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Storage and Viability Testing of *Protea* Pollen

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Abstract. **The influence of storage temperature and humidity on pollen viability was studied in four *Protea* species. Pollen was stored at a range of temperatures and relative humidities for up to 1 year and tested for ability to germinate in vitro. Pollen of *P. repens* (L.) L. 'Sneyd', *P. eximia* (Salisb. ex Knight) Fourcade 'Fiery Duchess' and *P. magnifica* Link. clone T 84 07 05 stored at –196 °C and –14 to –18 °C retained a germination percentage as high as that of fresh pollen regardless of humidity. Humidity control became increasingly important at storage temperatures above 0 °C. The study showed that long-term storage of *Protea* pollen is not feasible at temperatures above 0 °C. The relationship between germinability and fluorochromasia (FCR) was studied during storage of 'Sneyd' pollen. The correlations between FCR and germinability were found to be low and nonsignificant. Fifteen-month-old cryopreserved 'Sneyd' pollen functioned in fertilization and seed set as effectively as fresh pollen.**

Protea is the genus of most commercial importance of the South African wild-flower (fynbos) industry and has an annual export value of about R20 million. To meet the requirements of fashion-conscious markets, systematic controlled breeding is essential to produce new cultivars.

Genetic improvement of *Protea* by breeding and selection is gaining momentum in a number of countries around the world. However, breeding has been limited by crossing incompatibility between species, lack of pollen storage techniques, and low hybrid seed set (Brits, 1992). Developing technology for long-term pollen storage would facilitate the crossing of species with differing flowering periods. In addition, stored pollen could have potential as long-term germplasm preservation, especially of unique genotypes.

Favorable conditions for pollen storage have been investigated for many agronomic and horticultural crops (Lee et al., 1985; Yates et al., 1991). In general, low temperature and low relative humidity maintain viability, but there are many exceptions (Stanley and Linskens, 1974). Viability of pollen from Proteaceae species was higher at 5 °C storage than –10 °C. Viability could be maintained at 5 °C for at least 1 month (Shchori et al., 1992). Fresh *Protea* pollen has been viable for up to 6 d of storage at room temperature and up to 6 weeks at 5 °C (Brits and Van den Berg, 1990). *Banksia menziesii* pollen viability decreased rapidly at ambient temperature and 90% was inviable after 24 h (Ramsey and Vaughton, 1991). Our preliminary field investigations demonstrated that *P. repens* 'Sneyd' pollen had viability of up to 70% after 7 d, but decreased to 18% after 9 d. Storing pollen for long periods at –20 °C would be practical in that conventional and readily available freezers could be used for storage (Pinney and Polito, 1990). The purpose of this study was to develop a practical method, using inexpensive, commonly available supplies and equipment, of storing *Protea* pollen for 1 year or more with sufficient viability to be used in controlled hybridizations. Since the ultimate goal for a breeder using stored pollen is to obtain viable seeds, the ability of stored *Protea* pollen to effect seed set in controlled pollinations in the field was also investigated in this study.

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Materials and Methods

Pollen collection. Experiments were conducted from 1992 to 1994 on four species planted in experimental plantations at Elsenburg (lat. 33°51'S, long. 18°50'E) and Riviersonderend (lat. 34°08'S, long. 19°54'E) in South Africa. Before the experimental period, all plants had been subjected to routine plantation management practices, including drip-irrigation during the summer. Harvesting of blooms in previous years served as the only form of pruning of the bushes. For the storage experiments, pollen of *P. magnifica* clone T84 07 05, *P. eximia* 'Fiery Duchess', *P. repens* 'Sneyd', and *P. aristata* Phill 'Aristocrat' was used. Five inflorescences per plant from five plants, with about one-half of the florets having undergone anthesis, were harvested and brought to the laboratory where the stems were placed in water. All the open florets were removed, and 16 h later all florets that had subsequently opened were harvested (about 25/inflorescence). The pollen from each plant (five inflorescences) was scraped off, thoroughly mixed, and used as a single replicate. All replicates were first dried for 24 h in a desiccator over silica gel (blue) at 5 °C, after which the pollen mixtures were divided into separate samples and placed in gelatine capsules for storage. The number of samples was large enough to ensure that each sample would be tested only once.

