GERMINATION OF STRESS-TOLERANT EUCALYPTUS POLLEN

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SUMMARY

Earlier reports have indicated that the pollen of Eucalyptus is mechanically robust and unusually resistant to the osmotic stress imposed by immersion in water. We have investigated some of the features of the germination mechanism in the pollen of E. rhodantha with a view to clarifying the role of pollen-wall specializations in determining this resistance. Cultured in vitro, the pollen showed erratic germination, with a scatter of germination times up to 24 h. This was associated with variation between individual grains in the rate of hydration and dispersal of the pectins of the oncus, the thickened outer component of the intine present at each aperture. The oncus is itself differentiated, with a refractive outer layer lying within a sporopollenin operculum and itself overlying the protein-bearing layer of the intine. The outer layer, interpreted as a compacted pectin, undergoes only slow dissolution in aqueous media after the lifting of the operculum, and it is this that apparently protects the grain from the effects of short-term osmotic stress. The rate of dissolution varies between grains, possibly as a consequence of minor differences in developmental rate in the final stages of differentiation in the anther, and this contributes to the wider scatter of germination times. The dehydrated pollen gave one-third of the potential germination after 24 h exposure to 60°C, and a small proportion survived 24 h at 70°C. This degree of heat tolerance must primarily reflect properties of the protoplast of the vegetative cell, not examined in the present study; but the wall specializations may well provide a guard against extreme desiccation, and it is noteworthy that the function of the germination mechanism is not prejudiced by exposure to high temperatures.

INTRODUCTION

Pollen of the genus Eucalyptus (Myrtaceae) has been described as 'robust' by Pryor (1976), and the literature on the topic, limited although it is, undoubtedly justifies the description. Griffin et al. (1982), for example, have described a method of processing eucalypt pollen for use in controlled pollinations that would certainly be less than favourable for many angiosperm pollens. Their method involves grinding the anthers in distilled water, first in a tissue grinder and then with a pestle, filtering, and finally desiccation over silica gel. Their results show that a high proportion of the grains survive this vigorous treatment intact and remain viable. This finding is testimony both to the unusual mechanical sturdiness of the pollen and to its capacity to withstand immersion, under stressful conditions, in hypotonic medium. A further characteristic of eucalypt pollen can be deduced from the floral biology of the group, namely a capacity to tolerate heat stress, at least in those

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species adapted to semi-arid conditions. After the shedding of the caducous cap formed by calyx and corolla together or by the calyx alone (Pryor & Knox, 1971), the androecium with partly or wholly dehisced stamens remains fully exposed in the flower, open without protection against heat and desiccation — environmental stresses that can be severe in some habitats of the genus (Florence, 1981).

A preliminary examination of pollen germination in several eucalypt species kindly supplied to us by Dr A. R. Griffin of the Division of Forest Research, CSIRO, revealed a number of remarkable features. Some showed unusual heterogeneity of behaviour during hydration in germination media, and most proved capable of tolerating immersion in distilled water at 27°C for up to 4 h with only limited loss of viability, in agreement with the findings of Griffin et al. (1982). These and other observations suggested that some of the properties of *Eucalyptus* pollen might arise from specializations of the germination mechanism, and led to the present study of the pollen of *E. rhodantha*, a plant of the semi-arid heathland of Western Australia (Erickson, George, Marchant & Morcombe, 1979).

The important part played by the various strata of the pollen-grain wall in regulating germination has been revealed in investigations of the Gramineae (J. Heslop-Harrison, 1979; J. Heslop-Harrison & Y. Heslop-Harrison, 1980). In the grasses, the single germination aperture is occluded in the unhydrated grain by a sporopollenin operculum, which overlies a thickened outer pectic layer of the intine, the Zwischenkörper (Christensen & Horner, 1974). This layer, the counterpart of which has been named the oncus in other pollens (Hyde, 1955), thins out away from the aperture but forms a continuous stratum, the Z-layer, covering the whole surface of the cellulosic inner layer of the wall. When the pollen hydrates preparatory to germination, the Zwischenkörper gelatinizes and lifts the sporopollenin operculum. The tube tip emerges first as an enlargement of the cellulosic inner layer of the intine, but with the transition to cylindrical growth a microfibrillar pectic component displaces cellulose in the wall at the extreme apex. We have observed similar germination mechanisms in other porate pollens, and also in forate, colporate and colpate types, suggesting that mechanisms of this kind are widespread. We show in this paper that in *Eucalyptus* the mechanism is more elaborate than in the grasses, in that an additional component is present in the oncus at the apertures, that may have some significance in determining the special properties of the pollen of the genus.

**Materials and Methods**

**Plant Material**

Inflorescences were collected from plants of *Eucalyptus rhodantha* Blakeley & Steedman (rose mallee) in cultivation in Melbourne, and from a natural population near Badgingarra, Western Australia.

The stamens were excised, and held under desiccating conditions over silica gel at ambient temperatures until the pollen was required. The dry pollen was released from the anthers by vigorously shaking the free stamens in polycarbonate vials. The shed pollen, which adheres electrostatically to the walls of the vials with this treatment, was then collected, free of anther fragments, with a dry, artist's brush.

