Breeding Systems and Pistil Structure in the Family Proteaceae

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Floral display including *Dryandra* (yellow); Lucia's, Central Market, Adelaide.
(Photo: R. Mibus)

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Abstract

A considerable body of literature exists on the Australian Proteaceae. However, studies of the proteaceous breeding system have focussed upon a small range of genera, notably the genus *Banksia*. Although these studies are comprehensive, they highlight the need for a broadening of investigation to include other genera and specific aspects of the breeding system. One such aspect is the structure of the pistil which has been shown to potentially limit seed set. This limitation is of concern to plant breeders as it potentially hinders the commercial development of proteaceous species. The literature also highlights the unique features held by the Proteaceae, such as reduced quantities of transmitting tissue relative to the rest of the style and the presence of transfer tissue. To determine the significance of these features, comparison with other angiosperms is required.

Propagation of the Australian Proteaceae for commercial purposes primarily relies upon germination from seed. For some species such germination can be problematic. Thus investigation of methods designed to promote germination efficiency are required. Temperature has been shown to promote germination in some Australian Proteaceae but again research has concentrated upon certain a limited number of species.

Therefore it was the primary aim of this study to broaden our understanding of the breeding systems operating in the Australian Proteaceae and to provide a preliminary study of the optimum requirements for promotion of seed germination in two lesser researched genera which have horticultural potential.

In particular, the breeding systems of two species of *Dryandra* (Proteaceae); *D. quercifolia* and *D. formosa* were investigated. These species are currently sold internationally as cut flowers. The timing and pattern of stigma receptivity was determined using a combination of techniques. Hand pollinations were performed and pollen tubes counted to establish the time of peak pollen germination. In addition, changes at the stigma in terms of stigmatic groove openness and exudate production were assessed using

an environmental scanning electron microscope. Results were combined to ascertain the physical and physiological timing of peak stigma receptivity. Stigmas of both species showed peak receptivity at two to six days post-anthesis. *Dryandra quercifolia* was receptive immediately after anthesis while *D. formosa* showed little germination until two days post-anthesis. A stigmatic exudate was produced by stigmas of both species, its production overlapping with the time of peak pollen germination and maximum groove openness. Each species showed a distinct pattern of exudate production and groove opening. For *D. quercifolia* these factors increased from day three post-anthesis until the end of the study period (day 12 post-anthesis). In contrast, maximum values for *D. formosa* were reached four days post-anthesis and decreased thereafter. These factors were consistently lower in *D. formosa* compared to *D. quercifolia*. In addition, exudate production was lower in pollinated than unpollinated pistils of *D. formosa*.

To determine the compatibility system operating in *D. quercifolia* and *D. formosa*, hand pollinations were performed (self, cross and open pollination) and the results assessed in terms of pollen tube counts and seed set. Both species showed a mixed breeding system; cross- and self-pollination was possible although self-pollination was less favoured and often resulted in post-zygotic abortion of at least one seed. *Dryandra formosa* was more self-compatible than *D. quercifolia*. Pollen tube counts of *D. quercifolia* pistils suggested a self-incompatibility mechanism operating in the upper style of this species. To complement the breeding system investigation of *Dryandra*, the temperature (5, 15, 25 or 35 °C) required for optimal seed germination was determined for three species of this genus, and for three species of the genus *Isopogon*. In addition, the rate and percentage emergence under glasshouse conditions (glasshouse; max/min. 27.9/20.1 °C) was determined for ten species of *Dryandra* and seven of *Isopogon*. Species selection was based on their current or potential use in the floriculture and amenity industries. These industries rely upon seed germination for propagation of many Australian species. Optimal percentage, and rates of germination for both genera were achieved after incubation at 15 °C. In addition, *Dryandra* species germinated at 25 °C, however germination was reduced and slower compared to the 15 °C treatment. Germination was inhibited at 5 °C and 35

°C. The emergence of seedlings in the glasshouse was slow for all species and numbers were low compared with germination at 15 °C. In some cases there was no emergence.

The family Proteaceae is characterised by a low fruit to flower ratio. To determine whether structural limitations within the flower, such as stigmatic cavity size and the amount of transmitting tissue within the style, contribute to this low fertility, pistil structure was investigated. An anatomical and morphological study of pollen presenters, styles and pollen was performed on species from the genera *Banksia, Dryandra, Hakea, Isopogon* and *Macadamia*. In particular, to determine whether the size of the stigmatic cavity restricted access of pollen grains to the stigmatic surface, pollen presenters were serially sectioned and cavity volume determined. In addition, the distribution and volume of tissues within the pollen presenter was quantified using image analysis software. A field emission scanning electron microscope and image analysis were used to calculate pollen grain volume, and in turn the maximum pollen grain holding capacity of the cavity. To assess the amount of transmitting tissue, pistils were transversely sectioned down their length and the number of transmitting tissue cells counted. There were three types of stigmatic cavity. A groove in which the stigmatic papillae were enclosed (*Banksia, Dryandra* and *Hakea*), a groove with protruding papillae (*Macadamia*) and a tube which enclosed the papillae (*Isopogon*). Anatomical studies revealed the pollen presenter to be internally complex, but similar in structure across species studied. Groupings could be formed based upon the presence or absence of transfer tissue and the presence or absence of sclerenchyma. These groups were not mutually exclusive. Transfer tissue was associated with transmitting tissue; the tissue through which pollen tubes grow, in the pollen presenter and upper style of all species except *Hakea bucculenta.* The presence of transfer tissue may contribute to the nutrition of the growing pollen tube. The physical dimensions of the cavity, namely its volume, length and diameter, restricted pollen grain access to the stigmatic papillae. The transmitting tissue tract narrowed significantly from the pollen presenter to the base of the style, at this point cell numbers were as few as eight in *Isopogon cuneatus.* There were three structural filters to pollen tube passage in the pistil. The first was at the stigma – a consequence of cavity dimensions – and the second

and third related to a narrowing of the transmitting tissue tract within the pollen presenter, and to a lesser extent, within the lower style.

To determine whether reduced the quantities of transmitting tissue observed at the base of the proteaceous style were unusual, a comparative study incorporating seventeen species from nine angiosperm families, including one monocotyledon, was performed. Specifically, stylar anatomy was examined to determine whether structural limitations at this point influence pollen tube number, and thus seed set. Serial sections above the ovary were taken, and transmitting and stylar tissue quantified using image analysis software. A comparison was made of numbers of transmitting tissue cells, pollen tubes and ovules. Overall stylar structure and tissue quantities were consistent within families, but differed significantly between families. The proportion of transmitting tissue to the total style was very low in the Proteaceae compared to other families. Pollen tube number was related to transmitting tissue cell number and to ovule number. Species with multiple ovules (≥12) had the greatest area of transmitting tissue and highest number of pollen tubes. The ratio of transmitting tissue cells to pollen tubes was approximately 1:1 for these species, a ratio much lower than for species with few ovules (≤2). For all species, pollen tube number was similar to ovule number suggesting that ovule number may have a strong influence upon the number of pollen tubes reaching the base of the style. *Triticum aestivum* was the only exception to this, multiple pollen tubes reported at the base of the style to fertilise one ovule. Species with multiple ovules were potentially structurally limited by the amount of transmitting tissue in their style, however no firm conclusions could be drawn as pollen tube number was generally sufficient to fertilise the ovules present. The long styles of the Proteaceae may increase the number of transmitting tissue cells required to support the growth of one pollen tube in comparison to other families.

This study has addressed two areas of research into the Australian Proteaceae which needed attention. Firstly, the investigation of the breeding system of the commercial *Dryandra* species has provided a valuable study for comparison to the extensive work on *Banksia*, *Dryandra's* closest relative. It has also provided information which can be used

to breed and improve these species for further commercialisation in the cut flower and garden industries. Secondly the study of pistil structure has further identified the potential of this structure to effect the capacity of a proteaceous flower to be fertilised. Aspects such as the morphology and size of the stigmatic cavity and the quantity of transmitting tissue in the style were highlighted as filters to pollen tube passage. It is noted that any study investigating low seed set in the family should consider pistil structure as a potential site for hindrance of pollen tube growth and thus seed set. The comparative study of the number of pollen tubes at the base of the style in the Proteaceae to other angiosperm species has confirmed the unusual structure of the pistil of the proteaceous flower. In particular, the reduced amount of transmitting tissue at this point relative to the rest of the style is a feature which distinguishes the Proteaceae. The study has shown that pollen tube number at the base of the style and ovule number appear to be related, and that species may differ in the amount of transmitting tissue required for the successful passage of a single pollen tube. Each of these results contribute to our general understanding of the angiosperm breeding system, and in particular the breeding system of the Australian Proteaceae.

The promotion of seed germination by exposure of seeds to controlled temperatures of 15 °C observed in this study is an important result, as it can be used to enhance the seed germination efficiency for commercial propagation of *Dryandra* and *Isopogon* species.

Each of these results contribute to, and potentially assist our ability to use species of the family Proteaceae for commercial cut flower and garden venture.

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Chapter One

General Introduction and Aims

Plant reproductive biology is a vast field incorporating a number of areas such as breeding systems, pollination ecology, the structure and function of the flower, and the process of fertilisation. Knowledge of these systems is of great importance to agriculture, floriculture, maintenance of plant diversity, and the understanding of fundamental plant processes.

The Proteaceae is a family of economic and environmental importance. In 1992-93 proteaceous species accounted for almost A$5 million of Australia's cut-flower exports (Plate 1.1) (RIRDC, 1994). They are also a prominent feature of Australian plant communities. Knowledge of their reproductive biology is essential to develop breeding strategies to foster cultivation for cut-flower production, and to maintain and preserve diversity within the family. In addition, the Proteaceae offer some unusual reproductive features, the study of which helps to broaden our understanding of the plant reproductive system.

The family is believed to be Gondwanan in origin. Fossil records have been found dating back to the Cretaceous period, 135 to 65 million years ago when the southern continents were joined (Johnson and Briggs, 1975). 1500 species are distributed across South America, South Africa and Australia; of these 800 are endemic to Australia. It is a family of trees and shrubs that are believed to have no close taxonomic relatives (Johnson and Briggs, 1975).

Carl Linnaeus first described the Proteaceae in 1735 when he classified the South African genus *Protea*, naming it after the Greek sea god, Proteus, who could change his shape at will. This name is a reflection of the morphological diversity that is characteristic of the

family. Flowers of most genera are bisexual and are arranged in capitula, racemes, heads or spikes (Plate 1.2a) (Collins and Rebelo, 1987). The flowers are commonly tubular and comprise a perianth to which the anthers are generally fused, and the pistil. Pistil structure is unusual. In many genera the tip of the pistil is modified to form a region known as the pollen presenter on which pollen is displayed (Plate 1.2b,c,d) (Howell et al., 1993; Sedgley et al., 1993; Ladd, 1994; Ladd et al., 1996). The stigma is confined to this region and is often localised to a cavity. Protandry is a common feature of the family (Ramsey and Vaughton, 1991).

In the past, much of the research into reproductive biology of the Proteaceae has focussed on pollination ecology (Paton and Turner, 1985; Collins and Spice, 1986; Collins and Rebelo, 1987; Lamont and Collins, 1988) and seed bank dynamics (Abbott, 1985; Gill and McMahon, 1986; Lamont and Barker, 1988). However, since the late 1980s researchers have begun to focus their studies on the breeding systems of individual species. To date the breeding system of a handful of species within limited genera – notably the genus *Banksia* – have been described. These studies have incorporated aspects of the timing and function of stigma receptivity (Sedgley et al., 1985; van der Walt and Littlejohn, 1996a), techniques for hybridisation of species (Sedgley et al., 1985; van der Walt and Littlejohn, 1996a; Fuss and Sedgley, 1991a), estimation of outcrossing rates (Sedgley et al., 1985; van der Walt and Littlejohn, 1996a; Fuss and Sedgley, 1991a; Scott, 1980) and the determination of self-compatibility or incompatibility using hand pollinations (Sedgley et al., 1990; Fuss and Sedgley, 1991b; Goldingay et al., 1991; Carthew et al., 1996). A feature found to be consistently present throughout the species studied is the very low fruit to flower ratio, a direct result of a large floral display and poor fruit set.

The low fruit to flower ratio has inspired a suite of investigations attempting to explain the cause(s) of the observed low fertility. Research has included investigation of the spatial limitations within the infructescence (Collins and Rebelo, 1987; Fuss and Sedgley, 1991a), the effect of floral position (Pyke, 1981), insect predation (Vaughton, 1990) and the effect of low nutrient supply (Stock et al., 1989) on seed set. Pollen limitation (Goldingay and Whelan, 1990), ineffective pollinator behaviour (Whelan and Goldingay, 1986; Vaughton

and Ramsey, 1991) and andromonoecy (Walker and Whelan, 1991) have also been investigated.

In the late 1980s to 1990s, researchers began looking towards the structure of the pistil as a possible limitation to fertility. There are five papers which have addressed this aspect in the Australian Proteaceae; for *Macadamia* spp. (Sedgley et al., 1985), *Grevillea banksii* (Herscovitch and Martin, 1989, 1990), *Banksia menziesii* (Clifford and Sedgley, 1993) and selected *Banksia* and *Dryandra* species (Ladd et al., 1996). The key observations arising from these papers were that the pollen presenter is complex in structure and its tissues may contribute to attrition of self-pollen tubes in the upper style. The column of transmitting tissue in the pistil narrows markedly down its length. This narrowing may be responsible for structurally limiting pollen tube access to the ovary, as often pollen tube numbers are low or zero, at this point. The identification of transfer tissue in association with transmitting tissue is another unusual feature. Transfer tissue functions in the absorption and secretion of substances. Its association with transmitting tissue, the tissue through which pollen tubes grow, may have implications to nutrient availability for the growing pollen tubes.

As a result of these studies, the following questions can be raised: what is the role of transfer tissue in the pollen presenter, and does its association with transmitting tissue implicate it in the recognition of self-pollen tubes or pollen tube nutrition related to long stylar length? What is the minimum number of transmitting tissue cells required to support the passage of one pollen tube?

Thus the literature points to a number of issues which need to be addressed in relation to the breeding system of the Australian Proteaceae. The first is a broadening of the narrow range of species for which detailed breeding system studies exist. Secondly the literature also highlights the need for a more extensive investigation of the potential limitation to seed set presented by the unusual external and internal structure of the pollen presenter and style. Current research has raised questions regarding the minimum number of transmitting tissue cells required for the passage of one pollen tube, and the relationship

between pollen tube number and ovule number. To investigate these issues, a detailed study of the pistil of Australian Proteaceae species is required, combined with a comparison to other angiosperms and investigation of the breeding system of specific species.

Aims

1) To broaden our knowledge of the breeding system of the Australian Proteaceae by conducting an investigation of one of the lesser known genera, *Dryandra,* particularly the floricultural species *D. quercifolia* and *D. formosa* and in particular, to study in detail aspects of the timing of stigma receptivity operating within these species.

2) To enhance efficient propagation of these and other commercial species of *Dryandra* and *Isopogon* by providing a preliminary study of the optimal temperature requirements for seed germination.

3) To investigate a broader range of genera to determine whether structural limitations to fertility exist in the pistil and to quantify these features and determine the extent of these limitations. Specifically, to determine whether the structural configuration of the style, such as the confinement of the stigma to a cavity, the complex arrangement of tissues within the pollen presenter and the observed narrowing of the transmitting tissue tract were responsible for, or contributed to, the observed low fertility in the family.

4) To determine whether proteaceous stylar structure was unusual, particularly in terms of the quantity and distribution of transmitting tissue, by comparison of proteaceous pistils to species of nine other angiosperm families. To then use these results in conjunction with pollen tube number to determine the number of transmitting tissue cells required for the passage of one pollen tube.

Plate 1.1

A commercial packing shed at Blewitt Springs, South Australia. Photograph shows *Dryandra formosa* (arrowhead) in the foreground and a number of *Banksia* species, including *B. coccinea* (arrow)

Plate 1.2

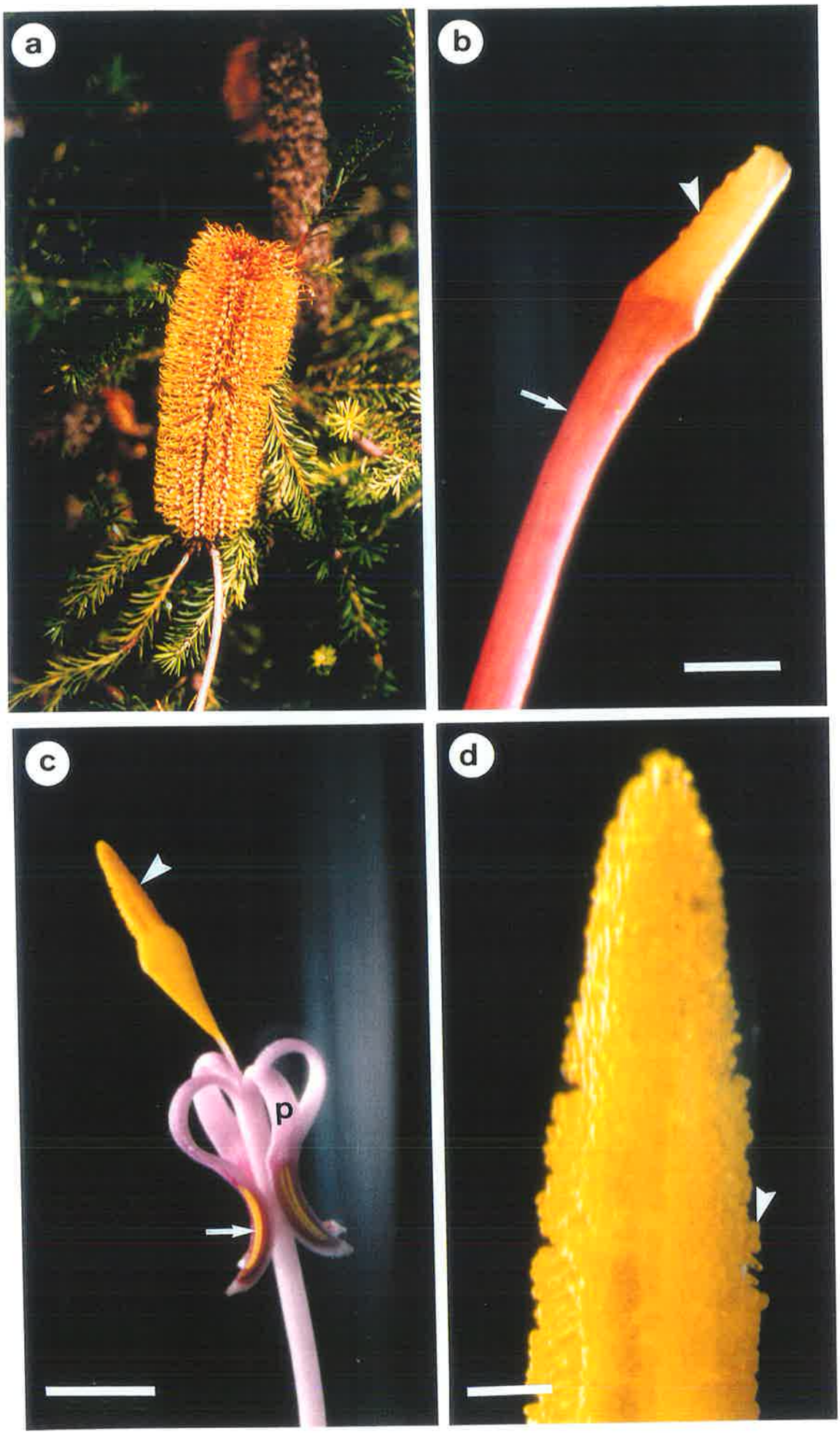
Examples of the proteaceous inflorescence, floret, pistil and pollen presenter

(a) Inflorescence of *Banksia ericifolia*

(b) Upper portion of a *Hakea bucculenta* pistil showing distinctive yellow pollen presenter region (arrowhead) and red style (arrow). Bar represents 1 mm.

(c) Floret of *Isopogon cuneatus* after anthesis. Note pollen presenter (arrowhead), curled pink perianth parts (p) to which the anthers are fused (arrow). Bar represents 2.5 mm.

(d) Upper portion of the pollen presenter of *I. cuneatus* showing pollen grains (arrowhead) that have adhered to the surface hairs of the pollen presenter. Bar represents 250 μm.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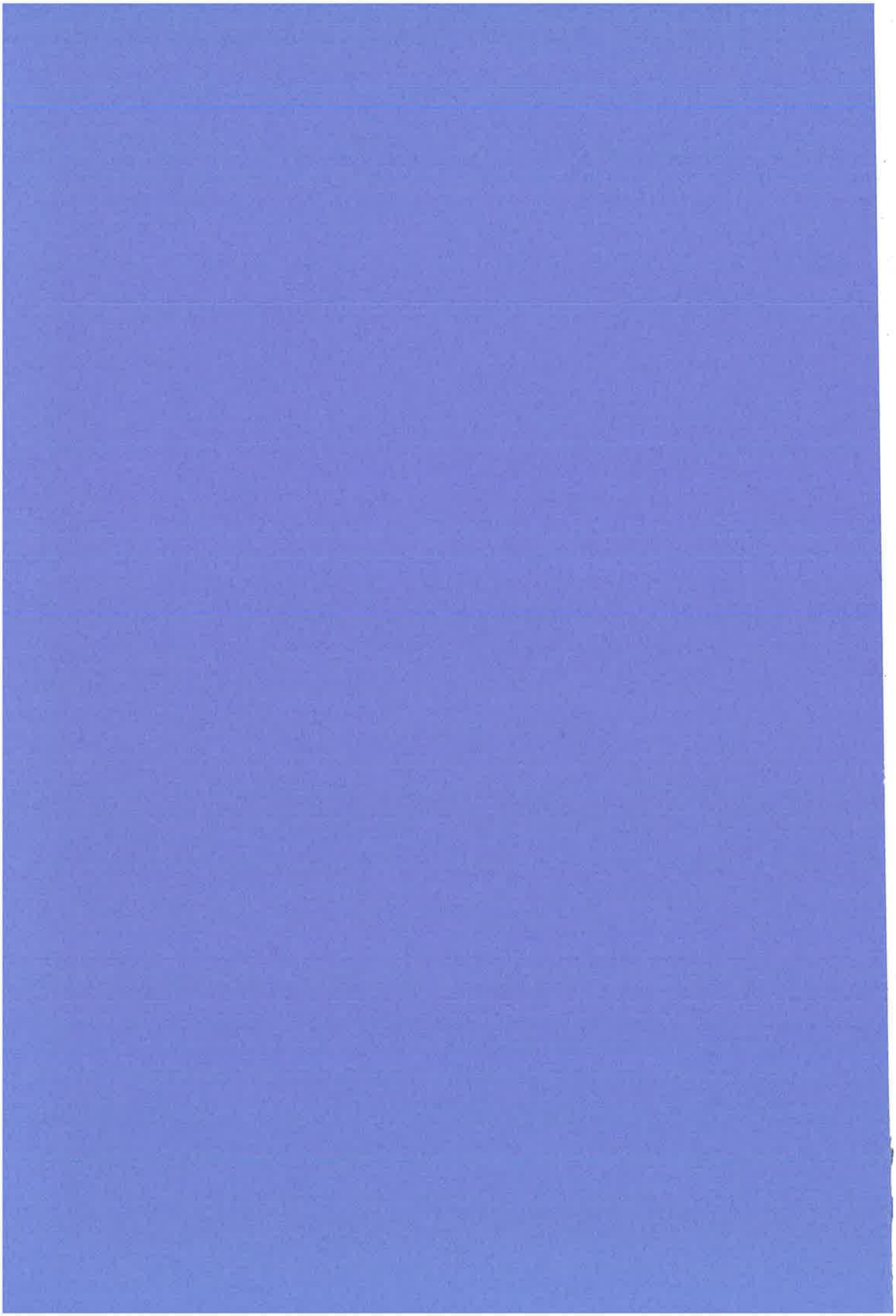


Chapter Two

Breeding System of *Dryandra quercifolia* and *D. formosa* (Proteaceae)

Abstract

The breeding system of one of the lesser studied genera of the Australian Proteaceae was investigated to help balance the numerous studies of the genus *Banksia*, thus allowing comparison of results between a broad range of genera. Hand pollinations were used to investigate the timing of stigma receptivity and the breeding system of two commercial cut flower species, *Dryandra quercifolia* Meiss. and *D. formosa* R. Br.. Stigmas of both species showed peak receptivity at two to six days post-anthesis, *D. quercifolia* was receptive immediately after anthesis while *D. formosa* showed little pollen tube germination until two days post-anthesis. Observation of fresh pistils with an environmental scanning electron microscope revealed the stigma of both species to be wet. For *D. quercifolia*, maximum groove dimensions and exudate area commenced three days post-anthesis and continued until day 12. A different pattern was observed for *D. formosa* stigmas; maximum groove dimensions and exudate area were reached four days post-anthesis and decreased thereafter. Both species showed overlap in the time of maximum observed pollen tube counts, groove openness and exudate area. These factors were consistently higher in *D. quercifolia* compared with *D. formosa*. Exudate production was lower in pollinated than unpollinated *D. formosa* stigmas. Pollen tube and seed set data indicated a mixed breeding system for both species, with self-pollination less favoured and often resulting in post-zygotic abortion of seeds. *Dryandra formosa was* more self-compatible than *D. quercifolia*.

Introduction

A recent review of *Dryandra* R. Br. taxonomy by (George, 1996) saw the genus increase from 57 species to over 90. First named by Robert Brown after the Swedish botanist, Jonas Dryander (1748-1810), *Dryandra* is endemic to the south-west of Western Australia. Preferring well drained lateritic, gravelly soils, its species are woody plants varying in size from prostrate undershrubs to small trees. The leaves are a diagnostic feature and are often prickly, toothed, lobed or pinnate and are rarely entire. Flowers are borne in dense heads surrounded by a ring of conspicuous bracts and are either terminal or on short lateral branches. They are generally yellow, but orange, pink and brown tones are also seen. Individual flowers are hermaphrodite and the perianth tube is more or less regular, splitting into four segments on maturity. The style of the majority of species is straight, often not exceeding the perianth tube in length. Curved styles are present in some species. Anthers are borne on the concave tip of each perianth segment and, like in other proteaceous genera, pollen is deposited pre-anthesis on a specialised portion of the distal style called the pollen presenter.

For its size, the genus *Dryandra* has been neglected in terms of breeding system research compared with *Banksia* L.f., its sister genus. In the past, studies have tended to focus on a wide range of proteaceous genera in which limited data on *Dryandra* were included (Grey, 1985; Collins and Rebelo, 1987), until Ladd et al. (1996) produced a comprehensive study of pollen presenter morphology and anatomy in both *Dryandra* and *Banksia.*

This chapter investigates stigma receptivity and the breeding system of two species of *Dryandra; D. quercifolia* and *D. formosa*. Both are sold as cut flowers in Australia and overseas. Although the genus currently represents only a small portion of Australia's native flower exports (0.95%; 0.55% cultivated and 0.4% bush picked) (RIRDC, 1994) the industry is gaining momentum and is sure to increase in future years. The total area in Australia planted with *Dryandra* is 22 Ha, 0.9% of the total land under the cultivation of Australian natives. Stigma receptivity is determined by examining pollen tube growth, change in the width of the stigmatic groove and the presence or absence of stigmatic

exudate at different times post-anthesis. The breeding system of each species is examined by controlled hand pollination with either self or cross pollen. Results are assessed in terms of pollen tube growth and seed set. This information is important in determining conservation strategies for *Dryandra* species, many of which occur in habitats threatened by land clearing and *Phytophthora* root rot. It is also essential information for the development of breeding strategies to foster cultivation for cut flower production, as an alternative to another conservation threat, bush picking.

Materials and Methods

Study species

Dryandra quercifolia Meiss. or Oak Leaf Dryandra (Subgenus *Dryandra*, Series *Ilicinae*) is found on rocky hills of quartzite, laterite or shale near the south coast of Western Australia from the Gairdner Range to East Mount Barren extending inland to the Ravensthorpe Range. It has yellow flowers and toothed obovate leaves (Plate 2.1).

Dryandra formosa R. Br. has been coined the Showy Dryandra (Subgenus *Dryandra*, Series *Dryandra*) due to its terminal golden flowers. It is a medium to large shrub with less pungent leaves than many other *Dryandra* species and its distribution is restricted mainly to the Stirling Ranges of Western Australia (Plate 2.1).

Experiments were conducted from May 1995 to September 1996 on *Dryandra quercifolia* (summer-autumn flowering, planted 1984) and *D. formosa* (winter-spring flowering, planted 1988) located on a commercial cut flower plantation in Blewitt Springs, South Australia (35°10' S, 138°34' E) (Plate 2.2). Prior to the experimental period all plants were subjected to routine management practices, including limited drip-irrigation to supplement rainfall and biannual fertilising in autumn and spring with slow release fertiliser (Complete D Mineral Mix, 1995 and Pivot Slow Release Fertiliser, 1996). Pruning of bushes took place following the harvest period. The mean maximum and minimum

temperature for the region (Kuipto Forest) during the experimental period was 26.6 °C and 4.3 °C respectively.

Pollen donors

For each pollination experiment two pollen donors were used consistently. Pollen from each donor was kept separated and inflorescences were pollinated by one donor only (Table 2.1). All pollen was collected on the day of pollination from newly opened florets of the specified pollen donor. Pollen of each donor was tested for viability using the fluorescein diacetate (FDA) test (Heslop-Harrison et al., 1984). All donors had greater than 80% viable pollen.

Preparation, pollination and observation of florets for pollen tube germination and growth

On selected inflorescences, all open florets were removed with fine dissecting scissors and a windowed polyester bag placed over the entire inflorescence (Plate 2.2). The bag was secured with a twist tie and left for 24 hours during which time a new row of florets opened. All remaining unopened florets were removed leaving only the row of known age (< 1-day-old, 10-20 florets). Self pollen was removed from these by inserting a looped synthetic pipe cleaner over the style to an area below the pollen presenter and dragging it upwards (Fuss and Sedgley, 1991a). Pipe cleaners were changed between plants and cleaned in ethanol before re-use. The inflorescences were re-bagged and left until pollinated.

The position of the stigmatic groove was established using a hand lens and pollen laden pistils from selected pollen donors applied to the groove until clumps of pollen could be seen on the tip of the style. The inflorescence was re-bagged until harvesting 7 days later. Harvested pistils were fixed in Carnoy's solution for 7-14 days at 5°C whereupon they were transferred to 90% ethanol for storage. Pistils were hydrated through a series of alcohol concentrations, softened (0.8M NaOH at 60°C for 50 min.) and stained overnight

with decolourised aqueous aniline blue (Martin, 1959). The following day pistils were bisected along their length for ease of viewing, squashed and mounted in 80% glycerol. Counts of germinated pollen grains and pollen tubes were made using an Olympus BHA microscope fitted with a reflective light fluorescence attachment and the appropriate excitation (420-490 nm) and emission filters (520 nm).

Plate 2.1

(a) *Dryandra quercifolia* inflorescence, Blewitt Springs, South Australia

(b) *Dryandra formosa* inflorescence, Blewitt Springs, South Australia


a


b

Plate 2.2

(a) Keith's property (Protea Summit) field site, Blewitt Springs, South Australia

(b) *Dryandra quercifolia* showing inflorescences that have been bagged (arrowhead) for hand pollination experiments (Blewitt Springs, South Australia)


a


b

Table 2.1: Summary of experiments carried out including replicates, pollen donors and month performed

Experiment	Species/ Plant number used (Female)	Pollen donors (Male)	Days unpollinated (unpoll.) or pollinated (poll.)	Inflorescences treated per plant	# Pistils observed	Technique for observation	Month(s) of experiment
Stigma receptivity (pollen tube counts)	*D. quercifolia* 1-5	1 and 2	Poll. 0, 1, 2, 3, 4, 6, 9 and 12 d post-anthesis	16 (8/pollen donor)	100 per day	Fluorescence microscopy	Mar.-Apr. 1996
	D. formosa 1-5	1 and 2	Poll. 0, 1, 2, 3, 4, 6, 9 and 12 d post-anthesis	16 (8/pollen donor)	100 per day	Fluorescence microscopy	August 1995
Stigma receptivity (groove and exudate)	*D. quercifolia* 1-5	-	Unpoll. 0, 1, 2, 3, 4, 6, 9 and 12 d post-anthesis	8 unpoll.	5 per day per plant	ESEM and NIH Image	April 1996
	D. formosa 1-5	-	Unpoll. 0, 1, 2, 3, 4, 6, 9 and 12 d post-anthesis	8 unpoll.	5 per day per plant	ESEM and NIH Image	September 1996
	D. formosa 1-5	3	Poll. 2 d post-anthesis	6 poll.	5 per day per plant	ESEM and NIH Image	September 1996
Breeding system (pollen tube counts)	*D. quercifolia* 1-5	1, 2 and self	Poll. 2 d post–anthesis	4 (2/pollen donor) 4 self	200 per pollen donor	Fluorescence microscopy	Mar.-Apr. 1996
	D. formosa	3, 4 and self	Poll. 2 d post anthesis	4 (2/pollen donor) 4 self	200 per pollen donor	Fluorescence microscopy	October 1995
Breeding system (seed set)	*D. quercifolia* 1-5	1, 2, self and open	Whole infl. poll.2 d post-anthesis	4 (2/pollen donor) 2 self, < 16 open	-	Weights and # infruct., foll. & seeds	July 1995
	1-8	1, 3, self and open	Whole infl. poll. 2 d post-anthesis	4 (2/pollen donor) 4 self, <16 open	-	Weights and # infruct., foll. & seeds	March 1996
	D. formosa 1-5	3, 4, self and open	Whole infl. poll. 2 d post-anthesis	4 (2/pollen donor) 2-4 self, < 9 open	-	Weights and # infruct., foll. & seeds	October 1995

Time of stigma receptivity

Pollen tube counts

Florets of *D. quercifolia* and *D. formosa* were pollinated on days 0 (anthesis), 1, 2, 3, 4, 6, 9 and 12 after floret opening. One inflorescence per plant was pollinated per time frame (Table 2.1). Pollen grain and pollen tube counts in the pollen presenter were performed on ten pistils per inflorescence totaling 100 pistils observed per time frame (50 per pollen donor).

Stigmatic groove and exudate production

Inflorescences of *D. quercifolia* were prepared as for pollination, but pollen was not removed and pistils were not pollinated. Inflorescences were harvested 0, 1, 2, 3, 4, 6, 9 and 12 days post-anthesis (Table 2.1). One inflorescence from each plant was harvested per time frame and transported immediately to the laboratory. Five pistils were positioned with their stigmatic groove facing upward and sandwiched between two pieces of double sided sticky tape. The `sandwich' was placed onto the chilled stage and each pistil viewed in the Electroscan environmental scanning electron microscope (ESEM) at 13 kV, 7 Torr at 7.5 °C. This microscope operates under very low vacuum, the electron current passing through water vapour. As a result, fresh material with exudates can be viewed with minimal interference. Each pistil was observed and the presence of stigmatic exudate noted. Specimens were photographed, a video image taken and the image saved to disc. The groove width (top, middle and bottom), groove length, and groove area were measured using the software package NIH Image. If exudate was present the area of the exudate cover was also measured. In some cases the presence of exudate obscured the rim of the groove making measurement of groove dimensions difficult, in these cases a measure of exudate cover alone was taken.

For *D. formosa,* both unpollinated and pollinated pistils were observed using the ESEM (Table 2.1). Unpollinated inflorescences were prepared and the pistils observed as for *D. quercifolia.* A second subset of inflorescences was pollinated at 2 days post-anthesis using

the pollen from one donor. Pollinated pistils were harvested for observation either on the day of pollination (day 2), or 3, 4, 6, 9 and 12 days after anthesis. Collection, viewing and recording of results was as for *D. quercifolia*. Results of pollinated and unpollinated pistils were compared for *D. formosa*.

Breeding system

Pollen tube growth

Inflorescences of *D. quercifolia* and *D. formosa* were prepared and pollinated on the day of peak stigma receptivity (day two) with either pollen from the same plant (self-pollination) or pollen from one of two pollen donors (cross-pollination). Ten pistils per inflorescence, that is 200 pistils per pollination treatment (self, cross pollen donor 1 or cross pollen donor 2), were viewed by fluorescence microscopy and pollen tubes counted in the pollen presenter, upper and lower style.

Seed set

Whole inflorescences of *D. quercifolia* and *D. formosa* were prepared and pollinated with either self- or cross-pollen (two pollen donors used, pollen not mixed) for analysis by seed set.

Fully closed inflorescences were selected, bagged and all florets progressively pollinated over subsequent days at time of peak stigma receptivity. Inflorescences of *D. quercifolia* contained on average 248±39 florets, *D. formosa*, 268±52 florets. All florets were pollinated at least twice with the relevant pollen treatment. Once pollinated, the inflorescence was re-bagged and left for one month until styles were shed, then the bag removed. In addition, a selection of inflorescences were tagged on each plant and left for natural (open) pollination. Ten months later the inflorescences were harvested. The number of infructescences produced by each pollination treatment was recorded and for each infructescence, follicle number and weight, and seed number and weight per follicle determined. The number of aborted seeds was also recorded. To release seeds from the

follicles, follicles were exposed either to an open flame (Gill, 1976; Lamont and Cowling, 1984) or they were placed onto a hot plate until the follicles split.

Statistical analysis

The package GENSTAT 5.3 was used for statistical analyses (Genstat Committee, 1993). Generalised linear models were fitted to the data unless otherwise stated. Analysis of deviance using a Poisson error distribution was used to test for differences between days in pollen tube and pollen grain number for stigma receptivity of *D. quercifolia* and *D. formosa*. Stigmatic groove dimensions and exudate area data were tested using the same model. In cases where exudate was present an analysis was performed to test for differences between pollinated and unpollinated flowers regarding the area of exudate cover. A log transformation was used to improve the distribution of these data. The presence or absence of exudate on pistils was tested using a Chi-square analysis. For both species a third order polynomial curve was fitted to combinations of variables obtained from pollen tube and ESEM data relating to stigma receptivity.

Analysis of deviance using a Poisson error distribution was used to determine the breeding system of *D. quercifolia* by comparing pollen tube data. This generalised linear model however could not be fitted to *D. formosa* breeding system data as pollen tube counts were too low. Instead a Chi-square analysis was performed to test for the difference in presence or absence of pollen tubes between pollination treatments. This test was also performed on *D. quercifolia* pollen tube data. The method of Residual Maximum Likelihood (REML) was used to analyse follicle and infructescence weights for unbalanced data. Analysis of deviance using a Poisson error distribution and a generalised linear model was performed to analyse the number of follicles for each seed set experiment. A Chi-square test was performed to ascertain differences between percentage seed set for each year. A pair wise comparison (z test) was carried to test for within pollination treatment differences between years of data for *D. quercifolia* (1995 and 1996, $z>3$).

Results

Time of stigma receptivity

Pollen tube counts

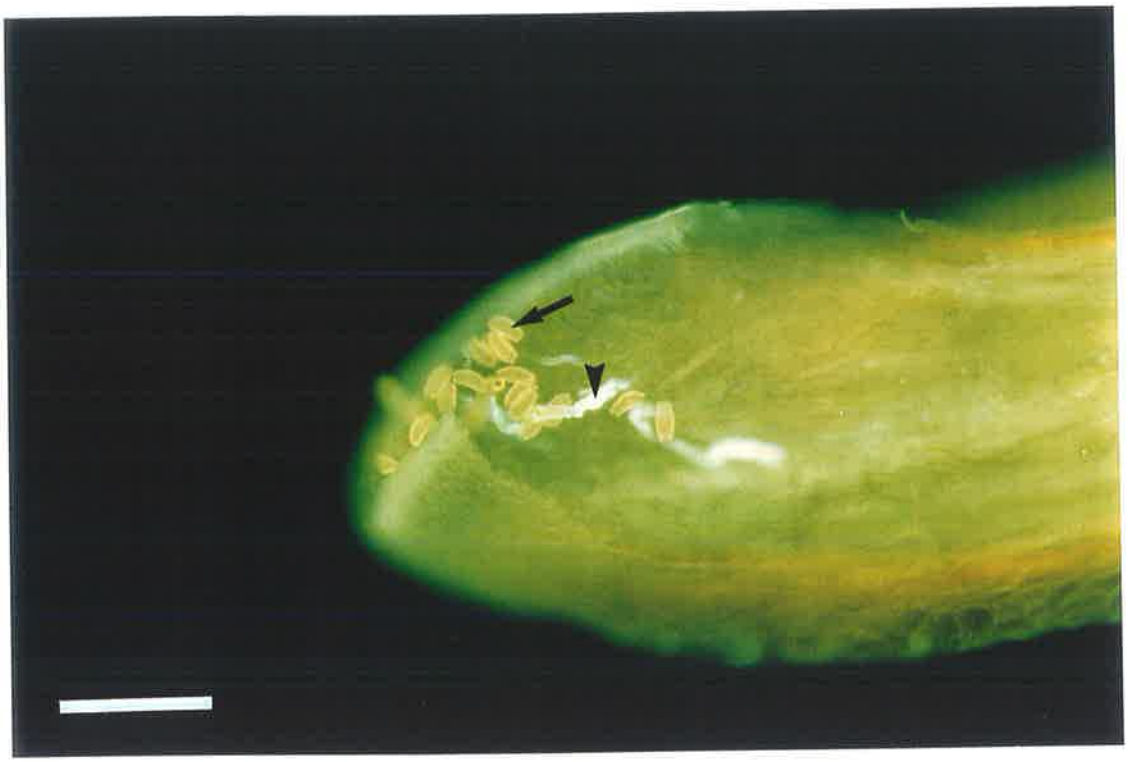
For *D. quercifolia* and *D. formosa,* peak stigma receptivity occurred two to six days post-anthesis. At this time, maximum numbers of pollen tubes and pollen grains in the pollen presenter were observed (Plate 2.3, Table 2.2). Pollen tube number varied significantly between days for both species as did the number of pollen grains for *D. quercifolia.* Results did not differ between pollen donors so data were pooled. For *D. quercifolia,* reasonable numbers of pollen tubes were observed immediately following anthesis suggesting weak protandry, the stigma becoming receptive with little delay following the release of the pistil from the perianth. The stigma of *D. formosa* showed delayed receptivity to pollen until two days post-anthesis, but some pollen tubes were observed prior to this.

