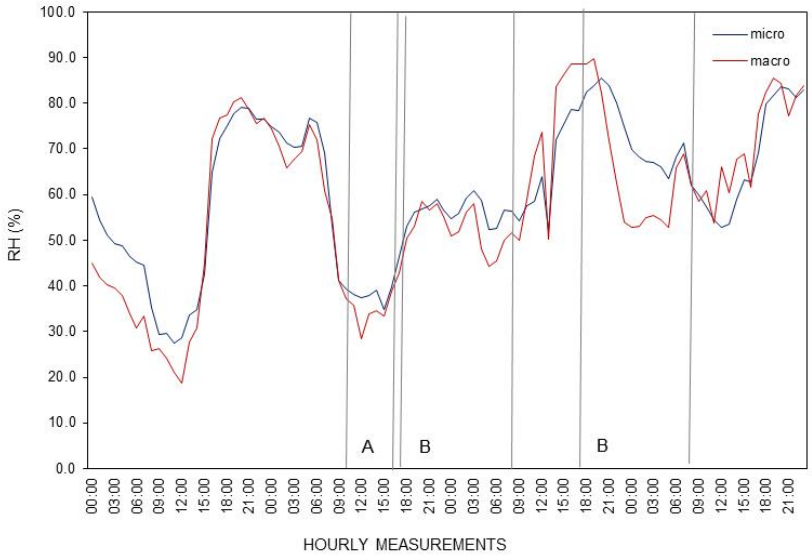

Figure 3.22: Average micro and macro RH for 2013 (A: pollination period; B: pollen droplet)

Environmental conditions at Sabie (Witklip, Tweefontein and Spitskop)

Climatic data outside the pollination bags were collected during the pollination season (May to June 2015) at three seed orchards (Witklip, Tweefontein and Spitskop) where the selected pine species investigated in this study have good seed set. During the pollination droplet period, Spitskop was slightly cooler (2 °C), while Witklip was approximately 3 °C warmer than Spitskop and Tweefontein. For DP, Tweefontein was on average 3 °C warmer during the pollen droplet and pollination period. Spitskop and Tweefontein had the same RH for the pollen droplet and pollination period, while Witklip was approximately 8 to 10 % higher. On average, RH differed not more than 10 % between the three seed orchards and less than 3 °C for both temperature and DP. Therefore, the average of the three seed orchards, namely Sabie, was calculated to compare with micro and macro-climatic data collected at Karatara.

Circadian (24-hour) models

Pollination bags placed in the top northern side of trees had the highest micro-temperature compared to other aspects and locations tested. In general, Kmac (top northern side) was up to 7 °C cooler than Kmic (top northern side) and only 2 °C cooler than Sabie during the day (Table 3.1, Figure 3.24). However, during the pollination period, Kmic was up to 7 °C warmer than that of Sabie and Kmac. During the night, temperatures at Sabie were between 4 and 5 °C warmer than that of Kmac and Kmic. Furthermore, less pronounced temperature fluctuations were observed at Sabie during the 24-hour temperature cycle than in the case of Kmac and Kmic.

DP values had smaller differences per hour intervals but with the same general pattern as temperature (Table 3.1, Figure 3.24). During the pollination period (10:00 and 16:00) Kmac and Sabie differed up to 4 °C, while Kmic was up to 7 °C warmer than Kmac and Sabie. Night DP of Kmic was warmer than Kmac, with Sabie

constantly warmer than both. During the pollen droplet period (02:00 to 04:00) Sabie was considerably warmer (4 to 5 °C) than Kmac and Kmic.

In general, RH had smaller differences and fluctuations per hour than temperature and DP (Table 3.1, Figure 3.24). During the pollination period (10:00 and 16:00) Kmac and Sabie differed between 2 and 10 %, while Kmic did not differ more than 2 % from Kmac, but up to 15 % from Sabie. Night RH of Kmic and Kmac differed less than 3 %, but Sabie was constantly lower during the early evening and higher in the morning (after 00:00). During the pollen droplet formation period (02:00 to 04:00) Kmic and Kmac did not differ more than 3 %, while Sabie was up to 10 % higher than Kmic and Kmac.

Table 3.1: Average temperature (°C), RH (%) and DP (°C) calculated for a 24-hour period at Karatara and Sabie

Time (hour)		Temperature (°C)			DP (°C)			RH (%)		
		Kmac	Kmic	Sabie	Kmac	Kmic	Sabie	Kmac	Kmic	Sabie
Day	08:00	15	19	15	5	10	9	57	56	71
	09:00	19	24	17	7	14	10	49	48	67
	10:00	19	26	19	7	14	10	49	46	61
	11:00	21	28	21	7	13	11	46	44	56
	12:00	21	28	22	8	14	11	49	46	54
	13:00	21	28	23	9	15	11	46	44	52
	14:00	21	28	23	8	14	11	50	47	52
	15:00	21	27	22	8	14	10	51	48	53
	16:00	19	23	21	8	12	10	55	52	54
	17:00	17	18	19	8	10	10	63	60	57
Night	18:00	13	12	18	7	7	9	71	70	60
	19:00	12	11	17	6	6	9	74	74	62
	20:00	11	11	16	6	6	9	75	76	63
	21:00	11	11	16	5	6	9	73	74	65
	22:00	11	10	16	5	6	9	71	73	66
	23:00	11	11	15	4	5	9	69	71	67
	00:00	10	9	15	3	5	9	64	67	68
	01:00	11	10	15	3	5	9	62	65	69
	02:00	11	10	14	3	5	9	62	65	70
	03:00	11	10	14	3	5	8	63	64	71
	04:00	11	10	14	3	5	8	61	63	71
	05:00	11	10	14	3	5	8	60	62	72
	06:00	11	10	14	3	4	8	62	63	72
	07:00	11	11	14	3	5	8	63	64	72

Kmac – Karatara macro; Kmic – Karatara micro; Sabie – average of Tweefontein, Spitskop and Witklip

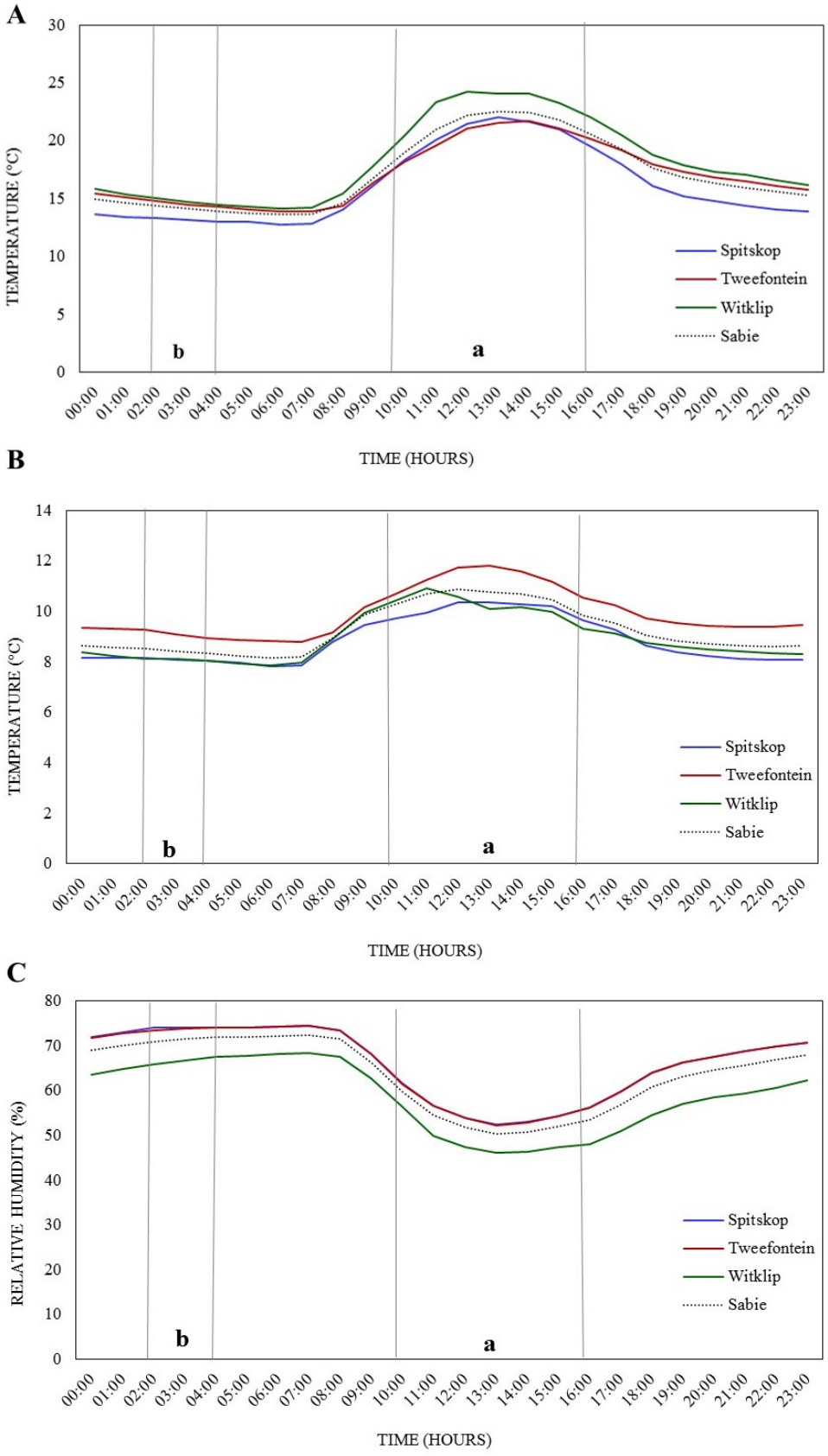

Figure 3.23: Average temperature (A), DP (B) and RH (C) compared at Spitskop, Tweefontein, Witklip and the combined Sabie for the 2015 pollination season (a: pollination period, b: pollen droplet)

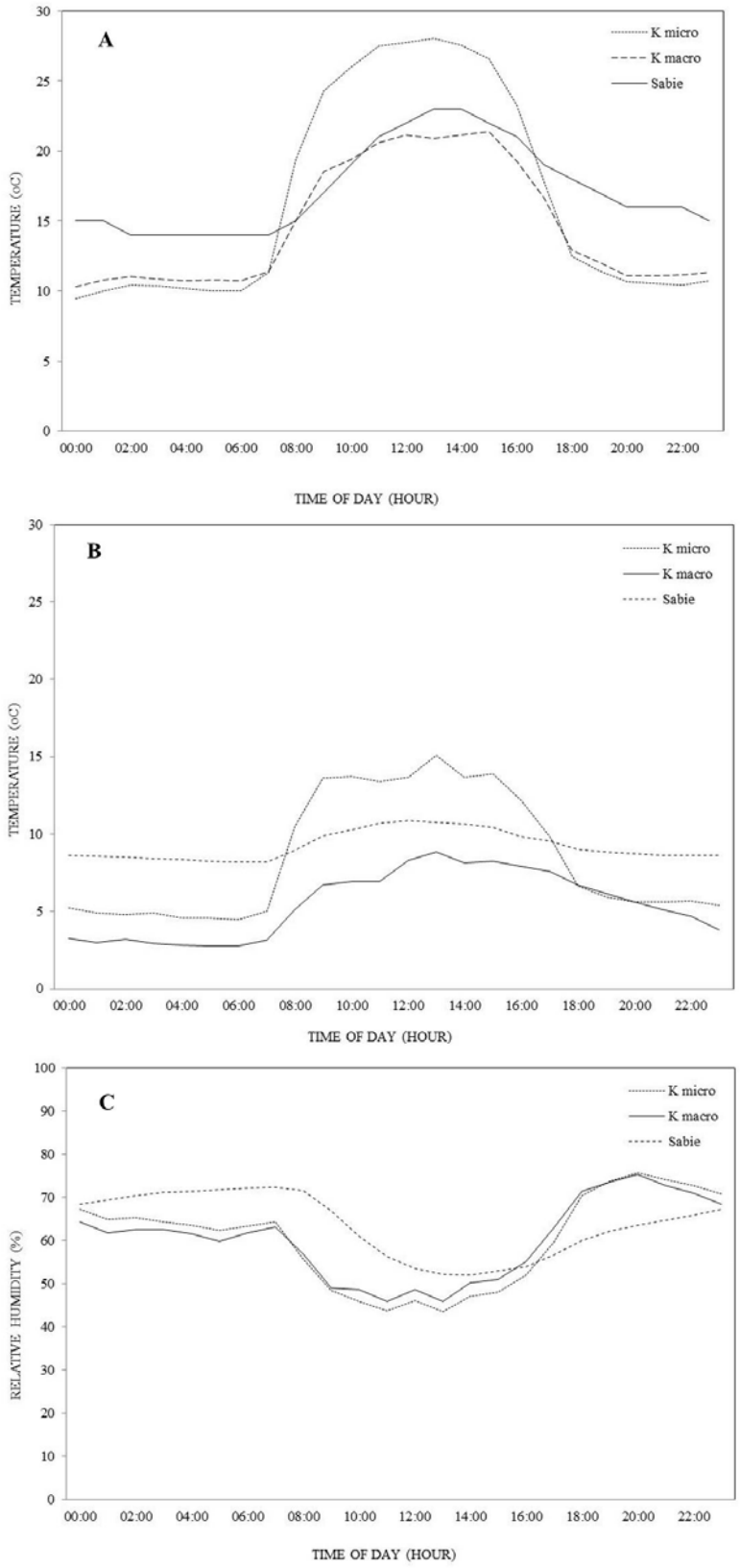

Figure 3.24: Comparison of the three circadian climatic models for Karatara micro, Karatara macro and Sabie (average of Tweefontein, Spitkskop and Witklip) for temperature (A), DP (B) and RH (C).

3.2 Natural growing conditions compared to Karatara

DA and PCA biplots were constructed to determine whether any of the five variables (altitude, precipitation, maximum, minimum and mean temperature) could be used to distinguish between species. Two separate biplot analyses, consisting of a DA and PCA, were performed: five variables (altitude, precipitation plus three temperature–related variables, Figures 3.25A and 3.25B); as well as temperature-related variables only (maximum, minimum and mean, Figures 3.25C and 3.25D). Clear groupings were evident in all the biplots (Figure 3.25, 95 % confidence interval, F_1 & F_2 = 96.8 %, 95.4 %, 98.1 %, 98.9 % for A, B, C and D respectively). Analysis per pollination month indicated that Month 2 was significantly cooler but drier than Month 1 (p<0.001; r^2=0.97; Appendix B).

Both analyses grouped the BIOCLIM (Karatara) and actual data collected during this study (Kmac) together, indicating a strong correlation between historic and actual data. *Pinus radiata*, *P. elliottii*, *P. taeda* and Karatara were grouped together in both analyses, indicating good site-species matching. However, the quartet of Karatara, *P. radiata*, *P. elliotti* and *P. taeda* differed significantly from the remaining species for all five variables (Table 3.2, Figures 3.25A and 3.25B). When only temperature is considered, *P. elliottii* and *P. taeda* were closely related to Witklip, Tweefontein, Spitskop, *P. radiata* and Karatara (Figures 3.25C and 3.25D). However, Kmic was more closely related to *P. maximinoi*, *P. tecunumanii* and *P. oocarpa* than *P. radiata*, Karatara and Kmac in the temperature only analysis. *Pinus maximinoi* and *P. oocarpa* grouped together in both analyses (all five variables and temperature only) and differed significantly from the other species, although *P. tecunumanii* was closer related. Furthermore, these three species showed a stronger correlation with temperature than altitude and precipitation. *Pinus patula* and *P. pringlei* grouped together and showed a closer correlation to Tweefontein, Witklip and Spitskop in both analysis (five variables and temperature only), indicating good site-species matching. In general, Karatara, *P. radiata*, *P. elliottii* and *P. taeda* had a stronger correlation with precipitation and minimum temperature than the other species.

Table 3.2: Comparison of five variables (altitude, precipitation and three temperature-related variables) per species and provenance as a mean for both pollination months

Region	Species	n	Altitude (m.a.s.l.)	Temperature (°C)			Precipitation (mm)
				Maximum	Minimum	Mean	
Meso-american	*P. radiata*	6	59 ^{E}	16 ± 1 ^{FG}	5 ± 1 ^{EFG}	10 ± 1 ^{EF}	84 ± 49 ^{AB}
	P. tecunumanii	38	927 ^{D}	25 ± 2 ^{A}	15 ± 2 ^{A}	20 ± 2 ^{A}	59 ± 39 ^{BC}
	P. oocarpa	136	1 222 ^{D}	25 ± 3 ^{AB}	12 ± 3 ^{AB}	19 ± 3 ^{AB}	26 ± 20 ^{D}
	P. maximinoi	52	1 308 ^{CD}	25 ± 3 ^{AB}	12 ± 2 ^{AB}	18 ± 2 ^{AB}	36 ± 29 ^{CD}
	P. elliottii	40	49 ^{E}	18 ± 3 ^{EFG}	5 ± 2 ^{FG}	11 ± 2 ^{DEF}	112 ± 23 ^{A}
	P. taeda	40	80 ^{E}	15 ± 4 ^{G}	2 ± 3 ^{G}	8 ± 3 ^{F}	111 ± 20 ^{A}
	P. pringlei	22	2 090 ^{AB}	24 ± 2 ^{ABC}	8 ± 3 ^{DE}	16 ± 2 ^{BC}	14 ± 8 ^{D}
	P. patula	56	2 371 ^{A}	20 ± 3 ^{DE}	5 ± 3 ^{EF}	12 ± 3 ^{DE}	23 ± 11 ^{D}
South Africa	Karatara	12	260 ^{E}	19 ± 0 ^{DEF}	7 ± 0 ^{DEF}	13 ± 0 ^{CDE}	65 ± 9 ^{BC}
	Kmac	2	239 ^{E}	22 ± 0 ^{BCD}	8 ± 0 ^{CD}	12 ± 2 ^{DE}	65 ± 11 ^{B}
	Kmic	2	239 ^{E}	26 ± 0 ^{A}	12 ± 1 ^{BC}	19 ± 2 ^{AB}	65 ± 11 ^{B}
	Witklip	10	1 152 ^{D}	21 ± 1 ^{CD}	7 ± 1 ^{DEF}	14 ± 1 ^{CD}	15 ± 1 ^{D}
	Spitskop	10	1 156 ^{D}	21 ± 1 ^{CD}	6 ± 1 ^{DEF}	14 ± 1 ^{CD}	13 ± 2 ^{D}
	Tweefontein	10	1 712 ^{BC}	19 ± 1 ^{DEF}	5 ± 1 ^{EFG}	12 ± 1 ^{DE}	13 ± 1 ^{D}
	r^2		0.91	0.99	0.99	0.99	0.97
	$p <$		0.001	0.001	0.001	0.001	0.001

n = number of provenances, Kmac – Karatara macro; Kmic – Karatara micro

Within a column, means with the same letter are not significantly different

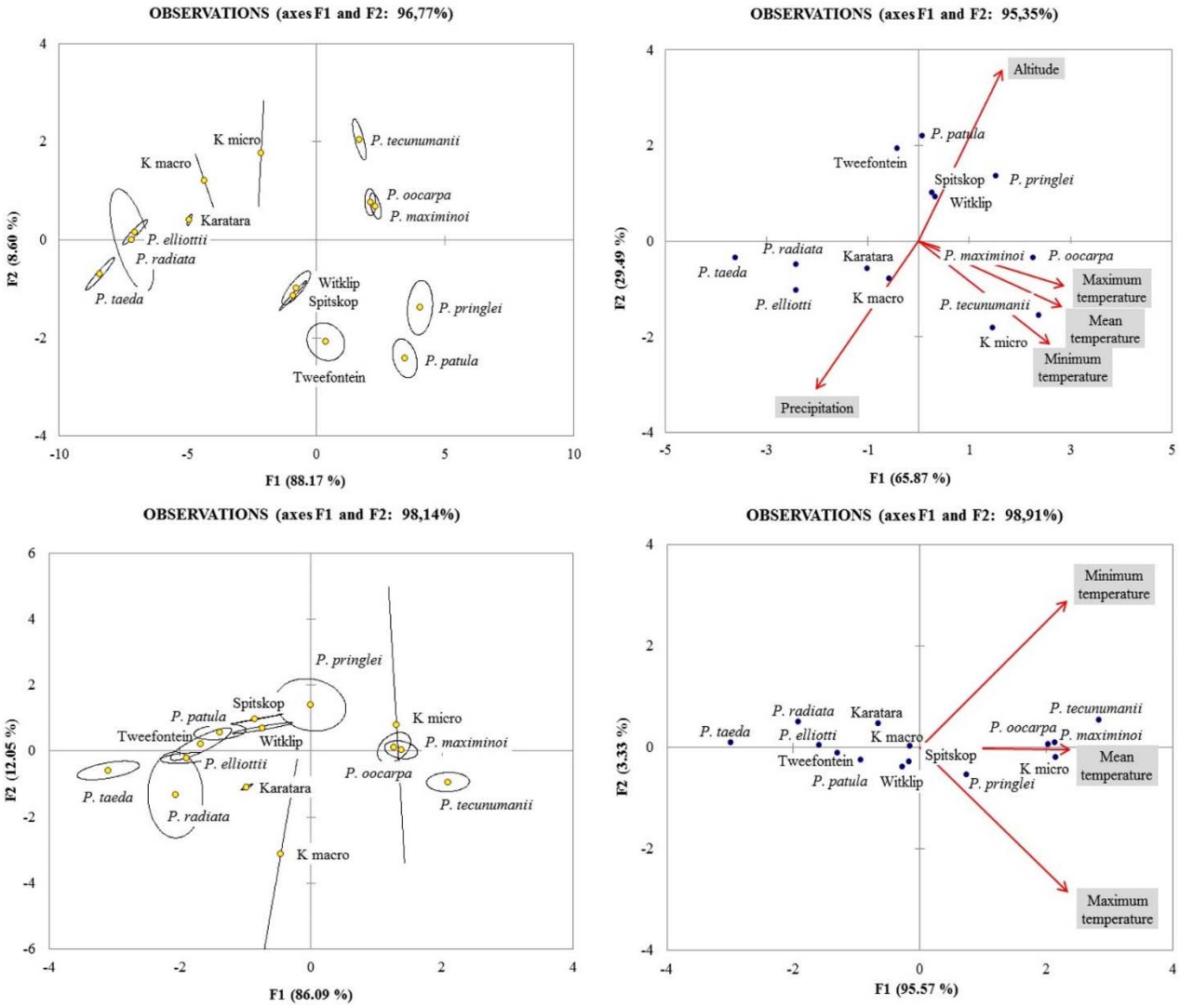

Figure 3.25: Comparison of environmental conditions between Karatara, Spitskop Tweefontein and Witklip with biplots (A: DA, B: PCA) for all five variables (altitude, precipitation and temperature (maximum, minimum and mean)) and only temperature-related variables (C: DA, D: PCA)

4. Discussion

Pinus radiata grows well in the Southern Cape, while the pine species (*P. maximinoi*, *P. patula*, *P. pringlei*, *P. oocarpa*, *P. tecunumanii*, *P. elliottii* and *P. taeda*) are evidently well adapted to the Sabie region (Tweefontein, Witklip and Spitskop). Although minimum temperatures never fall below freezing at Karatara, even during the coolest months of the year, interspecific crosses between *P. radiata* and some of these pine species have had limited success to date (Chapter 2, Dungey et al. 2003). As the time of flowering and pollination season differs between Karatara (July to August), Sabie (May to August) and the eight species, climatic conditions are different during male (catkins) and female (strobili or conelets) development (Burdon 1977; Boyer 1981; Greenwood and Schmidtling 1981; Dickson 1995). *Pinus radiata* grows at an optimum

temperature of 11 to 14 °C with low RH (winter rainfall) (Mead 2013), while the selected pine species are adapted to summer rainfall regions and climates typical to the tropics and subtropics. This gives rise to the question whether pollination success, as first step in the hybridisation process, will be higher if the micro-climate of pollination bags can be kept at temperatures and RH closer to the natural environmental variables experienced by the hybridisation partners in their native ranges.

The effect of the pollination bag on pollination success has been studied more intensely in angiosperms (for example effect of night temperatures) than in gymnosperms (for example Wyatt et al. 1992; Peet and Bartholemew 1996; Neal and Andersson 2004; He et al. 2006; Gruwez et al. 2014). These studies give some insights into key research questions that may be relevant to *Pinus* species. Therefore, this study investigated whether the environmental conditions (temperature, RH and DP) within and outside pollination bags and their position on the tree (top, middle, north and south) can affect pollination success. Collected data were compared to historical natural provenance climatic data (altitude, precipitation and temperature (maximum, minimum and mean)) of the eight pine species. A generic pollination protocol that were developed during a questionnaire survey (Chapter 2) were adapted for this study. Adaptions to limit pollen contamination included: comparing green (better interwoven cloth) and white cloth bags; daily sterilisation of breeding equipment; and regularly sealing the prickly holes caused by the bulb applicator. However, no pollination events took place and this study only collected climatic data. Although no differences in temperature, DP and RH were evident between the green and white cloth bags, the green cloth bags will be used in future. The practicality of sterilisation the breeding equipment seemed feasible and will be included in the generic pollination protocol in future.

Results indicated that position on tree could increase the temperature inside bags to levels that will be more suitable to tropical species, but the danger exist that too high temperatures and RH can negatively affect pollination success with *P. radiata* pollen. Placement of pollination bags in the top northern side of the trees increased daytime temperatures (up to 7 °C) during the pollination period to comparable levels with Sabie where it is known that *P. maximinoi*, *P. oocarpa*, *P. tecunumanii*, *P. pringlei*, *P. patula*, *P. elliottii* and *P. taeda* produce seed. However, nighttime temperatures, when the pollen droplet emerges, inside these pollination bags were substantially lower than at Sabie. Furthermore, the fluctuations between day and night temperatures were also more severe at Karatara than Sabie.

Previous studies have outlined the difference between the inside (micro) and outside (macro) temperatures (McWilliam 1959a; Nel and van Staden 2005), but limited information is available on the RH and DP. Pollination bags placed in the top northern side of trees resulted in not only a lower RH, but a higher temperature and DP than bags in the middle and southern side of the trees. When pollen is introduced into pollination bags with a high RH, it will become water saturated and be less effective, which can potentially result in lower germination. The risk of diseases or bacterial contamination might also increase (Nel 2002). Whereas, a lower RH and higher DP might affect the pollen droplet emergence negatively as the moisture difference between the inside and outside of the microstrobili may be too severe (Sweet et al. 1992). However,

if the air is too dry, it might induce abortions of microstrobili due to dehydration (Nel and van Staden 2003; Neal and Anderson 2004; Gruwez et al. 2014). McWilliam (1959b) also observed that extreme temperatures and limited air movement might lead to limited pollen germination success. Therefore, the balance between RH, DP and temperature seems to be delicate and might have a bigger impact on pollination success than previously considered.

Clear differences were evident between the circadian models of Sabie and Karatara. At Sabie the temperature regimes were as follows: approximately at 15 °C for 10 hours, eight hours between 15 and 20 °C, and six hours between 21 and 23 °C, while those at Karatara were around 10 and 15 °C for 15 hours, four hours between 15 and 20 °C, and five hours around 21 °C. The micro circadian model at Karatara also differed slightly as temperatures stayed around 10 °C for 14 hours, between 20 and 24 °C for three hours, and ranged between 26 and 28 °C for six hours. Circadian models also indicated that Sabie had lower fluctuations between maximum and minimum temperatures than micro and macro circadian models from Karatara. Temperature regimes were between 10 and 20 °C about 75 % of the day at Sabie, 80 % of the day at Karatara macro (outside the pollination bags), and 66 % of the day at Karatara micro (inside the pollination bags). These results supports the notion that the impact of the fluctuations between maximum and minimum temperatures in a 24-hour cycle (circadian model) on pollination success needs to be investigated further.

Biplots indicated a strong correlation between *P. radiata*, Karatara and the pollination bags placed in the northern top of trees at Karatara (Kmic and Kmac). Therefore, the data collected at Karatara not only correlates well with the BIOCLIM historic data, but also indicated that *P. elliottii* and *P. taeda* can be planted at Karatara although the duo are not genetic closely related to *P. radiata* (Dvorak et al. 2000; Mead 2013). The three seed orchards (Tweefontein, Spitskop and Witklip) representing Sabie in this study, grouped together and were closer correlated with *P. tecunumanii*, *P. oocarpa*, *P. maximinoi*, *P. pringlei* and *P. patula* than with *P. radiata*, *P. elliottii* and *P. taeda*. Although altitude is influenced by latitude, the combination of altitude and temperature does have an impact on the geographic distribution of a species. For example, *P. patula* is more closely related to the vector of altitude but less to precipitation than *P. radiata*. This is in agreement with Poynton (1979) that *P. radiata* does not perform as well in the Sabie region as *P. patula* due to the altitude and occurrence of frost and diseases. Also, the length of vectors altitude and precipitation are longer than temperature, indicating that the species in this study are more dependent on them than temperature alone (Young et al. 1993).