For the purpose of comparison, various parallel observations were made on the pollen of *Corylus avellana* L. (hazel) collected from natural populations in west Wales.
Pollen quality and germination

The state of the vegetative-cell protoplasts in pollen samples was assessed after fixation for 3 min in 2% glutaraldehyde in 20% sucrose followed by staining with the fluorochrome, Coriphosphine O (Gurr), 0.01% in 30% ethanol, for 15–20 min. A normal protoplast fluoresces conspicuously with this treatment, while deficient cells show little or no fluorescence apart from the autofluorescence of the exine. Samples of 300–400 grains were scored per pollen source. As a test of pollen quality, this technique is comparable with other largely non-specific staining procedures such as the venerable acetocarmine and acid fuchsins methods, but it has the merit of offering better discrimination, since the exine has relatively little affinity for the fluorochrome so that a stained vegetative cell is more readily distinguishable. In general, staining methods offer little guide to immediate pollen viability, but they have some value in indicating what might have been the likely maximum potential of the pollen when freshly shed.

Pollen state was assessed also by the fluorochromatic reaction (FCR). This is basically a test of the permeability properties of the vegetative cell membrane (J. Heslop-Harrison & Y. Heslop-Harrison 1970), and under the right conditions it can produce scores correlating well with germinability (Shivanna & Heslop-Harrison 1981; J. Heslop-Harrison, Y. Heslop-Harrison & Shivanna, 1984). However, Griffin et al. (1982) found it to be ‘inappropriate’ in application to Eucalyptus pollen, presumably because it yielded ambiguous results in their hands. In the present application, the medium was prepared by the semi-empirical method originally described (J. Heslop-Harrison & Y. Heslop-Harrison, 1970), with approx. 10^{-6} M-fluorescein diacetate in 20% sucrose. The reaction was allowed to proceed for 30–40 min at room temperature before scoring.

Germination of E. rhodantha pollen was obtained both in liquid and on semi-solid media, although the percentage achieved was almost certainly below the maximum potential, suggesting that the conditions were suboptimal. The liquid medium contained 10^{-3} M-H_3BO_3 and 10^{-3} M-Ca(NO_3)_2 with 20% sucrose. Pollen samples were cultured at the required temperatures in drops on microscope slides for short-term observation, or for longer periods in 100–200 μl medium in 1.5 ml vials with constant aeration on a rotator at approx. 80 rev/min. The semi-solid medium was of the same composition as the liquid, with the addition of 0.5% agar (BDH). The medium was cast in Petri dishes at a thickness of 1–1.5 mm, and pollen was sown on small segments cut out with coverslips and placed on microscope slides. The samples were incubated at the required temperatures in the dark at a relative humidity of 70–80%. The liquid medium was used in investigating the early events associated with germination, and the semi-solid for following pollen-tube growth.

Pollen and pollen-tube dimensions were measured using a camera lucida system with an Apple microcomputer and digitizer.

Pollen pretreatment

Lipidic surface materials were removed, when this was required, by brief rinsing in cyclohexane. This procedure is effective in stripping off much of the lipidic pollenkitt from pollen surfaces without affecting germinability (Shivanna, J. Heslop-Harrison & Y. Heslop-Harrison, 1983).

Temperature treatments were given in incubators regulating to an accuracy of ±1.5 deg. C. Pollen samples were dispersed on microscope slides, and then held over the appropriate media in polystyrene boxes or, for higher temperatures, in sealed glass capsules. The treatments were carried out at two levels of relative humidity, 5–10% RH (‘desiccating’) and 90–95% RH (‘humid’), monitored with hair hygrometers (Fischer), and also with the sample suspended in water in sealed vials. At the higher temperatures, pollen samples in high humidity were protected by glass shields from direct condensation.

Hydration and emission

The early events leading to germination were followed with pollen suspended in liquid medium on microscope slides at room temperature (21–23°C). In certain preparations, a small amount of carbon black was added to the medium to reveal emissions from the grains. Gelatinizing pectin displaces the carbon particles, rendering the diffusion shell visible (J. Heslop-Harrison, 1979; J.
Heslop-Harrison & Y. Heslop-Harrison, 1980). Estimates of the time course of events for individual grains were made by visual observation, and from photomicrographs taken at appropriate intervals. For more detailed analysis, continuous records were made with a Fisher VTR receiving a signal from a Hitachi CCTV camera mounted on the Vickers M17 microscope. Timings were calculated from the tapes, and linear dimensions and areas were derived from tracings taken directly from the monitor.

Pollen tubes in mounts covered for microscopy eventually cease growth due to anoxia, so the full course of hydration and germination could not be traced in individual grains. Later stages of germination were therefore followed in samples withdrawn from pollen growing in the agitated liquid medium, or on agar.

Preparation of intine ‘ghosts’

Intines were isolated for the observation of the changes associated with germination as described in earlier reports (J. Heslop-Harrison, 1979; Y. Heslop-Harrison & J. Heslop-Harrison, 1982). For the digestion of the exine and the removal of pectic components from the wall (Bailey, 1960; Bouveng, 1965), small pollen samples were suspended in 2-3 ml diethanolamine and maintained at approx. 90°C for 30-90 min. The dissolution of the sporopollenin of the exines was monitored by sampling at intervals until the bulk of the wall material staining with the fluorochrome Auramine O had been removed. The digest was then diluted several times with water, and the grains recovered by centrifugation. After two further water washes, the grains were suspended in 8 M-NaOH, and held just below boiling point until observation showed that the bulk of the cell contents had been removed. The sample was then recovered, washed free of NaOH, suspended in 0.1% aqueous Calcofluor White for 5 min, washed again and concentrated by centrifugation for observation by fluorescence microscopy.