Table 2.2. Stigma receptivity of *D. quercifolia* and *D. formosa*. Pollen grains at stigmatic groove and pollen tubes in pollen presenter. Pistils hand pollinated at varying times after anthesis and harvested seven days after pollination (mean±s.e.)

Day pollinated after anthesis	*Dryandra quercifolia*		*Dryandra formosa*	
	Pollen grains	Pollen tubes	Pollen grains	Pollen tubes
0	4.44±0.22	1.40±0.13	0.49±0.07	0.10±0.03
1	5.08±0.23	1.89±0.14	0.74±0.09	0.16±0.04
2	11.90±0.36	2.73±0.17	1.29±0.11	0.57±0.08
3	8.26±0.29	2.36±0.16	1.42±0.12	0.34±0.06
4	8.44±0.29	1.86±0.14	1.22±0.11	0.26±0.05
6	9.29±0.31	2.29±0.15	1.46±0.12	0.38±0.06
9	7.43±0.27	1.27±0.11	0.86±0.09	0.21±0.05
12	3.81±0.20	0.66±0.08	0.94±0.10	0.15±0.04
Probability	< 0.025	< 0.01	ns	< 0.01

Plate 2.3

Fluorescence micrograph of a longitudinally bisected *Dryandra quercifolia* pistil stained with aniline blue. Pollen grains (arrow) are present at the stigmatic groove region and pollen tubes are visible (arrowhead). Bar represents 240 μm.



Stigmatic groove and exudate production

On the day of anthesis for *D. quercifolia* (Plate 2.4) and *D. formosa* (Plate 2.5) the stigmatic groove was closed, except for a small opening at the tip of the *D. quercifolia* groove which resembled a keyhole (Plate 2.4a). Some exudate was recorded on this day for both species (Table 2.3). Groove length did not change significantly over the experimental period.

D. quercifolia and *D. formosa* each displayed a different pattern of change with regard to their groove dimensions and exudate production over the study period. Groove and exudate area reached high levels for *D. quercifolia* by day three, and continued at these high levels until day 12. For *D. formosa* however, maximum groove dimensions and area of exudate were reached four days post-anthesis and declined thereafter (Table 2.3, Plate 2.6). Both species showed significant differences between days with regard to groove area and percentage pistils with exudate. In addition, *D. formosa* displayed significant differences between days for area of exudate cover (Table 2.3).

A comparison of unpollinated and pollinated *D. formosa* pistils showed that each treatment displayed a similar pattern of groove opening over the time period. A significant interaction ($P < 0.025$) however was recorded between pollination and day for the area of stigmatic exudate. Unpollinated pistils produced significantly more exudate than pollinated pistils.

Plate 2.4

Environmental scanning electron micrographs of pollen presenters of *Dryandra quercifolia*

(a) Closed stigmatic groove (g) on day of anthesis. Note ungerminated pollen grains (p). Bar represents 50 μm.

(b) Stigmatic groove one day after anthesis with exudate (arrowhead) and pollen grains (p). Bar represents 100μm.

(c) Stigmatic groove two days after anthesis. Note exudate (arrowhead) containing pollen grains (p) oozing from open stigmatic groove (g). Bar represents 100 μm.

(d) Stigmatic groove three days after anthesis covered in a layer of exudate (arrowhead). Bar represents 50 μm.

(e) Pollen presenter six days after anthesis with exudate (e) covering groove. Pollen grains (p) and surface patterning present (arrowhead). Bar represents 50 μm.

(f) Pollen presenter nine days after anthesis with exudate (arrowhead) and pollen grains (p) covering the groove. Bar represents 100 μm.

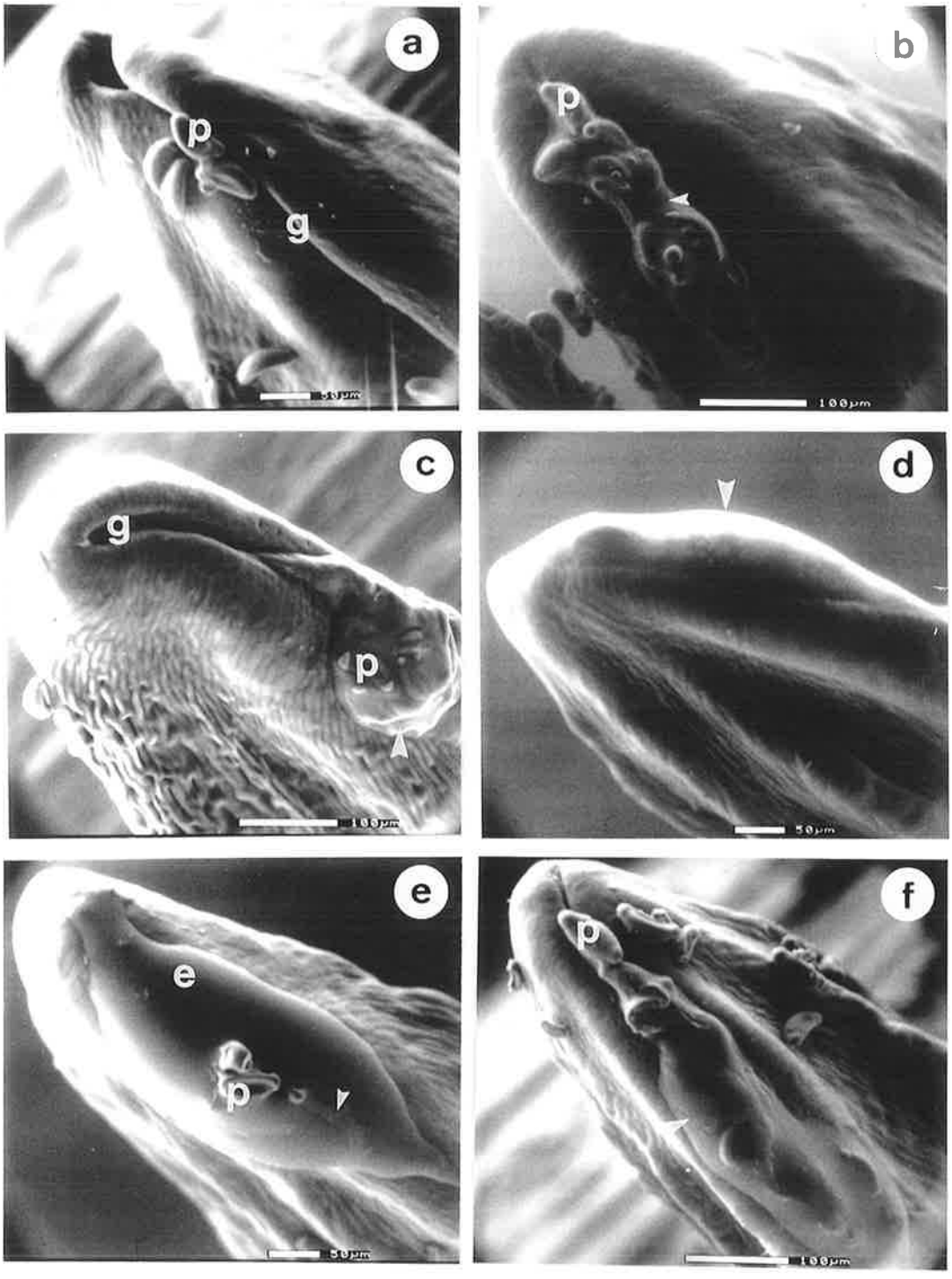

a
p
g
b
p
100µm
c
g
p
100µm
d
50µm
e
e
p
50µm
f
p
100µm

Plate 2.5

Environmental scanning electron micrograph of pollen presenters of *Dryandra formosa* at the stigmatic groove region from anthesis to three days after anthesis.

(a) Closed stigmatic groove (arrowhead) at anthesis with edges of groove abutting. Bar represents 50 μm.

(b) Closed stigmatic groove (arrowhead) one day after anthesis. Bar represents 50 μm.

(c) Open stigmatic groove (arrowhead) with ungerminated (u) and germinating pollen tube (g). Bar represents 50 μm.

(d) Pollen presenter three days after anthesis with stigmatic exudate (e) covering the groove. Bar represents 50 μm.

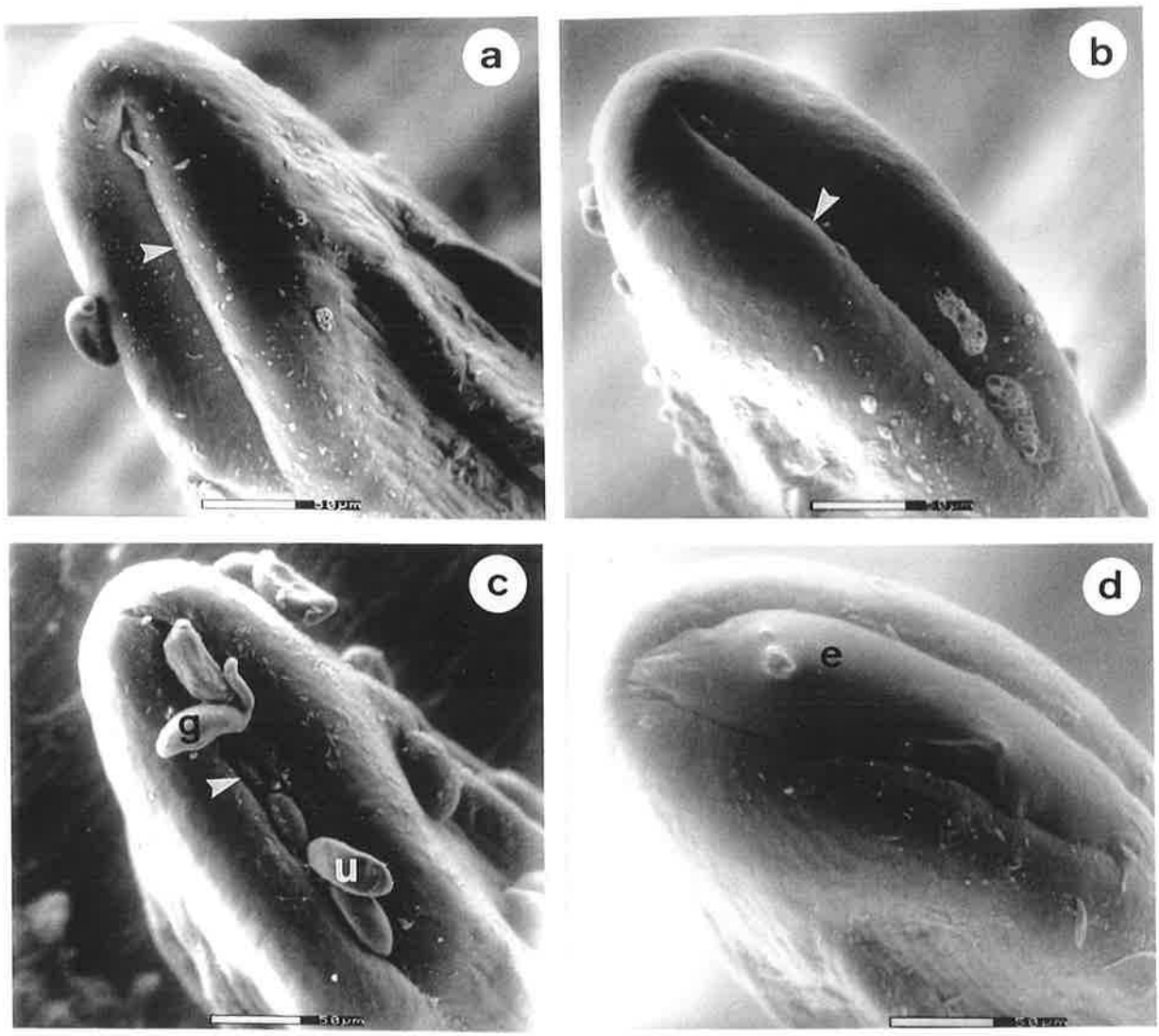

a
b
c
d
g
u
e

Plate 2.6

Environmental scanning electron micrograph of the pollen presenter of *Dryandra formosa* at the stigmatic groove region on days four, six, nine and twelve since anthesis.

(a) Pollen presenter four days after anthesis with stigmatic exudate (e) covering the groove. Bar represents 50 μm.

(b) Pollen presenter six days after anthesis showing almost closed stigmatic groove (arrowhead). Bar represents 50 μm.

(c) Closed stigmatic groove (arrowhead) nine days after anthesis. Bar represents 50 μm.

(d) Closed stigmatic groove (arrowhead) twelve days after anthesis. Bar represents 50 μm.

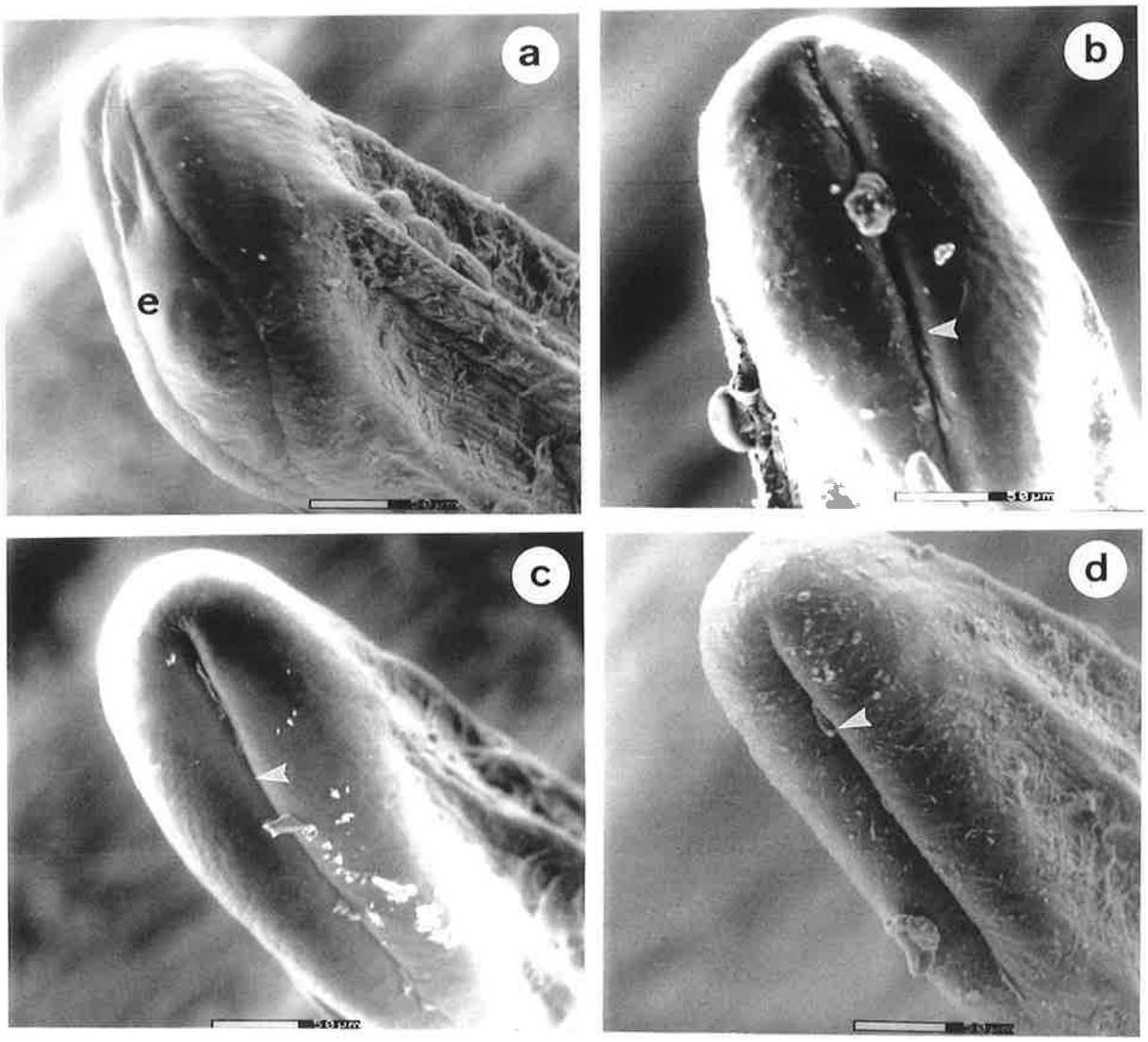

a
e
b
c
d

Table 2.3: Stigma receptivity of *D. quercifolia* and *D. formosa*. Dimensions of the stigmatic groove and exudate up to 12 days after anthesis (mean±s.e.)

Day post-anthesis	Width of stigmatic groove at mid-point (µm)		Length of stigmatic groove (µm)		Area of stigmatic groove (μm^2)		Area of stigmatic exudate (μm^2)		% Pistils with exudate	
D. quercifolia	Unpollinated	Pollinated	Unpollinated	Pollinated	Unpollinated	Pollinated	Unpollinated	Pollinated	Unpollinated	Pollinated
0	7.34±6.96	-	465.60±11.17	-	3522±1452	-	404±3078	-	8.3	-
1	24.49±7.67	-	427.79±11.89	-	3672±1546	-	3933±3277	-	24.0	-
2	18.91±6.42	-	430.11±9.04	-	6938±1248	-	5719±2539	-	53.8	-
3	35.38±9.07	-	432.61±12.77	-	11883±1996	-	11940±3392	-	59.0	-
4	19.03±10.15	-	462.23±16.28	-	8064±2117	-	7288±4488	-	47.4	-
6	31.12±8.65	-	436.45±11.89	-	10884±1724	-	8896±3277	-	50.0	-
9	31.46±9.07	-	447.47±15.35	-	12123±1996	-	13420±3664	-	57.8	-
12	50.80±7.96	-	425.44±12.77	-	16887±1728	-	10524±3520	-	46.3	-
Probability	< 0.025	-	ns	-	< 0.001	-	ns	-	< 0.0005	-
D. formosa										
0	2.02±2.05	-	238.89±5.26	-	561±372	-	194±1077	-	12.0	-
1	4.24±2.12	-	235.19±5.43	-	864±384	-	222±1111	-	22.0	-
2	8.57±1.94	9.85±2.32	242.44±4.97	235.92±5.24	1944±3518	1603±416	983±996	854±1076	32.0	44.9
3	13.54±2.00	21.46±2.24	235.99±5.15	240.56±4.89	2584±364	3619±389	2554±946	1941±1043	44.0	46.0
4	22.00±1.94	17.49±2.17	237.06±5.12	238.04±5.06	3837±373	2918±416	6130±946	1767±1043	58.0	47.5
6	16.19±1.94	15.25±2.24	233.51±4.52	240.14±4.74	2821±352	3085±416	3724±905	1273±983	54.00	52.1
9	9.18±1.80	6.61±2.11	239.02±4.74	234.57±4.62	2045±334	1168±378	2239±946	1028±985	52.0	32.0
12	4.56±2.35	8.06±2.76	220.73±6.03	238.24±5.94	1031±426	1743±494	517±1234	2577±1266	42.0	44
Probability	< 0.001	< 0.001	ns	ns	< 0.001	< 0.01	< 0.001	< 0.05	< 0.0005	ns

Regression analyses showed that groove area was largely a product of increases in the width of the groove at its mid-region (Table 2.4). This was the case for both species. Groove area and exudate area were also strongly correlated over time. Although the R^2 values for pollen tube number, groove and exudate area were small, there was overlap between the time of highest recorded pollen tube numbers, groove openness and presence of exudate. These correlations were chosen as they were the most logical given knowledge of the breeding system.

Table 2.4: Correlation between variable related to stigma receptivity over time (0-12 days from anthesis) for *D. quercifolia* and *D. formosa.* The regression model used was a third order polynomial.

Measurements		R^2	R^2
X Variable	Y Variable	*D. quercifolia*	*D. formosa*
Width of groove (mid-point)	Area of stigmatic groove	0.827	0.993
Area of stigmatic groove	Area of stigmatic exudate	0.911	0.974
Area of stigmatic groove	Pollen tubes in pollen presenter	0.656	0.548
Area of stigmatic exudate	Pollen tubes in pollen presenter	0.264	0.332

Breeding system

Pollen tubes

For *D. quercifolia* the percentage of pistils with a pollen tube present was high, more than 80% of pistils recorded at least one (Table 2.5). There was no significant difference between pollination treatments at the pollen presenter region. In the upper style however, although the percentage was still high, significantly less pistils were recorded with a pollen tube after self-pollination. There was a sharp decrease in the percentage of pistils in the lower style with a pollen tube and significant differences were recorded between cross-pollen donors, but not pollination treatments. These results indicated that possibly a mixed breeding system was operating, with partial self-compatibility.

For *D. formosa* there was no significant difference between pollination treatments at any point along the pistil (Table 2.5). The percentage of pistils containing a pollen tube was

very low compared with *D. quercifolia.* It appears that like *D. quercifolia*, a mixed breeding system is in operation, both self- and cross-pollination possible.

Seed set

The possession of a mixed breeding system by both *D. quercifolia* (Table 2.6, Plate 2.7) and *D. formosa* (Plate 2.8) was confirmed by seed set results which corresponded well to pollen tube data. At the level of the infructescence, the percentage of inflorescences producing follicles did not differ significantly between pollination treatments for either species. This indicates that both self- and cross-pollination successfully produce infructescences. At the seed level however, it appears that post-zygotic abortion of seeds has occurred after self-pollination in both species. The production of one seed per follicle is common after all pollination treatments, however, after self-pollination very few follicles contain the full complement of seeds and a very high proportion of the follicles produced are barren.

For *D. quercifolia* (Table 2.6), pollinations for seed set were carried out over two consecutive years, 1995 and 1996. Results from both years indicate a mixed breeding system for this species. Although very similar when considering the cross-pollination treatment and open-pollination across the years, results from self-pollination treatments differ significantly between years. In 1996, self-pollinations were more successful in their follicle and infructescence production than the previous year. There were also marked increases in seed numbers in 1996. For example, in 1995, self-pollination failed to produce follicles with the full complement of two seeds and half the follicles produced were barren, whereas in 1996 follicle production and weight after self-pollination were similar to the other treatments.

For *D. formosa,* the percentage of inflorescences that produced follicles did not differ significantly between treatments, all treatments produced a high percentage (>85%) of infructescences (Table 2.7). Cross- and self-pollination results were very similar regarding follicle weight and number, and infructescence weight. Open-pollination however, produced significantly more follicles than either self- or cross-pollination, and these

follicles were significantly heavier than either of the other treatments. The number of seeds per follicle after self-pollination differed from the other pollination treatments; fewer follicles contained two seeds and significantly more were barren.

Table 2.5: Breeding system of *D. quercifolia* and *D. formosa*. Percentage of pistils with a pollen tube present. Numbers in parentheses represent the sample size divided by the total observed for that treatment.

Pollen Donor	*D. quercifolia*			*D. formosa*		
	Pollen presenter	Upper style	Lower style	Pollen presenter	Upper style	Lower style
Cross 1	88.2 (75/85)	63.5 (54/85)	5.8 (5/85)	20.0 (22/110)	12.7 (14/110)	5.5 (6/110)
Cross 2	85.4 (82/96)	53.1 (51/96)	12.5 (12/96)	18.3 (30/164)	15.2 (25/165)	6.1 (10/164)
Self	83.7 (164/196)	41.3 (81/196)	4.1 (8/196)	13.6 (24/176)	7.4 (13/176)	1.1 (2/176)
Probability	ns	< 0.025	< 0.025	ns	ns	ns

Table 2.6: Breeding system of *D. quercifolia*. Seed set results for 1995 and 1996 (mean±s.e.). Numbers in parentheses represent the sample size divided by the total.

Measurement	Pollination Treatment								
	Cross 1		Cross 2	Cross 3	Self		Open		Probabilit
	1995	1996	1995	1996	1995	1996	1995	1996	1995
Number of follicles per infructescence	11.55±1.12[a]	11.82±1.92[a]	14.36±1.37[a]	13.12±1.25[a]	1.07±0.37[a]	8.97±0.67[b]	14.36±1.37[a]	13.12±1.25[a]	< 0.001
Total weight of infructescence (minus florets) (g)	9.97±2.1[a]	13.91±2.28[a]	13.48±2.1[a]	16.01±2.28[a]	3.69±2.1[a]	12.91±2.28[b]	13.48±2.1[a]	16.01±2.28[a]	< 0.0005
Total weight of follicles per infructescence (g)	2.92±0.96[a]	4.25±1.22[a]	3.84±0.96[a]	4.85±1.22[a]	0.05±0.96[a]	3.24±1.22[a]	3.84±0.96[a]	4.85±1.22[a]	< 0.0005
Weight of individual follicles (g)	0.33±0.04[a]	0.34±0.03[a]	0.33±0.04[a]	0.42±0.03[a]	0.21±0.04[a]	0.38±0.03[b]	0.33±0.04[a]	0.42±0.03[a]	< 0.0025
% inflorescence producing follicles	70.0 (7/10)	75.0 (6/8)	80.0 8/10)	77.8 (7/9)	22.0 (2/9)	63.6 (14/22)	48.7 (19/39)	74.1 (43/58)	ns
% follicles with 2 seeds	37.4 (37/99)	18.6 (18/97)	43.0 (49/114)	43.9 (47/107)	0.0 (0/8)	10.1 18/178)	43.0 (79/206)	52.4 (357/682)	ns
% follicles with 1 seed	54.1 (54/99)	53.6 (52/97)	33.3 (38/114)	48.6 (52/107)	50.0 (4/8)	68.0 (121/178)	33.3 (113/206)	42.7 (291/682)	< 0.001
% barren follicles	8.1 (8/99)	27.8 (27/97)	27.3 (27/114)	7.5 (8/107)	50.0 (4/8)	21.9 (39/178)	27.3 (14/206)	5.0 (34/682)	< 0.0005
Proportion of seeds per infructescence	12.8 (128/10)	11.0 (88/8)	13.6 (136/10)	16.2 (146/9)	0.9 (8/9)	7.5 (157/22)	6.8 (265/39)	18.6 (1005/54)	-
Proportion of follicles per infructescence	9.9 (99/10)	12.2 (97/8)	11.4 (114/10)	11.9 (107/9)	0.9 (8/9)	8.1 (178/22)	5.3 (206/39)	12.6 (682/54)	-

Numbers with a different letter (a,b) across a row within a pollination treatment are significantly different (z test, z>3. Overall probabilities of differences between treatments for a given year are presented in the final two columns.

Plate 2.7

Dryandra quercifolia infructescence and follicles

(a) Infructescence of *D. quercifolia* showing rows of enveloping bracts (b) and persistent florets (f)

(b) *D. quercifolia* infructescence after removal of dead florets showing seed containing follicles (arrowhead) embedded within the infructescence. Follicles that have been extracted from another infructescence are also shown (f).

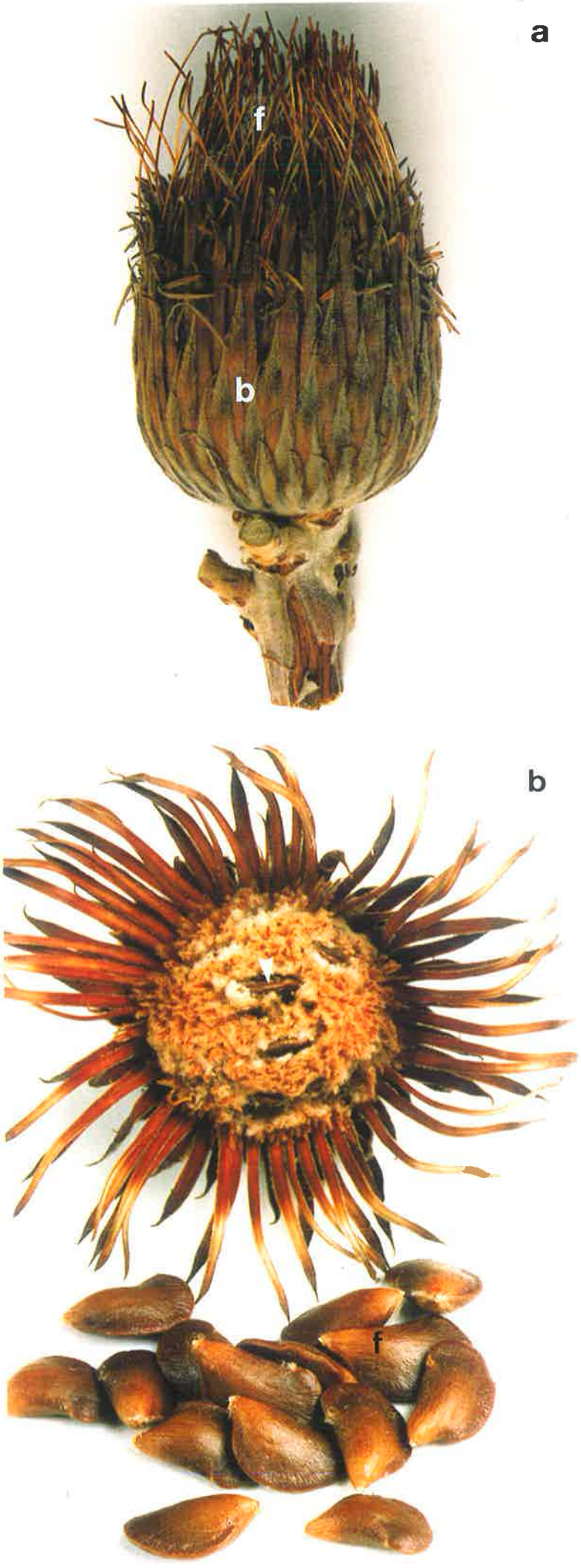

a
f
b
b
f

Table 2.7: Breeding system of *D. formosa*: seed set results for 1995 (mean±s.e.) (numbers in brackets represent the sample size divided by the total)

Measurement	Pollination Treatment				
	Cross 3	Cross 4	Self	Open	Probability
Number of follicles per infructescence	14.91±1.37[a]	13.42±1.39[a]	14.05±1.97[a]	21.79±0.88[b]	< 0.05
Total weight of infructescence (minus florets) (g)	2.69±0.39[a]	2.49±0.39[a]	2.88±0.39[a]	3.99±0.39[b]	< 0.0005
Total weight of follicles per infructescence (g)	0.69±0.15[a]	0.72±0.15[a]	0.67±0.15[a]	1.28±0.15[b]	< 0.0005
Weight of individual follicles (g)	0.045±0.003[a]	0.048±0.003[a]	0.046±0.003[a]	0.058±0.003[b]	< 0.0005
% inflorescence producing follicles	87.5 (7/8)	100.0 (7/7)	90.0 (9/10)	100.0 (32/32)	ns
% follicles with 2 seeds	27.3 (33/121)	23.1 (21/91)	4.7 (7/149)	20.0 (108/539)	< 0.0005
% follicles with 1 seed	70.2 (85/121)	70.3 (64/91)	65.1 (97/149)	71.6 (386/539)	ns
% barren follicles	2.5 (3/121)	6.6 (6/91)	23.5 (35/149)	8.3 (45/539)	< 0.0005
Proportion of seeds per infructescence	18.9 (151/8)	15.1 (106/7)	11.1 (111/10)	18.8 (602/32)	-
Proportion of follicles per infructescence	15.1 (121/8)	13.0 (91/7)	14.9 (149/10)	16.8 (539/32)	-

Numbers with a different letter (a,b) are significantly different (z>3), pairwise test across a row between pollination treatments

Plate 2.8

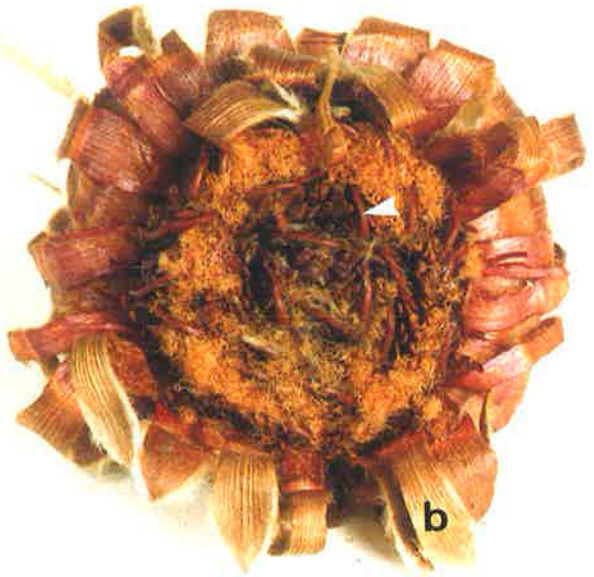
Dryandra formosa infructescence and follicles

(a) Infructescence showing embedded follicles (arrowhead) surrounded by enveloping bracts (b)

(b) Follicles (f) containing seeds

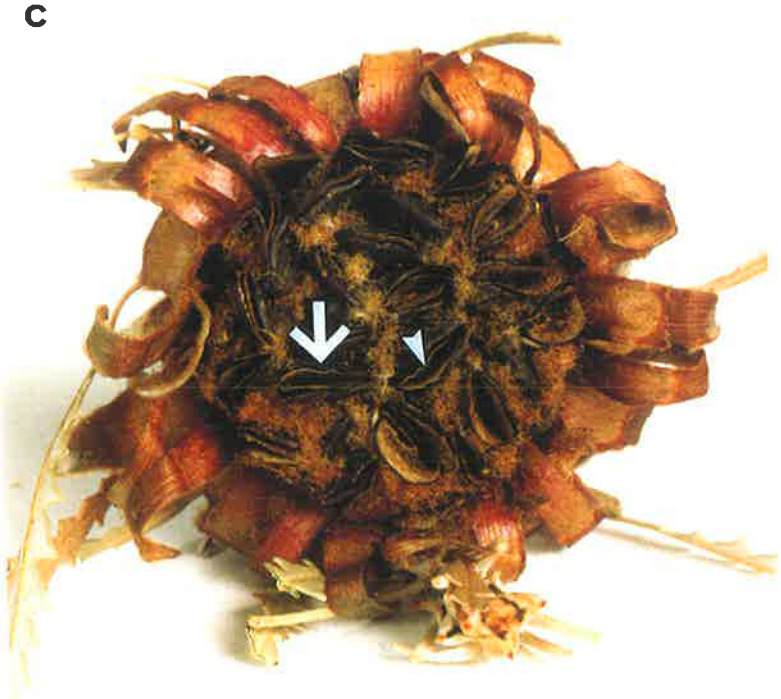
(c) Infructescence showing split follicles (arrow) and separator (arrowhead) that separates the two seeds

a



b

c



Discussion

Dryandra quercifolia and *D. formosa* are protandrous but not strongly so, in fact *D. quercifolia* shows some pollen tubes germinating at the stigma less than one day following anthesis. This observation is unusual compared with other proteaceous genera in which protandry is considered to be a fairly consistent feature of the family. For example *Banksia spinulosa* Smith var. *neoanglica* A. S. George (Vaughton and Ramsey, 1991), *B. menziesii* R. Br. (Fuss and Sedgley, 1991a; Ramsey and Vaughton, 1991), *Macadamia integrifolia* Maiden & Betche (Sedgley et al., 1985), *Protea repens* L. L. and *P. eximia* Salisb. ex Knight (van der Walt and Littlejohn, 1996a) all show protandry. Pronounced protandry is a feature of *B. spinulosa* var. *neoanglica*. This species also displays extended pollen longevity and slow opening of flowers. The authors suggest that these features may have been selected for in response to inefficient pollen removal by floral visitors and that the duration of the staminate phase may be related to pollen removal (Vaughton and Ramsey, 1991; Ramsey and Vaughton, 1991).

The period of peak pollen germination, two to six days post-anthesis, was accompanied by morphological changes at the stigma which were distinct for each species and included groove opening and the production of stigmatic exudate. For *D. quercifolia*, groove dimensions and exudate reached high levels by day three and continued at these high levels until day 12. In contrast, *D. formosa* showed a distinct peak in both these factors four days post-anthesis which declined thereafter. The pattern for *D. formosa* resembled that for *B. menziesii* which reached a maximum on day three and decreased thereafter (Fuss and Sedgley, 1991a). The timing of groove opening in both *Dryandra* species was markedly slower than many other proteaceous species in which the groove reached a maximum width within the first 4h, (*D. sessilis* Knight), or 15h (*B. prionotes* Lind.) of anthesis (Grey, 1985; Collins and Spice, 1986). Collins and Rebelo (1987) present a summary of groove opening, and for most species including *Dryandra*, maximum groove width and area were achieved within 24-36 h.

The stigmas of *D. quercifolia* and *D. formosa* are wet and the exudate observed displayed some surface patterning. This patterning superficially resembled that observed on watermelon stigmas which was a result of surface lipids over a carbohydrate rich exudate (Sedgley and Scholefield, 1980). Further work is required to characterise the exudate in *Dryandra*. Observation of a wet stigma type for *Dryandra* species challenges the classification of the Proteaceous stigma as dry papillate based on *Grevillea* and *Embothrium* stigmas (Heslop-Harrison and Shivanna, 1977). This challenge is further supported by the observation of exudate on *B. coccinea* R. Br. (Fuss and Sedgley, 1991b), *B. prionotes* (Grey, 1985), *M. integrifolia* (Sedgley et al., 1985) and *Grevillea wilsonii* A. Cunn. (Grey, 1985) stigmas. Heslop-Harrison and Shivanna (1977) do state however that some classified dry papillate stigmas may in fact be wet in those families showing strong protandry and a delay in exudate production until after anthesis. Heterogeneity of stigma type may also exist in the Proteaceae as *B. menziesii* stigmas are dry (Fuss and Sedgley, 1991b).

Significant differences in exudate production were observed between pollinated and unpollinated pistils of *D. formosa*, the quantity of exudate decreasing after pollination. This decrease may be a result of pollen grain hydration using the stigmatic secretion. This contrasts watermelon (Sedgley and Scholefield, 1980) and *Acacia* (Kenrick and Knox, 1981) stigmas in which exudate increased and is believed to be a response to pollination. Possibly due to the housing of the *Dryandra* stigma within a groove, the exudate may initially play a predominant role in pollen adhesion followed by hydration, versus watermelon and *Acacia* where the exudate is hypothesised to function primarily in pollen hydration. Further investigation of pollinated and unpollinated pistils of additional proteaceous species is required however before conclusions can be drawn as to the function of the stigmatic exudate on proteaceous pistils.

Stigma receptivity in the Proteaceae is a combination of structural and physiological changes which comprise the opening of the stigmatic groove allowing pollen deposition in the stigmatic cavity, and secretion of esterases, other enzymes and stigmatic exudates which assist pollen to germinate (Ramsey and Vaughton, 1991; Vaughton and Ramsey,

1991). Because it is not one factor alone, different workers have used different combinations of methods to determine its timing. For example, Vaughton and Ramsey (1991) measure pollen deposition and germination within the stigmatic groove of *B. spinulosa* var. *neoanglica* based on the assumption that groove opening indicates receptivity. Fuss and Sedgley (1991b) also use the change in groove dimensions as an indicator but base the timing of stigma receptivity on counts of pollen tubes in the pollen presenter. The esterase test has been used successfully on some genera within the Proteaceae for example *M. integrifolia* (Sedgley et al., 1985) and *B. prionotes* (Collins and Spice, 1986) but it has failed others such as *B. menziesii* (Ramsey and Vaughton, 1991). One then starts to question what is a true indicator of stigma receptivity - is it pollen germination, the width of the groove, exudate production or a combination of all of these? Is the stigma really receptive to pollen just because the groove is open? When determining stigma receptivity each of these factors should be considered where possible and combined in order to gain a truer picture of stigmatic behaviour associated with receptivity. In some cases such as *D. formosa* (this study), *P. repens* and *P. eximia* (van der Walt and Littlejohn, 1996a) and *B. menziesii* (Fuss and Sedgley, 1991a), the groove, even at maximum diameter is still narrower than the diameter of the pollen grain, thus limiting pollen deposition within the groove. Ladd et al. (1996) suggest that for some species timing of groove openness is irrelevant for pollination and that manipulation of the groove by pollinators is more important for these species.

