FACTORS INFLUENCING CONTROLLED POLLINATION OF *PINUS PATULA*

BY

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Submitted in fulfillment of the requirements for the degree of

MASTERS IN SCIENCE

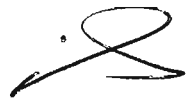
In the Research Centre for Plant Growth and Development
Faculty of Science and Agriculture
University of Natal, Pietermaritzburg

January 2002

PREFACE

The experimental work described in this thesis was conducted at the Sappi Shaw Research Centre, Tweedie and in the Research Centre for Plant Growth and Development, University of Natal, Pietermaritzburg, from June 1996 to December 2001 under the supervision of Professor J Van Staden.

The results have not been submitted in any other form to another University and except where the work of others is acknowledged in the text, are the result of my own investigation.



ANDRÉ NEL
JANUARY 2002

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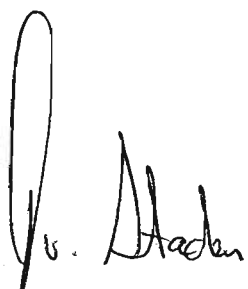

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ACKNOWLEDGEMENTS

I am grateful to Professor Van Staden for giving me the opportunity and the facilities in which to conduct my studies.

I also wish to thank Dr Nicky Jones for serving on my Research Committee and for her valuable input and assistance.

My thanks to Sappi for providing me with the opportunity, facilities and material to conduct my studies.

Thank you also to Dr Richard Barnes and Miss Kay Nixon for their input and suggestions throughout the study period.

I wish to thank Mr Lucky Zondi and Mr Christopher Komakech for their input with some of the technical work of this study.

I would also like to thank staff from the Tree Pathology Co-operative Programme (TPCP) based at the Forestry and Agricultural Biotechnology Institute (FABI) of the University of Pretoria for their input with the fungicide-treatment study and identification of fungi.

The assistance and advice of Mr Vijay Bandu of the Unit for Electron Microscopy of the University of Natal is gratefully acknowledged.

Thanks to my family, friends and colleagues for their encouragement and interest in my studies.

This thesis is dedicated to my wife, Rika, thank you for your love, support and encouragement.

ABSTRACT

A study of factors contributing to successful controlled pollinations of *Pinus patula* Scheide et Deppe was undertaken. The pollen morphology of *P. patula, P. oocarpa, P. greggii, P. elliottii, P. tecunumanii, P. caribaea* and *P. radiata* was studied and the mean size of pollen grains was determined for these species. Clonal differences in pollen size within *P. patula* were also determined.

The impact of pollen management practices on pollen viability was highlighted and a protocol for *in vitro* pollen viability testing of *P. patula* and other pine species was determined. A one percent agar solidified distilled water medium gave the best germination results after 72 hours incubation at 30 °C for a number of different *Pinus* species and *P. patula* clones. The addition of boric acid increase germination, although not significantly. The addition of sucrose to the pollen germination medium had a negative effect on pollen germination of *P. patula*, *P. greggii* and *P. caribaea.*

Re-hydration of pollen for two hours prior to *in vitro* germination testing improved germination significantly. Incubation temperatures of above 38 °C were detrimental to germinating pollen grains. Stored pollen with low humidity (less than 10 %) of *P. patula, P. greggii and P. caribaea* could tolerate temperatures of up to 70 °C while still retaining some level of viability. The initiation and growth of the pollen tube was also studied and differences in pollen tube-lengths germinated at 30 °C for 72 hours were found between species studied.

Flowering of different *P. patula* clones was monitored over seven seasons. Flowering periods varied in length between 4 and 14 days amongst five clones over the different seasons.

The best cone-survival after controlled pollination was achieved with breathable micro-fibre material. Seed yields were also highest when breathable material was used for controlled pollination. The role of pollen viability in controlled pollination was also determined in pollination studies with low viability resulting in low cone survival and low seed yields.

The temperature and relative humidity inside isolation bags were monitored and temperatures above 40 °C were reached inside bags constructed of non-breathable material. These temperatures were lethal to pollen germinating *in vitro*. Relative humidity of between 80 and 100 % was maintained in non-breathable bagging material, constituting a risk of diseases causing cone-mortality. The application of fungicide before, during and after controlled pollination was ineffective in improving cone survival.

CONTENTS

LIST OF COMMONLY USED ABBREVIATONS

ANOVA	Analysis of variance
d	Day
h	Hour
LSD	Least Significant Difference
min	Minute
NAA	Naphthalene acetic acid
RH	Relative humidity

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CHAPTER 1

INTRODUCTION

1.1. Forestry in South Africa

The first recorded establishment of an exotic tree species in South Africa can be traced back to 1670 when a plantation of oaks were planted at Newlands, Cape Town (OWEN and VAN DER ZEL 2000). According to POYNTON (1977) the first commercial plantations consisted of *Pinus pinaster and Pinus pinea* and were established between 1825 and 1830 at Genadendal. It is estimated that today a total area of about 1.5 million ha of South Africa (1.4%) is used for Forestry (Forest Owners Association 1998). Roundwood sales of 18.6 million m^3 generate revenue of about R1.7 billion per annum, as assessed in the year 2000. Commercial companies own approximately half of the land under afforestation. The public sector (30%) and private individuals (21%) own the rest of the afforested land. Softwoods in the form of various pine species make up about 52% of the afforested area (OWEN and VAN DER ZEL 2000).

1.2. Pinus patula Scheide et Deppe

Sir David Hutchins, conservator of Forests for the Cape, introduced *Pinus patula* Scheide et Deppe into South Africa in 1907 when a trial block was planted at Tokai plantation in the Western Cape Province (POYNTON 1977). Further introductions were made in 1908 when several arboretums were established at plantations near Tzaneen, Belfast and Lothair in the Mpumalanga and Northern provinces (LOOCK 1977). *P. patula* is the most important softwood species in commercial forestry in South Africa. Approximately 300 000 ha is afforested with this species by the different forestry companies and it is grown for a variety of timber and pulp products. It is the most extensively planted pine species on Sappi land and covers about 185 000 ha (STANGER 1999).

1.2.1. Species description

The genus *Pinus* comprises approximately 100 taxonomically distinct species and many hybrids, varieties and cultivars (POYNTON 1977). Their natural distribution includes most of the Northern Hemisphere and is almost entirely absent south of the equator. A few species have a more tropical distribution and crosses the equator. Two species, *Pinus halepensis* and *Pinus pinaster*, are represented in Africa north of the Sahara (POYNTON 1977).

Pinus patula Scheide et Deppe belongs to the *Pinaceae* family and the genus *Pinus*. Two different varieties occur in *P. patula*, var. *patula* and var. *longipedunculata*. The species is placed in the section *Serotinae* subsection *Oocarpae* (WORMALD 1975). Other species included in this subsection are *P. tecunumanii, P. oocarpa, P. greggii, P. muricata and P. pringlei*. The full taxonomic classification of *P. patula* is as follows:

Kingdom	Plant
Division	*Spermatophyta*
Subdivision	*Gymnospermae*
Order	*Coniferales*
Family	*Pinaceae*
Genus	*Pinus*
Sub-genus	*Diploxylon* (Hard pines)
Section	*Serotinae* (Closed-coned pines)
Sub-section	*Oocarpae*
Species	*Pinus patula* Scheide et Deppe
Common name/s	patula pine, Mexican weeping pine, spreading-leafed pine

(Adapted from PERRY 1991)

P. patula is indigenous to Mexico at altitudes of 1500 to 3100 m and at latitudes 16° N to 24° N with mean annual precipitation of between 600 and 2500 mm (WRIGHT 1994). Figure 1 shows the natural distribution of this species in Mexico.

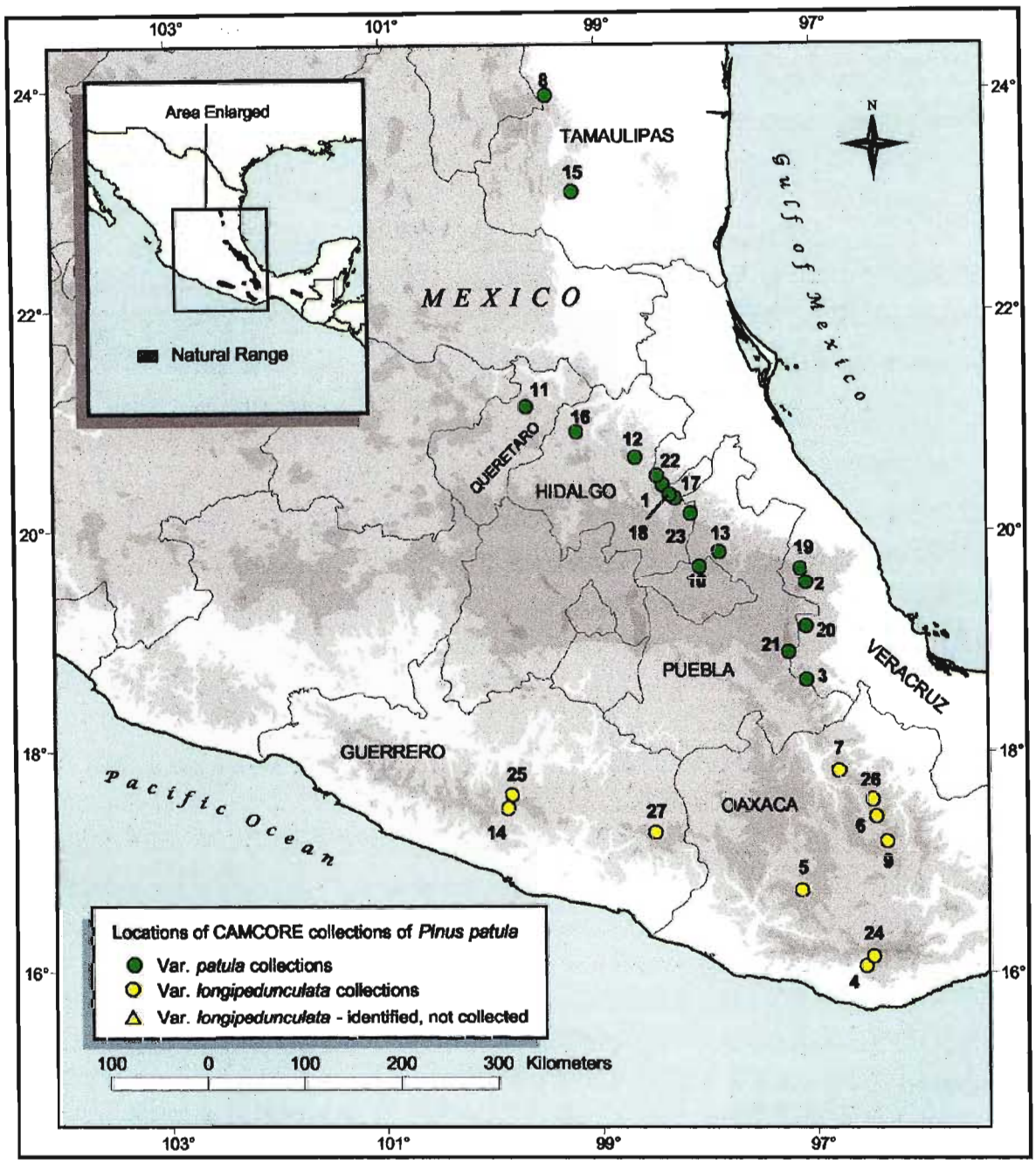
Within its native range it attains a height of 35 m and diameters of up to 80 cm (DVORAK, HODGE, KIETZKA, MALAN, OSORIO and STANGER 2000). *P. patula* is the most widely planted species in the *Oocarpae* subsection with an approximate 1.0 million ha established worldwide. The broad growth requirements for this species in South Africa are mean annual temperature (MAT) of <18°C and rainfall (MAR) of >700 mm at high altitudes and >950 mm at lower altitudes with well-drained soils (MORRIS and PALLETT 2000). Figure 2 shows the distribution of this species within South Africa.

The wood of *P. patula* is yellowish-white in colour, has a moderate wood-density, is low in extractives and is suitable for a number of wood and paper products (DVORAK, HODGE, KIETZKA, MALAN, OSORIO and STANGER 2000). These attributes and its fast growth in the summer-rainfall area make *P. patula* the most important and widely planted softwood in South Africa.

1.2.2. Flower morphology

Conifer species do not produce true flowers. Reproductive buds develop from dormant buds that are, according to their function either vegetative, giving rise to vegetative shoots, or reproductive leading to the development of reproductive structures. The reproductive buds develop into strobili or cones. The cones are of two kinds, the microsporangiate or male cone (catkin) that produce pollen, and the megasporangiate or female cone which produces the seed (OWENS and MOLDER 1983). When mature, the megasporangiate are the structures commonly recognized as cones.

Pines, like most conifers, are monoecious, i.e. they bear both types of cones on the same tree. Each cone is usually separate from the other kind and generally occurs on separate branches. Male cones are small structures that last for only a few weeks. After shedding their pollen, they dry out and fall off the tree. Female cones are of longer duration and in *P. patula* they develop over a period of about two years.



(DVORAK, HODGE, KIETZKA, MALAN, OSORIO and STANGER 2000)

Figure 1. Map of natural occurrence of *P. patula* in Mexico. Collections from different provenances in Mexico are indicated for the two varieties *patula* and *longipedunculata*.

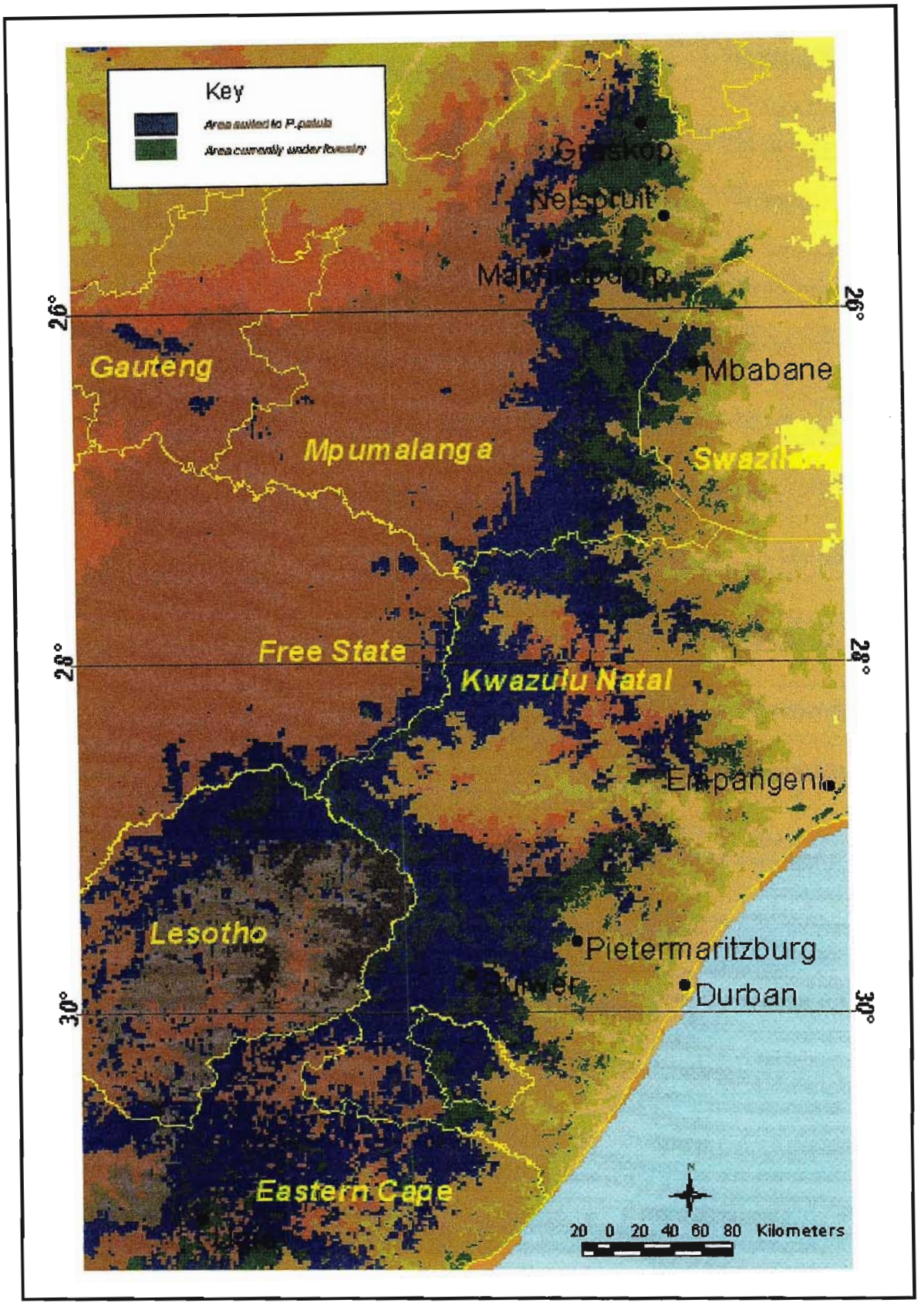


Figure 2. Map showing suitable areas for establishment (blue) and actual occurrence (green) of *P. patula* var. *patula* in the eastern part of South Africa. Image produced by Sappi Research Department.

Fertilization of the ovule by the pollen grain only occurs one year after pollination. When pollen is released from the male cones, it is carried in the atmosphere and is spread by means of wind. At the peak of the flowering season, pollen is produced in large amounts and appears at maturity as yellow dust. When it reaches the female cone, the pollen grains fall among the scales, reaching the ovules located at their base. When receptive, an ovule secretes a drop of resinous fluid that eventually dries out and pulls the pollen grains through the micropyle (OWENS and MOLDER 1983).

After maturity, female cones may fall off, disintegrate or remain on the tree for several years, even after shedding the seeds (OWENS and MOLDER 1983). *P. patula*, a closed-cone pine, does not shed its seeds while the cone is attached to the tree and can retain the mature cones on the tree for a number of years (DVORAK, HODGE, KIETZKA, MALAN, OSORIO and STANGER 2000).

The average number of seeds per cone of *P. patula* is approximately 125 in its natural habitat and there are about 118 000 seeds per kilogram of seed (DVORAK, HODGE, KIETZKA, MALAN, OSORIO and STANGER 2000). A recovery rate of between 64 000 and 96 000 germinated seedlings per kilogram of *P. patula* seed-orchard grade seed can be produced assuming 70–85 % germination in the nursery (BAYLEY, NIXON and SMITH 2000).

1.2.3. Flowering period

The flowering times of the commercial pine species vary between species and to some extent, between families or clones within a species. This necessitates the collection, processing and storage of pollen of some species so that pollen is available for the making of inter-specific and intra-specific hybrid crosses weeks or months later when the female partner-species is flowering. Table 1 lists the flowering times and cone-growth periods of some of the commercial pine species grown in South Africa.

Table 1. Flowering periods of some commercial *Pinus* species under South African conditions.

Species	Pollen production	Female flowers Receptive	Cone growth period	Cone harvest
P.patula	September	September	22 months	August
P.elliottii	July	July	19 months	February
P.caribaea	June	June	19 months	February
P.tecunumanii	September	September	22 months	August
P.oocarpa	July	August	21 months	May
P.teada	July	July	22 months	May

1.3. Controlled Pollination

Controlled pollination constitutes one of the most important aspects of any tree improvement programme (CRESPELL 1998). This technique is used to produce families of known parentage for the creation of advanced generation breeding populations and the evaluation of selections in production populations (BRAMLETT and O' GWYNN 1981). Controlled pollination is also used to create genotypes that may not occur under natural conditions, such as inter-specific hybrids (VERRYN, HETTASCH, PIERCE, SNEDDEN and STEYN 2000). Controlled pollination has been applied in the plant-breeding field for centuries, but was applied to forest tree species more recently. According to BRAMLETT and O'GWYNN (1981), the first use of controlled pollination on conifers in forestry was documented in 1937 and it was applied on a larger scale in 1948.

With controlled pollination of conifers, pollen from a selected tree is collected, processed and stored, and is then applied to isolated female flowers of another selected tree. Female cones are usually isolated with some type of isolation bag to prevent wind-borne pollen from reaching the selected female cones (BRAMLETT 1998). This produces seed of known pedigree and can be used to verify the genetic value of the parent trees. Tree breeders in South Africa report very high levels of conelet abortion after controlled pollination of *P. patula*. HAGEDORN

(2000) reports abortion levels as high as 40 to 90% and low seed yields from a survey among South African tree breeders from various forestry companies.

1.4 Aims of the Study

As outlined above, controlled pollination provides a valuable tool to the Tree Breeder and is an indispensable part of any tree improvement programme. Due to the high levels of conelet-abortion after controlled pollination and low seed-yields, the production of specific crosses is very expensive and takes several flowering seasons to complete. This often delays the release of superior genetic material and translates into a big loss in potential revenue.

The major objective of this study was to investigate some of the components of controlled pollination and to develop or adapt current techniques to devise an optimal protocol for successful controlled pollination of *P. patula*. The approach involved:

i) Testing various isolation bags made of different material and determining their effect on conelet survival and seed-yield;
ii) Studying the environment inside the different isolation bags to gain a better understanding of the conditions experienced by germinating pollen and developing conelets;
iii) Studying the pollen morphology of *P. patula* and other relevant species;
iv) Determining the role of pollen viability during the controlled pollination process; and
v) Determining a reliable method of pollen viability testing for *P. patula*.

CHAPTER 2

LITERATURE REVIEW OF FLOWERING AND CONTROLLED POLLINATION OF *P. PATULA*

2.1 Introduction

Conifers evolved from the progymnosperms in the late Devonian period and were at their most diverse during the Mesozoic Era (OWENS, TAKASO and RUNIONS 1998). Gymnosperms have naked seeds, which mean they are not completely enclosed within another structure, but are attached at the tip of a shoot, or on the surface of a bract or scale. Reproductive buds develop into strobili or cones of two kinds, the microsporangiate (male) and the megasporangiate (female) which produces pollen and seed respectively (OWENS and MOLDER, 1983). Conifers consist of a small group of gymnosperms that dominate north temperate forests and are all wind-pollinated. A number of different mechanisms have evolved to increase pollination success, such as pollination drops secreted by the ovule to aid in scavenging for pollen and pollen with wings or sacci (OWENS, TAKASO and RUNIONS 1998).

Most conifer tree improvement and gene conservation programmes make use of controlled pollination for the development of elite populations (COTTERILL 1984; WILLIAMS and HAMRICK 1996). The purpose of a tree improvement programme is to maximize the genetic gain and the most common way to achieve this is by mating known parents by means of controlled pollination. The making of inter-specific hybrids is also totally reliant on successful controlled pollination (SEDGLEY and GRIFFIN 1989). The success of controlled pollination depends on a detailed knowledge of the breeding systems, timing of floral development and how the environment influences these factors (SEDGLEY and GRIFFIN 1989).

