CHAPTER 3

THE INFLUENCE OF STORAGE AND MORPHOLOGY ON THE VIABILITY OF *PINUS CARIBAEA* POLLEN

3.1 Introduction

The hybrid between *P. elliottii* and *P. caribaea* displays many promising and desirable characteristics, such as rapid growth combined with excellent wood quality (Denison and Kietzka, 1993; Stanger *et al.,* 1999). These characteristics are mainly attributed to the hybrid vigor obtained during the cross and is the main reason why *P. elliottii x P. caribaea* hybrids are favored above improved parent species for commercial planting (Bester, 2000). However, a number of problems are associated with the *P. elliottii x P. caribaea* hybrid. Firstly, the *P. caribaea* pollen ripens approximately three months before the *P. elliottii* ovules are receptive (Mather, 2000); there is therefore a need to optimize the long-term storage conditions of the *P. caribaea* pollen. The second major problem facing the hybrid is one of incompatibility between *P. elliottii* and *P. caribaea,* which results in low seed set and a low number of viable hybrid seeds (Mather, 1996).

The odds of successful cross-pollination are increased by random wind dispersal and the millions of pollen grains that are produced (Baker and Baker, 1979). This ensures that at least a small percentage will reach its destination and result in pollination thereby ensuring the proliferation of the species. Of these millions of pollen grains a percentage will be sterile, others will be malformed and the vast majority will end up being dispersed by the wind and never even come near the pollination droplet (Singh, 1978; Wright, 1976). Other factors that have been found to influence pollination are male fertility (Schoen and Cheliak, 1987), pollen viability and pollen tube growth rates (Ottavariano *et al.,* 1980) and non-random embryo abortion (Sorensen, 1982).

Pollen viability is perhaps the most important factor known to influence reproduction in plants. Pollination and fertilization are directly dependent on pollen viability (Singh, 1978). According to Pacini (1996) cytoplasmic carbohydrates and sucrose are involved in protecting the pollen during exposure and dispersal, while Van Bilsen *et al.* (1994) found that a direct correlation exists between pollen viability and lipid degeneration during storage.

The entire reproductive cycle is dependent on the ability of the pollen grain to germinate once it is captured in the pollination droplet. Germination requires energy as well as the elaboration of cellular and structural materials within the pollen tube as it is formed (Baker and Baker, 1979). Pollen tube growth consists of two phases *in vivo*, namely an initial autotrophic phase followed by a heterotrophic phase (Bellani *et al.*, 1985). During the autotrophic phase energy and building blocks must be provided by the reserves, such as oils, sugars and starches in the pollen grain itself, as only limited additional material may be absorbed from the surrounding stylar tissue (Baker and Baker, 1979; Bellani *et al.*, 1985; Delph *et al.*, 1997; Willemse, 1968). During the heterotrophic phase polysaccharide reserves in the pistil are mobilized and enzymes for carbohydrate metabolism are induced in the vicinity of the growing pollen tube (Roggen, 1967).

Two broad aims were established for this investigation. The first aim was to optimize the storage conditions for the *P. caribaea* pollen during the three month storage period. The second aim was to investigate the relationship that exists between the morphology and viability of *P. caribaea* pollen.

3.2 Materials and Methods

3.2.1 Plant Material

Eighteen *P. caribaea* pollen types, which were provided by SAFCOL, South Africa, were used in this study. These eighteen types included pollen from: Ach 22, Ach 24, Ach 29, Ach 31, Ach 33, Ach 49, Ach 57, Ach 65, Ach 91, Ach 93, Ach 114, Ach 271, Acb 60, Acb 62, Pch 23, Pch 69, Pch 88 and Pch 105.

3.2.2 Influence of environmental factors on *P. caribaea* pollen viability

Ten of the eighteen *Pinus caribaea* pollen types (Ach 24, Ach 29, Ach 31, Ach 65, Ach 93, Ach 114, Pch 23, Pch 69, Pch 88 and Pch 105), which were provided by SAFCOL, Dukuduku, South Africa, were used in the germination trials to determine the effects of environmental conditions on viability. The factors investigated included the effects of pollen age, the temperature and humidity at which the pollen was stored and the developmental stage of the pollen cone at the time of harvesting on the viability of the pollen (Table 3.1). In order to facilitate germination, the pollen was placed on 3% (w/v) agar containing 10% (w/v) sucrose and left at room temperature for 72 hours, as described by Wright (1976). After 72 hours microscope slides were prepared and the samples were investigated using an Axiovert 35 28436 inverted light microscope. Germination percentage was determined by counting the number of germinated pollen grains per microscope field, at 20 X magnification, and by repeating the procedure over ten random microscope fields per pollen type.

Table 3.1. Environmental conditions investigated.

	Pollen age	Pollen cone developmental stage	Time of harvesting	Environmental conditions
***P. caribaea* Pollen**	• Fresh (1 – 2 weeks) • 3 months old • old (ca. one year)	• Young cones (ca. 10mm) • Medium cones (ca. 20 mm) • Old cones (ca. 35 mm)	• 08:30 • 12:00 • 16:30	• 4°C • 20°C • 20°C Low Humidity (LH) • 20°C High Humidity (HH) • -20°C • -80°C

3.2.3 The influence of environmental factors on the ultrastructure of *P. caribaea* pollen

P. caribaea pollen, Pch 23, Pch 29 and Pch 53, which were collected at various times of the day and which were exposed to various environmental conditions during storage, as described in section 3.2.2, was prepared for transmission electron microscopy. The pollen was fixed for two weeks in 3% (v/v) glutaraldehyde in 75 mM sodium phosphate buffer (pH 7.5). After the initial fixation the pollen was rinsed three times in 75 mM sodium phosphate buffer (pH 7.5) and then fixed further in 2% (w/v) aqueous osmium tetroxide for 2 hours, before being rinsed three times with distilled water. The pollen was subsequently stained overnight with 0.5% (w/v) uranylacetate-dihydrate, then rinsed

twice with distilled water and heated for a few seconds in a water bath at 65°C. Heated agar was subsequently added and the tubes covered with parafilm. Once the agar had cooled and solidified it was cut into small cubes. These pollen containing agar squares were then dehydrated in 30%, 50%, 75%, 95%, and twice in 100% ethanol for 30 minutes per dehydration step. The ethanol was subsequently replaced with 1.2 X propylene oxide and the samples were stored while the plastic embedding solution (10% (w/v) VCD, 26% (w/v) NSA, 6% (w/v) DER 736, 0.4% (w/v) DMAE) was prepared according to Spurr (1969). The pollen containing agar squares were then embedded in the plastic solution, which was cast in molds and left at 60°C for eight hours to allow polymerization. Excess plastic was trimmed from around the embedded pollen material and the material was subsequently sectioned, in Amstrong diameter, using the LKB ultrotome III ultramicrotome. The sections were caught up in a loop and transferred to 200-grid mesh. The section containing grids were then stained in 5% (w/v) uranylacetate for 30 minutes followed by staining with lead citrate as described by Reynolds (1963). The sections were examined using a Phillips CM 100 transmission electron microscope at 60 kV.

3.2.4 *P. caribaea* pollen morphology

3.2.4.1 Scanning electron microscopy (SEM)

The eighteen *P. caribaea* pollen types were prepared for Scanning Electron Microscopy according to the procedures outlined by Coetzee and Van der Merwe (1999). The procedure involved fixation of the pollen in 2.5% (v/v) glutaraldehyde in 75 mM sodium phosphate buffer (pH 7.5) for at least one hour. After fixation the pollen was rinsed three times in sodium phosphate buffer (pH 7.5) and fixed further in 0.25% (w/v) aqueous osmium tetroxide for approximately half an hour. The pollen was rinsed three times in distilled water and dehydrated for 15 minutes in 70% ethanol, followed by three times 15 minutes in 100% ethanol. The dehydrated pollen was then critical point dried in liquid carbon dioxide. Once dry, the pollen was mounted on a stub and sputtered with a colloidal gold solution (Duff *et al.*, 1993) using the Biorad SEM coating system. The prepared pollen stubs were examined using a Jeol Winsem JSM 6400 scanning microscope at 5 kV and 1 000 X magnification. Twenty images, in a side on view and twenty in a distal view of each of the eighteen pollen types were scanned in. As it was impossible to obtain a "size" estimate, such as total area or volume of individual pollen grains, five dimensions (representative of the size and shape of the pollen grains and their airbags) were measured using the UTHSCSA *Image Tool* programme to obtain a data set applicable for statistical analysis (Figure 3.1).