Dryandra quercifolia and *D. formosa* display a mixed breeding system. Pollen tube results indicate that both self- and cross-pollination are possible. In *D. quercifolia* the percentage of pistils with a pollen tube present does not differ significantly between treatments at the pollen presenter, however upon passage to the upper style the percentage pistils with a pollen tube present after self-pollination drops significantly. This decrease could infer a self-incompatibility mechanism operating at this point. Such an inference has been made for *M. integrifolia* and *B. coccinea*, the authors suggesting gametophytic self-incompatibility (Sedgley et al., 1985; Fuss and Sedgley, 1991b). Further investigation is needed to confirm this observation in *D. quercifolia*. There was no such occurrence in *D.*

formosa, the percentage pistils showing no significant difference at any point down the style. This lack of pre-zygotic discrimination between pollination treatments has been observed in the upper style in other species such as *B. ericifolia* and *B. spinulosa* (Carthew et al., 1996). The possession of a mixed breeding system is further supported by seed set results for both species. At the infructescence level self-pollen was as effective as cross-pollen at producing infructescences and follicles. Species of *Dryandra* have the potential to produce a maximum of two seeds per follicle and it was at this level, the number of seeds per follicle, that the differences in pollination treatments became apparent. It was found that self-pollination produced significantly fewer follicles with two seeds and significantly more barren follicles than either cross- or open-pollination

The production of comparable numbers of infructescences and follicles after each pollination treatment suggests that fertilisation is successful independent of the pollination treatment for *D. quercifolia* and *D. formosa*. However, following fertilisation discrimination between pollination treatments results in the post-zygotic abortion of at least one selfed seed. It is not an unusual occurrence in hermaphrodite flowering plants to reduce seed set after selfing (Burbidge and James, 1991) and it often produces an increase in the fitness of the surviving progeny (Latta, 1995).

The degree to which both *D. quercifolia* and *D. formosa* share a mixed mating system was not identical. *Dryandra formosa* appears to be slightly more self-compatible than *D. quercifolia*, although the potential for *D. quercifolia* to become more self-compatible was observed between the two years studied. This difference in breeding system is not surprising. Differences have been observed within genera many times in the Proteaceae, within *Banksia*, species range from completely self-incompatible (highly outcrossing) (Carthew et al., 1988; Goldingay and Whelan, 1990; Ramsey and Vaughton, 1991; Carthew et al., 1996) to self-compatible (Vaughton, 1988) with others in between (Fuss and Sedgley, 1991b). Carthew et al. (1996) presented very interesting findings relating to mate choice. Due to the size of the inflorescence of *Banksia* and multiple visits by pollinators to its flowers, the flowers inevitably have a mixture of self- and cross-pollen. These authors found in *B. spinulosa* and *B. ericifolia* that when pollen mixtures (self and

cross) are placed on flowers that the cross pollen is favoured by the maternal plant, however the filtering mechanism is not known.

This study has investigated one of the lesser researched, but economically important genera of the Proteaceae. It complements and broadens our current knowledge of the breeding systems of the Australian Proteaceae and raises some important considerations when determining stigma receptivity. In particular, this study highlights the importance of the combined effect of the physical and chemical factors that effect stigma receptivity such as groove openness, exudate production and time of greatest pollen germination. These factors each contribute to the success of controlled pollinations, an important manipulative tool used by plant breeders to improve characters of a given species to increase its desirability in the horticultural industry, or to produce new varieties for this industry. In particular, to select for, or against specific features possessed by the species or variety. In addition the new information presented in this chapter can also be used to help conserve these, and other species, by assisting in the effective production of seeds for a seed store, or in better managing native habitats.

Chapter Three

Seed germination of *Dryandra* and *Isopogon*: germination in the glasshouse, and temperature optimum for germination

Abstract

The Australian horticultural industry primarily relies upon seed germination for propagation of its native species. Knowledge of the factors which promote seed germination of native species is therefore essential to promote efficient propagation of these species. Thus the optimum temperature for germination, 5, 15, 25 or 35 °C, was determined for three species of *Dryandra* and three of *Isopogon*. In addition, the rate and percentage emergence under glasshouse conditions (glasshouse, max/min: 27.9/20.1 °C) was determined for ten species of *Dryandra* and seven of *Isopogon* and used as an indirect comparison to germination at specific temperatures. Species selection was based on their current or potential use in the cut flower and amenity industries. All species showed optimal percentage and rates of germination after incubation at 15 °C. This temperature coincided with the period of winter rainfall in the areas where these species were native. *Dryandra* species also displayed germination at 25 °C, however it was reduced compared with germination at 15 °C, and the germination rate was slower. Germination was inhibited at temperatures of 5 and 35 °C for all species studied. *Dryandra polycephala* displayed a wider range of germination temperatures than other species, which may be related to its wider geographic distribution. Seedling emergence was slow in the glasshouse for all species tested and low compared with 15 °C. In some cases there was no emergence. These results demonstrate that seed germination of selected species of *Dryandra* and *Isopogon* may be promoted by exposure to a controlled temperature of 15 °C, a result which can be directly used by the horticulture industry for efficient propagation of these species.

Introduction

Kingsley Dixon (1994) aptly highlights the importance of seed germination to the horticultural industry in his statement that "most of the 20 % of the 25,000 species of flowering plants native to Australia which are in horticultural use are disseminated and propagated from seed." The reliance on seed propagation is not confined to the horticultural industry alone, mine site rehabilitation (Sonia and Heslehurst, 1978) and conservation of rare and endangered species (Roche et al., 1994) also rely heavily on this. Thus until other methods of propagation, such as micropropagation, cuttings and grafting, are perfected and used widely, propagation by seed will continue to dominate these areas and knowledge of methods to promote efficient seed germination will continue to be important.

The true physiological definition of seed germination is that germination begins with the imbibition of water (water uptake) and ends with the start of elongation by the embryonic axis, usually the radicle (Bewley and Black, 1994). The factors that influence seed germination are complex. In order for a seed to germinate its chemical and physical environment must be suitable. These environments incorporate the presence of water for imbibition, oxygen for respiration, and an absence of inhibitory substances. The temperature must be appropriate to promote germination and in some cases light quality and quantity is important. However even in favourable conditions seed sometimes will not germinate due to seed dormancy, a state that requires a particular set of conditions for it to be broken. Dormancy can be controlled internally, relating to the embryo and seed coat, or externally, relating to temperature and light. For different species different treatments will break dormancy. For example, in quandong (*Santalum acuminatum*) germination responds well to infiltration of the embryo with the plant hormone gibberellin (Loveys and Jusaitis, 1994). Species of the family Asteraceae varied in their response to different treatments, *Leucochysum stipiatum* germination benefited from seed coat scarification, *Brachyscome iberidifolia* from exposure to gibberellic acid and *Rhodanthe stricta* germination was stimulated by light (Bunker, 1994) . . In *Eremophila maculata*

(Myoporaceae) leaching of inhibitory substances; soluble aromatic glycosides, from fruit in heavy rain promoted germination of seeds (Richmond and Ghisalberti, 1994). The breaking of dormancy by chilling is also a good stimulator of germination, especially for species in the northern hemisphere (Bewley and Black, 1994).

Within the Proteaceae, propagation from seed is the primary source of material for commercial cut flower properties and nursery stock. Studies have investigated methods to promote germination and to overcome seed dormancy in proteaceous species. For example, one such method is the application of gibberellic acid (GA_3); a plant growth hormone believed to be essential for seed germination, playing an important role in the mobilisation of endosperm reserves and growth of embryonic tissue (Jones and Stoddard, 1977). In *Protea eximia* and *P. neriifolia* imbibition of GA_3 by the seed served to overcome the requirement of low temperatures to break seed dormancy (Perez, 1995). In addition, treating seeds of *Leucospermum cordifolium* with hydrogen peroxide; a method used to increase the oxygen tension surrounding the seed, was found to significantly increase seed germination in this species (Brits and Niekerk, 1986). However the genus *Banksia*, unlike many other proteaceous genera, requires no germination pretreatments such as plant hormones or increased oxygen tension, to break dormancy. Instead exposure to low temperatures promotes seed germination (Sedgley, 1996). For example, seed germination of *Banksia integrifolia, B. serrata* and *B. aemula* responded most strongly to temperature, the effect of seed coat scarification, light exposure and the application of GA_3 and potassium nitrate having little influence (Sonia and Heselhurst, 1978).

Thus it was the aim of this study to preform a preliminary investigation of optimum temperature requirements for the seed germination of a selection of horticultural species from two of the lesser known genera of the Proteaceae, *Dryandra* and *Isopogon.* For comparison, a broader range of species was selected and germinated under glasshouse conditions. *Isopogon* or "drumsticks" are reasonably widespread, species occurring in both eastern and western Australia, although mainly concentrated in the west. The flower colour of many *Isopogon* species is purple, and this distinguishes them from other Proteaceae, makes them highly desirable horticulturally. Species chosen for this study

were either currently used, or have the potential for use in the cut flower or amenity industries.

Materials and Methods

Plant material

Based upon their current or potential floricultural use a selection of species from the *Dryandra* and *Isopogon* genera was made. Seed (Plate 3.1) was purchased from Nindethana Seed Services, Western Australia in 1994. The following species were chosen:

Note: species descriptions are derived from Wrigley and Fagg (1989).

Dryandra

D. carduacea Lindl.: Endemic to south-west Western Australia. A tall erect shrub up to 4 m high and 2 m wide. Pale yellow flowers are borne on short lateral branches. Excellent cut flower which dries well and lasts indefinitely, also a colourful screening plant. Propagation usually from seed but some cuttings successful.

D. carlinoides Meiss.: Endemic to south-west Western Australia, it is a rounded compact shrub (1.5 m high) with terminal well displayed pink/cream flowers and has potential as a cut flower and garden plant (Plate 3.2).

D. formosa R. Br.: see species description in Materials and Methods, Chapter Two (page 10)

D. hewardiana Meiss.: Endemic to gravelly soils in open *Eucalyptus wandoo* woodland, Western Australia. A tall open shrub (5 m high, 2 m across) with yellow flowers on short axillary branches. This species is fast growing and flowers within two years. It is a useful cut flower (fresh/dry) and can be grown from cuttings (Plate 3.2).

D. nivea (Labill.) R. Br.: The most widespread of the prostrate *Dryandra* undershrubs growing in many habitats of Western Australia. It has cream to orange terminal flowers. This species makes a good rockery plant and is hardy in cultivation. **Note**: at time of purchase, seed of the species *D. nivea* was requested and supplied by Nindethana Seed Services. Since that time the taxonomy of this species has been revised and now consists of a number of species including *D. lindleyana* (George, 1996) whose distribution is more widespread and encompasses prostrate forms. Information is unavailable as to the original seed source, but it is thought that it was most likely collected from plants of *D. lindleyana,* as *D. nivea* is now considered to be a restricted species.

D. nobilis Lindl.: Erect plant up to 3 m high with very large orange-gold flowers borne along stems on short axillary branches. A good garden plant growing well in winter rainfall areas and in sandy or heavy soils in south-west Western Australia.

D. polycephala Benth.: The distribution of this species is widespread and is found growing on gravel or gravelly soils. It is a tall, sometimes open shrub (4 m high, 3 m wide). Its yellow flowers are borne terminally on short lateral branches. This species would make an excellent garden plant if a little more were known about methods of cultivation (Plate 3.2).

D. praemorsa Meiss.: A rounded to erect medium sized shrub found in jarrah forests of Western Australia. Flowers are terminal, large and yellow/green or pink. One of the easier species to cultivate (Plate 3.2).

D. proteoides Lindl.: Grows on gravelly soils in the south-west of Western Australia. A small to medium sized shrub possessing the largest inflorescences of the genus. It's foliage is attractive and it is worth growing for this reason.

D. quercifolia Meiss.: see species description Materials and Methods, Chapter Two (page 10)

Isopogon

I. axillaris R. Br.: Found in the lower south-west of Western Australia. It is a medium-sized, erect shrub and showy when in flower. It is rare in cultivation and requires good drainage. Is best suited to areas of winter rainfall.

I. anethefolius (Salisb.) Knight: An erect bushy shrub that grows well in sclerophyll forests in coastal southern New South Wales. Its yellow flowers are held in terminal globular heads. Currently cultivated in the UK and USA and is one of most reliable *Isopogon* species in cultivation.

I. cuneatus R. Br.: Grows in stony lateritic hills or on gravelly sand in shrub lands of south Western Australia. It is a bushy shrub which may grow to 2 m high. Its mauve-pink flowers are large and borne terminally. It has been in cultivation in the UK since 1829 and is grown in New Zealand. A prized cut flower that is being farmed to a limited degree.

I. dubius (R. Br.) Druce: This species grows in gravelly and granitic soils in *E. wandoo* and jarrah forests, Western Australia. It is a dense well rounded shrub up to 1 m high and 1 m across. The mauve-pink flowers are borne terminally. It is one of the hardiest of the western species and has been in cultivation since 1840. Propagation is from seed.

I. formosus R. Br.: An erect or spreading Western Australian shrub. Flowers are borne terminally and in leaf axils and are mauve-pink. This beautiful shrub is readily cultivated in winter rainfall areas. Propagation is by seed and efforts should be made to graft onto more reliable eastern stock (Plate 3.3).

I. latifolius R. Br.: This species grows in sandy soils, often among rocks of the Stirling Ranges, Western Australia. It is a rounded shrub up to 2 m high. The terminal flowers are very large, showy and mauve-pink in colour. This is probably the most spectacular *Isopogon* and is highly prized as a cut flower. It has been successfully farmed in South Australia on acid sand over limestone.

I. trilobus R. Br.: Found in southern Western Australia this species is a compact, small shrub growing to about 1 m high. Its creamy flowers are borne terminally. This species is

in limited cultivation and is mainly of interest because of it's large fruits. Propagation is from seed.

Plate 3.1

Examples of seed of *Dryandra* and *Isopogon*

(a) *D. polycephala*

(b) *D. quercifolia*

(c) *I. cuneatus*

(d) *I. trilobus*

a

b

c

d

Plate 3.2

Examples of *Dryandra* species used for glasshouse seed germination experiment

(a) *D. carlinoides* (Photo: Alex George)

(b) *D. praemorsa* (Photo: Alex George)

(c) *D. hewardiana* (Photo: Alex George)

(d) *D. polycephala* (Photo: Alex George)

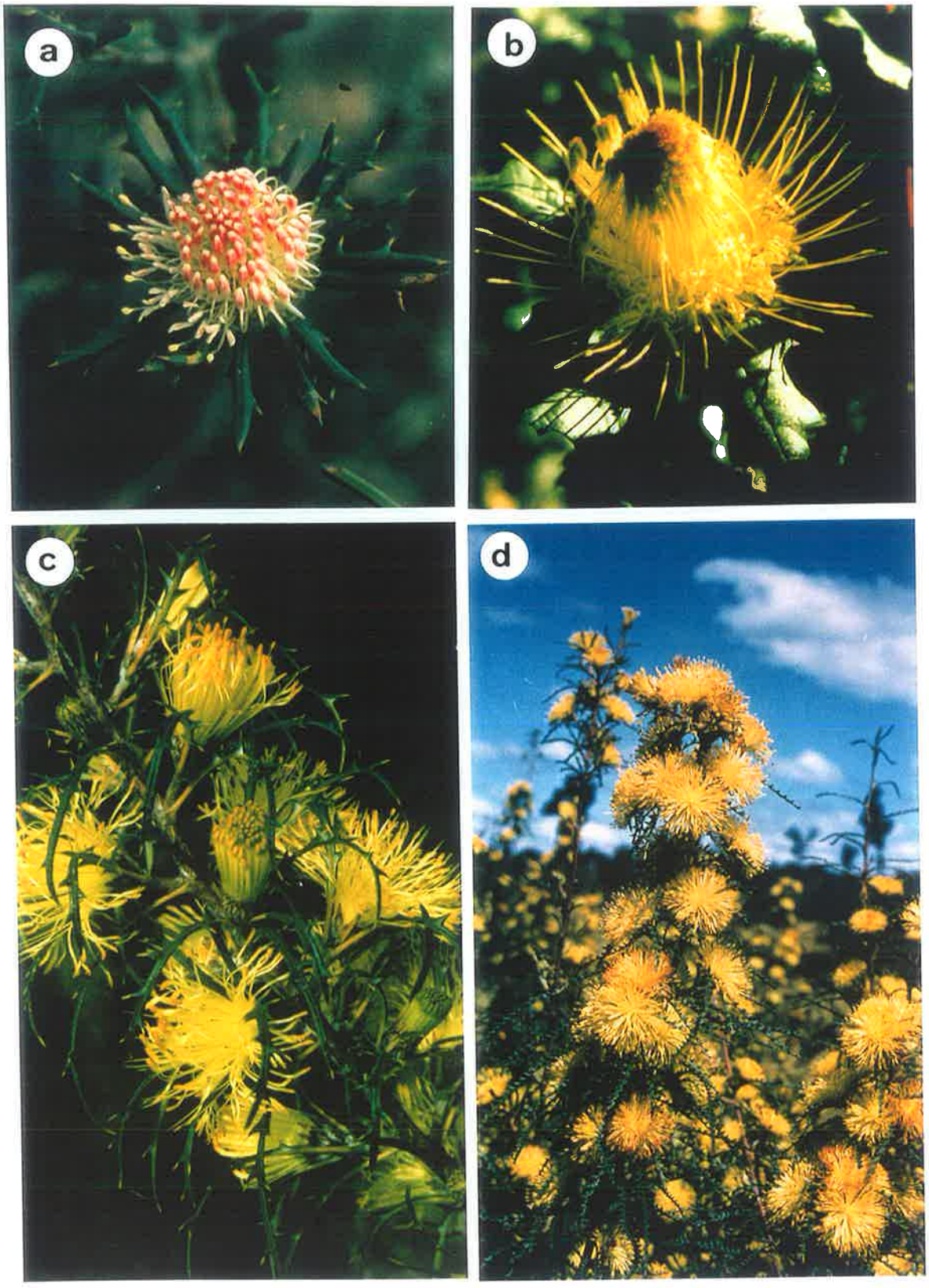

a
b
c
d

Plate 3.3

Isopogon formosus; species used for glasshouse seedling emergence experiment

Optimum temperature for seed germination of three species of *Dryandra* and three of *Isopogon*

In February 1995, eight hundred seeds of each of *D. formosa, D. polycephala, D. quercifolia, I. cuneatus, I. dubius* and *I. trilobus* were sterilised and placed in incubators set at: 5 °C, 15 °C, 25 °C or 35 °C.

Sterilisation of seeds was conducted in a laminar flow cabinet. Each seed was placed in 70 % ethanol for two minutes, 1% White King Household Bleach (4% available chlorine) for three minutes, then washed three times in millipure water. For each temperature and species twenty sterile petri dishes were prepared with autoclaved filter paper. Ten seeds were distributed evenly across the filter paper and watered using RO water (200 seeds/species/temperature). Petri dishes were wrapped in Parafilm to reduce water loss, covered in aluminium foil to exclude light and placed in cabinets set at the appropriate temperature. Light has previously been observed to have little influence on germination and so was excluded to reduce experimental variables (Sonia and Heslehurst, 1978; Bell et al., 1993). Every week petri dishes were checked, the number of seeds germinating recorded and the filter paper re-wetted. A seed was considered germinated once the rootlet could be seen emerging from the seed coat (>3 mm long). Seed affected by fungal infection was removed and the petri dish and filter paper replaced.

Results were collected for twelve weeks commencing the time of incubation and continuing until the number of seeds germinating failed to increase for three weeks. Results were assessed in terms of the cumulative germination of seeds over time for a given temperature and species and the percentage germination.

Seed germination of ten species of *Dryandra* and seven of *Isopogon* under glasshouse conditions

In February 1995, fifty seeds of each species were planted at 1 cm depth in sterilised seed flats containing sterile vermiculite. Approximately 200 ml of the fungicide Previcur (3 ml in 2 L water) was sprayed onto seeds and then gently watered in. Temperature readings

(maximum/minimum) within the glasshouse were recorded (Table 3.1). The number of seedlings emerging each day was recorded for ten weeks. Measurements ceased when this number failed to increase after three weeks.

Table 3.1: Mean maximum and minimum temperatures recorded in the glasshouse over the experimental period (mean±std. dev.)

Week	Maximum Temperature (°C)	Minimum Temperature (°C)
1	25.8±6.0	18.5±0.7
2	30.0±1.9	20.6±1.9
3	30.1±1.7	21.6±2.2
4	32.8±1.9	22.0±0.8
5	28.4±3.9	21.4±0.5
6	28.3±2.1	20.2±1.5
7	27.7±0.5	19.0±1.4
8	26.4±0.5	18.3±1.3
9	24.7±0.9	19.3±1.9
10	25.1±0.2	19.8±0.5
Mean	27.9±0.8	20.1±0.4

Statistical Analysis

For *Isopogon* the Kolmogarov-Smirnov Two-Sample Test was applied to determine significant differences between cumulative distribution functions of pairs of species. For *Dryandra* this test was also used to compare germination at either 15 °C or 25 °C for a given species and for pairs of species at a given temperature. A comparison of the effect of temperature compared to glasshouse germination was made for each species using a Chi-square analysis. This test was also used to compare the final percentage germination for each species within a given temperature.

Results

Optimum temperature for seed germination of three species of *Dryandra* and three of *Isopogon*

Dryandra

At 15 °C there was high seed germination for each species (Table 3.2). Germination was reduced at 25 °C and no germination was recorded at 5 or 35 °C. At 15 and 25 °C the rate of germination differed. At 15 °C by three weeks all species had significant germination (Figure 3.1), whereas at 25 °C germination was much slower. *Dryandra polycephala* (Table 3.2, Figure 3.2) was the only species to show a strong germination response at 25 °C but germination was delayed. Species showed significant differences in their pattern of cumulative seed germination (Table 3.3). Compared to the glasshouse results, germination numbers at least doubled at 15 °C for all species. *Dryandra polycephala* in particular showed marked improvement in germination at 15 °C compared to the glasshouse, although this species appears to have a relatively broad range of temperatures at which germination is possible (15-25 °C).

Isopogon

Germination of seeds for *Isopogon* was recorded at 15 °C only (Table 3.2). At this temperature *I. trilobus* showed the highest final percentage germination followed by *I. dubius* and *I. cuneatus*. High rates of germination commenced early (Figure 3.3) and there were significant differences in cumulative germination patterns between species (Table 3.3).

Table 3.2: Mean seed germination of three species of *Dryandra* and three of *Isopogon* at five different temperatures

Species and week since planting	% Germination or Emergence				
	5 °C	15 °C	25 °C	35 °C	Glasshouse (min/max: 20.1-27.9 °C)
D. formosa					
3	0.0	69.5	0.0	0.0	2.0
6	0.0	98.5	0.5	0.0	18.0
9	0.0	99.0	2.5	0.0	32.0
12	0.0	99.0	4.5	0.0	40.0
D. polycephala					
3	0.0	97.5	0.0	0.0	12.0
6	0.0	97.5	40.0	0.0	26.0
9	0.0	97.5	56.5	0.0	26.0
12	0.0	97.5	61.5	0.0	26.0
D. quercifolia					
3	0.0	35.0	0.0	0.0	4.0
6	0.0	81.0	3.0	0.0	22.0
9	0.0	82.5	8.5	0.0	40.0
12	0.0	82.5	13.5	0.0	40.0
I. cuneatus					
3	0.0	27.0	0.0	0.0	0.0
6	0.0	35.0	0.0	0.0	0.0
9	0.0	35.0	0.0	0.0	0.0
12	0.0	35.0	0.0	0.0	0.0
I. dubius					
3	0.0	64.5	0.0	0.0	0.0
6	0.0	69.0	0.0	0.0	14.0
9	0.0	69.5	0.0	0.0	34.0
12	0.0	69.5	0.0	0.0	40.0
I. trilobus					
3	0.0	4.0	0.0	0.0	0.0
6	0.0	64.5	0.0	0.0	6.0
9	0.0	75.0	0.0	0.0	36.0
12	0.0	76.5	0.0	0.0	38.0

Figure 3.1

Cumulative % seed germination for three *Dryandra* species at 15 °C. Maximum standard error 0.45.

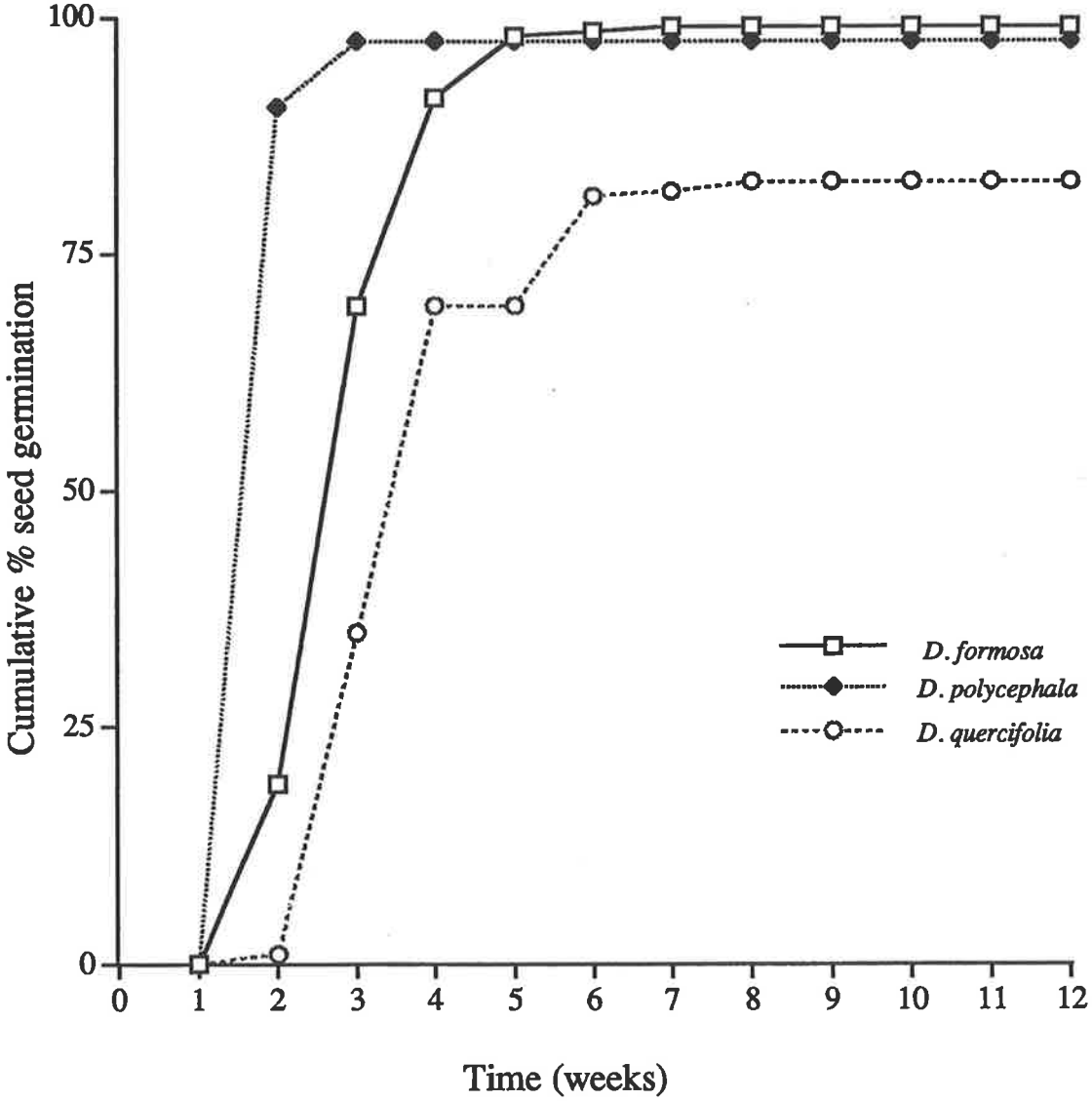

100
75
50
25
0
Cumulative % seed germination
0
1
2
3
4
5
6
7
8
9
10
11
12
Time (weeks)
D. formosa
D. polycephala
D. quercifolia

Figure 3.2

Cumulative % seed germination for three *Dryandra* species at 25 °C. Maximum standard error 0.41

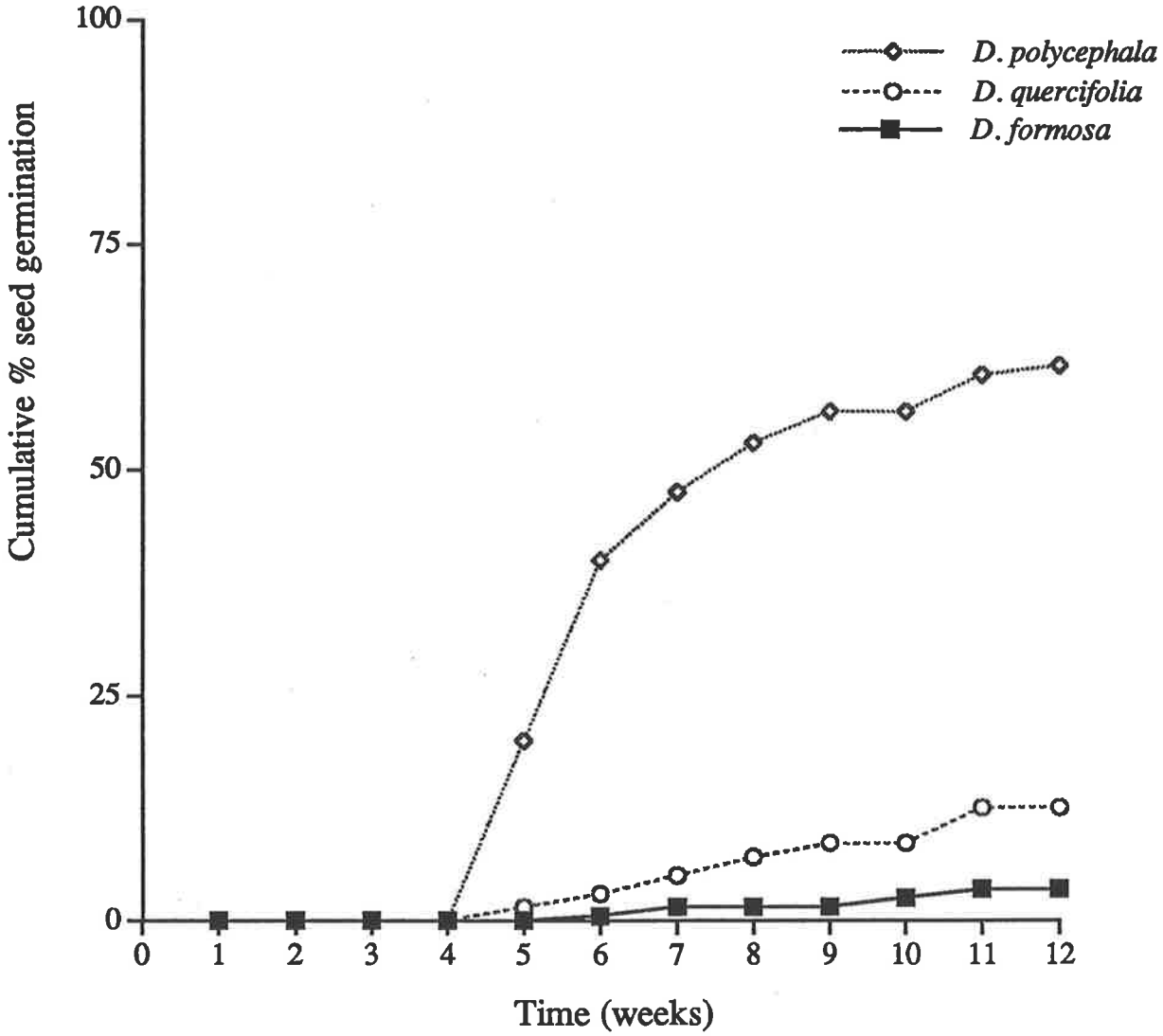
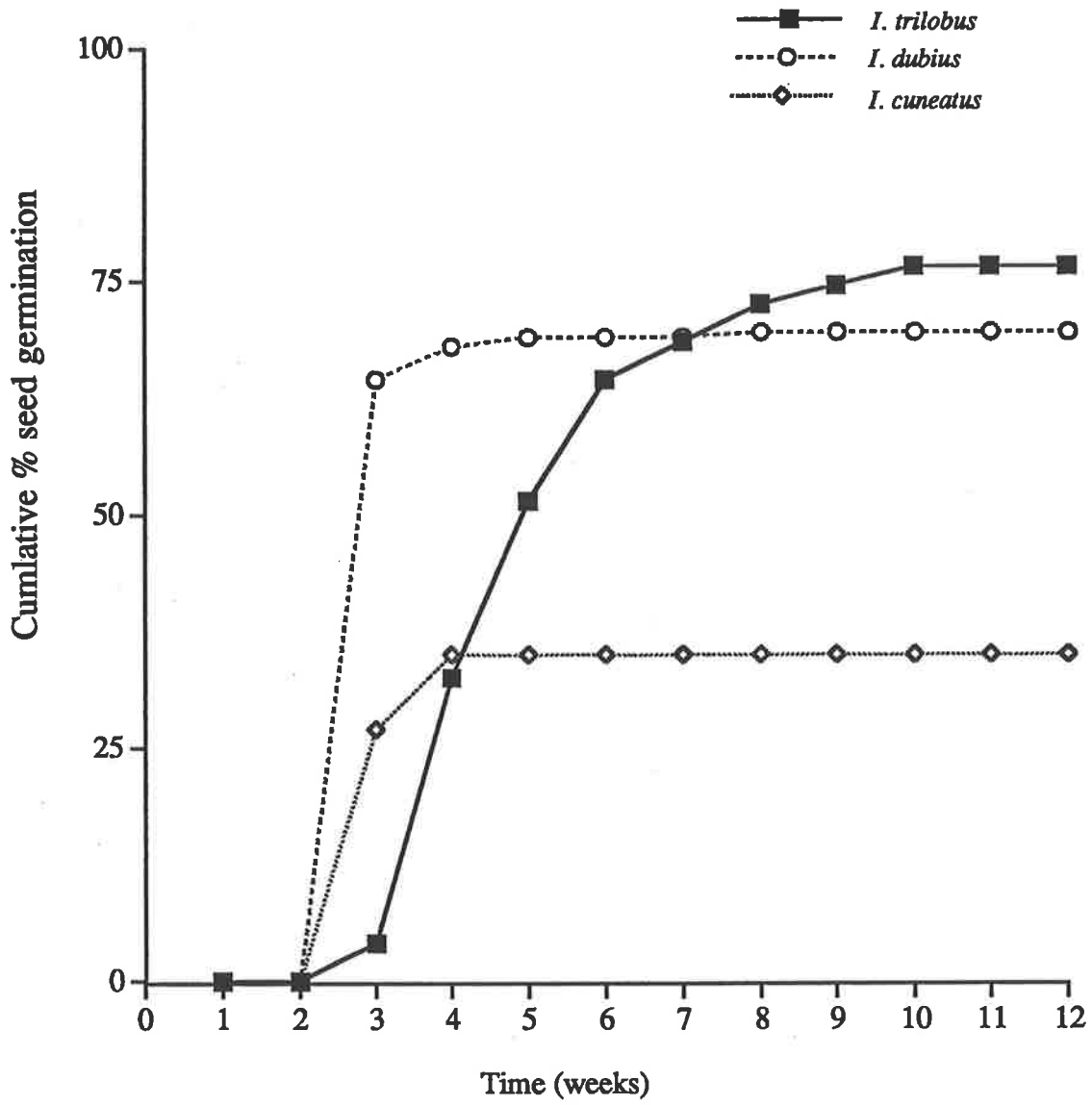

100
75
50
25
0
Cumulative % seed germination
0
1
2
3
4
5
6
7
8
9
10
11
12
Time (weeks)
D. polycephala
D. quercifolia
D. formosa

Table 3.3: Pairwise Kolmogarov-Smirnov Two Sample Test of seed germination for *Isopogon* at 15 °C and *Dryandra* species at 15 °C and 25 °C

Species pairs for comparison	P value
I. cuneatus; I. dubius	0.001
I. cuneatus; I. trilobus	0.02
I. dubius; I. trilobus	ns
D. formosa 15 °C; *D. formosa* 25 °C	0.0005
D. polycephala 15 °C; *D. polycephala* 25 °C	0.0005
D. quercifolia 15 °C; *D. quercifolia* 25 °C	0.01
D. formosa 15 °C; D. *polycephala* 15 °C	0.01
D. formosa 15 °C; D. *quercifolia* 15 °C	0.005
D. polycephala 15 °C; *D. quercifolia* 15 °C	0.0005
D. formosa 25 °C; *D. polycephala* 25 °C	0.005
D. formosa 25 °C; *D. quercifolia* 25 °C	0.05
D. polycephala 25 °C; *D. quercifolia* 25 °C	0.0005

Figure 3.3

Cumulative % seed germination of three *Isopogon* species at 15 °C. Maximum standard error 0.53


I. trilobus
I. dubius
I. cuneatus
Cumlative % seed germination
100
75
50
25
0
0
1
2
3
4
5
6
7
8
9
10
11
12
Time (weeks)

Seed germination of ten species of Dryandra and seven of Isopogon under glasshouse conditions

The temperature during the experimental period (Table 3.1) ranged from 33 - 25 °C (daily maximum) to 22 - 18 °C (daily minimum).

Dryandra

The final percentage emergence (Table 3.4) ranged from 0 % in *D. proteoides* to 40 % in *D. formosa, D. praemorsa* and *D. quercifolia*. Most emergence occurred 4-6 weeks after sowing (Figure 3.4). For those species with higher final percentage emergence (*D. formosa, D. praemorsa* and *D. quercifolia)*, seedlings continued to emerge until weeks nine and ten.

Isopogon

Three species of *Isopogon* failed to emerge over the experimental period (*I. axillaris, I. cuneatus* and *I. latifolius*) (Table 3.4) and the highest final percentage emergence occurred in *I. formosus*. This species commenced emergence at week three and continued until week seven (Figure 3.5).

Table 3.4: Final percentage emergence of ten *Dryandra* and seven *Isopogon* species after 10 weeks in the glasshouse

Species	Final % Emergence
D. carduacea	18
D. carlinoides	10
D. formosa	40
D. hewardiana	14
D. nivea	24
D. nobilis	10
D. polycephala	26
D. praemorsa	40
D. proteoides	0
D. quercifolia	40
I. axillaris	0
I. anethefolius	22
I. cuneatus	0
I. dubius	40
I. formosus	60
I. latifolius	0
I. trilobus	38

Figure 3.4

Emergence of seedlings of ten *Dryandra* species under glasshouse conditions. Zero seedling emergence was observed for *D. proteoides*. Note: the final percentage emergence for *D. quercifolia*, *D. formosa* and *D. praemorsa* was 40%, and for *D. nobilis* and *D. carlinoides* was 10%, the points have been stacked for ease of viewing. Standard errors have not been given as single values were obtained for each species on a given day.