DVORAK (2000) lists the reduction of *P. patula* breeding population generations to less than 10 years as an important challenge in the new millenium. Hybrid forestry involving *P. patula* and the related closed-cone pine species could also play an important role in achieving better genetic gain and should also be investigated further. This will involve the making of controlled crosses on a large scale (DVORAK 2000).

2.2 Flowering of Conifers

The floral buds of gymnosperms and conifers in particular are either female or male with the occasional rare occurrence of abnormal hermaphrodite buds (SEDGLEY and GRIFFIN 1989). In north temperate pines, a long-shoot terminal bud terminates lateral branches. Most of the axillary buds develop as dwarf-shoot buds or pollen-cone buds, but a few of the more distal axillary buds develop as seed-cone buds (HARRISON and SLEE 1992). Generally, vigorous shoots produce many bud scales that either develop into leaves and needles, or differentiate into a seed-cone apex. Less vigorous shoots produce fewer bud-scales and then transform into the pollen cone apex (OWENS and MOLDER 1974, 1976). Most conifers are monoecious, i.e. they bear both male and female structures on the same tree. Each structure is separate from the other and generally occurs on separate branches (OWENS and MOLDER 1974, 1976). Female flowers of *P. patula* are usually borne in the upper crown, and male flowers in the lower crown (WORMALD 1975).

2.2.1 Development of female and male reproductive structures

In the gymnosperms the ovule develops a nucellus which is surrounded by an envelope of integument tissue. In the *Pinus* genus, the integument primordium overgrows the nucellus and forms two arms that will allow access to the male gametes at fertilization. A ring of meristematic tissue develops at the tip of the nucellus leaving a shallow depression which serves as a pollen chamber (SEDGLEY and GRIFFIN 1989). At the tip of the ovule, the two arms of the

integument leave an opening called a micropyle. This allows the pollen grain entry and contact with the inner tissue of the young ovule, termed the megasporangium (OWENS and MOLDER 1974, 1976).

There is considerably more similarity between the gymnosperms and angiosperms in the development of the male reproductive structures. Pollen cones of the *Pinaceae* contain many microsporophylls, each of which bears two microsporangia (pollen sacs) on their lower surfaces (OWENS and MOLDER 1974, 1976). Pollen grains are initiated within the pollen-cone buds before the onset of winter dormancy.

The occurrence of extreme winter conditions serves to synchronize bud development in the temperate pines, while tropical climates are favourable for year-round growth. This leads to asynchronous bud development within and between trees of tropical pines (HARRISON and SLEE 1992). Temperate pine pollen-cones overwinter at the sporogenous tissue stage and meiosis, microsporogenesis, pollen development and pollination occur within a few weeks following winter dormancy (OWENS 1993). According to OWENS (1993) environmental damage to pollen and pollen- cones can be caused if low winter temperatures are experienced, especially to trees grown outside their natural range.

2.2.2 Time and age of flowering

In its native range in the central parts of Mexico, *P. patula* var. *patula* flowers during a four-month period from January to April (WORMALD 1975). Under natural conditions, conelets start developing 12 months after pollination and reach their full size of 55 to 100 mm from September to November. The collection of cones is generally conducted from December to March, approximately 24 months after pollination. Cones occur on branches and on the main stem in clusters of between 2 and 14. Cone crops are generally light and do not usually occur with regularity in natural stands before trees are 10 to 15 years old (DVORAK, HODGE, KIETZKA, MALAN, OSORIO and STANGER 2000).

Flowering of *P. patula* under southern African conditions is restricted to September and October, which coincides with the spring flush and rising temperatures (HAGEDORN 2000). BARNES and MULLIN (1974) reported that male and female flower production in Rhodesia was almost entirely confined to the spring flush in September and early October, with a subsidiary flush of female flowers occurring earlier in the year. *P. patula* starts flowering at a relatively early age in southern Africa. In Zimbabwe female flowers have been found on two-year-old plants. Female flowers are common at three years with male flowers appearing in the fourth year. At eight to ten years annual cone production is usually heavy (BARRETT and MULLIN 1968). Cones reach maturity about 22 months after pollination and are collected from July to September (SEWARD 1980).

2.2.3 Wind pollination (anemophily)

Unlike angiosperms, most of which are insect pollinated (entomophily), all conifers and the majority of gymnosperms are wind pollinated (anemophily) (OWENS, TAKASO and RUNIONS 1998). Anemophilous species rely on a simple system, which is not affected by population size or behavior, and is not reliant on any other vector organism. WHITEHEAD (1983) lists the following general features of the anemophilous species:

(i) they produce a large number of pollen grains with aerodynamic characteristics that facilitate airborne travel;

(ii) the male strobili are designed to maximize the probability of pollen entrainment in moving air. Conifers bear their strobili at or near the branch apices which are generally the more exposed parts of the crown. Pollen is shed when humidity is low and winds are strong enough to blow pollen out of the strobili;

(iii) the female strobili are designed to maximize pollen collection efficiency. There is aerodynamic compatibility between the morphological features of the female conifer strobili and the pollen; and

(iv) the floral phenology is tightly synchronized within a population to ensure a high density of pollen in the air at the time of female receptivity.

CRUDEN (2000) states that the primary relationship among floral traits of wind-pollinated plants is that between pollen number and the distance between putative mates. Environmental factors such as ambient temperature and humidity are important determinants of the time of pollen shed, which is normally diurnal with an afternoon peak (SEDGLEY and GRIFFIN 1989). The amount of conifer pollen-shed can vary greatly from day to day with the maximum shed occurring under warm, windy low-humidity conditions. Below-average temperatures retard, and above average temperatures advance the rate of development of most species (BOYER 1981).

2.2.4 Pollination mechanism and fertilization

The process of pollen capture and entrance of pollen or pollen tubes into the ovules of conifers is described by the term pollination mechanism. A detailed understanding of the pollination mechanism is critically important in determining the optimal time for pollination and in carrying out controlled pollination (OWENS 1993). The morphology of pollen and female strobili orientation plays an important part in the pollination mechanism of conifers (OWENS, TAKASO and RUNIONS 1998). The interaction between male and female strobili and the wind creates an aerodynamic environment in which pollen grains can contact and adhere to the ovule.

CRUDEN (2000) uses the pollen-ovule ratios (P/O) of plants to examine their breeding systems and pollination mechanism. The pollen-ovule ratios of wind–pollinated plants are substantially higher than those of animal-pollinated plants. Traditionally, anemophily has been viewed as an inefficient process because pollen to ovule ratios are in access of 10^6:1. This could be related to the fact that large numbers of pollen grains are lost in the turbulent air where random or chaotic-like patterns are associated with particle motion (ACKERMAN 2000). Anemophilous pollen grains are small in diameter relative to biotically pollinated

plants (20-60 µm versus ≤200 µm), their small size reducing the settling velocity and increasing the dispersal distance of pollen (ACKERMAN 2000). Conifer pollen varies in diameter from 20 µm to more than 100 µm and has a low moisture content of between 5-10%. Pollen of conifer species is generally larger than pollen from most angiosperm species, but it is light for its size and can be carried over distances of 300-1300 km (OWENS, TAKASO and RUNIONS 1998). In conifers, pollen mass is also reduced due to the presence of air-filled sacci or bladders, which reduces the density of the grain (OWENS and SIMPSON 1986; ACKERMAN 2000).

Factors such as pollen grain-size, gravity, wind speed and direction, and turbulence influence dispersal and the carrying distance of air-borne pollen grains (DI-GIOVANNI and KEVAN 1991). GEARY (1970) found that pollen *of P. patula* and *P. kesiya* dispersed predominantly in the direction of the prevailing wind, and isolation distances of several miles around a seed orchard probably would not be sufficient to prevent contamination. Locating orchards on the windward side of afforested areas would appear to reduce contamination from outside pollen to negligible levels (GEARY 1970).

Modern conifers have at least six different pollination mechanisms in two categories, those that include exudation of a pollination drop around the ovule and those that rely on some other pollen entrapment mechanism (OWENS 1993). The *Pinus* species rely on pollination drops, they have saccate pollen and inverted ovules at the time of pollination (OWENS, TAKASO and RUNIONS 1998). The cells at the tips of the integument produce small amounts of extra-cellular secretion before pollination and pollen adheres to these surfaces (SEDGLEY and GRIFFIN 1989).

In a number of gymnosperms which include *Pinus* species, a further much larger secretion is produced by the micropyle of the ovule, referred to as the pollination drop (OWENS, SIMPSON and MOLDER 1980 and 1981b; OWENS, SIMPSON and CARON 1987; TOMLINSON 1994). Pollen moves into the ovule by means of the pollination drop (RUNIONS and OWENS 1996). RUNIONS, RENSING,

TAKASO and OWENS (1999) state that there is a general misinterpretation of the role of saccate or winged pollen grains. The sacci of some of the *Pinaceae* species do not only aid in wind dispersal and pollen orientation on the nucellus, but the primary role of sacci is to function as floatation devices. Conifers with floating, saccate pollen have anatropous ovules that stand erect in ovulate cones during the pollination period. A pollination drop is secreted by the ovule from the micropyle that captures wind-blown pollen. Pollen adheres to surfaces near the micropyle and float upward into the ovule when it is exposed to the pollination drop (RUNIONS, RENSING, TAKASO and OWENS 1999). When this droplet recedes, the pollen grains are transported to the surface of the nucellus, where in the case of *Pinus,* germination occurs within several days (BROWN and BRIDGWATER 1987).

Pollen grains of most gymnosperms are deposited on the tip of the nucellus following the withdrawal of the pollination drop. In most species the pollen takes up moisture from the absorbing fluid present in the female tissue and germinates at the tip of the nucellus (SEDGLEY and GRIFFIN 1989). In conifers, pollen germination is normally defined by the growth of a pollen tube on the apex of the nucellus. With some gymnosperms, such as *Larix* and *Pseodotsuga*, no pollen tube is formed and germination takes place through the elongation of the pollen grain (CHRISTIANSEN 1972). The process of pollen germination and tube growth is commonly much slower in gymnosperms, taking between two weeks in *Picea engelmanii* and two months as in the case of *Pinus contorta* (OWENS, SIMPSON and MOLDER 1981a; SEDGLEY and GRIFFIN 1989).

The pace of pollen germination is determined by the slow development of the megagametphyte. Pollination occurs around the time of meiosis in the megaspore mother cell and the pollen tube must wait for the development of the multicellular megagametophyte before fertilization can take place (SEDGLEY and GRIFFIN 1989). In many species, the presence of pollen is an essential prerequisite for this development. In the case of *Pinus*, auxins produced by the pollen tube have been suggested as the trigger for megagametophyte development (SWEET and LEWIS 1969).

In *P. contorta*, the female gametophyte over-winters at the free nuclear stage and cell-wall formation occurs about 11 months after pollination. Fertilization takes place 12 months after pollination. Female gametophytes within the ovule abort before the onset of winter dormancy in all pines if the ovule is unpollinated (OWENS, SIMPSON and MOLDER 1982). The time interval from pollination to fertilization in *Pinus* ranges from 11.5 to 15 months (LILL 1976). The time-span between fertilization to the mature embryo can be from 2 months in the case of *Pinus contorta* and 20 months for *Pinus radiata* (OWENS, SIMPSON and MOLDER 1982; WANG, SMITH, OUTRED, ROWLAND and FOUNTAIN 2000).

The capacity of the micropyle limits the amount of pollen which can reach the nucellus and germinate. In the case of *Pinus sylvestris*, two to three grains and in the case of *P. radiata*, seven pollen grains are the maximum capacity of the micropyle (SEDGLEY and GRIFFIN 1989). Pollen tubes commonly form branches, but the main tube generally grows in the direction of the archegonia (OWENS 1993). Two sperm cells are produced at the tip of the pollen tube in most conifer species (OWENS and MOLDER 1983; OWENS, MORRIS and CATALANO 1994).

SEDGLEY and GRIFFIN (1989) define fertilization as the fusion of the female and male gametes. With gymnosperms, a single fertilization event takes place with one of the male gametes contained in the tip of the pollen tube fusing with the nucleus of the megaspore or egg cell (OWENS 1993). This results in the doubling of chromosomes, which restores their number to that characteristic of the specific species (OWENS and MOLDER 1983). In species where cone development takes longer than 12 months, fertilization only occurs in the year following pollination. Soon after fertilization, rapid division of the megaspore nucleus takes place followed by the rapid development of the seed-bearing cone (OWENS and MOLDER 1983).

2.3 Manipulation of Flowering

Flowering-promoting treatments that are applied to trees in seed orchards are based on the understanding of endogenous and exogenous factors influencing flowering (SWEET 1975). These factors include siting of orchard, grafting, stem and crown treatment, and the application of growth regulators. These treatments are over and above regular management treatments such as the application of fertilizer and irrigation during critical stages of shoot and bud development.

SWEET (1975) reports that siting of an orchard is the single most important factor contributing to flowering. Certain external conditions linked to the site may cause potential flowers in conifers not to develop florally, or that they may cease growth and abort or revert to the vegetative condition. Vegetative buds can also fail to develop vegetatively and be switched to develop florally due to changes in the environment or by the application of plant growth regulators (SWEET 1975; SCHMIDTLING and GREENWOOD 1993).

HAGEDORN (2001) found that altitude had a marked effect on the amount of female and male strobili of *P. patula* production and the seed potential of cones under South African conditions. Correct siting of orchards at altitudes between 1400 m and 1550 m ensured that female strobili production were at their optimum with adequate pollen production. WORMALD (1975) also report high female strobilii abortion due to poor synchronisation of male and female flowers at low altitudes between 900 m to 1500 m in South Africa. This effect of altitude on flower phenology of *P. patula* in southern Africa is also well documented by BARNES and MULLIN (1974).

It is important to ensure that male flowering is sufficient as increasing the amount of pollen produced in an orchard decreases contamination and selfing, and increases seed set and parental contribution (WHEELER, ADAMS and HAMRICK 1993; SCHMIDTLING and GREENWOOD 1993). It has also been reported that the

genetic composition of progeny depends heavily on the number of female and male strobili produced by each clone contained in an orchard (KANG and LINDGREN 1998).

Various root-pruning, stem-girdling and top-grafting treatments have been used to speed up flowering or to increase the amount of female and male flowering (SWEET 1975). Stem-girdling, the application of stem-injected growth regulator treatments and a combination of both treatments have yielded earlier and increased flowering in species like *Pinus taeda* and *Pinus radiata* (WHEELER and BRAMLETT 1991; DICKSON, RIDING and SWEET 1999). The most commonly used growth regulators are gibberellins ($GA_{4/7}$) and have been tested on a wide range of different *Pinus* species (BONNET-MASIMBERT 1987; PHARIS, WEBBER and ROSS 1987). Naphthalene acetic acid (NAA) has also been used, sometimes in combination with $GA_{4/7}$ (DICKSON, RIDING and SWEET 1999).

The grafting of scion onto root-stock can also influence the flower phenology of the grafted material and can speed up flowering. DICKSON, RIDING and SWEET (1999) found that the age and size of ramets onto which scion material was grafted affected the timing and differentiation of primordia. Larger ramets displayed earlier intiation and therefor flowering in *P. radiata*. Topworking involves the grafting of scion-material into the crown of reproductively-mature trees displaying heavy flowering. This is done in anticipation that this sexual competence would be transferred to the immature scion (GREENWOOD and SCHMIDTLING 1981). This technique has been used successfully *on Pinus taeda* for accelerated female and male flower production and to shorten the breeding cycle (BRAMLETT, WILLIAMS and BURRIS 1995; BRAMLETT and BURRIS 1995; BRAMLETT 1997). KNOETZE and DE JAGER (2000) also report success with this technique for *P. patula* in South Africa.

2.4 Pollen Management

Pollen Management involves the collection, processing and storage of pollen for later use in controlled or supplemental pollination programmes. Successful extraction, storage and evaluation of pollen are major requirements for genetic improvement programmes of plant crops (JETT and FRAMPTON 1990). In general, wind-pollinated species produce large amounts of pollen and present fewer difficulties than insect-pollinated hermaphrodite flowers (SEDGLEY and GRIFFIN 1989). The methods employed to collect, process and store pollen has a direct effect on pollen-quality, therefor successful methods of testing pollen is an integral part of pollen management (JETT, BRAMLETT, WEBBER and ERIKSSON 1993).

2.4.1 Pollen collection

The ideal time for pine pollen-collection is when the first pollen is released from the basal scales of the most advanced male cones or catkins (BEERS, BIVENS AND MOCHA 1981). There is, however, a danger of missing the opportunity of collection if the tree cannot be continually watched as pollen is often shed during a relatively short period of one to two days. Weather conditions can also hasten or prolong the shedding of pollen; therefor pollen is usually collected slightly earlier (SEDGLEY and GRIFFIN 1989). The best time to collect pine pollen is when male flowers turn yellow and produce very little liquid when squeezed between the fingers (BEERS, BIVENS AND MOCHA 1981; JETT, BRAMLETT, WEBBER and ERIKSSON 1993).

BRAMLETT and BRIDGWATER (1989) developed a six-stage pollen classification system for loblolly pine, which simplifies collection of pollen at the correct developmental stage. Collection of pollen for controlled pollination would take place at stage 3.6 to 3.9 when using this system, when very little fluid can be squeezed from the cone and microsporophylls start separating. Enclosing catkin-

bearing branch tips with sausage-casing bags can also speed up pollen maturation (BEERS, BIVENS AND MOCHA 1981). Catkin-bearing branches can also be harvested from trees and forced to mature under laboratory conditions (SEDGLEY and GRIFFIN 1989).

2.4.2 Processing of pollen

The aim of pollen processing is to extract pollen grains from the male flowers and process it so that it is ready for storage. Pollen should be extracted as quickly as possible after collection (MATTHEWS 1998). The simplest method of pollen extraction consists of drying clusters of male flowers in brown paper-bags in a forced air dryer at temperatures of 32° to 38° C and relative humidity below 50 percent (JETT, BRAMLETT, WEBBER and ERIKSSON 1993). Once pollen is released from the male flowers, it is filtered through a 100 µm mesh-screen to separate plant parts and insects. SPRAGUE and SNYDER (1981) obtained high viability pollen of *P. taeda* and *P. elliottii* with drying temperatures of 21° to 27° C and relative humidity between 20 and 40 percent. Extraction temperatures ranging between 20° and 30° C were tested and were found to have little impact on *P. radiata* pollen-germination of freshly extracted pollen (SIREGAR and SWEET 2000). Long-term storage, however, did require more specific extraction temperatures and in this study 25° C showed the best results. Supplemental drying in a desiccator over silica gel to moisture content-levels below 10 percent is also recommended for long-term storage of pine pollen (SPRAGUE and SNYDER 1981).

Freeze-drying of pollen of *P. monticola* have also proved to be effective for the removal of free water in the pollen whilst maintaining good viability (CHING and CHING 1964).

2.4.3 Storage of pollen

The efficient storage of pollen is a very important aspect of plant breeding as it provides flexibility to the plant breeder. Different species or genotypes often do not flower synchronously and would limit the range of crosses that can be attempted with fresh pollen (SEDGLEY and GRIFFIN 1989). Successful storage ensures against poor flowering years or accidental loss and fulfills a gene conservation function for threatened species (SEDGLEY and GRIFFIN 1989). BRAMLETT and MATTHEWS (1991) demonstrated that with *P. taeda* good pollen-viability could be retained for periods of up to ten years under appropriate storage conditions.

Three different methods are commonly used for pine pollen-storage, desiccator storage, freezer storage and vacuum drying and storage (MATTHEWS and KRAUS 1981). The latter two methods have gained in popularity as it was shown to maintain high levels of pollen viability and vigor for periods longer than one year (BRAMLETT and MATTHEWS 1991). Critical factors during storage are moisture content of pollen before storage, minimum fluctuation of humidity during storage, and storage-temperature (BRAMLETT and MATTHEWS 1991; SIREGAR and SWEET 2000). Longevity of stored pollen increases with decreasing moisture content as it lowers the metabolic activity of pollen and limits fungal and bacterial contamination (MATTHEWS and KRAUS 1981). The determination of pollen viability is an important aspect of pollen storage and should be routinely undertaken (JETT, BRAMLETT, WEBBER and ERIKSSON 1993). These and other methods of pollen storage of conifer species are summarized in Table 2.

Table 2. Pollen storage conditions for some conifer species.

Species	Storage conditions and pollen preparation	Storage period	Reference
Pinus monticola	Freeze-drying to –78 °C followed by vacuum drying	3 months	CHING and CHING (1964)
Pinus spp.	Desiccator storage at 4° C and moisture content 9 %	3 years	MATTHEWS and KRAUS (1981)
Pinus spp.	Freezer storage at –15° C and moisture content <6 %	>3 years	MATTHEWS and KRAUS (1981)
Pinus spp.	Vacuum drying and freezer storage at –15° C and moisture content <6 %	>3 years	MATTHEWS and KRAUS (1981)
Pinus radiata	Desiccator storage at 4° C and moisture content 7-10 %	1 year	SIREGAR and SWEET (2000)
Pinus taeda	Vacuum-sealed ampules stored at 3° C and moisture content <10 %	3 years	BRAMLETT and MATTHEWS (1991)
Pinus taeda	Freezer storage at –20° C and moisture content <10 %	10 years	BRAMLETT and MATTHEWS (1991)
Abies spp.	Cryogenic storage at –196° C or Freezer storage at -26° C and moisture content of 2-6 %	3 years	COPES (1987) WEBBER (1987)

2.4.4 Pollen viability and germination

The determination of pollen viability is an important aspect of any plant-breeding programme and should be routinely undertaken during processing and storage to check procedures (JETT, BRAMLETT, WEBBER and ERIKSSON 1993). Testing should also be undertaken before it is used in controlled pollination if seed production is to be successful (GODDARD and MATTHEWS 1981).

There are essentially five different methods of pollen viability testing for tree crops listed by SEDGLEY and GRIFFIN (1989). They appear in order of increasing reliability and required time to assay:

- Staining techniques involves treating pollen with chemicals that are reduced to coloured or fluorescent products by active enzymes;
- Measures of respiration or chemical conductivity of pollen leachates which estimates the rate of oxygen consumption of pollen in an aqueous medium, or from electrical conductivity of leachates;
- *In vitro* germination tests assesses pollen-germination in liquid medium or on a semi-solid agar medium;
- *In vivo* tests of germination and pollen tube growth involves microscopical examination of pollen germination by means of staining and fluorescence microscopy; and
- Capacity of applied pollen to effect seed set during the full development process.

