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Germ-furrow morphology and storage conditions determine the degree of viability of *Pinus caribaea* pollen

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This study has found a correlation between *Pinus caribaea* pollen morphology and viability. Eighteen different *P. caribaea* pollen families were screened to determine the effect of environmental conditions during storage on germination. 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. Scanning electron microscopy was used to investigate the dimensions of the 18 families. Upon statistical analysis of the dimensional data, the families were found to group into three clusters. Pollen families displaying narrow germ furrows clustered with those displaying wide germ furrows, while those pollen families displaying intermediate germ furrows clustered into a second group. When the clusters were compared with the germination data obtained it was found that the pollen families displaying highest germination percentages fell into the intermediate cluster, while the pollen families displaying low to intermediate germination percentages fell into the narrow/wide cluster.

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 favoured 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, Komatiland Forests Research, Research: Sabie, PO Box 574, Sabie, ZA 1260, pers. comm.); there is therefore a need to optimise the long-term storage conditions of the *P. caribaea* pollen. The second major problem is one of incompatibility between *P. elliottii* and *P. caribaea,* which results in low seed set and a low number of viable hybrid seeds (SAFCOL 1996).

Pollen viability is perhaps the most important factor that influences reproduction in plants. Pollination and fertilisation 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 a direct correlation 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, such as oils, sugars and starches, must be provided by the reserves in the pollen grain itself, as only limited additional material may be absorbed from the surrounding tissue (Willemse 1968, Baker and Baker 1979, Bellani *et al*. 1985, Delph *et al*. 1997). During the heterotrophic phase polysaccharide reserves in the pistil are mobilised 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 was to investigate the influence of various environmental factors on the viability of *P. caribaea* pollen and to use this information to optimise the storage conditions during the three-month storage period. The second was to investigate the possible relationship between the morphology and viability of *P. caribaea* pollen.

Materials and Methods

Plant material

Pollen obtained from 18 *Pinus caribaea* families, provided by SAFCOL, South Africa, were used in this study. Pollen from the different families was classified into pollen groups. The 18 families were: 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.

Germination

Ten of the 18 *Pinus caribaea* pollen families (Ach 24, Ach 29, Ach 31, Ach 65, Ach 93, Ach 114, Pch 23, Pch 69, Pch 88 and Pch 105) were used in 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. The germination percentages of fresh pollen (1- to 2-weeks-old), pollen that had been stored for three months, aged pollen (stored for ca. one year), pollen that had been stored at various temperatures and humidity (4°C, 20°C and 20°C + low humidity (>30%), 20°C + high humidity (<70%), -20°C and -80°C), and that of young (ca. 10mm), intermediate (ca. 20mm) and mature (ca. 35mm) pollen cones were investigated. To facilitate germination, the pollen was placed on 3% agar containing 10% sucrose and left at room temperature for 72h, as described by Wright (1976). After 72h, microscope slides were prepared and the samples screened 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 10 random microscope fields per pollen type.

Scanning electron microscopy

The *P. caribaea* pollen families were prepared for scanning electron microscopy (SEM) according to the procedures outlined by Coetzee and Van der Merwe (1999). The stubs were then examined using a Jeol Winsem JSM 6400 SEM at 5kV. Twenty images, in a side-on view and 20 in a distal view of each of the 18 pollen families 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 1: 1 and 2).

Transmission electron microscopy

Pinus caribaea pollen Pch 23 was prepared for transmission electron microscopy in order to investigate the general ultrastructure. The pollen was fixed in 3% phosphate-buffered glutaraldehyde and postfixed in 2% osmium tetroxide. The pollen was stained with 0.5% uranyl acetate and dehydrated in an alcohol series prior to embedding in Spurr's low-viscosity resin (Spurr 1969). The embedded pollen was sectioned using the LKB Ultratome III and the sections were subsequently stained with 5% uranyl acetate, followed by lead citrate (Reynolds 1963), and examined using a Phillips CM 100 electron microscope at 60kV.