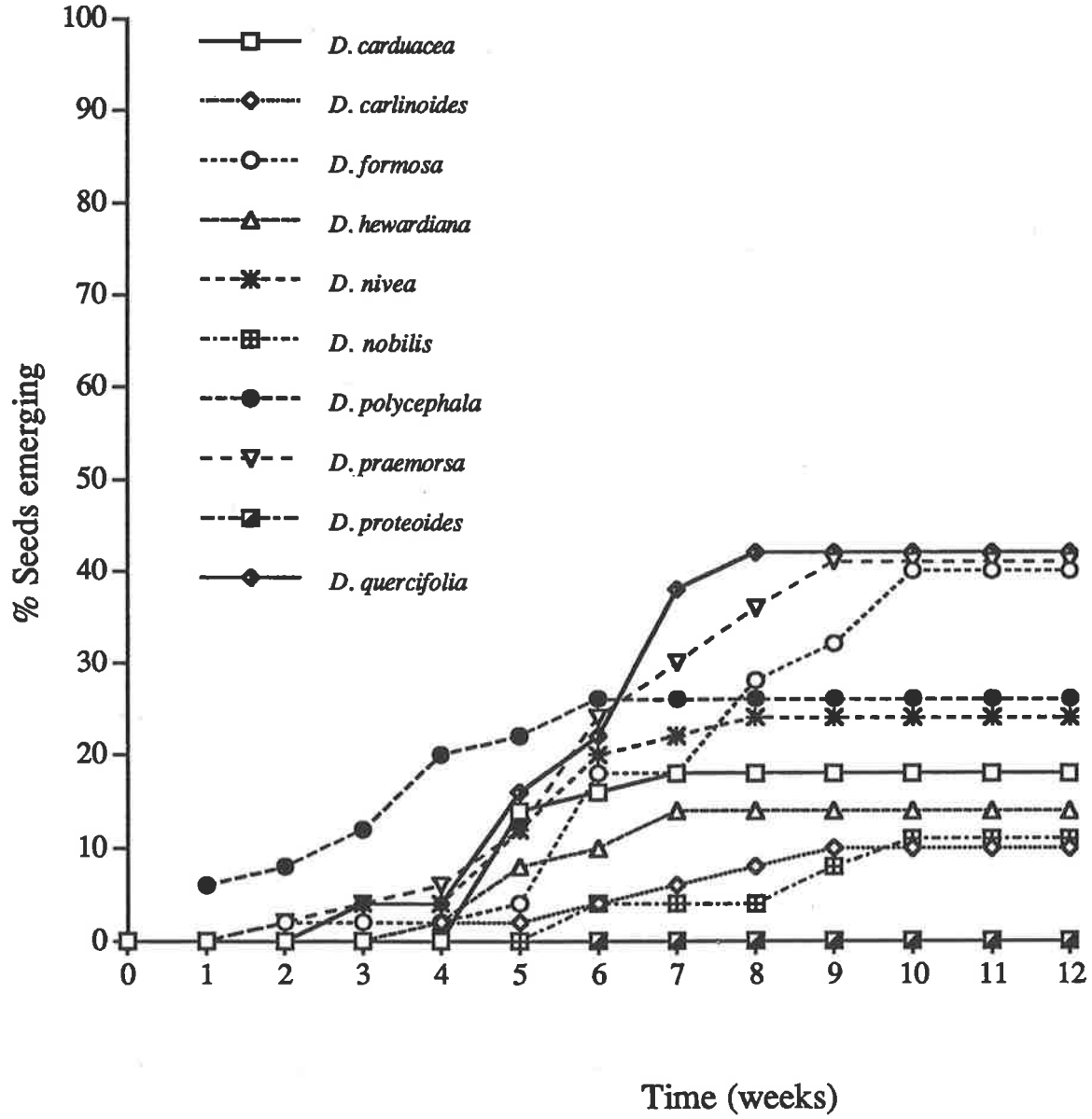
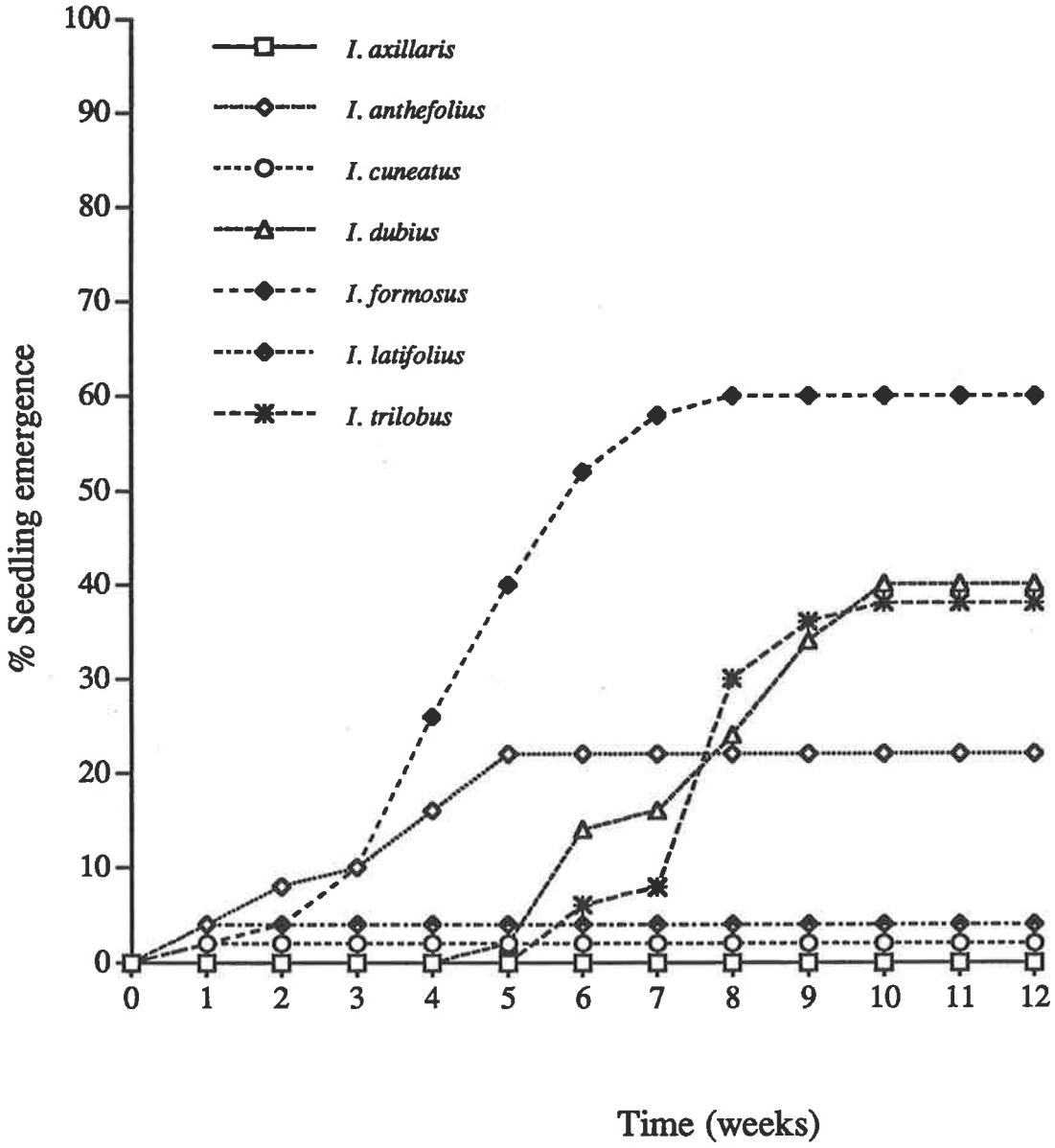

% Seeds emerging
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
11
12
D. carduacea
D. carlinoides
D. formosa
D. hewardiana
D. nivea
D. nobilis
D. polycephala
D. praemorsa
D. proteoides
D. quercifolia
Time (weeks)

Figure 3.5

Emergence of seedlings of seven *Isopogon* species under glasshouse conditions. Zero seedling emergence was observed for *I. cuneatus, I. axillaris* and *I. latifolius* however the points have been stacked for ease of viewing. Standard errors have not been given as single values were obtained for each species on a given day.


100
90
80
70
60
50
40
30
20
10
0
% Seedling emergence
I. axillaris
I. anthefolius
I. cuneatus
I. dubius
I. formosus
I. latifolius
I. trilobus
0
1
2
3
4
5
6
7
8
9
10
11
12
Time (weeks)

Discussion

Dryandra and *Isopogon* seed germination was promoted at 15 °C and to a lesser extent at 25 °C for *Dryandra* only, and was completely inhibited at 5 and 35 °C. Seeds subjected to constant temperatures of 15 °C showed the highest percentage germination and highest rates of germination compared to other temperatures.

After three weeks at 15 °C *Dryandra* seed germination reached high levels, with germination commencing soon after sowing. Growth curves for this genus displayed a generally sinusoidal pattern suggesting that within seed batch variation was low, most seeds germinating at the same time. High seed viability was reflected by the high final percentage germination. At 25 °C germination rates were much slower and *D. polycephala* was the only species to show significant germination at this temperature. Possibly germination of this species is effective over a wider range of temperatures due to its more northerly distribution compared to *D. quercifolia* and *D. formosa*, both of which occur on the south coast of Western Australia where lower temperatures are experienced. Differing temperature optima for germination relating to species distribution has been reported for three species of *Banksia*. Of these *B. aemula* had the highest temperature requirement for germination and correspondingly had the most northerly distribution, ranging as far as Proserpine in North Queensland (Sonia and Heslehurst, 1978).

Isopogon species also showed strong germination at 15 °C. No germination recorded at other temperatures. Seed germination was slower and more prolonged in the *Isopogon* species and did not reach as high final percentage germination as the *Dryandra* species at this temperature.

An optimum temperature of 15 ° C for seed germination has been reported for *Banksia* (Sonia and Heslehurst, 1978) and for some other Australian natives (Bell et al., 1993). This temperature coincides with periods of winter rainfall in the regions where these species are native. Germination occurs at a time when continual moisture is available thus favouring the subsequent survival of seedlings (Bell et al., 1993).

The glasshouse results cannot be directly compared with those from the constant temperature environments, as the glasshouse numbers reflect seedling emergence, a post-germination event under less controlled conditions. They can, however, be compared in terms of successful germination under commercial conditions. Germination success was much higher for seeds incubated at 15 °C for both genera compared with the glasshouse. *Isopogon cuneatus* is a very good example of promoted seed germination at this temperature. In the glasshouse this species did not germinate, where as 35% of seedlings germinated when incubated at 15 °C. Temperature has been shown to strongly influence germination numbers in this study, and high temperatures were found to inhibit germination. Thus the relatively high glasshouse temperatures may have caused the low percentage emergence of species tested.

Germination of *Dryandra* and *Isopogon* species in the glasshouse met with mixed success. *Isopogon formosus* was the only species to show strong seedling emergence, and for the majority of species seedling emergence was 40% or less. The percentage emergence values obtained for *Dryandra* species was low compared to the same species germinated by members of the *Dryandra* study group (SGAP, 1991). They obtained values of percentage emergence greater than or equal to 57%. These differences may be the result of different media or temperatures.

For most of the *Dryandra* seedlings in the glasshouse, there appears to be no dormancy as seeds germinated without treatment, however, three species of *Isopogon* failed to germinate at all. It is possible that these species require certain conditions to break dormancy, although the fact that *I. cuneatus* germinated at 15 °C suggests that this is not the case. Bewley and Black (1994) point out that when seeds with relative dormancy are tested at different temperatures, germination occurs only within a certain range. This may not be the temperature range for maximum germination, but rather the temperature range over which there is no dormancy. When dormancy is removed by chilling or some other means, then germination may occur over a substantially wider range of temperatures.

Other factors may also be important for germination of these species. Recently the importance of smoke for germination of Australian natives was reported (Roche et al.,

1994; Dixon et al., 1995; Brown and van Staden, 1997). In 45 of 94 difficult to germinate Western Australian species, exposure of seeds to cold smoke derived from burnt native vegetation promoted germination. The Australian vegetation has evolved alongside fire, but it is not yet certain which components of the smoke promote germination.

This information, that seed germination of *Dryandra* and *Isopogon* species is enhanced at temperatures of 15 °C, can be used to increase the efficiency of propagation of these species for the horticultural industry. Further studies on the effect of smoke on the germination of these and other species may also be beneficial to this industry.

Chapter Four

The Proteaceous Pistil: morphological and anatomical aspects of the pollen presenter and style of five genera and their possible relation to low fertility

Abstract

The extent to which structural limitations within the pistil affect fertilisation and thus seed set of Australian Proteaceous species is unknown. Structural limitations have been found to contribute to reduced seed set in *Banksia*, however further quantification of this observation is required for this genus, and additional genera before firm conclusions can be drawn. Thus the morphology and anatomy of pollen presenters, styles and pollen of several *Banksia, Dryandra, Hakea, Isopogon* and *Macadamia* species were studied. Serial sections of pistils and SEM images of pollen were quantified to determine whether the low fertility observed in the Proteaceae has a structural basis. In particular, pollen access to the stigma was determined. There were three types of stigmatic cavity. A groove in which the stigmatic papillae were enclosed was present in *Dryandra, Banksia* and *Hakea. Macadamia* had a groove with protruding papillae, and *Isopgon* had a tube which enclosed the papillae. Anatomical studies showed the pollen presenter to be structurally complex, but overall to have similar internal anatomy across the species studied. The species could be grouped according to presence or absence of transfer tissue and presence or absence of sclerenchyma, but these groups were not mutually exclusive. The stigmatic cavity limited pollen grain access to the stigmatic papillae as a result of its volume, diameter and length, as a consequence of pollen grain volume. The transmitting tissue tract narrowed significantly from the pollen presenter to the base of the style. In the pistil there were three structural filters to pollen tube passage. The first was at the stigma, as a consequence of groove dimensions. The second and third related to a narrowing of the transmitting tissue tract within the pollen presenter and in the lower style. The morphology and anatomy of the proteaceous pistil is complex and its influence on pollen tube passage is potentially significant.

Introduction

Some unusual features have arisen in the evolution of the proteaceous flower. The inflorescence consists of many individual flowers, yet it sets very few seeds. This is exemplified by the *Banksia menziesii* R. Br. inflorescence which consists of over 700 flowers and has a follicle set of only 0.4% (Clifford & Sedgley, 1993). Many theories have been proposed to explain the phenomenon of low seed set in the Proteaceae. Ayre and Whelan (1989) have incorporated these theories into the proximate and ultimate hypotheses. The proximate hypothesis includes four factors; pollen limitation (Goldingay & Whelan, 1990), pollen source, resource limitation (Harriss and Whelan, 1993) and predation (Vaughton, 1990). The ultimate hypothesis is concerned with adaptive reasons for the high flower to fruit ratio (Pyke, 1981). Further studies have found other factors to be involved. Spatially the inflorescence is unable to accommodate 100% follicle set and floral position may influence a flower's potential to produce seed (Collins & Rebelo, 1987; Fuss & Sedgley, 1991a; Vaughton, 1993). Competition between ovaries, within and among inflorescences, may also be influential. Andromonoecy has been reported in the Proteaceae (Johnson and Briggs, 1963; Collins and Rebelo, 1987), however Walker & Whelan (1991) do not believe that it is a widespread reason for the low fruit to flower ratios observed.

There may however be another possibility, that low fertility has a basis in the structure of the pistil. For this, the flower itself needs to be considered. The pistil consists of a woody style adorned at its tip by a morphologically distinct, and internally complex, pollen presenter which contains the stigma. The collar, the swelling at the pollen presenter base, together with the neck, the narrowing of the style below the pollen presenter, contributes morphologically to distinguish the pollen presenter from the rest of the style (Sedgley et al., 1993). Of the sixteen angiosperm families which possess pollen presenters (Howell et al., 1993), the Proteaceae has the greatest diversity (Ladd, 1994). Over 80% of species possess a pollen presenter, and at the family level there are six main types (Ladd, 1994) which can be further divided at the level of the genus (Sedgley et al., 1993; Ladd et al.,

1996). The pollen presenter has been given this name as, prior to anthesis, anthers dehisce in the mature bud and press their pollen onto the upper gynoecium. At anthesis (tepal split) pollen is displayed (presented) on this region, rather than in the anthers (Ladd et al., 1996). Despite the close proximity of self-pollen to the stigma most taxa are outcrossing, avoiding selfing by protandry or the precise placement of pollen away from the stigma. Often the stigma is confined to a cavity which is the result of an overgrowth of sterile stylar tissue (Ladd, 1994).

Enclosure of the stigma, a feature possessed by many genera of the Proteaceae, is a characteristic which distinguishes them from most other angiosperms. Commonly, the angiosperm stigma is a large secretory organ consisting of soft tissues, whose primary role is the capture and hydration of pollen grains. The stigma of the Proteaceae has the same function, however its location in a cavity brings into question the effectiveness of the stigma to carry out its function for these genera. Differences between species regarding stigmatic behaviour associated with receptivity have been observed in *Dryandra* (Matthews and Sedgley, in press, Chapter Two), and structural limitations within the pollen presenter and style, specifically the quantity of transmitting tissue, have been observed in *B. menziesii* (Clifford & Sedgley, 1993). Clifford and Sedgley (1993) concluded that pistil structure may limit fertility due to the progressive reduction of transmitting tissue to a point at the base of the style where only 11 transmitting tissue cells are present. These authors also observed the presence of transfer tissue within the pollen presenter. This tissue is known to function in the role of secretion and absorption of substances and may contribute to the growth of the pollen tube (Gunning and Pate 1974). As the pistil is vital to the process of fertilisation of the ovule features such as a stigmatic groove, limited transmitting tissue and the presence or absence of transfer tissue may affect the likelihood of the pollen tube reaching the ovary and fertilising the ovules, and thus may have a serious effect upon seed set. These features merit further investigation.

To this end eight Australian Proteaceous species were chosen which were either in current horticultural production, were rare and potentially threatened, or had unusual floral features. They included two *Banksia* species, *B. coccinea* and *B. ericifolia*, *Hakea*

bucculenta, *Isopogon cuneatus* and *Macadamia integrifolia.* To further broaden the study of *Dryandra* species, *D. quercifolia* and *D. formosa* were included and an additional *Dryandra* species, *D. nana.* This species has a very long style (≤7cm). This study investigates the possibility that the morphology and internal structure of the pollen presenter and style influences pollen grain access to the stigma, and the subsequent growth of the pollen tube. In particular, it aims to investigate stigmatic groove and pollen volume to determine the hypothetical maximum number of pollen grains that can be housed within the groove. It also quantifies the number of transmitting tissue cells present at seven regions down the style including the point at the base of the style near the ovary. This was carried out to determine whether a similar reduction in transmitting tissue such as that observed in *B. menziesii* occurs broadly over the Australian Proteaceae, a feature which is correlated to pollen tube number in Chapter Five. Investigations were carried out using measurements made from serial sections of the pistil and SEM images of pollen.

Materials and Methods

Study Species

A selection of Australian proteaceous species was made including both tropical and arid adapted species.

Current taxonomic positions within the Proteaceae

(Bentham and Mueller, 1870; Johnson and Briggs, 1975; Theile, 1993; George, 1996)

Subfamily: Proteoideae

Tribe: Conospermeae: Subtribe: Petrophilinae

Isopogon cuneatus R. Br. (Source: Fowles property, Blewitt Springs): In its natural environment in Western Australia this species grows on stony lateritic soils in the Stirling Ranges and heaths near Albany. It produces a bright floral display in spring when the large

pinkish-mauve flowers emerge. Both flower and foliage are attractive, making it a popular garden plant. Limited quantities are sold as cut flowers (Plate 4.1a).

Subfamily: Grevilleoideae

Tribe Grevilleeae

Hakea bucculenta C. A. Gardner (Source: Fowles property, Blewitt Springs): The common name of this species, Red Pokers, aptly describes the long red inflorescence which emerges in late winter and spring. Its leaves are thin and it grows as a tall rounded shrub in sandy soils. It often forms thickets in its natural habitat in south-west Western Australia. It is one of the most ornamental hakeas, having neat foliage and handsome flowers, and is grown as a garden plant (Plate 4.1b).

Tribe Macadamieae Subtribe: Macadamiinae

Macadamia integrifolia Maiden and Betche (Source: Waite Orchard): This sub-tropical species is the source of the commercial macadamia nut. Its natural habitat is the lowland rainforests of southern Queensland. It is a medium sized tree whose white flowers are borne in pendulous racemes emerging in winter and spring. The species is partially self-incompatible and its wet stigma is protandrous. It is now widely cultivated in Australia and Hawaii for its edible nuts and many cultivars have been developed (Plate 4.2a).

Tribe Banksieae Subtribe: Banksiinae

Banksia coccinea R. Br. (Section **Coccinea,** Series **Coccineae**) (Source: Keith's property, Blewitt Springs): The natural habitat of this protandrous, partially self-incompatible species is Western Australia, from Albany to the Stirling Ranges and east to the Young River. It grows in deep sand and produces conspicuous terminal blooms from June to January. It is a fast growing, erect, but little branching shrub which is widely cultivated for the cut flower industry (Plate 4.2b).

Banksia ericifolia L. f. (Section **Oncostylis,** Series **Spicigerae**) (Source: Fowles property, Blewitt Springs): An eastern Australian species, its natural distribution confined to New

South Wales, near the central coast and on adjacent ranges between Collaroy and Jervis Bay. In these regions it grows on deep sand, sandy loam or sand over limestone. During April to August it produces terminal golden brown to pale yellow flowers. It is protandrous and partially self-incompatible and is widely cultivated as a garden plant in eastern Australia (Plate 4.3a).

Dryandra formosa R. Br. (Subgenus **Dryandra**, Series **Dryandra**) (Source: Keith's property, Blewitt Springs): See description Chapter Two, Materials and Methods, page 10.

Dryandra nana (Subgenus **Dryandra**, Series **Pectinatae**) (Source: Alex George, material collected from 61 km north of Regansford, Band Highway, 30° 14' S, 115° 28' E, Herbarium Collection No. 17299): This rare prostrate species occurs on gravelly hills in south-west Western Australia. In spring, small yellow flowers appear, the perianth tube is short (25 mm), yet it has very long styles (80 mm) making these flowers unique in the genus. It is rare in cultivation but should be made more available for conservation reasons (Plate 4.3b).

Dryandra quercifolia Meiss. (Subgenus **Dryandra**, Series **Ilicinae**) (Source: Keith's property, Blewitt Springs): See description Chapter Two, Materials and Methods, page 10.

Sources of material

Blewitt Springs, South Australia (35°10' S, 138°34' E)

Keith's property: *Banksia coccinea, D. formosa* and *D. quercifolia* were collected from a commercial cut flower plantation in Blewitt Springs. Prior to the experimental period all plants were subjected to routine management practices, including limited drip irrigation to supplement rainfall and biannual fertilising in autumn and spring with slow release fertiliser (Chapter Two).

Fowles property: *Banksia ericifolia, I. cuneatus* and *H. bucculenta* were collected from a private garden.

Macadamia integrifolia was collected from the Waite orchard. *Dryandra nana* was collected by Alex George from its habitat in Western Australia.

SEM of pollen presenter morphology

For each species one inflorescence per plant from each of three plants was collected. From each inflorescence up to ten pollen presenters were collected and fixed in FPA50 (formalin: propionic acid: 50% ethanol, 5:5:90). Up to two weeks later pistils were transferred into 70% ethanol for storage until use. After post-fixing in osmium (1% OsO_4) material was dehydrated through an ethanol series (2 x 70%, 2 x 90%, 2 x 100%, 30 minutes each change, then 1 hour 100% ethanol), then infiltrated with acetone (1:1 100% ethanol and 100% acetone, one change, 15 minutes,) and finally 100% acetone for 15 minutes. The tissue was critical-point dried and sputter-coated with carbon and gold for scanning electron microscopy. A Phillips field emission scanning electron microscope (FESEM) (XL30) was used. Three pollen presenters of each species were placed horizontally on stubs with stigmatic cavities facing upwards, another three pollen presenters for each species were secured with Superglue into holes made in the stub enabling them to stand vertically. Pollen presenters were viewed at 5 kV using the FESEM and images collected for each pollen presenter.

Light microscopy of pollen presenter and style

Collection and preparation of tissues

Fresh plant material was collected two days post-anthesis from each of three plants per species, one inflorescence per plant. Pollen was wiped from up to ten pistils per plant and these whole pistils placed directly into 2.5% glutaraldehyde (Unilab, Auburn, Australia, 25% solution) in 0.025M sodium phosphate buffer, pH 7.0, for up to one week at 0-4 °C. After this time pistils were removed and total pistil and pollen presenter lengths determined

using a dissecting microscope with a graduated lens. After measurement, seven portions (2.5 mm long) were cut at specific positions along the style using a double-edged razor blade and placed in fresh glutaraldehyde. The remaining material was discarded. The following portions were cut and fixed (Figure 4.1):

UP	upper half of pollen presenter
LP	lower half of pollen presenter
US	upper 2.5 mm of style directly below LP
UMS	the mid-point between the upper style (US) and the mid-point of the whole style (MS) with 1.25 mm cut on either side of this mid-point
MS	the mid-point of the style with 1.25 mm cut on either side of it
LMS	the mid-point between the mid point of the style (MS) and the lower style (LS) with 1.25 mm cut on either side of it
LS	2.5 mm cut directly at the lower style above the ovary

In cases where the pollen presenter was less than 2 mm in length, pollen presenters were left whole.

After a period of up to a week pistil tissues were transferred to 0.025 M sodium phosphate buffer for storage. Following which, tissues were dehydrated through a series of alcohols: methoxyethanol, ethanol, propanol and butanol and transferred to 1:1 butanol and glycol methacrylate (GMA) (Sigma, St. Louis, USA, H-8633) (prepared by mixing 93ml of 2-hydroxylethyl methacrylate, 7 ml polyethylene glycol 400 and 0.6 g benzoyl peroxide). This was followed by two changes in 100% GMA. Infiltration continued over a week at 0-4 °C. Each tissue was carefully placed in a vertical position and supported by a folded piece of paper within a gelatine capsule (size '00', Panmedica Sydney). Capsules were labelled individually and polymerisation achieved overnight at 60 °C.

Transverse sections (3 µm in thickness) of one pollen presenter per plant were made using a Reichert-Jung 2050 Supercut Microtome. Every fifth section was collected so that sections were 15 µm apart. Sections were collected from the tip of the pollen presenter to ensure that a representation of the whole stigmatic groove was obtained. Sectioning continued below the stigmatic cavity for at least 200 µm. Sections were stained with Periodic acid-Schiff's reagent (PAS) for carbohydrates and counter stained with Toluidine Blue O (TBO) to provide details of cell contents (O'Brien and McCully, 1981). Stained sections were viewed using a Zeiss Axiophot Photomicroscope (D-7082).

The remainder of the stylar tissue (regions: US, UMS, MS, LMS and LS) (Figure 4.1) were transversely sectioned. For each piece of tissue 3µm sections were taken and every 10th section collected, so that sections were 30 µm apart. Sections were stained with Periodic acid-Schiff's reagent (PAS) and Toluidine Blue O (TBO) and viewed using the photomicroscope.

Three dimensional reconstruction of pollen presenter

A three dimensional (3D) reconstruction of the pollen presenter of *Banksia ericifolia* was generated. Digital images of the pollen presenter were obtained from physical slices (3 µm sections) used in the light microscopy study. Before collection of images by the image analysis package, VideoPro (Leading Edge P/L), sections were oriented in the same direction. The images were 3D rendered with the software package ImageVolumes (Minnesota Datametrics Corporation) on the Indigo 2 Silicon Graphics computer.

Quantification of pistil tissues

The quantity of tissue types within the pollen presenter were determined using the colour image analysis package, VideoPro 32 (Leading Edge P/L). A program was written using this package to determine the area of tissues within the pollen presenter (Appendix). The following features were quantified (Plate 4.4):

1) Stigmatic cavity

2) Transmitting tissue (staining pink)

3) Transfer tissue (staining pink)

4) Sclerenchyma, parenchyma and vascular bundles (no predominant colour)

5) Epidermis and polyphenol containing cells (staining dark blue)

Sections were viewed using a light microscope with a video recorder which translated an image to the computer for analysis. For each section a line was traced around the tissue or feature of interest and the area quantified. To determine the area of the cavity, the outline was traced as described in Figure 4.2.

This method was appropriate for all species except *M. integrifolia* and *I. cuneatus*. In *M. integrifolia* the stigmatic papillae protrude from the cavity making the groove difficult to define. For this species the stigmatic papillae were traced and joined by a line drawn (Figure 4.2a). For *I. cuneatus* the cavity is a hollow tube, and the inside of the tube was traced and quantified (Figure 4.2b)

The distance (μm) between each of the sections measured from the pollen presenter was used to obtain the average volume of a particular tissue by multiplying the distance between sections with the area value for that section (Figure 4.3). An average volume was calculated in two regions; at the cavity, and up to 200 μm below the cavity. These regions were compared to determine whether tissue arrangements differed in the presence or absence of the cavity. Volume better represents tissue quantities than area, due to the changing structure of the pollen presenter down its length. Counts of transmitting tissue cells for each region of the pollen presenter and style were made using a hand counter and an average number determined for each region.

Plate 4.1

(a) *Isopogon cuneatus*, Fowles property, Blewitt Springs, South Australia

(b) *Hakea bucculenta*, Fowles property, Blewitt Springs, South Australia


a


b

Plate 4.2

(a) *Macadamia integrifolia*, Waite Agricultural Institute Orchard, South Australia

(b) *Banksia coccinea*, Keith's property, Blewitt Springs, South Australia


a


b

Plate 4.3

(a) *Banksia ericifolia*, Fowles property, Blewitt Springs, South Australia

(b) *Dryandra nana*, Western Australia (Photo: Alex George). Note long style (arrow) and pollen presenter (arrowhead).


a


b

Figure 4.1

Schematic representation of a proteaceous pistil showing regions where material was collected for light microscopy study of pollen presenter (pp) and style (st) cut above the ovary (ov). Curved arrow shows stigmatic cavity. Shaded portions represent the area sampled for serial sectioning. Samples were collected from the upper half of the pollen presenter (up), the lower half of the pollen presenter (lp), the upper style (us), upper mid-style (ums), mid-style (ms), lower mid-style (lms) and the base of the style (ls) just above the ovary.

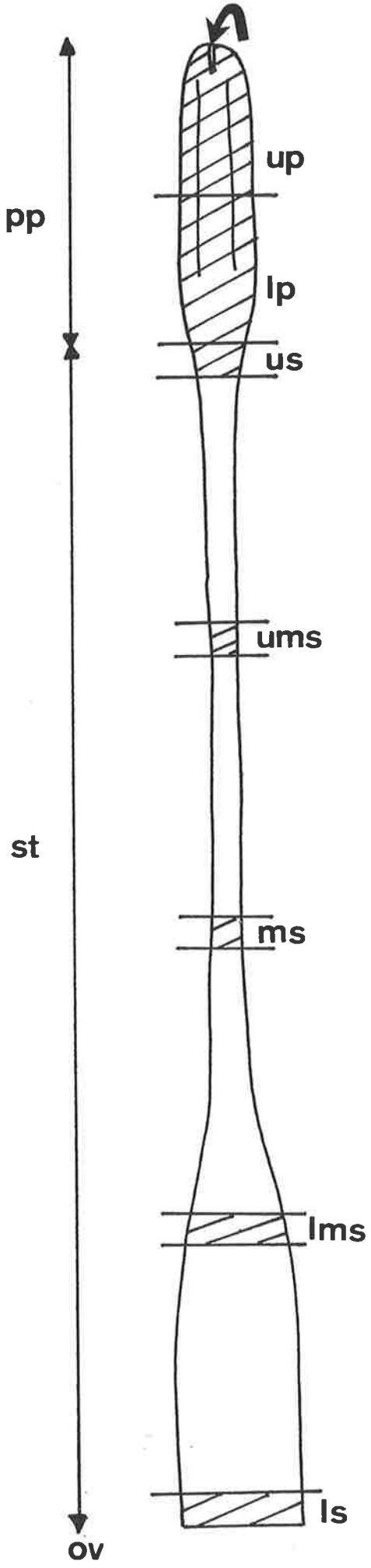

up
pp
lp
us
ums
st
ms
lms
ls
ov

Plate 4.4

Transverse section of the pollen presenters of *Dryandra quercifolia* and *Isopogon cuneatus* stained with PAS and TBO showing carbohydrates stained pink, lignins stained blue and polyphenols dark blue.

(a) *D. quercifolia*. Note cavity (g), transmitting tissue (t), transfer tissue (tt), polyphenol containing cells (arrowhead) and parenchyma/sclerenchyma (c).

(b) *I. cuneatus* Note starch grains (arrow) located in parenchyma cells and transmitting tissue (t).

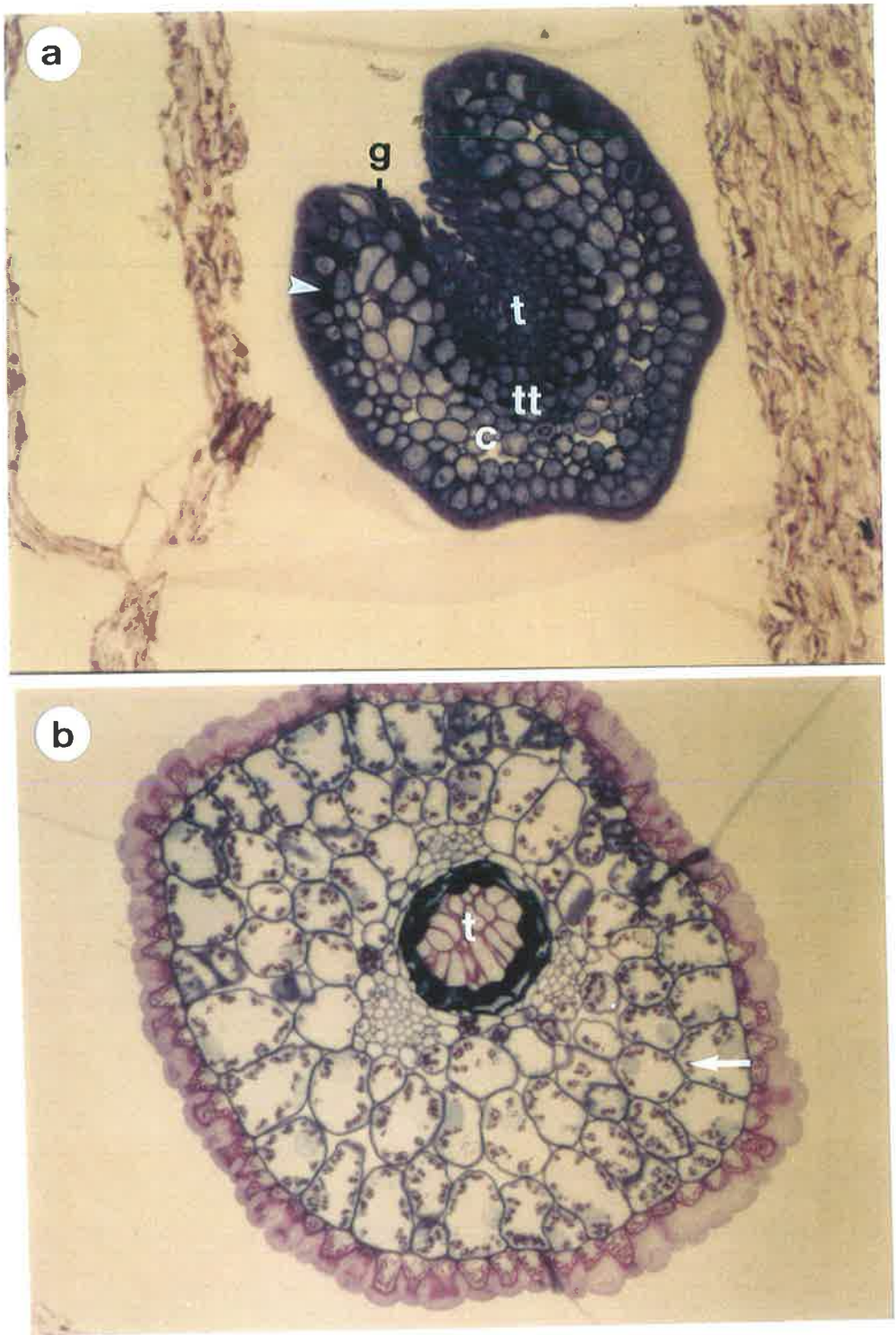

a
g
t
tt
c
b
t

Figure 4.2

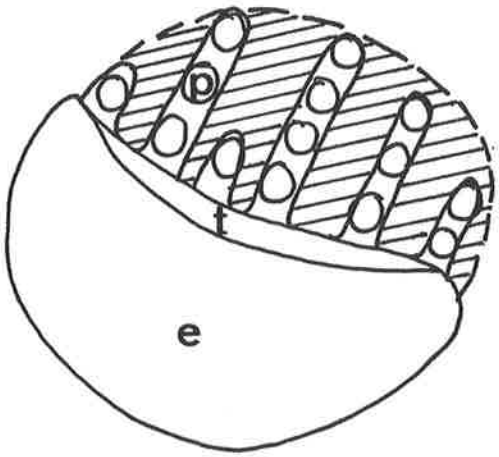
Schematic representation of the area traced to determine the area of the stigmatic cavity. Each diagram represents a transverse section of the pollen presenter in the region of the cavity. The hatched region represents the area of the stigmatic groove measured. Diagrams not drawn to scale.

(a) Open groove of *Macadamia integrifolia*. Diagram shows stigmatic papillae (p) in cross-section, the region of transmitting tissue (t) and epidermal/cortical area (e).

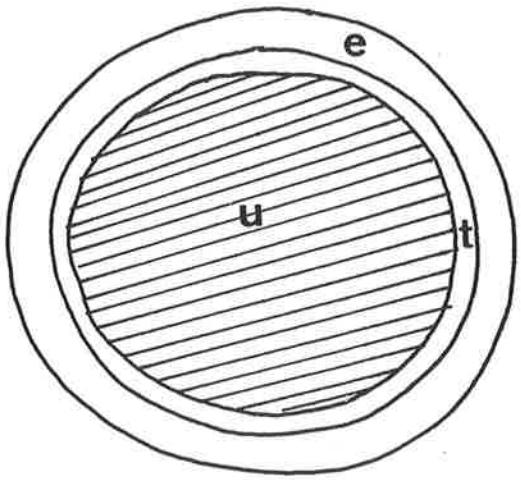
(b) Stigmatic tube of *Isopogon cuneatus*. Diagram shows the epidermis (e), papillae (t) and tube (u).

(c) Enclosed groove of *Banksia, Dryandra* and *Hakea*. Diagram shows the cortical region of the pollen presenter (e), papillae (t) and the stigmatic groove (g).

a



b



c

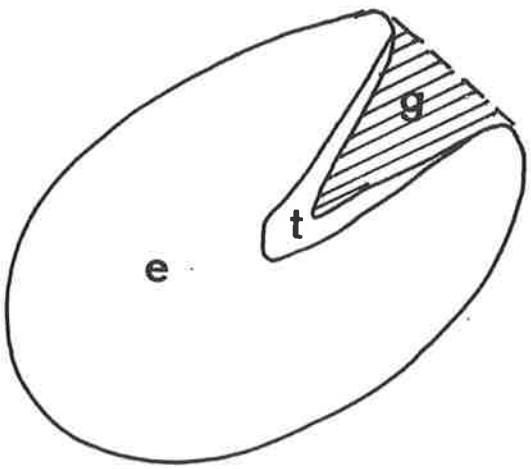


Figure 4.3

Schematic representation of the method used to determine the volume of a given tissue or cavity in the pollen presenter (not to scale)

(a) Diagram represents the pollen presenter of a given species with three transverse serial sections (s1, s2, s3).

(b) Side view of each of the three sections showing an example of the area measured on each section (a1, a2, a3) and the known distance between each section (d1, d2).

(c) Aerial view of each of the three sections showing the area measured (shaded).

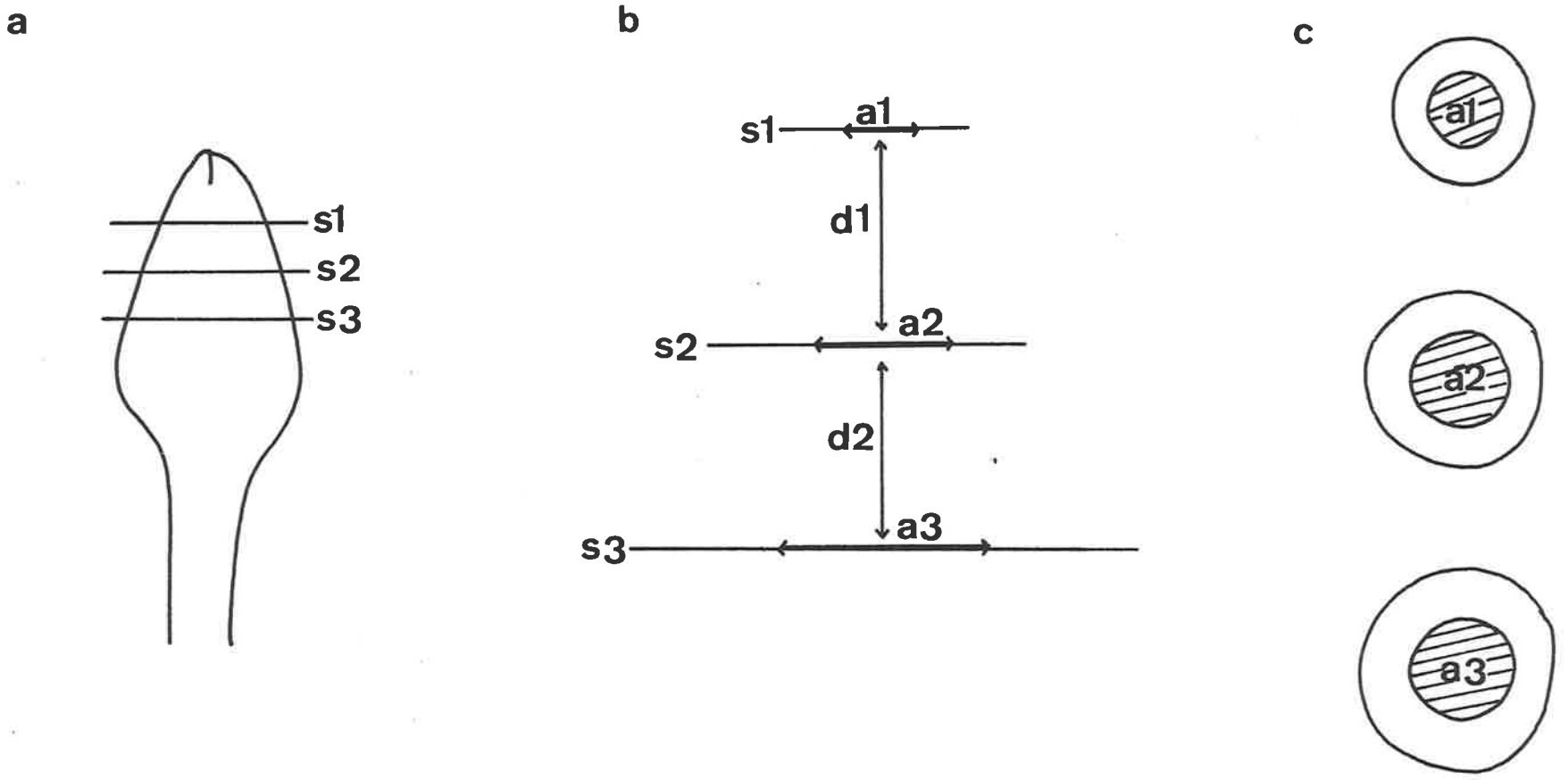
Calculations of the volume of the tissue were made using the following formula:

eg. Volume of tissue between sections 1 and 2

= area 1 (μm^2) x distance 1 (μm)

= volume 1 (μm^3)

The mean volume of a particular tissue was then calculated.


a
s1
s2
s3
b
s1
a1
d1
s2
a2
d2
s3
a3
c
a1
a2
a3

SEM of pollen grains

Collection and preparation of samples

For each species fresh pollen was collected from three plants, one inflorescence per plant except for *Macadamia integrifolia,* for this species one plant was sampled. A number of pollen laden pollen presenters were gently brushed over adhesive tabs on SEM stubs until coated with pollen. Samples were sputter coated with carbon and gold and viewed at 5 kV using the FESEM. Representative photographs of pollen morphology for each species were taken. *Dryandra nana* was not viewed, as fresh material was not available in South Australia.