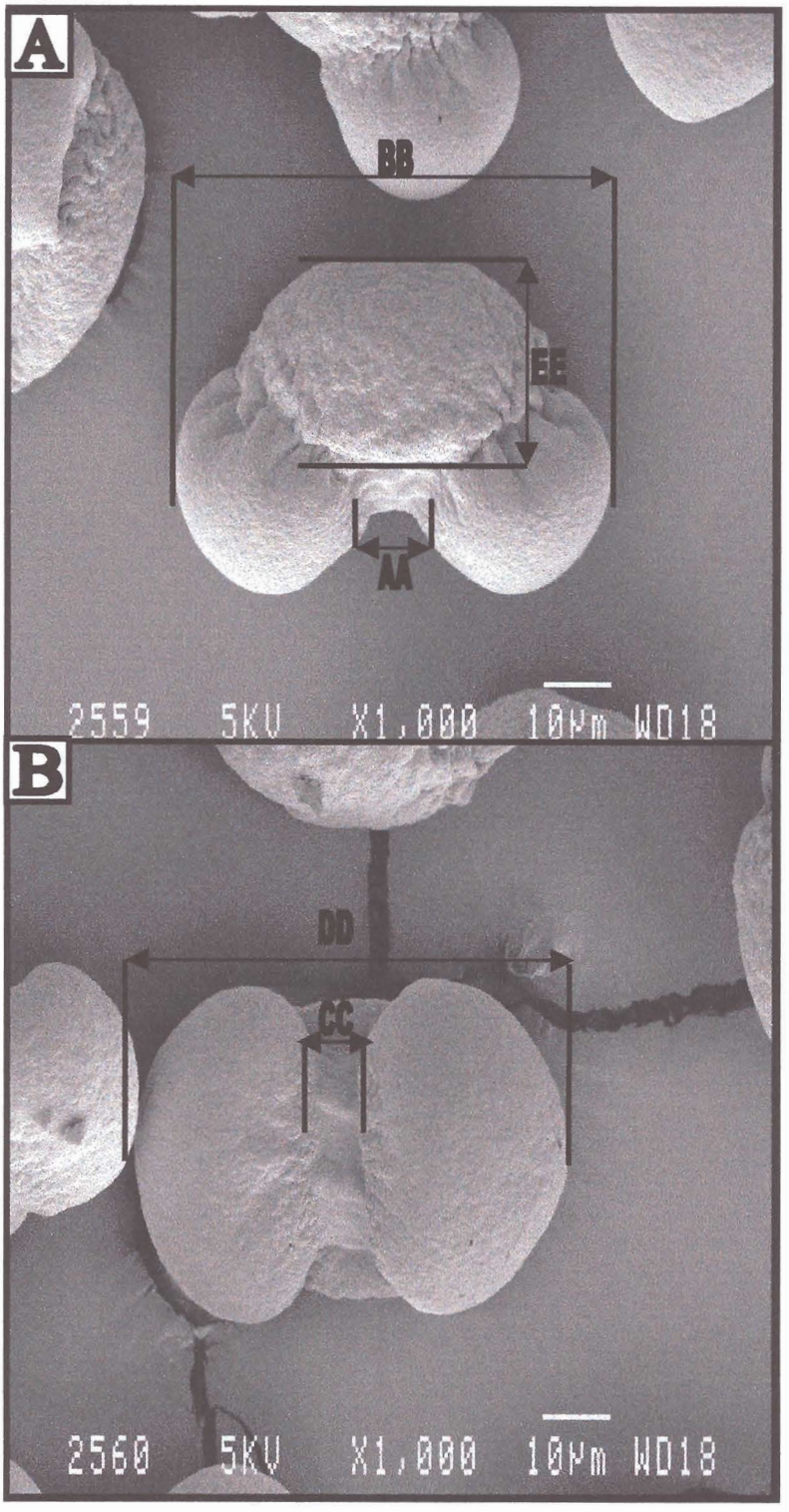

Figure 3.1. Five dimensions measured for each of the eighteen *P. caribaea* pollen types. (A). Side on view facilitating the measurement of dimensions AA, BB and EE. (B). Distal orientation facilitating measurement of dimensions CC and DD.

3.2.4.2 Transmission electron microscopy (TEM)

Seven *P. caribaea* pollen types (AP 30, AP 128, AP 168, AP 294, Pch 23, Pch 29 and Pch 53) were randomly selected and prepared for transmission electron microscopy as described in section 3.2.3.

3.2.5 Statistical analysis of *P. caribaea* germination vs. morphology

A number of questions arose from the cursory examination of the dimensional and germination data that had been obtained for the eighteen *P. caribaea* pollen types. In Table 3.2 these questions and the statistical analytic techniques that were used to investigate them are summarized.

Table 3.2. Statistical analytic techniques used to investigate the questions relating to the eighteen *P. caribaea* pollen types (SAS / STAT Users guide, 1989)

Question	Statistical technique
Are the eighteen types significantly different?	• Analysis of variance (ANOVA)
Is there an association between the dimensions of the types and their germination rate?	• Regression: Germination = dependent variable
Would the sum of the dimensions provide a reliable size variable?	• Cronbach coefficient alpha
If the sum of the dimensions is not reliable, could another aggregation technique help?	• Principle component analysis
Do the dimensions allow a clustering of the eighteen types into separate groups?	• Median Hierarchical cluster analysis • Ward's minimum variance cluster analysis

3.3 Results

3.3.1 Influence of environmental factors on *P. caribaea* pollen viability

The germination data displayed a direct correlation between pollen viability and the age of the *P. caribaea* pollen, the temperature and humidity at which the pollen was stored and the developmental stage of the pollen cone at harvesting. Highest germination percentages were observed the shorter the duration of storage (i.e. the fresher the pollen, Figure 3.2) and the lower the temperature at which the pollen was stored (Figure 3.3). High germination percentages were also observed for pollen that had been stored at room temperature and at low relative humidity (Figure 3.3).

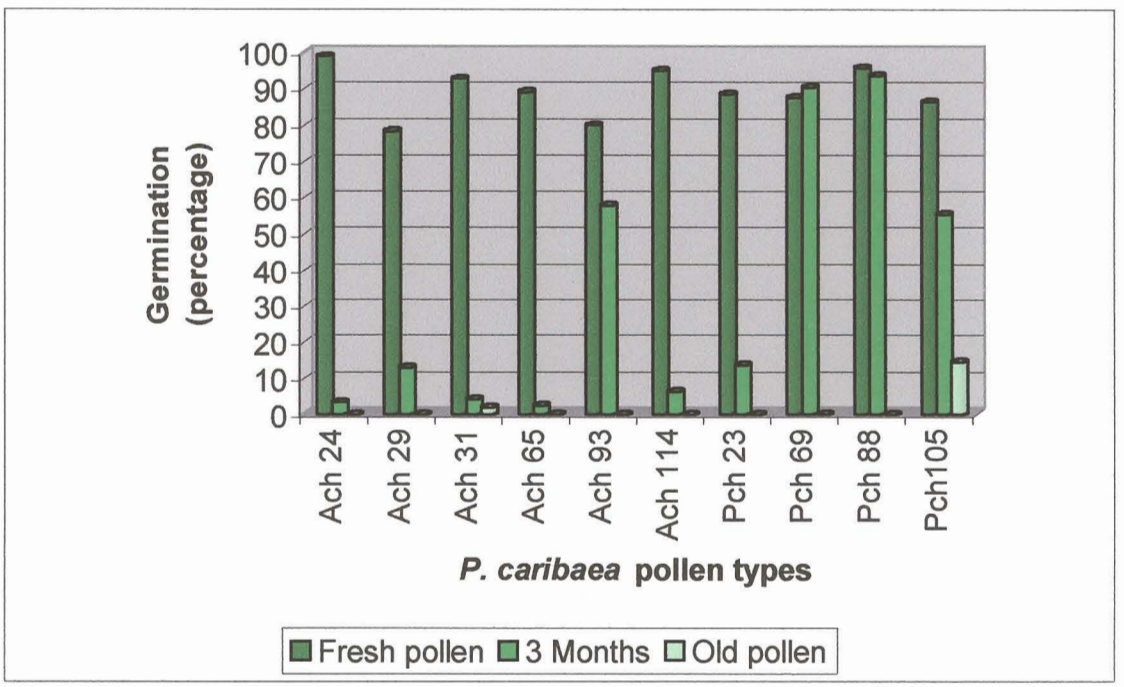

Figure 3.2. Germination percentages of *P. caribaea* pollen stored for various periods of time.