Freeze-sectioning

Unfixed pollen was encased in 15% gelatine, and sectioned at 10-12 μm at −15°C on a SLEE cryostat.

Fixation and resin embedding

Pollen samples were fixed without prior rehydration in 2% glutaraldehyde in 0.05 M-phosphate buffer at pH 7.2 with 20% sucrose for 2-4 h at room temperature, dehydrated through an ethanol series, and embedded in JB4 resin (Polysciences Inc.). Sections were cut at 1-2 μm with glass knives.

Cytochemistry

The investigation was aimed mainly at clarifying the role of the pollen walls and their associated incrustations in the germination process, and the cytochemical procedures were selected accordingly to provide information about the composition and behaviour of the wall materials. Notwithstanding the limitations imposed by the small size of the pollen of *E. rhodantha* (19.05 ± mean maximum diameter in the unhydrated grain, with a wall thickness of 1.2 μm), adequate resolution was obtained with most of the procedures to allow the main characteristics to be established, although various ambiguities remain.

The main staining procedures were as follows. (a) The periodic acid–Schiff (PAS) method for the general localization of polysaccharides with vicinal glycol groups (Pearse, 1972). (b) Calcofluor White (Polysciences Inc., 0.001% aqueous) for β-1,4- and mixed β-1,3- and 1,4-linked glucans (Maeda & Ishida, 1976; Takeuchi & Komamine, 1978). Calcofluor White stains alkali-resistant microfibrillar intine ghosts from which pectic materials have been extracted (Y. Heslop-Harrison & J. Heslop-Harrison, 1982), and is presumably mainly localizing cellulose-like polymers analogous with that described from lily pollen tubes by Herth et al. (1974). (c) Alcian Blue 8GX (R. A. Lamb; 1% in 3% acetic acid), and Ruthenium Red (TAAB, 0.02% aqueous) for the broad class of pectic polysaccharides, referred to hereafter simply as pectin. The mechanism of Alcian Blue staining has been discussed by Scott, Quintarelli & Dellovo (1964), Lev & Spicer (1964) and
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Pearse (1972). In plant cell walls, it appears mainly to stain polyanionic polysaccharides, including gel-forming pectic constituents and presumably also some glycoproteins. Ruthenium Red is the classical stain for plant pectins (Johanssen, 1940; Gurr, 1965), and has been used with success for the electron-microscopic localization of acidic plant-cell surface polysaccharides (Luft, 1976); its staining spectrum is, however, somewhat different from that of Alcian Blue, and it has much less affinity for the gelatinizing pectins of the pollen wall. (d) Decolourized Aniline Blue (BDH & Gurr; 0-05 % at pH 11) as a fluorochrome for the localization of callose (Linskens & Eser, 1957; Eschrich & Currier, 1964). (e) Auramine O (Gurr, 0-01 % in 0-05 M-Tris-HCl buffer at pH 7-2) and basic Fuchsin (R. A. Lamb, 0-01 % aqueous) for sporopollenin. Among the several basic fluorochromes that stain the pollen exine, Auramine O is one of the most sensitive, and can therefore be used for localizing thin strata (J. Heslop-Harrison, 1979). Basic Fuchsin is useful both as a dye for transmitted-light microscopy (Faegri & Iversen, 1964) and as a fluorochrome. With some exines it shows differential staining of sexine and nexine, and provides thus a means of differentiating the strata. The chemical basis for this remains obscure. (f) Coomassie Blue R250 (0-1 % in 7 % acetic acid and 30 % methanol) and the fluorochrome, 1-anilino-8-naphthalene sulphonlic acid (1-ANS, Nutritional Biochemicals Inc.) for protein. The latter was used at 0-001 % in 0-01 M-phosphate buffer at pH 6-8 containing 15 % methanol (J. Heslop-Harrison, Knox & Y. Heslop-Harrison, 1974). (g) Scarlet R (Scarlet R Michaelis, R. A. Lamb, saturated in 70 % ethanol) for the localization of surface lipids (Gurr, 1965).

Non-specific esterase was detected in intact and freeze-sectioned pollen with a-naphthyl acetate as a substrate and tetrazotized o-anisidine as a coupling agent (Pearse, 1972). Nuclei were located in pollen grains and tubes with the DNA-specific fluorochrome, 4,6-diamidinido-2-phenylindole (DAPI; approx. 0-001 % in germination medium).

RESULTS

Pollen dimensions and quality

Pollen samples of E. rhodantha from both sources revealed remarkable variability (Fig. 1). Assessed by Coriphosphine O stainability, the Melbourne sample showed 41-6 % normally filled grains, and that from Badgingarra, 49-8 %. The stainable grains were distinguishable by their greater size after hydration in the fixing medium (Fig. 2), and what were presumed to be the two classes, staining and non-staining, could readily be recognized after brief immersion in the aqueous germination medium (Figs 3, 4). The mean areas of the unhydrated population and the two size classes are shown in Table 1. The observations on stainability and size indicate a high level of inherent sterility in the pollen; the smaller class, which lacked a normal protoplast although with a well-formed exine, being infertile.

Following the standard FCR procedure, samples of pollen from both sources showed an unusual degree of variation, and a clear subdivision into 'positive' and 'negative' reactors could not be made. The samples were therefore assessed visually in three categories for the purpose of scoring: grade 0, where the observed intensity was essentially that established by the autofluorescence of the exine; grade 2, with a fluorescence intensity acceptable by analogy with other pollens as indicating high germinability, and grade 1, intermediates of uncertain status.

