In vitro pollen viability and pollen storage in *Eucalyptus marginata* (Myrtaceae)

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Summary

This study examines pollen germination in *Eucalyptus marginata*. *In vitro* pollen germination is best with a medium containing calcium and boron, such as the Brewbaker and Kwack medium (300 ppm hydrated calcium nitrate, 200 ppm hydrated magnesium sulphate, 100 ppm potassium nitrate and 150 ppm boric acid with 25% maltose). High levels of genotypic variability in pollen (10–60% viability) make it desirable to test the pollen quality of individual trees before use. Pollen of *E. marginata* has characteristics common to many other eucalypt species, but its adaptations to a relatively dry environment can be seen in its ability to germinate without prior desiccation, unlike pollen from cool temperate eucalypts. Daily changes in weather conditions have little effect on *in vitro* pollen germination in *E. marginata*. Pollen of *E. marginata* may be stored successfully at –20°C or –80˚C for at least one year.

Keywords: pollen germination; *in vitro*; viability; storage; anthesis; *Eucalyptus marginata*

Introduction

Successful controlled pollination requires viable pollens, and so techniques are needed for storing pollen and testing its viability. Eucalypts are long-lived trees with limited flowering times that may not overlap for trees which breeders wish to cross-pollinate. In this case it is necessary to store pollen successfully and to appreciate the impact of storage on its viability.

In terms of *in vitro* germination, eucalypt pollen appears similar between species and subgenera (Boden 1958; Pryor 1976; Griffin *et al.* 1982; Heslop-Harrison and Heslop-Harrison 1985; Potts and Marsden-Smedley 1989; Sedgley and Griffin 1989;Turner *et al.* 1994; Moncur 1995; Potts and Gore 2000). Most germination media use 20–30% sucrose, with the optimum concentration varying between species (Barnabas and Kovacs 1997; Potts and Gore 2000). Boron is an important trace element for pollen germination (Stanley 1973; Shivanna and Johri 1985; Potts and Marsden-Smedley 1989; Potts and Gore 2000). It has implications in the translocation and metabolism of carbohydrates, in indole-acetic-acid metabolism, and is required for efficient operation of membrane transport systems (Shivanna and Johri 1985). Potts and Marsden-Smedley (1989) added boric acid, while Heslop-Harrison and Heslop-Harrison (1985) added calcium nitrate as well as boric acid.

The importance of Ca^{2+} ions for promoting pollen germination has also been stressed (Brewbaker and Kwack 1963; Reiss and Herth 1978; Shivanna and Johri 1985; Taylor and Hepler 1997; Malho *et al.* 2000). The addition of calcium in the germination medium has been reported to increase fluorescence in the pollen tube tip (Reiss and Herth 1978), increase the length of the pollen tube in *in vitro* growth and assist in guiding the pollen tube towards the ovule (Malho *et al*. 2000). Although calcium is important for many species, Heslop-Harrison and Heslop-Harrison (1994) point out that pollen from some species will germinate in water-saturated air with or without Ca^{2+} ions. Among the *in vitro* pollen germination media examined for two clonal genotypes in *E. marginata*, 150 ppm boric acid with 25% maltose was found to be the most appropriate medium.

Eucalypt pollen can be successfully stored of for periods of up to three years at –16°C to –20°C or –80°C (Pryor 1976; Boden 1958 for *E. maculata*; Griffin *et al.* 1982 for *E. regnans*; Potts and Marsden-Smedley 1989 for *E. globulus, E. urnigera, E. morrisbyi* and *E. ovata*; Eldridge *et al.* 1993). Pollen of *E. camaldulensis* remained viable for eleven months at 4°C (Oddie and McComb 1998). For short-term storage of up to one month, eucalypt pollen may be stored successfully at room temperature or 4°C (Griffin *et al.* 1982; Turner *et al.* 1994; Moncur 1995). Pollen is usually partially dehydrated before storage as this results in longer-term viability (Barnabas and Kovacs 1997), but rehydration is necessary before germination testing.

Jarrah, *Eucalyptus marginata* Donn ex Smith (subgenus *Monocalyptus*), is a widespread, dominant forest tree that is restricted to the more mesic regions of south-western Australia. Breeding programs aimed at raising the level of resistance to *Phythophthora cinnamomi* (dieback) and jarrah leaf miner, and improving tree form, have been largely unsuccessful since hand pollinations have yielded very few seeds (M. Byrne and M. Stukely, Conservation and Land Management, Western Australia, *pers. comm.*).