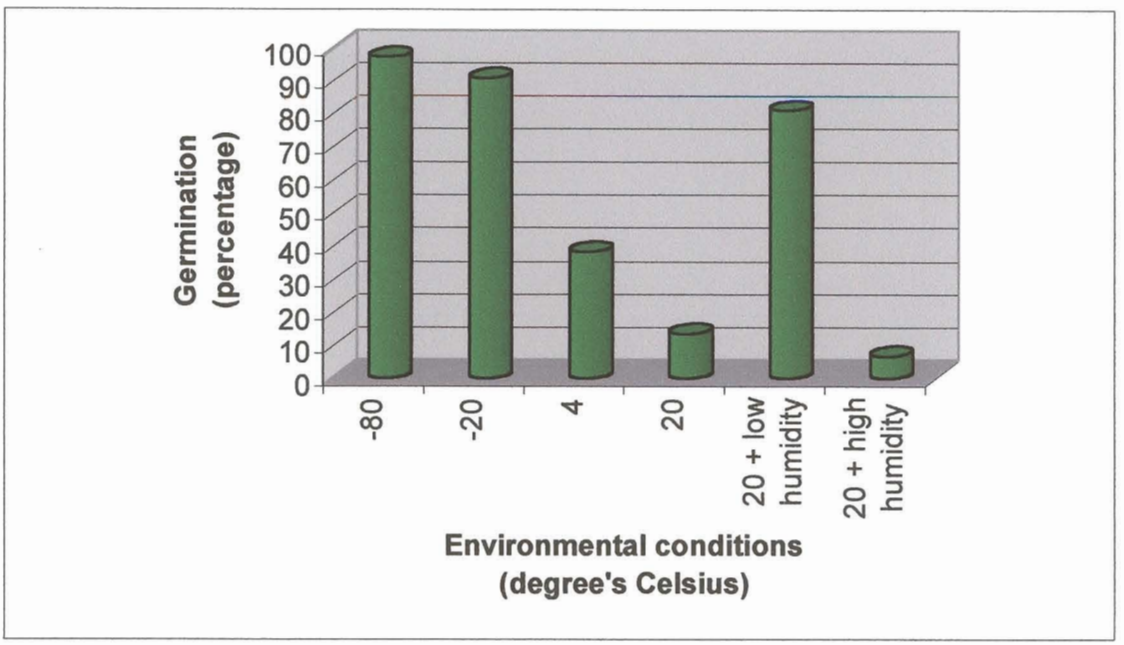

Figure 3.3. Germination percentages of *P. caribaea* pollen stored at various temperatures and humidity.

A correlation between the developmental stage of the pollen cone at the time of harvesting and pollen viability was also found to exist. High germination percentages were observed for the mature (medium and old) pollen cones and lowest germination percentages for young, immature pollen cones (Figure 3.4). The variable results obtained for the study on the effects of collection time on pollen viability indicated that this factor had little effect on the germination of the *P. caribaea* pollen. The results of this study were subjected to statistical analysis and were found to be significant.

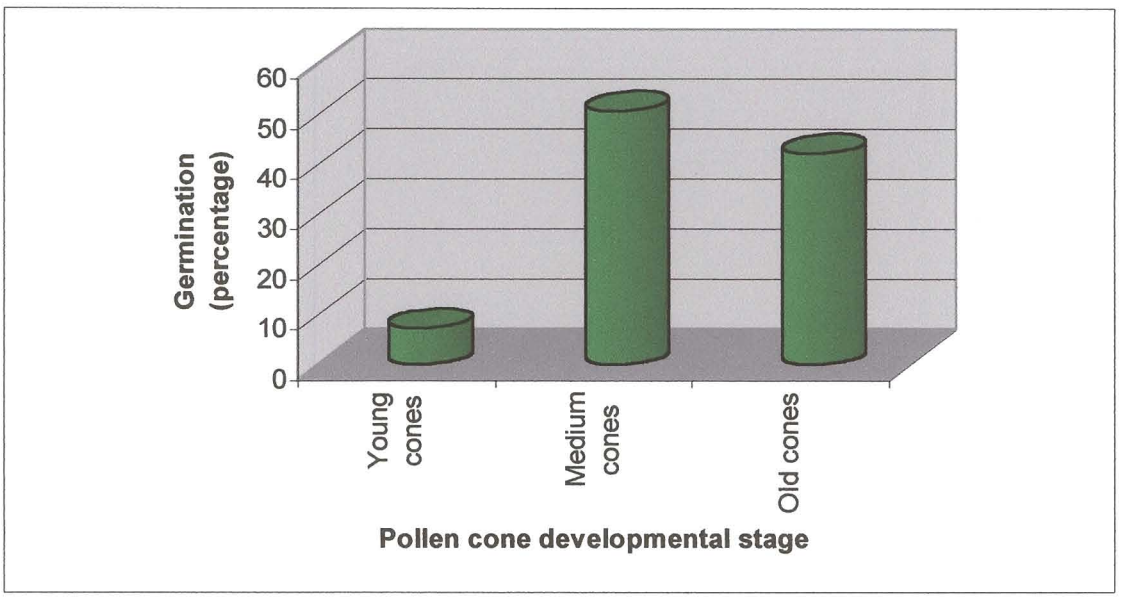

Figure 3.4. Influence of pollen cone developmental stage on germination rate of *P. caribaea* pollen.

3.3.2 The influence of environmental factors on the ultrastructure of *P. caribaea* pollen.

The results of the TEM investigation on the influence that environmental conditions exert on the ultrastructure of *P. caribaea* pollen were found to be variable and generally uninformative. No clear correlation was found which could link poor pollen viability with the time of harvest, the pollen cone developmental stage or even the temperature at which the pollen was stored.

3.3.3 *P. caribaea* pollen morphology

3.3.3.1 Scanning electron microscopy (SEM)

Based upon the SEM pictures it is possible to describe the general structure of *Pinus caribaea* pollen as being large, winged grains, which are strongly polarized, with proximal and distal regions. The wings are hemispheric, situated in the distal region and border on the germ furrow (Figure 3.5). The surface of the cap is sculptured, while the surface of the wings tends to be porous or pitted (Figure 3.6). *Pinus* pollen was similarly described by both Tomlinson (1994) and Pardi *et al.* (1996). The average size of the *P. caribaea* pollen based on the five dimensions, as described in Figure 3.1, is given in Table 3.3.

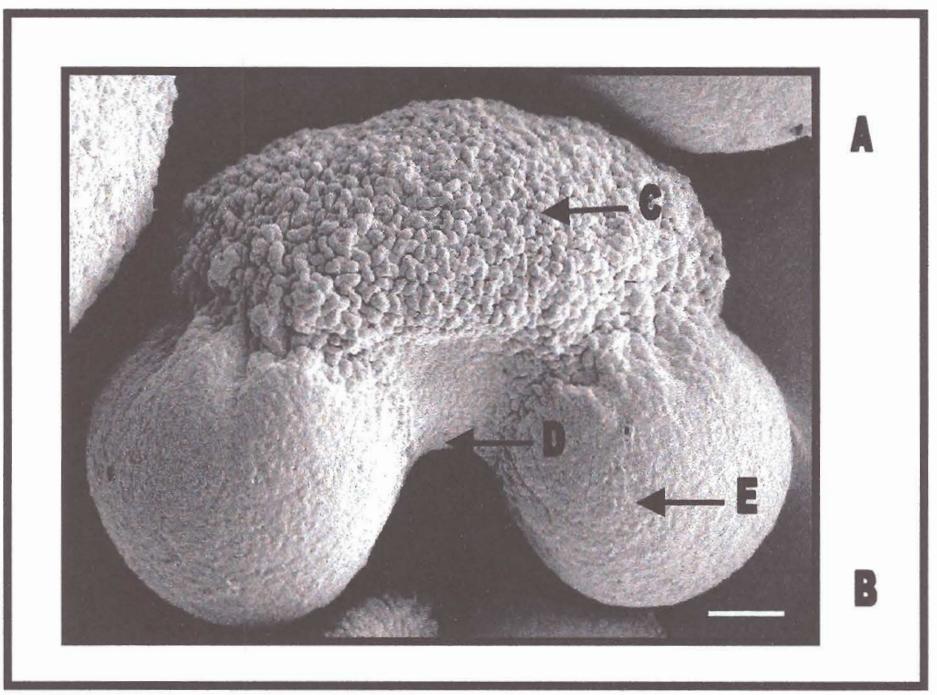

Figure 3.5. SEM picture of the general structure of *P. caribaea* pollen. (A) Proximal region. (B) Distal region. (C) Cap. (D) Germ furrow. (E) Hemispheric wing. 1 000 X Magnification, 10 μm Scale bar.