In Fig. 5, the percentages of FCR grades 1+2 and 2 observed in pollen samples exposed to various pretreatments are compared with the percentage germinability obtained from replicates. Hydration for 24 h reduced the grade 2 score and also reduced germinability. Water soaking, which has little effect on germinability of
Fig. 1. Unhydrated pollen of *Eucalyptus rhodantha* suspended in Whitemor oil. ~×480.

Fig. 2. Fluorescence micrograph, Coriphosphine O staining, showing grains with staining and non-staining vegetative cells. ~×500.

Fig. 3. As Fig. 1, unhydrated grain of the larger size class. ~×2000.

Fig. 4. As Fig. 1, unhydrated grain of the smaller, presumed sterile, class. ~×1900.

Table 1. *Areas of pollen of Eucalyptus rhodantha, as observed in polar view, before and after hydration*

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<tr>
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<th>Area (μm²)</th>
<th>Coefficient of variation (%)</th>
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<tr>
<td>Dehydrated, observed in</td>
<td>190·2±6·89</td>
<td>25·7</td>
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<tr>
<td>in Whitemor oil</td>
<td></td>
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<tr>
<td>Hydrated, vegetative</td>
<td>445·0±6·91</td>
<td>11·0</td>
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<tr>
<td>cell contents present</td>
<td></td>
<td></td>
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<tr>
<td>Hydrated vegetative</td>
<td>225·1±3·95</td>
<td>12·4</td>
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<td>cell contents absent or</td>
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<td>diminished</td>
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Stress tolerance of Eucalyptus pollen

Fig. 5. Effects of pollen pretreatments on germination after 24 h on semi-solid medium at 27°C, and on the fluorochromatic reaction (FCR), classes 1+2 and class 2, scored as described in the text. A. Cyclohexane rinsed; B, tested immediately following desiccated storage at ambient temperatures without pretreatment; C, stored 24 h at 90–95% RH and 27°C; D, suspended in distilled water 24 h at 27°C. Sample size, 200–400 in each case.

Eucalyptus pollen over periods of up to 4 h, reduced the grade 2 FCR score and eliminated germinability when protracted as long as 24 h.

Predictably, the class giving the highest grade 2 score in the desiccated sample contained the larger grains that in parallel observations showed high Coriphosphine stainability, but only some 40% of this group were germinable in the conditions of the experiment – that is to say, less than 20% of the total sample. Cyclohexane washing marginally increased both FCR score and germinability, and if the increases are indeed significant this may suggest that the lipidic surface materials are involved in regulating water passage through the apertures, a point considered further in a later section.

Response to temperature

The effect of temperature on FCR scores is shown in Fig. 6. The treatments, 24 h duration in each case, were given to the desiccated pollen, and the samples were restored to room temperature and tested immediately. The grade 2 scores may be compared with the germinability recorded in Fig. 7 for samples given similar temperature treatments. After exposure to 60°C, 37% of the sample tested for FCR gave grade 2 scores, and 6.7% of the total sample exposed to this temperature for 24 h proved germinable – that is to say, 33.7% of the maximum obtained from the
Fig. 6. Effect of temperature on the fluorochromatic reaction (FCR) classes 1+2 and class 2, scored as described in the text. Pollen held for 24 h over desiccant at each temperature before testing at room temperature (20–21°C). Sample size, 200–400.

Fig. 7. Effect of temperature on germination. Desiccated samples, held for 24 h at 5–10% RH at the temperatures shown. Hydrated samples, held for the same period in sealed chambers at 90–95% RH. Germination on semi-solid medium at 27°C tested immediately after restoration to room temperature. Sample size, 150–300.

Fig. 8. Cumulative germination curves over 24 h for *Corylus avellana* and *E. rhodantha* on semi-solid medium, the former at 21°C, and the latter at 27°C.

Fig. 9. Tube growth in *E. rhodantha*, cultured on semi-solid medium over a 24 h period at 27°C. The vertical bars indicate ± s.d.
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untreated sample. FCR grade 2 score fell to zero after 24 h exposure to 70°C, but even after this treatment a proportion of the grains, albeit very small, remained germinable. The retention of germinability in high temperatures is evidently a property of the desiccated pollen. As Fig. 7 shows, humid storage at 40°C reduced germinability substantially, and no grains survived humid storage at 60°C.

For comparison, the temperature tolerance of the pollen of *Corylus avellana*, a typical temperate mesophyte, was examined alongside that of *E. rhodantha*. With *C. avellana*, pre-hydration is necessary to achieve optimum germinability, and treatments of 2–3 h at 90–95 % RH were therefore given before testing for germination. Pollen collected from dehiscing anthers and tested immediately showed 87·0 % germination, and this fell to 63·5 % after storage for approximately 12 h at 18–20°C under desiccating conditions. In the same conditions, germinability was reduced to 30·8 % at 27°C, and eliminated altogether at 40°C.