Pollen storage. The gelatine capsules with predried pollen samples were stored at room temperature (22 to 27 °C), in a household refrigerator (2 to 7 °C), in a household freezer (–14 to –18 °C), and in liquid nitrogen (–196 °C). At all the temperatures, except in liquid nitrogen, which has negligible water vapor pressure, the pollen samples were stored at relative humidities (RHs) of 10%, 30%, and 60%. Humidities were maintained with different concentrations of sulfuric acid (Solomon, 1951; Weast, 1988). The atmospheres were produced in closed 1000-mL flasks and were equilibrated for at least 1 month before use. The freezing points of solutions were below the temperatures to which they were exposed in the freezer. Pollen of *P. repens* 'Sneyd' was stored at an additional humidity of 0%, maintained over silica gel (blue) in a desiccator. For storage at –196 °C, the predried pollen samples were individually placed in 5-cm^3 plastic cryovials, which were directly submerged in liquid nitrogen in a nitrogen storage vessel. No precooling or cryoprotectant was used to treat pollen before freezing.

Viability tests. As a control on storage procedures, each replicate was scored for in vitro pollen germinability before storage. Stored samples were thawed and rehydrated for 3 h at 25 °C in a 100% RH atmosphere chamber before testing with the hanging-drop technique of Van Tieghem (1869). Pollen stored at room

temperature was tested for germinability every 15 d, while the other treatments were tested after 30, 90, 180, 270, and 360 d of storage. The germination medium used in the storage experiments consisted of 0.4 M sucrose, 100 mg·L^{-1} H_3BO_3, 300 mg·L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 20 mg·L^{-1} $MgSO_4 \cdot 7H_2O$, and 100 mg·L^{-1} KNO_3 in distilled water. The pH was adjusted to 7.0 using 0.1 M HCl or KOH solutions. Germination was scored after 3 h with an Olympus BH-2 light microscope at a magnification of 200×. At least 200 randomly selected pollen grains in four different fields (about 50/field) were scored for germination. Only pollen grains producing tubes longer than the grain diameter were scored as having germinated.

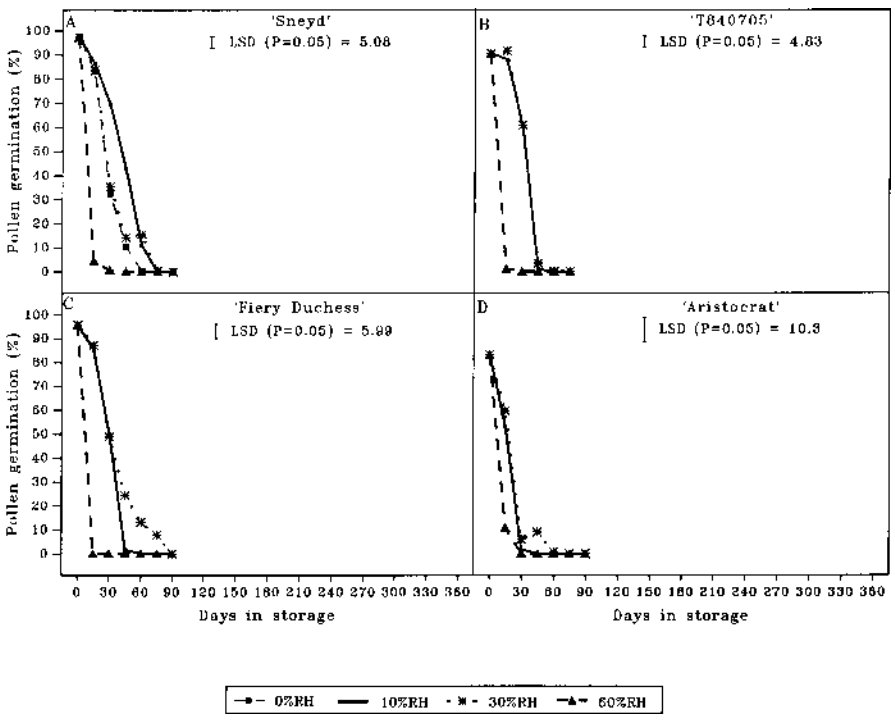
The in vitro germination test was compared with the fluorochromatic (FCR) test. Only stored pollen of *P. repens* 'Sneyd' was used for this purpose. The fluorochromatic (FCR) test procedure of Greissl (1989), using fluorescein-diacetate–propidium-iodide, was used. As a control, each pollen replicate was tested before storage with the FCR test procedure. Each stored pollen sample was divided into two subsamples, one being assayed for germination and the other using the FCR procedure at each of the testing times. Pollen grain fluorescence was determined with a Nikon Biophot microscope equipped with an episcopic-fluorescence attachment and a B-2A filter system consisting of a dichroic mirror (510 nm), a blue excitation filter (450 to 490 nm), and a barrier filter (520 nm). All pollen grains which fluoresced brightly were scored as viable. Viability percentages were determined, using five replicates of about 200 grains each.