As viability testing through germination requires at least one day, a quick test for viability is desirable. The fluoroscein diacetate test (Heslop-Harrison and Heslop-Harrison 1970) has been used successfully for many taxa. It depends on the integrity of the plasmalemma of the vegetative cell of the pollen grain (Heslop-Harrison and Heslop-Harrison 1970; Heslop-Harrison *et al.* 1984). This test has been used successfully on *Eucalyptus* species

by Heslop-Harrison and Heslop-Harrison (1985), but Griffin *et al*. (1982) reported that variable levels of fluorescence between grains made the fluorochromatic test ineffective in *E. regnans*. We have assessed the validity of the fluorochromatic test on *E. marginata*, using recommendations outlined by Heslop-Harrison and Heslop-Harrison (1985).

We report here protocols for effectively testing jarrah pollen viability and germination, and storage of pollen. This study tests the effectiveness of adding Ca^{2+} ions to the *in vitro* pollen germination medium for *E. marginata* compared to a sugar–boron medium, and also tests the hypothesis that individual trees differ in their pollen viability and vigour. This study focused on clonal populations of *E. marginata* resistant to dieback (McComb *et al.* 1994; Stukely and Crane 1994), in seed orchards, but included a number of wild trees.

Materials and methods

Trees used and pollen collection

Eucalyptus marginata trees used were either from a plantation of clones selected for their resistance to *Phythophthora cinnamomi*, or growing wild. Trees from four ten-y-old clones growing at Murdoch University were chosen (5JN119, 12JP72, 133JP38, and 503JP16). Six wild trees were located either growing undisturbed in the Harry Waring Marsupial Reserve at Wattleup (32°9′47″S, 115°49′22″E), or as roadside remnants (Wattle Grove and Kelmscott) within 18 km east or south-east of Murdoch University (32°S, 115°50″E) in south-western Western Australia. In the 2000 season, the clones flowered from the end of August to the end of October, while the roadside wild trees flowered in October–November. In the 2001 season, the clones flowered at the same times as the previous year, and the Wattleup trees flowered from mid November to the end of December.

Pollen was collected in 2000 by picking buds which were at operculum lift stage and were situated at mid height on the tree (Turner *et al.* 1994; Oddie and McComb 1998), and occupied positions that were partly shaded (observations not included here found buds that were high on the tree and exposed to the full sun were less fertile). The buds were desiccated overnight using silica gel, before sieving the pollen through a 200 mm steel mesh onto Alfoil and placing it into plastic Eppendorf tubes. The pollen was stored at 4°C in the vials for up to three days. During the 2001 season a comparison was made between pollen collected as above, and pollen from buds at the operculum lift stage used directly after excision of the flower, without desiccating and sieving. Sometimes branches of buds were kept with the base of the branch in water in the laboratory while the buds were collected over several days as they matured to the operculum lift stage (Turner *et al.* 1994).

Developing germination media

Maltose (25%) with 150 ppm boric acid ('BA medium') was used as a basic germination medium for *E. marginata* based on previous work by the second author. The addition of 300 ppm $Ca(NO_3)_2.4H_2O$ (hydrated calcium nitrate), 200 ppm $MgSO_4.7H_2O$ (hydrated magnesium sulphate) and 100 ppm KNO_3 (potassium nitrate) ('BK medium', after Brewbaker and Kwack 1963) was tested using repeated samples over the flowering season from clones 5JN119, 12JP72, and 503JP16, two remnant roadside trees and the three wild trees from Wattleup.

Pollen germination tests

Pollen was germinated *in vitro* using the wettable cellophane technique (Alexander and Ganeshan 1989) with four repeat samples for each test from the same pollen pool. Briefly, Petri dishes with four layers of filter paper were wetted (but not flooded) with the germination medium. Four 1-cm cellophane squares were placed between the third and fourth layer. Once saturated, the top layer of filter paper was removed leaving the wet cellophane squares ready for the pollen sample, which was placed on the square, using forceps if the sample was in a plastic vial, or by dabbing freshly picked anthers on the cellophane. The closed Petri dish was then placed in a plastic box containing damp paper. Cultures were incubated for 24 h in 2000, and for 48 h in 2001, as it was found that extending the incubation period gave a higher percentage of germination and allowed the pollen tubes to grow longer, making them easier to count. Scoring was by scanning each cellophane square completely, and then counting the percentage of germinated pollen grains in three representative sections. Each section had approximately 100 grains, from which the average percentage germination was calculated. This method was considered to partially compensate for the 'population effects' of clumped pollen (Shivanna and Johri 1985).