However, if altitude is left out of the equation, not only was *P. patula* grouped with *P. taeda*, *P. radiata* and *P. elliottii*; but Sabie and Karatara now correlate well. *Pinus maximinoi*, *P. oocarpa* and *P. tecunumanii* remained a separate group from *P. radiata*, although Kmic was now more closely related to the trio than Kmac. Length of vectors in the PCA biplot indicated that minimum and maximum temperature are more important than mean temperature (Young et al. 1993) for *P. radiata*, *P. patula*, *P. elliottii* and *P. taeda*. *Pinus pringlei*, *P. tecunumanii*, *P. maximinoi* and *P. oocarpa* were more closely correlated with the mean temperature which

might indicate fewer fluctuations between minimum and maximum temperatures as experienced in Sabie. Although the temperature biplots also indicated that *P. patula* and *P. pringlei* are grouped closely with *P. radiata*, previous crossability attempts yielded low seed viability between these species (Dungey et al. 2003), indicating that mean temperature might not be the only determining factor of pollination success.

McWilliam (1959b) postulated that the temperature in pollination bags (less than 10 °C) during the spring and early summer (or pollination season) can limit the germination of pine pollen. Limited studies on gymnosperms on the influence of temperature on pollen tube development are available and mostly for 96 hours after pollination events to test pollen viability. However, studies in both tomatoes and herbs indicated that the lower night temperatures, and 24-hour fluctuations between maximum and minimum temperatures, may have a more significant influence on pollen germination and tube elongation than higher average temperatures alone (Peet and Bartholemew 1996; He et al. 2006). Therefore, three circadian models (including temperature, RH and DP) representing Kmic, Kmac and Sabie for 24-hour period were developed during this study.

Reciprocal crosses between *P. radiata* and the seven pine species at Sabie might be a viable option to increase pollination success. However, overheating of female strobili in pollination bags of *Pinus rigida*×*taeda* was one of the main causes for pollination failure (Huyn 1976). Overheating of female strobili can also result in abnormalities of the female gametophyte. Studies during intraspecific crosses of *Gentiana straminea* (herb) indicated that bagged flowers tend to close by late afternoon or just before a thunderstorm when a change in RH and temperature are evident (He et al. 2006). The opposite were true when more hours of sunlight were experienced. This might be a reproductive protection mechanism to protect pollen against sudden changes in temperature and RH inside the pollination bags. Studies in milkweeds indicated that with a higher RH inside the pollination bags, nectar became more diluted with less pollination success (Wyatt et al. 1992). Therefore, future studies should explore the effect of the three circadian models on pollination success of the eight pine species and whether female strobili can also close scales as a protective mechanism.

In conclusion, the pollination protocol (Chapter 2) need to be adapted with daily sterilisation of breeding equipment, frequently seal openings created by bulb applicators, and green cloth bags should be used and replaced regularly. Although the pollination bag placements can decrease the climatic gap in temperatures between Karatara and Sabie for the duration of the pollination season, circadian models indicated that Sabie had a more stable (fewer fluctuations) climate than Karatara. PCA and DA biplots confirmed that the climate of Karatara and Sabie are different and a six-week period climate compensation by virtue of pollen bag placement might not be enough. *Pinus elliottii* and *P. taeda* were also grouped with *P. radiata* and Karatara in both sets of PCA and DA biplot analyses. PCA and DA biplots (temperature only) confirmed that minimum temperature is more important to *P. radiata* than mean temperature. However, if altitude is ignored as a vector, it might create superficial evidence suggesting *P. patula* as a potential hybrid partner for *P. radiata*. Future studies should thus investigate *P. elliottii* and *P. taeda* as potential hybrid partners with *P. radiata*. There is

also a need for more detailed *in vitro* pollen studies to investigate the effect of the circadian climate patterns on pollination success. These studies will be reported on in future chapters and will address the comparison of the circadian models developed during this study, under *in vitro* conditions.

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Chapter 4

Determine *in vitro* pollination success

1. Introduction

Interspecific hybridisation of *P. radiata* with various *Pinus* species (for example *P. oocarpa, P. pringlei, P. patula* and *P. tecunumanii*), has had limited success to date (Dungey et al. 2003). This might be either interspecific incompatibility (pre-zygotic barriers affecting pollination success) or inviability (post-zygotic barriers affecting fertilisation success; Hagman 1975; Dungey 2001). Climatic factors might also affect pollination success by influencing pollen grain dispersion (wind and humidity) or pollen tube development (temperature) (Cruden and Lyon 1985; Cruden 2000; Jürgens et al. 2012; Ham et al. 2017).

Pinus species are wind-pollinated, representing an aerodynamic balance between the donor (male pollen) and the recipient (female strobilus; Williams 2009). Female strobili will 'catch' the air-borne pollen grains, while the micropyle and droplet transport grains to the ovule (Songnuan 2013; Fernando 2014), filter-out non-conspecific taxa (Bramlett 1981; Dungey et al. 2003; Williams 2009), and hydrate the pollen grains to germinate within five days (Fernando et al. 2005). However, the mechanism for the exclusion of foreign particles (dust and unrecognised pollen) from the pollen chamber is poorly understood (Tomlinson 1994; Fernando et al. 2005; Jürgens et al. 2012).

Pinus pollen grains dehydrate to a water content of less than 10 % before shedding to ease wind distribution (Allen and Owens 1972; Fernando et al. 2005), but is temperature dependent (Dafni et al. 2005), especially with tropical pines (Fernando et al. 2005). Therefore, controlled-pollination with female strobili enclosed in micro-fibre pollination bags is used as a breeding technique to exclude other pollen, limit the effect of environmental conditions (temperature, relative humidity and precipitation) and ensure species specific crosses (Ham et al. 2017). Pollen management (harvesting, drying and storage) does not only influence the viability of *Pinus* pollen, but is the first step in successful controlled-pollination (Nel and van Staden 2005; Owens and Fernando 2007; Williams 2009). To increase viability, *in vivo* (in field) conditions are simulated by reducing the water moisture to below 10 % within 24 hours of harvesting (Bramlett and Matthews 1991; Jett et al. 1993; Fernando et al. 2005; Williams 2009). Also, pollen tube growth rate (PTGR) is an important indicator of gametophyte health or pollen vigour (Williams et al. 2016).

Pinus species have saccate pollen that will float on water inside the micropyle (Tomlinson 1994). During germination, the pollen grain swells and the exine of the spore wall splits open and elongates at the distal end to produce the pollen tube. Although elongation takes place over a considerable time period (± 12 months), it can abort when conditions are unfavourable (Allen and Owens 1972; Hagman 1975; Fernando et al. 2005; Songnuan 2013). The success of the reproductive cycle thus depends on: timely release of pollen grains, capturing of pollen grain within the droplet, germination of the pollen grain, pollen competition and pollen allelopathy (release of toxins that inhibit sexual reproduction) (Greenwood 1986; Dafni et al. 2005). Bramlet and Bridgewater (1989) developed a six stage protocol of pollen development and harvesting for *Pinus* species: Stage 1 – tiny male strobili enclosed in bud scales on the vegetative shoot; Stage 2 – emergence of individual

strobilus from bud scales; Stage 3 – male strobilus lengthens and exudes a clear liquid when pressed (harvest catkins when little or no fluid are exude when pressed); Stage 4 – pollen are released (harvesting of catkins); Stage 5 – maximum release of pollen; and Stage 6 – complete pollen release (strobili are dry, lightweight and brown). Although the timing of pollen shed is species specific, it can occur between stages 4 to 6 and might take between seven to 14 days and is the best time for pollen harvesting (Bramlet and Bridgewater 1989; Parker and Blush 1996; Williams 2009). Although fresh *Pinus* pollen can withstand temperatures of 50 °C within 24 hours after harvesting, viability decreases rapidly thereafter within weeks (Williams 2009). To increase viability, the water moisture content needs to be reduced to below 10 % within 24 hours after harvesting, and can be stored successfully at 3 °C in airtight containers for up to one year without affecting viability (Bramlett and Matthews 1991; Jett et al. 1993; Siregar and Sweet 2000; Williams 2009).

As the main components of pollination studies consist of pollen viability, pollen tube growth and ovule receptivity (Dafni et al 2005), the evaluation of pollen viability, size variation and PTGR (Williams 2012) is instrumental for understanding the reproductive cycle of individual pine species (Caron and Powell 1995). Pollen competition is always present and can also influence pollination success, pollen viability and pollen tube growth (Varis 2009). Pollination success is not only measured by counting the number of pollen grains attached to the micropyle or inside the ovules, but also by *in vitro* pollen germination and pollen tube growth tests (Owens et al. 2005; Owens and Fernando 2007; Williams 2012). Although it does not completely mimic *in vivo* (in-field) growth, it does give an accurate estimate (Taylor and Hepler 1997; Fernando et al. 2005). In previous studies, Taylor and Kepler (1997) stated that pollen tubes might only reach up to 30 or 40 % of *in vivo* lengths, while both Williams (2009) and Dafni et al. (2005) estimated *in vitro* experiments can overestimate pollen germination. In *Pinus* species, *in vitro* pollen studies are an important but underutilised experimental system as there is a strong positive correlation between *in vivo* and *in vitro* pollen germination and the early stages of pollen tube elongation (Fernando et al. 2005; Williams 2012). The main difference is that *Pinus* pollen germination can occur within hours under *in vitro* conditions, but might take a couple of days *in vivo* (Dawkins and Owens 1993; Fernando et al. 2005; Williams 2012). The low moisture content of dehydrated pollen grains might contribute to the difference in time between *in vivo* and *in vitro* germination (Dawkins and Owens 1993). Pollen viability can also be determined by staining pollen grains *in vitro* with Tetraxolium Chloride (Cook and Stanley 1960) or various other dyes (Rodriguez-Riano and Dani 2000; Nel 2002). Although these procedures can take up to 30 minutes, it is only an indication of viability and provides no indication whether the pollen tube will elongate or of pollination success. Best results are obtained when pollen grains are studied in their respective natural state with no adding of stains, sugars, artificially cleaned (acetolysed) and are re-hydrated (Hesse 2000).

This study will focus on the traits of the male strobilus of eight *Pinus* species (*P. maximinoi, P. oocarpa, P. tecunumanii, P. patula, P. elliottii, P. taeda, P. pringlei* and *P. patula*×*tecunumanii*) as possible interspecific hybrid partners for *P. radiata*. These traits will include pollen grain morphology and size (length and width), pollen viability, pollen germination rate, pollen tube size (length and width) and PTGR. Previous studies

concentrated on pollen germination and pollen tube growth at 72 hours (three days) after incubation at constant temperatures (ranged between 15 and 40 °C; Tanaka 1955; McWilliam 1959a, 1959b, 1959c; Critchfield 1975; Jett et al. 1993; de Win et al. 1996; Dumont-BéBoux and von Aderkas 1997; Dungey 2001; Major et al. 2005; Nel and van Staden 2005; Williams 2012). However, they excluded PTGR and pollen tube elongation under natural conditions. This study will simulate micro- (inside the pollination bag) and macro-climate (outside the pollination bags) conditions for a 24 hour cycle between one and seven days (24 to 168 hours) incubation period to compare pollen germination and PTGR. Objectives will address the following: is there a difference between de-hydrated and hydrated pollen grains; does pollen tube size and PTGR (length and width) differ between species.

2. Materials and methods

2.1 Plant material

Experiments were designed to investigate whether pollen tube elongation (length and width), PTGR (length and width) and pollen grain size (length and width for both dehydrated and re-hydrated pollen grains) differed between the eight pine taxa (*P. maximinoi, P. oocarpa, P. tecunumanii, P. elliottii, P. taeda, P. patula, P. pringlei* and *P. patula*×*tecunumanii*) and *P. radiata*. Nine temperature regimes (seven constant temperatures and two circadian models) were used in the experimental design. The two circadian models represent said natural conditions inside (micro) and outside (macro) the pollination bag in a *P. radiata* seed orchard (Chapter 3; Ham et al. 2017).

Pollen bearing micro strobili (catkins) were collected between July and October (2012 and 2013) at Stage 4 (see Chapter 2; Bramlett and Bridgewater 1989) from the nine species and dried in paper bags for approximately 48 hours at 25 °C. Pollen were sieved (100 µm sieve) to remove residues and placed in 65 mm glass Petri dishes on silica gel in sealed desiccators until water content was below 10 % (Chapter 2; Nel and van Staden 2005; Williams 2009). The moisture content percent of pollen was determined with a Sartorius Thermo Control YTC01L infrared dryer (calibrated before use). Approximately 2 ml of dried pine pollen was scooped into an aluminium foil dish to record fresh weight (g). Dry weight (g) was documented after exposure to an infrared beam for one minute. Percentage (%) moisture content (Z) was calculated as follow:

$$Z = \frac{\text{fresh weight (g) - dry weight (g)}}{\text{fresh weight (g)}} \text{ x } 100$$

2.2 Pollen tube elongation and growth rate

Pollen grain germination, tube size and PTGR were measured for the nine species under controlled laboratory (*in vitro*) conditions over a 7-day period (simulating one week after germination) at different temperature and

light regimes. As *in vitro* experiments were performed in Petri dishes on agar, it was not possible to simulate dew point and relative humidity. Therefore, only temperature as a climatic factor was investigated.

As the germination potential of pine pollen decreases over time, studies were completed within six months after harvesting. Pollen lots were taken from cold storage (4 ºC) and re-hydrated for two hours by placing approximately 2 ml stored pollen into 30 mm glass Petri dishes on distilled water saturated filter paper, in a sealed desiccator at 24 ºC (Nel and van Staden 2005; Williams 2009). After re-hydration, pollen lots were evenly dusted onto agar medium in 65 mm plastic Petri dishes without touching the edge of the dishes (Cook and Stanley 1960). The 1 % agar solidified medium contained 0.01 % boric acid with no sucrose as different sugars could induce species-specific effects on pollen germination, tube development and PTGR (Chira and Berta 1965). Boric acid was added to limit contamination during the incubation period (Goddard and Matthews 1981; Jett and Frampton 1990; Bramlett and Matthews 1991; Nel 2002). Hydrogen ion concentration (pH) of the germination medium was 6.0 prior to autoclaving. The medium was sterilised in an autoclave at 120 ºC and 103 kPA for 20 minutes where after approximately 20 ml of medium was poured into 65 mm plastic Petri dishes (Cook and Stanley 1960; Goddard and Matthews 1981; Nel and van Staden 2005). Pollen viability and water content were determined for all the pollen lots before experiments commenced (more than 90 % and less than 10 % respectively). Pollen tube growth rate experiments were divided into two different studies (pilot and simulation).

Pilot study

Two temperature regimes (24 and 32 °C), based on previous work of various *Pinus* species by Jett & Frampton (1990) and McWilliam (1959b), were followed. Pollen from seven *Pinus* species (*P. radiata, P. maximinoi, P. oocarpa, P. tecunumanii*, *P. taeda, P. elliottii* and *P. pringlei*) was exposed to a constant 24 and 32 °C temperature for seven days in a growth chamber (Scientific Manufacturing model 1400 LTIS). Considering that conelets close after pollination (no light penetrates), half of the Petri dishes used in these experiments were covered with aluminium foil to simulate dark conditions. The other half were exposed to fluorescent lights simulating 24 hour per day, light conditions (seven species at two temperature treatments and two light treatments for seven days each). During *in vitro* incubation of pollen studies, contamination needs to be limited or avoided as it has an impact on pollen competition, pollen germination, pollen tube elongation and accuracy of measurements. Fungal spores are very fine and can ‘cling’ to pollen grains even after drying and sifting (Tomlinson 1994; Williams 2009), resulting in visible contamination within 24 hours after incubation (Cook and Stanley 1960). As contamination was prominent in the light experiments, they were repeated and 0.025 % Benomyl (also marketed as Benlate) was added to the agar medium. However, contamination was still present (within 120 hours after incubation) and still severely affected the visibility and measurements of pollen tubes. As isolated fungal clones were general air-born species, pollen management is of the utmost importance to limit fungal contamination as well. Fungal colonies were isolated from contaminated agar gels and identified. Digital data loggers (Lascar EL USB 2) were regularly calibrated and placed with each treatment to record hourly temperature inside the incubator.

Simulation study

Temperature regimes selected were based on environmental conditions observed inside pollination bags (Chapter 3; Ham et al. 2017). Pollen from nine pine taxa (*P. radiata, P. maximinoi, P. oocarpa, P. tecunumanii*, *P. taeda, P. elliottii, P. patula, P. pringlei* and *P. patula*×*tecunumanii*) was used. Petri dishes were covered with aluminium foil to simulate dark conditions inside the conelets and Benlate was not added to the agar medium as in the pilot study. They were then placed in the growth chamber at different constant temperature regimes (10, 15, 20, 24, 28, 32 and 36 °C) and two circadian models, representing 24-hour temperature cyclic conditions both inside (micro) and outside (macro) the pollination bags (Figure 4.1; Chapter 3; Ham et al. 2017) for seven days per treatment. Digital data loggers monitored and recorded hourly temperature inside the incubator. In cases where fungal contamination was observed, the fungal colonies were isolated and identified.

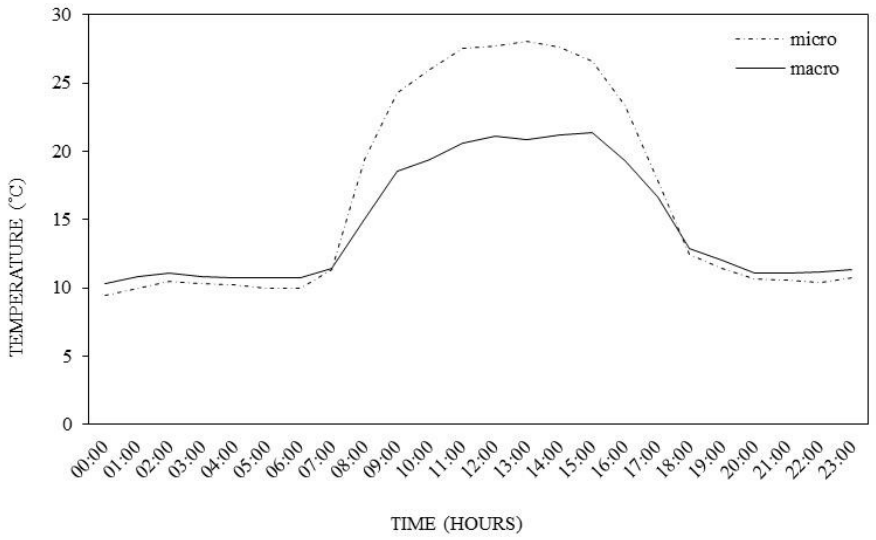

Figure 4.1: Two circadian models developed to mimic temperature inside (micro) and outside (macro) the pollination bag for a 24-hour cycle at a *P. radiata* seed orchard (adapted from Ham et al. 2017)

Two constant temperature and light treatments (pilot experiment) and nine temperature treatments (seven constant and two temperature regimes) during the simulation study were tested. For each temperature and light treatment, 35 Petri dishes (five replication × seven days with one Petri dish per replication) were sampled to determine pollen germination (viability) percentage and pollen tube size (length and width) without replacement per 24 hour interval per species (nine in total). Therefore, a total of 315 Petri dishes were sampled over a seven day period (one week after pollination). At each 24-hour sampling point, germination percentage, pollen tube development and pollen tube length and width were assessed. Percentage pollen viability was determined by counting the number of germinated pollen grains divided by the total number of pollen grains per microscope field with a Leica light microscope (DM 2500M) at 10 X magnification. Pollen grains were

considered germinated when the tube lengths were equal to, or exceeding grain diameter (Goddard and Matthews 1981; Nel and van Staden 2005; Williams et al. 2016; Figure 4.2). Although Kormutak et al. (2007) suggested measuring a total of 30 pollen grains per treatment, 10 measurements (for both pollen tube length and width) per petri dish per species, repeated for each of the five petri dishes per species (50 pollen grains in total for both length and width per species) for each temperature treatment were recorded per 24 hour (daily) interval (Cook and Stanley 1960). PTGR was determined per day (24-hour interval), by dividing total length or width by the number of hours since incubation (Anderhag et al. 2000; Williams 2012; Williams et al. 2016).

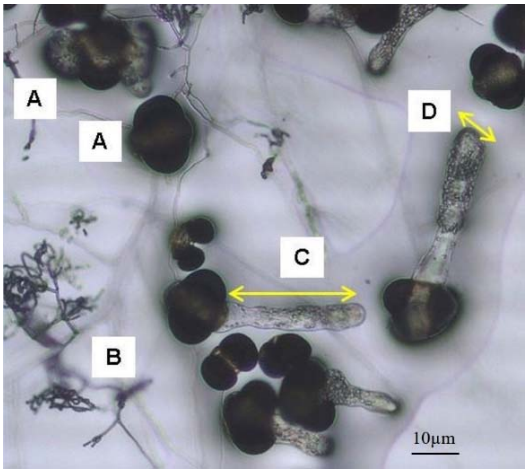

Figure 4.2: Example of *P. radiata* pollen tube measurement technique at 10 X magnification of a 96 hour (four day-old) culture (A: no germination, B: contamination, C: pollen tube length, D: pollen tube width)

The experimental design was a completely randomised design with five replicates for both studies. The treatment design was a factorial with two factors namely species with nine levels (*P. radiata, P. tecunumanii, P. oocarpa, P. maximinoi, P. pringlei, P. elliottii, P. taeda, P. patula* and *P. patula*×*tecunumanii*) and time with seven levels (24, 48, 72, 96, 120, 144, and 168 hours after inoculation). An experimental unit (species × time × replication) consisted of 315 Petri dishes per temperature treatment. The data were subjected to analysis of variance (ANOVA, Appendix B) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.2; SAS Institute Inc, Cary, USA). Shapiro-Wilk test was performed on the standardised residuals from the model to verify normality (Shapiro and Wilk, 1965). Fisher's least significant difference was calculated at the 5 % level to compare treatment means (Ott and Longnecker, 2001). A probability level of 5 % was considered significant for all significance tests.

As the length of a daily growth period is an important environmental-dependent trait (Yin et al. 2003), the rate of change in pollen tube length for each species at both temperature regimes was calculated with growth curves (linear and non-linear functions). Linear functions are characterised by a constant rate of change and describes the rate of change (pollen tube length) at a constant speed, but is not always constant. Therefore, non-linear and linear functions were fitted to the data of the two temperature regimes (micro and macro). A Shapiro Wilk

test for normality was conducted before the data could be assumed reliable. Two standard linear growth functions (Quadratic and Linear) and five non-linear functions (Natural Growth, Modified Exponential, Gempertz, Logistic and Sigmoid) with p=0.05 (5 %) were employed to compare treatment means (Shapiro and Wilk 1965; Ott and Longnecker 2001; Yin et al. 2003; Chijioke et al. 2010). A sigmoidal function with three parameters was applied to describe the development of pollen tubes over time for each temperature using the non-linear procedure (PROC NLIN) of SAS software (Version 9.2; SAS Institute Inc, Cary, USA). The sigmoidal function is describe by the following equation:

$$Y = \frac{\mathrm{a}}{(1 + \exp\,(-\,\frac{time - b}{c}))}$$

with observed pollen tube growth length as dependent variable (Y), maximum pollen tube length (a), exponential (exp), number of hours ($time$), PTGR length (slope) (b), and time at which PTGR length reaches maximum value (c).

2.3 Pollen grain morphology

Pollen grains de-hydrate to a water content of less than 10 % before distribution by the wind (Allen and Owens 1972; Fernando et al. 2005; Williams 2009). However, pollen grains are re-hydrated by the droplet before being transported inside the ovule (Greenwood 1986; Brown and Bridgewater 1987; Williams 2009). Therefore, pollen grain size (length and width) of both re-hydrated and de-hydrated grains was measured. Care was taken to use fresh pollen lots within two months after harvesting. Pollen lots (approximately 4 ml) were taken from cold storage (4 °C) and divided into two groups (approximately 2 ml per group). Group 1 (de-hydrated) was kept at room temperature (24 °C) (Nel and van Staden 2005) and group 2 (re-hydrated) was placed in 30 mm glass Petri dishes on distilled water saturated filter paper, in a sealed desiccator at 24 °C for two hours (Goddard and Matthews 1981; Fernando et al. 2005). After re-hydration, pollen was evenly dusted onto agar medium and incubated for 24- hours at micro and macro temperature regimes. Imaging was done with a Scanning Electron Microscope (SEM) and a secondary detector showed the surface structure of the pollen grains. Beam conditions during surface analysis were 7 kV and approximately 1.500 nA, with a working distance of 13 mm, a spot size of 150 and 1.200 kX magnification.

Pollen grains were measured across the distal region of the grain between the two air bladders (length) and from the distal pole including the bladders (width) as shown in Figure 4.3 (Cain 1940; Nel and van Staden 2005). A total of 6 750 measurements were taken: 125 grains (25 measurements with five replicates per species) for both pollen grain length and width with three treatments (dehydrated, re-hydrated micro and re-hydrated macro). Replicates were from different branches. Care was taken to measure only grains which were seen squarely in dorsal (backside of pollen), ventral (front) or lateral (attached to the side of an organ) view, avoiding obliquely viewed grains.

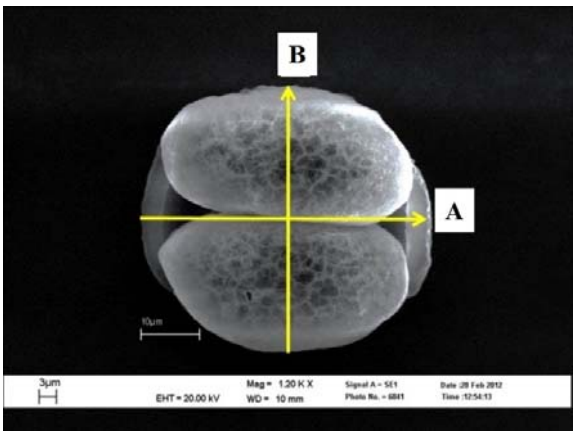

Figure 4.3: *Pinus radiata* pollen grain at 1.200 kX magnification indicating the grain length (A) and width (B) measurement technique

The experimental design was a completely randomised design with five replicates. The treatment design was a factorial with two factors namely species with nine levels (*P. radiata, P. maximinoi, P. oocarpa, P. tecunumanii*, *P. taeda, P. elliottii, P. patula, P. pringlei* and *P. patula*×*tecunumanii*) and size with two levels (length and width). The data were subjected to analysis of variance (ANOVA; Appendix B) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.2; SAS Institute Inc, Cary, USA). Shapiro-Wilk test was performed on the standardised residuals from the model to verify normality (Shapiro and Wilk, 1965). Fisher's least significant difference was calculated at the 5 % level to compare treatment means (Ott and Longnecker, 2001). A probability level of 5 % was considered significant for all significance tests.

2.4 Correlation between PTGR and pollen grain size

Discriminant Analysis (DA) and Principle Component Analysis (PCA) with Pearson correlations are multivariate techniques to determine the possible relationship between quantitative variables and are powerful tools for exploratory data analysis (Kakani et al. 2005). DA and PCA were performed to determine if there is an interaction between PTGR and pollen grain size (length and width for the micro and macro temperature regimes. As trends were evident, biplots were constructed to distinguish between natural and distinctive groups (DA), while quantitative variables were determined for correlations between multidimensional datasets (PCA; Rencher 2002; Kohler and Luniak 2005; Erasmus et al. 2016).

3. Results

3.1 Contamination

Although contamination was present at both pilot and simulation studies, it was more severe at the pilot study light treatments from day's five to seven. Despite adding Benlate, contamination still hampered visibility of pollen tubes and accuracy of measurements. Therefore, light treatments for both 24 and 32 °C of the pilot study were excluded from the analyses. Comparisons of pollen tube size (length and width) and PTGR were conducted up to day seven to simulate one week after pollination. A selection of contaminated agar gels from both light and dark treatments were analysed by the Plant Pathology Department at Stellenbosch University. Fungal colonies isolated were secondary fungal cultures that normally grow on agar growth media and are generally found in the air and on plant surfaces (cones, needles). They included species of *Epicoccum, Cladosporium, Aureobasidium, Alternaria* and *Trichoderma*.

3.2 Pollen moisture content and germination percentage

Pollen moisture content and germination percentage were measured for both pollen lots harvested in 2012 and 2013 (Table 4.1). In 2012, *P. oocarpa* had the lowest (12 %) and *P. pringlei* the highest (22 %) percentage water moisture content. *Pinus pringlei* and *P. maximinoi* had a 40 % germination percentage while all the other species had an 80 % germination percentage. Moisture content of pollen lots harvested in 2013 showed large variation for all pine taxa. The lowest moisture content was 9 % for *P. radiata* (Chile), while both *P. maximinoi* and *P. tecunumanii* had the highest water moisture content of 19 %. The *P. radiata* pollen lot received from Karatara in July 2013 had a moisture content of 47 % and 16 % after a second drying phase in the laboratory to lower the moisture content. However, pollen germination was only 30 % thereafter. Another *P. radiata* pollen lot was received from Karatara in November 2013 with a water moisture content of 21 % and germination of 0 %. A third pollen lot of *P. radiata* was collected a moisture content of 24 % and germination of 0 %. To improve pollen quality, pollen of *P. radiata* was then imported from Chile (January 2014) with a water moisture content of 9 % and germination of 90 %.