Viability of 15-month-old 'Sneyd' pollen stored in liquid nitrogen was tested further by pollination in the field, and the results were compared with those obtained with fresh pollen. The stored pollen was frozen and thawed once before this experiment. Thirty *P. repens* 'Sneyd' inflorescences, with about one-half of the florets having undergone anthesis, were labelled and all open florets were removed with scissors (Fuss and Sedgley, 1991). The inflorescences were covered with semi-rigid wire mesh cages (2 × 2-mm apertures) to exclude pollinators (Coetzee and Giliomee, 1985) and prevent contact between the florets and the pollination bag (Fuss and Sedgley, 1991). After 16 h, the cages were removed and all remaining unopened florets were removed, leaving a ring of between 15 and 35 open florets. Self-pollen was not removed from these florets. The inflorescences were recaged until pollination at three days after anthesis.

Ten inflorescences were pollinated with fresh 'Sneyd' pollen (self-pollinated); ten inflorescences with 15-month-old liquid nitrogen-stored pollen; and ten inflorescences unpollinated as controls. Florets were pollinated in the morning by touching their stigmas with pollen presenters from newly opened donor florets, which had also been caged to prevent contamination, or by touching stigmas with 15-month-old liquid nitrogen-stored pollen. Stored pollen was rehydrated before pollination, as previously described. Three replicates (nine inflorescences) were harvested 7 d after pollination when penetration of the ovule had occurred. The bottom part of the pollinated pistils, including the ovary, were carefully dissected from the involucral receptacles, fixed in Carnoy's solution for 24 h,, and transferred to 70% ethanol for storage at room temperature. Before processing ovaries for fluorescence microscopy (Kho and Baër, 1968; Martin, 1959), ovaries were transferred to 30% ethanol, washed in distilled water (×3), softened for 30 min in 0.8 N sodium hydroxide at 75 °C, washed again in distilled water (×3), and stained overnight in a 0.1% solution of water-soluble aniline blue dye in 0.1 N $K_3PO_4 \cdot H_2O$. To observe pollen tubes, the ovaries were placed on a slide and the cover slip was gently pressed to spread the material. Fluorescing pollen tubes were observed by means of a Nikon Biophot microscope equipped with a episcopic-fluorescence attachment and a UV-2A filter system consisting of a dichroic mirror (430 nm), an ultraviolet excitation filter (380 to 425 nm), and a barrier filter (450 nm). The number of pollen tubes in the ovary was recorded as well as the number of tubes that had penetrated the ovules. Seven replicates (21 infructescences) were harvested 7 months after pollination, when the achenes had matured (Van Staden, 1978), to record seed set for all the flowers pollinated previously. The infructescences were dried and the number of seeds per infructescence was recorded by dissecting the pollinated one-seeded achenes with a scalpel. The percentage seed set of pollinated florets per infructescence was calculated.