Calculation of pollen grain volume

There were two general pollen grain shapes amongst the species studied; biporate, crescent-shaped grains (*Banksia* and *Dryandra*) and triporate triangular grains (*Hakea, Isopogon* and *Macadamia*). Individual image analysis programs were written to quantify the dimensions of each pollen grain type using VideoPro 32(Appendix).

Digital images of pollen grains were collected in two positions, one in the vertical position with one germination pore facing upwards (equatorial view), and the second with pollen grains in a horizontal position (polar view). To measure volume, images of at least five vertical and ten horizontal pollen grains for each plant were collected. In addition, length and diameter measurements were made for up to ten horizontal pollen grains per plant.

Measurement of *Dryandra* and *Banksia* pollen dimensions

For each pollen grain positioned vertically (equatorial view) a measurement of the diameter of the pore (least diameter) and the diameter of the pollen grain at its widest point (greatest diameter) were taken. An average diameter was calculated for the grain. Pollen grain area was determined by tracing the outline of the horizontal grains (equatorial view) and these measurements used to calculate the theoretical volume of the grain (Figure 4.4a,b).

Measurement of *Hakea*, *Isopogon* and *Macadamia* pollen dimensions

The configuration of the triangular pollen grain when positioned vertically (equatorial view), allowed for the visualisation of one germination pore in front view, and the other two in side view (polar view). To obtain the volume, the diameter of the germination pore in front-view (equatorial view) was measured, as were the maximum grain diameter and the lengths of the other pores in side view (polar view). An average diameter was determined from these lengths. An area measurement was made on horizontal grains (polar view). The average length and area measurements were combined to give an approximate pollen grain volume (Figure 4.4c,d). In addition, the length of each side was measured and averaged.

Pollen grain holding capacity of the stigmatic cavity

The theoretical pollen grain holding capacity of the stigmatic cavity was calculated for each species. Groove volumes were combined with the pollen grain volumes to calculate the hypothetical maximum pollen grain holding capacity of the groove. Pollen arrangement within the groove and groove diameter were not considered in the calculation.

Figure 4.4

Schematic representation of the pollen grain dimensions used to determine the volume of the grain. Not to scale.

(a) Diagram of the side view (polar view) of a biporate pollen grain of *Banksia* and *Dryandra*. The region for which the area was calculated is depicted by shading.

(b) Diagram of the end view (equatorial view) of a pollen grain of *Banksia* and *Dryandra*. The diameter (L1) of the germination pore and diameter of the pollen grain (L2) at its widest point were measured.

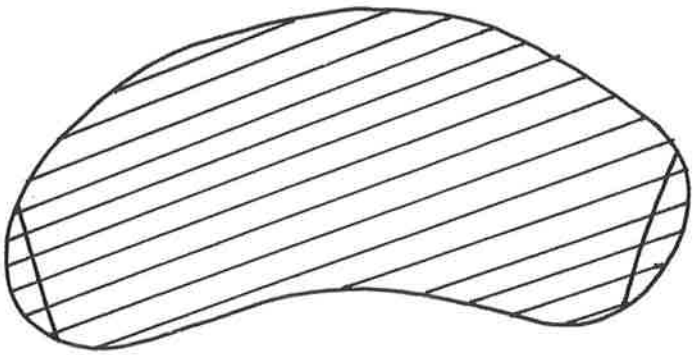
The average volume of the pollen grains of *Banksia* and *Dryandra* were calculated according to the formula : area x average (L1, L2)

(c) Diagram of side view (polar view) of a triporate pollen grain of *Hakea, Isopogon* and *Macadamia*. The region for which the area was calculated is depicted by shading.

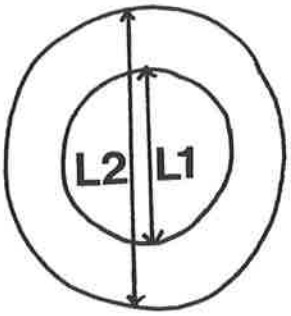
(d) Diagram of end view (equatorial view) of a pollen grain of *Hakea, Isopogon* and *Macadamia* showing an aerial view of the grain. The diameter of the central germination pore (L1), the two pores on their sides (L2, L3) and the maximum diameter (L4) of the grain were measured.

The average volume of the pollen grains of *Hakea, Isopogon* and *Macadamia* were calculated according to the formula: area x average(L1, L2, L3, L4)

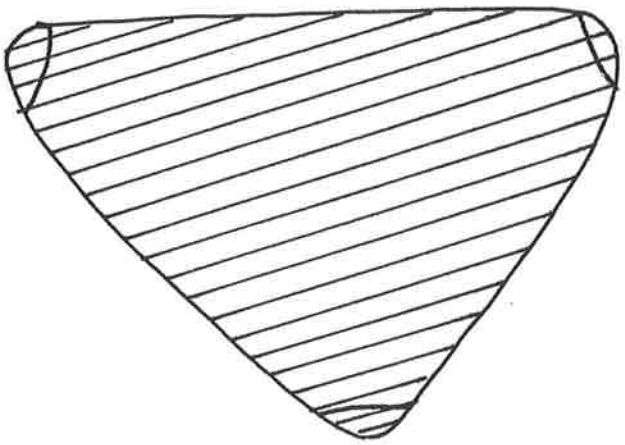
a



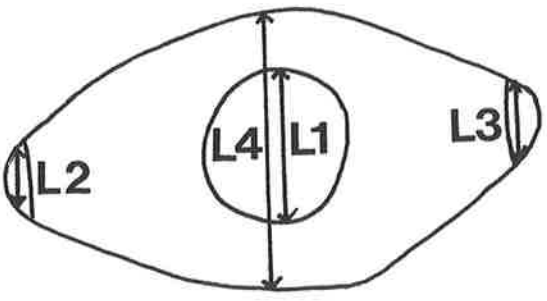
b



c



d



Statistical analysis

The method of Residual Maximum Likelihood (REML) was used for comparisons of transmitting tissue cell number between regions down the style and between species (Genstat Committee, 1993). For all remaining analyses programs from the BMDP statistical software package (Dixon, 1993) were used.

A Repeated Measures Analysis of Variance was used to test for significant differences between the grouping factor, species, for the within subject factors of pollen grain length, tissue volumes within the pollen presenter at and 200 μm below the stigmatic groove, and style and pollen presenter lengths. The overall effect of species was tested using the Wald's test for significance. Where a significant effect was determined a model was fitted to produce an estimated mean value for each species and their standard error. A pair wise comparison was made using the difference in estimated means relative to the estimated error of this difference. The result was recorded as a z score, which was assumed to be a standard normal deviate. Due to the number of potential pair wise comparisons, a z value of $z>2$, or $z>3$ was assigned as the critical level used to judge for significant differences between species. This procedure followed the logic of the Bonferroni Principal of adjusting for multiple comparisons. The program 5V (Unbalanced Repeated Measures) was used (Dixon, 1993).

For pollen grain volumes and areas a one-way Analysis of Variance was performed comparing species (Program 7D, Dixon 1993). Those species comparisons found to have a p value of less than $P<0.01$ were then compared in a pair wise fashion using the Tukey Studentized Range Method.

Results

Pollen presenter and style morphology

There was similarity of structure between the cylindrical pollen presenters of *Dryandra quercifolia* (Plate 4.5a,b) and *D. formosa* (Plate 4.8a,b). Both had eight ridges and an oblique terminal stigmatic groove. The lips of the *D. formosa* groove were more pronounced than those of *D. quercifolia*. Neck and collar were absent in both species thus making the pollen presenter almost continuous with the style. The *D. nana* pollen presenter was morphologically distinct, being conical in shape and its stigmatic groove was terminal (Plate 4.11). There were eight ridges and the pollen presenter diameter was greater than the diameter of the style. Neck and collar also absent.

Pollen presenters of *Banksia coccinea* (Plate 4.14) and *H. bucculenta* (Plate 4.17) were similar in appearance. Both had a conical pollen presenter with an oblique terminal groove. The pollen presenter lacked ridges, but had a collar and neck making the pollen presenter distinct from the rest of the style.

The *B. ericifolia* pollen presenter (Plates 4.20, 4.21) was ovoid and the stigmatic groove sub-terminal. Both neck and collar were absent, although the pollen presenter was wider in diameter than the style. The 3D reconstruction clearly showed the protruding lips of the stigmatic groove and the bulbous nature of the pollen presenter (Plate 4.21).

The pollen presenter of *I. cuneatus* (Plate 4.24) was the most ornate of the species studied and substantially differed in morphology from other species. It consisted of a stigmatic tube rather than a groove and eight vertical rows of epidermal hairs. Below the hairy region was a swollen area, the diameter of which decreased towards the style. The style below was narrow and unable to support the weight of the pollen presenter. This resulted in the suspension of the pollen presenter from the perianth tube which enclosed the style (Plate 1.2c, page 5).

The pollen presenter of *M. integrifolia* (Plate 4.28) was continuous with the style, its most prominent feature being the stigmatic papillae which protruded from the groove. There was a slight collar but a neck was absent.

Maximum style length was recorded for *D. nana*, significantly longer than the other species (Table 4.1). There was similarity in style length between the remaining *Dryandra* species and *Banksia*, and between *H. bucculenta* and *I. cuneatus*. The shortest style was recorded in *M. integrifolia*. The proportion of the style that was pollen presenter varied between species; *I. cuneatus* had the longest pollen presenter relative to its style and *B. ericifolia* the shortest.

Table 4.1: Style and pollen presenter length (±s.e.) of eight proteaceous species

Species	Pollen Presenter (mm)	Style minus pollen presenter (mm)	Style (including pollen presenter) (mm)	% Style length that is Pollen Presenter
B. coccinea	^{d}2.09±0.14	^{c}39.22±0.17	^{c}41.30±1.19	^{bc}5.06±0.66
B. ericifolia	^{a}0.52±0.14	^{c}39.16±0.17	^{c}39.67±1.19	^{a}1.34±0.65
D. formosa	^{e}2.71±0.05	^{c}39.03±0.42	^{c}41.73±0.43	^{cd}6.47±0.24
D. nana	^{e}2.74±0.14	^{e}74.80±1.17	^{e}77.54±1.19	^{ab}3.54±0.66
D. quercifolia	^{g}6.05±0.14	^{d}44.30±1.18	^{d}50.35±1.20	^{e}11.99±0.66
H. bucculenta	^{c}1.90±0.14	^{b}21.59±1.17	^{a}23.48±1.19	^{d}8.01±0.66
I. cuneatus	^{f}4.92±0.10	^{b}22.83±0.83	^{b}27.74±0.84	^{f}18.03±0.47
M. integrifolia	^{b}1.30±0.14	^{a}12.59±1.17	^{a}13.87±1.19	^{d}9.05±0.66
P value	<0.001	<0.001	<0.001	<0.001

Numbers with a different letter are significantly different (z>3). Pairwise comparisons have been made between species down a column

Pollen presenter and style anatomy

The pollen presenter

The stigmatic cavity

The nature and distribution of tissues at this region were determined by the type of stigmatic cavity. There were three distinct cavity types; a groove in which the stigmatic papillae were enclosed (*Dryandra quercifolia;* Plate 4.6, *D. formosa;* Plate 4.9, *D. nana;* Plate 4.12, *Banksia coccinea;* Plate 4.15, *Hakea bucculenta;* Plate 4.18, *B. ericifolia;* Plate 4.22) a groove with protruding papillae creating a stigmatic area or surface (*Macadamia integrifolia;* Plate 4.29) and a stigmatic tube enclosing the papillae (*Isopogon cuneatus;* Plate 4.26). Quantification of the relative cavity volumes for these species revealed that similar groupings were appropriate (Table 4.2). Those species with a enclosed groove had the smallest cavity. The volume of the stigmatic area of *M. integrifolia* with its protruding papillae was significantly large in comparison, but the largest cavity was that of *I. cuneatus* with its stigmatic tube leading down to the transmitting tissue. A similar pattern was observed for the proportion of pollen presenter represented by the cavity, in *Isopogon* it was significantly large, and correspondingly its proportion in *Banksia, Dryandra* and *Hakea* was significantly small (Table 4.3).

The arrangement and length of the stigmatic papillae varied. *Macadamia integrifolia* stigmatic papillae protruded some distance from the groove. They were loosely packed together creating an area of papillae with spaces between and transmitting tissue to one side (Plate 4.29). *Dryandra quercifolia, D. nana* and *B. coccinea* had elongated stigmatic papillae enclosed in the groove. In *D. quercifolia* they consisted of three to four rows of transmitting tissue cells (Plate 4.5c,d). Due to the terminal location of the *D. nana* groove, its cavity was enclosed by two lips, separated at the edges (Plate 4.12a). At groove opening the stigmatic papillae protruded into each other, but with distance down the groove the papillae were replaced by solid transmitting tissue in the centre with papillae restricted to the edges of the groove. For *B. coccinea*, papillae consisted of elongated cells proximal to the groove and more rounded cells distal to the groove (Plate

4.15). *Banksia ericifolia, H. bucculenta, D. formosa* and *I. cuneatus* had very short stigmatic papillae. *B. ericifolia* (Plate 4.22) and *H. bucculenta* (Plate 4.18) grooves were fully lined with transmitting tissue, as was the stigmatic tube of *I. cuneatus* (Plate 4.24). For *D. formosa* however the frontal lobes of the groove consisted of thick-walled polyphenol containing cells and epidermis with papillae confined to a position distal to the groove (Plate 4.9b). The pollen presenter also appeared to have anther locule contents surrounding it, the result of the close proximity of the anthers to the pollen presenter in the closed flower (Plate 4.9d).

The amount of transmitting tissue varied significantly between species (Table 4.2). *Hakea bucculenta* had significantly more transmitting tissue relative to other species. *Dryandra formosa* had the least volume of transmitting tissue and its volume in the pollen presenter was small. The three *Dryandra* species were similar in the proportion of transmitting tissue compared to the total tissue of the pollen presenter.

The most common arrangement of tissues in the pollen presenter at the stigmatic cavity was an area of transmitting tissue, partially enveloped by a layer of transfer tissue of up to four cells thick although this was not present in *H. bucculenta* or *I. cuneatus*. External to this was either polyphenol containing cells or ground tissue (either parenchyma alone, or a mixture of parenchyma and sclerenchyma) and the arrangement of these tissues varied between species as did their relative proportions. An epidermal layer(s) surrounded these tissues which was covered by a cuticle whose thickness varied between species. Vascular bundles tended to be absent at this point although some were observed for *D. quercifolia* at a location distal to the groove. Most species had starch grains within their cortical cells. The polyphenol containing cells distal to the *B. ericifolia* groove were very large and caused the bulbous appearance of the pollen presenter (Plate 4.22). Pollen grains were observed germinating in the stigmatic cavity of this species (Plate 4.22b).

Below the stigmatic cavity

Below the stigmatic cavity, tissues of the pollen presenter formed concentric rings around a central core of transmitting tissue. In most species the quantity of transmitting tissue

decreased dramatically from that present at the cavity (Tables 4.2, 4.3). Where transfer tissue was present, four to six layers of these cells surrounded the transmitting tissue completely, the thickened walls of the transfer tissue facing inwards. These tissues, either transfer or transmitting, (if transfer absent) were surrounded by rings of polyphenol containing cells or rings of ground tissue, the order of which was species dependent. *Banksia coccinea, B. ericifolia* and *I. cuneatus* had two to four layers of polyphenol containing cells enclosed within ground tissue which also enveloped the vascular bundles (Plates 4.15, 4.22, 4.26). The ground tissue which included parenchyma and sclerenchyma (*Banksia* and *Dryandra* only), was then enveloped by a second layer of polyphenol containing cells (*B. coccinea* and *B. ericifolia*) and an epidermal layer(s) or an epidermis alone (*I. cuneatus*). For *D. quercifolia* (Plate 4.6), *D. formosa* (Plate 4.9), *H. bucculenta* (Plate 4.18) and *M. integrifolia* (Plate 4.29) the polyphenol containing cells were confined to the outer ring of tissues inside the epidermis. In *D. nana* the polyphenol containing cells were scattered within the ground tissue, and associated with the epidermal layer (Plate 4.12). Where present, the vascular bundles were concentrated around the transmitting tissue distal to the cavity, or on either side of it in the case of *D. nana.* Sclerenchyma cells were present in the pistils of *Banksia* and *Dryandra* only.

The external surface of the pollen presenter of *I. cuneatus* was the most highly differentiated of all the species observed (Plate 4.26). Its epidermal cells showed considerable changes down the pollen presenter. At the tube region, the cells were heavily cuticularised and were small and oval shaped, further down they formed hairs, each hair covered with a thick cuticle (Plate 4.26). Beyond this the hairs were replaced by protruding "pear-shaped" cells with a thick cuticle (Plate 4.27a).

The most notable of the changes in tissue quantity between the cavity region and below was the decrease in transmitting tissue volume for all species except *H. bucculenta* and *B. coccinea* (Table 4.2). For these species, transmitting tissue volume increased significantly, corresponding morphologically to an increase in the diameter of the pollen presenter.

Transfer tissue was present in all species except *H. bucculenta* (Table 4.2). Beyond the groove its quantities increased, sometimes significantly (*D. formosa, D. nana* and *M.*

integrifolia). *D. nana* had more transfer tissue than the other species although the volume was also high for *M. integrifolia*. *I. cuneatus* had the lowest volume of transfer tissue in the pollen presenter. Proportionally the amount of transfer tissue increased beyond the groove for *B. coccinea*, *B. ericifolia* and *M. integrifolia* and decreased for the remaining species (Table 4.3). Also increasing in the region below the cavity was the quantity of ground tissue. This represented a decrease in the proportion of the pollen presenter dedicated to pollen tube passage and an increase in the tissues comprising the structure of the pollen presenter. The volume of these tissues was a large proportion of the pollen presenter, for all but *I. cuneatus* in which the greater proportion was made up of polyphenol containing and epidermal cells. For *B. coccinea*, *B. ericifolia*, *D. formosa* and *H. bucculenta* the proportion of these tissues decreased in the region below the cavity even though the volumes of ground tissues increased. For *D. quercifolia* there was no significant difference between regions and for *M. integrifolia* the proportion increased significantly in the region beyond the cavity. This was the only species for which an increase in the proportion of ground tissue directly related to an increase in the volume of this tissue. At the base of the pollen presenter the number of vascular bundles present remained constant for the length of the style for all species.

The style

The tissue distribution at the top of the style resembled that at the base of the pollen presenter. For *B. coccinea* (Plate 4.16) and *M. integrifolia* (Plate 4.30), the presence of transfer tissue ended here. In all except *H. bucculenta*, the transmitting tissue cell number was reduced. In some species polyphenol containing cells were scattered amongst the parenchyma and sclerenchyma (if present), and the proportion of polyphenol containing cells varied amongst the species. The tissues between the top and middle of the style were similar, although in *B. ericifolia* (Plate 4.23) and *D. nana* (Plate 4.13), transfer tissue was much reduced. There was significantly less transmitting tissue proportionally compared to the upper style. This transmitting tissue was surrounded by polyphenol containing cells or ground tissue with walls thickened to varying degrees. At the mid- and lower mid-style transfer tissue was no longer present, and the transmitting tissue was a small area in the

centre of the style. In all *Dryandra* species there was a concentration of small thick walled sclerenchyma cells to the outside of the three to five vascular bundles. The structure of the style of *I. cuneatus* was similar down its length, polyphenol containing cells surrounding a narrow core of transmitting tissue (Plate 4.27). There were no significant changes in tissue distribution at the base of the style compared to the other regions, and ground tissue dominated the structure. Polyphenol containing cells were scattered throughout and starch was present in the cells of some species. The transmitting tissue remained a small area of the stylar tissue.

Quantity of transmitting tissue down the pistil

Transmitting tissue cell number was greatest in the pollen presenter, with a substantial narrowing of the transmitting tissue tract down its length (Table 4.4). The greatest change in cell number for all species studied occurred within the pollen presenter, between its tip and base. The only species for which cell number increased significantly was *H. bucculenta.* For all other species it decreased to under one hundred files of cells at the base of the pollen presenter, regardless of the number in the top of the pollen presenter. A further decrease occurred between the base of the pollen presenter and the top of the style. Transmitting tissue cell numbers stabilised in the stylar region, with few significant changes occurring between regions for most species. For two species however, *D. nana* and *H. bucculenta,* the number of cells increased significantly from the mid-style to the base. Both these species had high transmitting tissue cell numbers in the pollen presenter and showed the most variation in cell number down their style.

There was a significant interaction between species and region of the style ($P<0.0005$) indicating that the pattern of change of transmitting tissue cell number from region to region differed between species. Figure 4.5 indicates however that most variation between species in transmitting tissue cell number occurs within the pollen presenter. In subsequent regions differences in transmitting tissue cell number between species became less significant, many species having similar amounts of transmitting tissue at a given region. The species with the most similarity of cell number between regions was *I. cuneatus*, this

species also had one of the lowest numbers of files of transmitting tissue cells down its style compared to the other species studied.

Pollen grains

Two pollen grain types were observed; biporate, elongated grains (*Banksia* and *Dryandra* species) and triporate, triangular grains (*Hakea*, *Isopogon* and *Macadamia* species).

Pollen of the *Banksia* species; *B. coccinea* (Plate 4.14c,d) and *B. ericifolia* (Plate 4.20d), although biporate differed morphologically. *Banksia coccinea* grains had highly patterned exines and textured germination pores. Pollen grain volume of *B. coccinea* was greater than that of *B. ericifolia* as was the diameter of the germination pore and area of the grain (Table 4.5). The pollen of *B. ericifolia* more closely resembled that of the *Dryandra* species in shape and texture, but its volume was smaller. There was little difference in the appearance of *D. quercifolia* (Plate 4.5c,d) and *D. formosa* (Plate 4.8c,d) pollen, both had pollen grains with slightly patterned, relatively smooth exines and a smooth germination pores. Their pollen volumes were similar. The pollen grains of *Isopogon* (Plate 4.25) and *Hakea* (Plate 4.17c,d), showed similarity in volume, size, and texture, each with three smooth protruding gemination pores and patterned exine. *Isopogon cuneatus* pollen grains were more rounded than those of *H. bucculenta*. The exine of the triangular pollen grain of *M. integrifolia* (Plate 4.28) was relatively smooth and the germination pores protruded only slightly. This species had the smallest grains of those studied. A smooth outer layer over the germination pore was observed for many species and this was breached as the grain germinated.

Pollen grain volume and pollen grain holding capacity of stigmatic groove

A simple division of the approximate volume of the grain into the volume of the cavity showed different theoretical pollen grain holding capacities of the cavity between species (Table 4.5). *Macadamia integrifolia* had the highest theoretical capacity for holding pollen due to the open cavity and small size of the grain. In contrast *B. coccinea* had a

very low capacity due to the enclosed cavity and the large size of the pollen grain. The holding capacity of the cavity of the *Dryandra* species were similar. Although the pollen grain volume of *Isopogon* and *Hakea* were similar, due to the difference in the size and type of the cavity, fewer pollen grains could theoretically be accommodated in the groove of *Hakea* in comparison to the tube of *Isopogon*.

Table 4.2: Tissue volumes (10^3 μm^3 ± s.e.) of pollen presenters of eight proteaceous species in the cavity region and 200 μm below the cavity

Species	Region	Cavity (10^3 μm^3)	Transmitting Tissue (10^3 μm^3)	Transfer Tissue (10^3 μm^3)	Parenchyma. Sclerenchyma and Vasc. Bundles (10^3 μm^3)	Epidermis and Polyphenol containing cells (10^3 μm^3)
B. coccinea	Cavity	[a]30.15±17.53	[a]346.11±69.72	[a]4.19±32.97	[a]246.67±102.91	[a]636.83±87.07
	Below Cavity	-	[b]661.26±107.39	[a]94.24±52.27	[a]833.90±166.91	[a]981.45±356.00
B. ericifolia	Cavity	[a]40.67±15.18	[a]488.54±60.48	[a]138.25±28.55	[a]441.46±89.12	[a]1913.42±75.40
	Below Cavity	-	[b]118.73±93.95	[a]188.89±45.27	[a]845.08±144.55	[b]2986.54±308.30
D. formosa	Cavity	[a]44.99±17.53	[a]168.94±69.72	[a]165.44±32.97	[a]167.81±102.91	[a]401.79±87.07
	Below Cavity	-	[a]111.65±107.39	[b]322.49±52.27	[a]654.47±166.91	[a]747.50±356.00
D. nana	Cavity	[a]40.58±17.53	[a]437.03±69.72	[a]416.11±32.97	[a]730.18±102.91	[a]859.72±87.07
	Below Cavity	-	[a]336.20±126.24	[b]899.36±63.30	[b]2602.51±202.98	[b]2685.34±435.38
D. quercifolia	Cavity	[a]61.75±17.53	[a]310.15±69.72	[a]282.83±32.97	[a]660.11±102.91	[a]451.34±87.07
	Below Cavity	-	[a]94.65±126.24	[a]332.66±63.30	[a]1005.54±202.98	[a]849.15±435.38
H. bucculenta	Cavity	[a]86.72±17.53	[a]580.72±69.72	[a]0.00	[a]225.01±102.91	[a]795.93±87.07
	Below Cavity	-	[b]1414.95±107.39	[a]0.00	[b]918.22±166.91	[a]1795.24±356.00
I. cuneatus	Cavity	[c]266.00±17.53	[a]209.85±69.72	[a]0.00	[a]3.37±102.91	[a]308.07±87.07
	Below Cavity	-	[a]101.43±107.39	[a]27.01±52.27	[a]157.25±166.91	[a]966.66±356.00
M. integrifolia	Cavity	[b]187.82±17.53	[a]564.09±69.73	[a]157.62±32.97	[a]354.78±102.91	[a]743.23±87.07
	Below Cavity	-	[b]266.56±107.39	[a]515.48±52.27	[b]1811.47±166.91	[b]2639.70±356.00
P value		<0.001	<0.001	<0.001	<0.001	<0.001

Numbers with a different letter are significantly different (z>2). Pairwise comparison between species for cavity volume, and between region within a species for other tissue volumes. Variables for which data is unavailable are represented by '-'

Table 4.3: Percentage volume (± s.e.) of each tissue type relative to the total pollen presenter volume of eight proteaceous species in the cavity region and 200 µm below the cavity

Species	Region	Cavity	Transmitting Tissue	Transfer Tissue	Parenchyma, Sclerenchyma and Vasc. Bundles	Epidermis and Polyphenol containing cells
B. coccinea	Cavity	[a]2.53±1.24	[a]23.52±2.62	[a]0.80±1.69	17.42±3.76	[a]56.68±3.71
	Below Cavity	-	[a]27.00±2.20	[a]3.21±1.51	30.98±4.00	[b]35.99±5.05
B. ericifolia	Cavity	[a]1.33±1.07	[b]15.67±2.27	[a]4.63±1.47	12.71±3.26	[a]66.26±3.21
	Below Cavity	-	[a]3.08±1.91	[a]5.29±1.30	26.28±3.53	[a]63.99±4.41
D. formosa	Cavity	[a]5.87±1.24	[a]15.28±2.62	[a]15.39±1.69	16.34±3.76	[a]48.72±3.71
	Below Cavity	-	[a]9.29±2.20	[a]16.92±1.50	29.89±4.00	[a]37.13±5.05
D. nana	Cavity	[a]3.36±1.24	[b]17.34±2.62	[a]15.88±1.69	26.88±3.78	[a]37.06±3.71
	Below Cavity	-	[a]6.69±2.65	[a]13.31±1.66	40.44±4.04	[a]37.67±5.70
D. quercifolia	Cavity	[a]3.51±1.24	[b]16.11±2.62	[b]16.59±1.69	35.69±3.78	[a]28.80±3.71
	Below Cavity	-	[a]7.60±2.65	[a]11.75±1.66	49.25±4.04	[a]29.85±5.70
H. bucculenta	Cavity	[a]6.11±1.24	[a]32.24±2.62	[a]0.00	12.92±3.76	[a]48.11±3.71
	Below Cavity	-	[a]35.81±2.20	[a]0.00	26.48±4.00	[a]40.17±5.05
I. cuneatus	Cavity	[c]35.11±1.24	[b]23.97±2.62	[a]0.00	0.89±3.76	[a]40.24±3.71
	Below Cavity	-	[a]12.02±2.20	[a]1.87±1.51	14.44±4.00	[b]70.02±5.05
M. integrifolia	Cavity	[b]11.60±1.24	[b]30.37±2.62	[a]7.70±1.69	15.64±3.76	[a]36.57±3.71
	Below Cavity	-	[a]5.37±2.20	[b]9.30±1.51	29.20±4.00	[a]47.62±5.05
P value		<0.001	<0.001	<0.001	<0.001	<0.001

Numbers with a different letter are significantly different (z>2). Pairwise comparison between species for cavity volume, and between region within a species for other tissue volumes. Variables for which data is unavailable are represented by '-'. No significant differences were observed between regions for parenchyma, sclerenchyma and vascular bundles.

Table 4.4: Average number of transmitting tissue cells in the pollen presenter and style of eight proteaceous species (mean±s.e.)

Species	Top Pollen Presenter	Bottom Pollen Presenter	Upper Style	Upper mid-Style	Mid-Style	Lower mid-Style	Lower Style	Probability
B. coccinea	$^{e}226.69\pm1.58^{x}$	$^{d}55.83\pm1.58^{v}$	$^{c}31.34\pm1.58^{v}$	$^{b}22.83\pm1.58^{vwx}$	$^{ab}17.97\pm1.58^{uvw}$	$^{ab}16.88\pm1.58^{uvwz}$	$^{a}11.35\pm1.58^{t}$	<0.01
B. ericifolia	$^{c}86.27\pm1.58^{v}$	-	$^{b}26.09\pm1.58^{uv}$	$^{ab}19.37\pm1.58^{uw}$	$^{a}14.56\pm1.58^{tw}$	$^{a}14.30\pm1.58^{tw}$	$^{a}13.15\pm1.58^{t}$	<0.01
D. formosa	$^{d}54.98\pm1.58^{u}$	$^{c}41.27\pm1.58^{u}$	$^{c}34.30\pm1.58^{v}$	$^{b}17.75\pm1.58^{uv}$	$^{ab}12.59\pm1.58^{tu}$	$^{ab}12.72\pm1.58^{tv}$	$^{a}8.30\pm1.58^{t}$	<0.01
D. nana	$^{e}263.28\pm1.58^{y}$	$^{d}69.05\pm1.58^{w}$	$^{c}56.00\pm1.58^{w}$	$^{a}22.88\pm1.58^{vxy}$	$^{a}20.22\pm1.58^{w}$	$^{a}22.41\pm1.58^{xyz}$	$^{b}32.05\pm1.58^{u}$	<0.01
D. quercifolia	$^{c}91.39\pm1.58^{v}$	$^{b}25.34\pm1.58^{t}$	$^{b}23.56\pm1.58^{u}$	$^{a}15.22\pm1.58^{tu}$	$^{a}12.93\pm1.58^{tv}$	$^{a}11.19\pm1.58^{tu}$	$^{a}9.82\pm1.58^{t}$	<0.01
H. bucculenta	$^{c}310.38\pm1.58^{z}$	$^{f}498.20\pm1.58^{x}$	$^{d}89.01\pm1.58^{x}$	$^{c}32.12\pm1.58^{z}$	$^{a}15.53\pm1.58^{uvw}$	$^{a}16.46\pm1.58^{uvwy}$	$^{b}26.22\pm1.58^{u}$	<0.01
I. cuneatus	$^{c}28.90\pm1.58^{t}$	$^{b}21.15\pm1.58^{t}$	$^{a}12.38\pm1.58^{t}$	$^{a}8.80\pm1.58^{t}$	$^{a}8.52\pm1.58^{t}$	$^{a}8.60\pm1.58^{t}$	$^{a}9.95\pm1.58^{t}$	<0.01
M. integrifolia	$^{d}201.76\pm1.58^{w}$	-	$^{c}52.79\pm1.58^{d}$	$^{b}27.08\pm1.58^{xyz}$	$^{a}18.89\pm1.58^{uvw}$	$^{a}16.05\pm1.58^{uvwx}$	$^{a}13.76\pm1.58^{t}$	<0.01
P value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Numbers with a different letter (a-e) are significantly different (z>3), pairwise test between regions within a species. Numbers with a different letter (t-z) are significantly different (z>3). Pairwise test between species within a region of the pollen presenter Variables for which data are unavailable are represented by '-'

Figure 4.5

Number of transmitting tissue cells with distance down the style for eight proteaceous species. Maximum standard error 1.58. Note: upper pp and lower pp are regions of the pollen presenter.

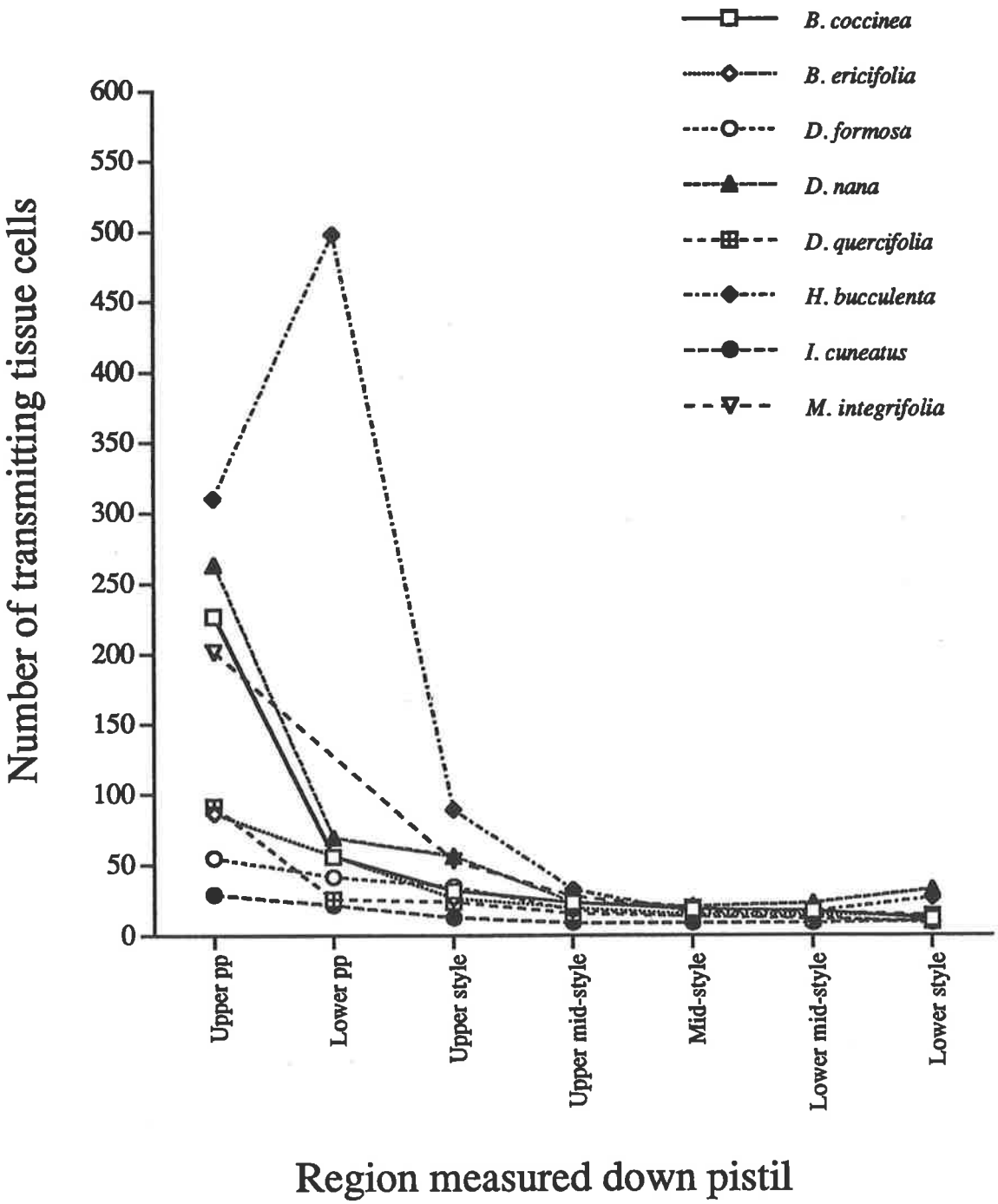

B. coccinea
B. ericifolia
D. formosa
D. nana
D. quercifolia
H. bucculenta
I. cuneatus
M. integrifolia
Number of transmitting tissue cells
600
550
500
450
400
350
300
250
200
150
100
50
0
Upper pp
Lower pp
Upper style
Upper mid-style
Mid-style
Lower mid-style
Lower style
Region measured down pistil

Table 4.5: Pollen grain and stigmatic cavity dimensions and pollen grain holding capacity of stigmatic Cavity for eight proteaceous species

Species	Number of germination pores	Pollen grain volume (10^3 μm^3)	Pollen grain length (μm)	Pollen grain diameter (μm)	Diameter germination pore (μm)	Cavity type & position	Cavity volume (10^3 μm^3)	Maximum # of pollen grains in cavity
B. coccinea	2	14.99±2.49	^{e}89.53±1.78	^{c}26.84±0.71	19.6	enclosed groove, oblique-terminal	^{a}30.15	2.01
B. ericifolia	2	3.42±0.46	^{b}40.38±1.77	^{a}18.74±0.70	12.1	enclosed groove, sub-terminal	^{a}40.67	11.89
D. formosa	2	6.31±1.66	^{c}49.71±1.84	^{b}22.55±0.68	11.62	enclosed groove, oblique terminal	^{a}44.99	7.19
D. nana	2	-	-	-	-	enclosed groove, terminal	^{a}40.58	-
D. quercifolia	2	6.93±0.53	^{cd}56.34±1.75	^{bc}23.95±0.86	13.30	enclosed groove, oblique terminal	^{a}61.75	8.91
H. bucculenta	3	16.23±0.09	^{d}63.02±1.71	-	36.00	enclosed groove, oblique terminal	^{a}86.72	5.34
I. cuneatus	3	18.88±6.05	^{c}49.85±1.72	-	35.34	tube, terminal	^{c}266.00	14.08
M. integrifolia	3	1.35±0.00	^{a}23.72±2.92	-	17.20	papillae protruding, terminal	^{b}187.82	139.13
P value	0.0216	<0.001	<0.001	<0.001	-	-	<0.001	-

Numbers with a different letter are significantly different (z>3), pairwise test between species.

Plate 4.5

Scanning electron micrographs (FESEM) of the pollen presenter and pollen of *Dryandra quercifolia*

(a) Pollen presenter showing longitudinal ridges (arrow) and stigmatic groove (arrowhead). Bar represents 1 mm.

(b) Stigmatic groove showing pollen grains (arrowhead) between groove edges. Bar represents 200 μm.

(c) Crescent-shaped pollen grain showing sculpturing of exine (s). Bar represents 20 μm.

(d) Pollen grain showing germination pore (p). Bar represents 10 μm.