The pollen viability-testing standard for the forestry industry is set out in GODDARD and MATTHEWS (1981) and is an *in vitro* germination test on a distilled water medium containing 0.5 percent agar. MOODY and JETT (1990) showed that *in vitro* germination using this standard test and pollen respiration of *P. taeda* were good predictors of pollen germination *in vivo*, while ultraviolet absorption and electrical conductivity tests were poorly correlated. KAPOOR and DOBRIYAL (1980) also obtained good pollen germination using this standard *in vitro* medium with sugar and boric acid additions with *P. patula* pollen. Table 3 summarizes pollen viability methods used for various species, with the emphasis on conifers.

Table 3. Pollen viability testing methods for some species.

Method	Species	Viability test protocol (% of w/w)	Results	Reference
In vitro germination	*Pinus banksiana*	Agar, 5 % sucrose and water	Good	CARON and POWELL (1995)
In vitro germination	*Pinus spp.*	0.5 % agar, with and without sucrose, and water	Good	GODDARD and MATTHEWS (1981)
In vitro germination	*Pinus elliottii* *Pinus nigra*	Hanging drop method In-vitro agar	Good	McWILLIAM (1959)
In vitro germination	*Pinus patula*	2 % Agar and 10-15 % sucrose and water	Good	KAPOOR and BOBRIYAL (1980)
In vitro germination	*Pinus patula*	0.5 % Agar and water	Good	HAGEDORN *et al.* (1997)
In vitro germination	*Pinus radiata*	1% Agar, 2 % sucrose and water	Good	SIREGAR and SWEET (2000)
In vitro germination	*Pinus taeda*	0.5 % Agar and water	Good	BRAMLETT and MATTHEWS (1991)
In vitro germination	*Pinus taeda*	0.5 % Agar and water	Good	JETT and FRAMPTON (1990)

Method	Species	Viability test protocol (% of w/w)	Results	Reference
In vitro germination	*Pinus taeda*	0.5 % Agar and water	Good	MOODY and JETT (1990)
In vitro germination	*Pinus caribaea*	3% Agar and water with 10% sucrose	Good	DOYLE (2001)
In vitro germination	*Pinus monticola*	0.5 % Agar and water with 8.5 % sucrose and 2.4 x 10^{-5} M boric acid	Good	CHING and CHING (1964)
In vitro germination	Various non-pine conifers	0.5 % Agar and water with 10 % sucrose and 2.5 x 10^{-5} M boric acid	Good	CHING and CHING (1976)
Leachate analysis	*Pinus taeda*	Correlated leachate analysis with agar germination	Poor correlation	MOODY (1988)
Respiration and conductivity	*Pinus clausa*	Electrical conductivity of pollen correlated to pollen germination	Highly variable	FRAMPTON *et al.* (1982)
Respiration	*Pinus taeda*	Correlated respiration with agar germination	Good correlation	MOODY and JETT (1990)
Staining	*Pinus clausa*	Liquid medium	Good	FRAMPTON *et al.* (1982)
Staining	*Pinus spp.*	Staining with Tetrazolium (TTC) and others	Highly variable	GODDARD and MATTHEWS (1981)

Method	Species	Viability test protocol (% of w/w)	Results	Reference
Staining	*Pinus and Lycopodium*	Malachite green and Orange G stains	Good	ALEXANDER (1969)
Staining	Various species	Staining with four different methods	Variable	RODRIGUEZ-RIANO and DAFNI (2000)
Staining	Cereals	Cotton Blue	Variable	D'SOUZA (1972)
Staining	Various Plant species	Staining with Tetrazolium (TTC) and others	Highly variable	DAFNI (1992)
Staining	*Pinus* species	Tetrazolium Chloride	Variable	COOK and STANLEY (1960)
Staining	*Pinus taeda*	Correlated nuclei staining with agar germination	Poor correlation	MOODY (1988)

2.5 Controlled Pollination Techniques

For genetic experiments and breeding strategies it is necessary to master techniques for manipulation of the pollination process in order to obtain progeny of known parentage (SEDGLEY and GRIFFIN 1989). Success depends on a detailed knowledge of the breeding system, floral development, pollination mechanism and environmental factors that may affect these factors. The most significant deviation from natural pollination is the isolation of female flowers to prevent contamination from non-target pollen. The nature of the isolation material will depend on the pollen-carrying vector, as it must form a barrier to pollen. Isolation bags made of plastic, paper and cloth have been evaluated for use in controlled pollination of pines (BRAMLETT and O'GWYNN 1981).

Results of controlled pollination experiments for pine species are often not published, or results are not adequately discussed in the literature (BESTER, VAN DER MERWE and MALEMA 2000). Results from trials where different types of isolation material was used in pine and fir species is reported in McWILLIAM (1959), MILLER (1983), HAGEDORN, RAUBENHEIMER and NEL (1997) and HAGEDORN (2000). Successful isolation material must in addition to keeping unwanted pollen out, also allow for movement of gases to prevent the build-up of heat, moisture and carbon dioxide (SEDGLEY and GRIFFIN 1989). McWILLIAM (1959) found in a study of conditions inside the isolation bag that the temperature of conelets of *P. elliottii* and *P. nigra* enclosed in sausage casing bags could increase drastically. Temperatures reached in excess of 51 °C inside unshaded bags while the outside temperature was 34.5 °C. Temperatures of 46 °C were lethal to germinating pollen.

Due to the high cost of bagging and the universally lower success rate with bagged controlled pollination, some attempts have been made at controlled pollination without isolation (SEDGLEY and GRIFFIN 1989; SLEE and ABBOTT 1990). The concept of "first come, first served" has been studied by SLEE and ABBOTT (1990) and has indicated that the pollen that enters the female scales first is most likely to effect fertilization. Either this can be due to the competitive position on the

nucellus or due to space limitations inside the ovule, that may deny entry to late-arriving pollen. The absence of a transport mechanism after pollen had entered the ovule may also prevent late arriving pollen from being effective (McWILLIAM 1958).

2.6 Cone Analysis and Seed Yields

The successful completion of pollinations on receptive flowers does not guarantee the success of good seed yields. Cones have to develop for up to 22 months in the case of *P. patula,* to reach maturity without damage or abortion, and seed yield and viability can only be determined when cones are mature (BRAMLETT and O'GWYNN 1981). Ovules of *Pinus* species must be pollinated before the megagametophyte will develop fully. A certain proportion of ovules (20 %) must also be pollinated before the cone will develop (OWENS, COLANGELI and MORRIS 1990).

In controlled pollinations, low quality pollen, poor timing and poor bag installation can cause cone or seed losses (BRAMLETT 1993). The activity of pollen vectors and of seed predators and parasites may also induce losses in seed production (HOULE and FILION 1993). OWENS, SIMPSON and MOLDER (1981a) also reported that not all ovuliferous scales are fertile in *Pinus* species, and in the case of *P. contorta* only 25 % of the scales bear fertile ovules. OWENS and MOLDER (1977) found that abortion of ovules was very low in naturally pollinated *P. monticola.*

Cone and seed analysis provides information to enable the breeder to evaluate seed production from seed orchards and controlled pollinations (BRAMLETT, BELCHER, DEBARR, HERTEL, KARRFALT, LANTZ, MILLER, WARE and YATES 1977). These authors and BRAMLETT (1993) list diagnostic observations, probable causes and procedures that may explain losses of cones, ovules and seeds from natural and controlled pollinations.

CHAPTER 3

POLLEN MORPHOLOGY AND VIABILITY TESTING

3.1 Introduction

Pollen management plays a crucial role in any plant-breeding programme where use is made of controlled pollination or supplemental pollination. Aspects of pollen management include collection, processing, storage and application in crossing programmes (CARON and POWELL 1995). These management processes may influence pollen viability and therefore the success of controlled pollination (JETT, BRAMLETT, WEBBER and ERIKSSON 1993). Controlled pollination of conifers, especially *Pinus* species, is a lengthy and costly process and therefore pollen quality is of the utmost importance. The evaluation of pollen viability and studying aspects of pollen morphology provide the tools to enable the plantbreeder to assess the effect of management processes on pollen quality (OWENS and SIMPSON 1986).

The first step in pollen-quality assessment is a study of the shape and size of pollen of the different species used in controlled pollination. This can provide a tool for identifying pollen of different species and understanding the pollination mechanism for pure species as well as inter-specific hybrids between species. SLEE and ABBOTT (1990) report that aerodynamic studies have suggested that pollen from one *Pinus* species may not readily enter cones of another species thereby hampering controlled pollination. In the tree-improvement programme of *P. patula*, inter-specific hybridization of *P. patula* with other important *Pinus* species such as *P. tecunumanii*, *P. radiata*, *P. greggii*, and *P. oocarpa* play a crucial role. DOYLE (2001) reported a

highly significant association between *P. caribaea* pollen morphology and viability and suggested that pollen morphology be used as a screening step before controlled pollinations are attempted.

An accurate and relatively quick method of testing pollen viability is needed to verify the quality of pollen before it is used in controlled pollination. Various methods of determining pollen viability are described in the literature, but the industry standard for *Pinus* species pollen is *in vitro* germination as described by GODDARD and MATTHEWS (1981). This procedure provides viability results within 48 to 72 h, requires only basic laboratory equipment, is relatively uncomplicated and can be performed by semi-skilled staff.

3.2 Materials and Methods

Pollen-bearing male cones were collected at developmental stage 3.9 as described by BRAMLETT and BRIDGWATER (1989). At stage 3.9, very little fluid is present in the cone, cones can be bent easily and spaces are visible between pollen-bearing sporophylls. Male cones were collected from several sites in South Africa. *P. patula* was collected from a clonal seed orchard near Howick in the Natal midlands. *P. greggii* var. *australis* was collected from a progeny trial near Helvetia in Mpumalanga. *P. tecunumanii* was collected from progeny trials near Melmoth, Kwazulu-Natal *and P. elliottii*, *P. caribaea* and *P. oocarpa* were collected from seed orchards and progeny trials near Matubatuba in KwaZulu-Natal. *P. radiata* pollen was obtained from Bioforest, a forestry company in Chile. *P. elliottii* and *P. caribaea* pollen were also included as both these species are used in inter-specific hybridization and would provide additional controls for comparison. Table 4 summarizes the information of the different collection sites.

Table 4. Summary of information of the different pollen collection sites in South Africa used in the pollen morphology and viability testing experiments.

Site	Species	Altitude (m)	MAT (°C)	MAP (mm)
Howick	*Pinus patula*	1100	16.7	998
Helvetia	*Pinus greggii*	1700	13.9	912
Melmoth	*Pinus tecunumanii*	880	18.0	798
Matubatuba	*Pinus caribaea*	60	21.7	1044
Matubatuba	*Pinus elliottii*	60	21.7	1044
Matubatuba	*Pinus oocarpa*	60	21.8	960

All pollen lots were collected and processed during the period from 1997 to 2000 using the procedures as set out by BEERS, BIVENS and MOCHA (1981) and SPRAGUE and SNYDER (1981). Male cones were harvested and dried in paper bags for 48 h in a growth chamber at 30 °C. The dried catkins were then placed over a 100 µm sieve to separate the pollen and catkin residue. Sieved pollen was placed on silica gel crystals in sealed containers to reduce the relative humidity to below 10 %. Pollen was stored after processing at 4 °C with relative humidity below 10 % in sealed 100 ml plastic bottles placed on silica-gel crystals in a larger sealed plastic container.

3.2.1. Pollen morphology and size

Pollen of five different *P. patula* clones was used as well as pollen of six other species; *P. tecunumanii, P. elliottii, P. caribaea, P. greggii, P. radiata* and *P. oocarpa*. All pollen-lots were taken from cold-storage (4 °C), pollen was re-hydrated and kept at room-temperature (25 °C) for 24 h before the study was conducted. A Phillips XL 30 Environmental Scanning Electron Microscope (ESEM) was used for the morphological study. Pollen was dusted onto microscope-mounts covered with double-sided black carbon-tape and was scanned using a low vacuum mode at 10

kV with a Large Field Detector. Magnification levels of 500 X, 800 X, 1000 X and 2500 X were used to scan pollen grains and images were captured for analysis.

For size determination, the diameters of intact pollen grains were measured across the distal region of the grain between the two air bladders on scanned slides (Figure 3). For the actual diameter determination, images at 500 X magnification were used. Five replicates of six grains each were assessed giving a total of 30 grains per clone or species.

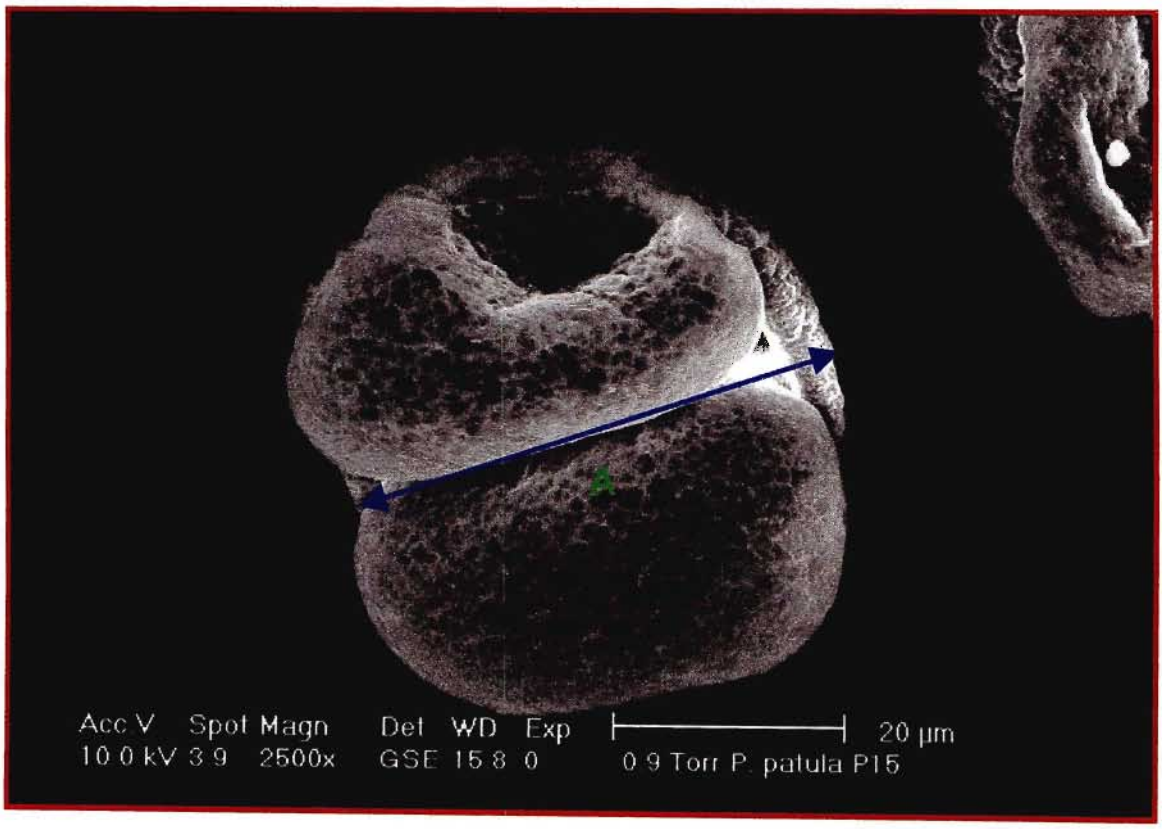


Figure 3. ESEM image of a *P. patula* pollen grain at 2500 X magnification. The line (A) represents the pollen grain diameter assessed across the distal region of the grain between the two air bladders.

3.2.2. Effect of germination media on pollen germination

The industry standard for pollen viability testing of *Pinus taeda* is *in vitro* testing on a distilled water and 0.5 % Agar solidified medium (GODDARD and MATTHEWS 1981). KAPOOR and BOBRIYAL (1980) reported successful germination of *Pinus patula* pollen on distilled water and 2 % Agar-containing medium with the addition of 12.5 % sucrose and 0.01 % boric acid. Germination-media treatments of distilled water and 1 % Agar-Agar were prepared containing different levels of sucrose and boric acid to test their effectiveness on a number of *P. patula* clones as well as some other species. Pollen of *P. patula, P. caribaea and P. greggii* were tested in Trial A. Five different *P. patula* clones (P11, P15, P29, P32 and P37) were tested in Trial B. The components of the different media treatments for the two trials are set out in Table 5.

Table 5. Components of different media treatments for pollen viability testing of *P. patula, P. caribaea and P. greggii* (Trial A) and five different *P. patula* clones (Trial B).

	Trial A		Trial B	
Treatment	Sucrose	Boric Acid	Sucrose	Boric Acid
1	0.0%	0.000%	0.0%	0.000%
2	10.0%	0.000%	5.0%	0.000%
3	12.5%	0.000%	10.0%	0.000%
4	15.0%	0.000%	15.0%	0.000%
5	10.0%	0.010%	5.0%	0.010%
6	12.5%	0.010%	10.0%	0.010%
7	15.0%	0.010%	15.0%	0.010%
8	10.0%	0.001%	5.0%	0.001%
9	12.5%	0.001%	10.0%	0.001%
10	15.0%	0.001%	15.0%	0.001%
11	0.0%	0.010%	0.0%	0.010%
12	0.0%	0.001%	0.0%	0.001%

The pH of all media treatments was adjusted to 6.0 prior to autoclaving. Medium was sterilized at 120 °C for 20 min in an autoclave. Approximately 15 ml of medium was poured into 65 mm plastic petri dishes (Labotec) under sterile conditions on a laminar flow bench. Pollen was taken from cold storage and was re-hydrated for two hours by placing 20-ml, open glass vials containing approximately 2 ml pollen on water-saturated filter paper, in a sealed plastic container. Re-hydrated pollen from the different pollen lots was dusted onto the medium with a fine paintbrush. Dishes were sealed with plastic cling wrap (Glad® Wrap) before incubation. Cultures were kept in the dark at 30 °C for 72 h, and were assessed for viability by viewing under a light microscope (Zeiss 47 3011-9901) at 100 X magnification.

3.2.3. Effect of pollen re-hydration on viability testing

JETT and FRAMPTON (1990) highlighted the importance of re-hydration of stored *P. taeda* pollen for at least 2 hours before *in vitro* germination. Stored pollen from two *P. patula* clones (P32 and P37) and two *P. caribaea* clones (C4 and C12) were used in the re-hydration study. These pollen lots were re-hydrated for different periods ranging from 1 to 8 h in hourly increments with a control of no re-hydration. Pollen was re-hydrated by placing approximately 2 ml stored pollen into 20-ml, open glass vials placed on water-saturated filter paper, in a sealed plastic container. After re-hydration, pollen was dusted onto medium consisting of distilled water and 0.01 % boric acid solidified with 1% Agar-Agar. The pH of the germination medium was adjusted to 6.0 prior to autoclaving.

Medium was sterilized at 120 °C for 20 min in an autoclave. Approximately 15 ml of medium was poured into 65 mm plastic petri dishes (Labotec) under sterile conditions on a laminar flow bench. Dishes were sealed with plastic cling wrap (Glad® Wrap) before incubation. Cultures were kept in the dark at 30 °C for 72 h, and were assessed for viability by viewing under a light microscope (Zeiss 47 3011-9901) at 100 X magnification.

3.2.4. Effect of temperature on dry stored pollen and germinating pollen

The effect of temperature was tested directly on low-humidity stored pollen and on pollen germinating on solid viability medium by exposure to different incubator temperatures. With the low-humidity stored pollen treatments, approximately 2 ml stored pollen was placed into 20-ml, open glass vials and was then treated at temperatures ranging from 30 to 90 °C in 10 °C increments for 2 h. Stored pollen with no temperature treatment was retained as a control. The pollen lots used in this study are outlined in Table 6. The treated pollen was then re-hydrated by placing the pollen in 20-ml glass vials on water-saturated filter paper, in a sealed plastic container.

Table 6. Pollen lots included in the low-humidity stored pollen temperature-treatment trial.

Species	Clone No
Pinus patula	P32, P37
Pinus caribaea	C4, C12
Pinus greggii	G46, G88

After re-hydration, pollen was dusted onto distilled water and 1% Agar solidified medium containing 0.01% boric acid. The pH of the germination medium was adjusted to 6.0 prior to autoclaving. Medium was sterilized at 120 °C for 20 min in an autoclave. Approximately 15 ml of medium was poured into 65 mm plastic petri dishes (Labotec) under sterile conditions in a laminar flow bench. Dishes were sealed with plastic cling wrap (Glad® Wrap) before incubation. Cultures were kept in the dark at 30 °C for 72 h, and were assessed for viability by viewing under a light microscope (Zeiss 47 3011-9901) at 100 X magnification.

With the second trial, pollen germinating on solid viability medium was exposed to different incubator temperatures. Pollen of four *P. patula* clones (P28, P35, P18 and P1098) and *P. elliottii, P. caribaea, P. greggii* and *P. tecunumanii* were used in this study. Pollen was re-hydrated as described previously. After re-hydration, pollen was dusted onto germination medium. Cultures were incubated at temperatures ranging from 30 to 46 °C in 2 °C increments and were kept in the dark at 30 °C for 72 h. Pollen was assessed for viability by viewing under a light microscope (Zeiss 47 3011-9901) at 100 X magnification. Pollen tube-lengths of the cultures incubated at 30 °C were also assessed to determine differences in growth rate between species and between several *P. patula* clones.

3.2.5. Data collection

For pollen-size determination, 500 X magnification ESEM images were used and pollen diameters were measured and expressed in µm. Five replicates of six grains each were assessed giving a total of 30 grains per clone or species. 2500 X magnification slides were used for visual morphology comparisons between different species.

Pollen viability was determined by assessing germination percentage. The number of germinated pollen grains were assessed out of 50 counted grains and was repeated over six random microscope fields per pollen lot. A pollen grain was counted as germinated if the pollen tube length was longer than the pollen grain diameter (GODDARD and MATTHEWS 1981).

Pollen tube-lengths were assessed using 50 X magnification microscope images captured with a Wild M5A Heerbrugg light microscope and JVC 3CCD KY-F55B Video Camera.

3.2.6. Data analysis

The data collected was analyzed using the GENSTAT 5 Release 3.2 statistical package. Percentage data was transformed using the Arcsine transformation procedure (ZAR 1984) prior to analysis of variance (ANOVA). ANOVA procedures were completed and treatment means, standard errors and least significant differences (LSD's) were calculated to determine statistically significant differences between treatments. Significant differences of percentage data were determined using transformed values and are indicated in tables with different letters. Actual (non-transformed) percentage values are, however, presented in all tables and figures.