Statistical methods. The four species in the storage experiments were investigated in separate experiments. Each experiment consisted of five replications in randomized blocks with a three-factor factorial design for treatments. The fluorometry experiment consisted of five randomized blocks with a split-plot design for treatments. Pearson product-moment correlations were calculated to measure the strength of the linear relationship between FCR percentage and germinability for each storage treatment. The pollination experiment consisted of randomized blocks. Analysis of variance (ANOVA) was performed for each part of the experiment separately and Pearson product-moment correlations were calculated to measure the strength of the linear relationship between different variables connected with pollen tube growth. For all experiments, ANOVA was performed using SAS statistical

Fig. 1. The effect of 22 to 27 °C storage at different relative humidities on pollen germination. (**A**) *Protea repens* 'Sneyd', (**B**) *P. magnifica* T84 07 05, (**C**) *P. eximia* 'Fiery Duchess', (**D**) *P. aristata* 'Aristocrat'.



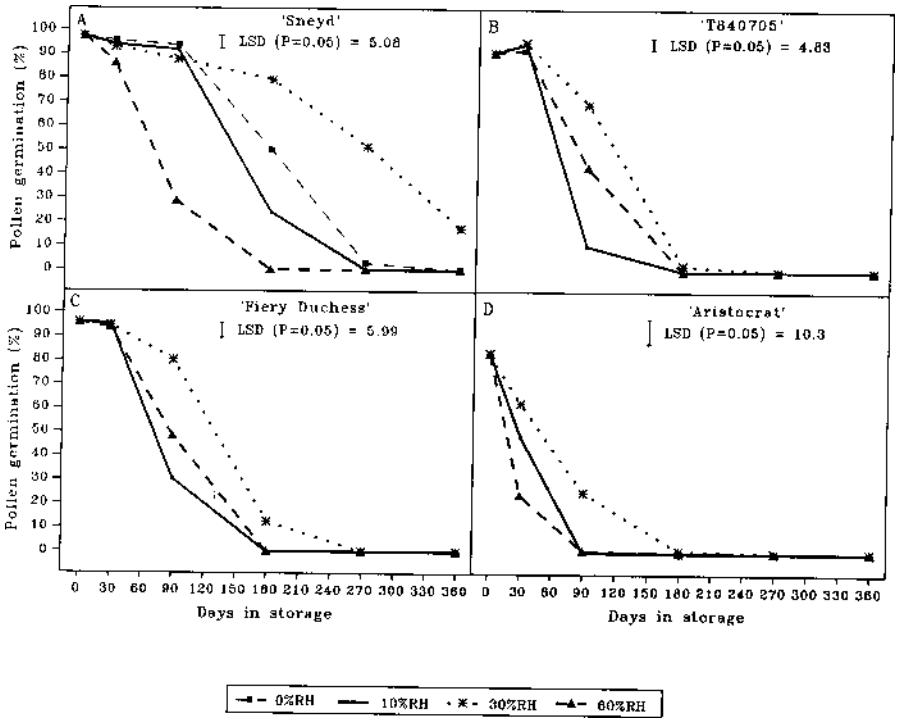


Fig. 2. The effect of 2 to 7 °C storage at different relative humidities on pollen germination. (**A**) *Protea repens* 'Sneyd', (**B**) *P. magnifica* T84 07 05, (**C**) *P. eximia* 'Fiery Duchess', (**D**) *P. aristata* 'Aristocrat'.

software version 6.08 (SAS Institute, Cary, N.C.). Student's least significant differences (LSD) were calculated at $P = 0.05$ to compare treatment means. For all other effects in the ANOVA, $P = 0.05$ was considered significant.

Results

In vitro germination. Pollen from four species of *Protea* had comparable responses to long-term storage conditions as determined by in vitro germination tests (Figs. 1 to 4). A significant three-factor interaction existed between method of pollen storage, RH, and storage time for each pollen species. The main effects of the analysis could therefore not be interpreted independently. All four species tested showed a very high (>80%) initial (fresh) germination percentage. The general response pattern of *P. aristata* pollen was similar to the other three species, but absolute germination percentages were lower for many treatments.