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

Using the UTHSCSA Image Tool programme the dimensional data pertaining to the eighteen *P. caribaea* pollen families was generated. These dimensional data in combination with the germination data were then subjected to statistical analy-

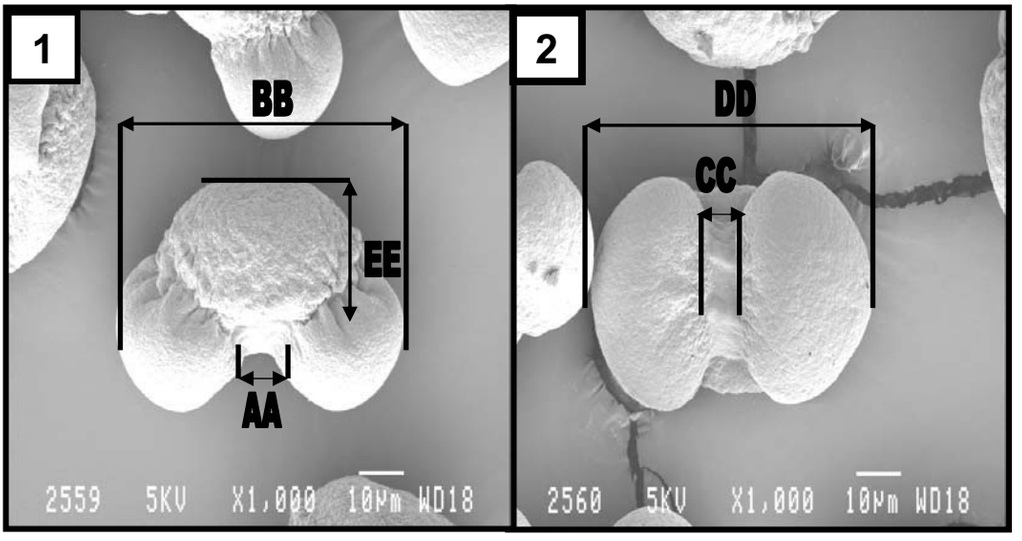

Figure 1: Five dimensions for each of the 18 *P. caribaea* pollen families. **1** Side-view, facilitating measurement of dimensions AA, BB and EE. **2** Distal view, facilitating measurement of dimensions CC and DD. Scale bar = 10µm

sis (SAS / STAT Users Guide, 1989).

Various statistical techniques were used to investigate questions that arose from the cursory examination of the dimensional and germination data. These statistical techniques included: i) Analysis of variance (ANOVA); ii) Regression, where germination was the dependent variable; iii) Cronbach coefficient-alpha; iv) Principal components analysis (PCA); and v) Median hierarchical and Ward's Minimum variance cluster analysis (SAS / STAT Users Guide, 1989).

Results

Germination

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 at the shortest duration of storage of the pollen (i.e. the fresher the pollen, ca. 80–99%) at the lowest temperature (ca. 90–97%). High germination percentages (ca. 80%) were also observed for pollen that had been stored at room temperature and at low relative humidity. A correlation between the developmental stage of the pollen cone at the time of harvesting and pollen viability was also found, as high germination percentages were observed for medium (ca. 50%) and old (ca. 42%) pollen cones.

Scanning electron microscopy

Based on SEM micrographs it was possible to describe the general structure of *Pinus caribaea* pollen as large, winged grains, with strongly polarised proximal and distal regions. The wings (Figure 2: E) are hemispheric, situated in the distal region and border on the germ furrow (Figure 2: D). The surface of the cap is sculptured, while that of the wings tends to be pitted. The average size of the *P. caribaea* pollen families based on the five dimensions, as described in Figure 1, is given in Table 1.

Transmission electron microscopy

Various features of a mature *Pinus* pollen grain can be distinguished in the electron micrographs. In Figure 3 (1 and 2) features such as the intine, exine and endexine of the pollen wall can be distinguished, as can the gas space in the wings and the germ furrow. The wall of the wings consists of a thin exine layer with many fingerlike projections that project inwards. 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 foot layer, tectum and infratectum.

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

The first question investigated related to whether the 18 pollen families could be shown to be significantly different, based upon the dimensional data. The results of the analysis of variance (ANOVA) showed that the term for differences between the pollen families was highly significant (P=0.0001).

The next question was to determine whether an association existed between the dimensional measurements and the germination rates of the 18 pollen families? The following function was fitted to the data:

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

where Y was the observed germination, β_1–β_5 were the regression coefficients and X_{AA} to X_{EE} were the dimensional measurements, as described in Figure 1 (1 and 2). This model was found to be significant at P<0.05 and the correlation coefficient squared was 0.8668.

Another point of interest was to determine the manner in which the dimensional measurements aggregated. A principal components analysis (PCA) was therefore done. The results of the PCA showed that the first principal component (PC) was 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 2).