Testing pollen viability at varying times of day and weather conditions

The three wild trees from Wattleup were tested at various times of the day during different weather conditions during the spring of 2001 to determine if weather affected pollen germination. Flowers were collected and pollen direct from the anthers was set up to germinate on cellophane (as above), within fifteen minutes of collection. Both the BA and BK germination media were used.

A three-way repeated measures ANOVA at the 0.05 significance level was conducted on the three wild trees at Wattleup to test for differences between the trees, the media, and the repeated pollen germination measurements taken at different times of the day and during different weather conditions from the same trees. Bartlett's test for homogeneity of variance was applied to the ten repeated pollen germination measurements, and treatments not meeting the homogeneity of variance test were removed before conducting the ANOVA. The appropriate Greenhouse–Geisser ε was used to correct for possible violation of the sphericity assumption.

Storage test

Storage was tested on a bulked pollen sample. In the 2000 flowering season, clones 503JP16, 5JN119 and 133JP38 were used; and in the 2001 season two roadside trees were used together with 12JP72 and 503JP16. Individual trees' pollen was tested for germination prior to inclusion in the bulked sample. The bulked pollen was sieved through a steel 200 mm sieve, thoroughly mixed, and placed into 26 plastic airtight Eppendorf tubes and germination-tested before storage. Pollen was not dried before germination-testing or before storage. The Eppendorf tubes

were placed into five larger plastic jars containing dried silica gel, and these were stored at room temperature, 4°C, –20°C, –80°C and –196°C. One vial from each storage regime was tested for germination after 1, 4, 13, 26 and 52 weeks. This experiment was initiated in the 2000 flowering season, using the BA germination medium, and repeated using fresh pollen in the 2001 flowering season with both the BK and the BA media. After removing the pollen from storage, it was left for at least two hours at room temperature in 2001, and in a humid environment at room temperature in 2002, before being processed for germination. This was to allow for some hydration of the pollen grains. From each vial four pollen samples were withdrawn and counted for germination in each of the two media. A three-way ANOVA at the 0.05 significance level was conducted on the 2001 data, using a pooled error (Statsoft 1999). This was not a repeated measures ANOVA since the pollen sample was bulked from a number of trees.

Table 1. Pollen germination percentage for *Eucalyptus marginata* using BK and BA media. Each test is from four samples drawn from the same plant germinated at the same time. Standard errors (s.e.) are in brackets.

Tree or clone	Mean germination (%)		No. of tests
	BK medium	BA medium	
5JN119	22.5 (5.42)	20.6 (4.66)	5
12JP72	18.4 (5.60)	14.4 (3.01)	5
503JP16	15.7 (3.09)	17.9 (4.18)	5
Wattle Grove	57.1 (2.55)	36.6 (4.14)	5
Kelmscott 1	40.4 (4.05)	35.0 (4.40)	5
Wattleup 1	59.3 (4.44)	53.3 (5.05)	10
Wattleup 2	10.4 (1.84)	8.7 (2.60)	10
Wattleup 3	32.1 (1.69)	21.1 (3.63)	10
Mean	32.0 (6.62)	26.0 (5.16)	

Viability testing

Fluorescein diacetate was used for testing viability (Heslop-Harrison and Heslop-Harrison 1970; Heslop-Harrison *et al.* 1984), by collecting fresh pollen and placing the sample on a slide with a droplet of the BA or BK germination medium containing 0.1% fluorescein diacetate, prepared from a stock solution of 5% fluorescein diacetate in acetone. This was allowed to stand for 30–40 minutes before the sample was covered with a cover slip and observed using fluorescent microscopy.

Results

Germination media

More pollen grains germinated, and pollen tubes were longer, in the BK medium containing Ca^{2+} ions than in the medium containing only maltose and boron (Table 1). The method of pollen germination (Alexander and Ganeshan 1989) gave consistent results when the 48-hour period for germination was used. There were significant differences in pollen germination between genotypes. Lack of consistency of results due to 'population effects' (caused by pollen clumping or by isolation of individual pollen grains) was reduced in the BK medium.