Pollen stored at 22 to 27 °C was influenced significantly by RH (Fig. 1). Pollen germination was reduced to <10% for pollen stored at 60% RH within 30 d for all four species. Lower humidities maintained germination at 50% or higher for *Protea* species, except *P. aristata*, for this length of time. Regardless of humidity conditions, pollen stored at 22 to 27 °C did not germinate after 90 d in any of the species.

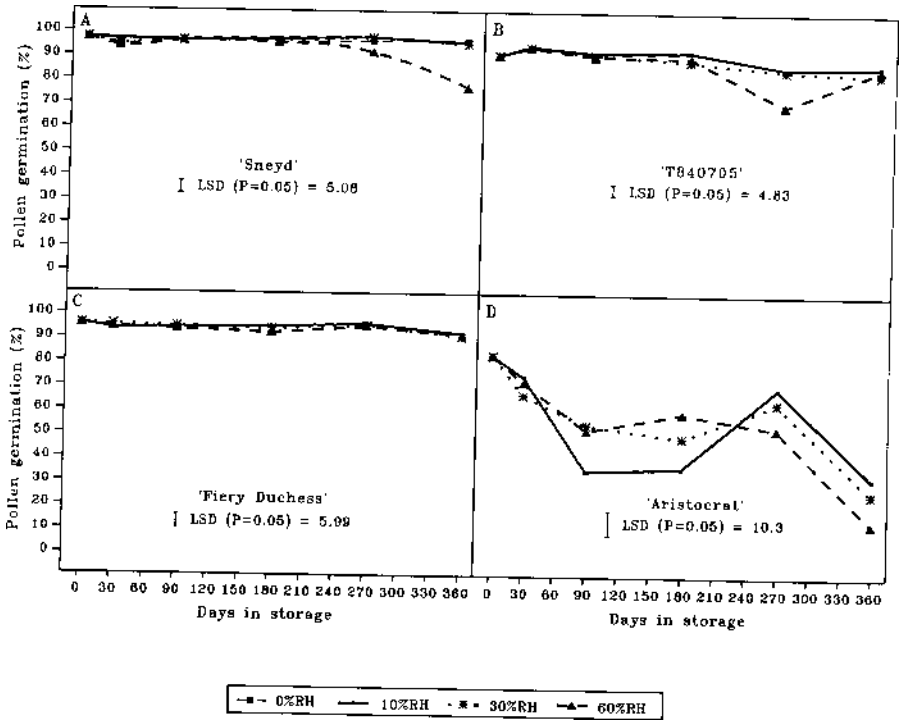
Pollen stored at 2 to 7 °C, like pollen stored at 22 to 27 °C, had a significant response to RH (Fig. 2). However, pollen viability for pollen stored at 2 to 7 °C was about three times longer than at 22 to 27 °C, regardless of humidity. At the optimum of 30% RH, >60% germination occurred after storage for 270 d for *P. repens*, 90 d for *P. magnifica*, 90 d for *P. eximia*, and 30 d for *P. aristata* (Figs. 2 A–D, respectively).

Pollen stored at –14 to –18 °C, only 'Aristocrat' showed a significant decline in germinability (50% to 70%) and differences between the RH treatments over the full length of time (Fig. 3). For this species, the 10% and 30% RH treatments were significantly better than the 60% RH after 360 d. In addition, a significant increase in germinability occurred at 270 d compared to 90 and 180 d in the 10% and 30% RH treatments; the reason for this is unclear.

Only 'Aristocrat' pollen showed a significant decline (25%) in germinability after 360 days of storage at –196 °C (Fig. 4). An unexplained increase in germinability at 270 d compared to 90 and 180 d again occurred for this species (Fig. 4D).

Fluorometry. The fluorescence of pollen grains stained with the contrast-staining technique (fluorescein-diacetate–propidium-iodide) varied from bright yellow to reddish-brown. Only reddish-brown grains were classified as nonviable. A significant four-factor interaction existed between method of pollen storage, RH, the time pollen was stored, and method of testing. The main effect of the analysis are therefore interpreted separately. For each storage treatment, correlations between FCR percentage and germinability are given in Table 1.