Table 4.1: Percentage moisture content (measured on arrival of pollen at Stellenbosch) and germination (at day seven) of pine pollen lots

Harvested at	Species	Moisture content (%)	Germination (%)
	2012		
Karatara RSA	*P. radiata*	16	80
Tweefontein RSA	*P. tecunumanii*	18	80
Tweefontein RSA	*P. oocarpa*	12	80
Kwambonambi RSA	*P. taeda*	18	80
Kwambonambi RSA	*P. elliottii*	19	80
Tweefontein RSA	*P. maximinoi*	19	40
Tweefontein RSA	*P. pringlei*	22	40
	2013		
Karatara RSA	*P. radiata* (July 2013)	47	0
Karatara RSA	*P. radiata* (July 2013: 2nd dry)	16	30
Karatara RSA	*P. radiata* (November 2013)	21	0
Karatara RSA	*P. radiata* (January 2014)	24	0
Chile	*P. radiata* (January 2014)	9	90
Tweefontein RSA	*P. oocarpa*	16	90
Tweefontein RSA	*P. tecunumanii*	19	90
Kwambonambi RSA	*P. elliottii*	9	90
Tweefontein RSA	*P. maximinoi*	19	90
Kwambonambi RSA	*P. taeda*	15	90
Tweefontein RSA	*P. pringlei*	16	90
Howick RSA	*P. patula*	14	90
Nooitgedacht RSA	*P. patula*×*tecunumanii*	16	90

Pilot study

Pollen from all species except *P. maximinoi* and *P. pringlei* started to germinate on day 1 and 2 for the 32 and 24 °C dark treatments respectively (Figure 4.4). *Pinus pringlei* did not germinate at 24 and 32 °C, while *P. maximinoi* germinated on day 7 at 32 °C and day 2 at 24 °C.

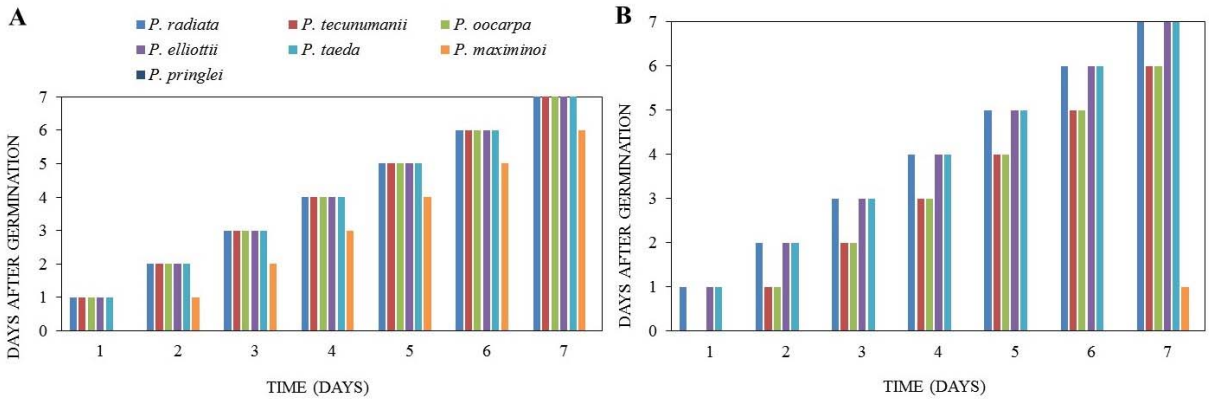

Figure 4.4: Pollen germination for the pilot study at 32 (A) and 24 °C (B) for the seven species tested

Simulation study

Germination of pollen lots at constant temperatures differed considerably (Figure 4.5). At 10 °C, five taxa (*P. tecunumanii*, *P. oocarpa, P. pringlei, P. maximinoi, P. elliottii* and *P. patula*×*tecunumanii*) did not germinate, while *P. taeda* and *P. patula* germinated at day 6, and *P. radiata* at day 7. Three species (*P. tecunumanii*, *P. oocarpa* and *P. patula*×*tecunumanii*) did not germinate at 15 °C, while *P. radiata, P. taeda* and *P. patula* germinated at day 3; *P. pringlei* at day 4; *P. maximinoi* at day 5; and *P. elliottii* at day 6. At 20 °C all the species were germinated by day 6. For 24, 28 and 32 °C, all the species germinated by day 3 or 4, but at 36 °C, *P. radiata* and *P. tecunumanii* germinated only at day 7. For the micro-temperature regime, *P. taeda* and *P. patula* germinated at day 2; *P. radiata* at day 3; *P. elliottii, P. maximinoi*, *P. pringlei* and *P. patula*×*tecunumanii* at day 4; while *P. oocarpa* and *P. tecunumanii* did not germinate at all. At the macro-temperature regime, *P. radiata, P. taeda* and *P. patula* germinated at day 3; *P. elliottii* at day 4; *P. maximinoi* and *P. pringlei* at day 5; *P. patula*×*tecunumanii* at day 6; while *P. oocarpa* and *P. tecunumanii* again did not germinate.

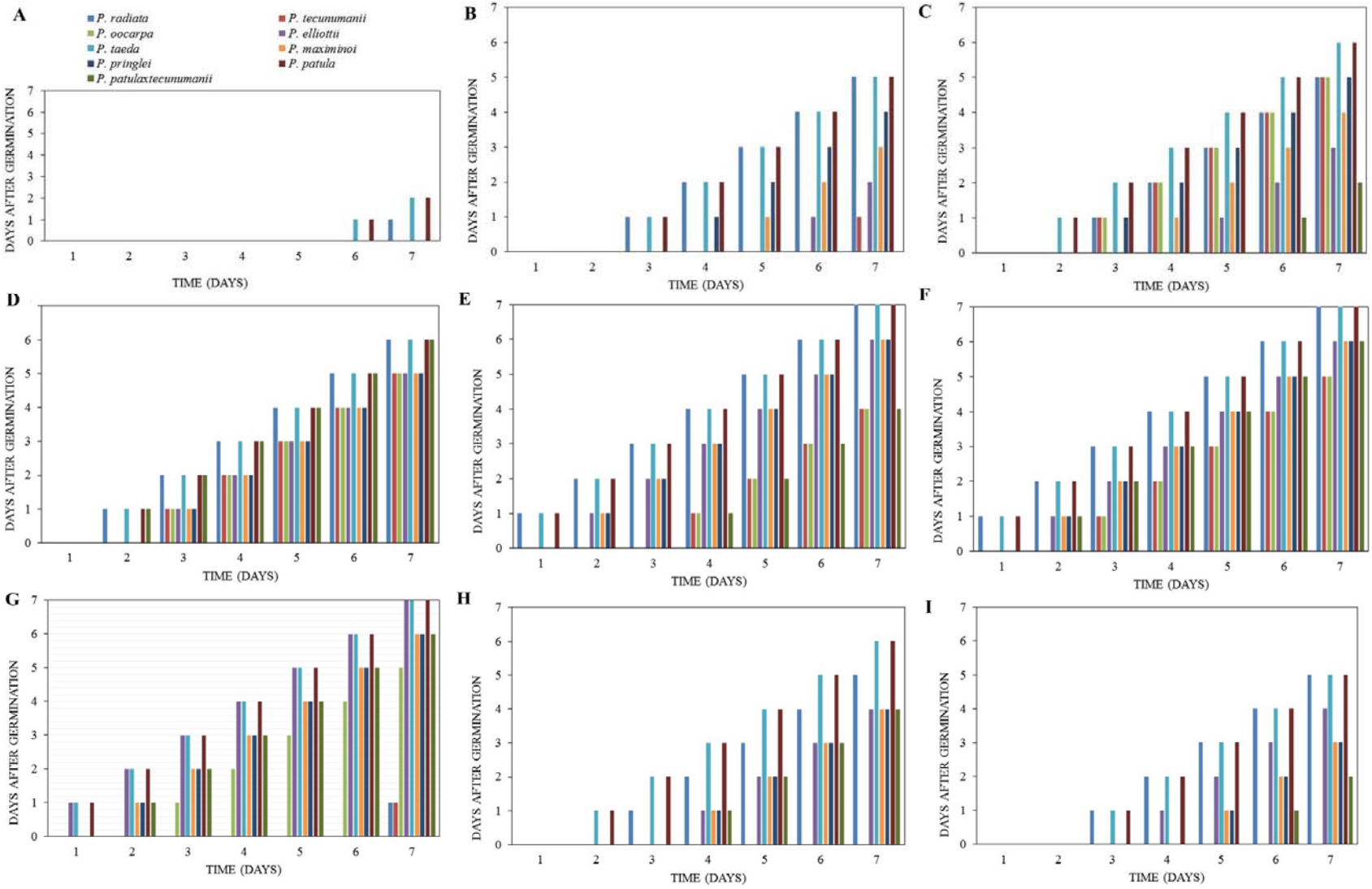

Figure 4.5: Pollen germination for the simulation study at 10 (A), 15 (B), 20 (C), 24 (D), 28 (E), 32 (F), 36 ºC (G), micro- (H) and macro-temperature (I) for the nine taxa tested (pollen lots were considered germinated with 90% or more at daily intervals)

3.3 Pollen tube size and PTGR

Pilot study

At 32 °C (Table 4.2) two groups were evident for pollen tube length and growth rate length as *P. radiata*, *P. tecunumanii* and *P. oocarpa* differed significantly (p<0.0001) from the other species. *Pinus radiata* and *P. tecunumanii* differed significantly (p<0.0001) from one another and the other species for pollen tube width and growth rate width. The differences in pollen tube length between species at 24 °C were less pronounced than at 32 °C except for *P. maximinoi* and *P. pringlei* that were significantly (p<0.0001) shorter than the other species. *Pinus radiata* and *P. taeda* had significantly (p<0.0001) wider pollen tubes than the other species at 24 °C. PTGR for length and width showed the same pattern as pollen tube length and width. Both *P. radiata* and *P. tecunumanii* had a PTGR length of more than 1 µm/h at 32 °C and less than 1 µm/h at 24 °C. All the species had a PTGR width of approximately 0.1 µm/h at both 32 and 24 °C.

Table 4.2: Average pollen tube size and PTGR at day 7 (168 hours) for both 32 and 24 °C (n=50; p<0.0001)

Species	32 ºC		24 ºC	
	Length (µm)	PTGR length (µm/h)	Length (µm)	PTGR length (µm/h)
P. radiata	170 ± 17.6 ^{A}	1.01 ± 0.1 ^{AB}	132 ± 8.9 ^{A}	0.78 ± 0.1 ^{A}
P. tecunumanii	189 ± 21.8 ^{AB}	1.12 ± 0.08 ^{AB}	123 ± 7.5 ^{AB}	0.74 ± 0.01 ^{AB}
P. oocarpa	153 ± 16.6 ^{B}	0.91 ± 0.08 ^{B}	112 ± 14.9 ^{AB}	0.67 ± 0.1 ^{AB}
P. elliottii	125 ± 14.3 ^{C}	0.74 ± 0.2 ^{C}	106 ± 14.2 ^{B}	0.63 ± 0.04 ^{B}
P. taeda	129 ± 15.4 ^{C}	0.76 ± 0.2 ^{C}	123 ± 38.6 ^{AB}	0.73 ± 0.2 ^{AB}
P. maximinoi	0.0 ± 0.0 ^{D}	0.0 ± 0.0 ^{D}	36 ± 7.7 ^{C}	0.22 ± 0.1 ^{C}
P. pringlei	0.0 ± 0.0 ^{D}	0.0 ± 0.0 ^{D}	0.0 ± 0.0 ^{D}	0.0 ± 0.0 ^{D}
	Width (µm)	PTGR width (µm/h)	Width (µm)	PTGR width (µm/h)
P. radiata	22 ± 2.9 ^{A}	0.13 ± 0.01 ^{A}	18 ± 1.8 ^{A}	0.10 ± 0.01 ^{AB}
P. tecunumanii	19 ± 1.0 ^{B}	0.11 ± 0.01 ^{B}	14 ± 1.2 ^{C}	0.08 ± 0.01 ^{C}
P. oocarpa	16 ± 0.9 ^{CD}	0.09 ± 0.01 ^{CD}	16 ± 2.3 ^{B}	0.10 ± 0.01 ^{BC}
P. elliottii	14 ± 1.3 ^{D}	0.08 ± 0.02 ^{D}	14 ± 1.9 ^{C}	0.08 ± 0.04 ^{C}
P. taeda	16 ± 2.3 ^{CD}	0.10 ± 0.01 ^{CD}	19 ± 2.8 ^{A}	0.11 ± 0.01 ^{AB}
P. maximinoi	0.0 ± 0.0 ^{E}	0.0 ± 0.0 ^{E}	15 ± 3.5 ^{BC}	0.09 ± 0.02 ^{C}
P. pringlei	0.0 ± 0.0 ^{E}	0.0 ± 0.0 ^{E}	0.0 ± 0.0 ^{D}	0.0 ± 0.0 ^{D}

Within columns, means with the same letter are not significantly different

Simulation study

Pollen germination was tested before each experiment and yielded more than 90 % with less than 10 % water content. For illustration purposes, only data of day seven (168 hours after incubation) for the nine temperature

regimes (micro, macro, 10, 15, 20, 24, 28, 32 and 36 °C) will be discussed as comparisons between species within temperature regimes.

Only *P. patula* (0.7 µm/h PTGR length and 0.2 µm/h PTGR width), *P. radiata* (0.4 µm/h PTGR length and 0.1 µm/h PTGR width) and *P. taeda* (0.6 µm/h PTGR length and 0.1 µm/h PTGR width) germinated and developed pollen tubes at 10 °C (Tables 4.3 and 4.4). At 15 °C, *P. radiata* (1 µm/h PTGR length and 0.2 µm/h PTGR width), *P. patula* (1 µm/h PTGR length and 0.1 µm/h PTGR width) and *P. taeda* (1 µm/h PTGR length and 0.1 µm/h PTGR width) gave similar results, while *P. elliottii* (0.7 µm/h PTGR length and 0.1 µm/h PTGR width), *P. pringlei* (0.7 µm/h PTGR length and 0.1 µm/h PTGR width) and *P. maximinoi* (0.7 µm/h PTGR length and 0.2 µm/h PTGR width) were comparable. *Pinus tecunumanii* (0.9 µm/h PTGR length and 0.1 µm/h PTGR width) and *P. patula*×*tecunumanii* (0.5 µm/h PTGR length and 0.1 µm/h PTGR width) had the slowest PTGR length, shortest and thinnest pollen tubes. *Pinus radiata* (2.3 µm/h PTGR length and 0.5 µm/h PTGR width) had the longest, widest and fastest PTGR at 20 °C, while *P. oocarpa* (0.7 µm/h PTGR length and 0.2 µm/h PTGR width) had the slowest PTGR, shortest and thinnest pollen tubes. At 24 °C, *P. radiata* (0.8 µm/h PTGR length and 0.1 µm/h PTGR width) and *P. taeda* (0.7 µm/h PTGR length and 0.1 µm/h PTGR width) again had similar results for length, width and PTGR's.

Pinus radiata had the longest and widest pollen tubes at 28 °C (250 µm long and 50 µm wide), 20 °C (385 µm long and 90 µm wide) and 15 °C (170 µm long and 33 µm wide; Tables 4.3 and 4.4). However, pollen tubes were more narrow and shorter at 10 °C (72 µm long and 20 µm wide) and 36 °C (82 µm long and 35 µm wide). *P. taeda* only had longer pollen tubes than *P. radiata* at 10 °C (99 µm), 15 °C (174 µm), 32 °C (185 µm) and 36 °C (100 µm). *Pinus maximinoi* (183 µm) and *P. elliottii* (248 µm) had longer pollen tubes at 32 °C than *P. radiata* (170 µm). At 36 °C, both *P. elliottii* (156 µm) and *P. maximinoi* (107 µm) again had longer pollen tubes than *P. radiata* (82 µm). In general, *P. radiata* had longer and wider pollen tubes than *P. oocarpa* and *P. tecunumanii*.

Although *P. oocarpa* and *P. tecunumanii* had a pollen viability of more than 90 %, no pollen tubes were visible during the micro and macro temperature regimes tested (Tables 4.3 and 4.4). Pollen viability of all the species was tested simultaneously to the micro and macro temperature regimes at 24 °C. Both species obtained more than 90 % pollen viability, indicating that the pollen were fresh and viable. In general, species responded differently to the micro and macro temperature regimes for pollen tube length, width and PTGR's. *Pinus taeda* had the longest and *P. maximinoi* the widest pollen tubes at micro and macro temperature regimes.

Table 4.3: Average pollen tube length and PTGR length for day 7 (168 hours) at different temperature regimes

Species	10 ºC		15 ºC		20 ºC	
	Length (µm)	PTGR length (µm/h)	Length (µm)	PTGR length (µm/h)	Length (µm)	PTGR length (µm/h)
P. radiata	72 ± 13.3 ^{B}	0.43 ± 0.19 ^{B}	170 ± 10 ^{A}	1.01 ± 0.06 ^{A}	385 ± 17.5 ^{A}	2.29 ± 0.10 ^{A}
P. tecunumanii	0 ± 0 ^{C}	0 ± 0 ^{C}	84 ± 7.9 ^{C}	0.50 ± 0.05 ^{C}	153 ± 11.6 ^{CD}	0.91 ± 0.07 ^{CD}
P. oocarpa	0 ± 0 ^{C}	0 ± 0 ^{C}	0 ± 0 ^{D}	0 ± 0 ^{D}	113 ± 16.4 ^{E}	0.67 ± 0.1 ^{E}
P. elliottii	0 ± 0 ^{C}	0 ± 0 ^{C}	120 ± 9.0 ^{B}	0.71 ± 0.05 ^{B}	170 ± 25.7 ^{C}	1.01 ± 0.15 ^{C}
P. taeda	99 ± 9.0 ^{A}	0.59 ± 0.08 ^{A}	174 ± 17.2 ^{A}	1.03 ± 0.10 ^{A}	250 ± 15 ^{B}	1.49 ± 0.1 ^{B}
P. maximinoi	0 ± 0 ^{C}	0 ± 0 ^{C}	112 ± 6.7 ^{B}	0.66 ± 0.04 ^{B}	155 ± 30.3 ^{CD}	0.92 ± 0.18 ^{CD}
P. pringlei	0 ± 0 ^{C}	0 ± 0 ^{C}	124 ± 32.2 ^{B}	0.74 ± 0.19 ^{B}	226 ± 14.1 ^{B}	1.35 ± 0.1 ^{B}
P. patula	114 ± 14.8 ^{A}	0.68 ± 0.13	167 ± 22.7 ^{A}	1.00 ± 0.13 ^{A}	166 ± 18.2 ^{CD}	0.99 ± 0.11 ^{CD}
P. patula×*tecunumanii*	0 ± 0 ^{C}	0 ± 0 ^{C}	80 ± 13.7 ^{C}	0.47 ± 0.08 ^{C}	143 ± 16.3 ^{D}	0.85 ± 0.1 ^{D}
	24 ºC		28 ºC		32 ºC	
P. radiata	132 ± 8.9 ^{C}	0.78 ± 0.05 ^{C}	514 ± 35.9 ^{A}	3.06 ± 0.14 ^{A}	170 ± 17.6 ^{CDE}	1.01 ± 0.1 ^{CDE}
P. tecunumanii	176 ± 12.4 ^{B}	1.05 ± 0.07 ^{B}	118 ± 7.7 ^{E}	0.70 ± 0.05 ^{E}	146 ± 14.4 ^{E}	0.87 ± 0.08 ^{E}
P. oocarpa	181 ± 15.6 ^{B}	1.08 ± 0.1 ^{B}	128 ± 15.7 ^{E}	0.76 ± 0.09 ^{E}	158 ± 13.4 ^{DE}	0.94 ± 0.08 ^{DE}
P. elliottii	175 ± 7.5 ^{B}	1.04 ± 0.04 ^{B}	167 ± 23.9 ^{C}	0.99 ± 0.14 ^{C}	248 ± 44.7 ^{A}	1.48 ± 0.3 ^{A}
P. taeda	123 ± 38.6 ^{C}	0.73 ± 0.23 ^{C}	210 ± 11.5 ^{B}	1.25 ± 0.07 ^{B}	186 ± 36.4 ^{BCD}	1.11 ± 0.2 ^{BCD}
P. maximinoi	226 ± 21.8 ^{A}	1.35 ± 0.13 ^{A}	136 ± 13.8 ^{DE}	0.81 ± 0.08 ^{DE}	183 ± 19.0 ^{BCD}	1.09 ± 0.1 ^{BCD}
P. pringlei	187 ± 17.8 ^{B}	1.11 ± 0.11 ^{B}	157 ± 19.5 ^{CD}	0.93 ± 0.12 ^{CD}	205 ± 15.2 ^{B}	1.22 ± 0.09 ^{B}
P. patula	222 ± 12.1 ^{A}	1.32 ± 0.07 ^{A}	215 ± 11.8 ^{B}	1.28 ± 0.07 ^{B}	158 ± 9.3 ^{DE}	0.94 ± 0.06 ^{DE}
P. patula×*tecunumanii*	174 ± 24.9 ^{B}	1.03 ± 0.15 ^{B}	121 ± 32.2 ^{E}	0.72 ± 0.19 ^{E}	198 ± 19.1 ^{BC}	1.18 ± 0.1 ^{BC}
	36 ºC		Micro-temperature regime		Macro-temperature regime	
P. radiata	82 ± 5.4 ^{DE}	0.49 ± 0.08 ^{DE}	180 ± 5.2 ^{C}	1.07 ± 0.03 ^{C}	191 ± 19.9 ^{B}	1.13 ± 0.11 ^{B}
P. tecunumanii	70 ± 11.8 ^{E}	0.41 ± 0.07 ^{E}	0 ± 0 ^{E}	0 ± 0 ^{E}	0 ± 0 ^{E}	0 ± 0 ^{E}
P. oocarpa	118 ± 7.2 ^{BC}	0.70 ± 0.04 ^{BC}	0 ± 0 ^{E}	0 ± 0 ^{E}	0 ± 0 ^{E}	0 ± 0 ^{E}
P. elliottii	156 ± 29.5 ^{A}	0.93 ± 0.17 ^{A}	176 ± 21 ^{C}	1.05 ± 0.12 ^{C}	124 ± 9.0 ^{C}	0.73 ± 0.05 ^{C}
P. taeda	100 ± 22.4 ^{CD}	0.59 ± 0.13 ^{CD}	239 ± 13.6 ^{A}	1.42 ± 0.08 ^{A}	211 ± 18.7 ^{A}	1.25 ± 0.11 ^{A}
P. maximinoi	108 ± 13.1 ^{BC}	0.64 ± 0.07 ^{BC}	167 ± 18.4 ^{CD}	1.00 ± 0.11 ^{CD}	115 ± 12.1 ^{C}	0.68 ± 0.07 ^{C}
P. pringlei	125 ± 14.3 ^{B}	0.74 ± 0.08 ^{B}	153 ± 15.4 ^{D}	0.91 ± 0.09 ^{D}	82 ± 8.9 ^{D}	0.49 ± 0.05 ^{D}
P. patula	152 ± 18.4 ^{A}	0.90 ± 0.1 ^{A}	210 ± 19.8 ^{B}	1.25 ± 0.11 ^{B}	195 ± 11.3 ^{AB}	1.16 ± 0.06 ^{AB}
P. patula×*tecunumanii*	116 ± 8.9 ^{BC}	0.69 ± 0.05 ^{BC}	153 ± 5.7 ^{D}	0.91 ± 0.03 ^{D}	118 ± 9.5 ^{C}	0.70 ± 0.05 ^{C}

Within columns, means with the same letter are not significantly different

Table 4.4: Average pollen tube width and PTGR width for day 7 (168 hours) at different temperature regimes

Species	10 °C		15 °C		20 °C	
	Width (µm)	PTGR width (µm/h)	Width (µm)	PTGR width (µm/h)	Width (µm)	PTGR width (µm/h)
P. radiata	20 ± 1.2 ^{B}	0.12 ± 0.007 ^{B}	33 ± 2.0 ^{A}	0.19 ± 0.01 ^{A}	91 ± 7.6 ^{A}	0.54 ± 0.04 ^{A}
P. tecunumanii	0 ± 0 ^{C}	0 ± 0 ^{C}	17 ± 1.6 ^{E}	0.10 ± 0.01 ^{E}	23 ± 2.4 ^{DE}	0.13 ± 0.01 ^{DE}
P. oocarpa	0 ± 0 ^{C}	0 ± 0 ^{C}	0 ± 0 ^{F}	0 ± 0 ^{F}	28 ± 1.6 ^{C}	0.17 ± 0.01 ^{C}
P. elliottii	0 ± 0 ^{C}	0 ± 0 ^{C}	21 ± 1.6 ^{CD}	0.12 ± 0.01 ^{CD}	26 ± 2.6 ^{CD}	0.16 ± 0.01 ^{CD}
P. taeda	22 ± 1.4 ^{AB}	0.13 ± 0.009 ^{B}	21 ± 3.6 ^{C}	0.12 ± 0.02 ^{C}	19 ± 2.4 ^{E}	0.11 ± 0.01 ^{E}
P. maximinoi	0 ± 0 ^{C}	0 ± 0 ^{C}	26 ± 2.4 ^{B}	0.15 ± 0.01 ^{B}	35 ± 3.0 ^{B}	0.21 ± 0.01 ^{B}
P. pringlei	0 ± 0 ^{C}	0 ± 0 ^{C}	20 ± 1.8 ^{CD}	0.12 ± 0.01 ^{CD}	27 ± 2.7 ^{C}	0.16 ± 0.01 ^{C}
P. patula	25 ± 2.6 ^{A}	0.15 ± 0.01 ^{A}	21 ± 1.2 ^{C}	0.13 ± 0.01 ^{C}	28 ± 1.8 ^{C}	0.17 ± 0.01 ^{C}
P. patula×*tecunumanii*	0 ± 0 ^{C}	0 ± 0 ^{C}	18 ± 1.4 ^{DE}	0.11 ± 0.01 ^{DE}	29 ± 3.3 ^{C}	0.17 ± 0.02 ^{C}
	24 °C		28 °C		32 °C	
P. radiata	18 ± 1.8 ^{D}	0.11 ± 0.01 ^{D}	109 ± 8.1 ^{A}	0.65 ± 0.04 ^{A}	22 ± 2.8 ^{E}	0.13 ± 0.01 ^{E}
P. tecunumanii	29 ± 2.1 ^{C}	0.17 ± 0.01 ^{C}	30 ± 2.0 ^{E}	0.18 ± 0.01 ^{E}	31 ± 3.3 ^{BCD}	0.18 ± 0.01 ^{BCD}
P. oocarpa	33 ± 3.0 ^{B}	0.19 ± 0.01 ^{B}	31 ± 1.5 ^{E}	0.18 ± 0.01 ^{E}	31 ± 2.8 ^{BCD}	0.19 ± 0.01 ^{BCD}
P. elliottii	27 ± 7.5 ^{B}	0.16 ± 0.04 ^{C}	30 ± 4.6 ^{E}	0.18 ± 0.02 ^{E}	28 ± 3.9 ^{CD}	0.17 ± 0.02 ^{CD}
P. taeda	19 ± 2.7 ^{D}	0.11 ± 0.01 ^{D}	38 ± 3.6 ^{BC}	0.23 ± 0.02 ^{BC}	27 ± 3.0 ^{DE}	0.16 ± 0.01 ^{DE}
P. maximinoi	37 ± 3.8 ^{A}	0.22 ± 0.02 ^{A}	43 ± 3.1 ^{B}	0.26 ± 0.01 ^{B}	43 ± 7.9 ^{A}	0.25 ± 0.04 ^{A}
P. pringlei	29 ± 3.0 ^{C}	0.17 ± 0.01 ^{C}	37 ± 2.7 ^{CD}	0.22 ± 0.01 ^{CD}	35 ± 5.5 ^{B}	0.21 ± 0.03 ^{B}
P. patula	33 ± 3.1 ^{B}	0.20 ± 0.01 ^{B}	37 ± 1.7 ^{CD}	0.22 ± 0.01 ^{CD}	33 ± 2.7 ^{BC}	0.20 ± 0.01 ^{BC}
P. patula×*tecunumanii*	33 ± 2.7 ^{B}	0.20 ± 0.01 ^{B}	32 ± 5.3 ^{DE}	0.19 ± 0.03 ^{DE}	35 ± 3.3 ^{B}	0.21 ± 0.02 ^{B}
	36 °C		Micro-temperature regime		Macro-temperature regime	
P. radiata	35 ± 2.9 ^{A}	0.21 ± 0.01 ^{AB}	41 ± 2.2 ^{C}	0.24 ± 0.01 ^{B}	32 ± 2.4 ^{A}	0.19 ± 0.01 ^{A}
P. tecunumanii	32 ± 4.3 ^{ABCD}	0.19 ± 0.02 ^{BCD}	0 ± 0 ^{E}	0 ± 0 ^{E}	0 ± 0 ^{C}	0 ± 0 ^{C}
P. oocarpa	27 ± 1.9 ^{E}	0.16 ± 0.01 ^{E}	0 ± 0 ^{E}	0 ± 0 ^{E}	0 ± 0 ^{C}	0 ± 0 ^{C}
P. elliottii	31 ± 4.1 ^{BCD}	0.18 ± 0.02 ^{CD}	30 ± 1.6 ^{C}	0.18 ± 0.01 ^{C}	25 ± 1.2 ^{B}	0.15 ± 0.01 ^{B}
P. taeda	30 ± 2.6 ^{DE}	0.18 ± 0.01 ^{DE}	25 ± 2.3 ^{D}	0.15 ± 0.01 ^{D}	24 ± 2.4 ^{B}	0.14 ± 0.01 ^{B}
P. maximinoi	34 ± 4.7 ^{ABC}	0.20 ± 0.02 ^{ABC}	48 ± 6.8 ^{A}	0.28 ± 0.01 ^{A}	35 ± 7.0 ^{A}	0.21 ± 0.04 ^{A}
P. pringlei	34 ± 2.2 ^{AB}	0.20 ± 0.01 ^{ABC}	29 ± 1.6 ^{CD}	0.17 ± 0.01 ^{CD}	20 ± 1.4 ^{B}	0.12 ± 0.01 ^{B}
P. patula	36 ± 2.3 ^{A}	0.21 ± 0.01 ^{A}	37 ± 2.9 ^{C}	0.22 ± 0.01 ^{B}	32 ± 5.4 ^{A}	0.19 ± 0.03 ^{A}
P. patula×*tecunumanii*	30 ± 2.2 ^{CDE}	0.18 ± 0.01 ^{DE}	30 ± 1.7 ^{C}	0.18 ± 0.01 ^{C}	23 ± 1.7 ^{B}	0.14 ± 0.01 ^{B}

Within columns, means with the same letter are not significantly different

Sigmoid growth curves

The sigmoid growth curves for micro- and macro-temperature regimes were constructed as a summary of pollen tube length over a seven-day period (measured per day for seven days). Pollen grains for both *P. oocarpa* and *P. tecunumanii* only germinated at day 8 and were eliminated from the sigmoid growth curves due to insufficient data points. The maximum pollen tube length at micro-climate indicated that *P. radiata* (203 µm), *P. elliottii* (297 µm) and *P. taeda* (295 µm) differed significantly ($p<0.001$; $r^2=0.73$) from the remaining species (Figure 4.6). Both *P. radiata* and *P. patula* had the same slope, but did differ significantly from the other species ($p<0.001$; $r^2=0.73$). However, *P. radiata* only differed significantly ($p<0.001$; $r^2=0.73$) from *P. patula*×*tecunumanii* for the time period to reach maximum pollen tube length.