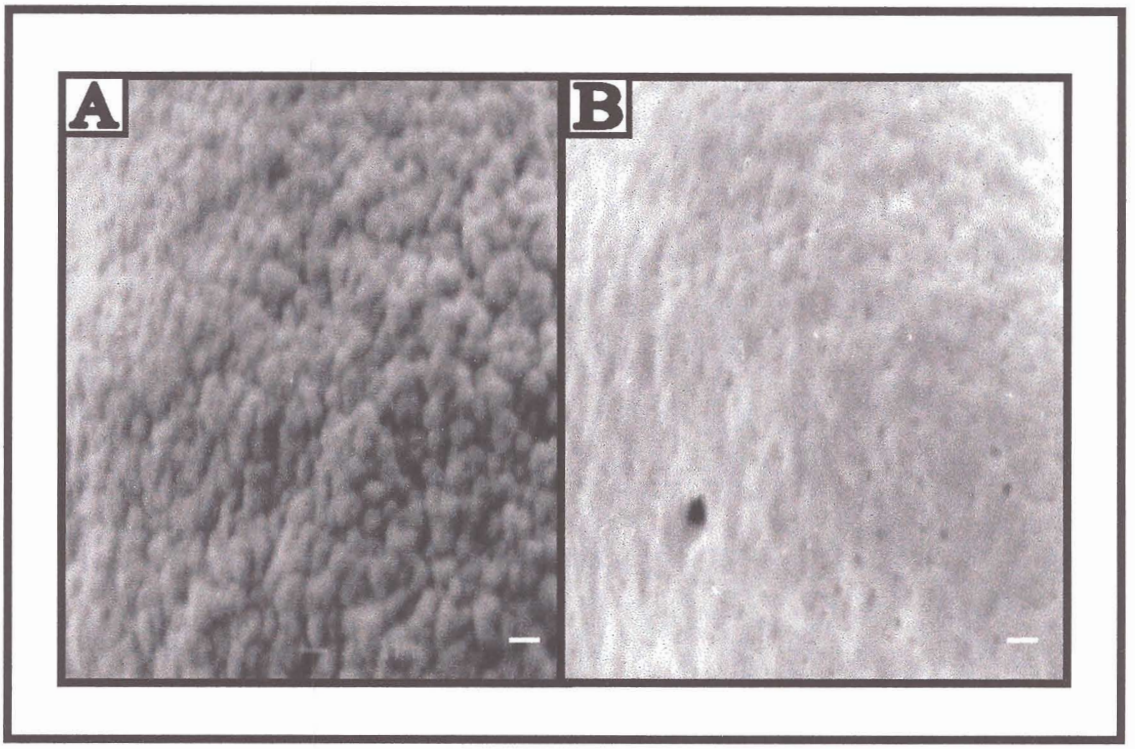

Figure 3.6. SEM pictures of the surface of *P. caribaea* pollen. (A) Sculptured surface of the pollen cap. (B) Pitted surface of the pollen wings. 2 000 X Magnification, 1μm Scale bar.

Table 3.3. Average size of *P. caribaea* pollen

	Dimensions in micrometers (μm)				
	AA	**BB**	**CC**	**DD**	**EE**
Mean	7.82	66.81	8.24	60.46	19.7
Standard deviation	1.96	3.42	2.26	3.94	1.8

3.3.3.2 Transmission electron microscopy (TEM)

The results of the TEM investigation correlated with the general ultrastructure of *Pinaceae* pollen grains as described by Tomlinson (1994) and Pardi *et al.* (1996). Various features of a mature *Pinus* pollen grain can be distinguished in the TEM micrographs. In Figure 3.7 features such as the intine, exine and endexine of the cell wall can be distinguished. The gas space in the wings and the germ furrow can also be clearly distinguished. The cell wall of the wings consists of a thin exine layer with many fingerlike projections that project inwards (Figure 3.8). The continuous exine is thicker in the proximal region, becoming thinner in the distal region from which the pollen tube will arise. The exine is made up of two distinct layers namely the endexine and the ektexine. The ektexine in turn is made up of three parts namely the footlayer, tectum and infratectum. In Figure 3.9 the TEM micrograph of the cell wall ultrastructure is given.

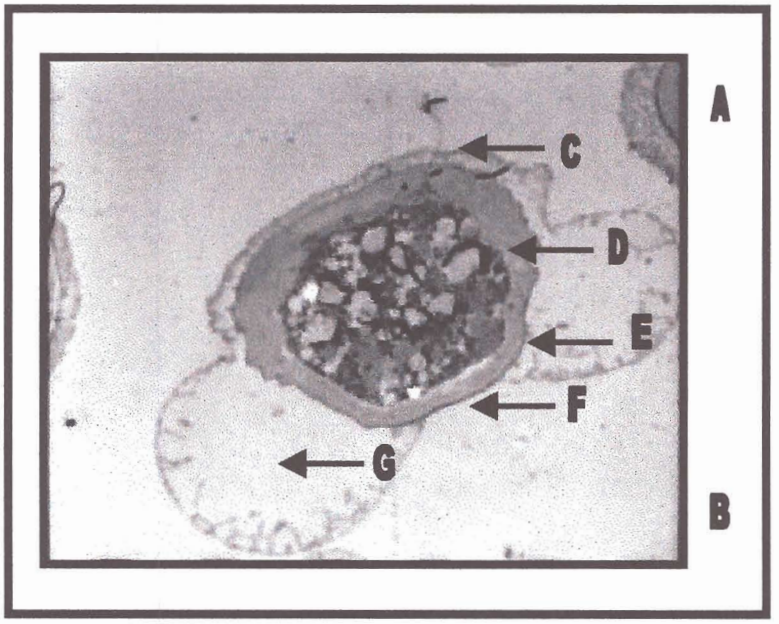

Figure 3.7. TEM micrograph of the general ultrastructure of *P. caribaea* pollen. (A) Proximal region. (B) Distal region. (C) Exine. (D) Intine. (E) Endexine. (F) Germ furrow. (G) Air space in wing. 360 X Magnification.

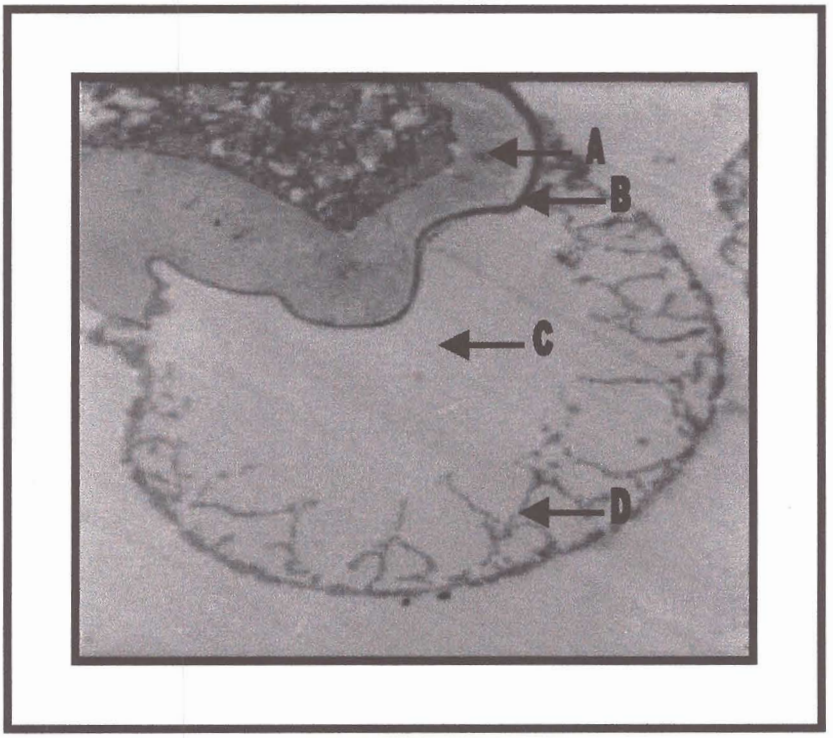

Figure 3.8. TEM micrograph of the ultrastructure of the *P. caribaea* pollen wing. (A) Intine. (B) Endexine. (C) Air space of wing. (D) Fingerlike projections of the exine. 870 X Magnification.