Fig. 3. The effect of –14 to –18 °C storage at different relative humidities on pollen germination. (**A**) *Protea repens* 'Sneyd', (**B**) *P. magnifica* T84 07 05, (**C**) *P. eximia* 'Fiery Duchess', (**D**) *P. aristata* 'Aristocrat'.



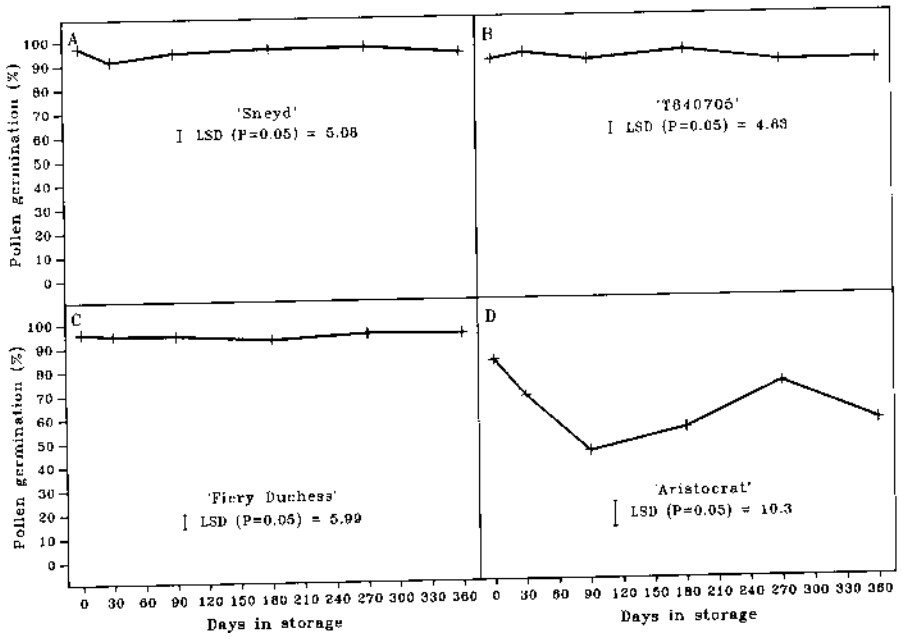


Fig. 4. The effect of –196 °C storage on pollen germination. (**A**) *Protea repens* 'Sneyd', (**B**) *P. magnifica* T84 07 05,(**C**) *P. eximia* 'Fiery Duchess', (**D**) *P. aristata* 'Aristocrat'.

Correlation coefficients (*r*) were low and nonsignificant, except for the 60% RH 2 to 7 °C treatment with $r = 0.89$ ($P < 0.05$).

Pollen stored at 22 to 27 °C, as in the germination test, had a significant response to RH (Fig. 5A). At the optimum of 10% RH, almost 50% FRC capacity was retained after 90 d storage. As for viability as measured by germinability (Fig. 4A), FCR capacity was completely lost within 30 d under 60% RH. Contrary to the results obtained with the germination test with pollen stored at 2 to 7 °C, the FCR test at this temperature indicated no significant differences or loss in viability for the different RH treatments, except at 60% RH after 360 d (Fig. 5B). At 60% RH pollen retained a FCR percentage exceeding 60% for only 120 d and was completely inviable after 270 d. The FCR test further indicated that 'Sneyd' pollen retained a FCR percentage as high as that of fresh pollen after 360 d of storage, regardless of humidity, at –14 to –18 °C and –196 °C (Fig. 5 C and D).

Pollination. Controlled hand self-pollination of 'Sneyd' florets with pollen cryopreserved for 15 months resulted in a normal seed set (80.2%), comparable to that derived from pollination with fresh pollen (75.3%) (Fig. 6). Moreover, no significant differences were observed between cryopreserved and fresh pollen with regard to the percentage of florets containing pollen tubes, the percentage of florets with ovule penetration, and the mean number of pollen tubes per floret (Fig. 6). The control (unpollinated) differed highly significantly from the two hand-pollination treatments in all four of the variables tested. Correlation analysis between the three pollen tube variables indicated that correlation coefficients (*r*) were strong and highly significant ($P < 0.01$).