The multiple regression of the scores from the PC's rep-

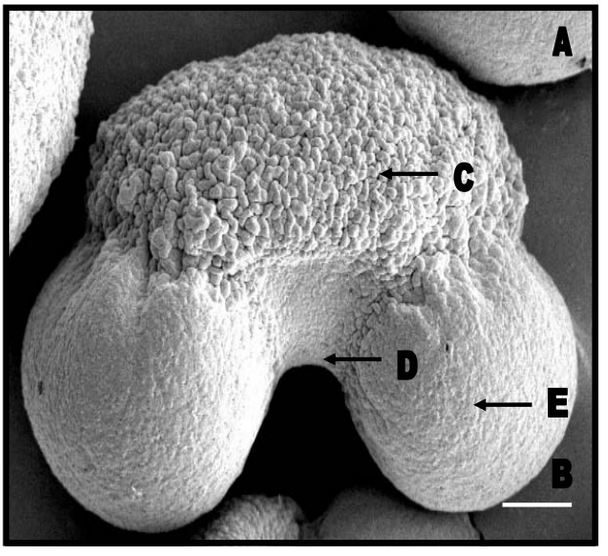

Figure 2: Scanning electron micrograph of the general structure of a *P. caribaea* pollen grain. A, proximal region; B, distal region; C, cap; D, germ furrow; E, hemispheric wing. Scale bar = 10µm

Table 1: Average size of eighteen *P. caribaea* pollen families

	Dimensions in micrometers (µm)*				
	AA	BB	CC	DD	EE
Mean	7.8	66.8	8.2	60.5	19.7
Standard deviation	2.0	3.4	2.3	3.9	1.8

* Dimensional measurements described in Figure 1

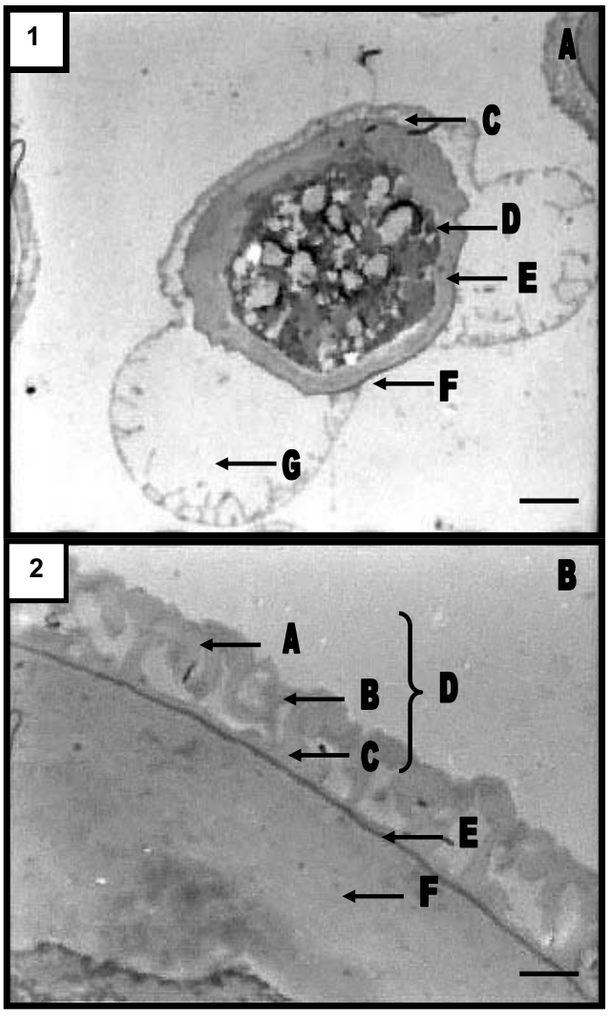

Figure 3: Transmission electron micrographs of *P. caribaea* pollen showing ultrastructure. **1** General ultrastructure. A = proximal region; B = distal region; C = exine; D = intine; E = endexine; F = germ furrow; G = air space of wing. Scale bar = 10µm. **2** Ultrastructure of the pollen wall. A = tectum; B = infratectum; C = foot layer; D = ektexine; E = endexine; F = intine. Scale bar = 73µm

resenting the dimensions AA, BB, CC, DD and EE as predictors of germination were found to be highly significant ($P<0.05$) and the regression coefficients (correlation coefficient squared = 0.8691) was 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 determine whether it is possible to cluster the *P. caribaea* pollen families. Using the Median hierarchical cluster analysis it was determined that the eighteen *P. caribaea* pollen tended to fall into only two clusters (Table 3). Using Ward's minimum variance cluster analysis three clusters were chosen to keep the data analysis as simple as possible (Table 4).