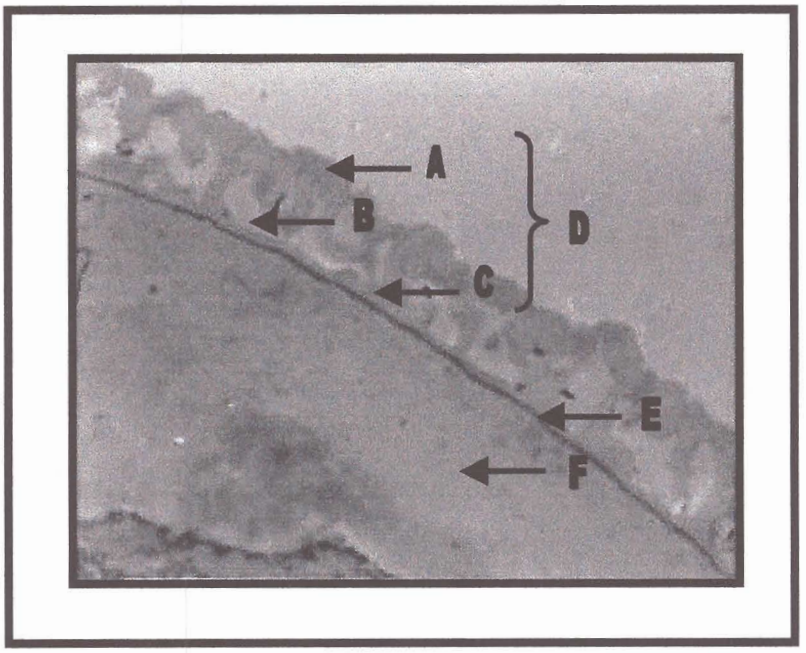

Figure 3.9. TEM micrograph of the ultrastructure of the *P. caribaea* pollen cell wall. (A) Tectum. (B) Infratectum. (C) Footlayer. (D) Ektexine. (E) Endexine. (F) Intine. 2 650 X Magnification.

3.3.4 Statistical analysis of *P. caribaea* germination vs. morphology

Using the UTHSCSA *Image Tool* programme the dimensional data pertaining to the eighteen *P. caribaea* pollen types was generated. This dimensional data in combination with the germination data (Figure 3.2) was then subjected to statistical analysis (SAS / STAT Users guide, 1989).

The first question investigated related to whether the eighteen pollen types could be shown to be significantly different, based upon the dimensional data. The results of the ANOVA (Appendix B) showed that the term for differences between the eighteen pollen types was highly significant (Pr = 0.0001).

The next question was to determine whether an association existed between the dimensional measurements and the germination rates of the eighteen *P. caribaea* pollen types. The following function was fit to the data:

$$Y_{Germination} = \beta_1 X_{AA} + \beta_2 X_{B\beta} + \beta_3 X_{CC} + \beta_4 X_{DD} + \beta_5 X_{EE}$$

where Y is the observed germination, β_i are the regression coefficients and X_{AA} to X_{EE} are the dimensional measurements, as described in Figure 3.1. This model was found to be significant at $Pr < 0.05$ and the correlation coefficient squared was 0.8668 (i.e. 86% of variation is determined by the model).

The third question involved an examination of the sum of the dimensional measurements for the eighteen pollen types. The analysis for consistency, which was done using the Cronbach coefficient (alpha), was found to be less than 0.7. Thereby indicating that the sum of the dimensional measurements was not representative of the size of a specific *P. caribaea* pollen type.

The forth point of interest was to determine the way in which the dimensional measurements aggregated. A principle component analysis (PCA) was therefore done. The results of the PCA showed that the first principle component (PC) is dominated by factor 1 (EE), the second by factor 2 (AA) and the rest by factors 3, 4 and 5 (DD, BB and CC, respectively) (Table 3.4).

Table 3.4. Results of PCA – Variance explained by each factor

Dimensional measurement	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
AA	0.07	**0.93**	0.16	0.22	0.24
BB	0.19	0.22	0.18	**0.93**	0.13
CC	0.08	0.24	0.27	0.13	**0.92**
DD	0.08	0.16	**0.93**	0.19	0.26
EE	**0.99**	0.06	0.07	0.17	0.07

The multiple regression of the scores from the PC's representing the dimensions AA, BB, CC, DD and EE as predictors of germination were found to be highly significant ($Pr < 0.05$) and the regression coefficients (correlation coefficient squared = 0.8691) were also

highly significant. These variables therefore contribute jointly to the size of the pollen grain and they are good predictors of germination.

The next step was to look whether it would be possible to cluster the *P. caribaea* pollen types. Two different clustering techniques were investigated. These techniques were Median hierarchical cluster analysis and Ward's minimum variance cluster analysis.

Using the Median hierarchical cluster analysis it was determined that the eighteen *P. caribaea* pollen types could be divided into three clusters, although the majority of the pollen lines tended to fall into only two clusters (Table 3.5). Using Ward's minimum variance cluster analysis three clusters were chosen in order to keep the data analysis as simple as possible (Table 3.6).

Table 3.5. Results of Median hierarchical cluster analysis

	Cluster 1		**Cluster 2**	
***P. caribaea* pollen lines**	Ach 22	Ach 24	Ach 57	Ach 65
	Ach 29	Ach 31	Ach 93	Ach114
	Ach 33	Ach49	Ach 271	Pch 23
	Ach 91	Acb 60	Pch 69	Pch 88
	Acb 62	Pch 105		

Table 3.6. Results of Ward's minimum variance cluster analysis

	Cluster 1		**Cluster 2**		**Cluster 3**	
***P. caribaea* pollen lines**	Ach 33	Ach 29	Ach 22	Acb 62	Ach 24	Ach 114
	Ach 91	Ach 49	Pch 105		Ach 93	Ach 57
	Ach 31	Acb 60			Ach 65	Ach 271
					Pch 23	Pch 69
					Pch 88	

When the results of the Median hierarchical cluster and Ward's minimum variance cluster analysis were compared basic similarities were observed. Both methods cluster Ach 29, Ach 31, Ach33, Ach 49, Ach 91 and Acb 60 in one group (Group A, Figure 3.10) and Ach 57, Ach 65, Ach 93, Ach 114, Ach 271, Pch 23, Pch 69 and Pch 88 in a separate group (Group B, Figure 3.11). When these results were compared with the original SEM images it was found that all the pollen types clustering in group B displayed intermediate dimensions (Figure 3.12 A & B), while all the pollen types clustering in group A either displayed smaller than average dimensions (Figure 3.12 C & D) or displayed larger than average dimensions (Figure 3.12 E & F). When these results were compared with the germination results obtained it was found that the pollen types which clustered into group B were amongst those types which displayed high germination percentages and those which clustered into group A were amongst those types which displayed low to intermediate germination percentages (Figure 3.13).