Discussion

Protea pollen stored above 0 °C maintained viability for less than one year as reported for other genera (Ganeshan, 1985; Polito and Luza, 1988; Yates et al., 1991). Viability at temperatures above 0 °C declines rapidly, especially when the humidity of the storage atmosphere is not controlled (Luza and Polito, 1985).

Controlling humidity at temperatures below 0 °C can have a marked effect on retention of pollen germinability for some plants (Allan, 1963; Hanna, 1990; Luza and Polito, 1985; Polito and Luza, 1988). However, controlling humidity below 0 °C had no advantage for many *Protea* species, similar to results with pear, apple, tomato and azalea (Visser, 1955). Our results provide evidence that *Protea* pollen may be stored relatively cheaply in a household freezer without humidity control for periods adequate for breeding purposes. Furthermore, satisfactory preservation of *Protea* pollen was obtained by cryotechnology simply by immersion into liquid nitrogen. Theoretically, pollen stored in liquid nitrogen should retain viability indefinitely (Stanley and Linskens, 1974), provided precautions are taken to reduce pollen moisture before storage (Yates et al, 1991).

Low germination of stored 'Aristocrat' pollen over all storage treatments suggests that extrapolation of pollen longevity results from one *Protea* species or clone to another could be unwarranted. Differences in pollen longevity among cultivars are known to occur (Parfitt and Almehdi, 1984). Therefore, more *Protea* species or clones should be tested before the results of this study can be applied to the entire genus.

The finding that the FCR test does not correlate well with the in vitro germination test indicates that this assay is not suitable for viability determinations of stored *Protea* pollen. Similar results have been reported by Widrlechner et al. (1983) for azaleas. Heslop-Harrison et al. (1984) experienced similar problems when the method was used on stored pollen. However, many other studies (e.g., Greissl, 1989; Jansson and Warrington, 1988; Pinney and Polito, 1990; Radicati di Brozolo et al. 1990) have shown that the FCR test can provide an excellent guide to pollen germinability in a number of different species. Inspection of our graphs indicates that germinability scores are always lower than the FCR scores except where they lie within the margins of error. According to

Table 1. Correlations between fluorochromasia (FCR) percentage and germination percentage in vitro based on the data sources used for the text figures listed.

Treatment		Source	*r*
Temp (°C)	RH[z] (%)	of data	(N = 6)
22 to 27	0	Fig. 1A, 5A	–0.42[NS]
	10	Fig. 1A, 5A	–0.52[NS]
	30	Fig. 1A, 5A	–0.55[NS]
	60	Fig. 1A, 5A	–0.24[NS]
2 to 7	0	Fig. 2A, 5B	0.80[NS]
	10	Fig. 2A, 5B	0.05[NS]
	30	Fig. 2A, 5B	–0.45[NS]
	60	Fig. 2A, 5B	0.89*
–14 to –18	0	Fig. 3A, 5C	–0.16[NS]
	10	Fig. 3A, 5C	–0.62[NS]
	30	Fig. 3A, 5C	–0.77[NS]
	60	Fig. 3A, 5C	0.45[NS]
–196	---	Fig 4A, 5D	0.70[NS]

[z]RH = relative humidity.
[NS,*]Nonsignificant or significant at $P = 0.05$, respectively.