When the results of the median hierarchical cluster and Ward's minimum variance cluster analysis were compared basic similarities were observed. Both methods cluster *P. caribaea* pollen families Ach 29, Ach 31, Ach 33, Ach 49, Ach 91 and Acb 60 in one group (group A) and Ach 57, Ach 65, Ach 93, Ach 114, Ach 271, Pch 23, Pch 69 and Pch 88 in a separate group (group B). When these results were compared with the original SEM images it was found that all the pollen families clustering in group B displayed intermediate dimensions (Figure 4: 1 and 2), while all the pollen families clustering in group A either displayed smaller than average dimensions (Figure 4: 3 and 4) or displayed larger than average dimensions (Figure 4: 5 and 6). When these results were compared with the germination results it was found that the pollen families that clustered into group B were amongst the families that displayed high germination percentages and those that clustered into group A were amongst those displaying low to intermediate germination percentages.

Discussion

Pollen viability is one of the most important limiting factors of reproductive success. The viability of gymnosperm pollen is very vulnerable due to its airborne dispersal and slow maturation (Wright 1976), factors which facilitate the exposure of the pollen to various environmental pollutants (Pardi *et al*. 1996). However, the viability of the pollen is 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

Table 2: Results obtained with PCA-variance explained by each factor

Dimensional measurements*	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

*Dimensional measurements described in Figure 1

Table 3: Results obtained with median hierarchical cluster analysis

	Cluster 1		Cluster 2	
P. caribaea pollen families	Ach 22	Ach 24	Ach 57	Ach 65
	Ach 29	Ach 31	Ach 93	Ach 114
	Ach 33	Ach 49	Ach 271	Pch 23
	Ach 91	Acb 60	Pch 69	Pch 88
	Acb 62	Pch105		

humidity during storage. These results agree with those of 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 de-esterification 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 pollen cones of medium size. This would suggest that medium-sized 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 having entered into the phase of declining viability. Based on these results it is recommended that the pollen should be dried prior to storage. The pollen should be stored in small quantities, in vacuum-sealed containers in a dry place and at the lowest possible temperature.

According to Snow and Spira (1991) and Stephenson *et al.* (1988) only the most vigorous pollen tubes achieve fertilisation under conditions of intense pollen competition. Pollen competition can occur when more pollen is deposited on the stigma than is required for fertilisation of all the megasporophyll'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 for the controlled crosses to use only the most vigorous *P. caribaea* pollen, which display the fastest germination rate and pollen tube growth rate.

Pollen performance rates cannot only be attributed to genetic factors, but non-genetic factors such as temperature during pollination (Elgersma *et al.* 1989), location of the pollen on the nucellus (Thomson 1989), and the competitive environment within the nucellar tissue (Cruzan 1986, 1990) may also play an important role on a seasonal basis (Charlesworth and Charlesworth 1992). According to Delph *et al*. (1997) if the plant is exposed to unfavourable 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.

Generally the results of the statistical analysis indicated that a highly significant relationship existed between germination and morphology of the *P. caribaea* pollen. The results of the PCA showed that the first principal component (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.

The PC's represent independent aggregations of the dimensions and allow aggregate values, called scores, to be calculated for each entry. 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 was found to be highly significant. As much as 86.91% of the variation could be determined by these predictors. These variables, thus contribute jointly to the size of the pollen grain and they are good predictors of germination. The results clearly confirm that there is an association between morphology and germination. The association, however, is not a simple one.

Having determined that the eighteen *P. caribaea* pollen families 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 determine if whether it would be possible to cluster the pollen families. 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 families into two distinct groups, i.e. group A and group B.

When the results of the cluster analysis were compared with the original SEM images and the germination results, it was found that all the pollen families clustering into group B displayed intermediate dimensions and high germination percentages. Conversely the pollen families clustering into group A displayed either smaller than average dimensions or larger than average dimensions and low to intermediate germination percentages. This would suggest that a highly significant association exists between *P. caribaea* pollen morphology and viability.