A three-way repeated measures ANOVA, using the first five tests from eight trees (Table 1), showed a significant interaction between the trees, media and repeated measures (df = 28,192, $F = 5.82$, $P < 0.001$). However, Bartlett's test indicated unequal variances in four out of the five tests. When the two-way ANOVA was conducted using the second test, the two main effects of tree and medium were significant (tree: df = 7,48, $F = 9.65$, $P < 0.001$; medium: df = 1,48, $F = 10.94$, $P < 0.005$).

Weather conditions and times of day

Germination levels were again significantly different between genotypes. Weather conditions and time of day caused little

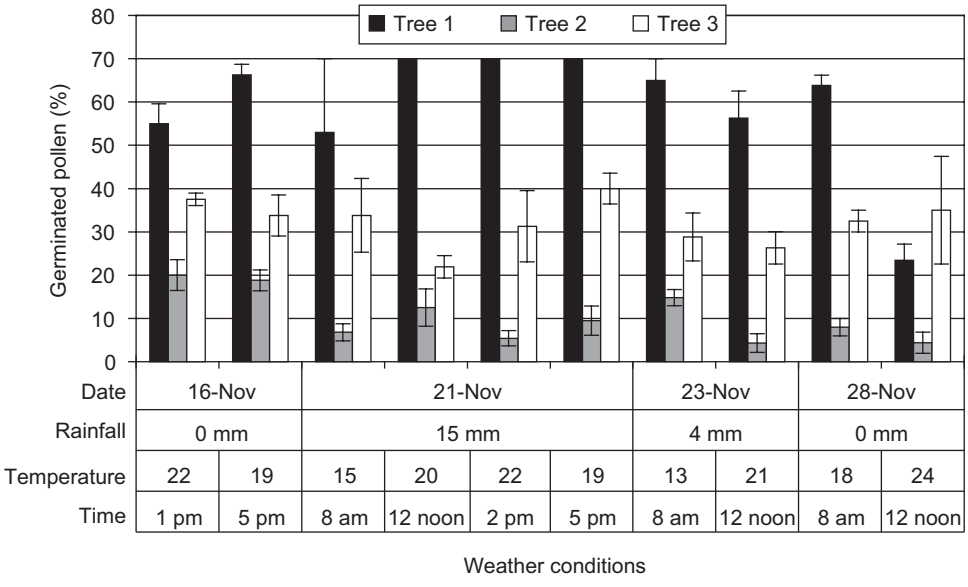

Figure 1. Variation of pollen germination for three wild individuals of *Eucalyptus marginata* for varying times of day and climatic conditions, using the BK germination medium.Vertical bars on columns represent standard error of means.

variation in the pollen germination (Fig. 1). Variation in germination levels between trees was the main observed effect in a three-way repeated measurements ANOVA using measurements 1, 2, 4, 6, 8 and 9 testing the trees, media and repeated measures (df = 10, 90, $F = 2.71$, $P < 0.02$ after Greenhouse–Geiser), but no significant difference was found between the repeated measures of varying time of day, rainfall and temperature (df = 5,90, $F = 0.46$, $P = 0.1$). The final measurement (10) was at the end of the flowering season for wild tree 1, and pollen germination fell rapidly at this time.

Storage

Pollen collected in both 2000 and 2001 retained a satisfactory germination percentage for a period of one year at –20°C, –80°C, and –196°C (Fig. 2). In contrast, pollen stored at room temperature for one week had a germination of only 8% (in 2001 using the BK medium) and pollen stored at 4°C lost most of its germination after one month (germination of 0.6% in 2001 using the BK medium). A significant difference in pollen germination rates was found between the BA and BK germination media (2001 data) using a three-way ANOVA (Fig. 2b and 2c) with a pooled error (Statsoft 1999) (df =1,20, $F = 62.3$, $P < 0.001$). There were also significant differences in the storage time (df = 5,20, $F = 37.2$, $P < 0.001$), the storage temperature (df = 4,20, $F = 19.3$, $P < 0.001$), and in the interaction of medium and temperature (df = 4, 20, $F = 5.0$, $P < 0.01$). The statistical analysis used a pooled error because there was only one repetition of the experiment (trees did not flower in 2002, so the experiment could not be repeated).