**Time course of germination and tube growth**

Germination of *E. rhodantha* pollen on the semi-solid medium over a 24 h period at 27°C is shown in Fig. 8, together with the germination of freshly collected, pre-hydrated *C. avellana* pollen on the same medium at 21°C. The striking feature is the wide dispersal of germination times in the *E. rhodantha* sample. While some grains initiated pollen tubes within 2 h of transfer to the medium, germination was just beginning in others at 12 h. The behaviour of the pollen of *C. avellana* is in marked contrast; in the sample tested, germination was completed within 2 h. Fig. 9 shows the mean lengths of pollen tubes of *E. rhodantha* in a succession of samples abstracted from the culture and fixed for measurement over a 24 h period. The extent of the variation, obviously a consequence of the scatter of germination times, is shown by the high standard deviations recorded.

**Pollen wall organization: the exine**

Exine structure in *E. rhodantha* differs in no fundamental respect from that described for the two species of *Eucalyptus*, *E. phoenicea* and *E. spathulata*, examined by Gadek & Martin (1982), who typified the grains as triangular, angulaperturate and tricolporate. A polar view of an *E. rhodantha* grain is shown in Fig. 17, and this may be compared with the equatorial views of Figs 12 and 13. Sexine and nexine stain differentially with basic Fuchsin (Fig. 10), and as noted by Gadek & Martin (1982), the sexine (ektexine in their terminology) is thickened along the margins of each colpus, a feature observed also by Wodehouse (1932) and Pike (1956). In suitable preparations, the endoaperture is visible as a ragged slit with its long axis at right-angles to the colpus (Fig. 11). Figs 12 and 13 provide a comparison of the colpial areas of the exines of unhydrated and partly hydrated grains as observed with Auramine O staining.

Pollen grains of the larger size class frequently, although not invariably, show a prominence at the aperture sites (Fig. 3), and this enlarges during initial hydration. The prominence, formed by the partly emergent intine, is enclosed by a thin
Auramine-staining cap or operculum (Fig. 14A,B), readily observed in semi-thin sections (Fig. 15). This layer, which may be composed of sporopollenin although it appears to be too delicate to survive acetolysis, probably corresponds to the 'colpus membrane' referred to by Gadek & Martin (1982), and the image in Fig. 15 indicates that it may represent an extension of the foot-layer of the sexine as suggested by these authors. Its fate during germination is described further below.

The exine cavities of *E. rhodantha* pollen carry sparsely distributed lipidic pollenkitt, which disperses gradually in attenuated droplets in the ethanolic Scarlet R medium (Fig. 16A,B). Concentrations are often seen along the colpial slits in unhydrated grains, and residues remain at the margins and over the operculum as hydration proceeds.
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Pollen wall organization: the intine

Two strata can be resolved in the non-apertural intine with the optical microscope, an inner cellulosic layer, PAS-reacting and with an affinity for Calcofluor White (Fig. 18), and an outer pectic layer staining intensely with Alcian Blue, but less so with Ruthenium Red (Figs 19, 20). It is the inner, cellulosic layer that survives in the preparation of intine ghosts, the pectic layer and the overlying exine being dispersed. Evidence of the microfibrillar substructure is apparent in the isolated cellulosic stratum even with optical microscopy (Fig. 24).
The apertural organization is complex. The most notable feature is the thickening of the pectic layer, which forms an umbonate oncus (Fig. 19); the cellulosic layer extends under this (Fig. 18), and is itself somewhat thickened in the vicinity (Fig. 25). The oncus itself is stratified, and the more readily recognizable differentiations are summarized in Fig. 27. A highly refractive, domed stratum lies immediately beneath the operculum (seen, e.g., in Fig. 16A). It stains lightly with Ruthenium Red (Fig. 20), but scarcely at all with Alcian Blue nor with other polysaccharide stains. The ambiguity of its cytochemical properties means that no firm conclusion as to its nature can be drawn, but the fact that it undergoes dissolution in oxalate–citrate buffer and more slowly during water soaking and in natural germination suggests that it is probably a highly compacted pectin.

Beneath the refractive layer lies the main accumulation of intine protein, lenticular in form in the partly hydrated grain, and showing the usual staining reactions (Figs 21, 22) and enzyme activity (Fig. 23). The protein zone stains both with Ruthenium Red and Alcian Blue (Figs 19, 20), indicating that the protein inclusions are embedded in the usual manner in a pectic matrix (for a review, see Knox, 1984).
This zone is sealed below by a continuous layer of pectin, interposed between it and the cellulosic stratum (Fig. 22).

Sterile pollen

As we have seen, sterile grains of the type illustrated in Fig. 4 lack a normal protoplast, but most have extremely well developed onci, which form hemispherical domes beneath each aperture site. These stain intensely with Alcian Blue (Fig. 28). The cellulosic stratum of the intine runs under the onci (Fig. 29). In intine ghosts prepared from sterile grains, the cellulosic layer usually evaginates at each aperture site (Fig. 30), showing no evidence of the thickening evident in these locations in ghosts prepared from the presumed fertile grains (cf. Figs 24, 25).

Hydration, early emissions and the initiation of the pollen tube

Pollen of the lesser size class, presumed to be sterile, underwent some initial enlargement in both liquid and semi-solid germination media and then stabilized, while the mean area of the larger, putatively fertile, grains increased substantially within the first hour of hydration, before the onset of germination (Table 1). Noteworthy, however, was the considerable variation in the behaviour of individual grains of this class. In those achieving the most rapid expansion in the liquid medium, the VTR records showed an enlargement of area in polar projection of as

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Fig. 17. Equatorial view of an intact grain partly hydrated in the liquid germination medium. In this focal plane, the onci are seen as collar-like thickenings at the apertures, extending as prominent bosses through the sexine, but still capped by the opercula. DIC; ~\times 1800.