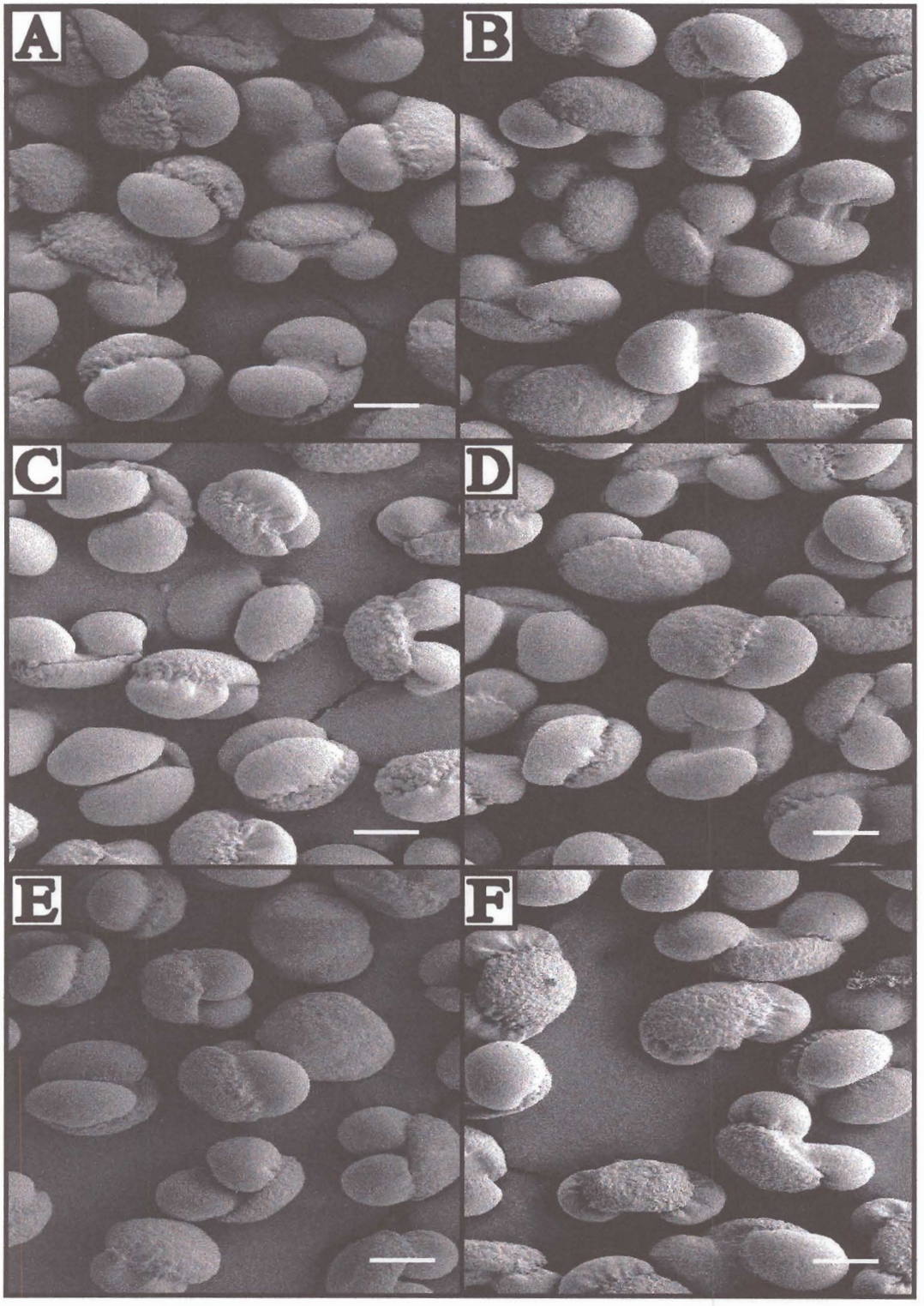

Figure 3.10. *P. caribaea* pollen types clustered into Group A. (A) Ach 29. (B) Ach 31. (C) Ach 33. (D) Ach 49. (E) Ach 91. (F) Acb 60. 250 X Magnification, 100 µm Scale bar.

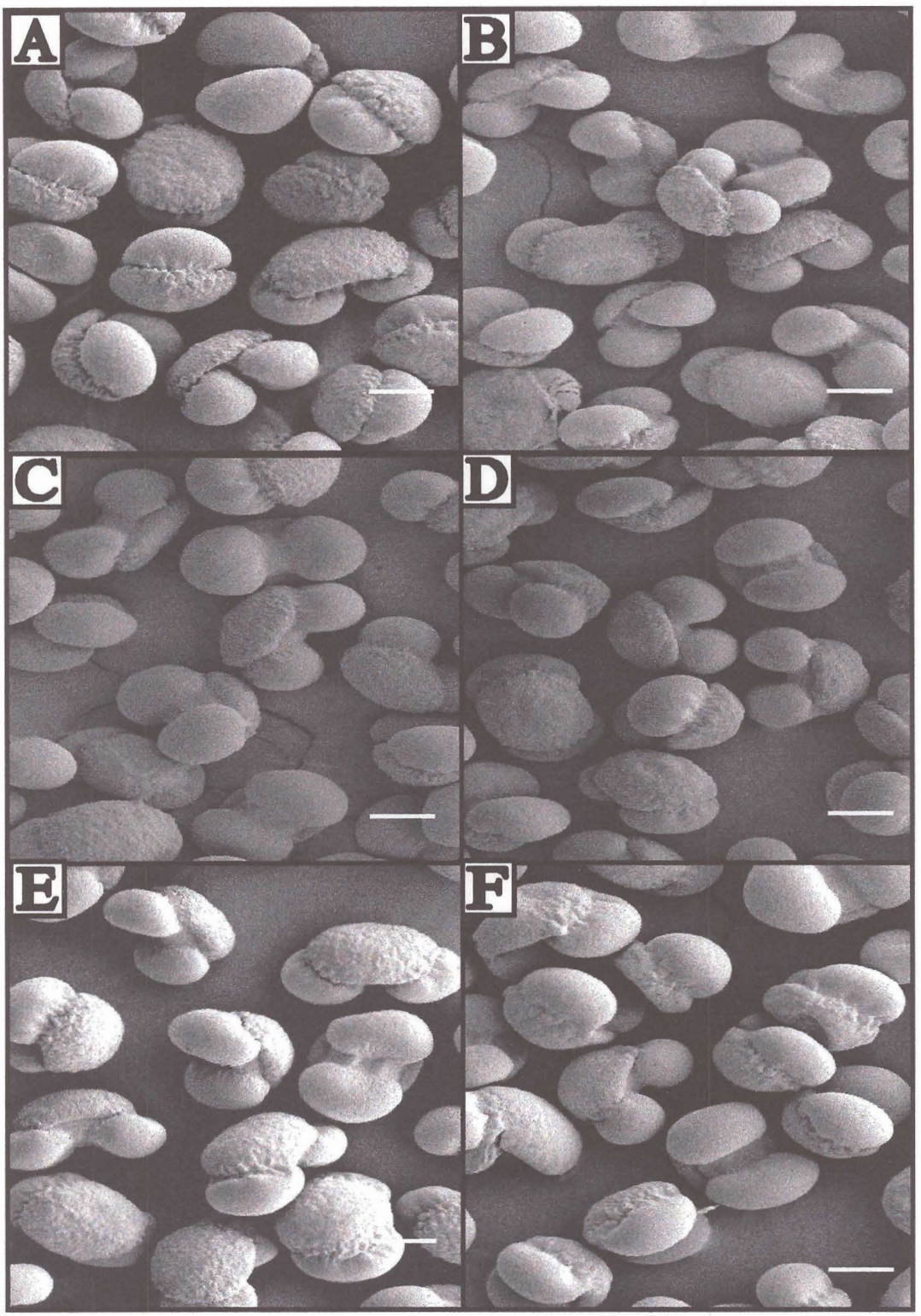

Figure 3.11. *P. caribaea* pollen types clustered into Group B. (A) Ach 57, (B) Ach 65, (C) Ach 93, (D) Ach 114, (E) Ach 271, (F) Pch 88. 250 X Magnification, 100 µm Scale bar.