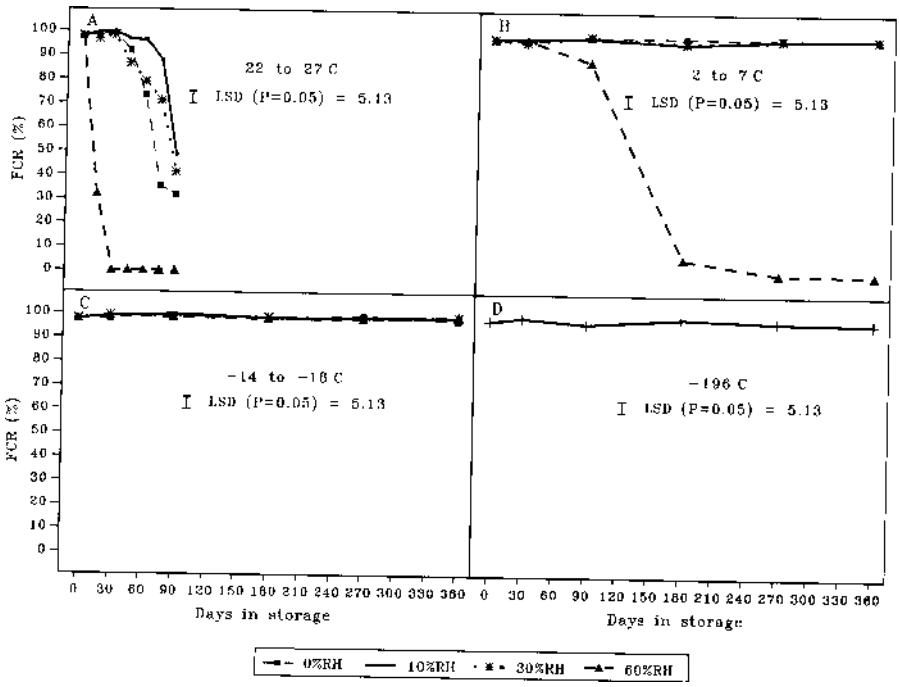


Fig. 5. Fluorochromatic reaction (FRC) in pollen samples of *Protea repens* 'Sneyd' stored at different temperatures and relative humidities. (**A**) FCR after 22 to 27 °C storage, (**B**) FCR after 2 to 7 °C storage, (**C**) FCR after –14 to –18 °C storage, (**D**) FCR after –196 °C storage.

Heslop-Harrison and Heslop-Harrison (1970), this could be expected, because a pollen grain showing FCR has at least the potential for germination, whereas one that does not certainly lacks this.

Several investigators (e.g., Ganeshan and Alexander, 1990; Griggs et al., 1971; Sedgley, 1981) have shown that even though pollen is able to germinate in vitro, fertilization and seed production may yet fail. However, the ability of *Protea* pollen to effect fertilization and produce seed does not appear to be influenced by cryopreservation. Similar results have been obtained with cryogenically stored pollen of jojoba (Lee et al., 1985), papaya (Ganeshan, 1986), pecan (Yates and Sparks, 1990), narcissus (Bowes, 1990), and rose (Marchant et al., 1993). Whether or not seed set can be obtained with viable *Protea* pollen stored at higher temperatures requires further investigation. Seed produced by

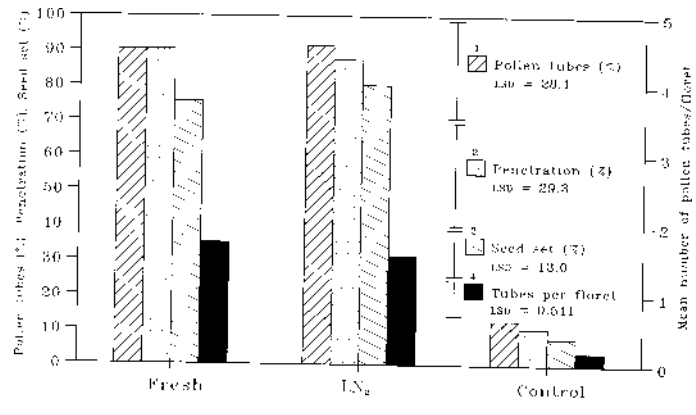


Fig. 6. Fertilization success of fresh and 15-month-old liquid nitrogen-stored *Protea repens* 'Sneyd' pollen after hand pollination, measured by 1) percentage of florets with pollen tubes, 2) percentage of ovules penetrated by a pollen tube, 3) percentage seed set, and 4) mean number of pollen tubes per floret.

cryopreserved broccoli pollen germinated poorly (Crisp and Grout, 1984), and this should be investigated for *Protea* pollen as well.

In summary, freezer and liquid nitrogen storage can be successfully employed for the long-term storage of *Protea* pollen to assist breeding programs. Viability assessments of *Protea* pollen based on germination and the FCR are not correlated. In vitro germination is the most accurate and routine method for determining viability of *Protea* pollen held in long-term storage.

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