3.3 Results

3.3.1. Pollen morphology and size

Scanned 2500 X magnification ESEM slides were used to describe the morphological structure of pollen of *P. patula* and other pine species included in this study. TOMLINSON (1994) described the structure of pollen of *Pinaceae* species as featured in Figure 4. Pollen grains are relatively large (42 to 50 µm), they have two strongly polarized wings (also referred to as sacci or bladders), with proximal and distal regions. The wings have a porous texture, are hemispheric, and are situated in the distal region on either side of the germ furrow. The cap of the pollen grain is situated in the proximal region and has a sculptured texture (DOYLE 2001). *P. patula* pollen grains exhibited the typical conifer morphology consisting of porous wings and sculptured cap (Figure 5).

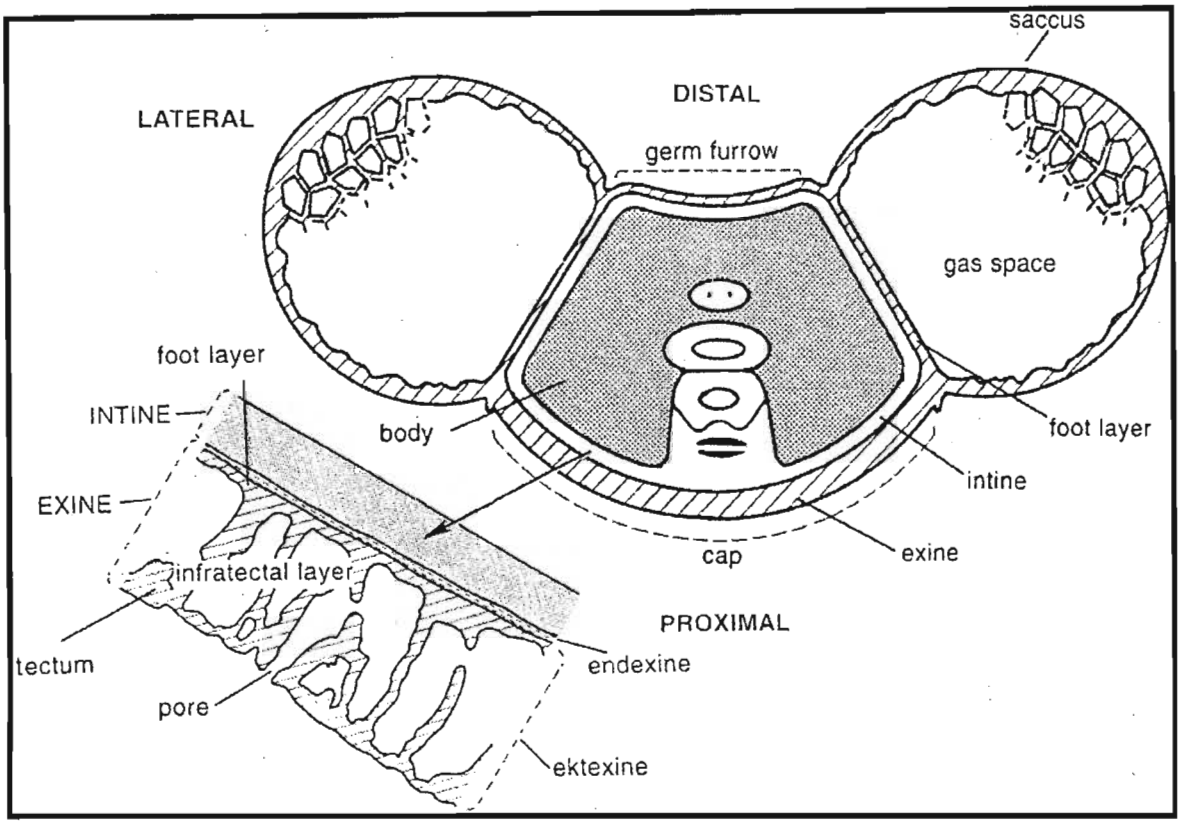


Figure 4. Diagramme showing the features of a mature saccate pollen grain found in *Pinaceae* species as reported by TOMLINSON (1994).

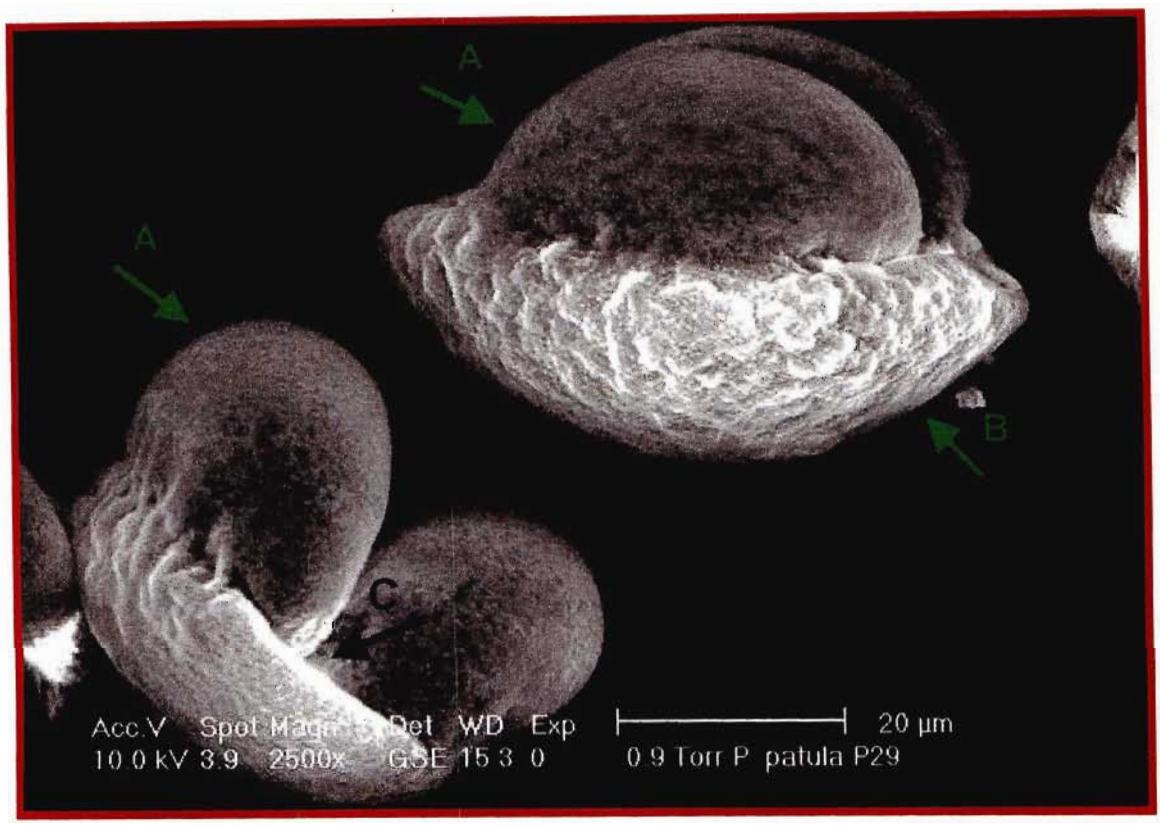


Figure 5. ESEM image of *P. patula* pollen grains at 2500 X magnification showing the porous wings (A) in the distal region of the grain and the sculptured cap (B) in the proximal region. The germ furrow (C) is also visible between the wings.

The analysis of pollen-diameter data obtained from ESEM scanned slides indicated significant differences in pollen diameter, both between species as well as between *P. patula* clones. *P. radiata* pollen grains had the largest mean diameter of 50.06 µm followed by *P. oocarpa, P. greggii* and *P. elliottii* all above the species mean of 45.62 µm. *P. patula* had a mean diameter of 43.22 µm and *P. caribaea* the smallest mean pollen diameter of 42.87 µm (Figure 6).

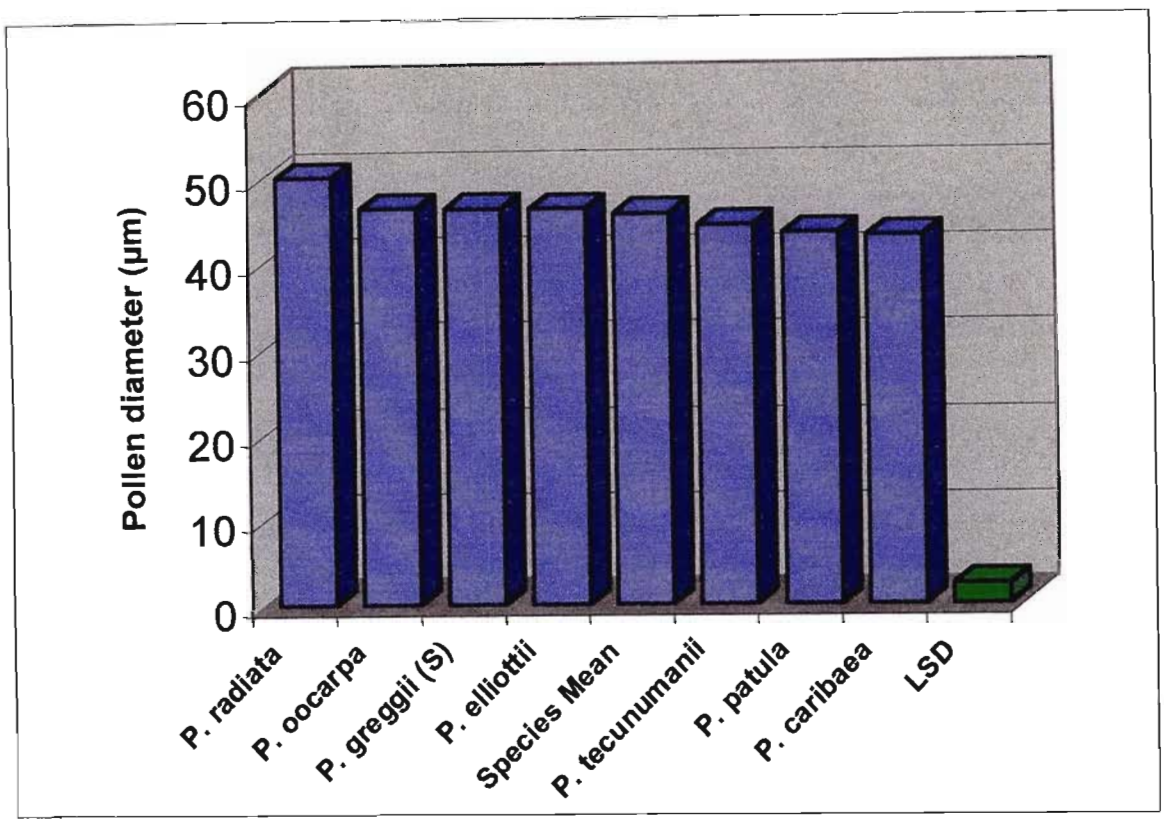


Figure 6. Mean pollen diameter (µm) of *P. patula* and six other pine species. The green bar indicates the least significant difference (LSD) at the 95% confidence level.

There were also significant differences in mean pollen grain diameter between five different *P. patula* clones (Figure 7). Clones P15, P32 and P37 were ranked above the mean diameter of all five clones and had significantly larger mean pollen diameters than clones P29 and P11. Clone P15 had the largest mean pollen diameter (47.36 µm) versus clone P11 which had the smallest mean grain diameter (42.01 µm).

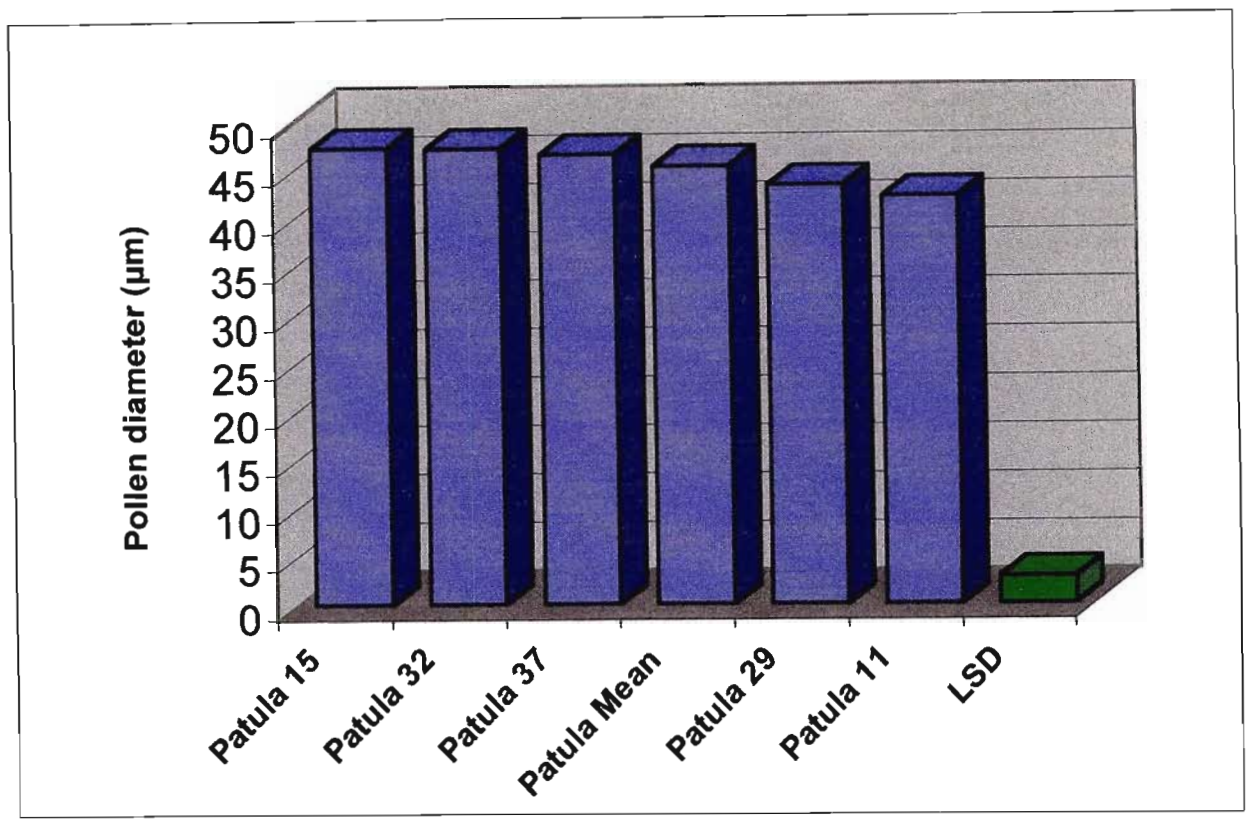


Figure 7. Mean pollen diameter (µm) of five different *P. patula* clones. Green bar indicates the least significant difference (LSD) at the 95% confidence level.

Figures 8A to 8F present scanned ESEM slides at 2500 and 3500 X magnification levels showing the morphological detail that can be observed with electron microscopy. Pollen grains of *P. patula*, *P. caribaea, P. tecunumanii*, *P. greggii, P. radiata and P. oocarpa* are shown. There are no apparent differences in the basic shape or structure of the pollen grains other than the reported size differences amongst species.

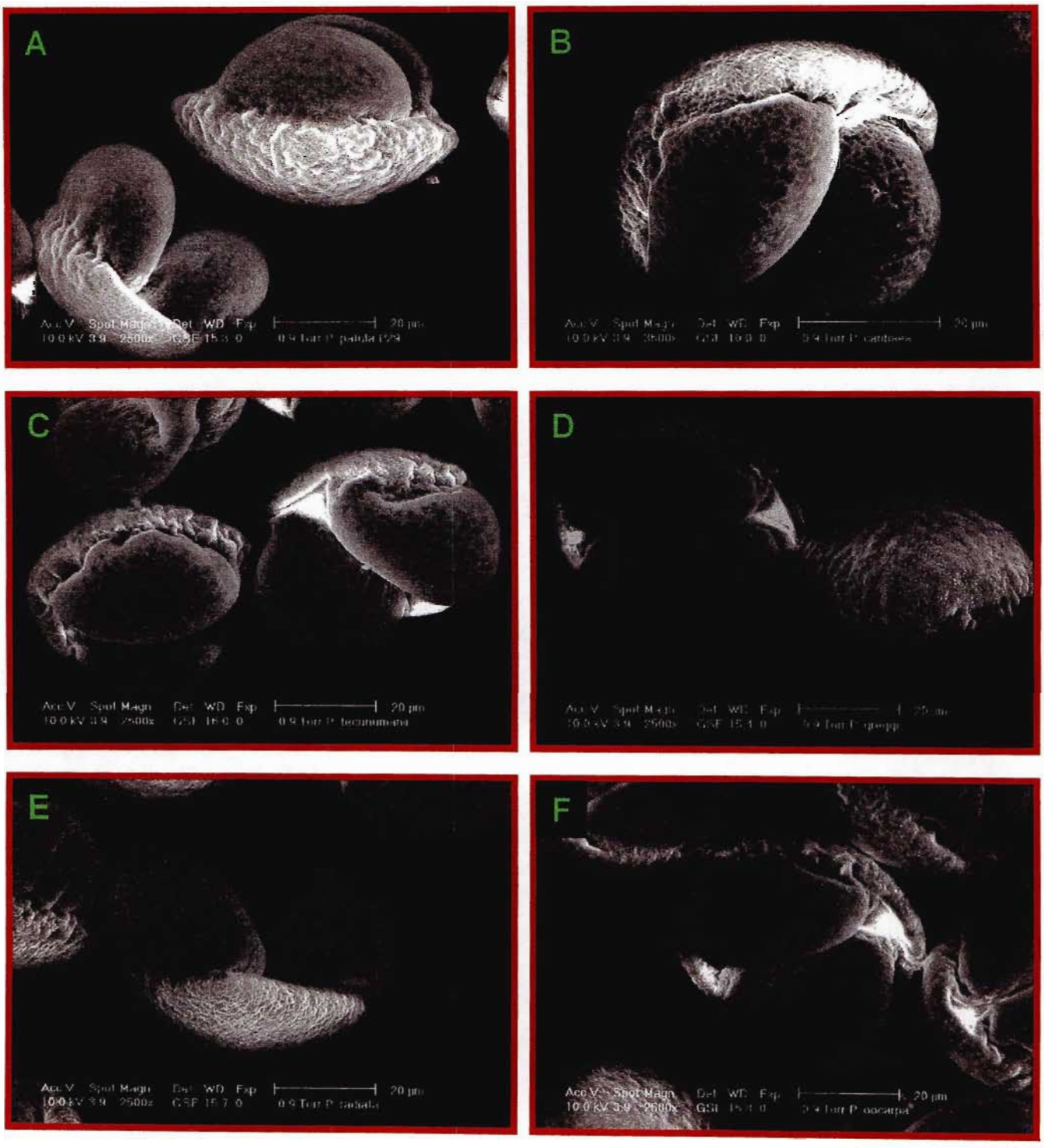


Figure 8. Scanned slides of pollen grains of *P. patula* (A), *P. caribaea* (B), *P. tecunumanii* (C), *P. greggii* (D), *P. radiata* (E) and *P. oocarpa* (F) viewed at 2500 X magnification (*P. caribaea* at 3500X) with a Phillips XL 30 ESEM microscope.

3.3.2. Effect of germination media on pollen germination

The results from germination trials testing the effect of germination media containing different levels of sucrose and boric acid showed that there were highly significant differences ($P<0.001$) in pollen germination between the different levels of sucrose (Table 7 and 8). All media treatments containing sucrose seemed to have a detrimental effect on pollen germination, and this effect increased with increasing amounts of sucrose. This effect was more pronounced for *P. patula*. Sucrose in combination with boric acid also had a negative effect on pollen germination. The addition of sucrose to the germination medium promoted fungal and bacterial growth, which reduced visibility and made assessments difficult and inaccurate. There may also be a negative effect on germination caused by the fungal and bacterial growth, but this could not be quantified.

Table 7. The effect of different levels of sucrose and boric acid (Trial A) included in an Agar and water medium on pollen germination of *P. patula, P. caribaea* and *P. greggii*. Pollen was germinated at 30 °C for 72 h. Data presented in mean germination percentage.

		Boric Acid		
Species	Sucrose	0%	0.01%	0.001%
P. patula	0%	33.67 a[1]	53.33 a	31.33 a
	10%	3.33 b	1.00 b	0.33 b
	12.5%	2.00 b	0.33 b	1.00 b
	15%	1.33 b	1.67 b	2.33 b
P. caribaea	0%	75.33 a	66.67 a	85.33 a
	10%	14.33 b	7.33 b	10.00 b
	12.5%	3.67 c	2.67 c	1.67 c
	15%	3.33 c	1.33 c	2.00 c
P. greggii	0%	65.00 a	73.67 a	64.00 a
	10%	8.33 b	3.67 b	2.33 b
	12.5%	1.33 b	1.67 b	2.00 b
	15%	0 b	3.00 b	0.33 b

[1] Significant differences ($P<0.05$) between sucrose levels are indicated with different letters.

Table 8. The effect of different levels of sucrose and boric acid included (Trial B) in an Agar and water medium on pollen germination of five *P. patula* clones. Pollen was germinated at 30 °C for 72 h. Data presented in mean germination percentage.

		Boric Acid		
Species	Sucrose	0%	0.01%	0.001%
P. patula 11	0%	29.00 a[1]	28.00 a	26.00 a
	5%	0 b	0 b	3.00 b
	10%	0 b	0.67 b	0 b
	15%	0 b	2.00 b	0 b
P. patula 15	0%	39.00 a	34.33 a	41.33 a
	5%	0 b	3.00 b	10.00 b
	10%	0 b	0 b	0 b
	15%	0 b	0 b	0 b
P. patula 29	0%	70.33 aa[2]	87.33 aa	57.67 ab
	5%	0 b	6.33 b	0 b
	10%	0 b	0 b	0 b
	15%	0 b	0 b	0 b
P. patula 32	0%	49.33 ab	73.67 aa	53.00 ab
	5%	0 b	0 b	0 b
	10%	0 b	0 b	0 b
	15%	0 b	0 b	0 b
P. patula 37	0%	69.00 ab	85.67 aa	82.00 aa
	5%	0 b	0 b	0 b
	10%	0 b	0 b	0 b
	15%	0 b	0 b	0 b

1 Significant differences ($P<0.05$) between sucrose levels are indicated with different letters.

2 Significant differences ($P<0.05$) between boric acid levels are indicated by second letters.

The boric acid levels had a beneficial effect, although not significant in most cases, on pollen germination with the 0.01% level providing the best germination result in almost all the species and clones tested (Table 7 and 8). Highly significant differences ($P<0.001$) were found between boric acid levels with three of the *P. patula* clones (P29, P32 and P37) tested in Trial B (Table 8). The negative effect of any level of sucrose and the positive effect of boric acid at 0.01 or 0.001% was found for all three species tested as well as the five different *P. patula* clones (Figure 9). No significant interactions between sucrose and boric acid were found during the analysis.