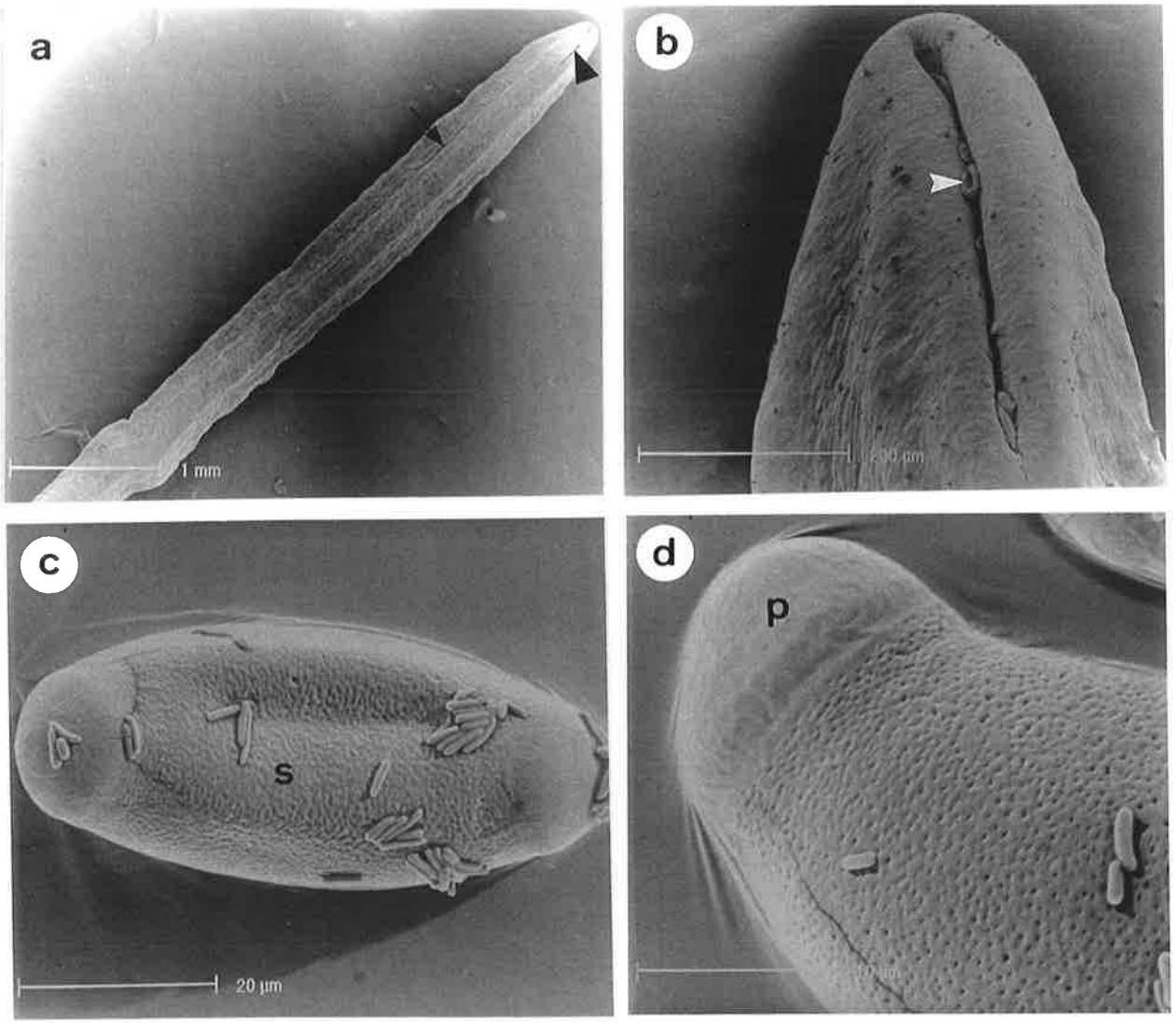

a
1 mm
b
c
s
20 µm
d
p

Plate 4.6

Light micrographs of the pollen presenter of *Dryandra quercifolia* in transverse section stained with PAS and TBO

(a) Tip of the pollen presenter showing the stigmatic groove (g), transfer tissue cell (tt) and stigmatic papillae (arrowhead). Bar represents 50 µm.

(b) Pollen presenter showing stigmatic papillae (arrowhead) consisting of files of cells, transmitting tissue (t) surrounded by transfer cells (r) and a cortex of parenchyma (a) and sclerenchyma (s). Note thick cuticle surrounding pollen presenter (arrow). Bar represents 50 µm.

(c) Stigmatic papillae (arrow) lining stigmatic groove (g) of pollen presenter. Bar represents 20 µm.

(d) Arrangement of cells at the distal end of the pollen presenter. Note transfer cells (r), loose arrangement of sclerenchyma (s) and parenchyma (p) and thick walled epidermal cells (e) surrounded by a thick cuticle (arrow). Bar represents 50 µm.

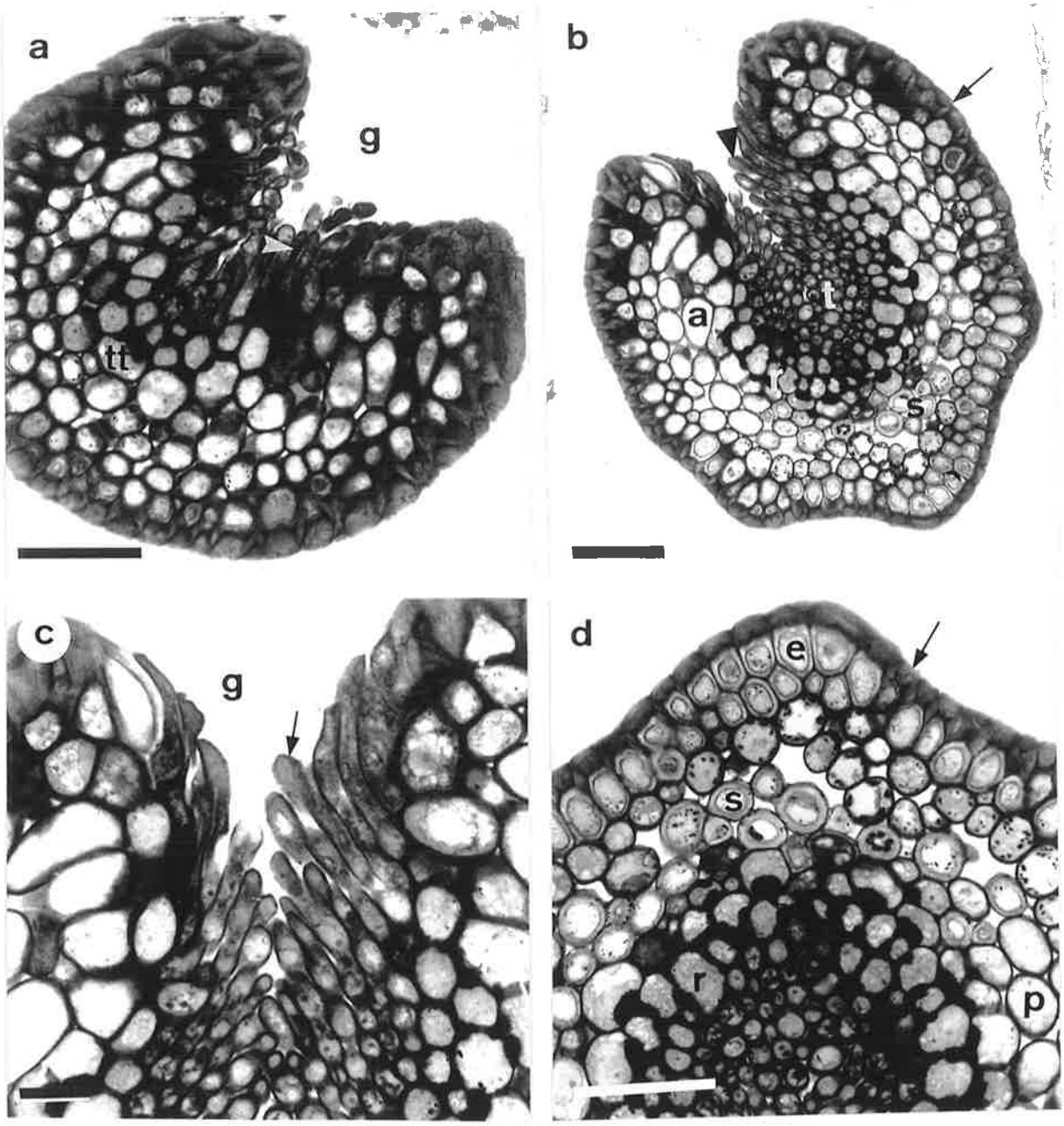

a
g
tt
b
a
t
r
s
c
g
d
e
s
r
p

Plate 4.7

Light micrographs of the pollen presenter, and style down its length of *Dryandra quercifolia* in transverse section stained with PAS and TBO

(a) Pollen presenter at its base showing ridges (arrow) comprised of polyphenol containing epidermal cells (e), cortex of sclerenchyma tissue (c) and three layers of transfer tissue (r) surrounding transmitting tissue (t). Bar represents 200 µm.

(b) Upper style showing transmitting tissue (t) surrounded by two to three layers of transfer tissue (r). Note stomatal pore (arrow). Bar represents 100 µm.

(c) Upper mid-style showing transmitting tissue (arrowhead), cortical cells containing starch grains (c) and polyphenol containing cells associated with five vascular bundles (v) and epidermal layer (e). Bar represents 100µm.

(d) Mid-style showing transmitting tissue (arrowhead) and heavily lignified sclerenchyma cells of cortex (c). Bar represents 100 µm.

(e) Lower mid-style showing transmitting tissue (arrowhead) and rounded cells of cortex (c). Bar represents 200 µm.

(f) Lower style showing transmitting tissue (arrowhead). Bar represents 200 µm.

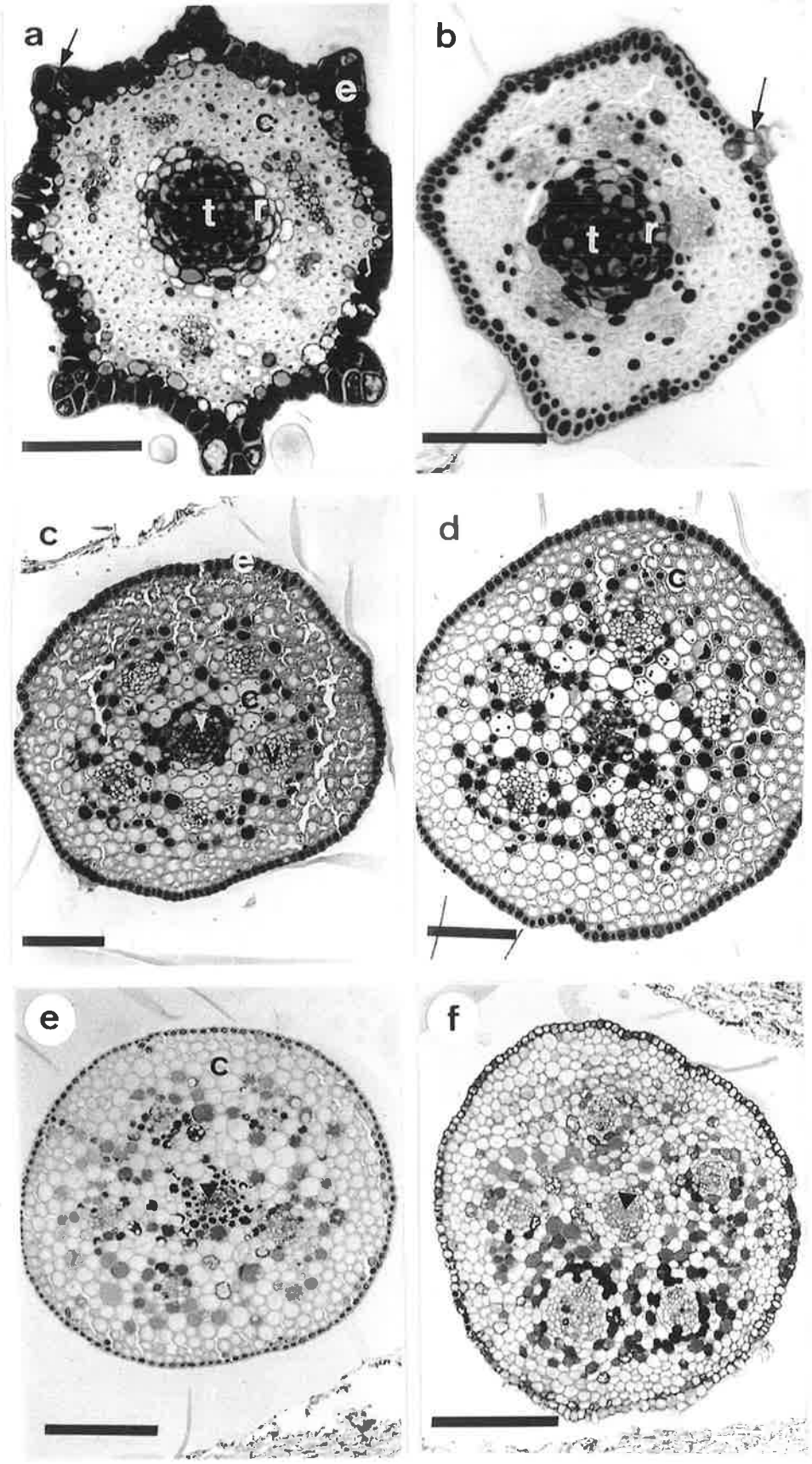


Plate 4.8

Scanning electron micrographs (FESEM) of the pollen presenter and pollen of *Dryandra formosa*

(a) Pollen presenter showing stigmatic ridges (arrow) and stigmatic groove (arrowhead). Bar represents 500 μm.

(b) Closed stigmatic groove (arrowhead) showing pronounced stigmatic lips (l). Bar represents 100 μm.

(c) Crescent-shaped pollen grain showing light sculpturing of exine (s). Bar represents 10 μm.

(d) Pollen grain showing germination pore (p). Bar represents 5 μm.

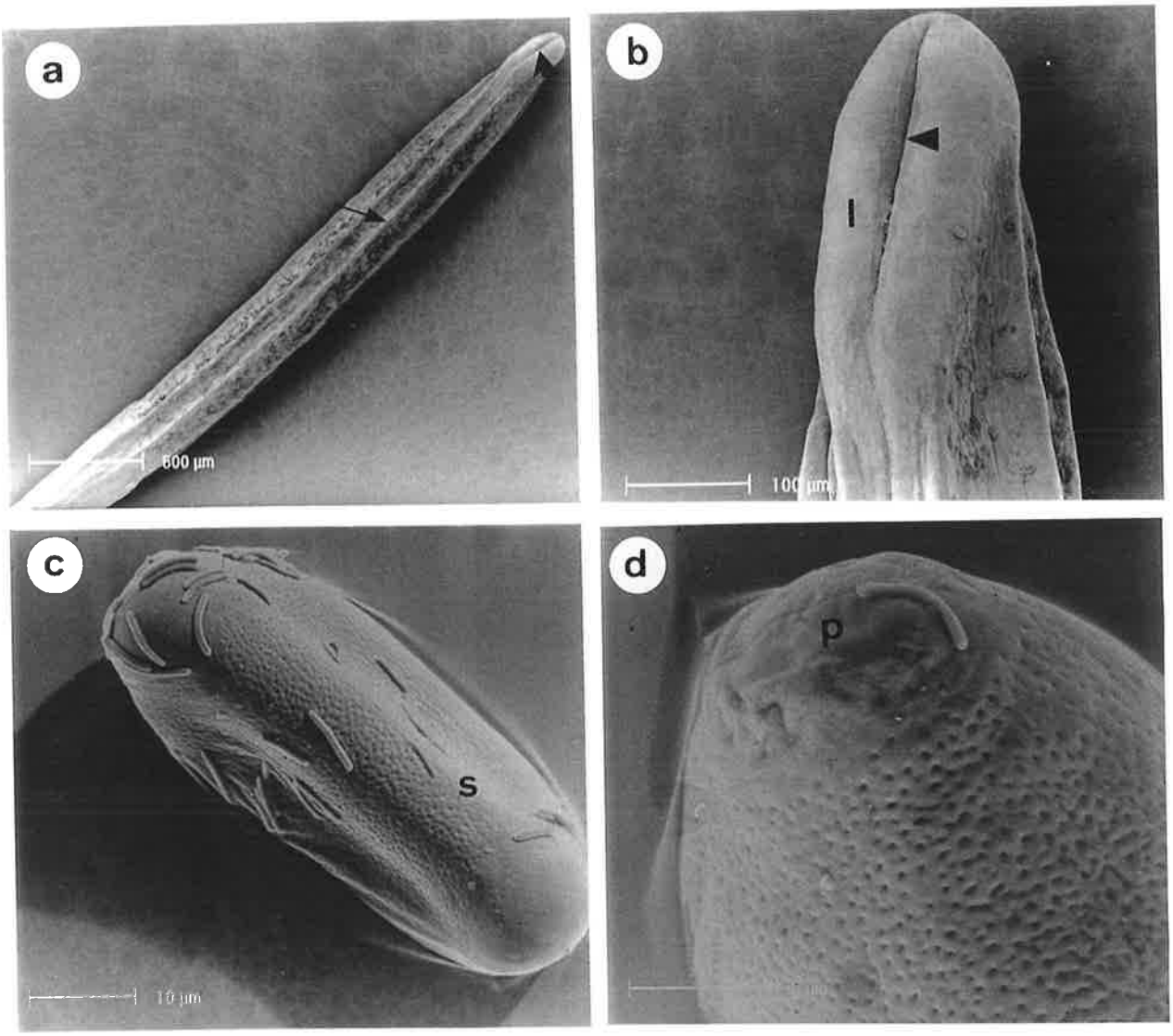

a
600 µm
b
l
100 µm
c
s
10 µm
d
p

Plate 4.9

Light micrographs of the pollen presenter of *Dryandra formosa* in transverse section stained with PAS and TBO

(a) Pollen presenter sectioned near its tip showing stigmatic groove (g) and polyphenol containing cells (p). Bar represents 50 µm.

(b) Pollen presenter showing stigmatic lips (arrow), transmitting tissue (t), transfer tissue (r) and stigmatic ridges (arrowhead). Bar represents 50 µm.

(c) Pollen presenter below stigmatic groove showing stigmatic ridges (arrowhead), vascular bundles (v), central core of transmitting tissue (t) surrounded by transfer tissue (r) and sclerenchyma tissue (s). Note also the pollen grains associated with the pollen presenter (p). Bar represents 50 µm.

(d) Portion of the pollen presenter showing anther locule contents surrounding pollen (a) associated with the outer surface of the pollen presenter. Bar represents 20 µm.

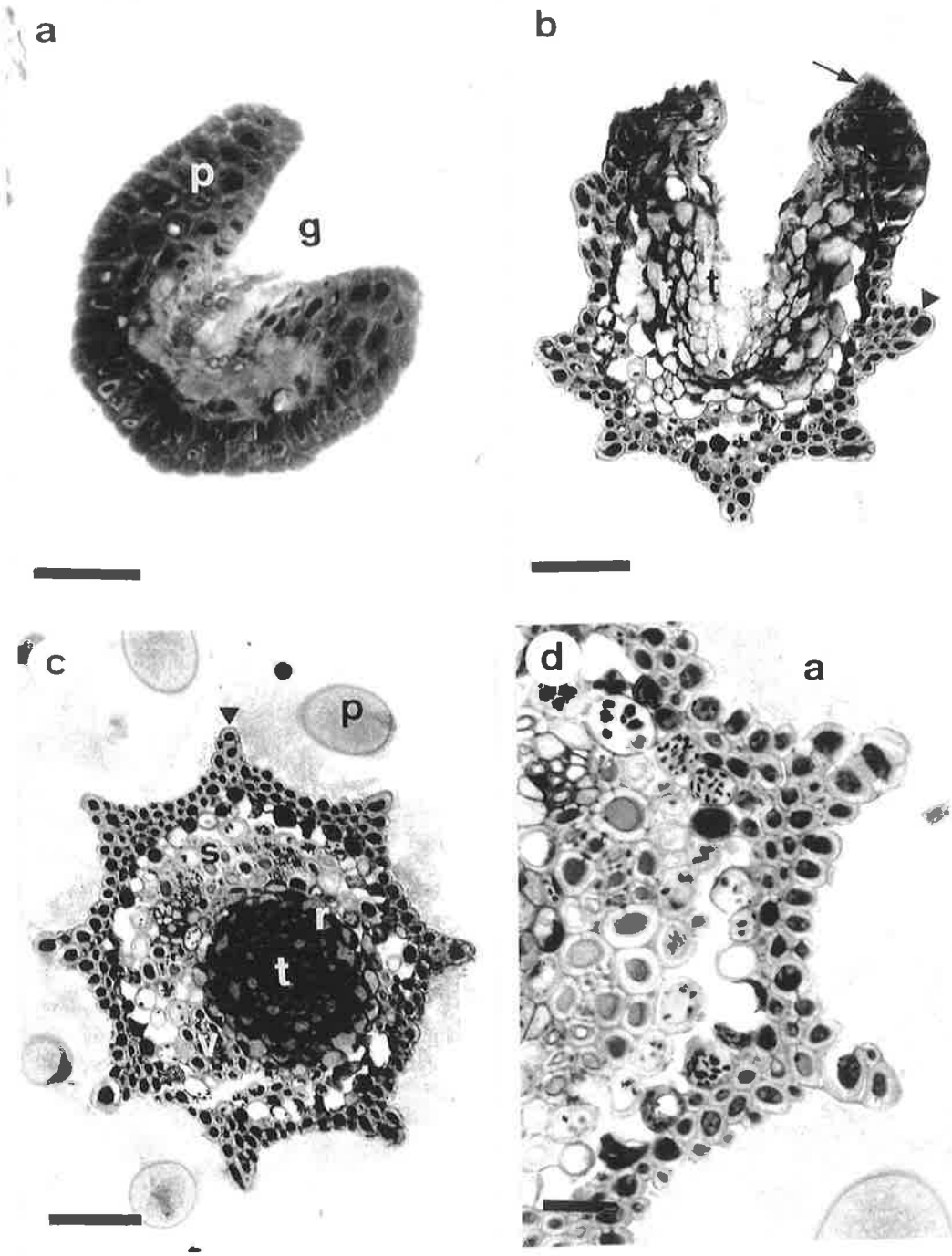

a
p
g
b
t
c
p
s
r
t
v
d
a

Plate 4.10

Light micrographs of the style down its length, of *Dryandra formosa* in transverse section stained with PAS and TBO

(a) Upper style showing inner core of transmitting tissue (t) surrounded by three layers of transfer tissue cells (r). Note thickened walls of cortical cells (c). Bar represents 200 µm.

(b) Upper mid-style showing transmitting tissue (arrowhead). Bar represents 200 µm.

(c) Mid-style showing transmitting tissue (arrowhead) and five vascular bundles (v). Bar represents 200 µm.

(d) Lower mid-style showing transmitting tissue (arrowhead). Bar represents 200 µm.

(e) Lower style showing transmitting tissue (arrowhead). Bar represents 100 µm.

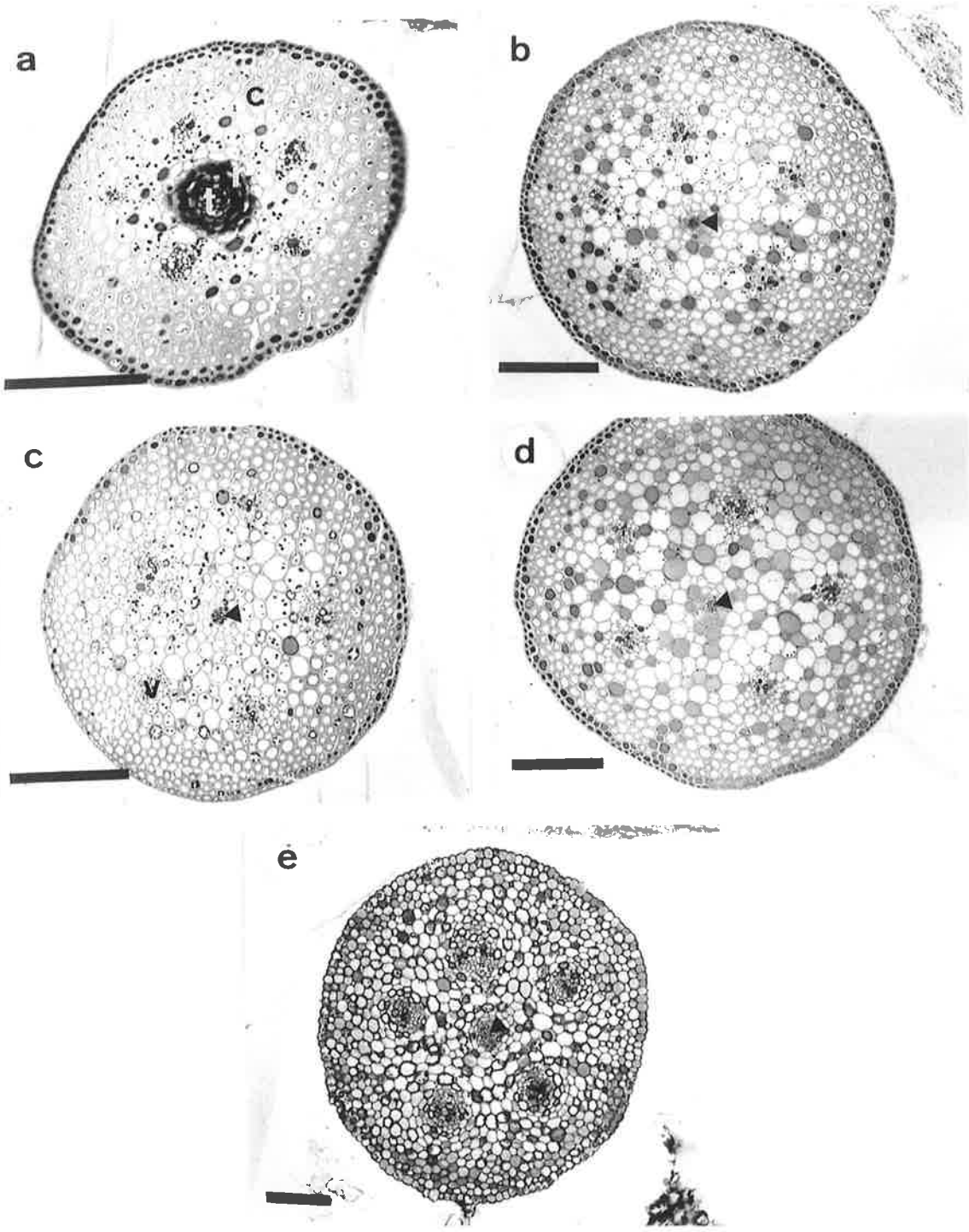

a
c
t
b
c
v
d
e

Plate 4.11

Scanning electron micrographs (FESEM) of the pollen presenter of *Dryandra nana*

(a) Pollen presenter showing ridges (arrow) and terminal stigmatic groove (arrowhead). Bar represents 1 mm.

(b) Terminal stigmatic groove (arrow). Bar represents 200 µm.

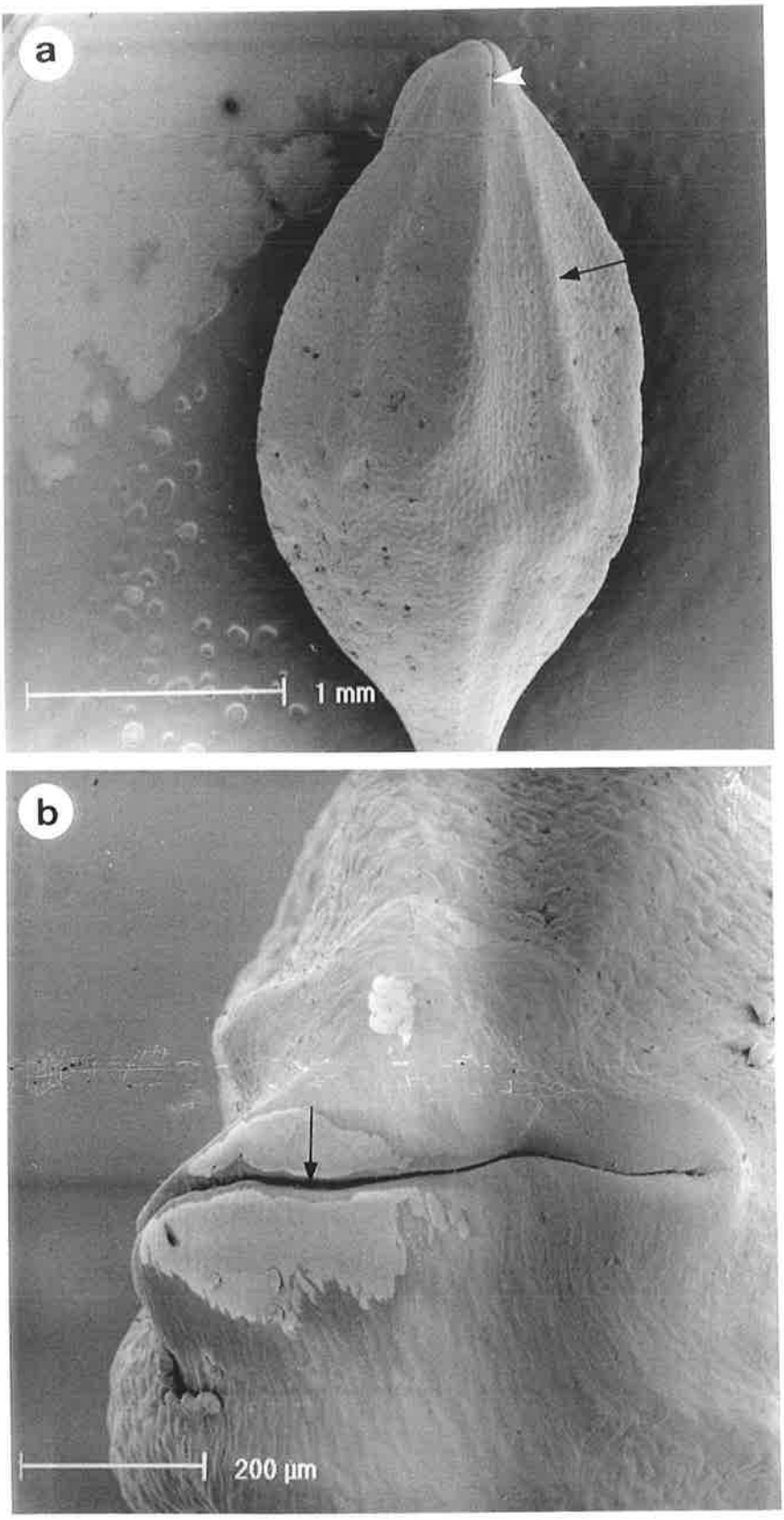

a
1 mm
b
200 µm

Plate 4.12

Light micrographs of the pollen presenter of *Dryandra nana* in transverse section stained with PAS and TBO

(a) Tip of the pollen presenter showing the two halves of tissue (arrows) surrounding the terminal stigmatic groove. Note the transmitting tissue (t) and pollen grains (p) within the groove. Bar represents 50 μm.

(b) Pollen presenter further down its length showing the joining of the transmitting tissue at its centre (t), transfer tissue (r) and polyphenol containing cells (p). Bar represents 50 μm.

(c) Pollen presenter at the base of the groove showing sclerenchyma (s), polyphenol containing cells (p), transmitting tissue (t) and transfer tissue (r). Bar represents 100 μm.

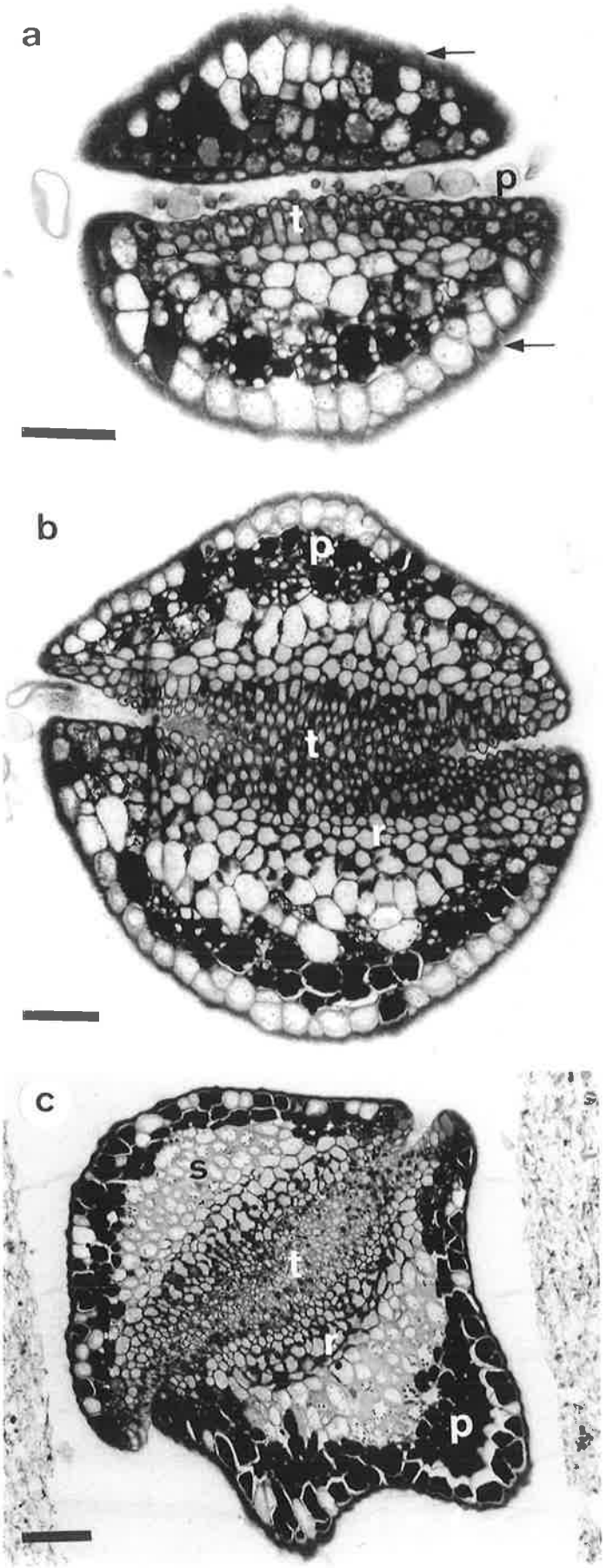

a
p
t
b
p
t
r
c
s
t
r
p

Plate 4.13

Light micrographs of the pollen presenter, and style down its length of *Dryandra nana* in transverse section stained with PAS and TBO

(a) Pollen presenter below stigmatic groove showing central core of transmitting tissue (t) surrounded by layers of transfer tissue (r) and heavily lignified cortex (c) with outer layers containing polyphenols (p), including epidermis. Bar represents 200 μm.

(b) Upper style showing polyphenol containing cells (arrowheads) associated with central core of transmitting tissue (t), five vascular bundles (v) and the outer tissues (p). Bar represents 100 μm.

(c) Upper mid-style showing central core of transmitting tissue (arrowhead) and cortex (c) with scattered polyphenol containing cells (dark staining). Bar represents 100 μm.

(d) Mid-style showing central core of transmitting tissue cells (arrowhead). Bar represents 100 μm.

(e) Lower mid-style showing central core of transmitting tissue (arrowhead). Bar represents 200 μm.

(f) Lower style showing transmitting tissue (arrowhead) and cortex (c). Bar represents 200 μm.

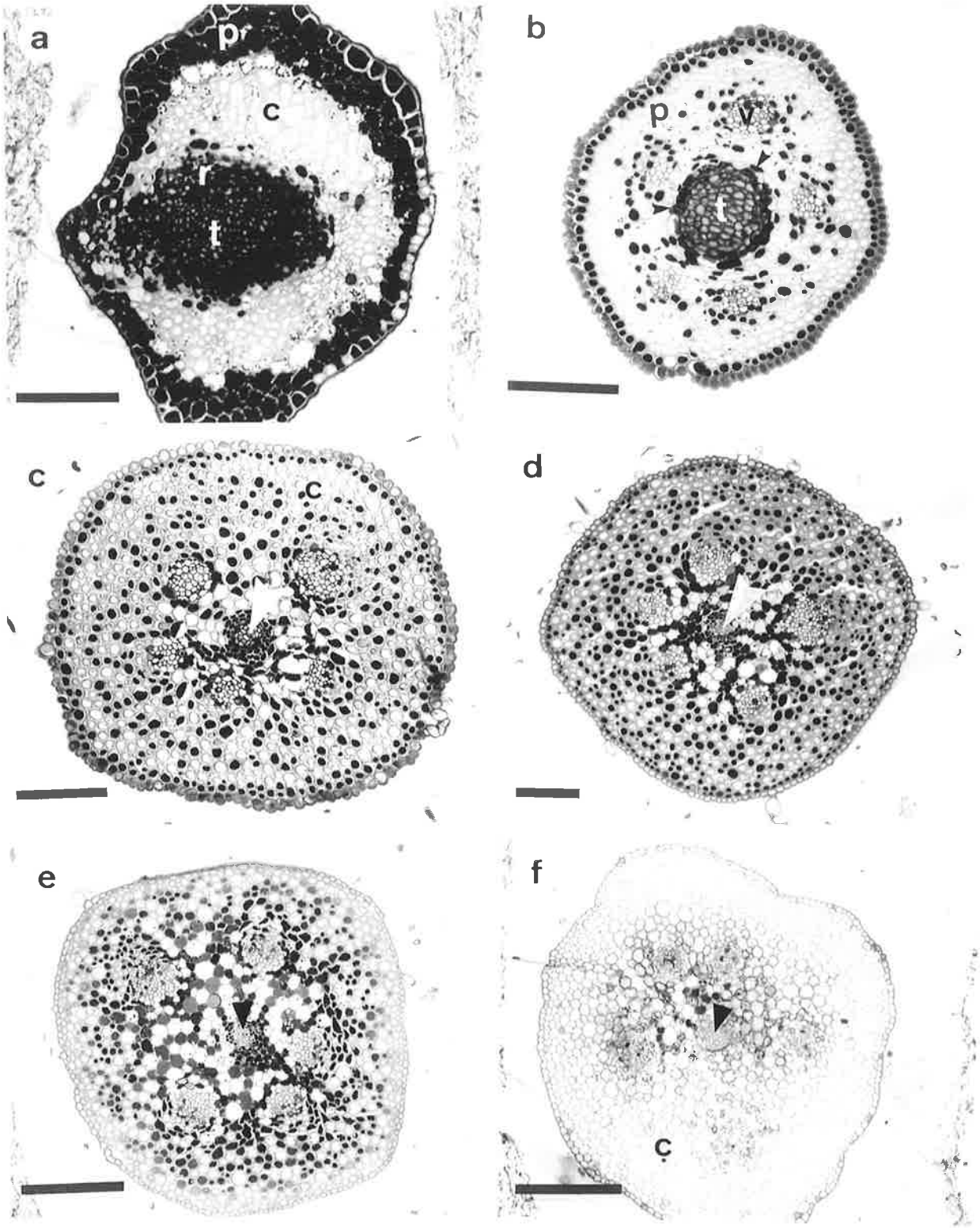

a
pr
c
r
t
b
p
v
t
c
c
d
e
f
c

Plate 4.14

Scanning electron micrographs (FESEM) of the pollen presenter and pollen of *Banksia coccinea*

(a) Pollen presenter showing stigmatic groove (arrowhead), collar (c) and neck (n). Bar represents 500 μm.

(b) Pollen presenter showing open stigmatic groove (arrowhead). Bar represents 100 μm.

(c) Pollen grain showing heavy sculpturing of exine (s). Bar represents 20 μm.

(d) Pollen grain showing germination pore (p). Bar represents 10 μm.

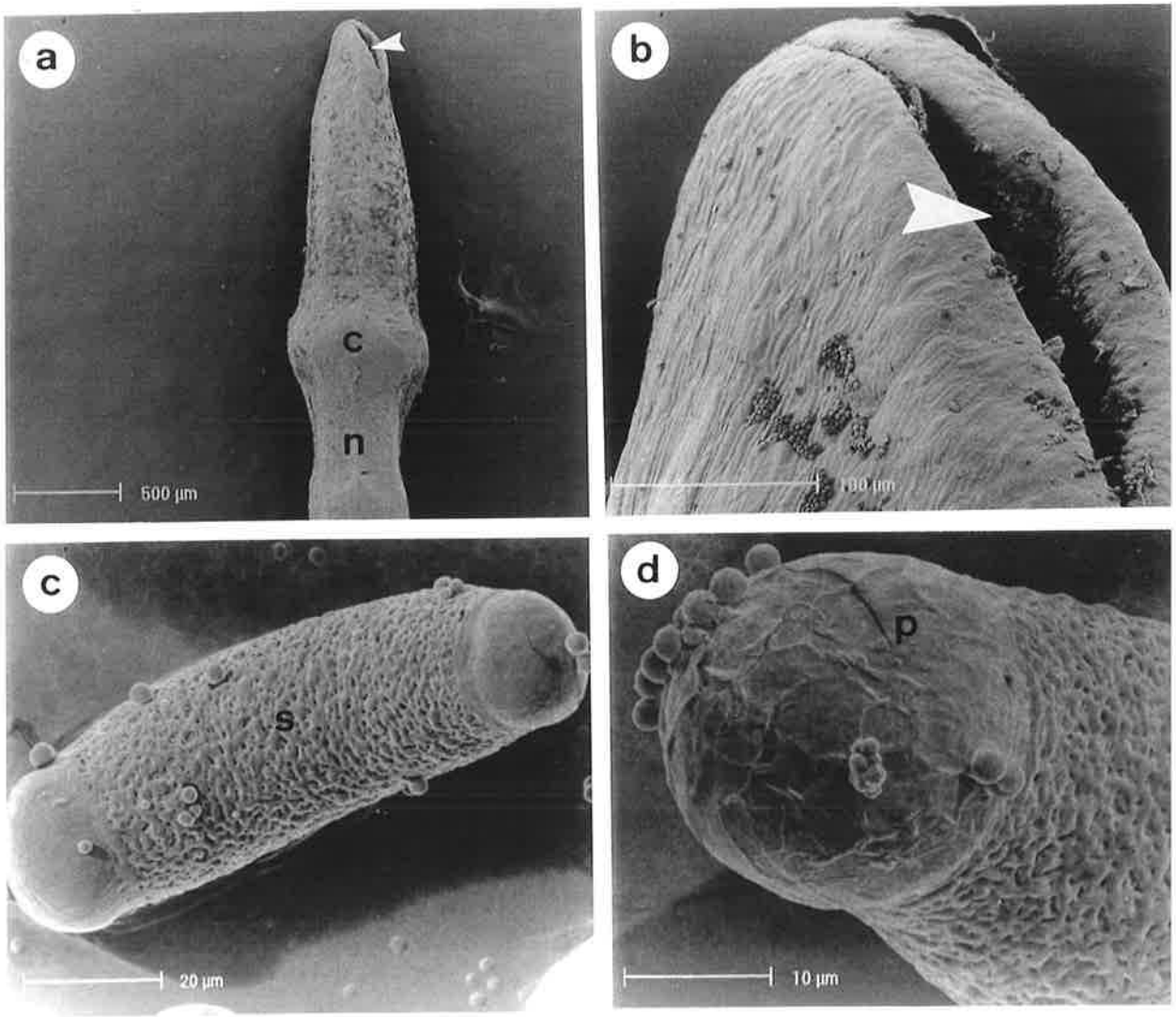

a
c
n
500 µm
b
c
s
20 µm
d
p
10 µm

Plate 4.15

Light micrographs of transverse sections of the pollen presenter of *Banksia coccinea* stained with PAS and TBO

(a) Pollen presenter at the tip of the stigmatic groove region showing the stigmatic groove (g), elongated stigmatic papillae cells (s) and polyphenol containing cells (p). Bar represents 50 μm.