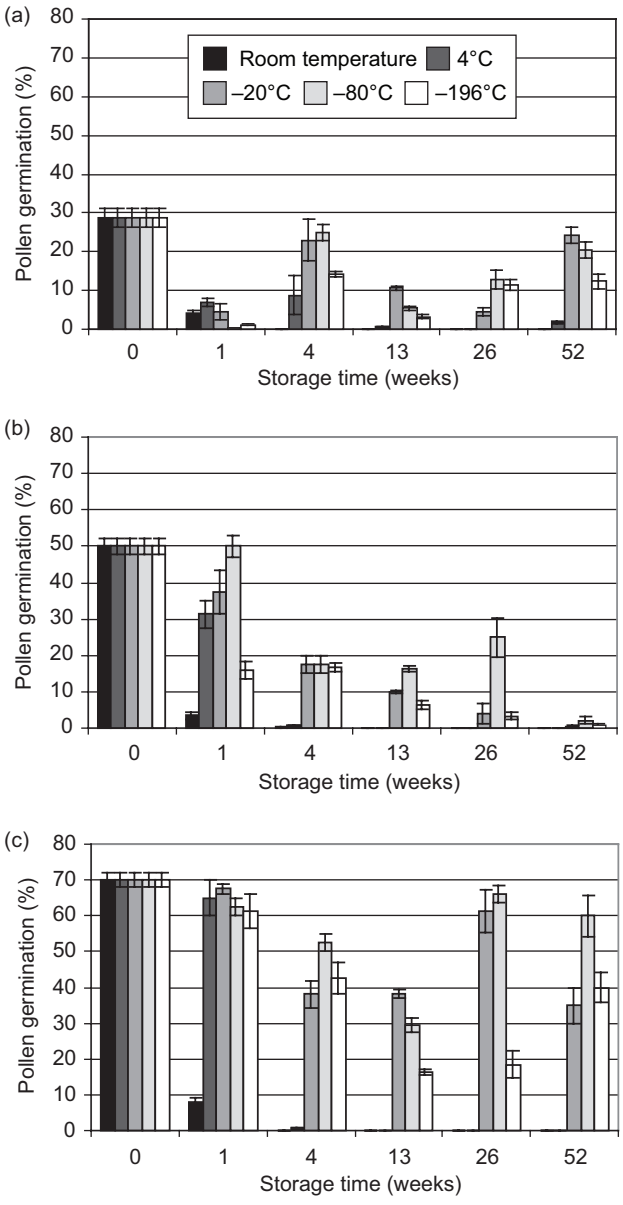

Figure 2. Germination of stored *E. marginata* pollen: (a) using BA germination medium (2000 flowering season); (b) using BA germination medium (2001 flowering season); (c) using BK germination medium (2001 flowering season). Vertical bars on columns represent standard error of means.

Viability testing methods

The results of the fluorochromatic test were difficult to assess using *E. marginata* pollen. When the BA medium was used, even within the same flower, fluorescence varied from none to bright (Fig. 3). Extending the staining time before scoring, as recommended by Heslop-Harrison and Heslop-Harrison (1985), made no noticeable difference. On one occasion, pollen grains germinated very quickly in the BA solution, and little consistency was noted between the level of fluorescence and whether or not the grain was capable of germination. One observation recorded many pollen grains that were not fluorescing but germinated rapidly. When the BK medium was used with fluorescein diacetate, there was no fluorescence although pollen germinates well in this medium.

Discussion

Tests conducted on eight different genotypes showed that the addition of calcium, magnesium and potassium to the pollen-germination media induced greater levels of pollen germination of *E. marginata* pollen than a germination medium containing

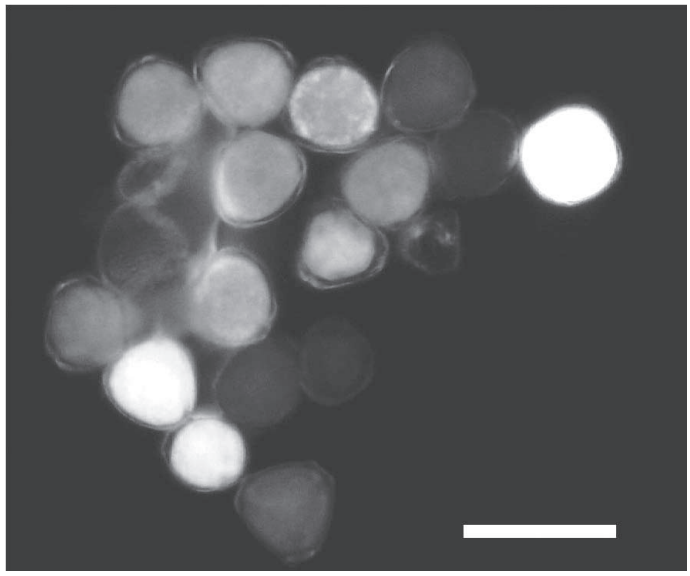

Figure 3. Fluorochromatic test of *E. marginata* pollen viability using fluorescein diacetate showing variable levels of fluorescence between the pollen grains. Scale = 25 μm.