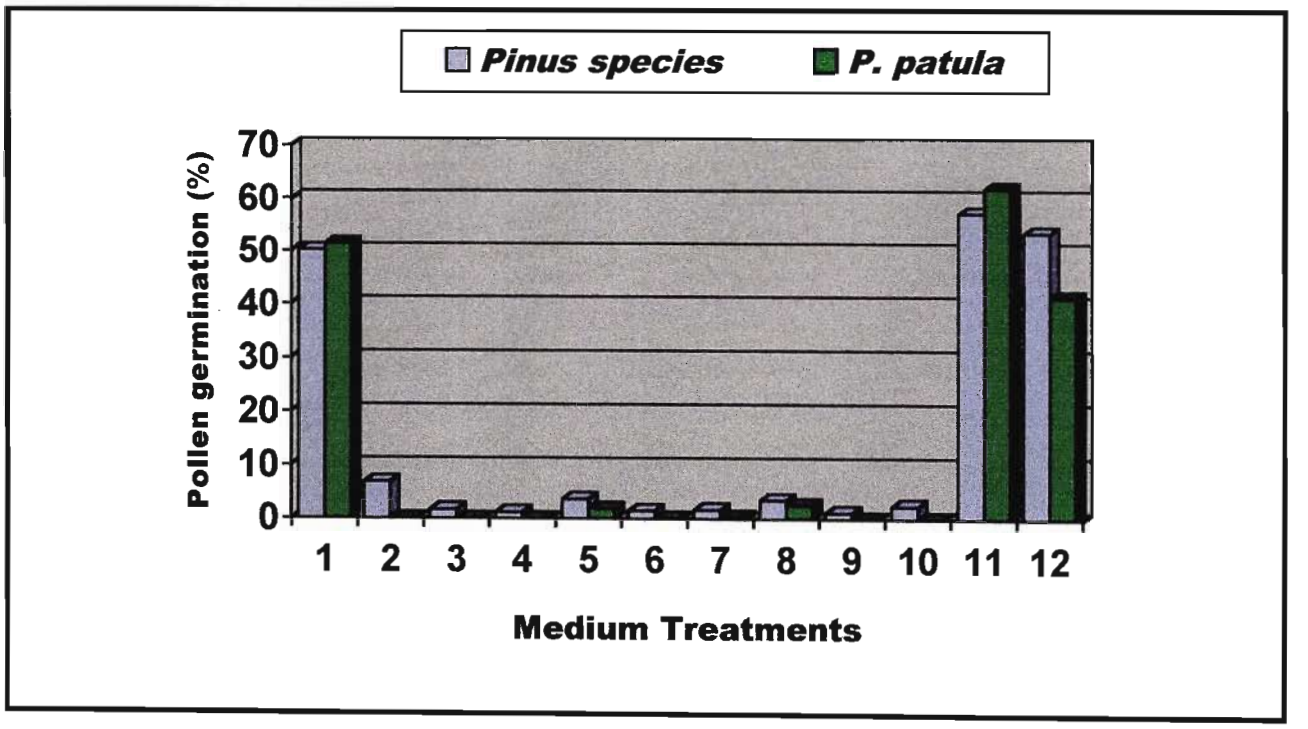


Figure 9. Mean pollen germination (%) of three pine species (*P. patula, P. caribaea* and *P. greggii*) and five *P. patula* clones. Pollen was germinated at 30 °C for 72 h on germination medium treatments (Trial A) containing different levels of sucrose and boric acid (see Table 5).

3.3.3 Effect of pollen re-hydration on viability testing

Pollen re-hydration and subsequent germination data showed that pollen re-hydration of *P. patula* and *P. caribaea* has a positive effect on pollen germination. There were highly significant differences between the control and the re-hydration treatments, as well as between the 1 and 4-h and the rest of the treatments (Table 9). The 2-h pollen re-hydration treatment seems to be adequate for both species tested although, apart from the 4-h treatment, there were no significant differences between the two-hour and the other treatments.

Table 9. The effect of different periods of re-hydration on *P. patula* and *P. caribaea* pollen prior to germination at 30 °C for 72 h on distilled water and 1% Agar solidified medium containing 0.01% boric acid.

	Pollen germination (%)			
Re-hydration Period (hrs)	*P. patula*	*P. caribaea*	Species Mean	
0	13.83	56.33	35.08	a[1]
1	24.67	82.00	53.33	b
2	27.50	91.33	59.42	c
3	30.00	87.67	58.83	c
4	24.67	82.17	53.42	b
5	25.67	87.17	56.42	c
6	27.67	87.33	57.50	c
7	25.83	86.00	55.92	c
8	26.67	87.83	57.25	c

[1] Significant differences ($P<0.05$) between re-hydration periods are indicated with different letters.

3.3.4 Effect of temperature on dry stored pollen and germinating pollen

The study of the effect of applying various temperature treatments on dry (humidity less than 10 %) stored pollen and on *in vitro* germination showed that all pollen lots tested could tolerate relatively high temperatures. There was no significant difference between the stored, 30 and 40 °C treatments (Table 10). The steady decline in pollen germination began at 50 °C with the lowest viability levels being recorded at temperatures of 80 °C for all species. There appeared to be differences in tolerance between the different species, but this was difficult to confirm as the species had different inherent germination as indicated by the germination of the stored pollen. *P. greggii* still maintained relatively good germination up to 70 and even 80 °C.

Table 10. The effect of different temperature treatments on *P. patula, P. greggii* and *P. caribaea* pollen germination prior to germination at 30 °C for 72 h on distilled water and a1% Agar solidified medium containing 0.01% boric acid.

	Pollen germination (%)				
Treatment Temperature (°C)	*P. patula*	*P. greggii*	*P. caribaea*	Species Mean	
Stored	35.83	83.67	89.83	69.78	a[1]
30	34.00	83.33	88.17	68.50	a
40	35.00	85.17	91.33	70.50	a
50	27.50	83.83	83.50	64.94	b
60	19.33	69.50	77.67	55.50	c
70	11.50	60.17	40.50	37.39	d
80	1.17	33.83	19.67	18.22	e
90	0	0	0	0	

[1] Significant differences ($P<0.05$) between temperature treatments of the species mean are indicated with different letters.

Data from the study of the effect of different incubation temperatures on pollen germination indicated that all species tested still germinated reasonably well at 36 °C with the best germination being achieved at 32 °C (Table 11). All species tested except, *P. caribaea,* showed a rapid decline in germination at 38 °C. *P. caribaea* still showed very good germination at 38 °C. An incubation temperature of 42 °C was lethal to the pollen of all *Pinus* species tested. High levels of fungal and bacterial contamination were also observed at the higher incubation temperatures (> 36 °C).

Table 11. The effect of incubation temperature on germination of *P. patula, P. greggii, P. elliottii, P. tecunumanii* and *P. caribaea* pollen. Pollen was germinated at different temperatures for 72 h on distilled water 1% Agar-solidified medium containing 0.01% boric acid.

	Pollen germination (%)						
Incubation Temperature (°C)	*P. patula*	*P. greggii*	*P. elliottii*	*P. tecunumanii*	*P. caribaea*	Species Mean	
30	82.67	80.33	69.33	73.67	95.33	75.75	a[1]
32	87.33	91.33	84.00	92.33	86.00	87.50	b
34	76.00	89.00	64.67	85.00	82.00	77.42	a
36	75.67	81.67	73.00	78.00	89.00	76.58	a
38	32.00	66.00	55.67	42.67	90.00	56.96	c
40	3.33	0	0	4.00	25.67	15.62	d
42	0	0	0	0	0	0	e
44	0	0	0	0	0	0	e

[1] Significant differences (P<0.05) between incubation temperatures of the species mean are indicated by different letters.

Table 12. The effect of incubation temperature on pollen germination of four *P. patula* clones. Pollen was germinated at different temperatures for 72 h on a distilled water 1% Agar-solidified medium containing 0.01% boric acid.

	Pollen germination (%)					
Incubation Temperature (°C)	*P. patula* P1098	*P. patula* P18	*P. patula* P28	*P. patula* P35	*P. patula* Mean	
30	82.67	41.00	83.33	80.00	71.75	a[1]
32	87.33	74.00	89.33	65.67	79.08	b
34	76.00	52.67	82.33	87.67	74.66	a
36	75.67	50.67	78.67	86.00	72.75	a
38	32.00	30.00	48.00	91.33	50.33	c
40	3.33	0	27.67	64.33	23.83	d
42	0	0	0	0	0	e
44	0	0	0	0	0	e

[1] Significant differences (P<0.05) between incubation temperatures of the *P. patula* mean are indicated by different letters.

There were significant differences in germination between the *P. patula* clones at all incubation temperature treatments. This is most probably because these selected clones had different inherent viability levels. The four *P. patula* clones tested also performed best at 32 °C and three of the clones still maintained some viability at 40 °C (Table 12). None of the *P. patula* clones could tolerate an incubation temperature of 42 °C. Clone P35 showed a higher level of tolerance to the higher incubation temperatures.

The analysis of pollen tube-lengths of different *Pinus* species and *P. patula* clones incubated at 30 °C for 72 h indicated significant differences. The pollen-tube growth of *P. caribaea* was the most vigorous and was significantly different to that of the other species and clones tested (Figure 10). Pollen-tube growth of *P. greggii*, *P. elliottii*,

P. tecunumanii and two *P. patula* clones (P98 and P35) were not significantly different from each other. The mean pollen tube-length of two *P. patula* clones (P18 and P28) were significantly shorter than that of the other *Pinus* species and *P. patula* clones.

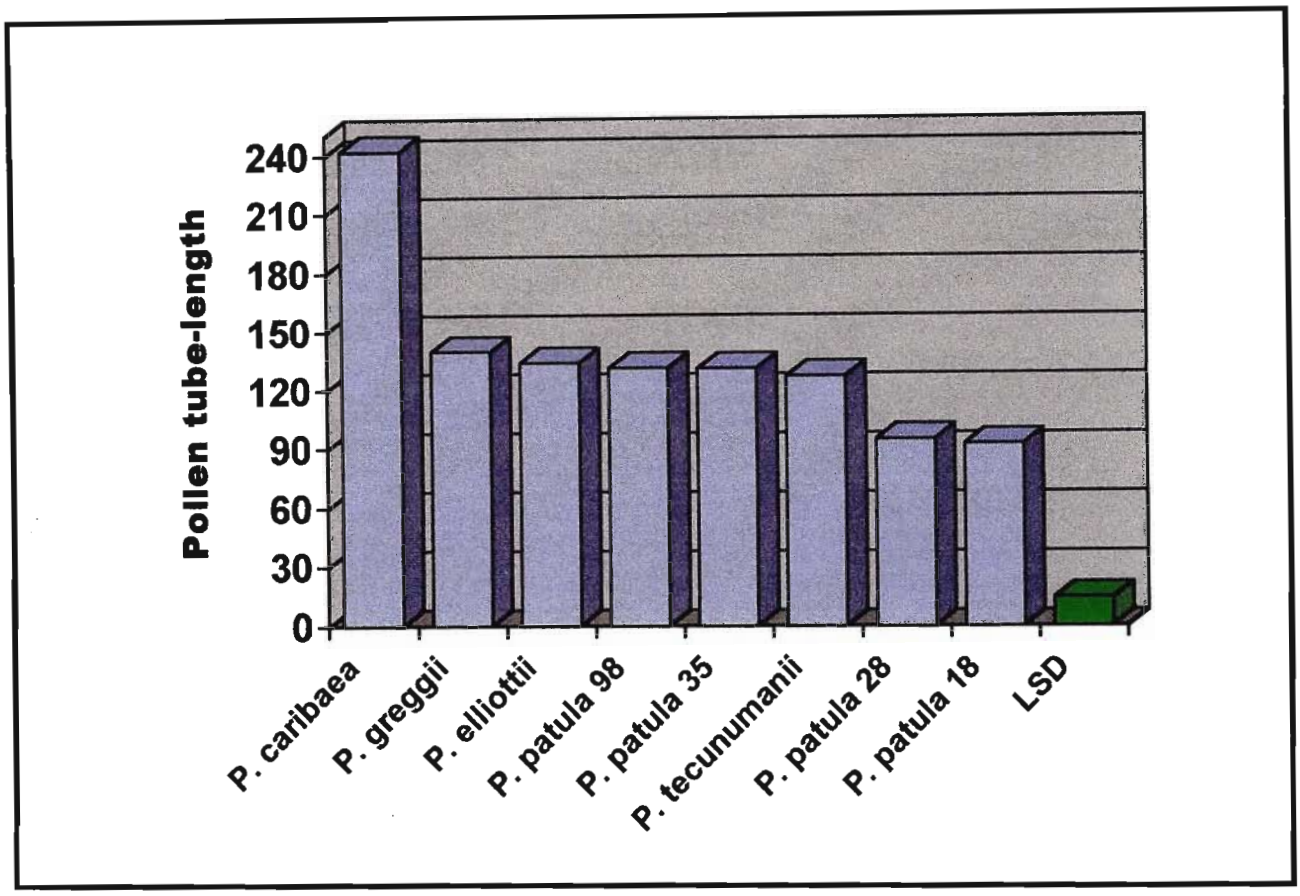


Figure 10. Mean pollen tube-length (µm) of four *P. patula* clones compared with four *Pinus* species germinated at 30 °C for 72 h on a distilled water 1% Agar solidified medium containing 0.01% boric acid. Green bar indicates the least significant difference (LSD) at the 95% confidence level.

ESEM images taken of germinating pollen incubated at 30 °C show the initiation of the pollen tube from the germ furrow between the two pollen-wings (Figures 11A to D). Pollen tubes are initiated after 24 h on germination medium (Figure 11A).

Pollen tube-growth is rapid over the next 24 to 48 h and is in excess of the pollen grain-diameter after 48 h (Figure 11B). The differences in pollen tube-length between *P. patula* and *P. elliottii* after 72 h are indicated in Figures 11 C and D.

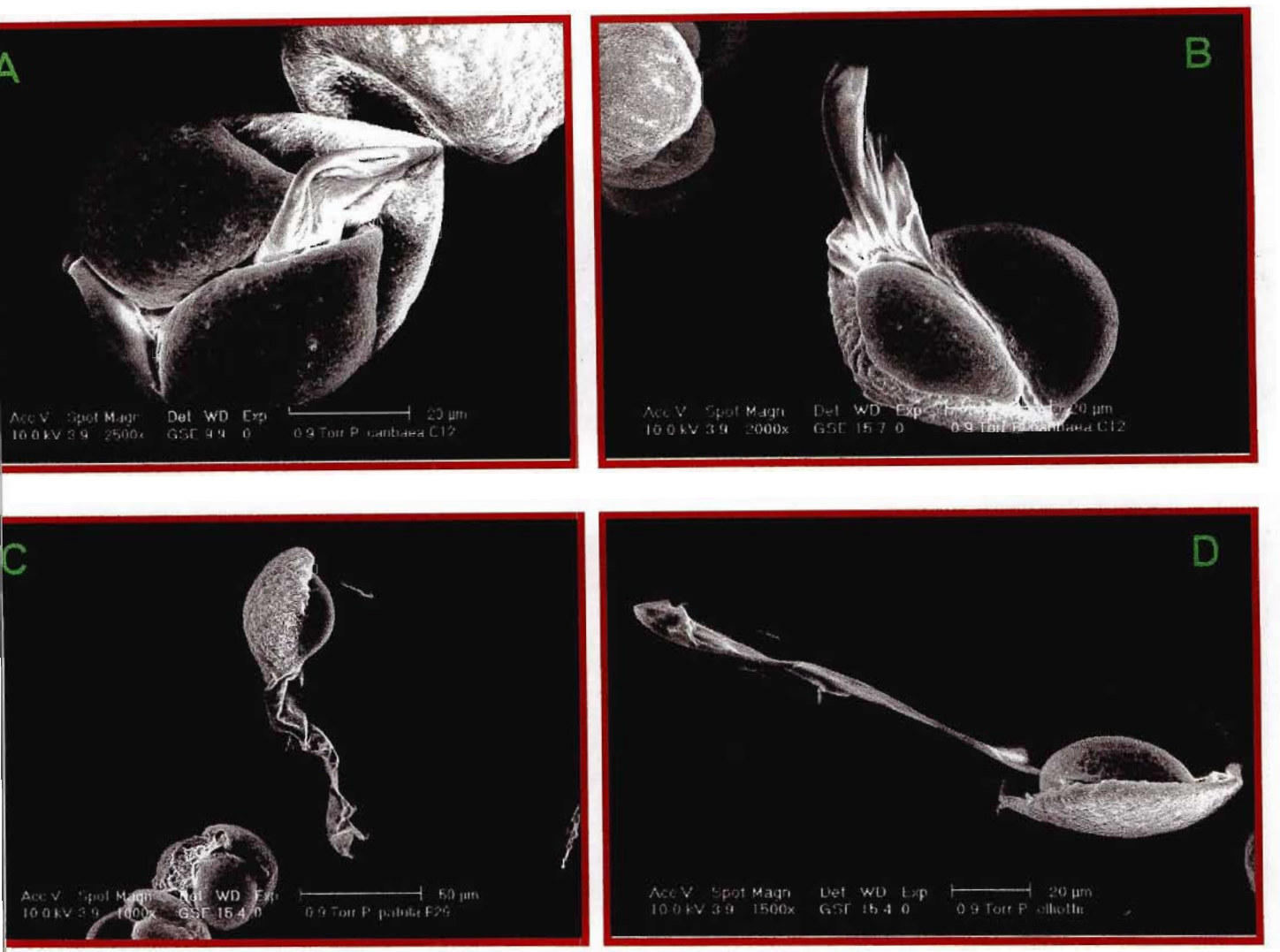


Figure 11. ESEM scanned slides at different magnification levels of germinating pollen grains of *P. caribaea* (A and B), *P. patula* (C) and *P. elliottii* (D) showing the initiation and growth of the pollen tube viewed with a Phillips XL 30 ESEM microscope. Pollen grains were incubated at 30 °C. Slide A was taken 24 h after placement, slide B after 48 h, and slides C and D after 72 h.

3.4 Discussion

Results from the pollen morphology study indicated that there are differences in pollen grain size between different *Pinus* species and within *P. patula* clones. The pollen of *P. patula* and the other *Pinus* species studied displayed the typical structure of a modern conifer with saccate pollen as described by TOMLINSON (1994) and confirmed for *P. caribaea* by DOYLE (2001). Scanning electron microscopy investigations suggest that apart from the differences in size between the species studied, the general structure and surface morphology of pollen grains of the species studied are very similar. It is therefore not possible to distinguish between different *Pinus* species by studying the structure and morphology of the pollen grain.

The wide range of pollen sizes within a species, as was found with *P. patula* also precludes size-comparison as a tool to identify or confirm pollen identity for a specific species. Knowledge of the expected size of pollen grains is, however, of the utmost importance when controlled pollination is attempted. This information will help to determine suitable porosity of isolation material to use in controlled pollinations to ensure the exclusion of undesirable pollen. The use of breathable isolation material will be covered in more detail in Chapter 4.

The germination medium used for pollen viability testing has a significant impact on pollen germination. Addition of sucrose to the medium had a detrimental effect on germination for all *Pinus* species tested. MOODY (1988) states that better pollen germination and tube growth was achieved in sucrose-free medium using *P. taeda*, *P. elliottii*, *P. palustris* and *P. echinata* pollen. GODDARD and MATTHEWS (1981) also mention the use of sucrose for as a consideration for pollen testing of some conifer species, but do not include any sucrose in their testing medium. KAPOOR and DOBRIYAL (1980), however, report good germination (above 60%) of *P. patula* fresh pollen on germination medium containing 12.5% sucrose in combination with boric acid. DOYLE (2001) also made use of an Agar-based medium containing 10 % sucrose to test pollen of *P. caribaea* and obtained good results, especially with fresh pollen. SIREGAR and SWEET (2000) showed positive

results on medium containing 2 % sucrose. The pollen lots used in this study were all kept in storage for between one and three years and this storage period could explain the different results to studies using fresh pollen. The high levels of fungal and bacterial contamination experienced with media containing sucrose could also have had a detrimental effect on germination, but could not be quantified. The excellent results obtained without the addition of sucrose does however suggest that sucrose was not needed for viability testing for the *Pinus* species tested in this study.

The addition of an anion to the germination medium in the form of boric acid (0.01%) was shown to have a positive effect on pollen germination. GODDARD and MATTHEWS (1981) have reported on the beneficial effect of boric acid on pollen germination for certain hardwoods. CHING and CHING (1964, 1976) found similar results for pollen of *Pinus monticola, Pseudotsuga menziesii, Abies procera* and *Tsuga heterophylla*.

Re-hydration of stored pollen for 16 h before viability testing forms part of the industry standard *in vitro* testing procedure of pollen viability described by GODDARD and MATTHEWS (1981). Numerous re-hydration procedures have been described in the testing of pollen viability in various *Pinus* species (McWILLIAM 1959, MOODY 1988, MOODY and JETT 1990, BRAMLETT and MATTHEWS 1991, SIREGAR and SWEET 2000). The re-hydration study conducted as part of this research indicated that 2 h was adequate to ensure maximum *in vitro* pollen germination in *P. patula* and *P. caribaea*. JETT and FRAMPTON (1990) also obtained similar results in a study on the required length of re-hydration for *P. taeda* pollen. In their study, different re-hydration periods were tested ranging from 0 to 16 h, and a period of approximately 2 h proved adequate.

Results from studies testing the impact of temperature on pollen showed very different responses to temperature between stored and germinating pollen. Stored pollen has a relatively low humidity (less than 10%) and some *Pinus* species tested could tolerate temperatures of up to 80 °C with low levels of pollen viability being retained. Most species tested could tolerate temperatures of 50 °C, which is well

above ambient temperatures experienced during the pollination season for these species. BRAMLETT and MATTHEWS (1991) found that *P. taeda* pollen exposed to temperatures of up to 65 °C still maintained 41 % viability *in vitro*.

The response of germinating pollen to increasing temperatures was rather different to the tolerance shown by non-germinating pollen. The optimum germination for all species occurred at 32 °C, and germination ceased for all species tested at an incubation temperature of 42 °C. *P. patula* maintained high germination at 36 °C, but germination dropped-off rapidly at 38 °C. *P. caribaea* seem to be the least affected by higher incubation temperatures and still maintained above 85 % viability at 38 °C. This is a sub-tropical species and may be able to tolerate higher temperatures than the other more temperate species. There was, however, a rapid drop-off at 40 °C and no germination was observed at 42 °C. McWILLIAM (1959) found a similar upper temperature threshold for germinating pollen of *P. nigra* with optimum germination at around 30 to 32 °C. The effect of temperature on pollen germination may have a major effect on the success of controlled pollinations and this aspect will be covered in more detail in Chapter 4.