(b) Pollen presenter at the stigmatic groove region showing reduction in the diameter (arrow) of the groove and rounded transmitting tissue cells (t). Bar represents 50 μm.

(c) Pollen presenter below the stigmatic groove showing transmitting tissue (t) and sclerenchyma (s). Bar represents 100 μm.

(d) Pollen presenter at the base just above the style showing vascular bundles (arrow), central core of transmitting tissue (t), transfer tissue (arrowhead), polyphenol containing cells (p) and cortex of sclerenchyma and parenchyma (s). Bar represents 200 μm.

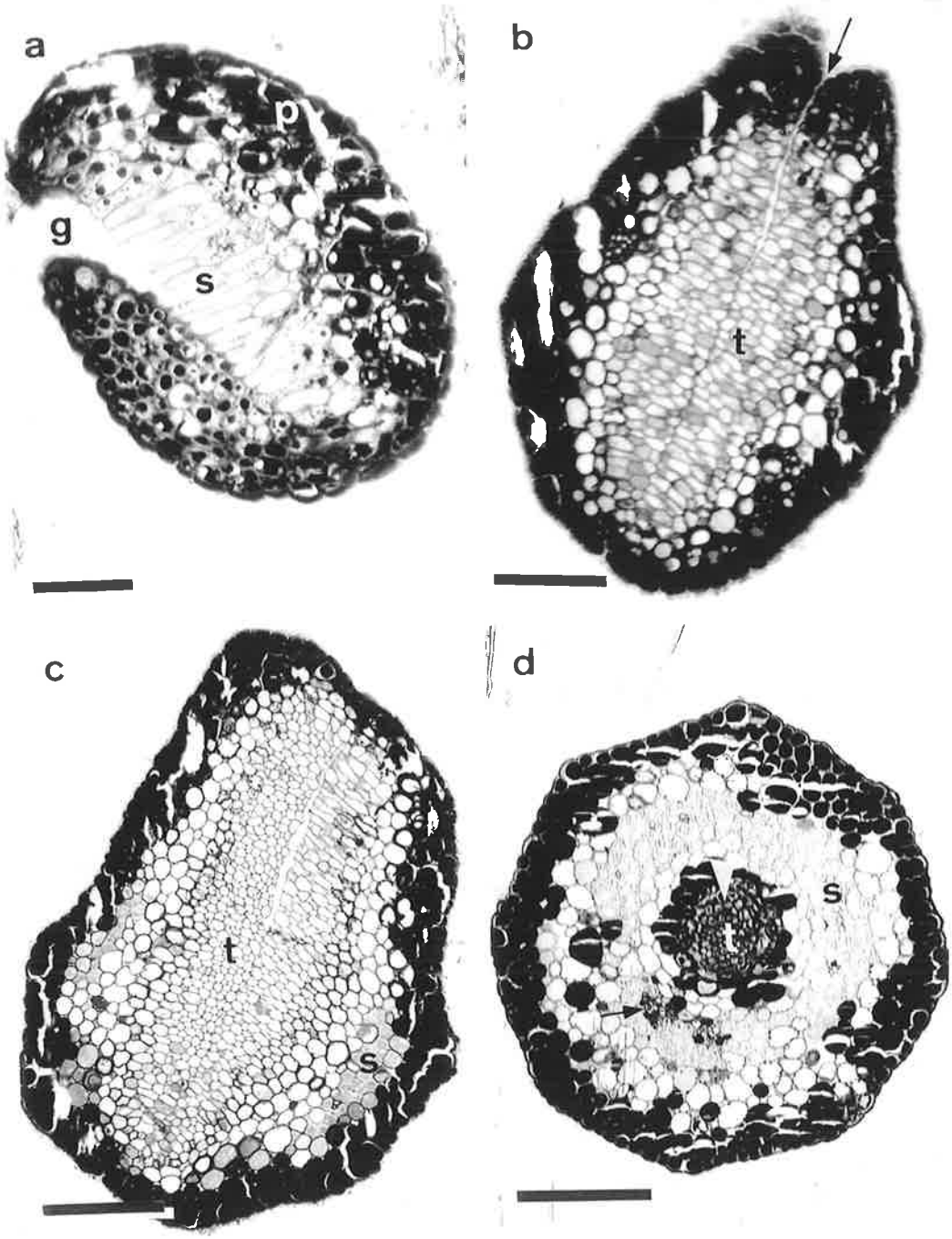

a
p
g
s
b
t
c
t
s
d
s

Plate 4.16

Light micrographs of the style down its length, of *Banksia coccinea* in transverse section stained with PAS and TBO

(a) Upper style showing central core of transmitting tissue (t) surrounded by transfer tissue (r). Note inner and outer layers of polyphenol containing cells (p), vascular bundles (v), and cortex of parenchyma (x) and sclerenchyma (s). Bar represents 100 µm.

(b) Upper mid-style showing reduced core of transmitting tissue (arrowhead), multiple vascular bundles (v) concentration of sclerenchyma outside the ring of vascular tissue and a single layer of epidermal cells containing polyphenols (e). Bar represents 200 µm.

(c) Mid-style showing large cortical area (s) of parenchyma and sclerenchyma cells and reduced area of transmitting tissue (arrowhead). Bar represents 200 µm.

(d) Lower mid-style showing transmitting tissue (arrowhead). Bar represents 200 µm.

(e) Lower style showing transmitting tissue (arrowhead) and epidermal layer containing polyphenols (u). Bar represents 100 µm.

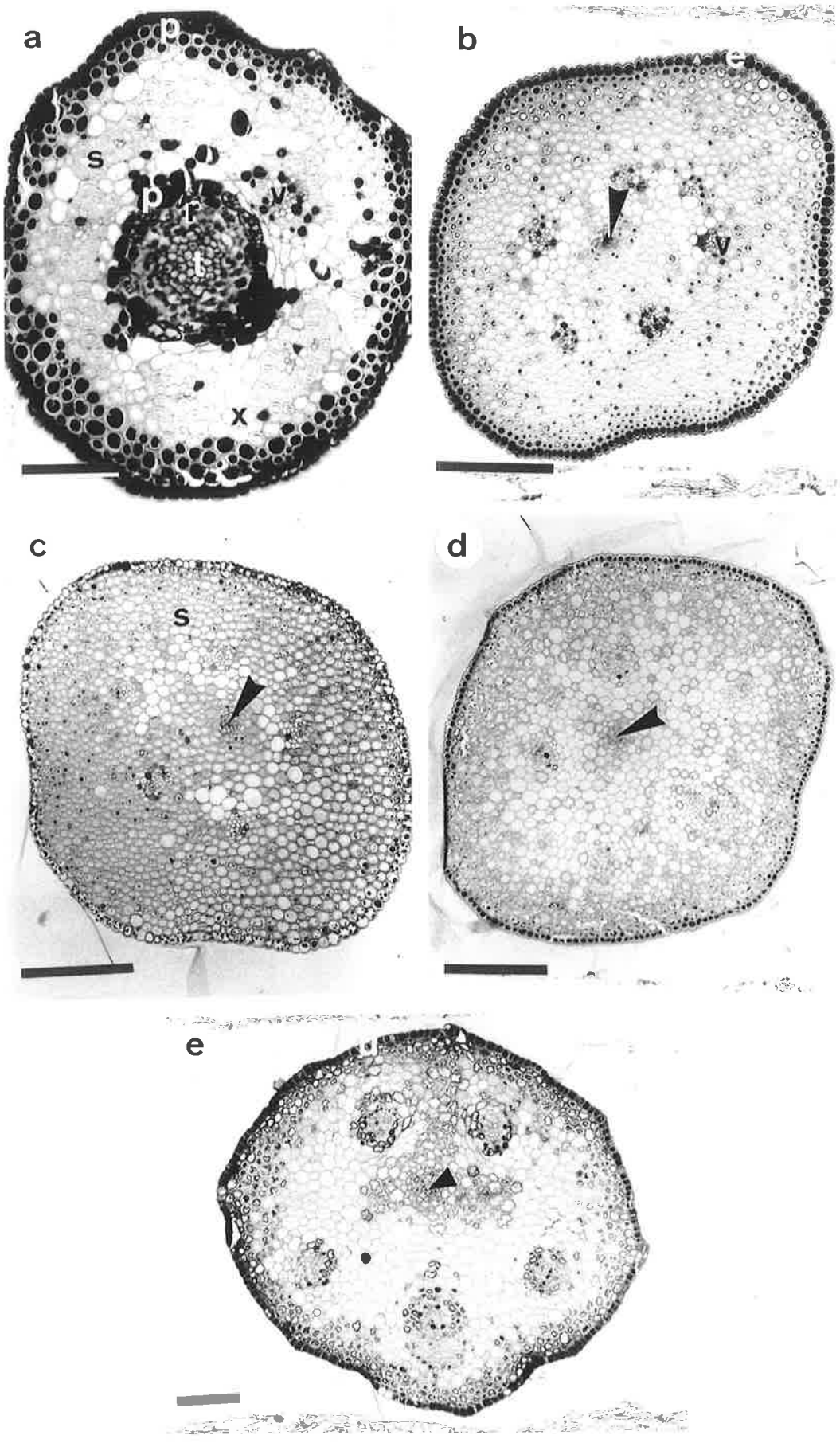

a
p
s
p
r
v
t
x
b
e
v
c
s
d
e

Plate 4.17

Scanning electron micrographs (FESEM) of the pollen presenter and pollen grains of *Hakea bucculenta*

(a) Pollen presenter showing stigmatic groove (arrowhead) and collar (c) and neck (n). Bar represents 500 μm.

(b) Open stigmatic groove (s) showing pollen grains and debris within (arrow). Bar represents 100 μm.

(c) Triangular pollen grains with highly sculptured exines (e). Bar represents 50 μm.

(d) Pollen grain showing one of the three germination pores (p). Bar represents 10 μm.

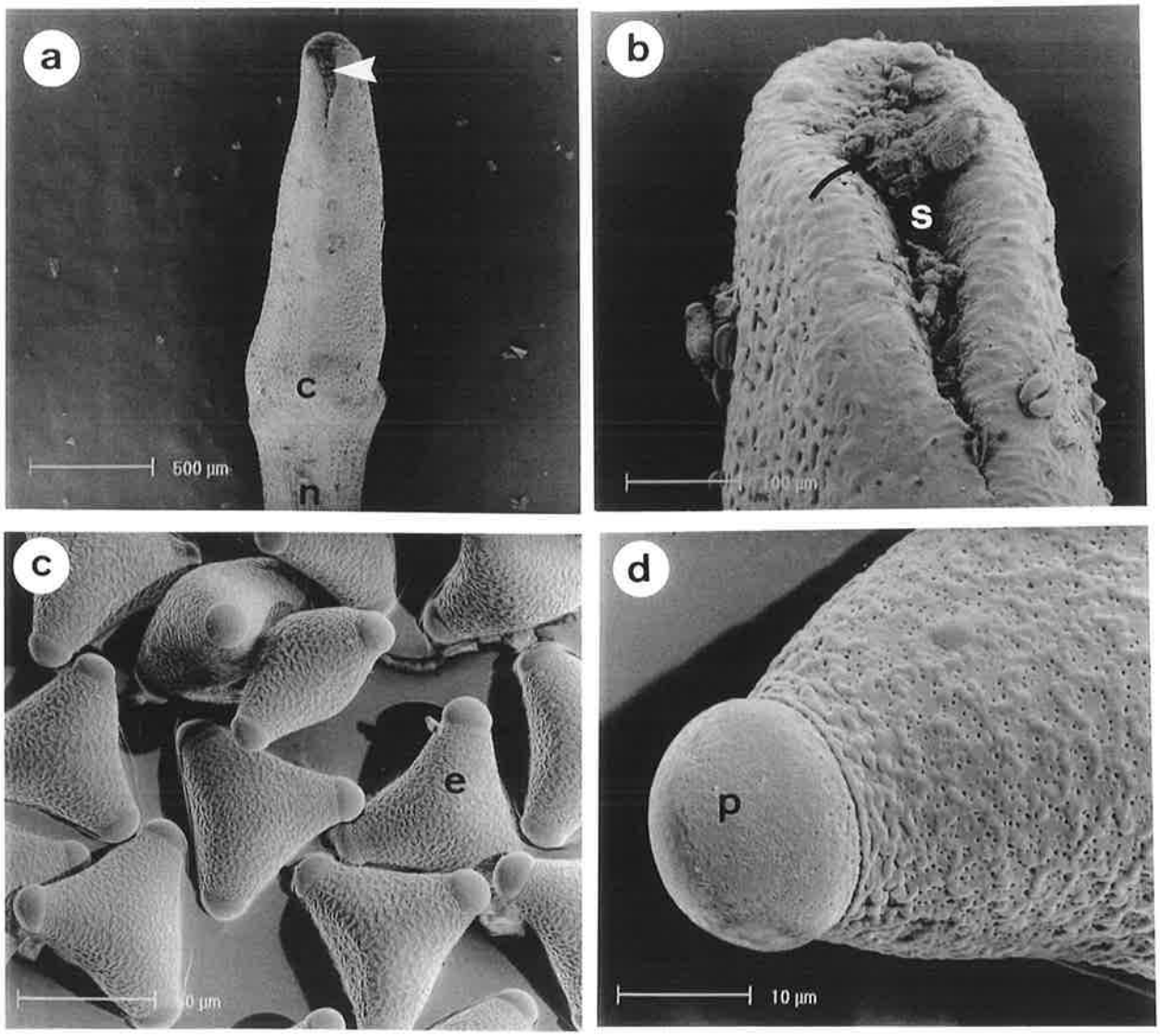

a
c
n
500 µm
b
s
100 µm
c
e
d
p
10 µm

Plate 4.18

Light micrographs of the pollen presenter of *Hakea bucculenta* in transverse section stained with PAS and TBO

(a) Pollen presenter at the stigmatic groove (g) region. Note layer of polyphenol containing cells (x) near the epidermis, transmitting tissue (t) and vascular bundle (v). Bar represents 100 µm.

(b) Pollen presenter below stigmatic groove. Note cortex of thin walled parenchyma (p) cells surrounding transmitting tissue (t). Bar represents 100 µm.

(c) Loosely packed transmitting tissue (t) within the pollen presenter below the stigmatic groove. Bar represents 50 µm.

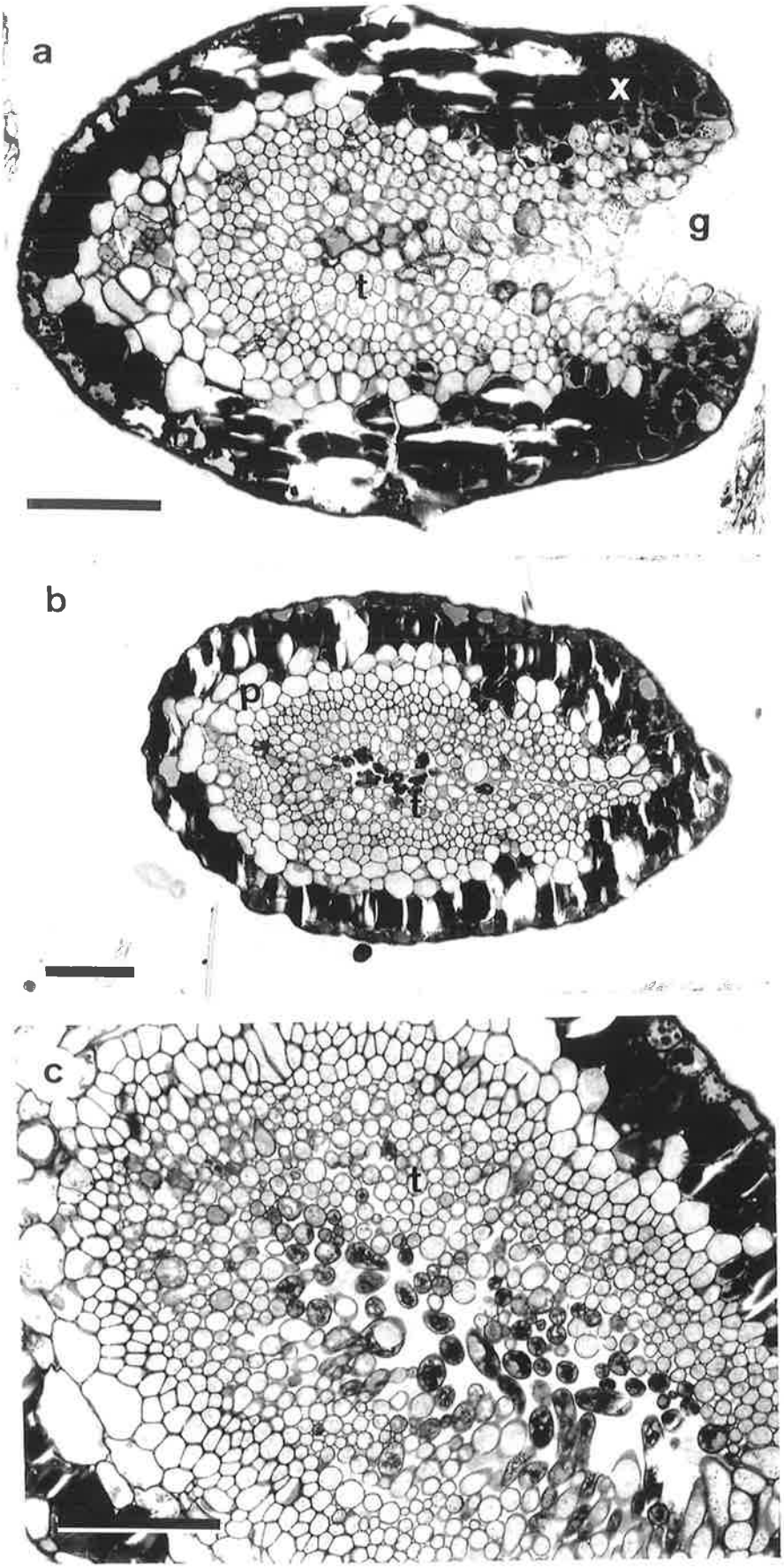

a
x
g
t
b
p
t
c
t

Plate 4.19

Light micrographs of the style down its length, of *Hakea bucculenta* in transverse section stained with PAS and TBO

(a) Upper style showing outer layers of cortex containing polyphenols (e), including epidermis, inner cortex of large parenchyma cells (x) and three vascular bundles (v) and inner core of transmitting tissue (t). Bar represents 200 µm.

(b) Upper mid style showing transmitting tissue (arrowhead) and five vascular bundles (v). Bar represents 100 µm.

(c) Upper mid-style showing reduced transmitting tissue (arrowhead), cortex (x) and outer two rows of cells containing polyphenols (p). Bar represents 100 µm.

(d) Lower mid-style showing transmitting tissue (arrowhead). Bar represents 200 µm.

(e) Lower style showing outer layers of polyphenol containing cells (p) and transmitting tissue (arrowhead). Bar represents 200 µm.

(f) Lower style showing polyphenol containing cells (arrowhead) associated with transmitting tissue. Bar represents 200 µm.

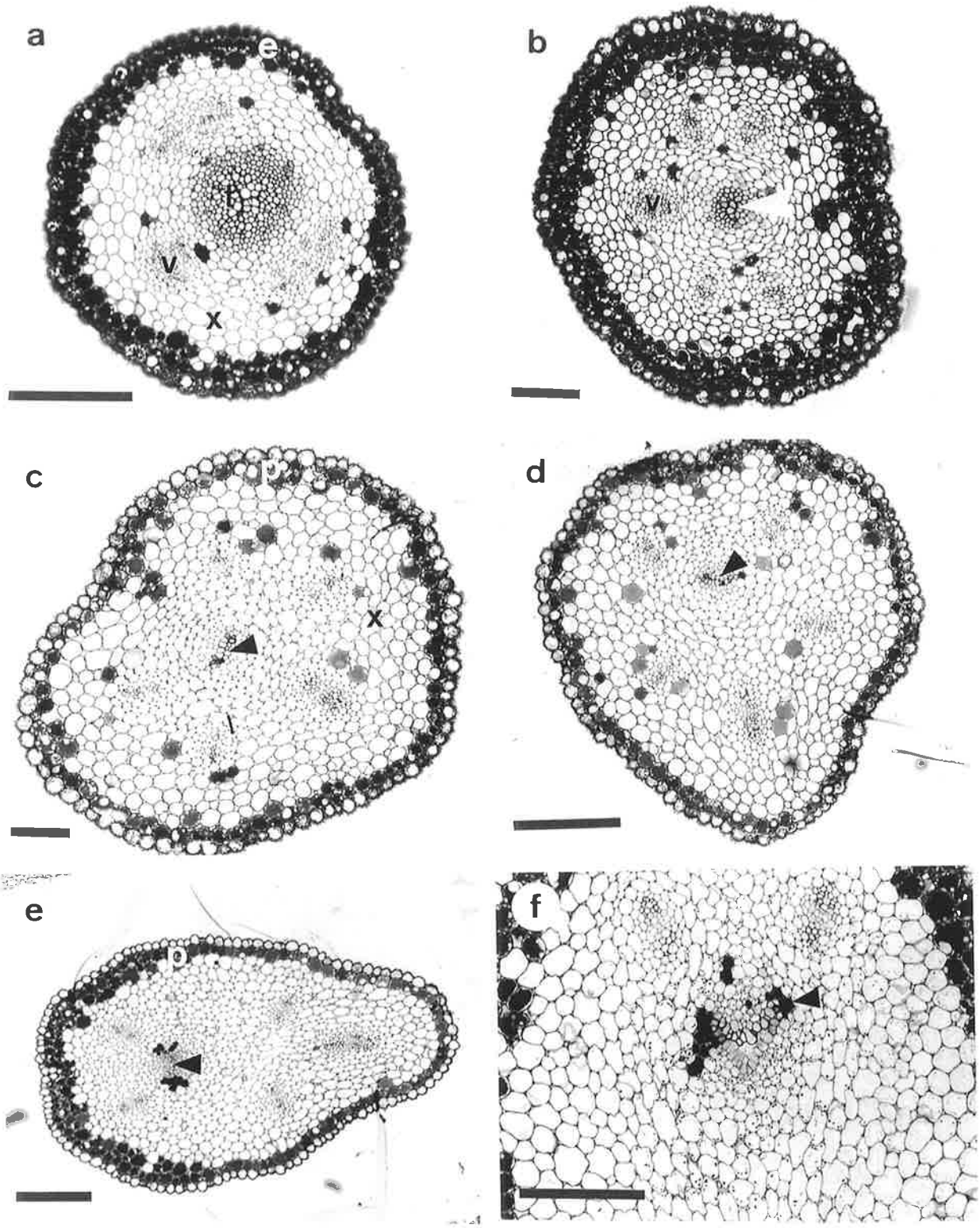

a
e
p
v
x
b
v
c
p
x
d
e
p
f

Plate 4.20

Scanning electron micrographs (FESEM) of the pollen presenter and pollen grains of *Banksia ericifolia*

(a) Pollen presenter showing the sub-terminal stigmatic groove (arrowhead). Bar represents 200 μm.

(b) Side view of a pollen presenter showing the protruding lips of the stigmatic groove (arrowhead). Bar represents 200 μm.

(c) Pollen presenter showing an open stigmatic groove (arrowhead) and the distribution of pollen grains (p). Bar represents 100 μm.

(d) Crescent-shaped pollen grain showing light sculpturing of exine (e) and germination pore (p). Bar represents 10 μm.

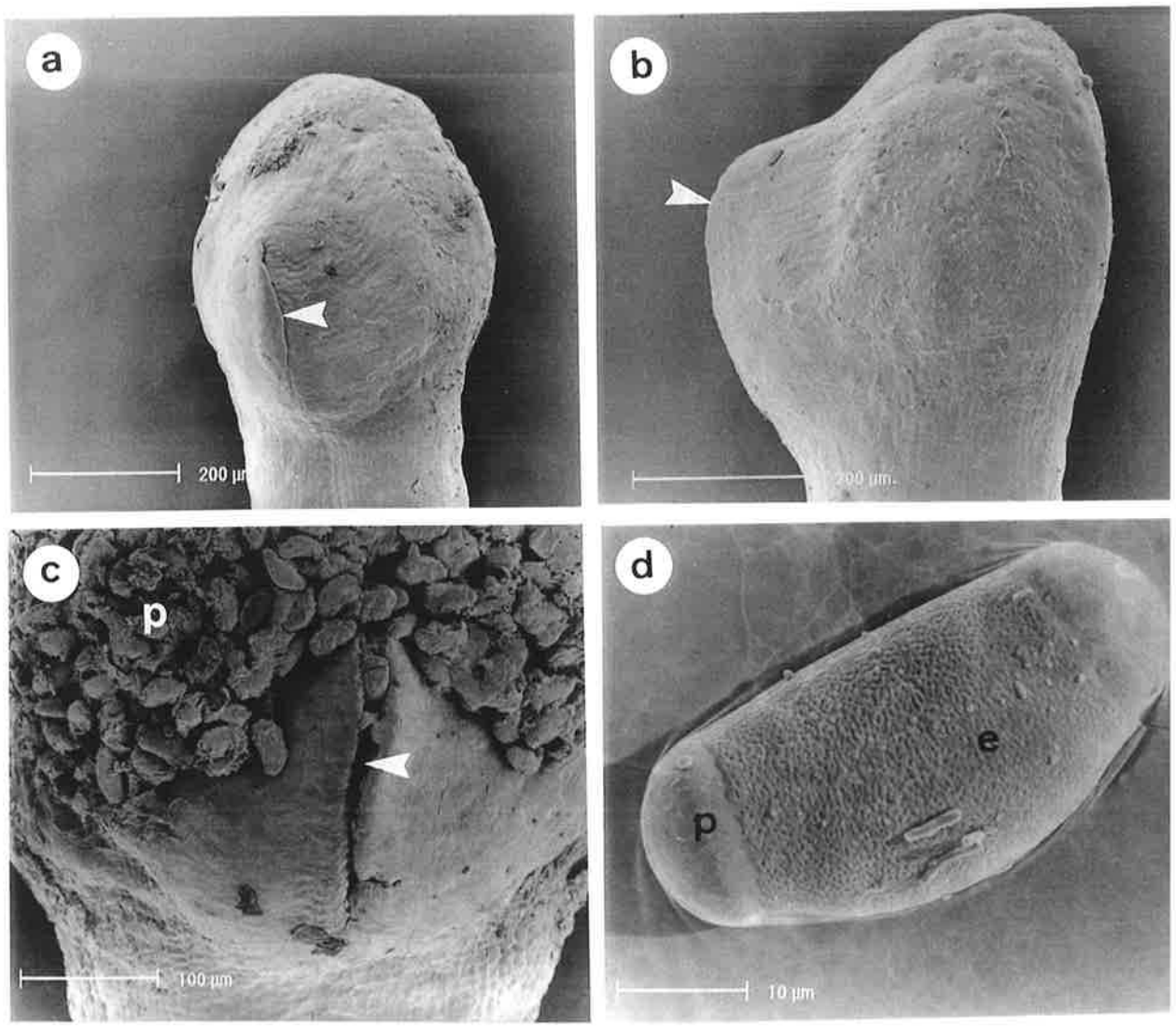

a
200 µm
b
200 µm
c
p
100 µm
d
e
p
10 µm

Plate 4.21

Three dimensional (3D) reconstruction of the pollen presenter of *Banksia ericifolia*. Bar represents 200 μm.

(a) Pollen presenter in front view showing the stigmatic groove (arrow).

(b) Pollen presenter in side view. Note stigmatic groove (arrow).

(c) Pollen presenter in front view. Note stigmatic groove (arrow).

(d) Pollen presenter in side view. Note protruding lips of stigmatic groove (arrow).

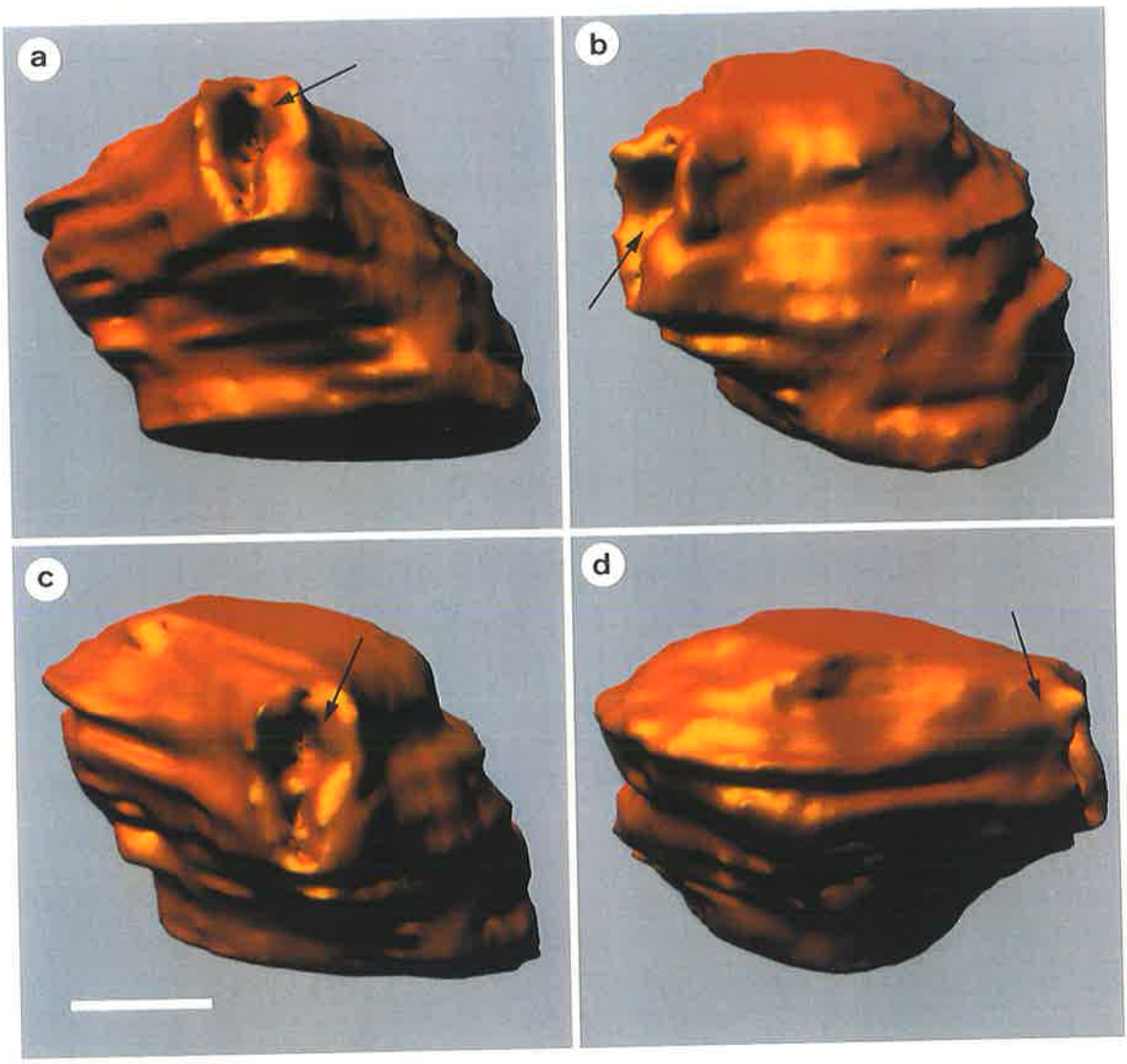

a
b
c
d

Plate 4.22

Light micrographs of the pollen presenter of *Banksia ericifolia* in transverse section stained with PAS and TBO

(a) Pollen presenter showing stigmatic groove (arrow), transmitting tissue (t), sclerenchyma (s) and parenchyma (p) cells containing polyphenols. Bar represents 100 µm.

(b) Stigmatic groove of pollen presenter (g) showing a pollen grain (p) germinating and growing into the transmitting tissue. Bar represents 50 µm.

(c) Pollen presenter at the lower extreme of the stigmatic groove showing a central core of transmitting tissue (t) beginning to form surrounded by transfer cells (r). Note the uneven thickness of the cell walls that is characteristic of transfer cells. Bar represents 100 µm.

(d) Pollen presenter below the stigmatic groove showing enlarged epidermal cells which give the bulbous form of the pollen presenter its shape (b). Note also the central core of transmitting tissue (t), transfer cells (r), layer of sclerenchyma (s) and starch containing (arrow) parenchyma cells Bar represents 100 µm.

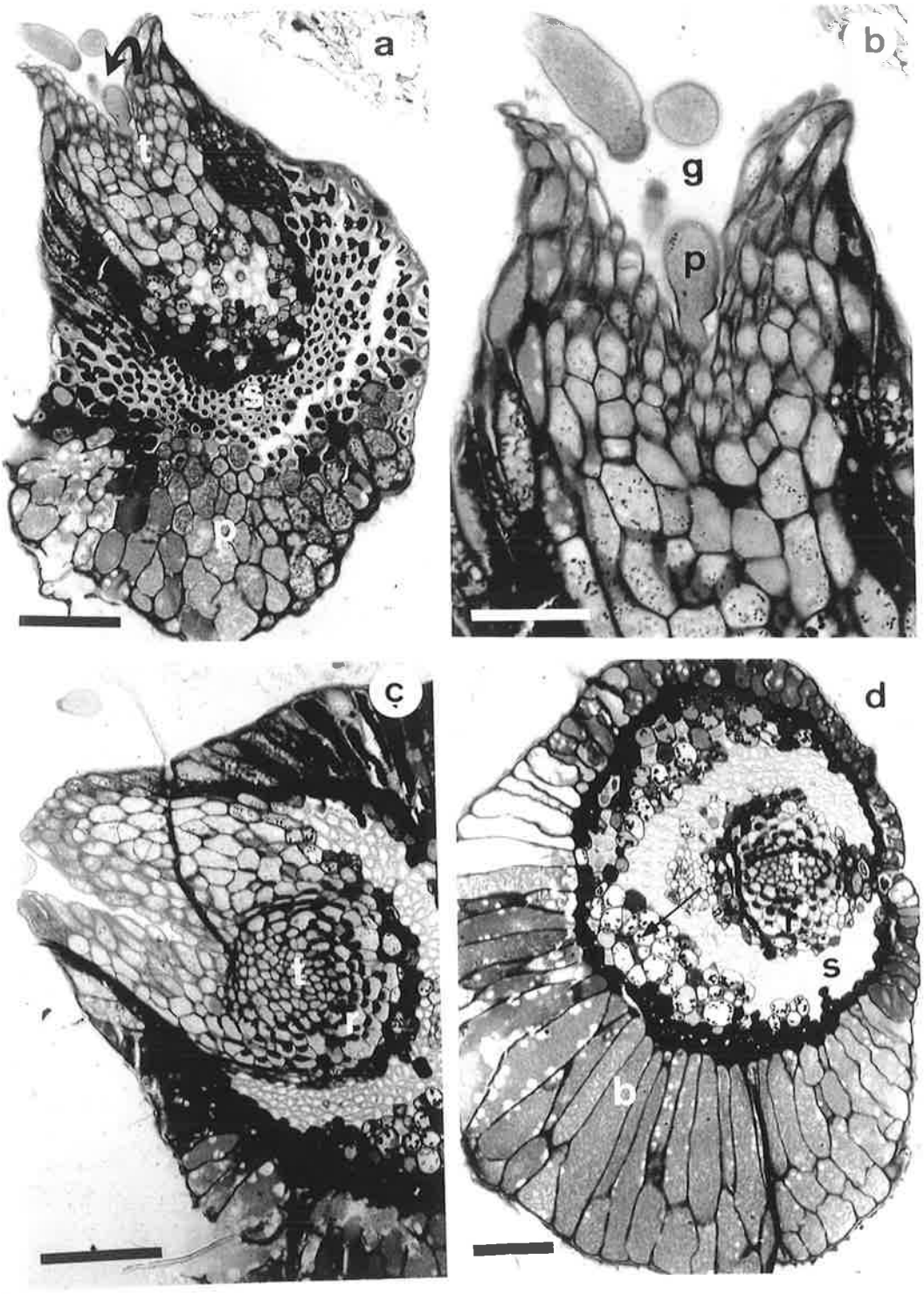

a
t
p
b
g
p
ç
t
l
d
s
b

Plate 4.23

Light micrographs of the style down its length, of *Banksia ericifolia* in transverse section stained with PAS and TBO

(a) Upper style showing central core of transmitting tissue (t) surrounded by three cell layers of transfer tissue (arrowhead) and five vascular bundles (v). Note cortex with a predominance of thick walled sclerenchyma cells (s) and outer two layers of cells including epidermis containing polyphenols (e). Bar represents 100 µm.

(b) Upper mid-style showing transmitting tissue (arrowhead) surrounded by a few transfer cells and cortex of mainly sclerenchyma (s). Bar represents 100 µm.

(c) Mid-style showing central core of transmitting tissue (arrowhead), cells of inner layer of cortex with starch grains (s), five vascular bundles (v) and outer two layers of cells containing polyphenols (e). Bar represents 200 µm.

(d) Lower mid-style showing reduced transmitting tissue (arrowhead) and large, rounded cortical cells (x). Bar represents 200 µm.

(e) Lower style showing layers of polyphenol containing cells (p) surrounding central core of transmitting tissue (arrowhead), and associated with vascular bundles (v). Bar represents 200 µm.

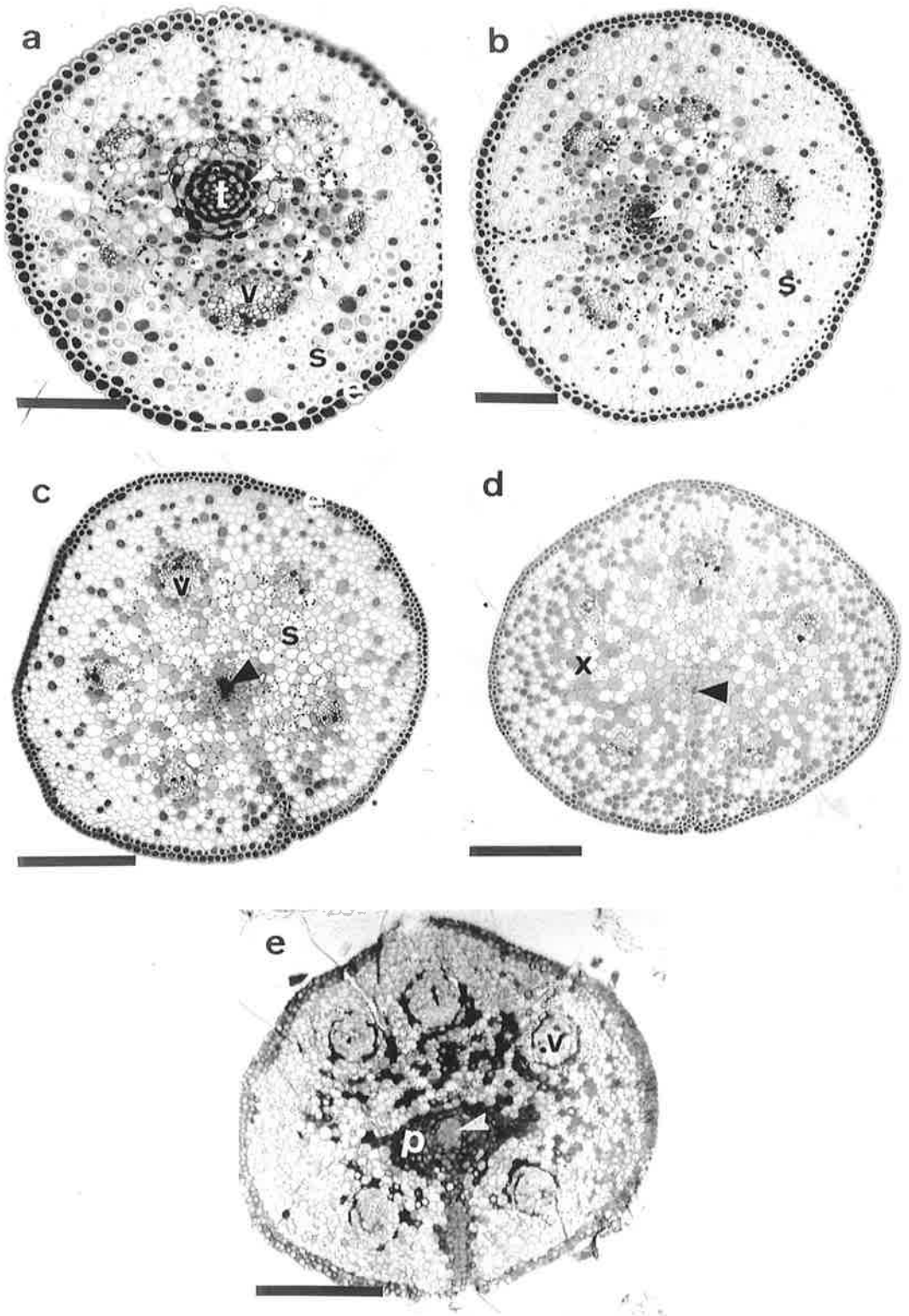

a
t
v
s
b
s
c
v
s
d
x
e
v
p

Plate 4.24

Scanning electron micrographs (FESEM) of the pollen presenter and pollen of *Isopogon cuneatus*

(a) Pollen presenter showing rows of hairs (h) and stigmatic tube (arrow). Bar represents 1 mm.