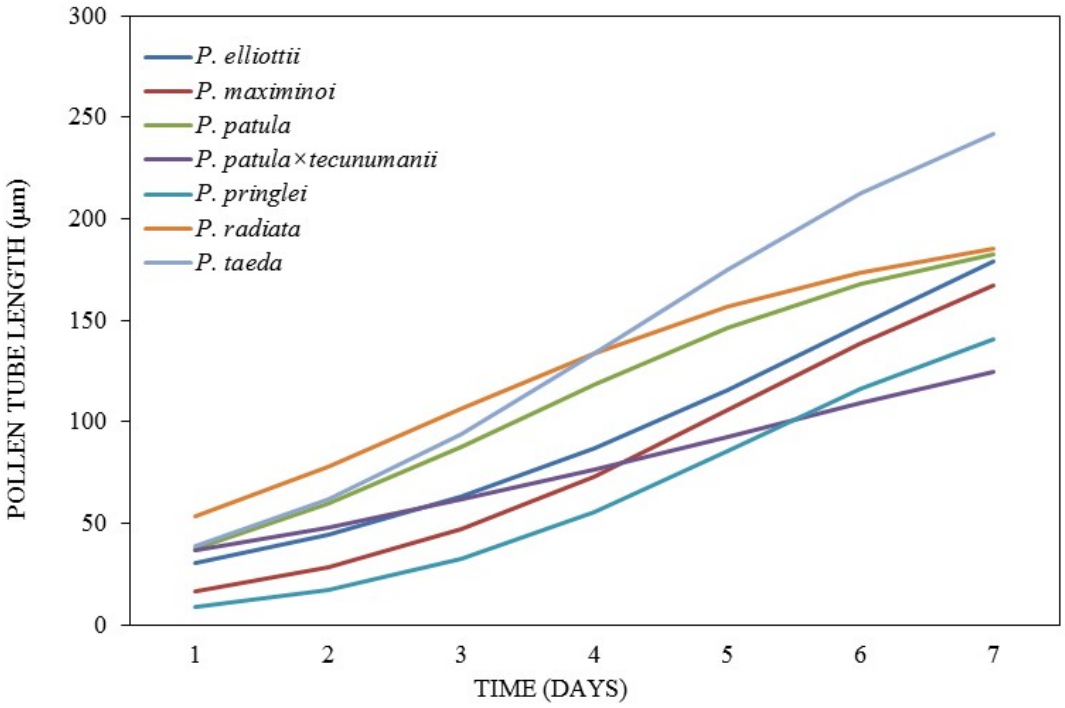

Figure 4.6: Pollen tube length sigmoid growth curve at micro-temperature regime for seven days

Macro-temperature regime sigmoid growth curves indicated (Figure 4.7) that *P. radiata* (236 µm) differed significantly ($p<0.001$; $r^2=0.73$) from *P. patula*×*tecunumanii* (146 µm) for maximum pollen tube length, while it differed significantly ($p<0.001$; $r^2=0.73$) from *P. elliottii* and *P. pringlei* for the slope of the graph. The time period to reach maximum pollen tube length indicated no significant difference between the seven species.

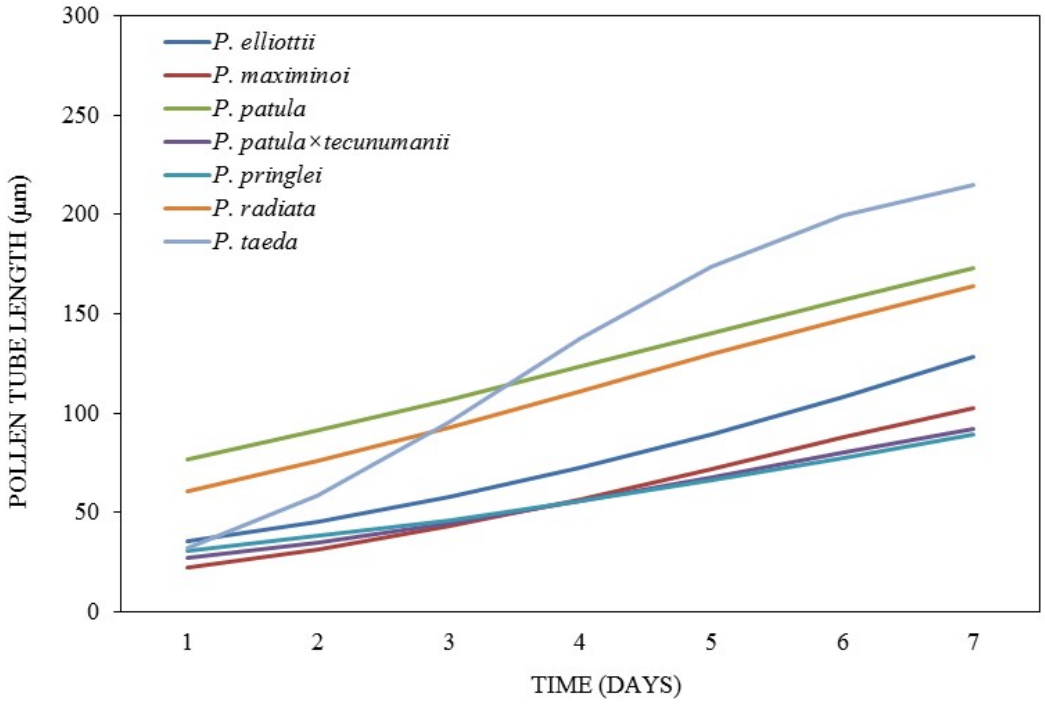

Figure 4.7: Pollen tube length sigmoid growth curve at macro-temperature regime for seven days

3.4 Pollen grain morphology

Pollen grain length (dehydrated) of *P. radiata* (55 µm) and *P. taeda* (52 µm) differed significantly (Table 4.5; $p<0.001$; $r^2=0.61$) from *P. elliottii* (49 µm), *P. tecunumanii* (47 µm), *P. pringlei* (44 µm), *P. maximinoi* (41 µm) and *P. oocarpa* (37 µm). However, it did not differ significantly from *P. taeda, P. patula* and *P. patula×tecunumanii. Pinus radiata* (48 µm), *P. tecunumanii* (49 µm) and *P. patula×tecunumanii* (49 µm) width (dehydrated) differed significantly ($p<0.001$, $r^2=0.64$) from *P. taeda* (52 µm), *P. patula* (54 µm), *P. elliotii* (42 µm) *P. pringlei* (43 µm) and *P. maximinoi* (42 µm). *Pinus oocarpa* had the shortest (37 µm) and narrowest (38 µm) pollen grains of all the species when dehydrated. The pollen grain length of *P. patula×tecunumanii* compared to parent species (*P. patula* and *P. tecunumanii*) was not significantly wider than those of *P. patula* but both *P. patula* and *P. patula×tecunumanii* pollen grains were significantly longer than that of *P. tecunumanii*. *Pinus patula* had a significantly larger pollen grain width than *P. tecunumanii* and *P. patula×tecunumanii*, while the latter species did not differ significantly in pollen grain width. Results from this study compared well with previous studies on pollen grain size by Williams (2009), Nel & van Staden (2005) and Cains (1940) (Table 4.6). However, Cain's study (1940) on fossil pollen (*P. taeda)* indicated a bigger pollen grain length, while the grain width was smaller than in this study.

Re-hydrated pollen grain size (length and width) were measured 24 hours after incubation at both micro- and macro-temperature regimes to compare with de-hydrated pollen grains (Table 4.7). At micro temperature regime, re-hydrated pollen grains of *P. oocarpa* (74 µm), *P. radiata* (71 µm), *P. taeda* (70 µm), *P. maximinoi*

(70 µm) and *P. patula*×*tecunumanii* (70 µm) had significantly (p<0.001; r^2=0.26) longer pollen grains than *P. elliottii* (67 µm) and *P. tecunumanii* (66 µm). However, *P. radiata* (48 µm) only differed significantly (p<0.001; r^2=0.26) from *P. tecunumanii* (43 µm) and *P. elliottii* (53 µm) for pollen grain width. Pollen tube length at macro-temperature regime showed less variation as *P. radiata* (70 µm) differed only significantly (p<0.001; r^2=0.26) from *P. patula* (64 µm). For pollen tube width, *P. radiata* (45 µm) differed only significantly from *P. elliottii* (50 µm), *P. taeda* (49 µm) and *P. patula*×*tecunumanii* (50 µm). In general, pollen grain length of *P. radiata* did not differ between micro (48 µm) and macro (45 µm) temperature regimes, but it did differ slightly for width. There were more differences between dehydrated and re-hydrated length and width for all the species, than between micro and macro-temperature regimes. Although *P. oocarpa* had the shortest and narrowest pollen grains when dehydrated, it had the longest and widest grains when re-hydrated.

Table 4.5: De-hydrated pollen grain length and width for nine *Pinus* species

Species	Average pollen grain size (n = 25)	
	Length (µm)	Width (µm)
P. radiata	55 ± 4.8 ^{AB}	48 ± 3.7 ^{B}
P. tecunumanii	47 ± 2.7 ^{D}	49 ± 2.6 ^{B}
P. oocarpa	37 ± 3.5 ^{G}	38 ± 3.5 ^{D}
P. elliottii	49 ± 2.9 ^{CD}	42 ± 3.8 ^{C}
P. taeda	52 ± 4 ^{A}	52 ± 4.7 ^{A}
P. maximinoi	41 ± 4.5 ^{F}	42 ± 4.2 ^{C}
P. pringlei	44 ± 4.2 ^{E}	43 ± 4.1 ^{C}
P. patula	50 ± 4.2 ^{BC}	54 ± 3.2 ^{A}
P. patula×*tecunumanii*	51 ± 4.9 ^{ABC}	49 ± 4.2 ^{B}

Within columns, means with the same letter are not significantly different

Table 4.6: Comparison of de-hydrated pollen morphology between previous and this study

Species	Literature				Results from this study		
	Type of pollen	Grain length (µm)	Grain width (µm)	*Reference	Type of pollen	Grain length (µm)	Grain width (µm)
P. taeda	fossil unknown	66 – 72 mean 53	45 - 48	a b	fresh	46 – 61	41 – 60
P. radiata	fresh	34 – 59		c	fresh	43 – 60	42 – 55
P. oocarpa	fresh	34 – 52		c	fresh	30 – 46	30 – 45
P. patula	fresh	31 – 59		c	fresh	42 – 60	48 – 60
P. tecunumanii	fresh	38 – 53		c	fresh	42 – 53	45 – 55

a: Cain 1940; b: Williams 2009; c: Nel and van Staden 2005

Table 4.7: Re-hydrated pollen grain size (length and width) for nine *Pinus* species at macro- and micro-climate

Species	Micro-climate (n = 25)		Macro-climate (n = 25)	
	Length (µm)	Width (µm)	Length (µm)	Width (µm)
P. radiata	71 ± 6.9 ^{AB}	48 ± 7.1 ^{BC}	70 ± 5.8 ^{AB}	45 ± 5.2 ^{CD}
P. tecunumanii	66 ± 5.7 ^{DE}	43 ± 5.4 ^{D}	68 ± 4.6 ^{B}	47 ± 6.9 ^{BC}
P. oocarpa	74 ± 5.6 ^{A}	47 ± 5.6 ^{BCD}	72 ± 5.2 ^{A}	47 ± 6.8 ^{ABC}
P. elliottii	67 ± 5.4 ^{CD}	53 ± 6.7 ^{A}	70 ± 5.6 ^{AB}	50 ± 5.6 ^{A}
P. taeda	70 ± 5.9 ^{ABC}	47 ± 8.9 ^{BC}	72 ± 7.0 ^{A}	49 ± 7.4 ^{AB}
P. maximinoi	70 ± 6.9 ^{ABC}	49 ± 5.9 ^{B}	72 ± 6.2 ^{A}	47 ± 6.3 ^{ABC}
P. pringlei	64 ± 4.0 ^{F}	46 ± 6.6 ^{CD}	67 ± 4.4 ^{BC}	45 ± 6.1 ^{CD}
P. patula	63 ± 6.3 ^{EF}	45 ± 5.2 ^{CD}	64 ± 5.4 ^{C}	42 ± 4.3 ^{D}
P. patula×*tecunumanii*	70 ± 5.8 ^{BC}	48 ± 5.6 ^{BC}	69 ± 5.0 ^{AB}	50 ± 6.5 ^{A}

Within columns, means with the same letter are not significantly different

3.5 Correlation between PTGR and pollen grain size

As PTGR was expressed as pollen tube elongation over time (hours), PCA and DA analyses were performed only on PTGR (length and width) at both micro- and macro-temperature regimes. Distinct groups were evident in both biplots for PTGR's (Figure 4.8; 99 % confidence interval). PTGR width (micro and macro) is in the lower right quadrant and PTGR length (micro and macro) in the upper right quadrant. Both PCA and DA biplots statistically grouped *P. maximinoi*, *P. elliottii*, *P. radiata* and *P. taeda*, indicating significant differences from the other species.

Pollen grain size (hydrated and dehydrated) for micro- and macro- temperature regimes were analysed (Figure 4.9; 95 % confidence interval). Species were statistically grouped together as *P. patula*×*tecunumanii*, *P. radiata* and *P. taeda*, while the other species differed significantly. Length and width for dehydrated (left upper quadrant) and hydrated width for micro- and macro-climates (right upper quadrant) differed significantly as shown in Tables 4.6 and 4.8.

A strong positive correlation was evident between PTGR and pollen grain size (Figure 4.10; 97 % confidence interval). *Pius tecunumanii* and *P. oocarpa* (left quadrant) differed significantly from the other taxa. *Pinus maximinoi*, *P. elliottii, P. taeda* and *P. patula*×*tecunumanii* grouped closely with *P. radiata*, indicating that interspecific hybridisation between these taxa might be possible.

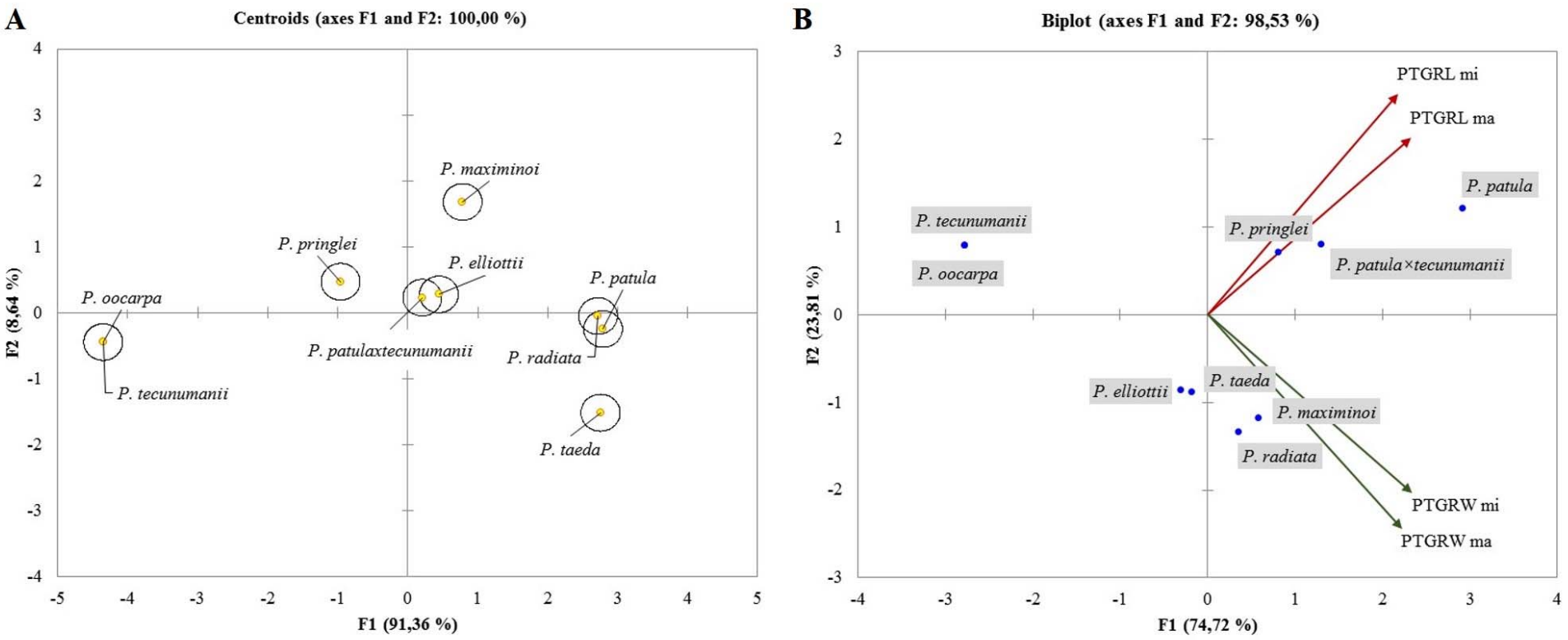

Figure 4.8: DA (A) and PCA (B) biplots indicating significant differences for PTGR (length and width) at micro- and macro-temperature regimes for the nine pine taxa (WPTGR ma: width PTGR macro; WPTGR mi: width PTGR micro; LPTGR ma: length PTGR macro; LPTGR mi: length PTGR micro)

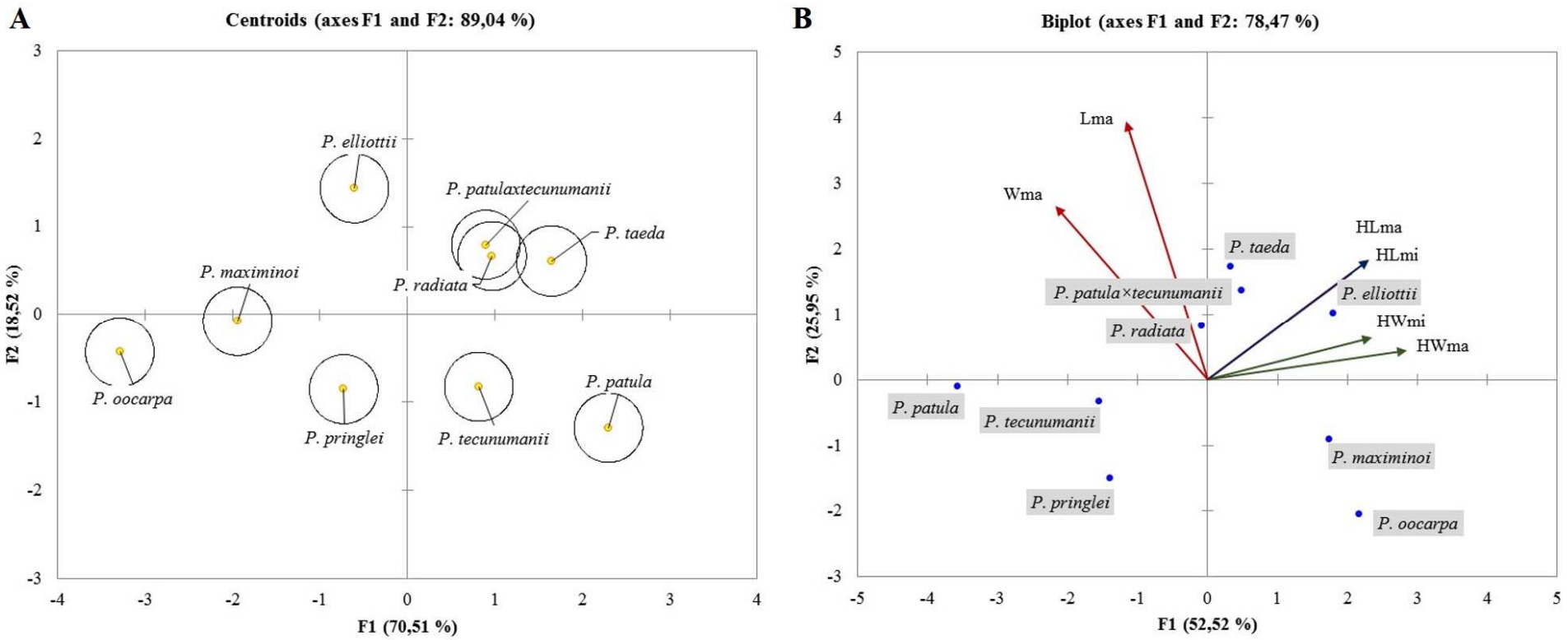

Figure 4.9: DA (A) and PCA (B) biplots indicating significant differences for hydrated and de-hydrated pollen grain size (length and width) at micro- and macro-temperature regimes for the nine pine taxa (Wma: width macro; Lma: length macro; HLmi: hydrated length micro; HLma: hydrated length macro; HWmi: hydrated width micro; HWma: hydrated width macro)

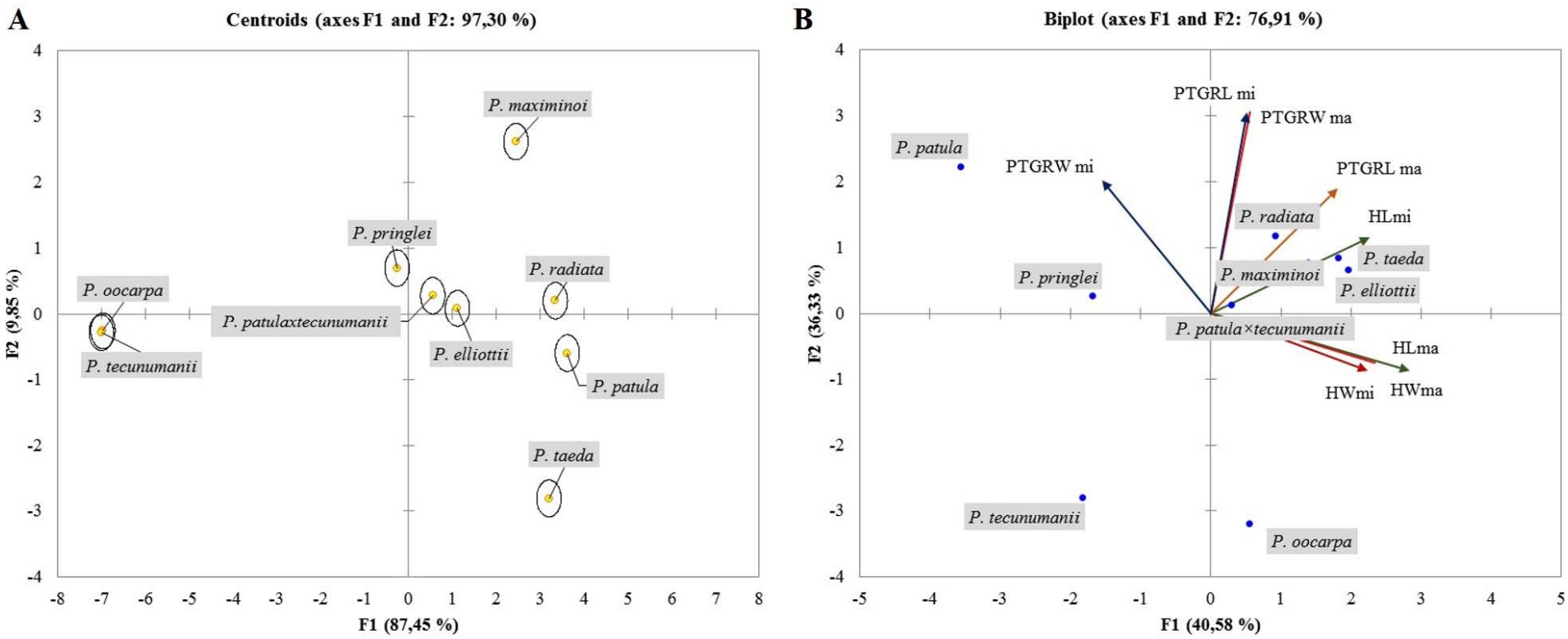

Figure 4.10: DA (A) and PCA (B) biplots indicating significant differences for the Pearson correlation between PTGR (length and width) and hydrated pollen grain size (length and width) at micro- and macro-temperature regimes for the nine pine taxa (HLmi: hydrated length micro; HLma: hydrated length macro; HWmi: hydrated width micro; HWma: hydrated width macro; WPTGR ma: width PTGR macro; WPTGR mi: width PTGR micro; LPTGR ma: length PTGR macro; LPTGR mi: length PTGR micro)

4. Discussion

Pollen tube development and PTGR are dynamic processes that determine pollination success and varies among *Pinus* species (McWilliam 1959b; Chijioke et al. 2010; Ellstrand 2014; Williams et al. 2016). Studies in Angiosperms indicated that retarded growth of pollen tubes in *Betula verrucosa×pubescens* hampered pollination success (Hagman 1975), while overly large pollen grains might clog the openings in the stigma depth and too short pollen tubes might limit pollination success (Cruden 2000; Potts and Dungery 2004). As these phenomena might also exist in *Pinus* species (McWilliam 1959a; Hagman 1975; Owens et al. 2005), it was investigated. Therefore, it was hypothesised that *P. radiata* hybrid partners need to have pollen tube elongation, PTGR and grain size comparable to that of *P. radiata*.

Although pollen tube elongation and pollen grain size are controlled by genetic factors (Stanton and Preston 1986), non-genetic factors (temperature and humidity during pollination) can also have an impact on pollination success (Elgersma et al. 1989; Slee and Abbott 1990; Kelly et al. 2002; Chijioke et al. 2010). Although pollen tube growth represents viability (Mulcahy and Mulcahy 1987; Derksen 2002; Anderhag et al. 2002; Fernando 2005), pollen tube development is more sensitive to temperature (Varshney and Varshnye 1982). Other factors impacting on *in vitro* pollen tube development and germination specifically, are pollen age, water moisture content, genetic variations between species (Lanner 1966; Greenwood 1980; Bramlett and Matthews 1991; Jett et al. 1993; Fernando et al. 1997, 2005a; Dafni et al. 2005; Williams 2012), and agar gels (pH, sucrose content, hydration of pollen) (Webber 1995). Therefore, care was taken during the *in vitro* experiments to limit the effect of non-genetic factors on the results of pollen tube size and PTGR. Although water moisture content varied between pollen lots, pollen germination was still high (more than 90 %) and was consistent with studies by Owens & Fernando (2007), Dumont-BéBoux & Aderkas (1997) and Varshney & Varshney (1981).