Pollen tube-growth also varied between the different *Pinus* species tested with *P. caribaea* displaying rapid and significantly longer tube-growth than the other species tested. The *P. patula* clones tested also showed significant differences in tube-growth and this may be an indication that pollen with shorter tube-growth may be less viable and less successful when used in controlled pollinations.

CHAPTER 4

CONTROLLED POLLINATION OF *PINUS PATULA*

4.1 Flowering of *Pinus patula*

4.1.1. Introduction

The flowering times of the commercial pine species vary between species and to some extent, between families or clones within a species. This variation also occurs from year to year and is strongly affected by climatic conditions preceding the flowering event (HARRISON and SLEE 1992). A good understanding of the flowering times of clones within a species plays a crucial role in seed orchards where synchronized flowering among clones is needed for out-crossing (SWEET 1975). Even in the best designed seed orchards, based on good flowering data, some level of variance occurs with some clones being very late or early in developing flowers (SWEET 1975).

When controlled pollination is attempted, this understanding is even more crucial. Different flowering times among clones of the same species or between species may necessitate the collection, processing and storage of pollen prior to controlled pollination. This will ensure the availability of pollen for conducting inter-specific and intra-specific hybrid crosses weeks or months later when the female partner-species is flowering. Knowledge of when clones of a particular species flower is also important to ensure that female flowers are isolated at the correct time for controlled pollinations, especially in isolated locations.

BARNES AND MULLIN (1974) found evidence of variability in flowering times of *P. patula*, *P. teada*, *P. kesiya* and *P. elliottii* grown in southern Africa. Clonal differences in the times at which flowering peaked were observed while within-clone flowering was usually synchronous and spread over a shorter period. This within-clone synchronicity was pronounced enough to warrant using a single reference-ramet as representative of a clone to monitor both male and female flowering. Optimum flowering peaks between clones were reached within three weeks of each other while within a clone flowering usually peaked during a two-week window (BARNES and MULLIN 1974).

4.1.2. Materials and Methods

The Research Department of Sappi Forests started with annual controlled pollinations at the Shaw Research Centre near Howick in 1992. Controlled pollination registers are kept recording data of each pollination and are updated on a 4 to 6-monthly basis during the 22-month cone-development period. An attempt was made to use this data as an index of general flowering with the aim of determining differences in flowering times between *P. patula* clones planted in the clonal seed orchard as well as between different flowering seasons.

Clones were selected from the registers that were used in each of the annual controlled pollination programmes during September and October covering the years 1992 to 1998. Five *P. patula* clones were selected from the available list (P6, P10, P12, P16 and P27) for analysis. The following annual parameters were recorded for each clone; date of first and last isolation, date of first and last pollination and date of first and last removal of isolation bags. The data was not subjected to statistical analysis. Available data was plotted to determine if any meaningful observations could be made to determine if clonal differences in flowering was apparent.

4.1.3. Results

Of all the parameters studied the date of first flower-pollination per clone for each study year was the most meaningful to consider as an indicator of flowering time (Figure 12). In controlled pollination, flowers are pollinated when the female cone-scales have opened and are receptive to introduced pollen, by means of an applicator. The results show that the length of the flowering period for the five clones varied from season to season and there were some indication that certain clones consistently flowered earlier or later than others (Figure12). The length of the flowering period amongst the different clones varied from 4 days in 1992, 1997 and 1998 to 14 days during 1993 and 1994. Clone P6 seemed to flower earlier than some of the other clones for all years studied apart from 1993 when it flowered in the middle of the flowering period.

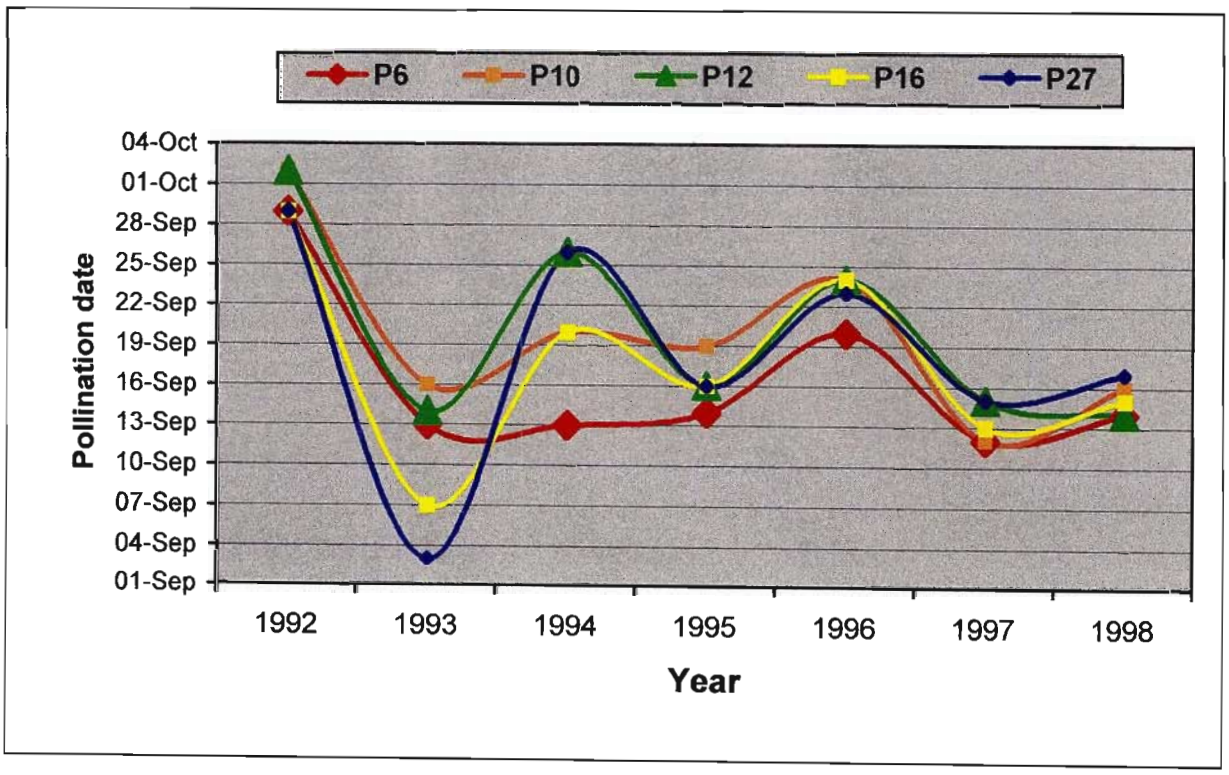


Figure 12. Use of the first date of controlled pollination as an indicator of the length of flowering period of five *P. patula* clones (P6, P10, P12, P16 and P27) for each flowering season from 1992 to 1998.

4.1.4. Discussion

During controlled pollination, female flowers are isolated before they become receptive and pollination is attempted when the female flowers are most receptive. BRAMLETT and O'GWYNN (1981) described a 6-stage development system for classifying female flowers of pines. Isolation would take place at stage 2 and pollination would be most effective when implemented at stage 5.

The stage at which female flowers are receptive to pollen was used as an indicator of general flowering periods for all clones. This is the most critical part of the controlled pollination process and needs to be conducted timeously. More flexibility is usually tolerated with early isolation or late debagging operations in a biological process where time is limiting. Using the latter data would therefore be less accurate when used as an indicator of general flowering.

The flowering period of the selected clones compared well to that found by BARNES and MULLIN (1974) for a much larger number of *P. patula* clones in Zimbabwe. This study, however, should be carried out on a much larger scale covering a larger number of clones and better record keeping of annual flowering is required to make this type of assessment more accurate. Climatic data could also be used to explain a particular flowering year and possibly predict future flowering. This could be crucial to ensure proper out-crossing in clonal seed orchards and to help plan future controlled pollination programmes.

4.2 CONTROLLED POLLINATION BY MEANS OF ISOLATION

4.2.1. Introduction

Controlled pollination constitutes one of the most important aspects of any Tree Improvement program (CRESPELL 1998). This technique is used to produce families of known parentage for the creation of advanced generation breeding populations and the evaluation of selections in production populations (BRAMLETT and O' GWYNN 1981). Controlled pollination is also used to create genotypes that may not occur under natural conditions, such as inter-specific hybrids (VERRYN 1994). Controlled pollination has been applied in the plant-breeding field for centuries, but was only applied to forest tree species more recently. According to BRAMLETT and O'GWYNN (1981), the first use of controlled pollination on conifers in forestry was documented in 1937 and it was applied on a larger scale in 1948.

With controlled pollination of conifers, pollen from a selected tree is collected, processed and stored, and is then applied to isolated female flowers of another selected tree. Female flowers are usually isolated with some type of isolation bag to prevent wind-borne pollen from reaching the selected female cones (BRAMLETT 1998). This produces seed of known pedigree and can be used to verify the genetic value of the parent trees. Tree breeders in South Africa report very high levels of conelet abortion after controlled pollination of *P. patula*. HAGEDORN (2000) reports abortion levels as high as 40 to 90 % and low seed yields from a survey among South African tree breeders from various forestry companies.

For genetic experiments and breeding strategies it is necessary to master techniques for manipulation of the pollination process in order to obtain progeny of known parentage (SEDGLEY and GRIFFIN 1989). Success depends on a detailed knowledge of the breeding system, floral development, pollination mechanism and the impact of environmental factors that may affect these factors. The most significant deviation from natural pollination is the isolation of female flowers to prevent contamination from non-target pollen. The nature of the isolation material

depends on the pollen-carrying vector, as it must form a barrier to pollen. Isolation bags made of plastic, paper and cloth have been evaluated for use in controlled pollination of pines (BRAMLETT and O'GWYNN 1981).

Results of controlled pollination experiments for pine species are often not published, or results are not adequately discussed in the literature (BESTER, VAN DER MERWE and MALEMA 2000). Results from trials where different types of isolation material was used in pine and fir species is reported by McWILLIAM (1959), MILLER (1983), HAGEDORN, RAUBENHEIMER and NEL (1997) and HAGEDORN (2000). Successful isolation material must, in addition to excluding unwanted pollen, also allow for movement of gases to prevent the build-up of heat, moisture and carbon dioxide (SEDGLEY and GRIFFIN 1989). In a study of conditions inside the isolation bag McWILLIAM (1959) found that the temperature of enclosed conelets of *P. elliottii* and *P. nigra* could increase drastically. Temperatures reached in excess of 51 °C inside unshaded bags while the outside temperature was 34.5 °C. Temperatures of 46 °C were lethal to germinating pollen.

4.2.2. Materials and Methods

4.2.2.1. Bagging-material studies

The controlled pollination experiments were conducted at the Shaw Research Centre of Sappi Forests near Howick, Kwazulu-Natal during September and October of each study-year. The *P. patula* clonal seed orchard was established in 1989 and consists of 44 different clones with a varying number of ramets each. The pollen used for these studies was collected during the preceding flowering season, and was processed and stored following the same procedures set out in Chapter 3.2. A ten-clone pollen mix (polymix) was made consisting of equal parts of ten different clones. The ten clones included in the polymix for the 1997 study (Trial A) consisted of the following clones: P9, P21, P29, P31, P33, P34, P35, P36, P39 and P44. The polymix used during the 1998 study (Trial B) included the following ten clones: P13, P16, P18, P21, P22, P24, P29, P30, P42 and P44.

Female flowers were identified on five *P. patula* clones (P15, P22, P24, P25 and P26) for the 1997 study (Trial A). The 1998 study (Trial B) included six clones: P6, P10, P20, P25, P35 and P41. Female flowers in clusters of between 3 and 5 were identified and were isolated at stage 2 following the BRAMLETT and O'GWYNN (1981) 6-stage development system using the different bagging materials. Bagging treatments were randomly assigned to ramets within each of the clones. Isolation bags constructed of polythene, micro-fibre and cellulose material were included with an open and supplemental pollinated control (Table 13). Pollinations were conducted using a bulb applicator, applying approximately 1 ml of pollen per bag when flowers became receptive (stage 5). Isolation bags were removed once female flowers reached stage 6 and their survival was monitored during the 22-month development period.

Table 13. Bagging material and controls included in the *P. patula* bagging material trials conducted in 1997 and 1998.

Treatments included	Description
Trial A (1997)	
Sappi sponge	Clear polythene casing with foam rubber stoppers
Sappi polythene	Clear polythene casing heat-sealed at one end
Green micro-fibre	Green densely woven cloth allowing air exchange, with clear window
White micro-fibre	White densely woven cloth allowing air exchange, with clear window
Open pollination control	Natural "open" pollination
Supplemental pollination control	Applying supplemental pollen on non-isolated flowers
Trial B (1998)	
Sappi polythene	Clear polythene casing sealed at one end
Green micro-fibre	Green densely woven cloth allowing air exchange with clear window
Cellulose casing	Clear cellulose casing sealed at one end allowing air exchange
Open pollination control	Natural "open" pollination
Supplemental pollination control	Applying supplemental pollen on non-isolated flowers

4.2.2.2. Pollen study

The pollen study consisted of pollen treatments of differing viabilities and other treatments applied in controlled pollinations (Table 14). The study was conducted during September and October 1998 in the *P. patula* clonal seed orchard at the Shaw Research Centre of Sappi Forests near Howick, KwaZulu-Natal. Female flowers of four *P. patula* clones (P6, P17, P25 and P32) were used and were isolated with Sappi polythene bags as previously described.

A ten-clone pollen mix that did not include any of the four clones used as females, was constituted consisting of equal portions of the following clones: P13, P16, P18, P21, P22, P24, P29, P30, P42 and P44. These pollen-lots were collected during 1997 and were kept in storage at 4 °C until 1998. This pollen mix constituted the "viable" pollen treatment. A portion of the pollen mix was subjected to a 90 °C heat treatment for 2 h and was used as the "0 %" viable treatment. Equal portions of these two treatments were mixed together to constitute a theoretical "50 %" viable treatment. The *in vitro* germination of these pollen lots was determined to confirm their validity.

Table 14. List of pollen treatments included in a controlled pollination trial *of P. patula* clones.

Treatment No	Treatment
1	Viable pollen
2	50% viable pollen
3	0% viable pollen
4	Self-pollination
5	No pollen
6	Natural "open" pollination

A self-pollination treatment was included where pollen of each of the four female clones was collected and applied to corresponding isolated flowers. An isolation-without-pollination treatment was also included where all pollen was excluded from isolated flowers of the four clones. These treatments were compared with natural "open" pollination controls of each clone used.

Pollinations were carried out using a bulb applicator applying approximately 1 ml of pollen per bag when flowers became receptive (stage 5). Isolation bags were removed once female flowers reached stage 6 and their survival was monitored during the 22-month development period.

4.2.2.3. Cone studies

The 1997 bagging material and 1998 pollen studies were subjected to a detailed cone study at the end of the 22-month development period. Harvested cones of the various applied treatments were used in this study. Some of the cone-analysis procedures described by BRAMLETT, BELCHER, DEBARR, HERTEL, KARRFALT, LANTZ, MILLER, WARE and YATES (1977) were used to generate data. Cone-scales were removed from the apex (Fig. 13 A and B) and classified into three scale-categories (Fig. 13 C to F). Aborted ovules were also classified according to whether they occurred in the first or second year of development (Fig. 13 E and F). The number of seeds produced per cone was also recorded.

The number of germinating seeds per treatment was determined *in vitro* for the 1998 pollen study. Seeds were imbibed in distilled water for 24 h at 25 °C. Seeds were then placed onto moist vermiculite in 90 mm glass petri dishes and incubated at 25 °C in the dark and monitored for germination over a 20-d period.

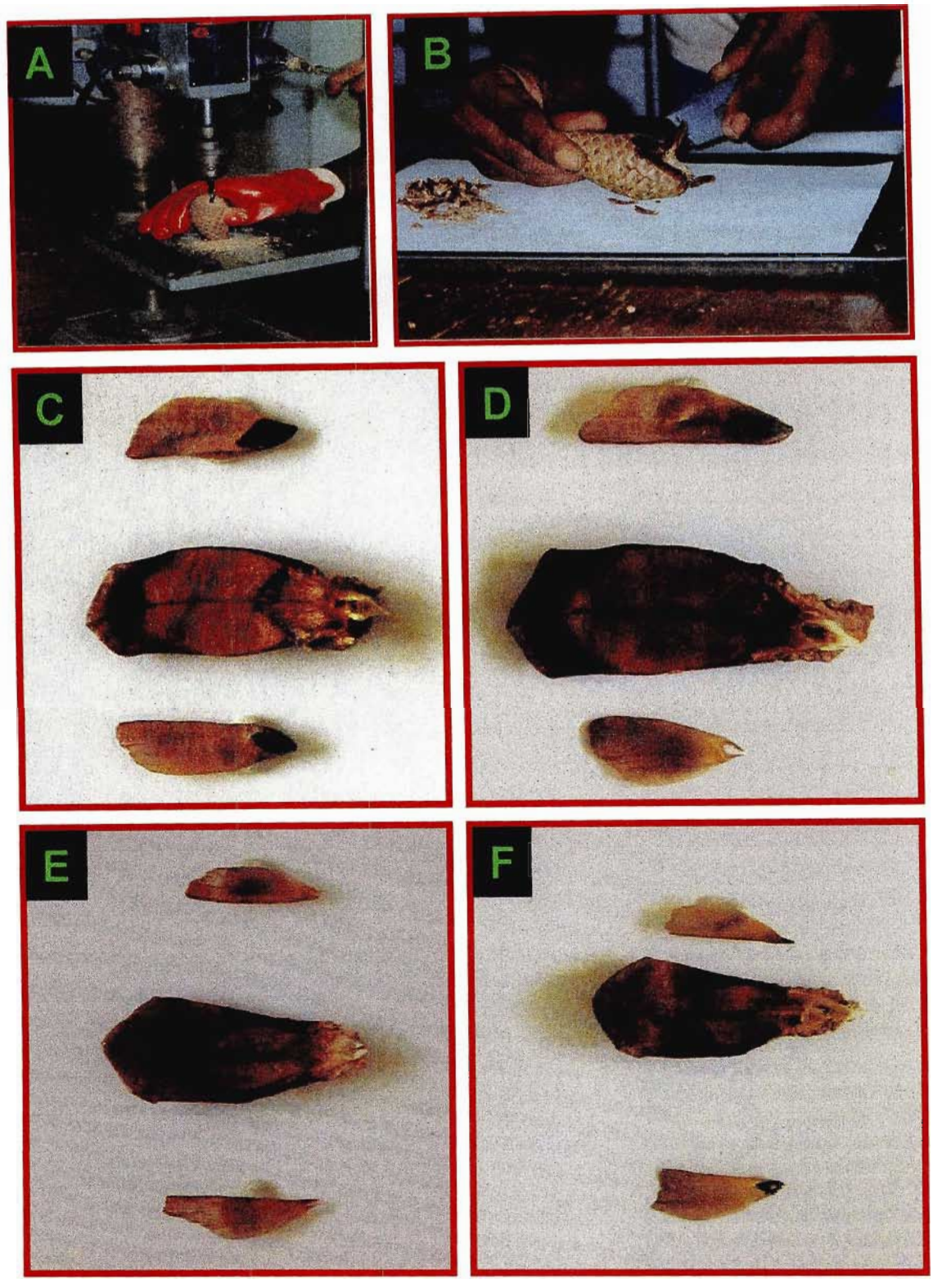


Figure 13. Cone-analysis procedure followed for *P. patula* bagging-material and pollen-studies. Cone-scales were removed from the cone-apex (Fig. 13 A and B) and classified into 2-cavity scales (Fig. 13C), 1-cavity scales (Fig. 13D) and no-cavity scales (Fig. 13 E and F). Aborted ovules were also classified as first or second year abortions (Fig. 13 E and F).

4.2.2.4. Bag-temperature and humidity study

The temperature and relative humidity (RH) experienced by female strobili inside pollination bags was measured on single trees of *P. patula*. These parameters were recorded inside bags constructed of different isolation material and used in the bagging-material study as summarized in Table 13. Ambient conditions of non-isolated flowers in similar positions to those in bags were also recorded as a control. Measuring points on trees that received an average of 2 h direct sunlight, daily were selected.

Measurements were made at 10-min intervals using Hobo® H08-003-02 (temperature and RH) and H08-001-02 (temperature only) loggers capable of measuring and storing data. The Hobo® loggers measure 60 x 50 x 20 mm in size and were placed inside ventilated 300 ml sealed round, plastic containers. The containers were used to prevent possible condensation damage to the loggers due to high humidity or rain. The loggers were then placed next to female flowers inside isolation bags and non-isolated branches.

Six loggers were used in the study, three of which could measure both temperature and RH. Loggers were calibrated before use by placing them (with and without their individual protective containers) in one sealed plastic container and exposing the container to the normal daily range of temperatures during a 24-h period. The data recorded by the loggers were then compared to ensure that loggers were recording similar values with little variation. The container was also placed next to the Shaw Research Centre weather-station and recorded values were compared to determine variation. Variation in temperature and RH readings amongst loggers were very small (0.2 °C and 6 % respectively).

The study covered a period of 12 days in October 2000 during which time the two types of loggers were rotated to measure temperature and RH readings for all the different treatments.

4.2.2.5. Data collection

All controlled pollination studies were assessed for cone-survival at 6-monthly intervals during the 22-month development period. The initial number of cones pollinated for each treatment was recorded, as well as the number present when the isolation-bags were removed. The cone survival percentage among clones was calculated based on these survival figures. Cones were harvested after the 22-month development period.

The mean number of cone scales and aborted ovules in the different categories were calculated for all treatments in the cone study. Seed viability was calculated for the pollen study based on *in vitro* germination under controlled conditions.

Temperature and RH data inside different isolation materials was downloaded from Hobo ® loggers and mean hourly values were calculated from the 10-min interval data and compared amongst isolation-material treatments.

4.2.2.6. Data analysis

The data collected was analyzed using the GENSTAT 5 Release 3.2 statistical package. Percentage data was transformed using the arcsine transformation procedure (ZAR 1984) prior to analysis of variance (ANOVA). Cone-study data, which consisted of scale and seed-counts and contained 0-value data, was transformed using the square root transformation (ZAR 1984). ANOVA procedures were completed and treatment means, standard errors and least significant differences (LSD's) were calculated to determine statistically significant differences

between treatments. Significant differences in percentage data were determined using the transformed values and are indicated in Tables using different letters. Actual (non-transformed) values are, however, presented in all Tables and Figures. A two-sample t-test was performed to determine if cone mortality in the first and second year differed significantly for all bagging-material and controlled pollination studies.

Temperature and RH data was not subjected to statistical analysis, but recorded values were plotted to show differences between treatments.