Fig. 18. View of an intact grain corresponding to that of Fig. 17, fluorescence micrograph following Calcofluor White staining. The brightly fluorescent inner cellulosic layer of the intine is seen to invest the vegetative cell, passing under the onci, where it is somewhat thickened. ~\times 1800.

Fig. 19. Light micrograph of a semi-thin (1-1.5 μm) section of a grain in equatorial view. JB4 resin embedment, Alcian Blue staining. A single aperture has been intersected. The thin but heavily staining pectic stratum, corresponding to the Z-layer of the grass pollen grain (J. Heslop-Harrison, 1979), extends without break over the inner cellulosic part of the intine, thickening at the aperture to form the oncus. ~\times 1900.

Fig. 20. Intact grain, corresponding to that of Fig. 17, in polar view, Ruthenium Red staining. The staining of the onci is less intense than that produced by Alcian Blue, and is mainly restricted to the outer zones. ~\times 1950.

Fig. 21. Section corresponding to that of Fig. 19, Coomassie Blue staining for protein. Although there has been some degree of diffusion, the main protein-bearing zone is seen to be in the outer part of the oncus, more or less corresponding with the zone staining most heavily with Ruthenium Red (cf. Fig. 20). ~\times 2000.

Fig. 22. As Fig. 21, fluorescence micrograph, 1-ANS staining for protein. The protein-bearing zone is here more precisely defined as a cap in the prominent part of the oncus, below the consolidated refractive zone (r). ~\times 1800.

Fig. 23. Esterase localization in the aperture region of a freeze-sectioned partly hydrated grain. The reaction product lies in the exserted part of the oncus, beneath the refractive zone (r), which is well-defined in this micrograph as a layer extending beneath the operculum. The light flecks are gas bubbles released by the enzymic reaction. ~\times 2200.
Figs 24–26. Fluorescence micrographs of isolated cellulosic layers (intine 'ghosts') prepared from intact grains.

Fig. 24. Surface view of ghost from a putatively fertile grain. From the analogy with similar isolated layers from other pollens (Y. Heslop-Harrison & J. Heslop-Harrison, 1982), the striations are likely to represent bundles of oriented microfibrils. ×1850.

Fig. 25. As Fig. 24, equatorial focal plane, revealing the thickening of the cellulosic layer at the aperture sites. On release from the restraint imposed by the exine and the outer pectic layer, this stratum of the intine evaginates at the aperture sites (compare directly with Fig. 18). ×1700.

Fig. 26. Ghost isolated from a germinating grain, showing the prominence formed by the emergent cellulosic layer before the establishment of the pectic wall at the tube tip. ×1600.

Fig. 27. Wall stratification at the margin of a germination aperture of the pollen of *E. rhodanica*.

Much as 55% in 48 s. However, expansion was much slower in most of the grains of the class, suggesting some appreciable impedance to the entry of water. In general, it appeared that hydration was most rapid in grains in which the intine was prominently exserted at the apertures at the time of first immersion.

Because of the variation between grains in the rates of hydration and germination,
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Fig. 28. Intact sterile grain in polar view, Alcian Blue staining. The onci are seen as enlarged, deeply staining hemispherical masses at each aperture. ~×2000.

Fig. 29. As Fig. 28, fluorescence micrograph following Calcofluor White staining. The cellulosic layer extends around the shrunken and empty vegetative cell, passing under the onci at the apertures. ~×2000.

Fig. 30. Fluorescence micrograph of an intine ghost released from a grain corresponding to that of Fig. 29. Here the evaginated parts of the cellulosic layer formerly lying beneath the onci are less thickened than in the ghosts prepared from fertile grains (compare Fig. 25). ~×1900.

it was not feasible to draw up any general timetable for events in the population as a whole. Nevertheless, the sequence of phases in pollen germinating in the liquid medium could be pieced together from the timed samplings and the VTR records, and in the following paragraphs approximate timings are given for the more conspicuous events observed in the most rapidly germinating grains.

(a) Early pectin emission (5—10 min). Hydration is followed by the gelatinizing of pectins of the oncus at each aperture, evidenced in Fig. 31 by the displacement of carbon particles.

(b) Lifting of the operculum (10—30 min). The continued gelatinization of the apertural pectins in germinating grains is associated with the displacement of the operculum, usually at one aperture — that from which the tube will emerge — but sometimes at two or all three. The process is seen in polar and equatorial views in Figs 32 and 33.

(c) Later pectin emission and dispersal of the refractive layer of the oncus (10—60 min). The more rapidly released pectins form a diffuse and continuously enlarging halo generally at all three apertures (Fig. 35). In due course, often only at the aperture destined to provide passage for the tube tip, a second rather more clearly defined cap of material emerges, derived by the slow gelatinization of the consolidated refractive layer of the oncus (Fig. 34). Unlike the earlier, more-diffuse emission, this exudate, which survives fixation, stains with Alcian Blue.
Fig. 31. Intact, living grain suspended in germination medium with carbon black, showing the early emission of gelatinized pectin from the three apertures. ~x1050.

Fig. 32. Polar view of a germinating grain at a somewhat later stage than that of Fig. 31. The operculum (o), lifted by the gelatinizing pectin, is visible at one of the apertures, and the emerging compacted layer of the oncus (r) may be seen below. DIC; ~x1350.

Fig. 33. As Fig. 32, grain in equatorial view. DIC; ~x1350.