The effect of pollen competition and allelopathy on pollen germination, pollen tube size and PTGR were not investigated during this study. The study of pollen competition or density under *in vitro* conditions is more complicated as there are only pollen grains of one species on the agar gel (Callaham 1966). However, during the *in vitro* experiments, it was noted that when pollen grains are closer together on the agar gel, pollen germination was higher with much variation in pollen tube elongation. These areas were avoided when recording data. In angiosperms, when many pollen grains reach a receptive stigma simultaneously (pollen competition), grains that germinate the fastest might outcompete slower ones to fertilise ovules (Stanton and Preston 1986). However, in *Pseudotsuga menziesii* (Douglar-Fir) studies, pollen competition did not affect pollination success but rather embryo abortion (Nakamura and Wheeler 1992). The effect of pollen competition on pollen germination during *in vitro* experiments need to be investigated in future studies.

At constant temperatures *P. taeda* and *P. patula* performed comparable to *P. radiata* for pollen germination, PTGR and pollen tube size. The micro-temperature regime indicated that for pollen tube and PTGR length,

P. elliottii and *P. maximinoi* to a lesser extent, performed comparable to *P. radiata*. This might be as the temperature inside the pollination bags were closer to Sabie (Chapter 3; Ham et al. 2017). However, for pollen tube and PTGR width at micro-temperature regime, *P. patula* performed comparable to *P. radiata*. At macro-temperature regime, *P. patula* and *P. taeda* performed comparable to *P. radiata* for pollen tube and PTGR length, while *P. patula* and *P. maximinoi* compared well to *P. radiata* for pollen tube and PTGR width. Both sigmoid growth curves (micro- and macro-temperature regimes) indicated that *P. patula* performed comparable to *P. radiata* for PTGR length. As both *P. radiata* and *P. patula* are highly susceptible to *F. circinatum*, *P. patula* is not a feasible hybrid partner. Furthermore, limited success was obtained with *P. patula* in previous studies when hybridised with *P. radiata* (Dungey et al. 2003).

As pollen studies should be investigated under conditions as close to *in vivo* as possible (Hesse 2000), mimicking the temperature fluctuations inside (micro) and outside (macro) the pollination bags for a 24-hour cycle, could improve the understanding of temperature as a possible barrier to pollination success (Ham et al. 2017). Two circadian models representing these 24-hour temperature regimes during a pollination season at a *P. radiata* seed orchard (Karatara, South Africa) that were developed (Ham et al. 2017), indicated fluctuation in temperatures and night-time temperatures might be too low for *P. tecunumanii* and *P. oocarpa* (Chapter 3; Ham et al. 2017). These results correlate with previous studies in both tomatoes and herbs, which indicated that the lower night temperatures and 24-hour fluctuations between maximum and minimum temperatures, have a more significant influence on pollen germination and tube elongation than higher average temperatures alone (Peet and Bartholemew 1996; He et al. 2006).

Pollen tube size and PTGR of *P. radiata* deteriorated at 32 and 36 °C. Previous studies indicated that high temperature (more than 30 °C) had a limiting factor on the germination of *P. nigra* (temperate pine) pollen (McWilliam 1959b), while temperature more than 46 °C were lethal to *P. patula* (Nel 2002). However, no pollination for *P. nigra* were evident at temperatures lower than 15 °C (McWilliam 1959c). Tanaka (1955) germinated *P. densiflora* (temperate to subtropical pine) pollen between 20 and 34 ºC, indicating that both germination and tube growth were better at higher temperatures. Limited studies are available on the PTGR length and width of *Pinus* species, but *P. radiata* compared well to *P. sylvestris* PTGR length at all nine temperature regimes tested. Previous studies indicated that PTGR (length) of gymnosperms (for example *P. sylvestris* 1 µm/h) are slower than angiosperm (for example *Austrobaileya* species 271 µm/h) due to different morphology and evolutionarily more primitive characteristics (de Win et al. 1996; Anderhag et al. 2000; Fernando 2005; Williams 2012).

As pollen grains are dehydrated (water content less than 10 %) before being shed (Fernando et al. 2005) and re-hydrated to float when captured by the droplet (Fernando et al. 2005; Chichiriccò and Pacini 2008), re-hydrated pollen grain size (length and width) were also recorded for the micro- and macro-temperature regimes to simulate the natural state (Hesse 2000). Pollen morphology studies on re-hydrated *Pinus* pollen grains are

not available. This might be as previous studies focused on differences in size, shape, surface sculpture, stratification, presence or absence of obvious apertures for taxonomic purposes.

De-hydrated pollen grain size (length and width) of *P. patula* was the most comparable to *P. radiata*. However, no single species were comparable with *P. radiata* during the re-hydration study that simulated micro- and macro-temperature regimes. *Pinus taeda* was the closest related during the micro-temperature regime, while *P. elliottii* and *P. pringlei* at the macro-temperature regime for PTGR length and width respectively. In general, all the taxa showed an increase of 10 to 20 % between de-hydrated and re-hydrated pollen tube length, except *P. oocarpa* that increased by at least 50 %. This is a clear indication that although *P. oocarpa* had the smallest de-hydrated pollen grain size, the effect of re-hydration during the capturing in the pollen droplet (size recognition) cannot be ignored and need to be investigated further. However, the effect of species recognition (chemical or hormonal) can still hamper pollination success.

The dehydrated (air dried) pollen grains were generally flatter (smaller in length) in appearance than re-hydrated ones. This might be due to the middle intine layer that disappears or falls flat during dehydration (Hesse 2000; Chichiriccò and Pacini 2008). As the pollen grains absorb water during re-hydration, the intine layer will inflate and the pollen grain will have a more rounded shape (Hesse 2000; Chichiriccò and Pacini 2008). This confirms that saccate *Pinus* pollen grains have unique hydrodynamics, assisting with exclusion of foreign particles from the pollen chamber or micropyle (Tomlinson 1994). Reserves inside the pollen grain might also assist with the PTGR or the survival of the pollen tube for 12 months inside the ovule in the time between pollination and fertilisation. Future studies should investigate differences in pollen morphology between dehydrated and re-hydrated pollen grains in terms of pollen wall stratification, intine, pollen tube formation and correlation with pollination.

A Pearson correlation by means of biplots (DA and PCA) were performed to determine whether the combined and separate effect of pollen grain size and PTGR can predict pollination success between *P. radiata* and the eight pine taxa (seven pure species and one hybrid). The biplots confirmed the notion that *P. tecunumanii* and *P. oocarpa* might not be good male hybrid partners for *P. radiata* in terms of PTGR, pollen grain size and both PTGR and pollen grain size. Previous experiments by Dungey et al. (2003) also support this notion. Although the biplots grouped *P. radiata* and *P. taeda* together for pollen grain size, *P. radiata* grouped with *P. patula* for PTGR. However, the combined effect of PTGR and pollen grain size, *P. radiata* grouped with *P. taeda* and *P. elliottii*. This is consistent with a previous study where climatic regimes in native conditions of provenances of *P. taeda* and *P. elliottii*, grouped with both Karatara and *P. radiata* (Chapter 2).

In conclusion, the reproductive process in plants represents not only a critical phase in their life-cycle, but understanding the reproductive cycle and investigating incompatibility (reproductive barriers) is imperative to pollination success. Temperature fluctuations between day and night had an impact on pollen grain size (re-hydration) and tube growth during micro- and macro-temperature regimes. However, PTGR, pollen tube and

grain size cannot provide an accurate prediction whether a *Pinus* species is a good hybrid partner for *P. radiata*. It was however possible to distinguish between groups of *Pinus* species with similar growth patterns at various temperatures. The tropic species performed the same but differed significantly from *P. radiata*. Pollen grain size also varied considerable between de-hydrated and re-hydrated grains. The interaction of the pollen grain size, pollen tube size and PTGR cannot be established with *in vitro* studies as with angiosperms due to the complex interactions between pollen grains, pollen droplet and the nucellus of the ovule. There are various chemical or hormonal interactions that not only filter-out pollen from unknown species, but also re-hydrate the pollen grains for successful pollination and tube growth. Although this study provided some answers on pollen grain size, pollen tube size, PTGR and the limited success of previous interspecific hybrid attempts with *P. radiata*, it is clear that *in vitro* experiments will not provide all the answers in the absence of chemical and hormonal interaction of *in vivo* conditions.

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Chapter 5

Determine *in vivo* pollination success

1. Introduction

Studying the reproductive processes of *Pinus* species not only has practical application in breeding programmes, but provides a better understanding on how to increase pollination success (Bramlett and O'Gwynn 1980; Greenwood 1980; Williams 2009; Fernando 2014; Santos-Del-Blanco and Climent 2014). Pollination success is not only measured by counting the number of pollen grains attached to the micropyle or inside the ovules, but also by *in vitro* pollen germination and pollen tube growth tests (Chapter 4; Owens et al. 2005; Owens and Fernando 2007). However, pollination success often become more dominant when the genetic distance between species (evolutionary history) is large (Chapter 1), and might also be influenced by environmental conditions inside pollination bags (Chapter 3; Ham et al. 2017), suboptimal pollen management (Chapter 4), or abnormal female strobili and conelet development (Huyn 1976, Nel and van Staden 2003, Solga et al. 2014).

The reproductive biology of *Pinus* species can be divided into three general phases: first year seasonal growth (pollination); second year seasonal growth (fertilisation); and a period of cone maturation (Sweet and Bollmann 1970; Williams 2009; Fernando 2014). Therefore, the different stages of the female flower development are defined as female strobilus maturation up to pollination, conelet development between pollination and fertilisation, and cone elongation after fertilisation until harvesting (Williams 2009). As pines are wind-pollinated, the ovule represents the ultimate effective pollen trap while the morphology is adapted to partially filter-out pollen of non-conspecific taxa (McWilliam 1958; Schwendemann et al. 2007; Funda et al. 2014). During pollination, pollen sifts between the open scales of the female strobilus and some will land on the ovule's micropylar arms (Tomlinson 1994; Williams 2009; Fernando 2014). During the receptivity of the female strobilus, generally lasting a few days, ovules exude a localized pollen droplet at night that recedes before daybreak (Slee and Abbott 1990; Owens et al. 2005; Williams 2009; Fernando 2014).

The pollen droplet is neither a water droplet nor a product of guttation in the adult tree (O'Leary and von Aderkas 2006), but an aqueous, protein-rich solution (Tomlinson et al. 1997) that consists of three sugars (sucrose, glucose and fructose) and has a total concentration of approximately 1.25 % (McWilliam 1958). Rainwater has been found to be an effective substitute for the pollen droplet in *P. taeda* (Greenwood 1986; Brown and Bridgwater 1987). The pollen droplet fills the micropylar opening (Tomlinson 1994; Lee 1995), capturing the pollen grain, hydrates it and provides transport to the chamber inside the ovules (Greenwood 1986; Brown and Bridgewater 1987; Slee and Abbott 1990; Varis et al. 2008), where the spongy nucellus serves as a medium for pollen grain germination and tube elongation (Williams 2009). Pollen grains can remain outside the female strobili until early morning when the pollen droplet emerges to capture it (Owens et al. 1991; Cresswell et al. 2007). This process ensures that only a few pollen grains reach the ovule even though heavy quantities of pollen were released during wind pollination (Williams 2009). Therefore, if pollen droplets play an important part in the recognition of pollen grains, then the number of pollen grains outside and inside

the ovules, as well as the number of pollen tubes that elongated, should differ significantly between different interspecific cross combinations compared to pure species.

Pinus radiata exhibits a high degree of reproductive incompatibility with the commercially important pine species (Moran and Griffin 1985), which are genetically distant from it (Dvorak et al. 2000). Previous studies have reported that interspecific crosses between *P. radiata, P. oocarpa, P. maximinoi* and *P. tecunumanii* yielded insignificant success (Dungey et al. 2003). This study aimed to investigate whether the pollen grains of *P. maximinoi, P. oocarpa* and *P. tecunumanii* do enter ovules via the micropyle of *P. radiata* as an indication of pollination success within the first seven weeks after pollination. This was done through histology studies which consist of a fixation-dehydration-embedding sequence (Feder and O'Brien 1968), while CT and microCT scans were used to determine when fertilisation happens and whether a 3D model on intact cones can be used to determine seed set and viability. The frequency and production of droplets and the entry of late arriving pollen to the cone and micropyle, as well as evidence of fertilisation success, were not part of this study.

2. Materials and methods

Pinus radiata female strobili were pollinated with freshly collected pollen polymixes from *P. maximinoi* (*P. radiata*×*maximinoi*), *P. tecunumanii* (*P. radiata*×*tecunumanii*), *P. oocarpa* (*P. radiata*×*oocarpa*) and *P. radiata* (*P. radiata*×*radiata*) during two consecutive pollination seasons (2012 and 2013) in a *P. radiata* seed orchard at Karatara (33 ° 54 ' 0 " South, 22 ° 50 ' 0 " East). The generic pollination protocol developed in Chapter 2 was employed and adapted as discussed in Chapter 3. Although cross-pollinations were performed, destructive sampling between the pollination event and seven weeks after pollination resulted in no seed production.

To determine pollination success, conelets from all four hybrid crosses were used to count the number of visible ovules, number of visible pollen grains inside and outside the ovules, as well as the number of visible pollen tubes within the ovules. Prior to pollination, water content was less than 10 % and viability was more than 90 % for all the pollen lots. Micro-fibre pollination bags (green cloth with a clear window) were placed over female cone clusters and tied to the branch. To limit pollen contamination, breeding equipment were sterilised and new green cloth bags were used (Chapter 2). Pollinations were performed daily between 10:00 and 16:00 in the top and northern side of trees to increase the temperature inside the pollination bags (Chapter 2; Ham et al. 2017*a*). A total of 272 *P. radiata* female strobili were initially pollinated to study pollination success at weekly intervals until seven weeks after pollination. To test the pollination protocol, only female strobili of *P. radiata*×*radiata* were harvested within 24 hours after pollination to investigate whether pollen grains sifted between the open scales (Tomlinson 1994; Williams 2009; Fernando 2014).

Pollination success varied between the cross combinations over the seven-week period. The control (*P. radiata*×*radiata*) had an 86 % pollination success, while *P. radiata*×*maximinoi* had 50 % and both *P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii* approximately 33 % (Table 5.1). Due to logistical constraints (strong winds hampering harvesting with cherry pickers), conelets could not be collected during Week 6. The experimental unit was adjusted to compensate for the lower levels of pollination success and lack of data for Week 6 to three conelets per cross combination harvested at weekly intervals for Weeks 1 to 5 and 7 after pollination (Slee and Abbott 1990). Climatic data were collected for the cross combinations as described in Chapter 3 and will be compared to Sabie data.

Table 5.1: Number of pollinated *P. radiata* female strobili with pollen lots *of P. radiata, P. maximinoi, P. oocarpa* and *P. tecunumanii* over a seven week period with the number of harvested conelets per cross combination

Time interval	Number of pollinated female strobili					Number of conelets				
	rxr	rxm	rxo	rxt	Total	rxr	rxm	rxo	rxt	Total
Week 1	10	10	10	10	40	10	6	4	3	23
Week 2	10	10	10	10	40	10	7	3	5	25
Week 3	10	10	10	10	40	10	5	4	4	23
Week 4	10	9	10	10	39	10	6	3	4	23
Week 5	10	10	9	10	39	10	6	4	3	23
Week 6	9	10	10	9	38	0	0	0	0	0
Week 7	9	9	9	9	36	9	5	5	3	22
Total	68	68	68	68	272	59	35	23	22	139

r×r: *P. radiata*×*radiata*, r×m: *P. radiata*×*maximinoi*, r×o: *P. radiata*×*oocarpa*, r×t: *P. radiata*×*tecunumanii*

2.1 Paraffin wax method

Histology studies are based on the examination of specimens preserved by fixation, dehydrated, embedded in a solid matrix, sectioned and stained (Feder and O'Brien 1968). The paraffin wax method is considered the most suitable for routine preparation, sectioning and staining (Winsor 1994) resulting in live-like permanent specimens (Feder and O'Brien 1968; Coura et al. 2005). However, quality of structural preservation seen in the final stained and mounted section is largely determined by the choice of fixative and embedding medium (Winsor 1994). Throughout the paraffin wax method it is important to note that structural damage can happen during any step of the fixation-dehydration-embedding sequence and cannot be rectified (Feder and O'Brien 1968; Coura et al. 2005). Some tissues or elements can have cracks or loose shape (disfigure) even with the best methods available (Feder and O'Brien 1968). Therefore, during preservation, distortion and shrinkage should be limited (Winsor 1994; Coura et al. 2005).

Over the seven-week period, a total of 75 conelets were collected and fixated in 50 % Formalin-Acetic-Alcohol (FAA) solution in sealed glass bottles that consisted of 40 % formalin, glacial acetic acid, ethanol (96 %) and

distilled water (10:5:50:35 v/v) (Sass 1958; Coetzee 1982; Mert and Soylu 2006). The FAA fixated conelets were prepared for microscopic morphologic assessments using the tertiary butanol method (Feder and O'Brien 1968; Coetzee 1982; Winsor 1994; Coura et al. 2005). Conelets were de-hydrated in 50 % ethanol at room temperature for three hours, followed by a series of chemical solutions in preparation for wax embedding. The procedure can be summarised as follows. The conelets were placed in distilled water, 100 % ethanol and butanol (45:45:10 v/v) for one hour at room temperature; followed by distilled water, 100 % ethanol and butanol (30:50:20 v/v) for 12 hours at room temperature. The conelets were then transferred to distilled water, 100 % ethanol and butanol (15:40:35 v/v) for one hour at room temperature; followed by distilled water, 100 % ethanol and butanol (5:40:55 v/v) for one hour at room temperature; where after conelets were placed in 100 % ethanol and butanol (25:75 v/v) for one hour at room temperature. The conelets were placed in tertiary butanol for two hours at 40 °C; where after the solutions were replaced with new tertiary butanol and left for 18 hours at 40 °C. The conelets were then transferred to tertiary butanol and benzene (50:50 v/v) for 24 hours at 40 °C; followed by placing them in Benzene for 12 hours minimum at 40 °C. New Benzene and a few pieces of paraffin wax were added and left uncovered for a minimum of 12 hours at 60 °C; where after the conelets were placed in melted pure paraffin wax for 48 to 72 hours at 60 °C prior to embedding conelets in paraffin wax. Before placing the conelets with an identification code into the moulds, the ice cube moulds were placed on a cold surface and filled with melted paraffin wax (60 °C). Care was taken to avoid air pockets in the wax. The ice cube moulds were left for approximately 24 hours to allow coagulation where after the paraffin wax blocks were removed and cut to size for microtome cuts. The entire preparation process for each conelet sample took approximately eight days.

The 2012 pollination season samples were cut with a freeze microtome and cuts less than 10 µm that distorted the wax samples. Two colouring methods were tested (starch and Alcian Blue) but did not yield good results. The methods for the 2013 pollination season samples were then adjusted. Permanent microscope slides of each sample was made by cutting the moulded samples into wax ribbons of 10 µm with a rotary microtome (Leica RM2245). Conelets are not symmetrical and were therefore divided into middle-left and middle-right zones (Figure 5.1). Four microtome cuts were randomly selected from the large number of cuts made per zone. Therefore, eight wax ribbon cuts per conelet per cross-pollination per week (three replicates per hybrid) gave 24 microtome cuts per cross-pollination per week (510 cuts in total) that were fixated as permanent microscope slides with Haupt adhesive (Coetzee 1982).

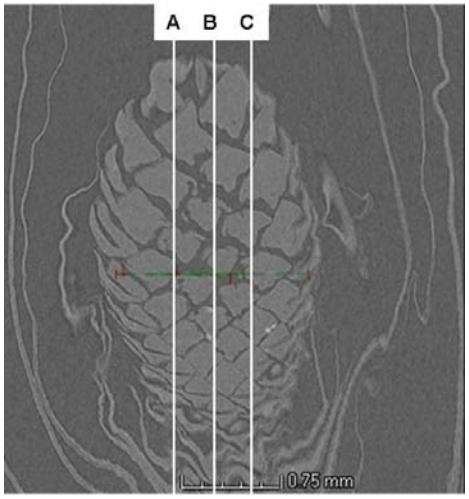

Figure 5.1: Conelet indicating the microtome cuts as middle-left (A to B) and middle-right (B to C)

Slides were coloured using Alcian Blue Safranin (ABS) method to provide a one-step double-staining procedure. Alcian Green Safranin (AGS), which is often used on microscope slides was replaced with Alcian Blue (Tjoel 1983). The ABS method is frequently used as carbohydrates, including cell walls, micilages and polysaccharide cells appear blue; lignified, cutinised and suberized walls, as well as tannin, lipid material, nuclei, chloroplast and some organelles appear red (Tjoel 1983; Trigiano and Gray 2011). As pollen grains consist of carbohydrates they were clearly visible to count manually (Williams 2009) and observe differences between cross combinations. Alcian Blue 8 GX (1 g) was dissolved in 100 ml distilled water with 0.1 ml acetic acid and 2 ml of 40 % formalin. Safranin O (1 g) was dissolved in 50 % ethanol (Tjoel 1983). Slides were coloured at room temperature as previously described (Coetzee 1982). The slides were placed in Xylene for 30 minutes; then in a solution consisting of xylene and 100 % ethanol (50:50 by volume) for five minutes. The slides were then placed in a series of 100, 96, 70 and 50 % ethanol for five minutes each, where after they were placed in ABS solution for 10 minutes. Where after the slides were again placed in a series of 50, 70, 96, and 100 % ethanol for five minutes each; followed by a solution consisting of 100 % ethanol and xylene (50:50 by volume) for five minutes; then in xylene for 30 minutes (repeated three times with new xylene). The slides were removed from xylene and placed on dotting paper to drain excess xylene. Microscope slides were then ready to be mounted. A drop of mounting medium (Permount) was placed on each slide and a glass coverslip gently lowered with tweezers. Air bubbles were removed by gently pressing down the glass coverslip and slides were left for 48 hours to dry.

The 15 best microtome cuts (according to visual inspection) of each cross-pollination per week were selected (as replications) and assessed (Figure 5.2; McWilliam 1959; Bramlett 1981; Moody and Jett 1990; Slee and Abbott 1990; Owens and Fernando 2007). Data was collected with a light microscope (Leica DM2500M) at a series of magnifications (4, 10, 20 and 40 X). The total number of assessments per slide at each magnification was 180. Room temperature was kept at a constant 24 °C to improve the cooling down of paraffin wax moulds and ensure best results with colouring of permanent microscope slides.

The morphologic traits assessed at different magnifications to determine pollination success were: a) number of visible ovules, b) pollen grains outside the ovules; c) pollen grains inside the ovules; and d) number of pollen tubes inside the ovule (Figure 5.2). Pollen grains that germinated, formed an elongated tube and were counted when the length was double the depth of the pollen width.

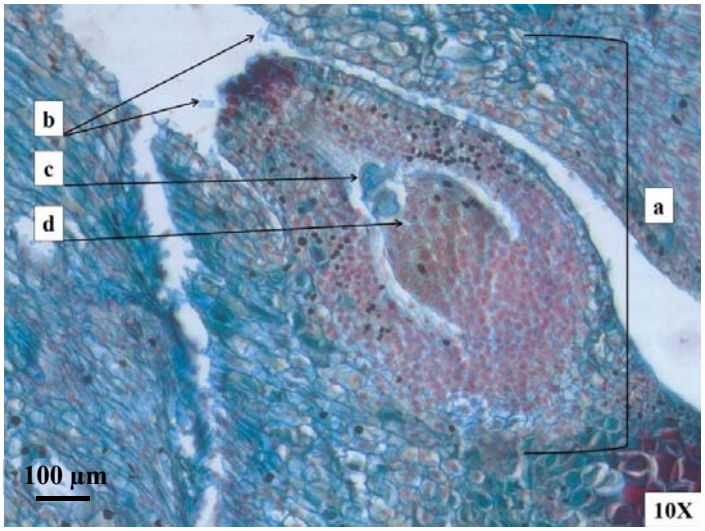

Figure 5.2: Data collected from conelets (permanent microscope slides) at weekly intervals for the four cross combinations for (a) number of visible ovules, (b) pollen grains visible outside ovules, (c) pollen grains visible inside ovules, and (d) pollen tube visible inside ovules

Statistical analysis for number of ovules, pollen grains outside ovules, pollen grains and tubes inside ovules, as well as comparison of interaction of time and species for the four hybrid cross combinations had a completely randomised experimental design with 15 replications (microtome cuts per cross combination per week). The data were subjected to analysis of variance (ANOVA; Appendix B) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.2; SAS Institute Inc, Cary, USA). Shapiro-Wilk test was performed on the standardised residuals from the model to verify normality (Shapiro and Wilk, 1965). This was followed by a split plot experimental design. The main plot was four cross-pollination combinations and the subplot time (seven weeks) with repeat measurements over a week considered as subplot factor. Best group of genotypes were identified with the Best test macro of SAS/STAT® (Little and Hills 1978; Ott and Longnecker 2001). Fisher's least significant difference was calculated at the 5 % level to compare treatment means (Ott and Longnecker 2001). A probability level of 5 % was considered significant for all significance tests.

Verification of pollination success was conducted by comparing the size of pollen grains inside and outside the ovules of the four cross combinations with pollen grain sizes of *P. radiata, P. oocarpa, P. tecunumanii* and *P. maximinoi* as determined in Chapter 4. Size of pollen grains was measured as length across the distal region

of the grain between the two air bladders, while grain width was measured from the distal pole including the bladders if they overhung the body (Figure 5.3; Cain 1940; Owens et al. 1981; Nel and van Staden 2005). Care was taken to measure only grains which were seen squarely in dorsal (back side of pollen), ventral (front) or lateral (attached to the side of an organ) view, ignoring obliquely (asymmetric) grains (Cain 1940; Caron and Powell 1995; Major et al. 2005).

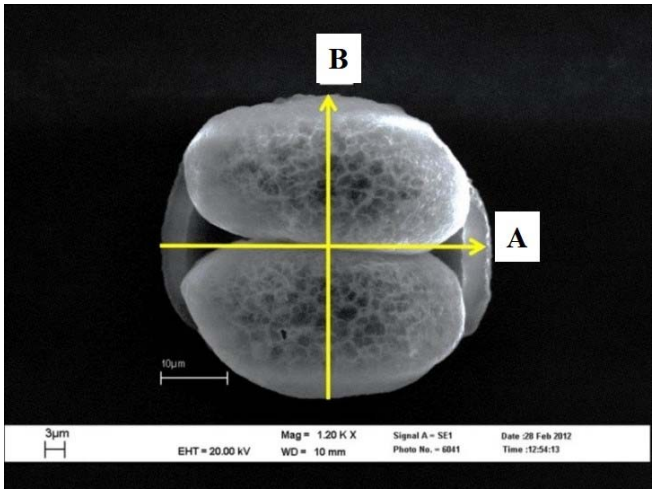

Figure 5.3: *Pinus* pollen grain at 1.200 kX magnification indicating the grain length (A) and width (B) measurement technique (Chapter 4)

The experimental design was a completely randomised design with five replicates. The treatment design was a factorial with two factors namely species with four levels (*P. radiata*×*radiata, P. radiata*×*maximinoi, P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii*) and size with two levels (length and width). The data were subjected to analysis of variance (ANOVA; Appendix B) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.2; SAS Institute Inc, Cary, USA). Shapiro-Wilk test was performed on the standardised residuals from the model to verify normality (Shapiro and Wilk, 1965). Fisher's least significant difference was calculated at the 5 % level to compare treatment means (Ott and Longnecker, 2001). A probability level of 5 % was considered significant for all significance tests.

2.2 MicroCT scans

The reproductive cycle of *P. radiata* is up to 28 months long (Rimbawanto et al. 1988), and fertilisation occurs around 16 months after pollination (Chapter 4; Sweet and Bollmann 1970). During seed and cone maturation, density differences between different tissues are more evident; for example, the formation of the hard seed coat (Bramlett et al. 1977). Previous studies indicated that mature seed are visible in X-ray radiograph pictures (Bramlett et al. 1977). These images are two-dimensional (2D) and can be used for diagnosis but are limited in contrast and cannot be used for quantitative analysis (Tigabu and Odén 2003). In contrast, microcomputed tomography (MicroCT) scans provide a three-dimensional (3D) model, which generates more accurate and

reliable data (du Plessis et al. 2016; Guelpa et al. 2016). The number of seed and viability depends on the type of crosses performed. Interspecific crosses, for example, yield far fewer viable seeds than do intraspecific crosses (Williams 2009).

During microCT scanning, thousands of 2D X-ray images are combined to construct an accurate 3D model. The data, based on differential X-ray attenuation, are analogous to those otherwise obtainable only by serial sectioning of plant tissues (Stuppy et al. 2003; van der Niet et al. 2010; Staedler et al. 2013). It requires no staining, sectioning or fixing, but produces a 3D digital map (viewed from arbitrary angle) of the specimen that allows measurements and visualisations (Stuppy et al. 2003; van der Niet et al. 2010; Staedler et al. 2013), for example, of metabolite content, pollination status or crop yield (van der Niet et al. 2010; Staedler et al. 2013). Previous experiments have been able to distinguish between pith, xylem, cortex, vascular bundles, leaf bases, seeds and ovuliferous scales (Pika-Biolzi et al. 2000). This is due to contrasting plant tissues (soft versus hard) absorbing X-rays differently due to different thicknesses of cell walls and cell contents (Stuppy et al. 2003). Previous studies have classifed *P. patula* seeds as viable or not using near-infrared transmittance (energy in the region of the electromagnetic radiation spectrum at wavelengths longer than visible light, but shorter than radio waves) and reflectance spectroscopy (study of light as a function of wavelength) (Tigabu and Odén 2003). Qualitative and quantitative investigation of the internal morphology and histology of plants is a potential application of microCT scans (Stuppy et al. 2003) as it gives higher definition than standard CT scans (Chapter 2).