4.2.3. Results

4.2.3.1. Bagging material studies

Data analysis showed that there were significant differences in cone survival between different bagging-material treatments at most of the monitoring intervals. Of the bagging-material treatments used in Trial A, the green micro-fibre performed the best and was not significantly different from the open and supplemental controls across all monitoring intervals (Table 15). At 20 months, the green micro-fibre cone-survival was 42.5 % compared to 57.0 and 65.7 % in the open and supplemental controls. The white micro-fibre bag was not significantly different to the sponge and polythene treatments at the 12, 18 and 20-month intervals.

Highly significant differences ($P<0.001$) were found between first and second-year mortality. This indicated that in all treatments the highest mortality rate occurred during the first 12 months following pollination across all treatments (Figure 14). The supplemental pollination control also gave consistently better results than the open pollinated control across all monitoring intervals. No clone by bagging-material treatment interaction was apparent. Table 15 and Figure 14 represents the same data, statistical significance is presented in Table 15 and the survival trend is displayed in Figure 14.

Table 15. Survival of *P. patula* controlled pollinated cones isolated using different bagging-material treatments (Trial A) monitored during the 22-month development period. Data presented is mean cone survival (%) at 6-monthly intervals and prior to harvest.

	Cone survival (%)							
Bagging material	6 months		12 months		18 months		20 months	
Sponge	64.9	a[1]	41.2	a	41.2	a	36.6	a
Polythene	49.7	a	36.4	a	29.3	a	18.8	c
Green micro	82.9	b	53.7	b	47.6	b	42.5	ab
White micro	70.4	b	39.8	a	39.1	a	35.5	a
Open control	72.2	b	62.2	b	60.5	b	59.0	b
Suppl. Control	82.7	b	76.4	b	68.4	b	65.7	b

[1] Significant ($P<0.05$) differences between bagging treatments at each of the assessment intervals are indicated by different letters.

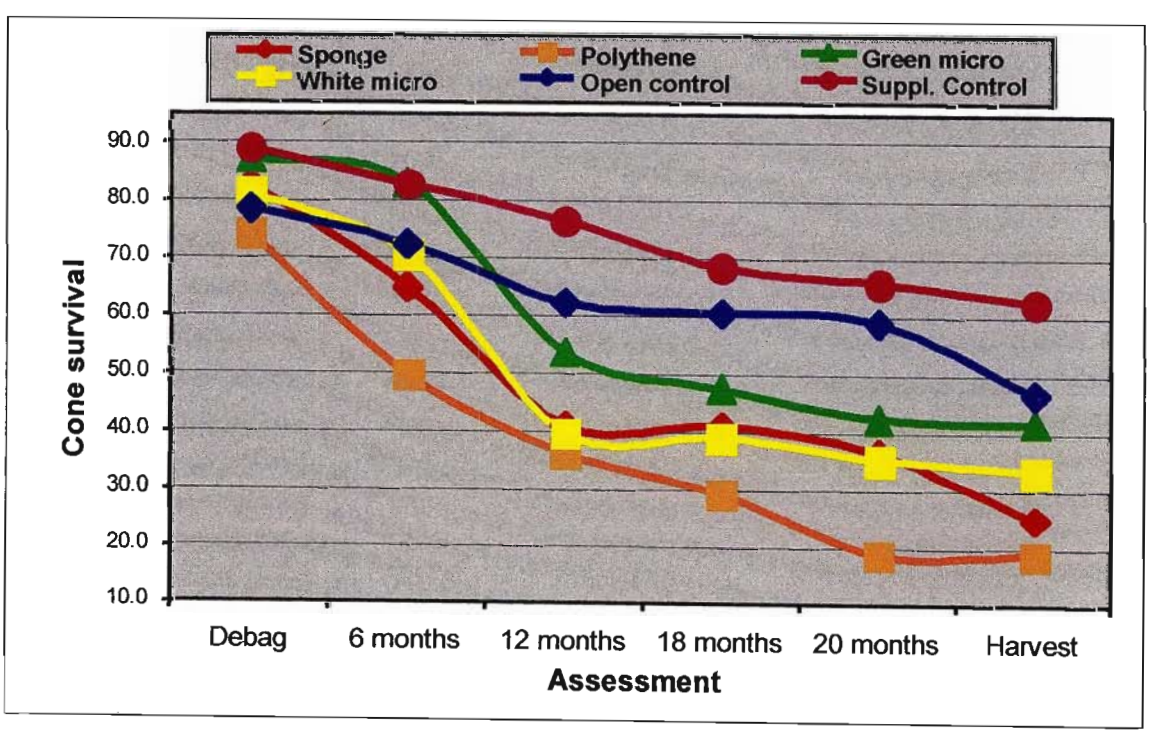


Figure 14. Survival of *P. patula* controlled pollinated cones isolated using different bagging-material treatments (Trial A) monitored during the 22-month development period. Data presented is mean cone survival (%) at all the assessment intervals.

The treatments tested in Trial B displayed similar results to trial A, although the green micro-fibre treatment was not significantly different to the open and supplemental controls. In this trial, green micro-fibre was compared with polythene and cellulose bagging treatments and the same two controls (Table 16). At 20 months, the mean survival of the treatments was 32.4 % (polythene), 45.6 % (green micro-fibre), 31.3 % (cellulose), 77.8 % (open control) and 66.8 % (supplemental control). No significant differences were found between the three bagging-material treatments and the two controls were significantly different from these treatments.

Highly significant differences ($P<0.001$) were found between first and second-year mortality, again an indication that most cone abortion occurred during the first 12 months following pollination. This was apparent across all treatments. Figure 15 plot the survival of the different treatments across the monitoring intervals and highlight the high mortality occurring during the first 12 months. Trial B differed from Trial A in that the supplemental pollination treatment gave consistently poorer results than the open control across all monitoring intervals. Furthermore, the green microfibre treatment proved to be the most successful isolation material used in both trials. Table 16 and Figure 15 represents the same data, statistical significance is presented in Table 16 and the survival trend is displayed in Figure 15.

Table 16. Survival of *P. patula* controlled pollinated cones isolated using different bagging-material treatments (Trial B) monitored during the 22-month development period. Data presented is mean cone survival (%) at 6-monthly intervals and prior to harvest.

	Cone survival (%)							
Bagging material	**6 months**		**12 months**		**18 months**		**20 months**	
Polythene	61.4	a[1]	45.5	ab	39.7	ab	32.4	a
Green micro	62.9	a	51.4	a	50.5	a	45.6	a
Cellulose	48.8	a	34.0	b	34.0	b	31.3	a
Open control	88.7	b	83.0	c	80.9	c	77.8	b
Suppl. Control	82.4	b	70.7	c	70.7	c	66.8	b

[1] Significant ($P<0.05$) differences between bagging treatments at each of the assessment intervals are indicated by different letters. Significance based on LSD analysis.

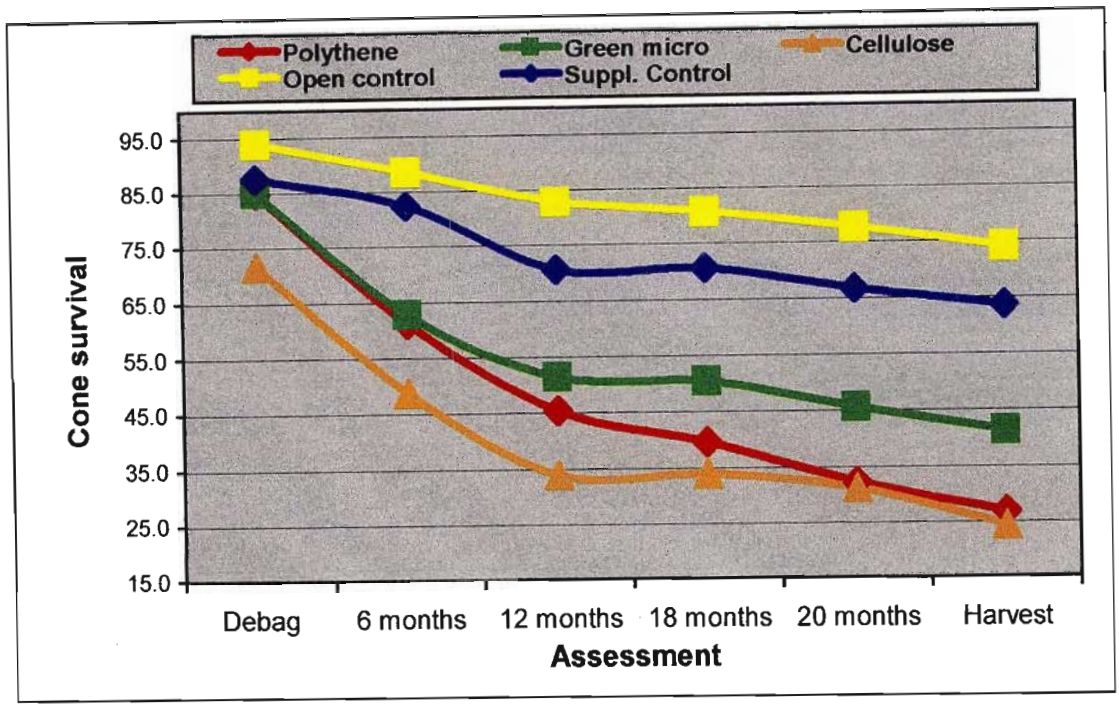


Figure 15. Survival of *P. patula* controlled pollinated cones isolated using different bagging-material treatments (Trial B) monitored during the 22-month development period. Data presented is mean cone survival (%) at all the assessment intervals.

4.2.3.2. Pollen study

In vitro germination results indicated that the viable pollen, 50 % and 0 % viability pollen lots had germination percentages of 84, 45 and 0 % respectively. Results from the pollen study showed that highly significant differences in cone survival between the viable pollen and all the other pollen treatments at all the monitoring intervals (Table 17). The survival of the 50 % viable, 0 % viable, self-pollination and no-pollination treatments did not differ significantly from each other. All pollen treatments were significantly ($P<0.05$) worse than the open pollination control, which resulted in 80 % cone survival at harvest. All controlled-pollinated pollen treatments caused a rapid drop in cone survival during the first six months following pollination with relatively low mortality during the rest of the development period (Figure 16). No clone by pollen treatment interaction was apparent. Table 17 and Figure 16 represents the same data, statistical significance is presented in Table 17 and the survival trend is displayed in Figure 16.

Table 17. Survival of *P. patula* controlled pollinated cones using different pollen treatments monitored during the 22-month development period. Data presented is mean cone survival (%) at 6-monthly intervals.

	Cone survival (%)							
Pollen treatments	6 months		12 months		18 months		Harvest	
Viable pollen	54.0	a[1]	50.0	a	42.4	a	39.3	a
50% viable pollen	43.4	b	31.4	b	26.4	b	15.8	b
0% viable pollen	36.7	b	23.2	b	19.7	b	15.8	b
Self-pollination	26.1	b	22.9	b	18.4	b	14.1	b
No pollen	39.4	b	26.6	b	21.8	b	10.3	b
Open pollination	91.7	c	91.7	c	87.7	c	81.5	c

[1] Significant ($P<0.05$) differences between pollen treatments at each of the assessment intervals are indicated by different letters. Significance based on LSD analysis.

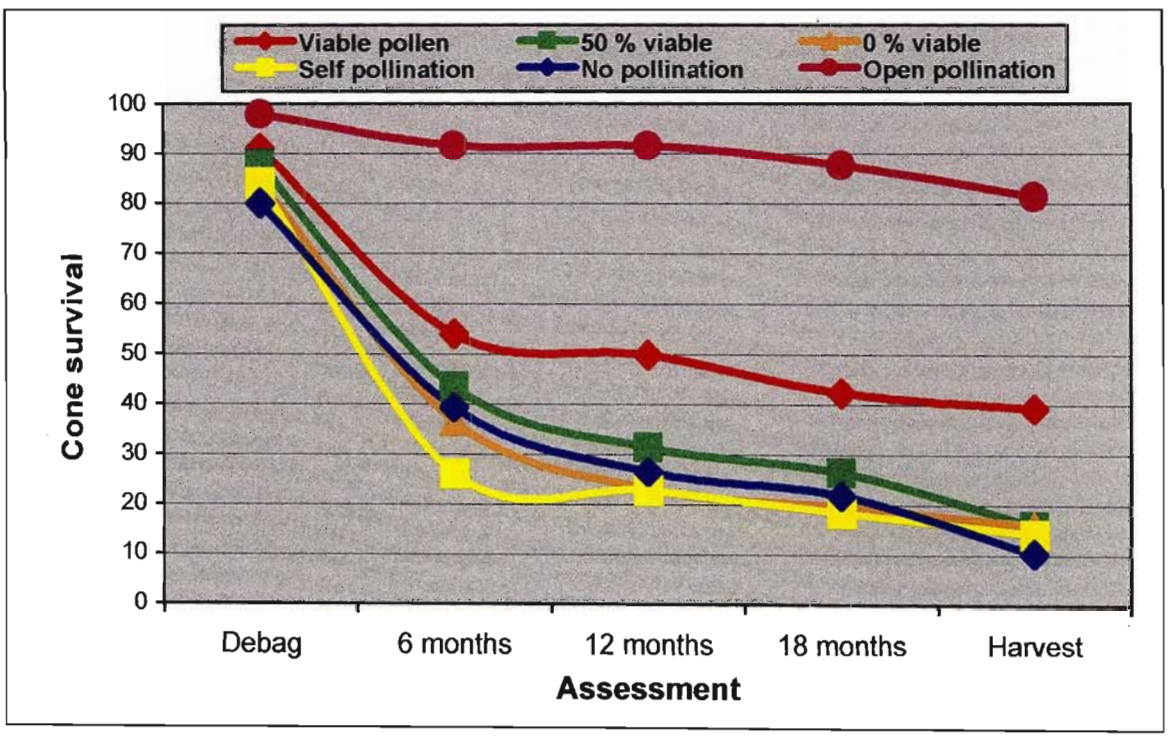


Figure 16. Survival of *P. patula* controlled pollinated cones pollinated using different pollen treatments compared with open controls and monitored during the 22-month development period. Data presented in mean cone survival (%) at all the assessment intervals.

4.2.3.3. Cone study

A sample of cones from the 1997 bagging-material and 1998 pollen studies were subjected to a cone analysis when harvested in 1999 and 2000 at the end of the cone development period. The number of 2-cavity (2 seeds) and 1-cavity (1 seed) scales determine the number of developed seeds per cone. Data from different clones used in the two studies were bulked together to increase the sample size.

The mean number of developed seeds for the bagging-material study indicated that there were no significant differences between the open-pollinated control and white micro-fibre treatments, and that of the remainder of the treatments (Table 18). Poor pollination would result in fewer fertilized ovules which would produce larger numbers of 1-cavity or 0-cavity scales. All bagging-treatments displayed relatively high numbers of 0-cavity scales. Increased numbers of 0-cavity scales resulted in lower numbers of 2-cavity scales, with a resulting decrease in developed seed. The significantly higher seed abortion-rate during the first year over that of the second year of development is also evident in all treatments. The lower third of cones from all treatments including the open pollinated control do not contain any seed.

Table 18. Summary of cone-analysis data of bagging-material Trial A. Data represents mean values of five cones per treatment for each category calculated for two clones (P22 and P25) bulked together.

	Cone scale data			Extracted seed data			
Bagging material	**2-cavity scales**	**1-cavity scales**	**0-cavity scales**	**1st-year abortion**	**2nd-year abortion**	**Developed seed**	
Sponge	15.8	23.9	111.8	127.6	45.2	55.5	a[1]
Polythene	24.2	17.7	103.0	136.1	25.7	66.5	a
Green micro	22.0	19.9	112.1	157.1	36.4	64.0	a
White micro	36.9	23.9	96.8	136.3	16.2	98.3	b
Open control	50.5	11.1	89.4	122.8	14.2	112.3	b
Suppl. Control	31.2	14.1	80.1	103.0	8.4	74.2	a

[1] Significant ($P<0.05$) differences between bagging-material treatments for developed seed are indicated by different letters. Significance based on LSD analysis.

The effect of different pollen treatments on cone and seed development was also evident in the cone analysis study. The open pollinated control produced the highest number of developed seeds and the best germination percentage (Tables 19 and 20). There was a steady decline in the mean number of developed seeds between the viable (57.3), 50 %-viable (36.3) and 0 %-viable (10.7) treatments. There was also a corresponding increase in 0-cavity scales and a decrease in 2-cavity scales between these treatments.

The self-pollination treatment also produced a larger than expected number of developed seed while the no-pollen treatment produced a low number of non-germinating infertile seed. The increased rate of abortion during the first year over that of the second year was less pronounced, but still significantly different ($P<0.01$).

Table 19. Summary of the cone-analysis study from the pollen viability trial. Data represents mean values per cone for each category calculated for two clones (P6 and P32) bulked together.

	Cone scale data (No)			Extracted seed data (No)			
Pollen treatments	**2-cavity scales**	**1-cavity scales**	**0-cavity scales**	**1st-year abortion**	**2nd-year abortion**	**Developed seed**	
Viable pollen	21.7	13.8	102.8	49.2	39.4	57.3	ab[1]
50% viable pollen	8.8	17.5	118.1	84.0	36.2	36.3	b
0% viable pollen	2.0	4.5	68.8	26.2	18.2	10.7	c
Self-pollination	11.3	17.1	105.1	24.9	48.9	36.5	b
No pollen	3.3	2.3	35.9	46.2	2.7	7.3	c
Open pollination	24.5	34.0	84.9	30.6	43.9	82.4	a

[1] Significant ($P<0.05$) differences between pollen treatments for developed seed are indicated by different letters. Significance based on LSD analysis.

Table 20. Summary of data from cone-analysis of pollen study. Data represents mean seed germination values (%) monitored over two periods calculated for two clones (P6 and P32) bulked together.

	Seed germination (%)			
Pollen Treatments	10-day assessment		20-day assessment	
Viable pollen	7.2	bc	52.2	a[1]
50% viable pollen	9.5	cd	55.8	ac
0% viable pollen	3.2	ab	4.9	b
Self-pollination	13.3	cd	58.2	ac
No pollen	0.0	a	0.0	b
Open pollination	19.2	d	72.8	c

[1] Significant ($P<0.05$) differences in germination between pollen treatments monitored at two intervals are indicated by different letters. Significance based on LSD analysis.

4.2.3.4. Temperature and relative humidity study

The mean annual temperatures during September and October when *P. patula* flowers, reach between 25 and 30 °C (Figure 17). Daily temperatures in excess of 30 °C are common during these months and relative humidity also begins to increase. McWILLIAM (1959) showed that temperatures inside the isolation bags, used for controlled pollination, could reach 10 °C higher than ambient temperatures. This would represent temperatures of over 40 °C, which was shown to be lethal to pollen germination of all *Pinus* species studied in Chapter 3.

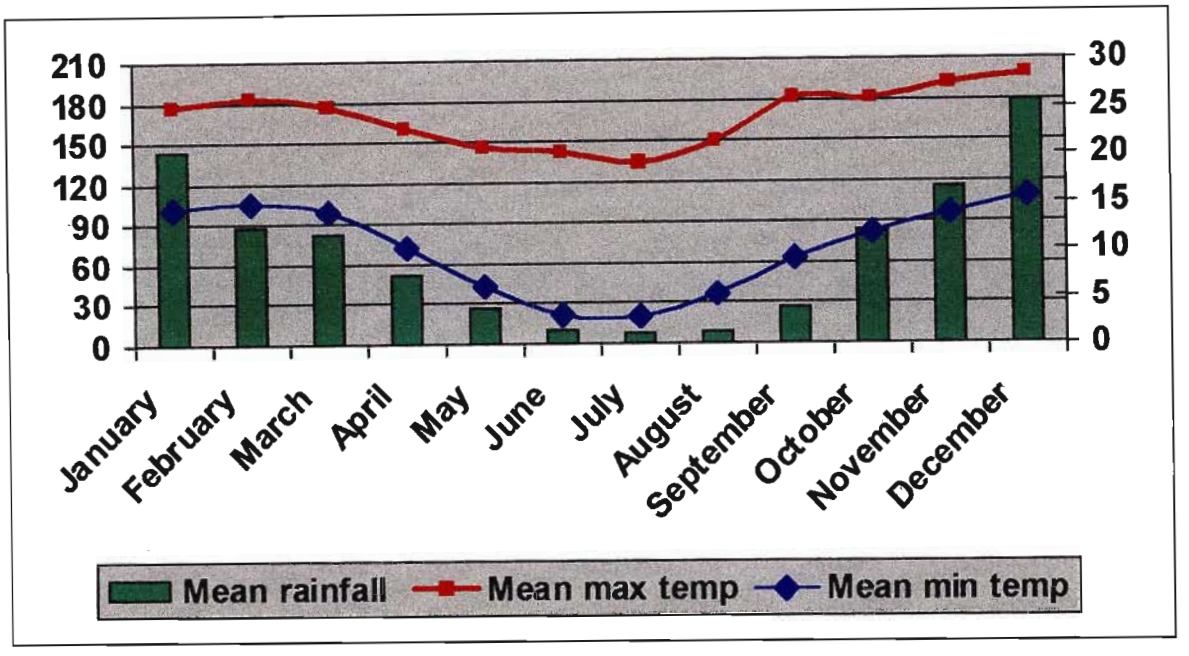


Figure 7. Mean annual rainfall, mean maximum and minimum temperatures measured at the Shaw Research Centre near Howick during the period 1992 to 2001.

The average hourly temperatures for each type of isolation material used in the bagging material study are plotted in Figure 18. Very little difference in internal bag temperatures occurred during the colder part of the day and during night-time hours. During the hottest hours of the day, which occurred from 9h00 to 15h00, however, larger temperature differences between treatments were apparent.

All types of material gave rise to internal bag-temperatures above 30 °C during this time-period. The sponge treatment had the highest temperature of 44.4 °C compared to the ambient temperature of 33.5 °C at 10h00 (10.9 °C increase). The breathable material treatments (green micro, white micro and cellulose) all displayed temperatures 3 to 5 °C higher than ambient at 13h00.