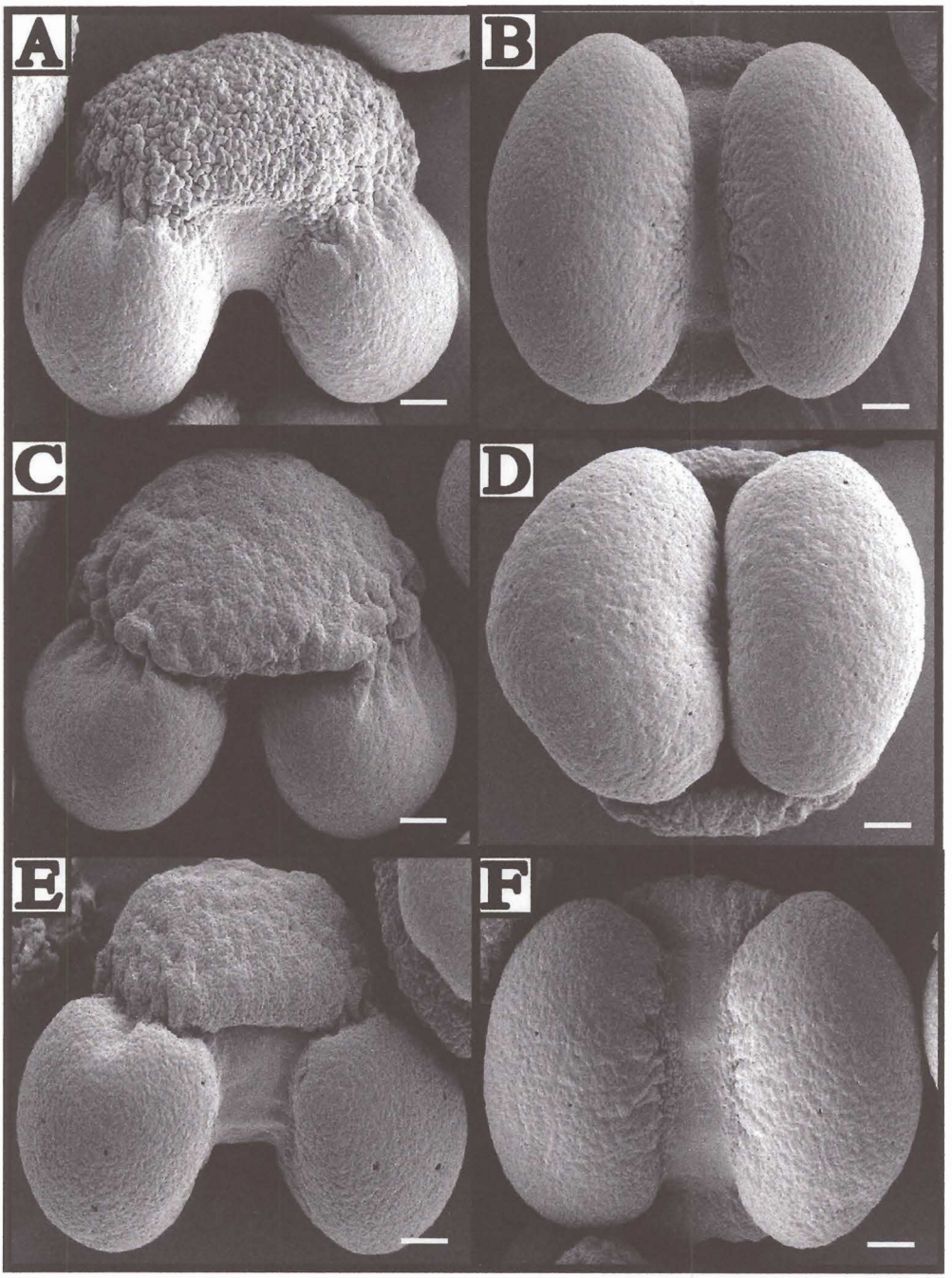

Figure. 3.12. SEM pictures of *P. caribaea* pollen clusters. (A). SEM picture of pollen with intermediate germ furrow in side view. (B). SEM picture of pollen with intermediate germ furrow in distal view. (C). SEM picture of pollen with narrow germ furrow in side view. (D). SEM picture of pollen with narrow germ furrow in distal view. (E). SEM of pollen with wide germ furrow in side view. (F). SEM of pollen with wide germ furrow in distal view. 1 000 X Magnification, 10 μm Scale bar

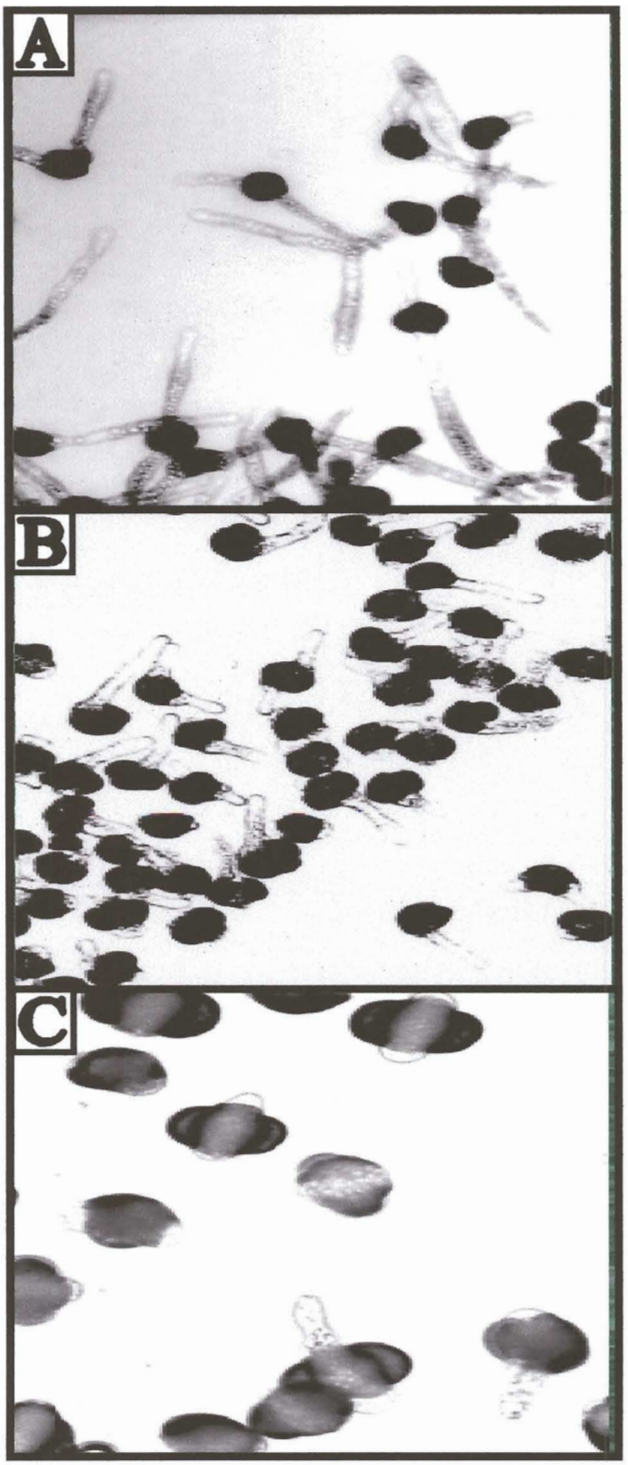

Figure 3.13. Light microscope pictures of *P. caribaea* pollen germination. (A) High germination (10 X Magnification). (B) Intermediate germination (10 X Magnification). (C) Low germination (20 X Magnification).

3.4 Discussion

Pollen viability is one of the most important limiting factors of reproductive success (Singh, 1978). The viability of gymnosperm pollen is very vulnerable due to its airborne dispersal and slow maturation (Wright, 1976), which facilitates the exposure of the pollen to various environmental pollutants (Pardi *et al.*, 1996). The viability of the pollen is, however, not only determined by atmospheric agents, but also by internal factors such as lipid (Van Bilsen *et al.*, 1994) and carbohydrate reserves (Pacini, 1996) and the duration and conditions under which it is stored (as found in this study).