This study investigated microCT scans as an alternative non-destructive method in *Pinus* breeding programmes. Three experiments were conducted to determine whether CT and microCT scans can be used to confirm at what time (weeks after pollination) fertilisation occurred as compared to histology studies; whether seed viability can be assessed by scanning multiple seeds together, and can viable seed be counted in a closed *P. radiata* cone. MicroCT has progressed to the point where service facilities are available worldwide and especially in university departments, making the method easily accessible and more cost-effective. Its use in non-destructive testing of various types of materials has made it a valuable tool in materials sciences (Maire and Withers 2014), geosciences (Cnudde and Boone 2013), agricultural and food sciences (Schoeman et al. 2015). The method is very diverse and can provide quantitative information on density of biological materials such as maize kernels (Guelpa et al. 2015) to determine, for example, milling quality. It has also proved possible to scan large numbers of samples in a single operation; making high-throughput analysis feasible for this type of application (Guelpa et al. 2016).

Experiment 1: CT scans as an alternative to paraffin wax embedding

Pinus radiata intraspecific crosses (*P. radiata*×*radiata*) were made according to the adapted pollination protocol developed (Chapter 3). Three conelets per week interval were harvested for both wax embedding and CT scans each (total of six conelets per week interval). In total, 186 conelets were harvested over a period of 104 weeks (weekly intervals for the first eight weeks after pollination and thereafter every fourth week) and

fixated in FAA (see Staedler et al. 2013). Week 104 represents 22 months after pollination when cones are considered mature (Bramlett and O'Gwynn 1981). Conelets were prepared for histology studies and CT scans performed within seven days after harvesting. The fresh weight, length and width of conelets and cones were documented before scanning (see Sweet and Bollmann 1970).

Conelets were subjected to X-ray CT scans (General Electric Phoenix V|Tome|X L240/NF180) with X-ray settings of 100 kV and 200 µA, acquired 1 700 images in a full rotation at image acquisition time of 500 ms per image, with no averaging and no skipping of images. Detector shift was activated to minimize ring artefacts. Background calibration was performed and the scan time was approximately 60 minutes per scan. Reconstruction was done with system-supplied Datos reconstruction software. Analysis was performed with Volume Graphics VGStudio Max 2.1 or Visualization Sciences Group Avizo Fire 8.0 commercial 3D analysis software packages.

Experiment 2: Number of viable seed by scanning groups of seeds in three species

Seed from *P. radiata, P. patula* and *P. pinaster* were obtained from commercial nurseries. These seeds had been winnowed and empty seeds had been removed. Thirty seeds from each species were subjected to microCT scans (total of 90 seeds) to determine percentage porosity. Seeds were subjected to microCT scans with 60 kV and 240 µA for the seed-scans of 30 seeds in one scan, at a voxel resolution of 24 microns. After microCT scans, the seeds were subjected to a water-floating test to verify viability. Seeds were placed overnight in glass beakers filled with distilled water at room temperature (25 °C). The number of submerged seeds were counted and taken as viable. Seeds were then placed between moistened filter papers at room temperature and natural light (16:8 hours light:dark). Seeds were taken as germinated when the radicle was at least 10 mm. Germination percentages were documented for four weeks.

Samples were subjected to X-ray microCT scans using a Phoenix V|Tome|X L240/NF180 scanner (General Electric, USA). X-ray settings for a single scan were 100 kV and 200 µA, with 1 700 images being acquired in a full rotation at image acquisition time of 500 ms per image, with no averaging and no skipping of images (Broeckhoven et al. 2016; du Plessis et al. 2016). Dose rate was measured in kV based on a previous study conducted by Broeckhoven et al. (2016). Reconstruction was undertaken with system-supplied Datos reconstruction software. Analysis was performed with VGStudio Max 2.1 and 2.2 software (Volume Graphics, Heidelberg, Germany).

Image analyses for experiments 1 and 2 were done with VGStudioMax 2.2 software (Volume Graphics). The basic processing used involved an adaptive Gauss filter to remove noise and to select the material of interest using region growing and morphological operations. This process involves the processing of microCT slice images by selecting the background air with a "flood fill" tool in 3D. After viewing a number of slice images to ensure the selection was done properly, the air was removed. Similarly, each seed is then selected to include its internal air volumes (Matthews and du Plessis 2016). None of this is required for a basic viewing of slice

images in a typical non-destructive analysis viewing, but for 3D analysis and quantitative volume measurements as reported here. During the two experiments, seeds were numbered with a permanent marker to compare results between experiments.

Experiment 3: Number of viable seed by scanning an intact *Pinus radiata* cone

One arbitrarily chosen mature *P. radiata* cone (closed) with seeds was harvested from the top of a tree and scanned to investigate the potential for *in-situ* seed viability assessment. The pine cone was subjected to X-ray microCT scans at 120 kV and 150 µA at a voxel resolution of 80 microns. Image averaging and skipping was used to enhance image quality. Detector shift was activated to minimise rotation artifacts and automatic scan optimisation was activated. Reconstruction was done with system-supplied software. The scan times were 1 hour each including setup time.

3. Results

3.1 Paraffin wax embedding

Only results from the 2013 pollination season will be reported on. When pollen grains are captured by the pollen droplet, it is re-hydrated before entering the ovule. Therefore, the pollen grain sizes were measured (Chapter 4) and compared to re-hydrated pollen grains at micro-temperature regime (inside the pollination bag; 24 hours after pollination). This was done in an attempt to confirm that *P. radiata* female strobili were indeed pollinated with *P. maximinoi, P. oocarpa* and *P. tecunumanii* pollen (Table 5.2) and that the adapted pollination protocol limited pollen contamination (Chapter 2). However, pollen grains were disfigured, serrated, not clearly visible, cut in half or out of focus for all four cross combination (Figure 5.4). Although significant differences were evident ($p<0.001$; $r^2=0.52$) over the seven weeks between species, and measurements closely resembled the pollen size of pure species (Chapter 4), variation was too severe to deem results reliable.

Table 5.2: Pollen grain size measurements per hybrid cross combination per week, compared to micro-temperature regime measurements of the male parent (Chapter 4; *n*=90)

Hybrid combination	Week	Pollen grain size			
		Microscope slides		Micro-temperature regime (re-hydrated)	
		Length	Width	Length	Width
P. radiata×*radiata*	1	58.63 ± 3.4 ^{A}	33.79 ± 4.9 ^{A}	71.1 ± 6.9	48.21 ± 7.1
	2	70.83 ± 3.8 ^{B}	36.27 ± 4.5 ^{B}		
	3	70.37 ± 4.3 ^{AB}	40.30 ± 4.4 ^{B}		
	4	69.73 ± 12.7 ^{AB}	37.17 ± 7.7 ^{A}		
	5	72.66 ± 9.3 ^{A}	41.91 ± 6.0 ^{A}		
	7	67.57 ± 4.3 ^{B}	40.98 ± 9.2 ^{A}		
P. radiata×*maximinoi*	1	0.0 ± 0.0 ^{B}	0.0 ± 0.0 ^{B}	70.4±6.6	49.3±5.9
	2	64.25 ± 4.5 ^{A}	33.45 ± 5.4 ^{B}		
	3	68.05 ± 6.5 ^{B}	34.34 ± 6.2 ^{C}		
	4	63.33 ± 6.9 ^{B}	34.68 ± 5.5 ^{A}		
	5	67.39±10.4 ^{A}	39.76±3.2 ^{A}		
	7	0.0±0.0 ^{C}	0.0±0.0 ^{C}		
P. radiata×*oocarpa*	1	61.57 ± 9.8 ^{A}	37.12 ± 5.9 ^{A}	73.6 ± 5.6	46.5 ± 5.6
	2	61.86 ± 7.4 ^{B}	34.58 ± 6.7 ^{B}		
	3	68.46 ± 6.8 ^{B}	38.37 ± 2.1 ^{BC}		
	4	0.0 ± 0.0 ^{C}	0.0 ± 0.0 ^{C}		
	5	0.0 ± 0.0 ^{C}	0.0 ± 0.0 ^{C}		
	7	67.71 ± 6.1 ^{B}	41.42 ± 5.0 ^{A}		
P. radiata×*tecunumanii*	1	71.26 ± 5.2 ^{A}	37.31 ± 8.5 ^{A}	65.5 ± 5.7	43.3 ± 5.4
	2	72.18 ± 7.7 ^{A}	41.53 ± 5.1 ^{A}		
	3	76.55 ± 9.8 ^{A}	48.43 ± 3.8 ^{A}		
	4	73.24 ± 6.4 ^{A}	36.80 ± 8.0 ^{A}		
	5	73.58 ± 11.5 ^{A}	37.85 ± 6.2 ^{A}		
	7	81.67 ± 6.0 ^{A}	43.90 ± 3.9 ^{A}		

Means per week interval with the same letter are not significantly different

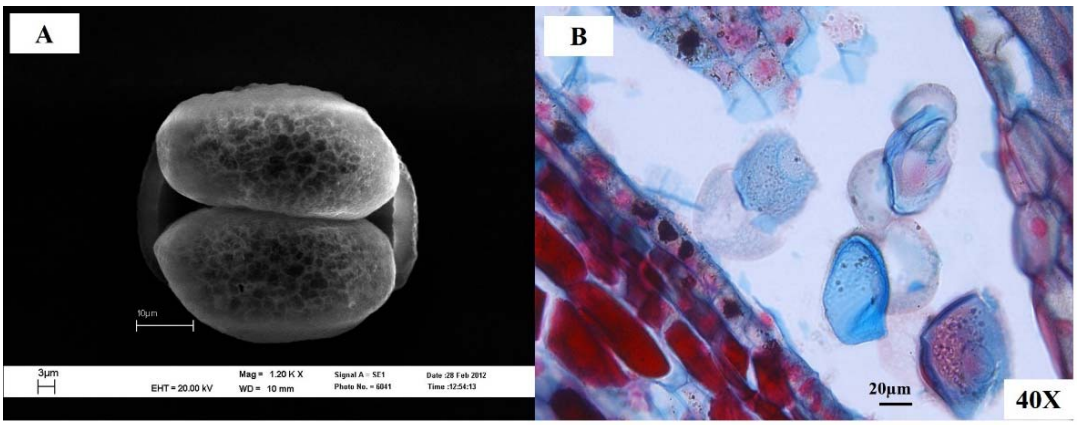

Figure 5.4: An example of an intact *P. maximinoi* pollen grain (A) measured with a SEM and distorted *P. maximinoi* pollen grains (B) during the paraffin wax embedding process

Ovules were mainly visible within the top and middle parts of conelets, while pollen grains and tubes were visible inside and outside the ovules as bright blue circles with white wings (Figure 5.5). In the bottom 25 % of the female strobilus, a number of brown and seemingly de-hydrated ovules were noted in the *P. radiata*×*maximinoi, P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii* cross combinations.

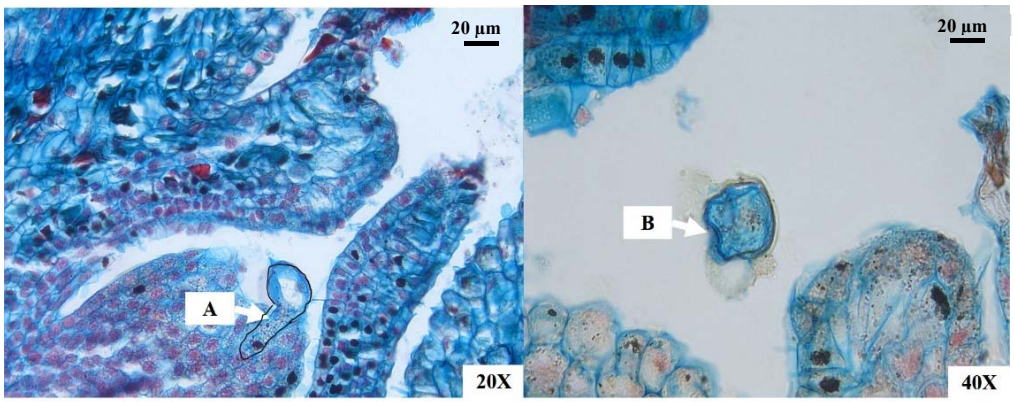

Figure 5.5: *Pinus radiata*×*radiata* conelet (week 1) indicating the pollen tube (A) and pollen grain (B)

Actual data collected within 24 hours of pollination (Week 0) for the control (*P. radiata*×*radiata*) indicated a total of 114 visible ovules, 40 visible pollen grains outside the ovule with only two visible pollen grains inside the ovule (Table 5.3). For Week 1, *P. radiata*×*radiata* had a total of 112 visible ovules, 25 pollen grains inside the ovules and 8 pollen tubes, but *P. radiata*×*oocarpa* had the most visible pollen grains outside the ovules (105). Significant differences were thus evident for the total number of ovules (p<0.0001, r^2=0.42), pollen grains outside ovules (p=0.00017, r^2=0.23), pollen grains inside ovules (p=0.06, r^2=0.12) and pollen tubes (p=0.02, r^2=0.15) between the four cross combinations at Week 1 (Table 5.4).

Fourteen days (Week 2) after pollination, *P. radiata*×*maximinoi* had the most visible pollen grains outside the ovules (165), but *P. radiata*×*radiata* had the most visible ovules (121), visible pollen grains inside the ovules (15) and visible pollen tubes (5; Table 5.3). As in the case of with Week 1, significant differences were evident for the number of ovules (p<0.0001, r^2=0.58), pollen grains outside ovules (p=0.09, r^2=0.11), pollen grains inside ovules (p=0.11, r^2=0.11) and pollen tubes (p=0.23, r^2=0.10) between the four cross combinations (Table 5.4). During Week 3 (21 days after pollination), *P. radiata*×*radiata* had the most visible pollen grains outside the ovules (165), pollen grain inside ovules (27) and pollen tubes (16). Although *P. radiata*×*maximinoi* had the most visible ovules (130), *P. radiata*×*radiata* had the second highest number (121). Significant differences were also evident for number of visible ovules (p=0.009, r^2=0.19), pollen grains outside ovules (p<0.0001, r^2=0.66), pollen grains inside ovules (p=0.005, r^2=0.21) and pollen tubes (p=0.0003, r^2=0.28) between the four cross combinations.

Twenty-eight days (Week 4) after pollination, *P. radiata*×*tecunumanii* had the highest number of visible pollen grains outside the ovules (118), pollen grains inside ovules (10) and pollen tubes (5; Table 5.3). *Pinus radiata*×*oocarpa* had the most visible ovules (109), while *P. radiata*×*radiata* had the second most visible ovules (95). As with previous weeks, significant differences were evident for number of visible ovules (p=0.01, r^2=0.17), pollen grains outside ovules (p<0.0001, r^2=0.53), pollen grains inside ovules (p=0.04, r^2=0.13) and pollen tubes (p=0.19, r^2=0.10) between the four cross combinations (Table 5.4). However, at Weeks 5 and 7 *P. radiata*×*radiata* had the highest number of visible pollen grains outside the ovules (80 and 116 respectively), pollen grains inside ovules (32 and 12 respectively) and pollen tubes (26 and 11 respectively). *Pinus radiata*×*radiata* had the most visible ovules at Week 5 (150) but the second lowest number of visible ovules at Week 7. At Week 5 significant differences were evident for number of visible ovules (p<0.0001, r^2=0.51), pollen grains outside ovules (p<0.0001, r^2=0.41), pollen grains inside ovules (p=0.0003, r^2=0.28) and pollen tubes (p=0.0001, r^2=0.30) between the four cross combinations. However, significant differences obtained at Week 7 were weaker for number of visible ovules (p=0.0005, r^2=0.27), pollen grains outside ovules (p<0.0001, r^2=0.49), pollen grains inside ovules (p=0.27, r^2=0.10) and pollen tubes (p=0.33, r^2=0.10) between the four cross combinations.

Macro-temperature data indicated that temperatures were approximately 3 °C cooler than Sabie during the pollen droplet period (Figure 5.6A) and up to 6 °C lower during the pollination period. Micro-temperature data were approximately 2 °C cooler than Sabie with pollen droplet emergence (Figure 5.6B). However, during the pollination period, temperatures at Sabie were up to 4 °C lower than inside the pollination bags of the four cross combinations. During the micro- and macro-temperature regimes (Chapter 4) comparing the germination and pollen tube growth between *P. oocarpa, P. maximinoi, P. tecunumanii* and *P. radiata*; there was no germination of the viable pollen lots of *P. oocarpa* and *P. tecunumanii* seven days after inocubation. This is consistent with data collected during this study (Table 5.3) As indicated in Chapters 3 and 4, temperature might have contributed to the limited pollination success.

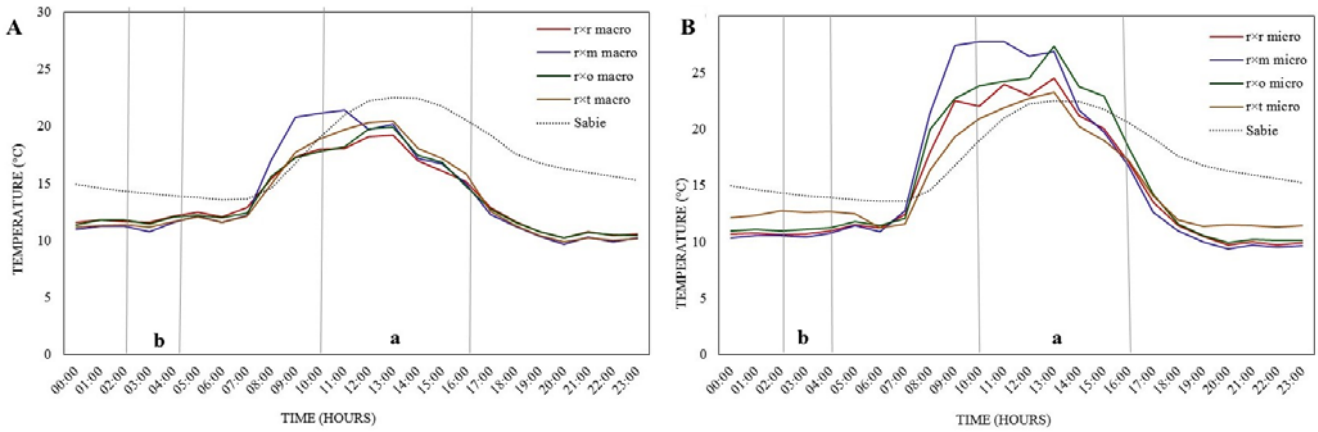

Figure 5.6: Macro- (outside pollination bag; A) and micro (inside pollination bag; B) temperature regimes compared to the Sabie circadian model (Chapter 3; a: pollination period, b: pollen droplet; r×r: *P. radiata*×*radiata*, r×m: *P. radiata*×*maximinoi*, r×o: *P. radiata*×*oocarpa*, r×t: *P. radiata*×*tecunumanii*)

Time by hybrid combination interaction over Weeks 1 to 7 indicated highly significant differences for number of visible ovules (p<0.0001, r^2=0.53), pollen grains outside ovules (p<0.0001, r^2=0.73), pollen grains inside ovules (p=0.0031, r^2=0.30) and pollen tubes (p<0.0001, r^2=0.38) between the four cross combinations (Figure 5.7). In Figure 5.6A the trend between time and hybrid combination appears very erratic. For all four factors, statistical analyses grouped *P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii* together. However, *P. radiata*×*radiata* was statistically grouped with *P. radiata*×*maximinoi* for only pollen grains visible outside the ovules. For both number of pollen grains visible inside the ovules and pollen tubes, *P. radiata*×*radiata* was statistically identified as a unique cross combination.

Reputable data could not be collected for wax embedded conelets after week 8 as conelets become woodier making it more difficult to make 10 µm cuts. Although microtome cuts up to 100 µm were not disfigured or serrated, visibility was zero due to no penetration of light from the light microscope. Staining was more intense and darker, further limiting visibility.

Table 5.3: Actual data collected from conelets of four cross pollinations to determine pollination success (week 0 *n*=15 and weeks 1 to 7 *n*=90)

Number of visible	week 0	week 1				week 2			
	r×r	r×r	r×m	r×o	r×t	r×r	r×m	r×o	r×t
ovules	114	112	77	43	61	121	45	93	78
pollen grains outside ovules	40	85	70	105	50	138	165	144	123
pollen grains inside ovules	2	25	1	8	7	15	7	8	4
pollen tubes	0	8	6	1	0	5	2	0	2
		week 4				week 4			
ovules		121	130	151	110	95	44	109	82
pollen grains outside ovules		165	139	77	50	10	56	0	118
pollen grains inside ovules		27	5	9	11	3	5	0	10
pollen tubes		16	3	3	6	3	2	0	5
		week 5				week 7			
ovules		150	64	88	104	103	128	111	80
pollen grains outside ovules		80	30	5	4	116	15	45	30
pollen grains inside ovules		32	17	7	5	12	11	8	4
pollen tubes		26	12	5	0	11	10	8	4

r×r: *P. radiata*×*radiata*, r×m: *P. radiata*×*maximinoi*, r×o: *P. radiata*×*oocarpa*, r×t: *P. radiata*×*tecunumanii*

Table 5.4: Pollination success determined by counting the number of visible ovules, pollen tubes, pollen grains inside and outside ovules for weeks 1 to 7 for the four cross pollinations (*n*=90)

Number of visible	week 1				week 2			
	r×r	r×m	r×o	r×t	r×r	r×m	r×o	r×t
ovules	7.5 ± 1.2 ^{A}	5.1 ± 2.5 ^{B}	2.9 ± 1.0 ^{C}	4.1 ± 2.8 ^{BC}	8.1 ± 1.7 ^{A}	3.0 ± 1.5 ^{C}	6.2 ± 2.0 ^{B}	5.2 ± 1.0 ^{B}
pollen grains outside ovules	1.1 ± 0.2 ^{A}	0.9 ± 0.2 ^{AB}	1.2 ± 0.4 ^{A}	0.7 ± 0.4 ^{B}	2.3 ± 0.8 ^{B}	2.9 ± 0.2 ^{A}	2.4 ± 0.8 ^{AB}	2.3 ± 0.9 ^{B}
pollen grains inside ovules	1.7 ± 2.2 ^{A}	0.9 ± 0.9 ^{AB}	0.5 ± 0.6 ^{B}	0.5 ± 0.9 ^{B}	1.0 ± 1.1 ^{A}	0.5 ± 0.9 ^{AB}	0.5 ± 0.6 ^{AB}	0.3 ± 0.4 ^{B}
pollen tubes	0.5 ± 0.8 ^{A}	0.4 ± 0.6 ^{AB}	0.1 ± 0.2 ^{BC}	0.0 ± 0.0 ^{C}	0.3 ± 0.6 ^{A}	0.1 ± 0.5 ^{AB}	0.0 ± 0.0 ^{B}	0.1 ± 0.3 ^{AB}
	week 3				week 4			
ovules	8.1 ± 2.2 ^{B}	8.7 ± 2.5 ^{AB}	10.1 ± 2.0 ^{A}	7.3 ± 1.7 ^{B}	6.3 ± 1.1 ^{A}	4.1 ± 2.4 ^{B}	7.3 ± 3.9 ^{A}	5.5 ± 2.3 ^{AB}
pollen grains outside ovules	3.0 ± 0.0 ^{A}	2.3 ± 0.8 ^{B}	1.1 ± 0.9 ^{C}	0.7 ± 0.4 ^{C}	0.1 ± 0.3 ^{C}	1.2 ± 1.0 ^{B}	0.0 ± 0.0 ^{C}	2.0 ± 1.1 ^{A}
pollen grains inside ovules	1.8 ± 1.6 ^{A}	0.3 ± 0.6 ^{B}	0.6 ± 0.9 ^{B}	0.7 ± 0.9 ^{B}	0.2 ± 0.4 ^{AB}	0.5 ± 0.8 ^{A}	0.0 ± 0.0 ^{B}	0.7 ± 1.0 ^{A}
pollen tubes	1.1 ± 0.7 ^{A}	0.2 ± 0.4 ^{B}	0.2 ± 0.4 ^{B}	0.4 ± 0.6 ^{B}	0.2 ± 0.4 ^{AB}	0.3 ± 0.4 ^{AB}	0.0 ± 0.0 ^{B}	0.3 ± 0.6 ^{A}
	week 5				week 7			
ovules	10.0 ± 2.5 ^{A}	4.3 ± 1.7 ^{C}	5.9 ± 2.5 ^{B}	6.9 ± 1.6 ^{B}	6.9 ± 1.6 ^{B}	8.5 ± 2.2 ^{A}	7.4 ± 1.4 ^{AB}	5.3 ± 2.3 ^{C}
pollen grains outside ovules	1.1 ± 0.5 ^{A}	0.4 ± 0.5 ^{B}	0.1 ± 0.2 ^{B}	0.3 ± 0.4 ^{B}	1.9 ± 1.0 ^{A}	0.2 ± 0.4 ^{B}	0.6 ± 0.5 ^{B}	0.3 ± 0.6 ^{B}
pollen grains inside ovules	2.1 ± 2.1 ^{A}	1.1 ± 1.1 ^{B}	0.5 ± 0.7 ^{BC}	0.1 ± 0.2 ^{C}	0.8 ± 1.0 ^{A}	0.7 ± 0.7 ^{A}	0.5 ± 0.7 ^{A}	0.3 ± 0.4 ^{A}
pollen tubes	1.7 ± 1.6 ^{A}	0.8 ± 1.0 ^{B}	0.3 ± 0.7 ^{BC}	0.0 ± 0.0 ^{C}	0.7 ± 0.9 ^{A}	0.7 ± 0.7 ^{A}	0.5 ± 0.7 ^{A}	0.3 ± 0.4 ^{A}

r×r: *P. radiata*×*radiata*, r×m: *P. radiata*×*maximinoi*, r×o: *P. radiata*×*oocarpa*, r×t: *P. radiata*×*tecunumanii*

Means with the same letter in a row per time interval are not significantly different

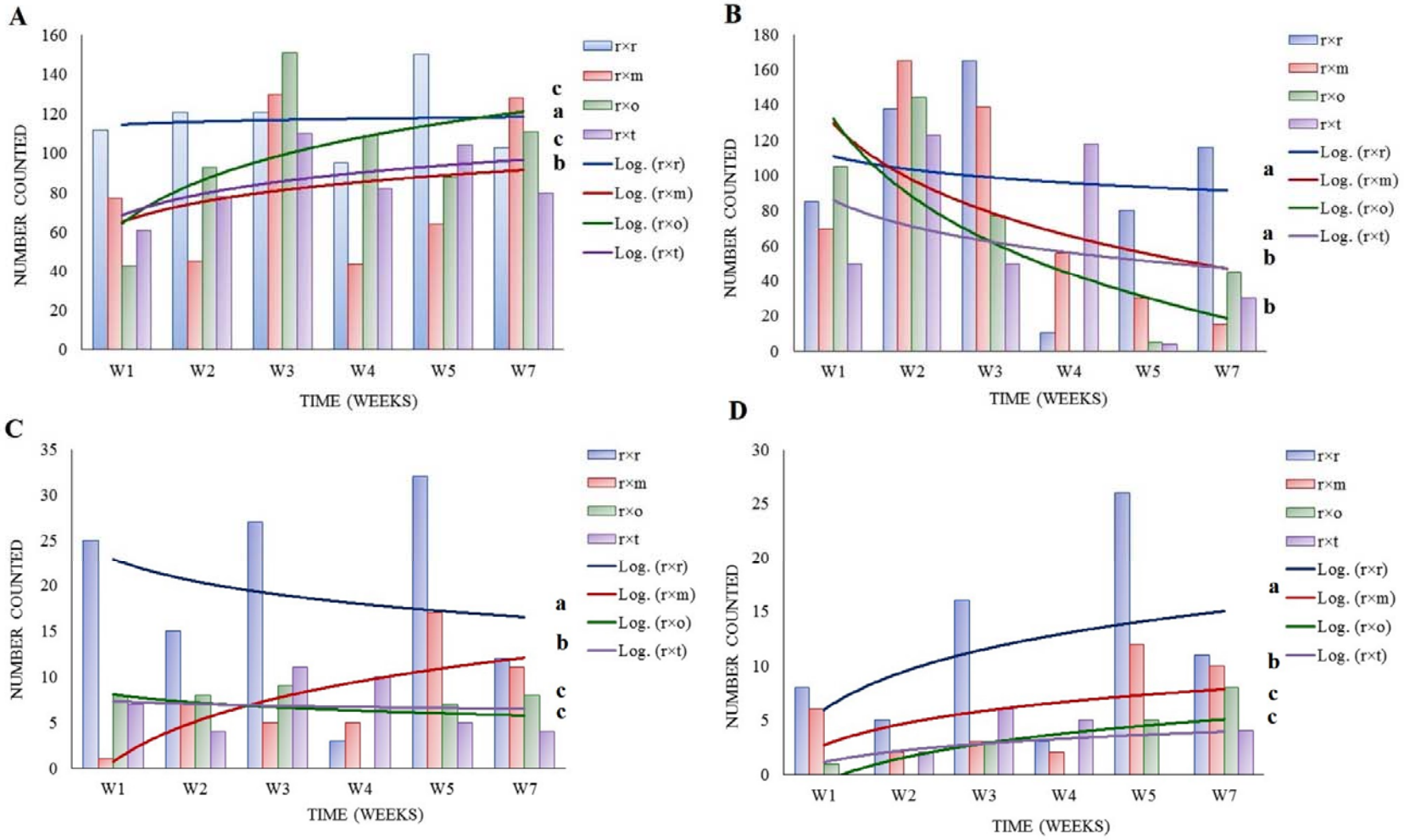

Figure 5.7: Time by species interaction for all four-hybrid combinations from Week 1 to 7 (lines with the same letter do not differ significant) with the number of visible ovules (A), visible pollen grains outside ovules (B), visible pollen grains inside ovules (C), and visible pollen tubes (D; bars represent actual data; r×r: *P. radiata*×*radiata*, r×m: *P. radiata*×*maximinoi*, r×o: *P. radiata*×*oocarpa*, r×t: *P. radiata*×*tecunumanii*)

3.2 MicroCT scans

Experiment 1: CT scans as an alternative to paraffin wax embedding

During the scans, 2D X-rays were used to observe conelets (after pollination and before fertilisation) and cones (after fertilisation) non-destructively at a high resolution and contrast. The conelets and mature cones were exposed to collimated (parallel) X-rays and the absorbed radiation was measured at repeated angles to allow 3D image reconstructions. Although this technique can be classified as non-destructive sampling, conelets are 'destroyed' in the sense that they cannot be grafted back onto the trees to develop into mature seed cones. However, reconstructed images create a permanent record and life-like image for future analysis, comparison or prediction studies.