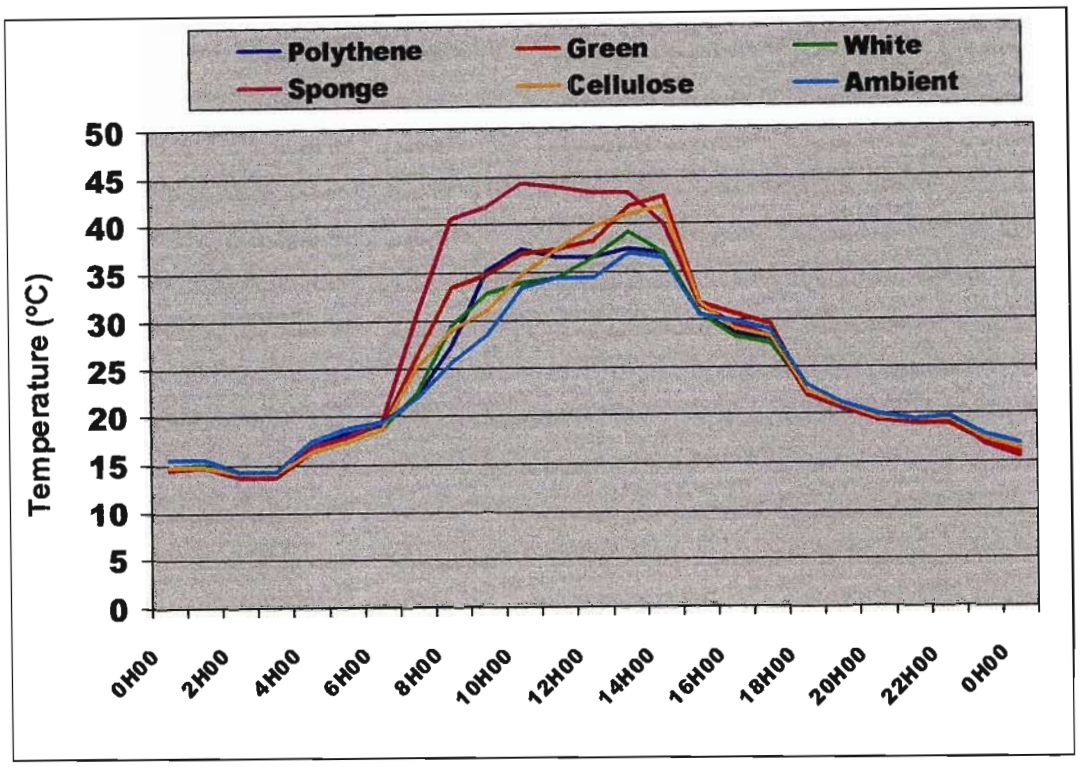


Figure 18. Temperatures measured inside different types of isolation bags compared against ambient (light-blue) conditions during a 24-hour period in October 2000.

Relative humidity (RH) levels were assessed during two different periods as only three of the loggers could measure RH. During each of the two periods, the polythene treatments was included as a control to make cross-comparisons more meaningful (Figures 19 A and B). The breathable material treatments displayed the ability to displace moisture from inside the bag while the polythene treatments could not do so and maintained high (> 80 %) humidity levels throughout the test-period.

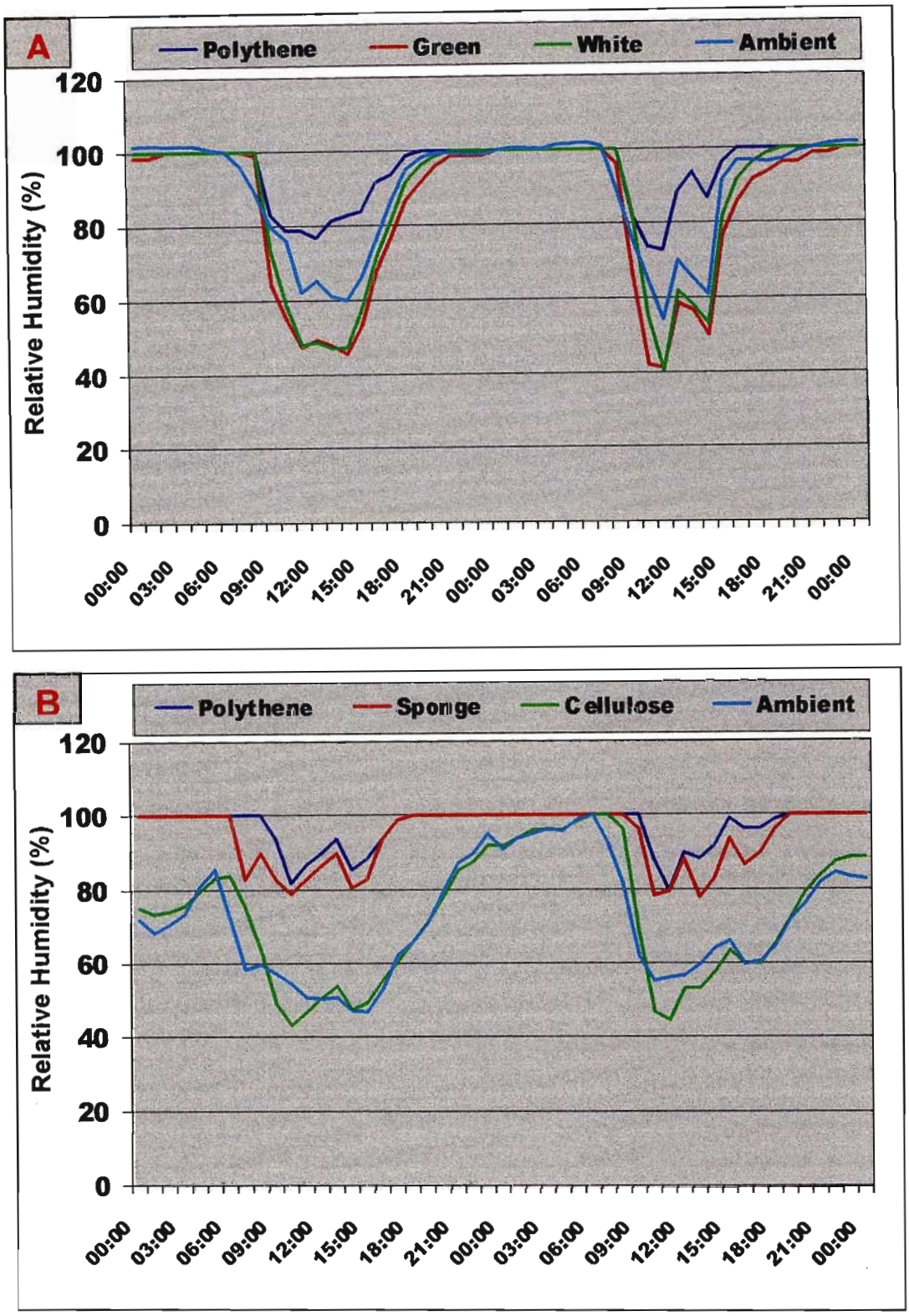


Figure 19. Relative humidity (%) levels inside various isolation bags compared with ambient conditions during two different 48-hour periods. Fig. A monitored from 7 – 8 October 2000 and B from 14 to 15 October 2000.

4.2.4 Discussion

The high cone-mortality of *P. patula* controlled pollinations in South Africa is well documented (BESTER, VAN DER MERWE and MALEMA 2000; HAGEDORN 2000). BESTER, VAN DER MERWE and MALEMA (2000) reported that the average cone-survival, covering five years of controlled pollination studies of *P. patula* was only 31.7 %. SARVAS (1962) found that the dropping of conelets was caused by insufficient pollination and was a common phenomenon in the *Pinus* genus. Results from the isolation-material studies showed that treatments consisting of breathable material (green and white micro-fibre) gave consistently better cone survival than the polythene-base treatments. The only exception to this was the cellulose material, which performed very poorly.

No clone by isolation-treatment interaction could be detected and differences in cone-survival between clones were more likely linked to differences in their inherent cone-producing ability. High cone mortality also occurred with the open-pollinated and supplemental pollination controls, which may point to off-site planting of the *P. patula* clonal orchard. HAGEDORN (2000) also reported similar results of increased cone survival with bagging-treatments of breathable material and low survival in open pollination treatments in an experiment conducted in the same orchard. The use of supplemental pollination in the bagging-material studies gave conflicting results. In Trial A, supplemental pollination caused an increase of 6.7 % in cone-survival and in Trial B, a decrease of 11 %. The different responses could be due to lower-viability pollen being used in trial B compared to Trial A or by incompatibility between the clones used in Trial B and those included in the pollen mix.

The temperature and relative humidity monitoring indicated large differences in the ability of different bagging-material treatments to regulate conditions inside the isolation-bags. The breathable treatments closely matched the ambient conditions in temperature and relative humidity while the cellulose treatment deviated from this response by substantially exceeding the ambient temperature. MILLER (1983) reported similar results with the use of cellulose bags and found them to be

unsatisfactory for use in controlled pollination of *Abies fraseri* due to the heat build-up within the bag. The sponge stoppers used in the polythene-sponge treatment were not effective and did not permit gaseous exchange.

The effect of high temperature on pollen viability was investigated in Chapter 3 and temperatures above 38 °C were found to be lethal to pollen germination. The temperature monitoring study indicated that on days with ambient temperatures between 30 and 35 °C, internal bag temperatures could exceed 38 °C, particularly in the non-breathable bags. Days with high ambient temperatures occur regularly during the flowering season of *P. patula* during September and October. The high humidity inside the non-breathable bags further exacerbated the unfavorable conditions. When pollen is introduced into bags with high relative humidity, pollen becomes saturated and would be less effective. High humidity could also increase the risk of disease, but this effect could not be quantified.

The pollen study which consisted of pollen-lots of different viability levels, self-pollination and no-pollination treatments, indicated the important role that pollen plays in controlled pollination. The use of 50 %-viable pollen caused significantly higher cone-mortality, reducing cone survival from 39.3 % when viable pollen was used to 15.8 %. It was also interesting that no-pollination and self-pollination treatments also produced small numbers of mature cones. It was noted that most of these cones appeared smaller than cones produced from the viable, 50 % viable and open pollinated treatments.

BRAMLETT (1993) lists inadequate pollination as the cause of under-developed cones in southern pines. Female flowers that do not receive adequate pollen normally abort during the first year, but some clones will hold non-pollinated cones to maturity with no viable seed being produced. The majority of cone-mortality in the bagging-material and pollen studies took place during the first 12 months following controlled pollination, which points to poor pollination as the most probable cause. Mechanical damage caused by rubbing against isolation-bags is also given as a cause of abortion following controlled pollination, but this would not explain differences in survival between isolation-material treatments (BRAMLETT 1993).

The cone study further quantified the effect of poor pollination. The analysis of cones from the isolation-material studies indicated that the positive effect of using breathable materials also extended to seed production. The white micro-fibre material produced a mean number of developed seed that was not significantly different from that of the open pollinated control. The supplemental pollination treatment produced significantly less developed seeds per cone than the open control and the white micro-fibre material and was not significantly different to that of the sponge, polythene and green micro-fibre treatments. This may be linked to poor pollen viability.

BRAMLETT, BELCHER, DEBARR, HERTEL, KARRFALT, LANTZ, MILLER, WARE and YATES (1977) list the number of aborted ovules and the year in which they occur as an important diagnostic tool. High rates of aborted ovules during the first year following pollination is caused by a lack of viable pollen, while second year abortions are caused by insect-damage (OWENS, SIMPSON and MOLDER 1982). Treatments in the isolation-material and pollen viability studies that produced low cone-survival also had the highest number of aborted ovules during the first year of development.

The self-pollination treatment used in the pollen study produced a higher number of second year aborted ovules and also produced a high number of germinating seed. KOSINSKI (1987) lists self-pollination as an important factor limiting seed production of *Larix decidua* that may contribute up to 30 % higher seed-mortality. SARVAS (1962) and FRANKLIN (1981) found that one effect of selfing is a large reduction in yield of filled seeds in comparison with yields using cross-pollinations. The high number of germinable seeds produced through the self-pollination treatment is surprising. This could be explained by a mix-up of clonal identity discovered in 2001, which occurred in the *P. patula* seed-orchard at establishment during 1988. This resulted in some of the pollen used in the study as a single clone, actually consisting of more than one clone. The pollen, therefore, was not genetically identical to the trees used for female flower-isolation. This problem was fortunately limited to this particular pollen study.

4.3. FUNGICIDE APPLICATION DURING CONTROLLED POLLINATION

4.3.1. Introduction

Results from the temperature and relative humidity study described in Chapter 4.2 indicated that measurements inside isolation bags used in controlled pollination, were abnormally high. Temperature-increases of up to 10 °C higher than ambient and RH values of close to 100 % occur, creating ideal conditions for pests and diseases. During the controlled pollination process, pine-needles surrounding the flowers are cut back to accommodate isolation bags, thus providing an entry point for diseases and pathogens. Although no evidence existed to support that disease was responsible for cone mortality, a study was undertaken to investigate whether the abnormal climatic conditions inside the isolation bags were disease-associated and thus causing high mortality in controlled pollinated cones.

4.3.2. Material and Methods

The trial-design provided for both the monitoring of cone-survival over time, and for the identification of any occurring diseases by destructively sampling cones and branches for identification of diseases. The study was conducted during September and October 2000 in the *P. patula* clonal seed orchard at the Shaw Research Centre of Sappi Forests near Howick, KwaZulu-Natal. Female flowers of three *P. patula* clones (P15, P25 and P32) were used and the bagging-treatments were isolated with polythene bags as previously described. A five-clone pollen mix that did not include any of the three clones used as females, was constituted consisting of equal portions of the following clones: P3, P6, P9, P33 and P35. These pollen-lots were collected during 1999 and were kept in storage at 4 °C until 2000. Treatments were replicated over three ramets of each clone.

Two different methods of controlled pollination were attempted, the traditional method of female-flower isolation and pollination, and pollination of non-isolated flowers where no bagging material was used. Furthermore, fungicide treatments were applied to both pollination methods. Pollinations were done using a bulb applicator applying approximately 1 ml of pollen per bag or open flower-cluster when flowers became receptive (stage 5). Isolation bags were removed once female flowers reached stage 6 and their survival was monitored for the first 14 months of the 22-month development period.

The systemic fungicide used in the study was Benlate® containing 500 g/kg benomyl active ingredient in the form of a wettable powder. The treatments consisted of cover-sprays of branches and female cones. A summary of the treatments is given in Table 21.

Table 21. Fungicide treatments applied in a *P. patula* controlled pollination trial.

Treatment	Description
Control	No fungicide applied
Treatment A	Fungicide applied at a rate of 10 g per 1000 ml 1-2 days before flower-isolation and repeated 1-2 days before pollination.
Treatment B	Fungicide applied at the same rate after isolation bags were removed as three monthly-sprays (26 October 2000, 30 November 2000 and 5 January 2001), at the same application rate.

4.3.3. Data Collection

Cone survival was monitored over the first 14 months of the cone-development period. The initial number of cones pollinated for each treatment was recorded, as well as the number present at the time when isolation-bags were removed. Non-isolated treatments were also assessed at the same time. The percentage survival of treatments at each monitoring interval was calculated based on these figures.

Destructively sampled collections covering all treatments were also made periodically and sent to the diagnostic clinic of the Tree Pathology Co-operative Programme (TPCP) based at Pretoria University for disease identification.

4.3.4. Data Analysis

Data collected was analyzed using the GENSTAT 5 Release 3.2 statistical package. Percentage data was transformed using the Arcsine transformation procedure (ZAR 1984) prior to analysis of variance (ANOVA). ANOVA procedures were completed and treatment means, standard errors and least significant differences (LSD's) were calculated to determine statistically significant differences between treatments. Significant differences in percentage data were determined using transformed values and are indicated in the Tables with different letters. Actual (non-transformed) values are, however, presented in all Tables.

Disease identification based on the samples sent to the TPCP diagnostic clinic was summarized and no attempt was made to analyze this data, as there was no certain method of determining any definite association between identified diseases and cone-mortality.

4.3.5. Results

Monitoring of cone survival in this trial was limited to the first 14 months of the development period. The results of the controlled pollination study where different fungicide treatments were applied showed that there were significant differences in cone survival between clones and pollination method (Table 22). The significant differences in survival between isolation treatments displayed the same trend as in the bagging-material studies (results described in section 4.2.3.1 of this Chapter). These differences were also observed from the earliest monitoring (2 months).

Table 22. Cone survival (%) of clonal, isolation and fungicide treatments of a *P. patula* controlled pollination study at different monitoring intervals.

	Cone survival (%)							
Treatments	**2 months**		**4 months**		**10 months**		**14 months**	
Clones								
15	60.4	a[1]	60.4	a	56.9	a	52.3	a
25	49.6	b	48.3	b	42.7	b	38.5	b
32	73.8	c	72.3	c	67.2	c	63.4	c
Isolation								
Bagged	56.8	a	55.9	a	49.0	a	41.8	a
No bag	65.7	b	64.7	b	62.3	b	60.9	b
Fungicide								
None	62.6	*NS*[2]	61.7	*NS*[2]	57.5	*NS*[2]	54.5	*NS*[2]
Treatment A	61.1		60.0		55.5		51.4	
Treatment B	60.0		59.3		53.9		48.1	

1 Significant ($P<0.05$) differences in cone survival between treatments monitored at four intervals are indicated by different letters. Significance based on LSD analysis.

2 No significant differences ($P>0.05$) between fungicide treatments. Significance based on LSD analysis.

Differences between clones are expected as cone production is genetically driven and differences in production do occur. No significant difference in cone survival between the fungicide treatments was apparent at all of the monitoring intervals that spanned the 14-month test period. There was also no significant interaction between the isolation and fungicide levels.

The identification of fungi isolated from samples taken across all treatments of the study showed that a wide range of different species occurred on the cones and branches (Table 23). All treatments displayed some level of pathogen infestation, including the fungicide treatments. An association between pathogen and cone-mortality could however not be made as species such as *Sphaeropsis sapinea* can occur as endophytes in healthy pine tissue and although it can cause tree mortality, do not necessarily cause cone mortality.

Table 23. Summary of identified fungi isolated from samples taken from treatments of the *P. patula* controlled pollination study.

Sample date	Isolation treatment	Pollination treatment	Fungi isolated from branches	Fungi isolated from cones
October 2000	Bagged	Control	*Penicillium*	*Penicillium, Pestalolsiopsis*
		Fungicide A	*Penicillium*	*Pestalolsiopsis*
		Fungicide B	*Penicillium*	*Penicillium*
	Open	Control	*Penicillium*	None
		Fungicide A	*Penicillium, Pestalolsiopsis*	*Pestalolsiopsis*
		Fungicide B	*Penicillium*	None
December 2000	Bagged	Control	*Spharopsis sapinea*	*Penicillium*
		Fungicide A	*Penicillium*	None
		Fungicide B	*Penicillium*	None
	Open	Control	*Alternaria*	*Penicillium*
		Fungicide A	*Alternaria*	*Spharopsis sapinea*
		Fungicide B	*Alternaria*	None
March 2001	Bagged	Control	*Pestalolsiopsis*	*Alternaria*
		Fungicide A	*Pestalolsiopsis, Fusarium*	*Penicillium, Alternaria*
		Fungicide B	*Penicillium, Alternaria*	*Alternaria,*
	Open	Control	*Penicillium*	*Pestalotiopsis, Alternaria*
		Fungicide A	*Pestalotiopsis, Alternaria*	*Penicillium*
		Fungicide B	*Pestalotiopsis, Alternaria*	*Alternaria, Penicillium*
August 2001	Bagged	Control	None	None
		Fungicide A	*Sphaeropsis sapinea*	*Sphaeropsis sapinea*
		Fungicide B	*Sphaeropsis sapinea*	*Sphaeropsis sapinea*
	Open	Control	*Sphaeropsis sapinea*	*Sphaeropsis sapinea*
		Fungicide A	*Sphaeropsis sapinea*	*Sphaeropsis sapinea*
		Fungicide B	*Sphaeropsis sapinea*	*Sphaeropsis sapinea*
December 2001	Bagged	Control	*Alternaria*	*Fusarium*
		Fungicide A	*Fusarium*	*Aspergillus*
		Fungicide B	*Pestalotsiopsis*	*Spaeropsis, Alternaria*
	Open	Control	*Penicillium*	*Aspergillus*
		Fungicide A	None	*Fusarium, Pestalotsiopsis*
		Fungicide B	*Alternaria*	*Alternaria, Aspergillus*

4.3.6. Discussion

The fungicide application study showed that there was no significant impact on controlled pollination success with the introduction of fungicide applications. Significant differences between clones and isolation treatments used in the study occurred and were expected as the other controlled pollination studies had shown. No significant differences, however, occurred between the fungicide treatments and therefore no interaction between fungicide applications and isolation treatments could be shown.

A number of fungal species were isolated and identified from samples taken from the study and could have had an effect on cone-mortality. WINGFIELD and SWART (1994) describe *Sphaeropsis sapinea* as being an important disease that can cause damage to trees. This disease is associated with hail damage and normally occurs after hailstorms when trees had been wounded. A study of the microclimate favourable to *Spaeropsis* led to the conclusion that most severe damage occurred under conditions of reduced air movement and higher humidity (WINGFIELD and SWART 1994).

The effect of these diseases on cone-mortality, however, could not be quantified and no association could be shown.

CHAPTER 5

CONCLUSIONS

The pollen morphology study indicated that little difference in pollen size was apparent between different *Pinus* species studied. There were size differences amongst species and within *P. patula* clones studied. Comparing pollen size and structure, therefore, do not provide an accurate method of distinguishing between different species. Testing pollen viability *in vitro* on Agar-solidified medium incubated at 30 °C provide good results within 72 h. Re-hydration of stored pollen for at least 2 h prior to germination testing is crucial for good germination. Stored, low-humidity pollen can also tolerate relatively high temperatures of 70 °C, whereas germinating pollen is far more sensitive and temperatures above 38 °C proved lethal to most *Pinus* species.

Differences in the length of the flowering period between *P. patula* clones also occur from season to season and can vary between four and 14 days in length. A more detailed study consisting of a larger number of clones is crucial for gaining a proper understanding of flowering in a *P. patula* clonal orchard and would have major benefits for both seed production and controlled pollination programmes.

The monitoring of temperature and relative humidity has provided some insight to observed differences in cone-survival with use of various bagging material for strobili isolation during controlled pollination. Breathable material caused less mortality than polythene-based bags due to lower increases in temperature and RH inside bags. The polythene-based bags produced internal conditions that proved to be lethal to pollen germinated *in vitro*. The increased production of viable seeds per cone was also possible with breathable material.

The effect of different levels of pollen viability in controlled pollination was also shown. Low levels of viability caused increased cone-mortality and lower seed yields. When pollen was excluded during pollination, a small number of cones and seed was produced, but the seed produced was not viable. The production of seed from the self-pollination treatment is most probably due to the clonal identity mix-up recently discovered in the orchard and is therefore not a true reflection of this treatment. Pollen used for the self-pollination treatment may, therefore, have consisted of other genotypes.

The application of fungicide treatments during controlled pollination did not have a significant effect on cone survival and was therefore not effective. A number of fungal species, which could have caused cone-mortality, were isolated and identified on samples taken from the fungicide treatment study. This association, however, could not be shown with this study.

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