The results of the investigation on the effect of environmental conditions indicate that long term pollen viability can be maintained if the pollen is stored under specific conditions. 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 paternal parent. This screening step would

Table 4: Results obtained with Ward's minimum variance cluster analysis

	Cluster 1		Cluster 2		Cluster 3	
P. caribaea pollen families	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	

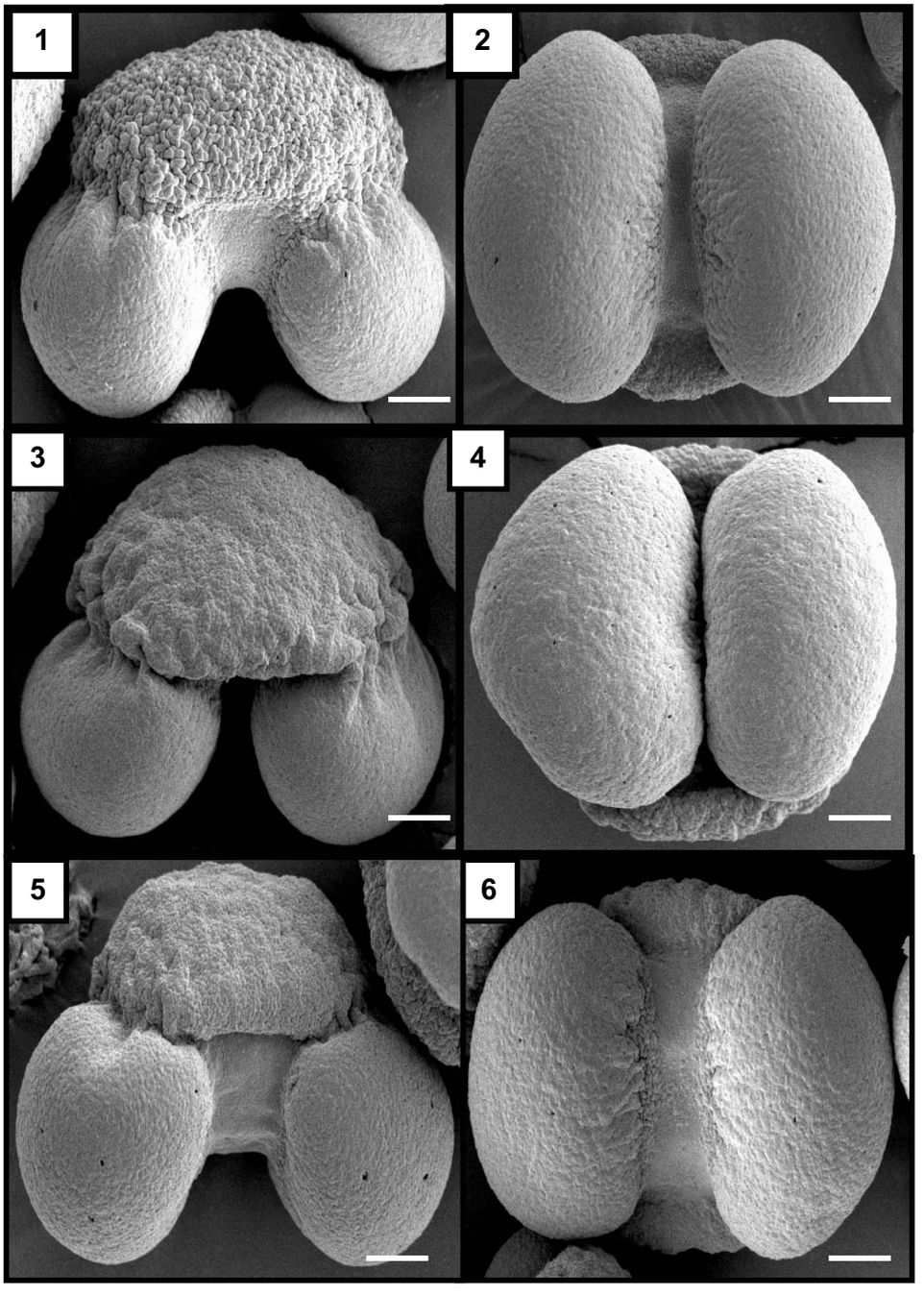

Figure 4: Scanning electron micrograph of *P. caribaea* pollen clusters. **1** Pollen with intermediate germ furrow in side-view. **2** Pollen with intermediate germ furrow in distal view. **3** Pollen with narrow germ furrow in side-view. **4** Pollen with narrow germ furrow in distal view. **5** Pollen with wide germ furrow in side-view. **6** Pollen with wide germ furrow in distal view. Scale bar = 10µm

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. 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 in turn should result in increased fertilisation.

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