Control conelets scanned at week 0 (24 hours after pollination) had a uniformed grey colour equivalent to an herbaceous flower. Ovules, nucellus, megagametophyte, micropyle, pollen grains or central axis were not visible or clearly distinguished. Only air pockets were visible as white (Figure 5.8A) or blue (Figure 5.8B) colour during the defect analyses. Conelets scanned at week 7 (approximately 2 months after pollination) had a darker grey colour indicating an increase in density and development (Figure 5.8C). However, ovules, nucellus, megagametophyte, micropyle, pollen grains or central axis were still not visible or clearly distinguished. Air pockets were visible as a blue colour due to density differences in the defect analyses.

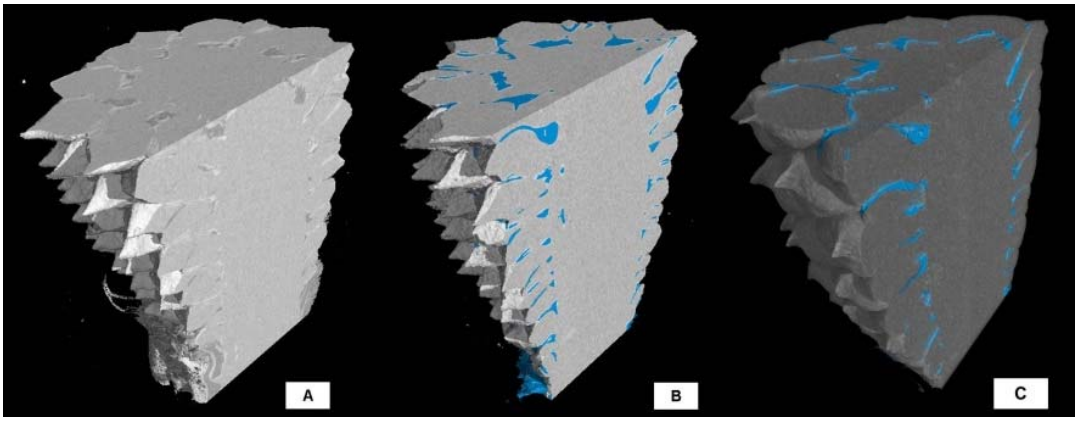

Figure 5.8: Conelets scanned (3D longitudinal cuts) at week 0 (A and B) and week 7 (C) with air pockets in blue

Scans performed at week 60 (approximately 14 months after pollination) had the same shade of grey observed in week 7 but still within the herbaceous range. Air pockets were less visible while ovules, nucellus, megagametophyte, micropyle, pollen grains or central axis were still not visible or clearly distinguished (Figure 5.9A). However, red patches (defect analyses) indicated that the central axis became woodier (higher density; Figure 5.9B, and 5.9C).

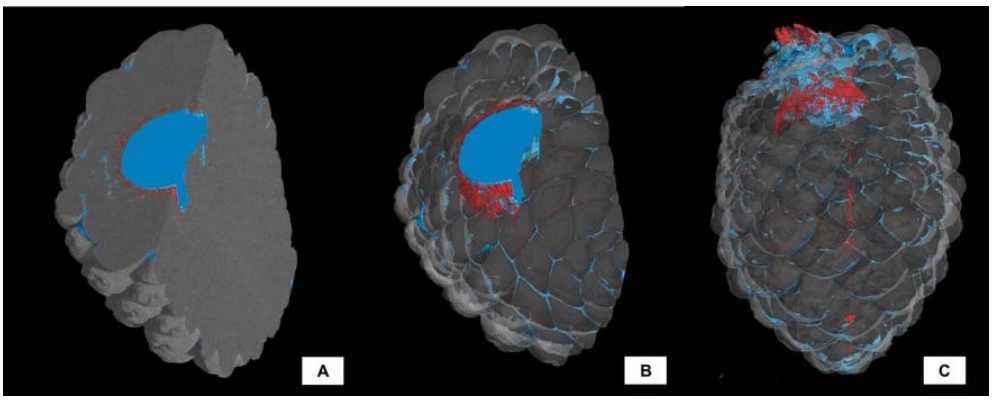

Figure 5.9: Conelets scanned at week 60 showing density differences in blue and red

Conelets scanned at week 64 (approximately 15 months after pollination) did not differ from previous scans indicating no changes in the density and structure of the conelet. The grey colour was still within the herbaceous range and no ovules, nucellus, megagametophyte, micropyle, pollen grains or central axis were visible or clearly distinguished (Figure 5.10A). However, seeds, central axis and ovuliferous scales are visible at scans performed during week 68 (approximately 15.5 months after pollination) indicating that fertilisation occurred between week 64 and 68 (Figure 5.10B). Cones scanned at week 72 (approximately 16.5 months after pollination), 76 (approximately 17.5 months after pollination) and 80 (approximately 18.5 months after pollination) presented various stages of seed development (Figures 5.10C, 5.10D, 5.10E). Filled seed are clearly visible (black) and seed set could be calculated without destructive sampling. Seed with wings (blue) were clearly visible on scans performed at week 104 (approximately two years after pollination; Figure 5.10F).

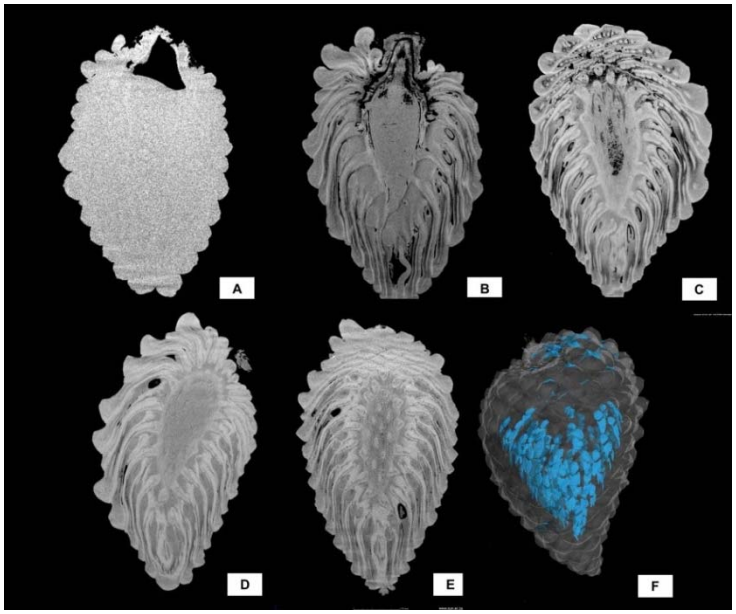

Figure 5.10: Conelets and cones (*P. radiata*×*radiata*) scanned at week 64 (A), week 68 (B), week 72 (C), week 76 (D), week 80 (E) and week 104 (F)

The *P. radiata*×*radiata* cones had a steady increase in fresh weight (g), length (mm) and width (mm) of between weeks 60 and 80 (Figure 5.11). Conelets harvested at week 72 had a significant increase in fresh weight and length compared to week 68. Furthermore, fresh weight, length and width of weeks 76 and 80 seem lower and slightly more skew compared to week 72.

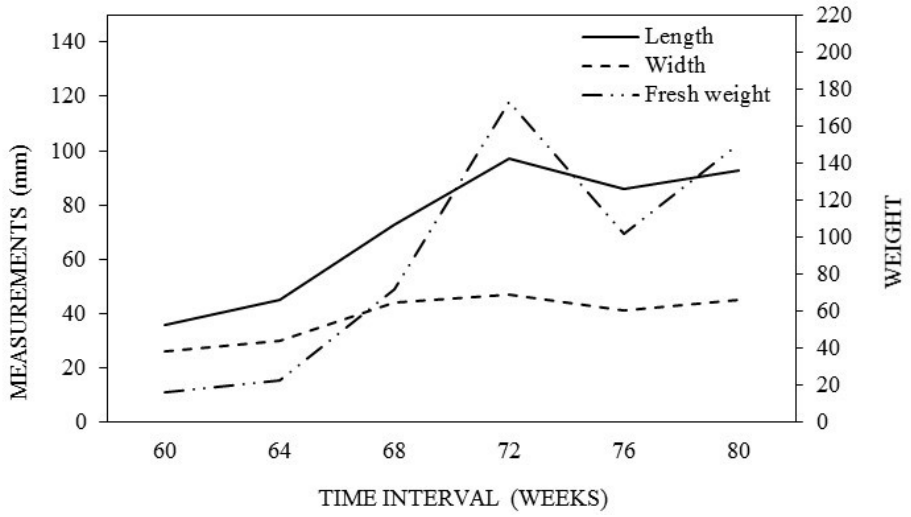

Figure 5.11: Weight and size of cones harvested between weeks 60 and 80 after pollination

Experiment 2: Number of viable seed by scanning a cluster of seed

During the water-soaking test, 30 seeds of *P. radiata*, 29 of *P. patula* and 28 of *P. pinaster* submerged, indicating a seed viability of 100, 97 and 93 % respectively. Germination percentage of seeds kept in moistened filter papers (up to four weeks) matched the results of the water-soaking test. The data collected with the microCT scans also mirrored the results from the water-soaking test and germination filter-paper test (seeds were numbered). Dark seeds had lower weights and floated on water, indicating a large air volume (Figures 5.12 and 5.13), and these seeds proved to be inviable. Using colour-coding to represent air volume also indicated that non-viable seed had an air volume higher than 20 mm^3 (shown in pink in Figure 5.12). The percentage porosity (air inside seed) of the seeds that did not submerge was more than 50 %. Viable seeds of *P. radiata* had an average porosity of 17.3 %, *P. patula* 22.7 % and *P. pinaster* 17.5 %.

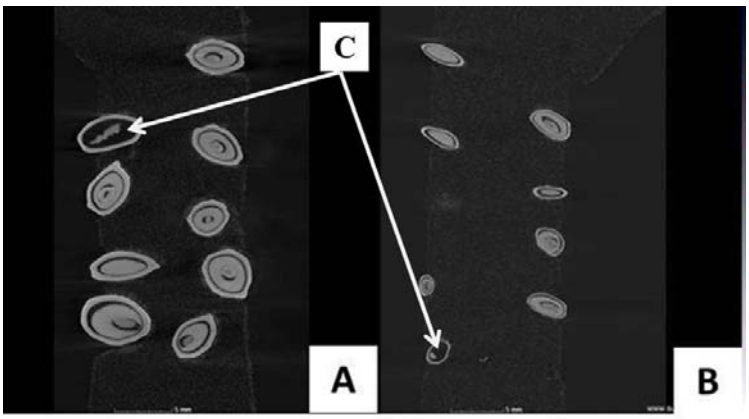

Figure 5.12: A microCT slice image showing *Pinus pinaster* (A) and *P. patula* (B) seeds, with one seed in each case having a large void or black space (C) indicating it is non-viable

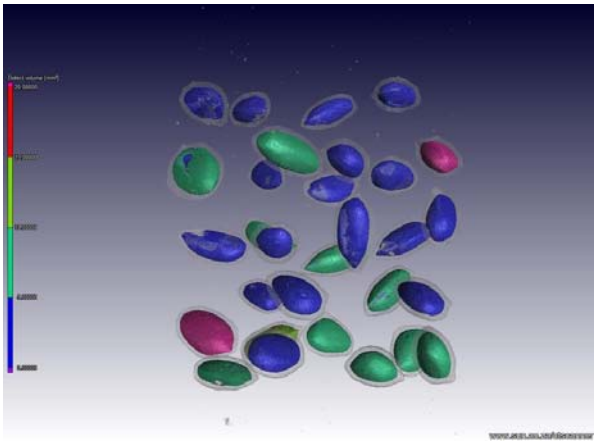

Figure 5.13: A 3D image of 30 *Pinus pinaster* seeds with the voids colour-coded, indicating two non-viable seeds with voids larger than 20 mm^3 (pink). The colour coding is based on volume; therefore, blue and green seeds are intermediate volumes according to the colour bar

Experiment 3: Number of viable seed by scanning an intact *Pinus radiata* cone

Images from the *P. radiata* cone scanned with intact seed indicated non-viable seeds which were easily distinguished from viable seeds (Figure 5.14). Unfortunately, the seeds were not able to be numbered *in situ*, so it was difficult to distinguish between viable and non-viable seeds after shaking from the cone. Care should be taken to extract all the seed as some non-viable seed seemed to get trapped inside the cone (bottom of cone with unopened scales). However, the number of viable seeds corresponded with the water soak test.

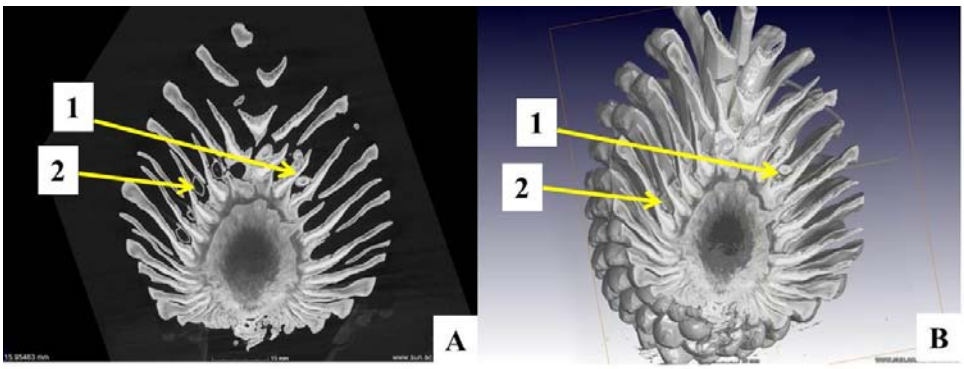

Figure 5.14: Demonstration of intact pine cone (week 104) microCT scan in slice (A) and 3D virtual cut-open (B) images, with non-viable seeds upper left (1) and viable seeds top right (2) as indicated by their voids

4. Discussion

Mature seed cones from interspecific hybrid crosses are the first 'product' in the sequential process of tree breeding as it contains the F_1 progeny (seed). The number of seed harvested is generally considered an indication of pollination success (Johnsson 1976). Previous studies used radiography to determine whether mature seed can be viable (Bramlett et al. 1977; Tigabu and Odén 2003). However, they could only determine whether seeds are full, partially full or empty, and could not predict with certainty whether these seed might be viable. Full seeds were thus classified as potentially sound seed but damaged full seeds (insects, aborted ovules etc.) could not be distinguished from healthy full seeds. This study investigated whether pollination success can be determined by the number of visible ovules, pollen grains inside and outside the ovule, as well as pollen tubes inside the ovule with paraffin wax embedding. CT and MicroCT scans were also employed to narrow down when fertilisation happens in *P. radiata*×*radiata* and whether 3D images of intact cones can be used to determine seed set and viability.

Across the four hybrid combinations, ovules were mostly visible in the top and middle areas and weekly significant differences were evident. This is consistent with previous studies on the hybrid between *P. elliottii* and *P. caribaea* (Slee and Abbott 1990). A higher number of deformed and un-pollinated ovules (brown in colour) were observed in *P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii* than *P. radiata*×*maximinoi,* while none for *P. radiata*×*radiata.* This might be an indication that the ovules in the *P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii* crosses had a higher abortion rate or more sensitive to temperature opposed to *P. radiata*×*maximinoi* and *P. radiata*×*radiata* crosses. The notion of ovules aborting if not pollinated within the receptive period might thus be valid. In northern pines, 50 to 70 % of fertile ovules that were pollinated, still resulted in conelet abortions (Owens and Fernando 2007). However, conelet abortion might also be influenced by pollen droplet recognition (Stephenson 1981).

Pollen was visible both outside and inside the ovules of the *P. radiata*×*radiata* control harvested 24 hours after pollination. This provides good evidence that: pollen grains were able to enter the conelet and accumulate at the micropyle; in some instances actually entering into the ovule; and that pollen grains did sift through the scales within 24 hours after pollination. As expected, *P. radiata* pollen grains were thus recognised by the droplet and the pollen grain size was sufficient to enter the micropyle. As no pollen tubes were visible, it indicates that pollen tube elongation only appears after 24 hours. This is consistent with Fernando et al. (2005), indicating that pollen grains can germinate inside the ovules within five days after pollination and a tube will elongate towards the nucellus. Throughout the seven weeks of the study *P. radiata*×*radiata* had significantly more visible pollen grains inside the ovules than *P. radiata*×*maximinoi, P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii*. This again supports the notion than although high quantities of pollen grains are available due to wind-pollination, only a few reach the ovule due to recognition by the pollen droplet and micropyle. Various studies indicated that the number of pollen grains per ovule varies between species, clones and the type of study (see Sarvas 1962; Lill 1976; Sweet 1977; Bramlett 1981; Griffin and Lindgren 1985; Slee and Abbott 1990; Owens et al. 2005; Owens and Fernando 2007; Varis et al. 2008), however, the higher the number of pollen grains per ovule, the higher the possibility of obtaining viable seed (Slee and Abbott 1990). On average, the pollen grains observed per ovule for *P. radiata*×*radiata* were between one and four, correlating with the study of Sweet (1977). The notion that only one pollen grain or tube is necessary for pollination and the effect of pollen competition were not part of this study.

Although the number of pollen grains inside the ovules for *P. maximinoi, P. oocarpa* and *P. tecunumanii* varied considerably, *P. maximinoi* resembled *P. radiata* the best. This can be due: to a chemical recognition mechanism between the micropyle, ovule and pollen grains; speed of pollen germination; number of ovules pollinated; pollen quality; pollen droplet size and number of re-appearance before closing. These barriers might be overcome with: mixed pollen lots with mentor (same species as female killed by heat) to ensure recognition by micropyle; more emphasis on pollen water content, viability, freshness and storage; and more regular pollination events to optimise the relationship between pollen droplet appearance and capturing of pollen grains. To address these, future studies can include: correlation between pollen droplet size and pollen grain size; determine whether pollen droplet size varies between *Pinus* species; and whether reciprocal crosses might increase pollination success.

However, as with pollen grains visible inside the ovules, *P. radiata*×*radiata* had significantly more visible pollen tubes than *P. radiata*×*maximinoi, P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii* over the seven weeks. Again, supporting the notion that the micropyle and pollen droplet appears to recognise pollen grains and may cause pollen tube arrest and abortion of unpollinated ovules (McWilliam 1959; Hagman 1975; Fernando et al. 2005; Williams 2009). Interaction of time by species also confirmed the notion of pollen recognition as both *P. radiata*×*radiata* and *P. radiata*×*maximinoi* had the highest number of pollen grains outside the ovules, and differed significantly from *P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii*. Also,

more visible pollen grains inside the ovules and pollen tubes were evident for *P. radiata*×*radiata* than for the interspecific crosses per week interval.

Actual temperature data collected for both micro and macro indicated that during the pollen droplet, temperatures were lower at Karatara than Sabie, but during the pollination period the temperatures are either too high (micro) or too low (macro) at Karatara than Sabie. Previous studies on *P. radiata* pollination success (Chpaters 3 and 4) indicated that the cooler night temperatures and fluctuations between day and night temperatures limit the pollen grain germination and pollen tube elongation of *P. maximinoi*, *P. oocarpa* and *P. tecunumanii,* compared to *P. radiata*. Interspecific crosses during this study were performed at Karatara in a *P. radiata* seed orchard. Therefore, the pollen lots of *P. maximinoi*, *P. oocarpa* and *P. tecunumanii* were exposed to lower night temperatures and greater temperature fluctuations than experienced within their natural ranges. Again confirming the notion that temperatures inside the pollination bags might have an impact on pollination success (Chapters 2, 3 and 4). *In vitro* data collected during the micro- and macro-temperature regimes (Chapter 4) indicated no pollen germination of the viable *P. oocarpa* and *P. tecunumanii* pollen lots. This was consistent with actual data collected during this study at week 1 after pollination. Again supporting the notion that temperature might have an impact on pollination success.

This could also have impacted on the number of pollen grains entering via the micropyle, pollination success and abortion of unpollinated ovules. Pollen grains were clearly visible inside and outside the ovules within 24 hours after pollination in the control (*P. radiata*×*radiata*). Therefore, the droplet recognised, hydrated and transported pollen grains through the micropyle to the ovules (McWilliams 1959; Hagman 1975; Fernando et al. 2005). As the number of visible pollen grains and tubes inside the ovule were significantly different per cross combination and time interval, the notion that the droplet and micropyle may (partially) exclude foreign pollen (*P. maximinoi, P. oocarpa* and *P. tecunumanii*) compared to *P. radiata* was supported. Thus, the visible pollen grains inside ovules and pollen tubes are a better indication of pollination success than pollen grains outside the ovules. Also, environmental conditions inside the pollination bags cannot be ignored and reciprocal crosses with *P. radiata* pollen need to be investigated in areas (for example Sabie) where *P. maximinoi, P. oocarpa* and *P. tecunumanii* yield viable seed (Chapters 3 and 4).

CT and microCT scans were applied in a *Pinus* tree breeding study with three experiments. In the first experiment, the fertilisation time of *P. radiata* was narrowed down by CT imaging to probably occur between weeks 64 and 68 after pollination. Full-sized seeds were visible at week 68, indicating initial seed set although not fully mature seeds. In the second experiment, quantitative porosity analyses of seeds were used to identify inviable seed. This method can be used for qualitative viewing for faster and lower-cost application, or more time-consuming quantitative analysis. The clarity of seed viability assessment by microCT scans should allow its use for breeding assessments or calibration of indirect measures. An experiment with a full cone with viable and inviable seed demonstrates the potential for *in-situ* seed viability assessment. However, care should be taken to shake out all the seeds as some can still be trapped between scales at the bottom of the cones.

Furthermore, the results of the conelet weight, length and width measurements also correlated with the study of Sweet and Bollmann (1970) who observed an increase in weight after fertilisation.

Previous studies indicated that X-rays are absorbed to varying degrees by different parts of the seed and helps to distinguish between different tissues, and injuries; although physiological changes are not visible (Simak 1957; Kriebel 1972). Very mild X-ray doses (~ 100 kV) do not injure physiologically sound (freshly collected) seed and the germination ability should remain high (Simak 1957; Gustafsson and Simak 1958; Ohba and Simak 1961), but might differ between species (Gustafsson and Simak 1958). As mature seed is also less affected by mild X-rays (Gustafsson and Simak 1958; Johnson 1976), this study was carried out only on mature seed with an X-ray dose of ~ 100 kV. Seed germination with moistened filter paper confirmed that mature seed scanned at ~ 100 kV X-rays doses were not damaged as germination was consistent with seed viability determined during Experiment 2 (seeds were numbered). Seedling survival was, however, not determined past four weeks after sowing. Broeckhoven et al. (2016) concluded that small doses of X-rays have no harming effect on live reptiles of various sizes. Previous studies also indicated that samples of up to 150 maize kernels yielded good survival results and allowed for the scanning of more than 1 000 maize kernels per day with semi-automated analysis (du Plessis et al. 2016; Guelpa et al. 2016).

Experiments 2 and 3 investigated whether MicroCT scans can be used as an alternative method to determine seed set and viability accurately and measurably (Kalathingal et al. 2007). Studies with microCT scans are very limited in plant sciences, due to plant tissues mostly consisting of light elements which display low X-ray absorptions. The results of the seed viability tests indicated clear differences between seed (viable versus non-viable) by quantitative porosity measurements indicated by the colour-coded 3D images. When mature seed cones are harvested in large quantities, this can be a useful tool to distinguish between cones with a high number of non-viable seed, lowering the cost of seed extraction.

In conclusion, significant differences were evident for the number of visible ovules, pollen grains inside and outside the ovules, as well as pollen tubes. Over the seven-week period, *P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii* were grouped statistically together and differed significantly from *P. radiata*×*radiata* for all four factors investigated. Only *P. radiata*×*maximinoi* grouped statistically closer to *P. radiata*×*radiata* although significant differences were evident between the duo. Results also supported the notion that the micropyle and droplet may have recognition properties which might include, but not be limited to, pollen grain size. Furthermore, pollen grains were visible inside and outside the ovules within 24-hours after pollination, and the applied methodology can be used as an early mechanism to detect pollination success although the fixation-dehydration-embedding sequence is a tedious process. Temperature differences between Sabie, micro and macro data collected at Karatara indicated that temperature might have an impact on pollination success that need to be considered. The paraffin wax method used in this study is however not suitable to study conelets eight weeks and older after pollination. CT scans can be used as an alternative method to paraffin wax as it is

more time and cost effective and yield information on conelet fertility 15 to 16 months after pollination. MicroCT scanning of conelets can assist in determining seed viability with 3D images.

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Chapter 6

Summary, conclusion and recommendations

1. Summary

Limited pollination success between *P. radiata* and selected pine taxa (*P. tecunumanii, P. oocarpa, P. maximinoi, P. elliottii, P. taeda, P. pringlei, P. patula* and *P. patula*×*tecunumanii*) might be a result of biological or reproductive barriers. These barriers maintain the integrity of a species over time by limiting gene flow. Therefore, differences in the karyotype and phylogenetic tree need to be considered as well. Previous studies indicated that the nine pine taxa are grouped in three distinct different subsections. Although there is no difference in the karyotype between them, the phylogenetic tree indicated significant differences between *P. radiata* and the other species. Therefore, this study employed the following objectives to investigate pollination success between the nine pine taxa:

- Determine and evaluate the current pine pollination protocol used by pine breeders
- Investigate and compare climate conditions inside pollination bags to natural provenance data
- Compare *in vitro* pollen viability, pollen tube elongation and pollen grain size between the nine pine taxa (eight pure species and one interspecific hybrid)
- Determine *in vivo* pollination success
- Investigate if Computed and MicroComputed Tomography can be employed in pine breeding programmes to generate 3D images to determine seed set and viability

A generic *Pinus* cross-pollination protocol was developed and tested by pollinating *P. radiata* female strobili with imported pollen lots of *P. oocarpa* and *P. tecunumanii*. Moisture content and germination percentage were less than 10 % and 50 % respectively. Pollination success was evaluated with pathology diagnostic tests and DNA fingerprinting. The low pollen germination might have played a critical role in the failed attempts to make *P. radiata*×*oocarpa* and *P. radiata*×*tecunumanii* hybrid crosses at the Karatara seed orchard. There might be a number of reasons, but subfreezing temperatures affecting the pollen tube development of the tropical pines was also not one of these. A DNA referencing sample for *P. radiata* was compiled, but unfortunately, all seedlings were assigned to the *P. radiata* population, suggesting that pollen contamination might also have played a role. The pathology tests indicated that all the tested seedlings were indeed infected and susceptible to *Fusarium circinatum* as would be expected from *P. radiata* pure species seedlings. Images (3D) obtained with computed tomography (CT) scans were used to determine seed set of intact cones at 104 weeks after pollination. Follow-up experiments were conducted to determine when fertilisation happens and whether seed viability could be determined with Microcomputed tomography (microCT) scans.

As pollination was unsuccessful, the following studies were initiated to find possible ways to overcome barriers that might limit said success:

- Investigate different bagging options and sterilisation of breeding equipment before, during and after pollination events.
- Placement of pollination bags inside the trees to manipulate number of sunshine hours.

- Determine the effect of environmental conditions (temperature, relative humidity, dew point) inside the pollination bag on pollination success.
- Compare Karatara climatic conditions to the natural provenances of the pine taxa chosen for this study.
- The effect of temperature on the pollen grain germination, pollen tube size and pollen tube growth rate (PTGR) with *in vitro* experiments mimicking *in vivo* conditions.
- Determine pollination success in conelets with *in vivo* experiments.