only sugar and boron. These results were supported by the statistically significant differences (Table 1), but further investigation is needed to confirm this finding. The Ca^{2+} ions are mobilised in the presence of magnesium and potassium (Brewbaker and Kwack 1963). Calcium, magnesium and potassium in the medium also reduced the 'population effects' reported by Shivanna and Johri (1985), and generally gave more consistency and greater pollen tube growth. The hanging-drop method of quantifying pollen germination (Potts and Gore 2000) prevents clumping of pollen grains, so that 'population effects' are not a problem, but it is more time consuming. It is possible that the BK medium may improve pollen germination in other eucalypts.

Optimum conditions for storage of *E. marginata* pollen are very similar to those found for eucalypts studied by Boden (1958), Pryor (1976), Griffin *et al.* (1982), Potts and Marsden-Smedley (1989) and Potts and Gore (2000), except that pollen from *E. marginata* did not remain viable at room temperature for more than one day, and for not more than one week at 4°C. Other eucalypt pollen remains viable at room temperature or at 4°C for longer periods. *Eucalyptus regnans* (*Monocalyptus*) pollen produced a mean of 36 pollen tubes per style after the pollen was stored at room temperature for 36 days (Griffin *et al*. 1982). The statistical analysis indicated that there were significant differences in germination rates between the two germination media, storage times and storage temperatures, and also between the germination media and storage temperatures together. Using the BA medium for germination tests greatly underestimates the pollen viability and obscures the effect of the storage conditions. This effect can be seen in Fig. 2b and Fig. 2c where the BK media produced better pollen germination rates at –20°C and –80°C. While pooling the error does not prove beyond doubt that the differences in germination rates are significant, the low *P*-values indicate differences in media, storage time and storage temperature are likely to be significant.

Significant differences in germination percentages were greater between genotypes than for any other reason (Figs 1, 2). This is similar to results from other taxa such as sesame (Pfahler *et al.* 1997). Short-term weather conditions during the flowering period had very little effect on *E. marginata* pollen germination.

Relative humidity testing of the air and the level of hydration of the pollen before testing pollen germination is considered critical by many researchers. Fresh pollen of some eucalypts will not germinate until it has been desiccated (Boden 1958; Griffin *et al*. 1982; Sedgley and Griffin 1985; Shivanna and Johri 1985 Turner *et al*. 1994; Moncur 1995). There was no such problem with *E. marginata*, and high germination rates using fresh pollen were consistently recorded (Fig. 1). *Eucalyptus marginata* flowers during dry, often hot conditions, and the pollen must have adaptations for these conditions. Although no testing of relative humidity and pollen hydration was undertaken on *E. marginata* pollen, it was likely to be partly desiccated at the time of anthesis (Heslop-Harrison 1979) and may have a low water content due to the dry surrounding environment (Barnabas and Kovacs 1997). The pollen was stored with silica gel, ensuring some desiccation in the stored state. The higher levels of germination in the 2001–2002 stored pollen experiment compared with those from the 2000–2001 were probably due to improved re-hydration of the pollen before germination testing, but seasonal and genotype differences may have contributed.

The fluorochromatic test for pollen viability using fluorescein diacetate was found to be inappropriate for *E. marginata*. This concurs with observations on other *Eucalyptus* spp. (Griffin *et al.* 1982) and shows recommendations by Heslop-Harrison and Heslop-Harrison (1985) are not uniformly applicable within *Eucalyptus*. Pollen must be hydrated for the fluorochromatic test, since it tests the integrity of the plasmalemma of the vegetative cell, which is not functional in a desiccated state (Heslop-Harrison and Heslop-Harrison 1970; Heslop-Harrison *et al.* 1984). Some of the variation in fluorescence (Fig. 3) may be a result of variable levels of hydration of the pollen grains in *E. marginata*, as has been found in other eucalypt species (Heslop-Harrison and Heslop-Harrison 1985).

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