Fig. 34. As Fig. 31, later stage of germination. The compacted, refractive layer of the oncus (r) is now expanding into the outer pectic cloud as it undergoes dissolution, and the emerging pollen-tube tip may be seen below. ~x2100.

Fig. 35. As Fig. 34. Enlarging masses of gelatinizing pectin can be seen at each aperture, with the emerging pollen-tube tip at one. Germinating pollen at this stage yields intine ghosts like that illustrated in Fig. 26. ~x950.

Fig. 36. Germinating grain at the same stage as that in Fig. 34, Coomassie Blue staining for protein. ~x1750.
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Figs 37-40. Pollen germinating in the standard liquid medium, viewed intact and without fixation. The staining was achieved by infiltrating the mounts from the side with the stain made up in the medium.

Fig. 37. Grain at a somewhat more advanced stage than that of Fig. 34; Calcofluor White staining. ~×2000. A. Bright field. The compacted layer of the oncus is seen in an advanced stage of dissolution, and the collar of pectic material within the apertural exine is already much reduced. B. Fluorescence micrograph, showing the emerging tube tip.

Fig. 38. As Fig. 37, at a still later stage. ~×2000. A. Bright field. The tube tip has displaced the last remains of the compacted layer of the oncus, and the collar of pectic material beneath the apertural exine is wholly dispersed. B. Fluorescence micrograph. Calcofluor White stainability is being lost from the tube tip as the cellulosic component is displaced by the new pectic wall at the tip.

Fig. 39. Grain with an emerging pollen tube; Alcian Blue staining. All pectic material has been lost from the interior of the grain, but a residual collar of material derived from the oncus remains around the base of the tube. The wall at the tube tip shows affinity for the stain. ~×1100.

Fig. 40. Grain comparable with that of Fig. 39; Calcofluor White staining. Stainability has been lost from the wall in the tip zone with the transition to cylindrical growth. ~×1550.
Figs 41–44. Generative cells and vegetative nuclei during hydration and early pollen-tube growth.

Fig. 41. Intact, partly hydrated grain, unfixed, viewed in a focal plane revealing both the generative cell (gc) and the vegetative nucleus (vn). DIC; ~×1300.

Fig. 42. Grain comparable with that of Fig. 41, fluorescence micrograph following GDA fixation and DAPI staining. The condensed generative cell nucleus (gn) lies in the proximity of the more diffuse vegetative nucleus (vn). ~×1300.

Fig. 43. Entry of the vegetative nucleus (vn) into an actively growing pollen tube. The generative cell (gc) is seen in the proximity of the vegetative nucleus, but has not entered the tube. No fixation. DIC; ~×950.

Fig. 44. Generative cell in an extending tube, DAPI staining following GDA fixation. A. Phase-contrast; B, fluorescence micrograph. ~×1650.
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(d) Protein emission (10–60 min). The release of the apertural intine proteins begins soon after the earliest pectin emissions, and the process continues throughout the early period of germination. The released protein is presumably dispersed throughout the pectin cloud at the aperture sites, the image of attenuated fibrils observed in the Coomassie Blue stain-fixing medium (Fig. 36) representing the denatured condensate.

(e) Emergence of the tube (90 min onwards). The emerging tip presses into the gelatinizing cap of oncus material, and eventually displaces it (Figs 37A, 38A). Initially, the wall at the extreme apex shows intense Calcofluor White stainability (Fig. 37B), and survives in intine ghosts prepared from grains at this stage of germination (Fig. 26). As the apex extends, Calcofluor White stainability is progressively lost from the wall at the tip (Fig. 38B).

(f) Assumption of cylindrical growth in the pollen tube (2 h + ). In the actively extending tube, the wall at the tip shows little or no stainability with Calcofluor White (Fig. 40), but has affinity for Alcian Blue (Fig. 39). In Fig. 39, a residual collar of Alcian Blue-staining material derived from the oncus is seen at the base of the tube.

The foregoing sequence can probably be taken as typical for a successively executed, normal germination in a liquid medium. The course of events is not always uninterrupted, and in fact in the pollen samples tested more than half of the grains of the larger size class – that regarded as potentially fertile – failed to complete the process in the medium used, even after protracted incubation (Fig. 8). The arrest usually occurred after the early emissions of pectin and protein and the lifting of the operculum, and was marked by a failure to define a tube tip. In the osmotically balancing medium, however, the grains did not burst. Aged and inviable pollen progressed through phases (a) to (c), but similarly failed to produce a tube.

Emergence of the vegetative nucleus and generative cell

The spindle-shaped generative cell usually, but not invariably, lies in close contact with the vegetative nucleus in the hydrated but ungerminated grain (Figs 41, 42). On the semi-solid medium, the earliest passage out of the grain was observed when the tube had reached a length of 60 μm (Fig. 43). It appears to be a matter of indifference whether the generative cell or the vegetative nucleus emerges first. The generative cell is readily observed in the tube, where it usually retains the spindle shape (Fig. 44A,B). Generative cell division was not seen in the tubes in culture.

DISCUSSION

A striking feature of the samples of E. rhodantha available to us was the variation in both grain size and shape. In part this was attributable to a high level of inherent sterility. Moran & Hopper (1983) remark of species of the series Macrocarpae, to which they refer E. rhodantha, that they tend to occur in small populations occupying specialized habitats and to have low fecundity. We found a sample of the pollen of another species of the group, E. caesia, supplied by the Division of
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Forestry Research, CSIRO, to be very variable also, although it gave a rather higher maximum germinability of 32.5% on the standard semi-solid medium.