The environmental conditions to which the pollen was exposed played a major role in germination and pollen viability. The results indicated that there was a direct decrease in viability with an increase in the age of the pollen, temperature at which the pollen was stored and exposure to high humidity during storage. These results correlate with those described by Van Bilsen *et al.* (1994) who found that the higher the relative humidity (i.e. 75% vs. 40%) during pollen storage the lower the viability. Van Bilsen *et al.* (1994) linked this decrease in viability to the deesterification of phospholipids that resulted in the degradation of membrane integrity. The pollen cone developmental stage at the time of harvesting was also found to influence viability as the highest germination percentages were observed for medium pollen cones. This would suggest that medium cones are more likely to contain mature, but fresh pollen. It is therefore proposed that the decrease in viability observed with the increase in pollen cone age is due to the pollen having passed it's optimal condition and entering into the declining viability phase. Based on these

results recommendations were made to SAFCOL, suggesting ways in which the viability of *P. caribaea* pollen could be prolonged. These recommendations are summarized in Table 3.7.

Table 3.7. Summary of recommendations pertaining to optimization of *P. caribaea* pollen storage conditions

Recommendations
The *P. caribaea* pollen should be:
• Dried prior to storage;
• Stored in small quantities;
• Stored at the lowest possible temperature;
• Stored in a dry place;
• Stored in sealed containers;
• Once opened, all left over pollen should be discarded.

The results of the TEM investigation on the influence that environmental conditions exert on the ultrastructure of *P. caribaea* pollen were found to be variable and generally uninformative. These results would seem to correlate with those obtained for the study on the effect of environmental factors on viability as those results were also found to be variable and uninformative. Of the environmental factors tested only temperature, humidity and the duration of storage were found to influence pollen viability. These differences in germination are, however, not clearly reflected in the ultrastructure of the

P. caribaea pollen. It is therefore proposed that a study needs to be done in which the effects of temperature, humidity and the duration of storage on the ultrastructure of *P. caribaea* pollen are the main focus. Such a study would help determine whether these results are a true reflection of the effects of environmental conditions on the ultrastructure of *P. caribaea* pollen or whether they are just a selectively skewed version of what is really happening.

According to Snow and Spira (1991) and Stephenson *et al.* (1988) only the most vigorous pollen tubes achieve fertilization under conditions of intense pollen competition. Pollen competition can occur when more pollen is deposited on the stigma than is required for fertilization of all the flower's ovules (Havens, 1994). The deposition of excess pollen occurs regularly during the controlled pollination of the *P. elliottii* ovules with the *P. caribaea* pollen. It would therefore be advantageous to only use the most vigorous *P. caribaea* pollen, which displays the fastest germination rate and pollen tube growth rate, for the controlled crosses.

As a cautionary note it must be included that pollen performance rates can not only be contributed to genetic factors, but that non-genetic factors such as: (i) temperature during pollination (Elgersma *et al.*, 1989), (ii) the location of the pollen on the stigma (Thomson, 1989) and (iii) the competitive environment within the style (Cruzan, 1986 and 1990), may play an important role on a seasonal basis (Charlesworth and Charlesworth, 1992). According to Delph *et al.* (1997) if the plant is exposed to unfavorable environmental conditions it may lead to a reduction in the resources available for pollen production and

ultimately to differences in the quality and / or the quantity of the storage products that are produced within those pollen grains.

In 1994 Tomlison described the general structure of a pollen grain based on five contributing factors. These factors included the size, overall shape, surface sculpture, sporoderm ultrastructure and the presence or absence of obvious apertures. The combined SEM and TEM investigations provide all the necessary information in order to be able to describe all these factors relating to *P. caribaea* pollen. The SEM investigation provided valuable information pertaining to four of the five factors, namely size, overall shape, surface sculpture and the presence of an obvious aperture in the form of the germ furrow. The SEM pictures were, however, limited to an external view of these factors using the scanning electron microscope. The TEM sections on the other hand provided the means necessary in order to investigate the sporoderm ultrastructure, as well as the other four factors on an internal level using the transmission electron microscope. In 1996 Pardi *et al.* similarly described the general structure of *Pinus pinea* and *Pinus pinaster* pollen.

Generally the results of the statistical analysis indicated that a highly significant relationship existed between germination and morphology of the *P. caribaea* pollen. To this end factors such as whether: (i) the eighteen pollen types were significantly different; (ii) an association between the dimensions of the pollen types and their germination existed; (iii) the sum of the dimensions would provide a reliable size variable; and (iv) the dimensions would allow the eighteen pollen types to be clustered into groups were investigated.

The first factor was investigated by doing an ANOVA of the dimensional data available. The results of the ANOVA indicated that the term for differences between the eighteen pollen types was highly significant, which suggests that the eighteen pollen types are significantly different from one another when their dimensions are taken into account.

Using regression a good association was found to exist between the dimensional measurements and germination of the pollen types, as it was possible to determine up to 86% of the variation. The association was therefore found to be highly significant.

The Cronbach coefficient (alpha) was used to determine whether the sum of the dimensions would provide a reliable size variable. The sum can only be accepted if the measures of the five dimensions add consistently to the total (i.e. their individual measurement error must be very small) (Steyn *et al.*, 1994). The analysis for consistency (i.e. the Cronbach coefficient) indicated that this was not the case and the sum of the dimensional measurements was therefore not representative of the size of a specific *P. caribaea* pollen type.

A principle component analysis (PCA) was carried out in order to determine the way in which the dimensional measurements aggregated. During a PCA the five dimensions are combined in a linear function (five linear functions are formed in this case), called a principle component (Steyn *et al.*, 1994). Differences in the size of the coefficients of the functions (PC's) indicate the relative importance of the dimensions. The results of the

PCA showed that the first PC was dominated by factor 1 (EE) and the second by factor 2 (AA) this means that of the five factors, factor 1 and factor 2 carry the most weight when the dimensions of the pollen grains are determined (Table 3.4).

The PC's represent independent aggregations of the dimensions and allow aggregate values, called scores, to be calculated for each entry (Steyn *et al.*,1994). These scores were used to regress on germination. The multiple regression of the scores from the PC's representing the dimensions AA, BB, CC, DD and EE as predictors of germination were found to be highly significant and up to 86.91% of the variation could be determined. It can therefore be concluded that these variables contribute jointly to the size of the pollen grain and they are good predictors of germination. These results clearly confirm that there is an association between morphology and germination, but also emphasize that the association is not a simple one.

Having determined that the eighteen *P. caribaea* pollen types were significantly different from one another (with reference to all five dimensions) and that there was a highly significant association between the dimension of the pollen and its germination rate, the next step was to look whether it would be possible to cluster the pollen types. Two different clustering techniques were investigated. These techniques were Median hierarchical cluster analysis and Ward's minimum variance cluster analysis. Using the Median hierarchical cluster analysis it was determined that the eighteen *P. caribaea* pollen types could be divided into three clusters, although the majority of the pollen types fall into only two clusters (Table 3.5). Using Ward's minimum variance cluster analysis

three clusters were chosen in order to keep the data analysis as simple as possible (Table 3.6). When the results of the Median hierarchical cluster analysis and Ward's minimum variance cluster analysis were compared it was found that many of the clusters overlapped, thereby confirming the clustering of the *P. caribaea* pollen types into two distinct groups (Group A, Figure 3.10 and Group B, Figure 3.11).

When the results of the cluster analysis were compared with the original SEM images and the germination results obtained it was found that all the pollen types clustering into group B displayed intermediate dimensions (Figure 3.12 A & B) and high germination percentages (Figure 3.13). Conversely the pollen types clustering into group A displayed either smaller than average dimensions (Figure 3.12 C & D) or larger than average dimensions (Figure 3.12 E & F) and low to intermediate germination percentages (Figure 3.13). This would suggest that a highly significant association seems to exist between *P. caribaea* pollen morphology and viability.

3.5 Conclusion

The results of the investigation on the effect of environmental conditions indicated that long term pollen viability can be maintained if the pollen is stored under specific conditions. The results indicated that only mature pollen from intermediate cones should be harvested and that if temperature and humidity are controlled during storage, then it should be possible to maintain *P. caribaea* pollen viability.

The highly significant association found to exist between *P. caribaea* pollen morphology and viability strongly suggests that a dimensional screening step would be beneficial during the selection of the pollen parent. This screening step would reduce the chances of inferior *P. caribaea* pollen parents from being used in crosses during hybrid production and therefore from entering into the hybrid performance trials in general. The correct storage of the *P. caribaea* pollen would ensure the viability of the pollen used in the cross and therefore result in increased pollination which should in turn result in increased fertilization.