The generic pollination protocol was adapted with daily sterilisation of breeding equipment, frequently sealing openings created by bulb applicators, and replaced white with green cloth bags. Data indicated that placing the pollination bags in the top northern side of *P. radiata* trees at the Karatara, might increase temperatures inside the pollination bag closer to Sabie temperatures where the eight pine taxa (seven pure species and one hybrid) yield viable seed. However, minimum (nighttime) temperatures (inside pollination bags) during the droplet period is much lower than at Sabie, which might be a possible barrier. The fluctuation (pattern) between day and night temperatures and number of hours at each temperature interval differed significantly between Karatara and Sabie for both inside (micro) and outside (macro) the pollination bag temperature regimes.

Three circadian models were developed simulating climatic conditions at Karatara (micro and macro) and Sabie (average between Tweefontein, Witklip and Spitskop). Although the pollination bag placements might decrease the gap in temperatures between Karatara and Sabie (pollination period), the models indicated that Sabie had a more stable (fewer fluctuations) climate than Karatara. Biplots (discriminant analysis (DA) and principal component analysis (PCA)), resembling the natural provenance climate data, grouped *P. elliottii, P. taeda* and *P. radiata* with Karatara, indicating good site species matching. When altitude is ignored as a vector, *P. patula* was grouped with the trio as well.

Therefore, hybridisation at Karatara is apparently not likely to be constrained by site factors as far as the mother tree's ability to produce seed is concerned. However, if the pollen partner is not adapted to the temperature regimes at Karatara, it might result in limited pollination success. Actual data collected at Karatara during this study and previous studies indicated that *P. radiata* is better adapted to drier summers, wetter winters and coastal sites with a sea breeze, opposed to the more tropical species investigated in this study.

Karatara micro- and macro-temperature regimes had an impact on pollen grain size (re-hydration), tube growth and PTGR. Although it was possible to distinguish between groups of *Pinus* species with similar growth patterns at various temperature regimes, PTGR, pollen tube and grain size cannot provide an accurate prediction whether a *Pinus* species might be a good hybrid partner with *P. radiata*. Barriers that were not investigated during this study, such as various chemical or hormonal interactions, that not only filter-out pollen from unknown species, but also re-hydrate the pollen grains, might have an impact on pollination success and pollen tube growth. Therefore, although this study provided some answers on pollen grain size, pollen tube

size, PTGR and the limited success of previous interspecific hybrid attempts with *P. radiata*, *in vitro* experiments will not provide all the answers in the absence of chemical and hormonal interaction of *in vivo* conditions.

Scanning electron microscope (SEM) images indicated that the pollen grains from the nine pine taxa displayed the structure typical of the Pinaceae with saccate pollen grains. To simulate the re-hydration effect of the pollen droplet on pollen grains, de-hydrated and re-hydrated pollen grain size (length and width) were compared between the nine pine taxa. Significant differences were evident between them, but none of the pine taxa had a size comparable to *P. radiata*.

In vivo pollination success between *P. radiata, P. tecunumanii, P. oocarpa* and *P. maximinoi* were investigated at various time intervals (week 1 to 7). Pollination success was determined by counting the number of visible ovules, pollen grains inside the ovules, pollen grains outside the ovules and pollen tubes. Data were collected with a standard fixation-dehydration-embedding histology sequence. Although hybrid status could not be confirmed by measuring the pollen grain size inside the ovules, pollen sizes were more closely correlated with the male than female species, indicating that the adapted generic pollination protocol might have limited pollen contamination. Over the seven-week period, *P. radiata×oocarpa, P. radiata×tecunumanii* and *P. radiata×maximinoi,* differed significantly from *P. radiata×radiata* for all four factors investigated, indicating that the micropyle and droplet may have recognition properties. The paraffin wax method was not suitable to study conelets older than eight weeks and CT scans were investigated as an alternative method.

CT scans confirmed that fertilisation occurs between 15 to 16 months after pollination. Seed were visible from week 68 after pollination and viability of seed could be determined with microCT scans combining quantitative porosity and defect analyses. These techniques are applicable when scanning multiple cones (high volume) at once to determine seed viability. The germination potential of seed were determined with the water float, correlating with results from the count of viable seed in the mature cones and confirmed that X-rays did not hamper germination potential.

Professor van der Walt (personal communication) hypothesised that pollination is analogous to a key (male) and lock (female). The size and shape of keys are important in opening doors. This theory was tested in this study (only as pilot study) as it has been demonstrated for *Geranium* species (angiosperms). However, follow-up studies are needed to confirm if this possible mechanism is applicable to the pine species investigated. The recognition mechanism of the pollen droplet and micropyle might come into play. But more than one pollen grain were visible inside the ovules (also supported by literature: for example Sarvas 1962; Lill 1976; Sweet 1977; Bramlett 1981; Griffin and Lindgren 1985; Slee and Abbott 1990; Owens et al. 2005; Owens and Fernando 2007; Varis et al. 2008), indicating that the PTGR might be more important or the reserves inside the pollen tube to sustain the growing process throughout the 12 months.

2. Conclusion

DA and PCA biplots (Figure 6.1) indicated significant differences for the Pearson correlation between natural provenance data (altitude, precipitation and temperature), PTGR (length and width) and hydrated pollen grain size (length and width) at micro and macro-temperature regimes for the eight pure species. Three distinct groups were evident in the DA biplot: (1) *P. radiata, P. elliottii* and *P. taeda;* (2) *P. maximinoi, P. pringlei* and *P. patula;* and (3) *P. oocarpa* and *P. tecunumanii* (Figure 6.1). Collectively, these groups correlated with results obtained during the smaller studies. As the data were collected in one geographical region during *in vitro* and *in vivo* experiments wherein micro and macro temperature regimes were mimicked at Karatara, a *P. radiata* seed orchard, reciprocal crosses should also be performed in more regions such as Sabie (seed orchard more suitable the selected *Pinus* taxa) before *P. radiata* as pollen partner can be ruled out. The higher temperatures at Sabie might also result in conelet or ovule abortions as *P. radiata* pollen has limited viability due to the higher temperature regimes. Most of the experiments indicated that *P. patula* might be the best pollen partner for *P. radiata*. But, due to altitude and *F. circinatum* sensitivity, *P. patula* is not a desired pollen partner. Therefore, an alternative site from Karatara seed orchard with ecological conditions intermediate to Karatara and Sabie could also be investigated for *in vivo* hybridisation studies.

This study could not provide a clear cut answer to the limited pollination success between *P. radiata* and selected pine species. But, the phylogenetic tree, environmental conditions inside pollination bags and data collected during this study, indicated that *P. elliottii* and *P. taeda* might be potential hybridisation partners for *P. radiata*. A generic pollination protocol was determined, investigated, adjusted and seems to limit pollen contamination during pollination at Karatara. Valuable information were collected that can help with pine breeding programmes, such as: pollen grain size, PTGR, climatic data, three circadian models, *in vivo* pollination success and microCT scanning as a tool for early detection of seed set and viability.

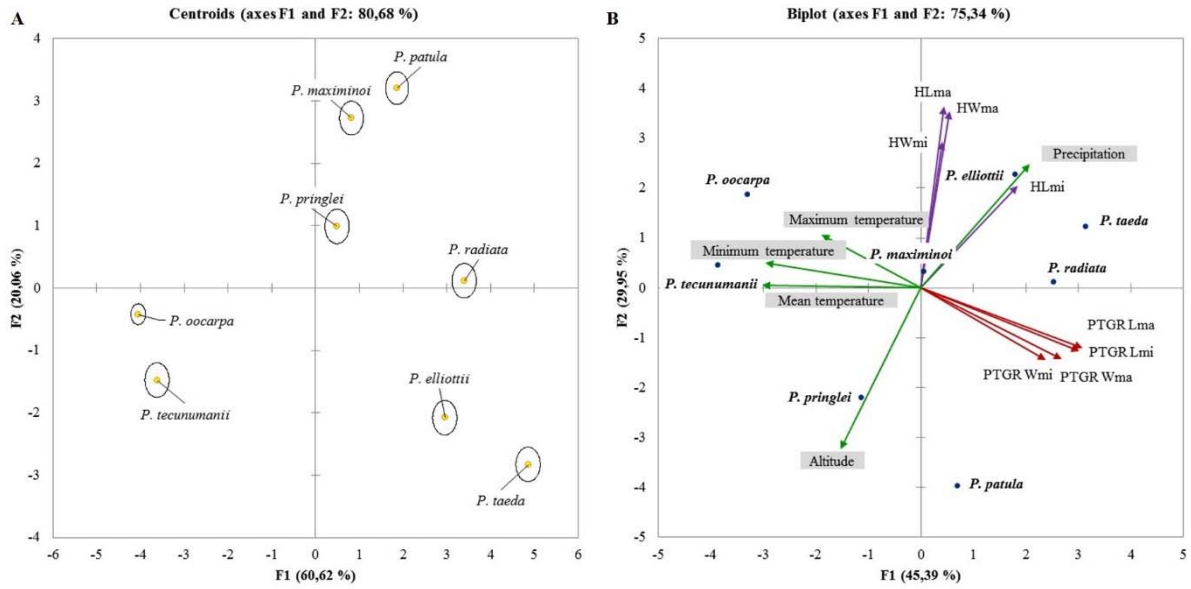

Figure 6.1: DA (A) and PCA (B) biplots indicating significant differences for the Pearson correlation between natural provenance data (altitude, precipitation and temperature), PTGR (length and width) and hydrated pollen grain size (length and width) at micro and macro-temperature regimes for the eight pure *Pinus* species (HLmi: hydrated length micro; HLma: hydrated length macro; HWmi: hydrated width micro; HWma: hydrated width macro; WPTGR ma: width PTGR macro; WPTGR mi: width PTGR micro; LPTGR ma: length PTGR macro; LPTGR mi: length PTGR micro; green lines: natural provenance variables; red lines: PTGR; purple lines: pollen grain size)

3. Recommendations

This study aimed to investigate some of the factors in the cross-pollination process that can be adapted slightly to improve pollination success between *P. radiata* and eight pine taxa. However, due to time limits and logistic constraints various aspects had to be excluded from the scope of this study, for example: pollen competition; chemical, hormonal and mechanical mechanisms affecting the recognition of the pollen grains by the pollen droplet and micropyle; and pollen allelopathy (release of toxins that inhibit sexual reproduction). All of these might constitute possible barriers limiting pollination success.

Considering the combined effect of the phylogenetic tree, climatic conditions at natural provenances, PTGR, pollen grain size and *in vivo* hybridisation attempts studied during this study, the following recommendations for future studies are made:

a) Future studies should investigate the use of non-traditional bagging materials, such as plastic, cellophane etc.

b) *P. elliottii* and *P. taeda* should be tested as interspecific hybridisation partners (male and female) with *P. radiata*.

c) The effect of chemical and hormone balances on the recognition of pollen grains, pollen tube development, PTGR and dormancy of pollen grains between pollination and fertilisation events need to be investigated as possible pollination barriers.

d) *In vivo* pollination success studies should investigate the ovules in more detail, to determine whether brown ovules are indeed aborted and whether abortion of ovules increase closer to fertilisation.

e) The *in vivo* experiments need to be repeated with enough repetitions to sample conelets and cones up to week 104 after pollinations in order to determine whether the adapted pollination protocol does improve pollination success.

f) Bigger sample size when doing *in vivo* studies could be used to determine when pollen tubes develop in different hybrid combinations. This might assist with determining the frequency and quantity of pollination attempts. Shorter time periods than seven days could be investigated. Care should be taken to perform experiments in the top northern part of trees.

g) A better method (using dyes) to determine or measure pollen grains inside ovules to confirm hybrid status needs to be developed.

h) *In vitro* and *in vivo* studies should include a wider variety of *P. radiata* clones and seed orchards.

i) Although *P. radiata* is not geographically adapted to the Sabie region, reciprocal crosses can be simulated *in vitro* with the Sabie circadian models to compare the PTGR and pollen grain size of *P. radiata* with *Pinus* species. Also, an alternative site from Karatara seed orchard with ecological conditions intermediate to Karatara and Sabie could also be investigated for *in vivo* hybridisation studies.

j) The effect of the frequency and quantity of pollination attempts during a pollination season should be clarified. Multiple applications of about 24 hours apart might improve pollination success.

k) Determine whether the mechanical properties of pollen tubes can contribute to pollination and fertilisation success.

Appendices

Appendix A

UNIVERSITEIT STELLENBOSCH UNIVERSITY

Confidential questionnaire

Analysis of current Pine breeding programmes with outcomes, successes and recommendations

The information in this questionnaire is only for information purposes as background study to a Camcore project. Intelectual Property (IP) of individual companies will be respected and confidentiality is guaranteed. No part of this questionnaire is for sale or will be sold to anybody. Copyright is with Stellenbosch University: Department of Forest and Wood Science.

Name of breeder and contact details:

Name:
E-mail:
Telephone number:

Company details:

Name of company:
Website address:

General information on breeding programme (Camcore and company focus):

Age of breeding programme (years):

Main focus and effort of breeding programme:

			Effort percentage (100% = full time)
1 Molecular breeding	Y	N	
2 Conventional breeding	Y	N	
3 GMT (genetic modified trees) research	Y	N	
4 List main species involved			

Please specify partners in breeding programme via funding or joint projects:

1 Universities
2 Camcore
3 Private companies
4 Research institutes (CSIR, ICFR, etc.)
5 Private consultants
6 Other

Please list the main objectives (and ranking) of your breeding programme:

			Ranking objectives (1 is low and 5 is high)
1 Volume production	Y	N	
2 Wood properties	Y	N	
3 Pulp quality	Y	N	
4 Stem form and branching	Y	N	
5 Pest and diseases	Y	N	
6 Mass propagation	Y	N	
7 Seed set and production	Y	N	
8 Provenances (GEI)	Y	N	
9 Hybridisation	Y	N	
10 Fire resistance	Y	N	
11 Drought/ frost/ snow tolerance	Y	N	
12 Other (please specify)			

If hybridisation is part of the main objectives of your breeding programme (Camcore and commercial), please specify the following:

1 Species involved

Ranking objectives (1 low and 5 high)

2 Main objectives	2.1 Volume production	Y	N
	2.2 Wood properties	Y	N
	2.3 Pulp quality	Y	N
	2.4 Stem form and branching	Y	N
	2.5 Pest and diseases	Y	N
	2.6 Mass propagation	Y	N
	2.7 Seed set and production	Y	N
	2.8 Provenances (GEI)	Y	N
	2.9 Fire resistance	Y	N
	2.10 Drought/ frost/ snow resistance	Y	N
	2.11 Other		

3 Please indicate out of 10 (10 high and 0 very low) the effort of hybridisation versus pure breeding

Has any research been conducted on the following disciplines/ techniques/ procedures:

1 Fertilisation & ovule studies	Y	N
2 Pollination and pollen studies	Y	N
3 Abortion of flowers/ cones	Y	N
4 Plant anatomy	Y	N
5 Plant morphology	Y	N
6 Wood properties	Y	N
7 Ecology and/ or GEI	Y	N
8 Propagation and physiology	Y	N

Please provide a brief overview of projects undertaken mentioned above. Please supply the references or outputs of the most important projects undertaken. References can be submitted as a separate document.

Outputs of breeding programme (Camcore and company focus):

Please provide a list of publications published with relevance to the pine breeding programme. *The list can be attached as a separate file to the returning mail.*

Please list the main discipline of articles (or closest related discipline):

1 Plant pathology	Y	N
2 Population genetics	Y	N
3 Quantitative genetics	Y	N
4 Molecular genetics	Y	N
5 Silviculture management	Y	N
6 Provenances (GEI)	Y	N
7 Wood properties	Y	N
8 Forest Management	Y	N
9 Forest Ecology	Y	N
10 Nursery practices	Y	N
11 Conservation of genetic resources	Y	N
12 Soil science	Y	N
13 Agroforestry	Y	N
14 Pulp quality	Y	N
15 Stem form and branching	Y	N
16 Volume production	Y	N
17 Fire resistance	Y	N
18 Seed set and production	Y	N
19 Propagation research	Y	N
20 Other:		

Future prospectives and focus on pine hybridisation (Camcore and company focus):

Briefly describe what your company see as future prospectives and focus of your pine breeding programme with special emphasis on hybridisation. Will the objectives and species involved change and how?

Pollination protocol for pine hybridisation (Camcore and company focus):

Briefly describe the process of pine pollination as practiced by your company under the following headings.

1) stage of collection of catkins
2) collection method
3) storage (method and time period) of pollen
4) preparation of pollen for crosses (drying etc.)
5) time and method (stage) of bagging flowers
6) pollination method
7) length of bagging period
8) what data are collected
9) monitoring method of pollination success
10) materials used (type of bag etc.)
11) placement of bags in trees (randomised, upper, lower, middle, sun or shade)

Successes of breeding programme (Camcore and company focus):

Please give a brief summary of the main successes of the breeding programme with special references to the following:

1 Number of new hybrids developed as part of Camcore research, for example 5*P. patula x P. tec.*
2 Number of new hybrids developed for commercial deployment, for example 5*P. patula x P. tec.*
3 Number of new hybrids with high potential for future pine plantations, for example 5 *P. patula x P. tec.*
4 Number of new hybrids under field experiments, for example 5*P. patula x P. tec.*
5 Number of new hybrids planted on commercial scale, for example 5*P. patula x P. tec.*
6 Number of new hybrids considered as a replacement for traditional pine species, for example 5 *P. patula x P. tec.*
7 New breeding techniques developed (IP will be protected)
8 New breeding equipment developed (IP will be protected)
9 New breeding strategies developed (IP will be protected)
10 Any other scientific successes that have a positive impact on the outcome of breeding programmes and hybridisation
11 Number of plant breeders' rights registrations (successful, pending etc.)
12 Any trademark registrations (successful, pending etc.)
13 Please explain what you consider as a successful hybridisation programme.
14 How do you measure the success of your pine breeding programme?

Recommendations (Camcore and company focus):

Please list any recommendations for future research projects connected to pine hybridisation.

1 Do you need more resources in terms of better qualified staff, funding opportunities etc.
2 Any bottlenecks in the process of hybridisation?
3 What is the impact of climate change on your breeding programme?
4 What can the forestry industry do to promote tree breeding?
5 Do you have easy access to scientific information (papers etc.), professional bodies, scientific resources etc.?
6 Do you have access to international funding opportunities?
7 Do you collaborate with international pine bodies? How, who and how regular?
8 Is there sufficient courses etc. available to improve general breeding knowledge and networking worldwide?
9 According to you, name five general recommendations that will improve pine breeding worldwide.

Thank you for your time and assitance

Appendix B

ANOVA tables of statistical analyses

Chapter 3

Provenance data of the selected pine species

Sources of variation	DF	Alt MS		Prec MS		MAT MS		MT MS		MIT MS	
Pollination Month 1:											
Provenance	67	226 261.78	*	825.30	ns	8.38	*	7.59	*	10.95	*
Species	6	6 699 391.13	**	8 193.24	**	124.10	**	181.47	**	262.13	**
Error	82	139 209.00		736.62		5.82		4.96		5.75	
Pollination Month 2:											
Provenance	67	226 261.78	*	351.02	ns	8.50	*	7.24	*	10.29	*
Species	6	6 699 391.13	**	2 143.91	**	116.58	**	163.48	**	231.97	**
Error	82	139 209.00		343.68		5.82		4.90		5.56	
Pollination Months 1 and 2:											
Provenance	67			1 067.20	**	16.81	**	14.78	**	21.19	**
Species	6			8 828.90	**	240.41	**	344.64	**	493.61	**
Provenance x species	82			962.77	**	11.59	**	9.83	**	11.25	**
Month	1			10 780.63	**	145.00	**	61.66	**	13.96	**
Month x species	6			1 434.95	**	0.39	**	0.39	**	0.57	**
Error	149			116.70		0.05		0.04		0.05	

Chapter 4

Pollen tube size and PTGR

Sources of variation	DF	PTL MS		PTGRL MS		PTW MS		PTGRW MS	
Temperature: 10°C									
Species	2	2 331.53	*	0.08	*	27.03	*	0.001	*
Error	12	158.70		0.01		3.62		0.0001	
Temperature: 15°C									
Species	7	17.04	**	0.25	**	124.58	**	0.0002	**
Error	32	290.50		0.01		4.54		0.01	

Sources of variation	DF	PTL MS		PTGRL MS		PTW MS		PTGRW MS	
Temperature: 20°C									
Species	8	33 924.77	**	1.20	**	2 330.18	**	0.0824	**
Error	36	366.93		0.01		12.37		0.0004	
Temperature: 24°C									
Species	8	6 000.84	**	0.21	**	205.83	**	0.0073	**
Error	36	397.49		0.01		7.50		0.0003	
Temperature: 28°C									
Species	8	77 400.96	**	2.74	**	3 141.95	**	0.1112	**
Error	36	449.81		0.02		17.52		0.0006	
Temperature: 32°C									
Species	8	4 867.67	**	0.17	**	175.87	**	0.0062	**
Error	36	562.97		0.02		18.55		0.0007	
Temperature: 36°C									
Species	8	4 100.69	**	0.15	**	46.32	*	0.0017	*
Error	36	265.08		0.01		10.36		0.0004	
Temperature: micro climate									
Species	6	4 903.45	**	0.17	**	324.16	**	0.0114	**
Error	28	236.52		0.01		10.73		0.0004	
Temperature: macro climate									
Species	6	12 389.88	**	0.440	**	153.64	**	0.0054	**
Error	28	181.95		0.006		14.09		0.0005	

Pollen grain size

Sources of variation	DF	D MS		MaH MS		MiH MS	
Pollen grain length							
Species	8	698.17	**	153.07	**	203.57	**
Error	216	16.75		31.05		34.88	
Pollen grain width							
Species	8	713.15	**	174.54	**	399.16	**
Error	216	14.97		38.96		41.94	

Sigmoid growth curve: pollen grain length

Sources of variation	DF	*a* MS		*b* MS		*c* MS	
Micro climate							
Species	6	11 923.84	**	6.08	**	1.38	ns
Error	27	1 514.78		0.51		0.78	
Macro climate							
Species	6	10 517.63	*	11.13	*	4.21	ns
Error	27	3 279.42		2.27		5.23	

Chapter 5

***In vivo* flower studies**

Sources of variation	DF	Ovules MS		PGOO MS		PGIO MS		PTIO MS	
Week 1									
Species	3	57.35	**	0.78	*	4.56	*	0.99	*
Error	56	4.32		0.14		1.78		0.29	
Week 2									
Species	3	66.82	**	1.39	*	1.44	ns	0.28	ns
Error	56	2.64		0.62		0.69		0.19	
Week 3									
Species	3	20.13	*	17.26	**	6.22	*	2.53	*
Error	56	4.72		0.47		1.29		0.35	
Week 4									
Species	3	27.75	*	13.40	**	1.39	*	0.31	ns
Error	56	7.05		0.65		0.49		0.19	
Week 5									
Species	3	87.71	**	2.82	**	12.24	*	8.51	*
Error	56	4.56		0.22		1.68		1.05	
Week 7									
Species	3	26.51	*	8.73	**	0.86	ns	0.64	ns
Error	56	3.83		0.48		0.64		0.55	
Time and hybrid combination interaction over seven weeks									
Species	3	98.76	**	5.61	**	10.15	**	4.90	**
Replication (species)	56	3.42	ns	0.39	ns	0.72	ns	0.35	ns
Time	6	83.82	**	31.69	**	5.59	**	3.77	**
Treatment	15	38.77	**	7.28	**	2.52	**	1.31	**
Error	3	4.70		0.43		1.12		0.43	

Confirming hybrid status

Sources of variation	DF	PGL MS		PGW MS	
Week 1					
Species	2	139.27	ns	13.53	ns
Error	11	68.69		40.27	
Week 2					
Species	3	250.73	*	127.59	*
Error	36	37.64		30.58	
Week 3					
Species	3	154.45	*	351.04	**
Error	36	51.19		23.64	
Week 4					
Species	2	252.37	*	18.13	ns
Error	27	84.02		52.19	
Week 5					
Species	2	40.11	ns	27.16	ns
Error	14	103.11		33.39	
Week 7					
Species	2	319.07	*	12.59	*
Error	18	28.12		53.73	

Declaration of candidate and co-authors

Declaration by the candidate

With regard to the published paper titled 'Climate as possible reproductive barrier in *Pinus radiata* (D. Don) interspecific hybridisation', the nature and extant of my contributions were as follows:

Nature of contribution	Extant of contribution
o Literature review	100 %
o Develop experimental design and execution of experiments	95 %
o Data collection, capturing and analyses	95 %
o Conceptualised and wrote the paper	95 %
o Formatting and editing of paper	95 %

The following co-authors have contributed to the paper titled 'Climate as possible reproductive barrier in *Pinus radiata* (D. Don) interspecific hybridisation', as follows:

Name	**[E]**	**Nature of contribution**	**Extent of contribution**
B du Toit	ben@sun.ac.za	Advice and editing	± 5 %
A Kanzler	arnulf.kanzler@sappi.com	Advice and editing	± 5 %
A-M Botha-Oberholster	ambo@sun.ac.za	Advice and editing	± 5 %

Signature of candidate:  Date: 18 August 2017

Declaration of co-authors:

The undersigned hereby confirm that

1. The declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authos to this paper,
2. No other authors contributed to this paper without recognition in acknowledgements, and
3. Potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in this paper.

Name:	**Signature:**	**Affiliation:**	**Date:**
B du Toit		Stellenbosch University	18 August 2017
A Kanzler		Sappi Forests	18 August 2017
A-M Botha-Oberholster		Stellenbosch University	18 August 2017

Declaration by the candidate

With regard to the accepted paper titled '*Pinus radiata* hybridisation: pollen tube elongation and pollen grain size as possible reproductive barriers', the nature and extant of my contributions were as follows:

Nature of contribution	Extant of contribution
o Literature review	100 %
o Develop experimental design and execution of experiments	95 %
o Data collection, capturing and analyses	95 %
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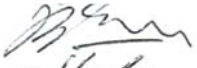
Name:	**Signature:**	**Affiliation:**	**Date:**
B du Toit		Stellenbosch University	18 August 2017
A Kanzler		Sappi Forests	18 August 2017
A-M Botha-Oberholster		Stellenbosch University	18 August 2017

Declaration by the candidate

With regard to the accepted paper titled '*Pinus radiata* interspecific hybridisation: case study', the nature and extant of my contributions were as follows:

Nature of contribution	Extant of contribution
o Literature review	100 %
o Develop experimental design and execution of experiments	95 %
o Data collection, capturing and analyses	95 %
o Conceptualised and wrote the paper	95 %
o Formatting and editing of paper	95 %

The following co-authors have contributed to the accepted paper titled '*Pinus radiata* interspecific hybridisation: case study', as follows:

Name	**[E]**	**Nature of contribution**	**Extent of contribution**
B du Toit	ben@sun.ac.za	Advice and editing	± 5 %
A Kanzler	arnulf.kanzler@sappi.com	Advice and editing	± 5 %
A-M Botha-Oberholster	ambo@sun.ac.za	Advice and editing	± 5 %

Signature of candidate:  Date: 18 August 2017

Declaration of co-authors:

The undersigned hereby confirm that

1. The declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authos to this paper,
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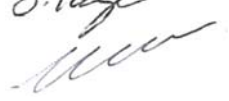
Name:	**Signature:**	**Affiliation:**	**Date:**
B du Toit		Stellenbosch University	18 August 2017
A Kanzler		Sappi Forests	18 August 2017
A-M Botha-Oberholster		Stellenbosch University	18 August 2017

Declaration by the candidate

With regard to the published paper titled 'Microcomputed tomography (microCT) as a tool in *Pinus* tree breeding: pilot studies', the nature and extant of my contributions were as follows:

Nature of contribution	Extant of contribution
o Literature review	100 %
o Develop experimental design and execution of experiments	80 %
o Data collection, capturing and analyses	80 %
o Conceptualised and wrote the paper	80 %
o Formatting and editing of paper	80 %

The following co-authors have contributed to the paper titled 'Microcomputed tomography (microCT) as a tool in *Pinus* tree breeding: pilot studies', as follows:

Name	**[E]**	**Nature of contribution**	**Extent of contribution**
A du Plessis	anton2@sun.ac.za	CT scanning, 3D imaging, selective writing and editing of paper	± 20 %
SG le Roux	ctscanner@sun.ac.za	CT scanning, 3D imaging, selective writing and editing of paper	± 20 %

Signature of candidate:  Date: 18 August 2017

Declaration of co-authors:

The undersigned hereby confirm that

1. The declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authos to this paper,
2. No other authors contributed to this paper without recognition in acknowledgements, and
3. Potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in this paper.
4. Microcomupted tomography (microCT) as a tool in *Pinus* tree breeding: pilot studies') of this dissertation.

Name:	**Signature:**	**Affiliation:**	**Date:**
A du Plessis		Stellenbosch University	18 August 2017
SG le Roux		Stellenbosch University	18 August 2017