Partial sterility cannot account for all of the variation observed in E. rhodantha pollen. Among those grains regarded as potentially germinable because of a positive FCR reaction and the possession of a stainable protoplast, the variation was expressed mainly in the extent of exertion of the intine beneath the operculum at each aperture and the degree of concavity of the amb, features that in large degree determine the area of the grain as observed in polar view, and presumably also the volume. Since these differences can be seen in pollen extracted from anthers before natural shedding, it evidently arises from developmental variation, probably during the final stage of dehydration, which is the prelude to anther dehiscence.

Turning to the mechanism of germination, the similarities to that described for the grass pollen grain (J. Heslop-Harrison, 1979; J. Heslop-Harrison & Y. Heslop-Harrison, 1980) are evident enough. Hydration leads to an expansion of the grain, and then the progressive gelatinization of the oncus pectins. This results in the displacement of the operculum at one or more apertures, and development of a prominence in the inner, cellulosic, layer of the intine, which is ultimately exerted, presumably as a consequence of pressure within the grain attributable to the matric potential of the protoplast. Up to this point the process may depend upon pre-existing adaptations, since the events occur in dead pollen, but the subsequent steps, involving the disappearance of the cellulosic layer and the establishment of a tenuous pectic wall at the tip (the events marking the assumption of cylindrical growth in the emerging tube), presumably require the involvement of a metabolically active vegetative cell.

In the grasses the sequence of events tends to progress synchronously throughout a pollen population, occupying only a matter of minutes. The corresponding processes in the pollen of hazel, used as a standard of comparison in the present study because of the certain structural similarities of the pollen with that of Eucalyptus, are accomplished more slowly, but again more or less in synchrony. In contrast, in E. rhodantha the succession is very much more leisurely, individual grains advancing at different rates.

Structural features of the pollen undoubtedly contribute to this peculiarity. The rapidity of dispersal of the pollenkitt at the apertures probably affects the rate of water ingress, but it is unlikely that this should be the most significant factor, and from the detailed study of the events during hydration and leading up to germination we have concluded that more importance attaches to the function of the outer refractive layer of the oncus, provisionally identified as a consolidated pectin, which has no counterpart in the grass pollen grain or in that of Corylus. This layer gelatinizes and ultimately disperses during germination, but the process is singularly slow in some grains, even when immersed in the liquid medium. It is this behaviour of the specialized stratum of the oncus that appears to provide the principal control over germination, differences in rate of dissolution contributing to the wide scatter of germination times. Variation in the state of the grains at the time of release is evidently also a factor, since those with exerted intines – probably more highly
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hydrated initially in any event – tend to expand more rapidly than those with more fully closed apertures, and accordingly reach the point of germination earlier.

The outer stratum of the oncus evidently also provides the principal guard against the early destruction of the vegetative cell in hypotonic media. Although the grains may expand rapidly when suspended in water, most survive the exposure without immediate bursting, and it is not until the refractive outer layer of the oncus has undergone dissolution and the wall at the aperture has been weakened in consequence that the protoplast of the vegetative cell ruptures. In osmotically balancing germination medium this would, of course, be the moment of emergence of an intact pollen-tube tip.

The foregoing relates to the behaviour of the pollen of *E. rhodantha* as observed in a liquid medium, or on the surface of a semi-solid agar medium, and the question arises as to the appropriateness of extending the conclusions to the situation on the stigma surface. The surface secretions of the *Eucalyptus* stigma have not as yet been characterized, but preliminary observations on the stigma of *E. rhodantha* and other species of the genus suggest that germination takes place in pollen partly or completely entrapped in a polysaccharide-rich aqueous surface film. The conditions of hydration may not therefore be radically different from those experienced by pollen in the synthetic media, although it would obviously be desirable now to compare the timing of the events in the two environments.

Assuming that the operation of the germination mechanism is essentially the same in the natural environment, one can perceive that it could confer various adaptive advantages, notably that of versatility. The pollen is presented in dehisced anthers of gaping flowers over a considerable period of time, and is thus subjected directly and without protection to vagaries of the weather, including not only desiccating heat but also the risk of periodic wetting through dew or rain. The present experiments suggest that the heterogeneity of the population would ensure that whatever the stresses during this period, a proportion would remain functional.

Undoubtedly heat tolerance could be significant in the natural environment of the species. Dehydrated pollen is one of a number of desiccated plant systems that can show resistance to heat stress (Levitt, 1980), but we have found no other example matching the tolerance of *E. rhodantha* pollen, which achieved over 30% of potential germination after 24 h incubation at 60°C, and retained some germinability even after the same period at 70°C. Such an ability to resist high temperatures must reflect primarily special properties of the protoplast of the vegetative cell, not considered in the present study. It is noteworthy, however, that the germination mechanism involving the wall specializations at the apertures is not prejudiced by exposure to high temperatures, since steps (a) to (c) in the sequence described above progress more or less normally even in the grains that fail to germinate after heat treatment.

Finally, we wish to emphasize that we do not suppose that the adaptations described in this paper for the pollen of *Eucalyptus* are likely to be unique; rather the contrary – we expect that many angiosperms adapted for the same kinds of environments and with the same type of floral biology will be found to possess comparable systems, once they are investigated.
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