(b) Stigmatic tube (t) showing pollen grain (arrow) within. Bar represents 50 μm.

(c) Pollen presenter showing rows of epidermal hairs (h). Bar represents 100 μm.

(d) Short epidermal hairs (h) at the base of the pollen presenter. Bar represents 200 μm.

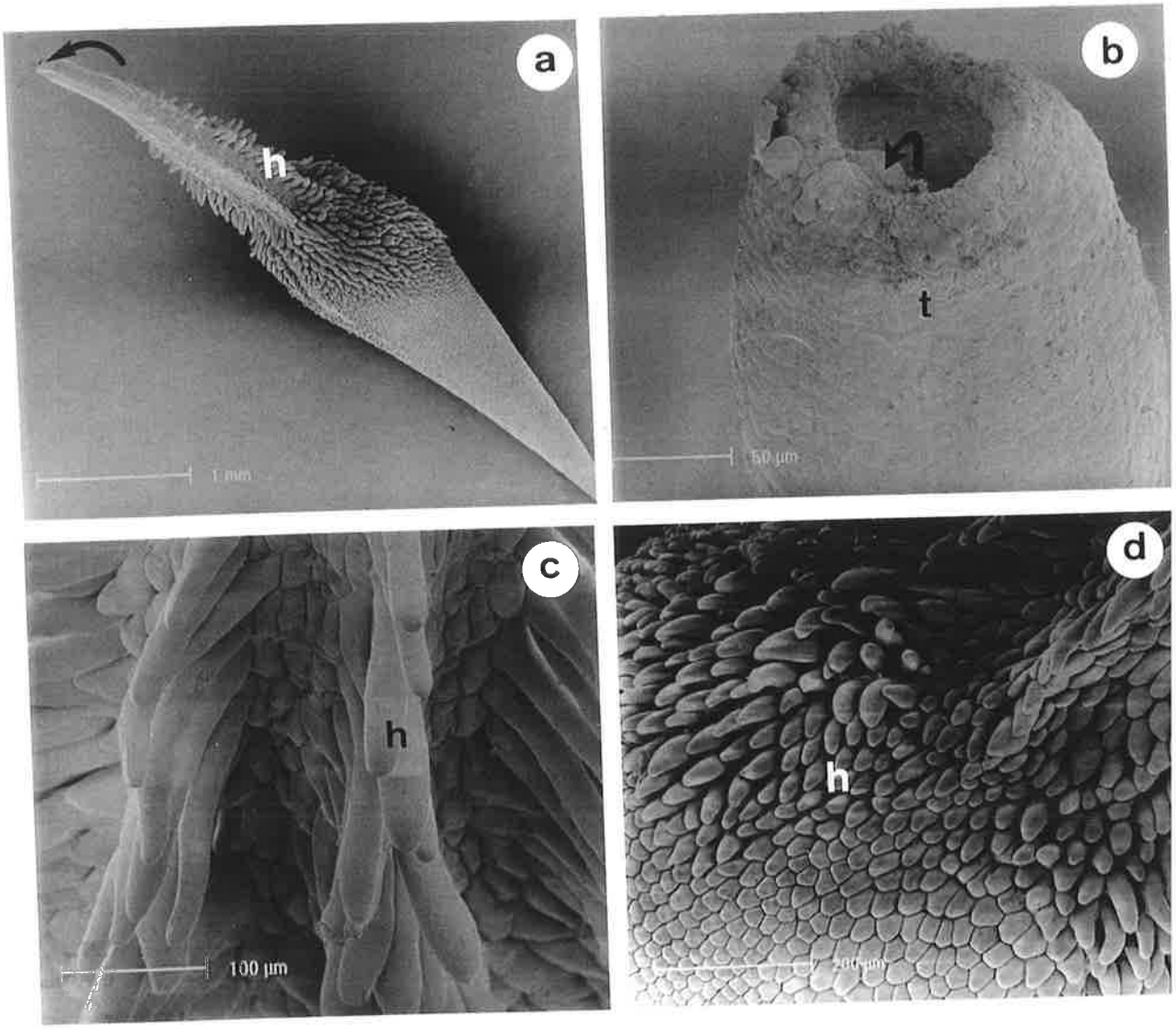

a
h
1 mm
b
t
50 µm
c
h
100 µm
d
h

Plate 4.25

Scanning electron micrographs (FESEM) of *Isopogon cuneatus* pollen grains

(a) Triangular pollen grain with highly sculptured exine (e). Bar represents 20 μm.

(b) Pollen grain showing germination pore (p). Bar represents 5 μm.

(c) Germinating pollen grain showing a pollen tube (arrow). Bar represents 20 μm.

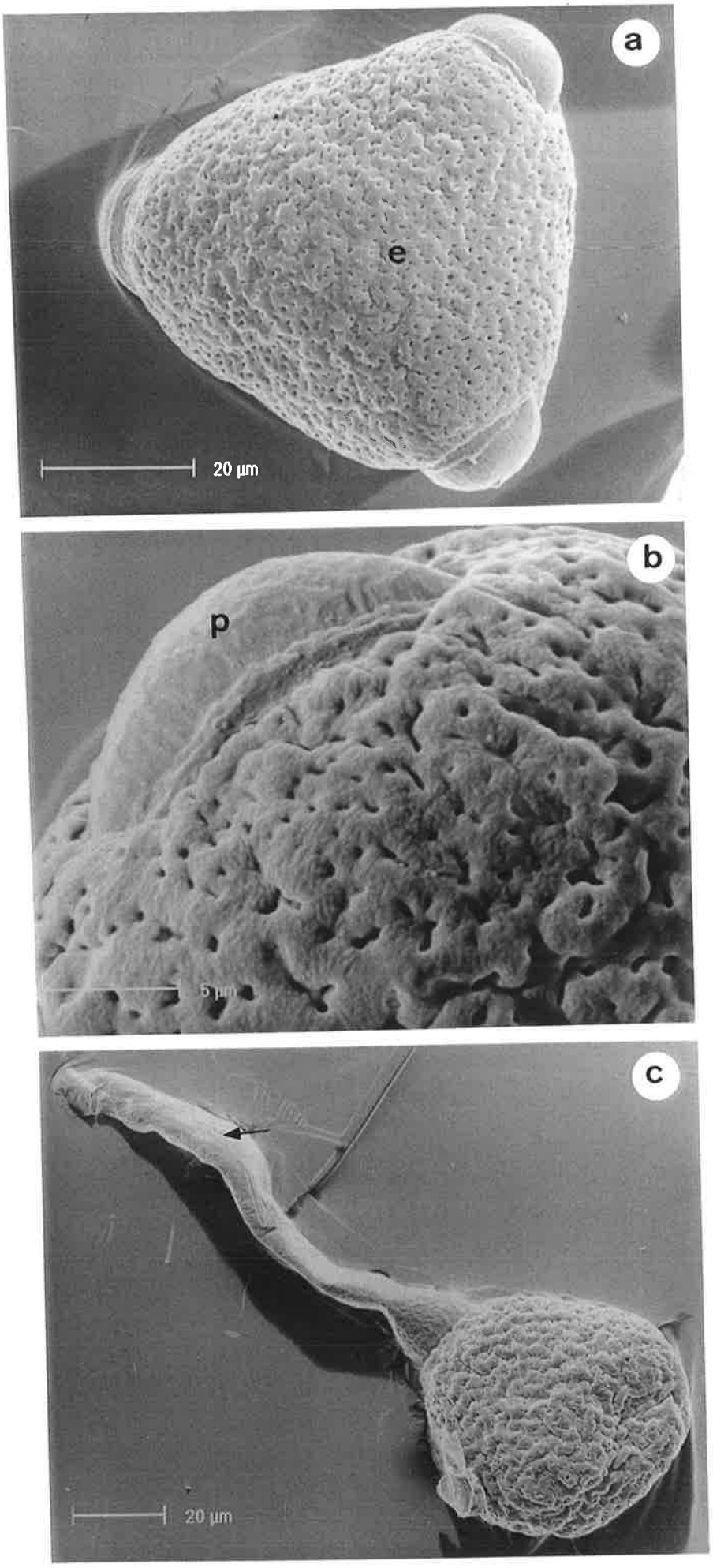

a
e
20 µm
b
p
5 µm
c
20 µm

Plate 4.26

Light micrographs of the pollen presenter of *Isopogon cuneatus* in transverse section stained with PAS and TBO

(a) Tip of the stigmatic tube containing three pollen grains (p) and a single layer of transmitting tissue (t) around the internal circumference. Note sculpturing of exine of pollen grains (arrow). Bar represents 50 µm.

(b) Stigmatic tube of pollen presenter with additional layers of transmitting tissue (t) surrounding a single pollen grain (p). Bar represents 50 µm.

(c) Pollen presenter below the hollow stigmatic tube. The structure is filled with transmitting tissue (t) and surrounded by polyphenol containing cells (p). Bar represents 50 µm.

(d) Pollen presenter just above the hairy region showing a central core of transmitting tissue (t) surrounded by a layer of polyphenol containing cells (o). Bar represents 50 µm.

(e) Pollen presenter at the hairy region. Note the central core of transmitting tissue (t) surrounded by transfer cells (arrowhead), the cortex comprised of large starch containing parenchyma cells (p) and enlarged epidermal cells forming hairs (e) on the outer surface coated by a thick cuticle (arrow). Bar represents 200 µm.

(f) Central core of transmitting tissue (t) surrounded by transfer cells (arrow). Bar represents 50 µm.

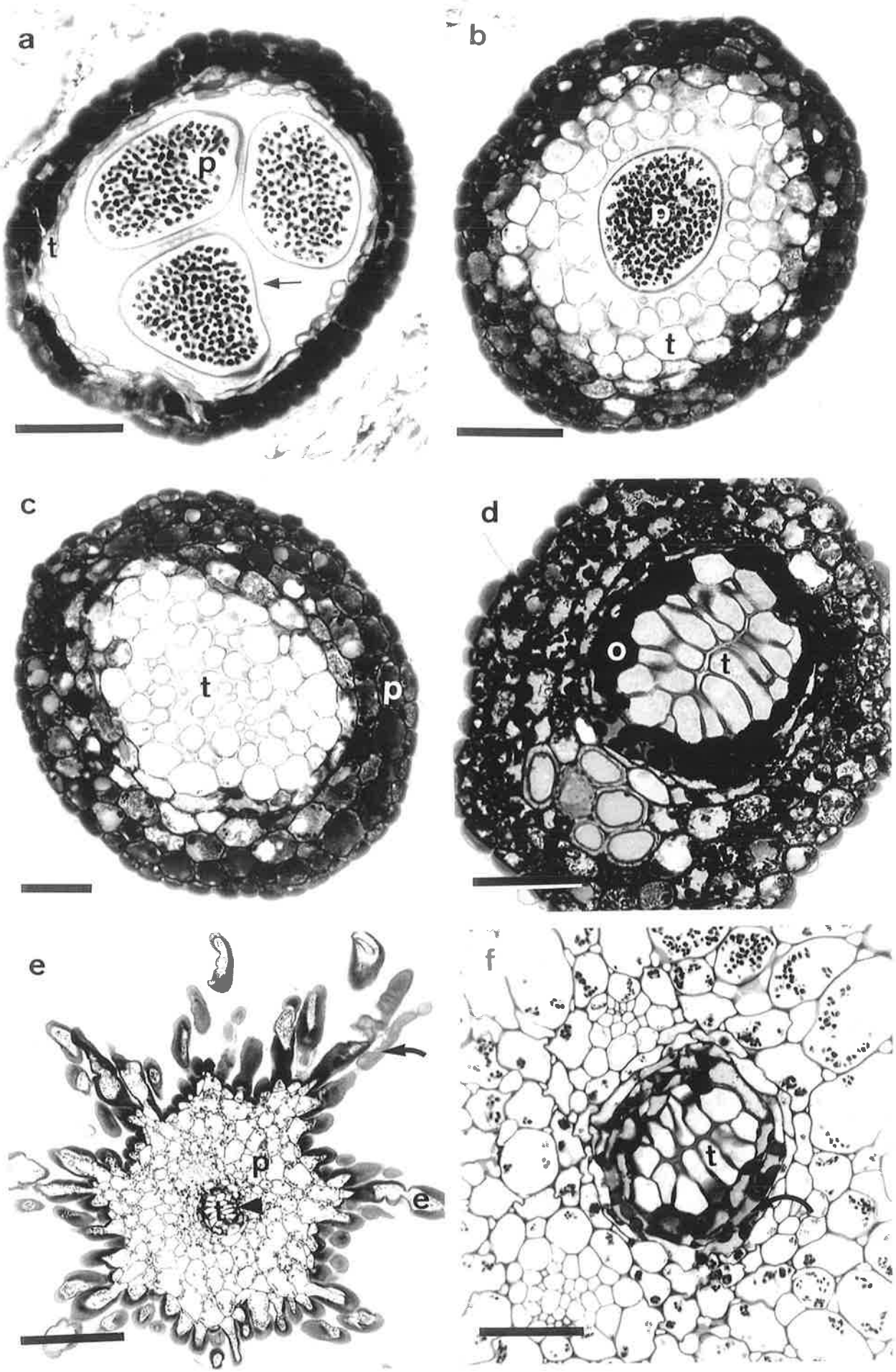

a
p
t
b
t
c
t
p
d
o
t
e
p
t
e
f
t

Plate 4.27

Light micrographs of the pollen presenter and style down its length, of *Isopogon cuneatus* in transverse section stained with PAS and TBO

(a) Base of the pollen presenter below the hairy region showing pear-shaped epidermal cells (e) covered with a thick cuticle (arrow). Note also starch grains (arrowhead) within thin walled cortical cells and central core of transmitting tissue (t). Bar represents 100 µm.

(b) Section through the upper style, below the pollen presenter. Note the three vascular bundles (v), central core of transmitting tissue (t) surrounded by polyphenol containing cells. Bar represents 50 µm.

(c) Section through the upper mid-style. Note transmitting tissue (t). Bar represents 50 µm.

(d) Mid-style showing central core of transmitting tissue (t). Bar represents 50 µm.

(e) Lower mid-style showing central core of transmitting tissue (t). Bar represents 50 µm.

(f) Base of style above ovary showing central core of transmitting tissue (t) surrounded by cortex (x) and epidermal layer containing polyphenols (p). Bar represents 50 µm.

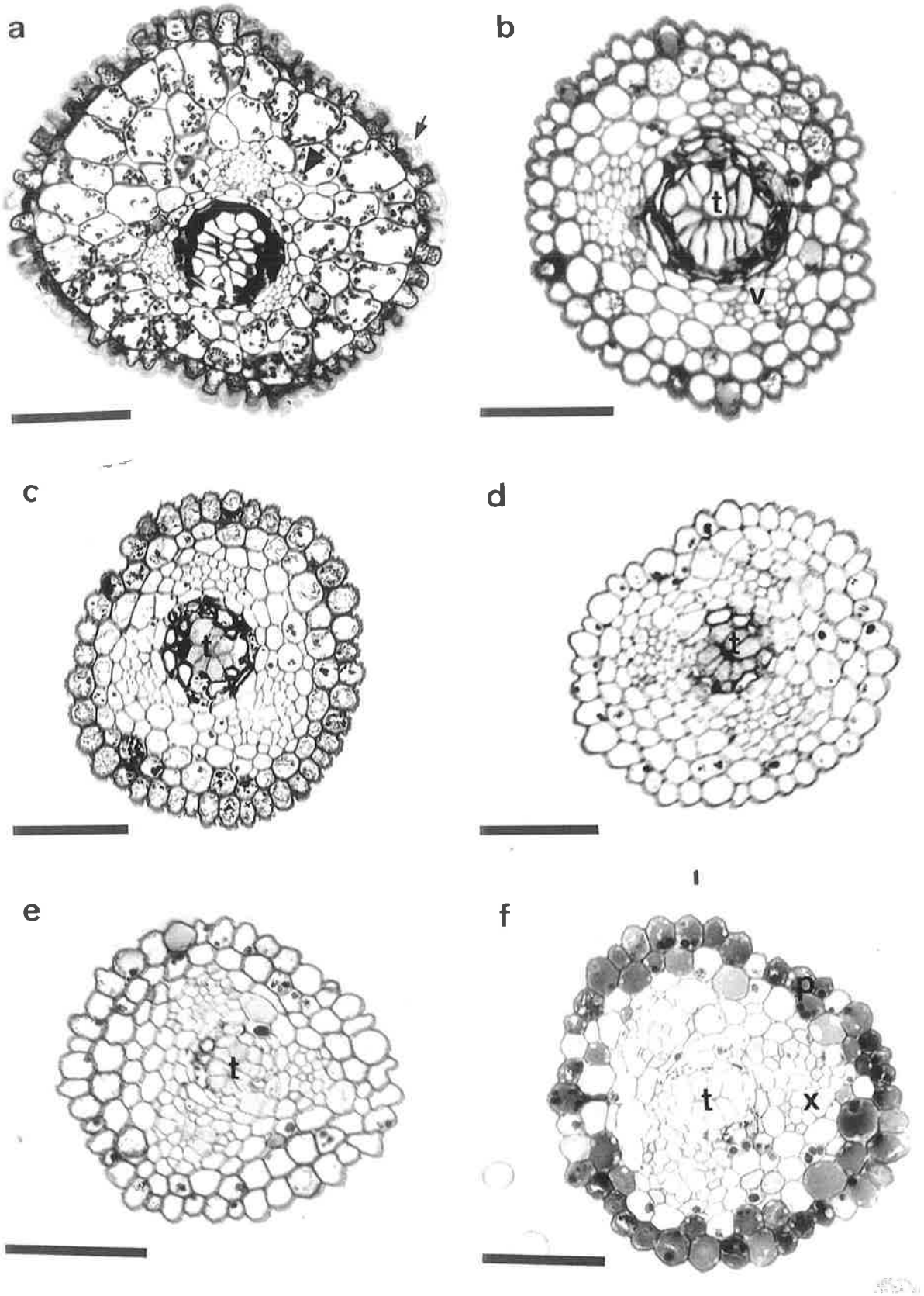

a
b
t
v
c
t
d
t
e
t
f
p
t
x

Plate 4.28

Scanning electron micrographs (FESEM) of the pollen presenter and pollen grains of *Macadamia integrifolia*

(a) Pollen presenter showing terminal stigmatic papillae (arrow) and collar (arrowhead). Bar represents 500 μm.

(b) Pollen presenter showing protruding stigmatic papillae (arrowhead). Bar represents 100 μm.

(c) Triangular pollen grains with lightly sculptured exine (e) and small germination pore (p). Bar represents 10 μm.

(d) Freshly prepared cryo-FESEM micrograph of the stigmatic region of pollen presenter showing stigmatic papillae (arrowhead) and pollen grains (p) and surrounding pistil tissue (t). Bar represents 20 μm.

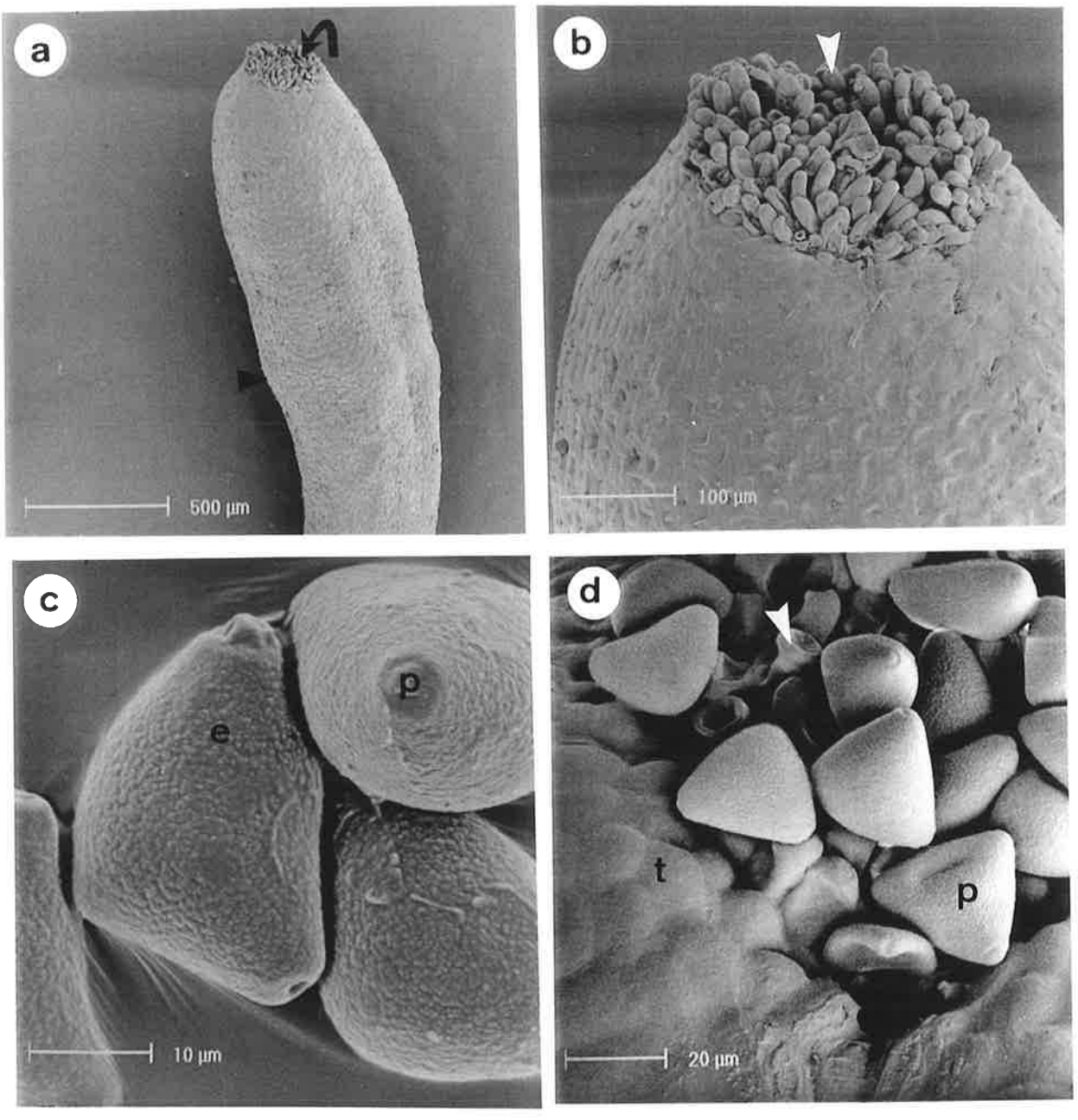

a
500 µm
b
100 µm
c
e
p
10 µm
d
t
p
20 µm

Plate 4.29

Light micrographs of the pollen presenter of *Macadamia integrifolia* in transverse section stained with PAS and TBO

(a) Tip of the pollen presenter showing the loose arrangement of stigmatic papillae (s) and surrounding tissue containing polyphenols (p). Bar represents 100 μm.

(b) Pollen presenter showing stigmatic papillae (s), transmitting tissue (t), transfer tissue (r), parenchyma (x) and outer layers of polyphenol containing cells (o). Bar represents 100 μm.

(c) Stigmatic groove region showing transmitting tissue (t). Bar represents 100 μm.

(d) Pollen presenter below stigmatic groove showing layers of polyphenol containing cells (o), parenchyma (x) containing starch grains (arrow) and transmitting tissue (t). Bar represents 100 μm.

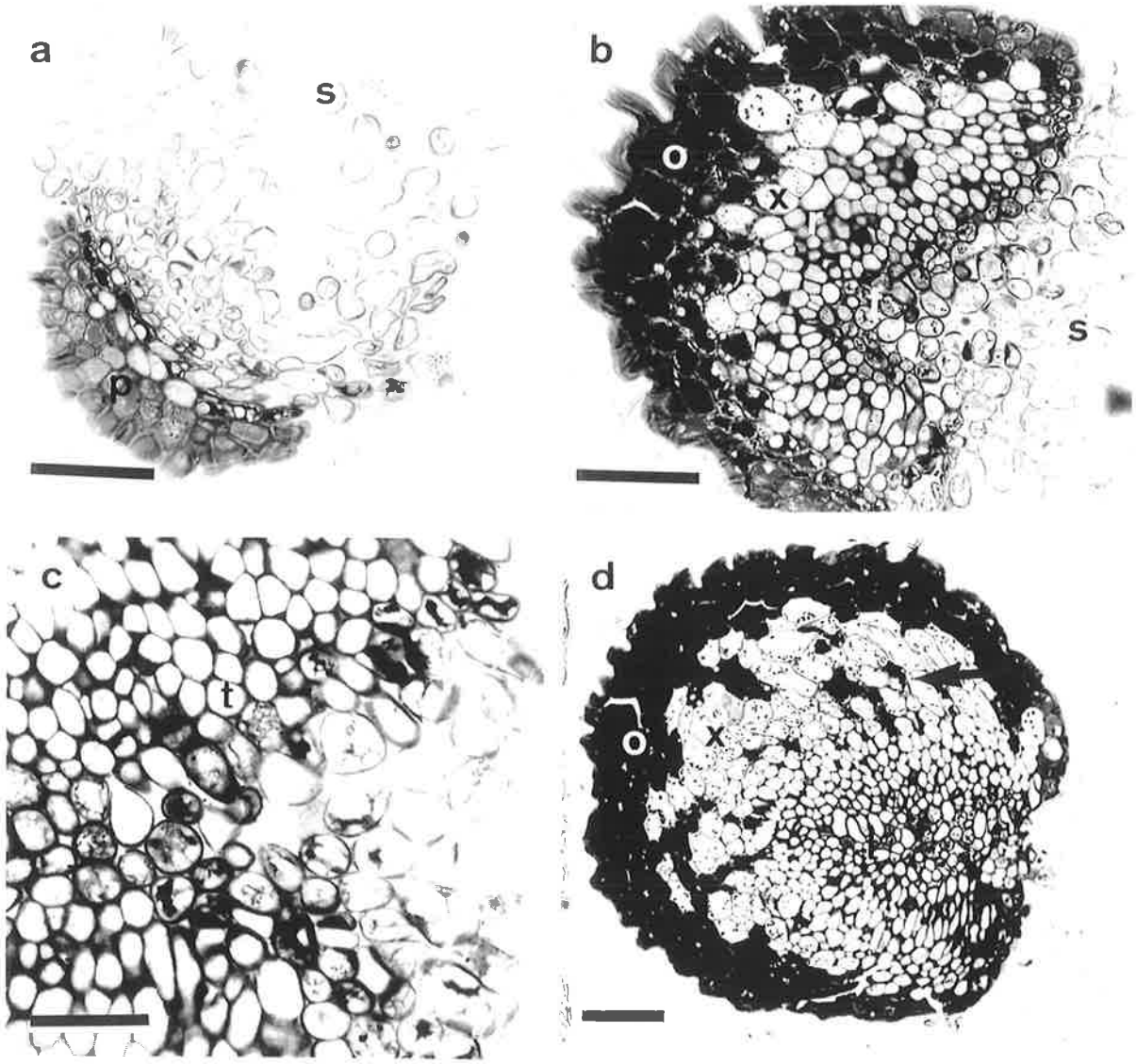

a
s
p
b
o
x
s
c
t
d
o
x

Plate 4.30

Light micrographs of the pollen presenter style down its length, of *Macadamia integrifolia* in transverse section stained with PAS and TBO

(a) Pollen presenter below the groove showing central core of transmitting tissue cells (t) surrounded by transfer cells (r), a cortex of heavily stained polyphenol containing cells (o) and parenchyma containing starch grains (arrow). Note the two large vascular bundles (v). Bar represents 100 μm.

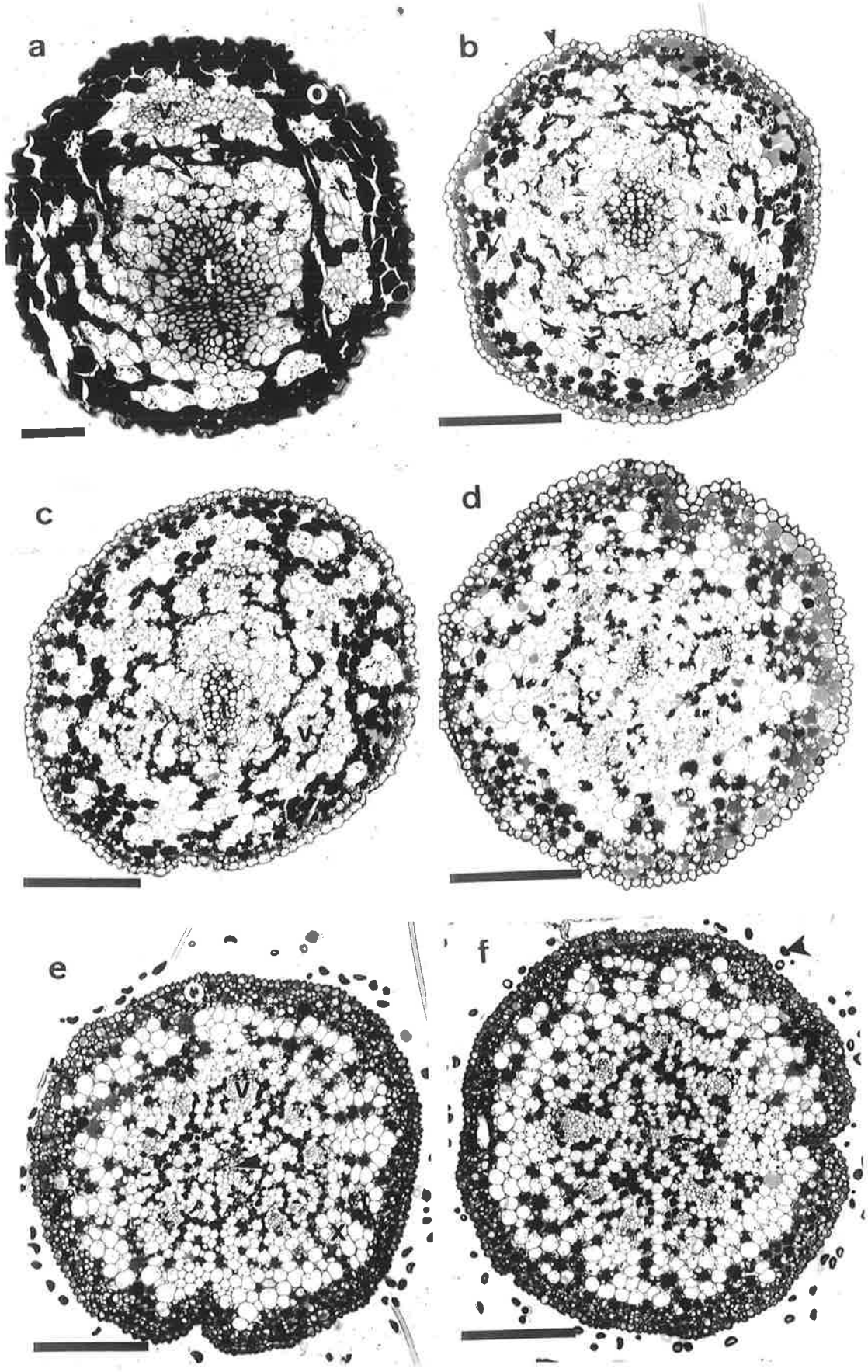
(b) Upper style below pollen presenter showing the transmitting tissue (t) and irregularly shaped epidermal layer (arrowhead). Note the large irregularly shaped parenchyma (x) of the cortex and the polyphenol containing cells, both which have starch grains (arrows) within. Bar represents 200 μm.

(c) Upper mid-style showing five vascular bundles (v) and transmitting tissue (t). Bar represents 200 μm.

(d) Mid-style showing transmitting tissue (t). Bar represents 200 μm.

(e) Lower mid-style showing transmitting tissue (arrowhead), cortex of parenchyma (x) and polyphenol containing cells (o) within cortex and forming rings to the outside of the vascular bundles (v) near, and including the epidermis. Bar represents 200 μm.

(f) Lower style showing transmitting tissue (arrow) and hairs (arrowhead) associated with the base of the style near the ovary. Bar represents 200 μm.


a
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Discussion

The complexity of structure of the proteaceous pollen presenter described for *Macadamia integrifolia* (Sedgley et al., 1985), *Banksia menziesii* (Clifford and Sedgley, 1993), *Protea repens* (van der Walt and Littlejohn, 1996b) and selected *Banksia* and *Dryandra* species (Ladd et al., 1996) has been confirmed by this study. The eight species studied had features which distinguish the proteaceous pollen presenter from most other angiosperm pistils. These features include the complete or partial enclosure of the stigma within a cavity, concentric layers of tissues within the pollen presenter which incorporate transmitting tissue, transfer tissue, polyphenol containing cells, vascular bundles, parenchyma and sclerenchyma. Internally and externally the pollen presenter is distinct and complex relative to the rest of the pistil.

There were two main discriminating characters between pollen presenters of the species studied; there were those with transfer tissue and those without, and there were those with sclerenchyma and those without. These features formed distinct, but not mutually exclusive groups. The other less definable difference between species was the arrangement of ground tissue and polyphenol containing cells.

The persistence of transfer tissue down the style varied. All but *H. bucculenta* possessed transfer tissue in the pollen presenter and top of the style, however for *M. integrifolia* and *B. coccinea* it ended here. Its occurrence in the upper mid-style was reduced to almost absent in *B. ericifolia* and *D. nana*, but was still present in the upper mid-styles of *D. formosa* and *D. quercifolia*. The differences between *Dryandra* species may be the result of the abnormally long style of *D. nana*.

In order to understand possible reasons for the distinct pattern of tissue distribution within the pollen presenter, one needs to consider their functions.

Transfer cells are characterised by pronounced invagination of their cell wall. This greatly increases the surface area of the plasmalemma which assists their primary function, to

absorb or secrete nutrients (Gunning and Pate, 1974). The location of the cell wall thickening can indicate the specific role of the transfer cell. In the species studied, the cell wall thickenings were towards the centre, the inner layer abutting the central core of transmitting tissue. Transmitting tissue cells are also known to have a secretory role, primarily to support growth of pollen tubes down the style (Jensen and Fisher, 1969; Cresti et al., 1976; Herrero and Dickinson, 1979; Herrero, 1992). Perhaps with the low numbers of transmitting tissue cells observed in many of the species studied here, transfer tissue is necessary to assist in the provision of nutrients for the growing pollen tube. This explanation is supported by *H. bucculenta*, a species lacking transfer tissue, but with proportionally more transmitting tissue than the others.

In contrast, however, *I. cuneatus* has very low proportions of transfer tissue and correspondingly low numbers of transmitting tissue cells in the pollen presenter. The structure of the pistil is distinct from the other species observed. The amount of transmitting tissue is very low for the entire length of the pistil, and the cells tightly enclosed within a ring of polyphenol containing cells for much of their length.

It has been suggested that the complex tissue distribution, particularly transfer tissue, in the pollen presenter plays a role in self-pollen tube recognition (Sedgley et al., 1985; Clifford and Sedgley, 1993). *Macadamia integrifolia* and *B. menziesii* display partial pre-zygotic self-incompatibility in the upper style, a region located just below the area of maximum cell complexity. *Dryandra quercifolia* also displays this phenomenon (Matthews and Sedgley, in press, Chapter Two). Ladd et al. (1996) do not support this conclusion and suggest further investigation of self-compatible and -incompatible species with regard to the presence or absence of transfer tissue. In addition, Ladd *et al* (1996) propose an alternative reason for the presence of transfer tissue in *Banksia* and *Dryandra* styles. These authors propose that the presence of transfer tissue is related to the long styles present in these genera, and that transfer tissue is required to facilitate the movement of sugars from the hydrolysis of starch to the growing pollen tube. The transfer tissue volumes and corresponding style lengths of the species observed in this study support a relationship between transfer tissue and style length. *Dryandra nana* has the longest style,

and correspondingly the greatest volume of transfer tissue present in the pollen presenter, and in contrast the shorter styled *Banksia* species had the least volume of transfer tissue. *Hakea bucculenta* is the only proteaceous species studied which lacked transfer tissue, however its breeding system is unknown. A study of the breeding system of this species would help shed light upon the possible role of transfer tissue in the self-incompatibility system of the Proteaceae or its relatedness to style length.

Secondary cell thickenings in the form of sclerenchyma cells were present in *Dryandra* and *Banksia* species only. Pollinators of these genera are often small marsupials or birds (Paton and Turner, 1985; Collins and Spice, 1986; Cunningham, 1991) and the floral structure of these genera provides a stiff and solid structure to support the weight of these pollinators. This contrasts with inflorescences of *I. cuneatus, H. bucculenta* and *M. integrifolia*, which have a more delicate structure. These genera are mainly insect and bird pollinated (Lamont, 1985; Vaughton, 1996). The presence or absence of sclerenchyma may be an adaptation to pollinator type.

The presence of polyphenol containing cells showed no clear pattern between species in their cell distribution or their association with transmitting tissue or the epidermis. Polyphenols are polymeric, and in plants they include such substances as tannins, melanins and lignins (Harborne, 1984). Lignins impregnate the plant cell wall improving its strength. The role of polyphenol substances in cell vacuoles of the pollen presenter is less clear, although polyphenolic substances are known to act as a deterrent to insect predators. Their location below the epidermis or surrounding the transmitting tissue may protect the pistil from such attack.

The taxonomic relationships of the species studied was reflected by similarities and differences in structure and morphology of the pistil and pollen. *Isopogon* had the most distinct pollen presenter of the species studied. Johnson and Briggs (1975) have speculated that the pollen presenter of the Proteaceae has arisen independently a number of times, one of these being in the Subtribe Petrophilinae to which *Isopogon* belongs. *Isopogon* was also the only genus studied from the subfamily Proteoideae. In contrast, the close taxonomic relationship between the sister genera *Banksia* and *Dryandra* was

reflected by the sharing of structural and morphological attributes between species of these genera. In particular internal pollen presenter anatomy, groove type and pollen morphology were very similar. The exception was the pollen morphology of *Banksia coccinea*. Recently this species was re-classified into a new section, Section Coccinea, based on its distinct morphological features and its pollen-pistil compatibility to other species of the genus (Maguire et al., 1996). The presence of transfer tissue in all but *Hakea bucculenta* suggested that this feature may have arisen early in Proteaceae evolution. The anatomical study of *Grevillea banksii* by Herscovitch and Martin (1989) did not report the presence of transfer tissue in this species however this study was not specifically interested in pollen presenter anatomy. *Grevillea* and *Hakea* are classified into the same tribe, and study of this tribe for the presence or absence of transfer tissue may provide further insight into the taxonomic relationships that exist within the Proteaceae.

Transmitting tissue volume decreased significantly from the pollen presenter to upper style and then progressively to the lower style for all species studied. This has been observed previously for *B. menziesii* (Clifford and Sedgley, 1993) and these authors suggest that the lower style is able to support the growth of only one pollen tube on average. Although not directly studied here, the potential for reduced pollen tube growth to the ovary due to a narrowing of the tract seems possible.

There were differences in the theoretical pollen holding capacity of the stigmatic cavity and many factors contribute to the actual number of grains that have access to the stigma. When one considers the volume of the cavity versus the volume of the grain, those species lacking an enclosed groove such as *Macadamia* have the highest holding capacity, as access to the papillae is not restricted by stylar walls. The tube of *Isopogon* also had a greater capacity to hold pollen grains, compared to the grooves. The size of the pollen grain is also a major contributor. In *B. ericifolia*, groove size is one third the volume of *D. quercifolia*, yet due to smaller pollen grains its holding capacity is greater. The calculation of the theoretical pollen holding capacity assumes that the cavity is at maximum diameter and that pollen grain diameter is smaller than cavity diameter, thus allowing access to the stigma. However in nature this is not always the case. For example, in *D. formosa*,

groove diameter was less than that of the pollen grain suggesting that access to the stigma would potentially restricted and result in low pollen tube germination. These differences between cavity and pollen size were observed by Ladd et al. (1996), however these authors conclude that for some species maximum cavity width is of no concern and should not be considered as a factor limiting fertility. This conclusion was based upon the observation that some cavities have the potential to be pushed open and pollen deposited inside by the probing action of the pollinator. This may well be the case for *D. formosa* which displayed very low pollen tube counts after careful hand pollination (Matthews and Sedgley, in press; Chapter Two).

The groove of *D. formosa* also shows other characteristics which may be responsible for the very low numbers of pollen tubes observed germinating in this species (Matthews and Sedgley, in press; Chapter Two). It produces little exudate compared with *D. quercifolia,* Its groove opens at maximum diameter for one day only, and the structural characteristics of the groove may limit pollen tube germination. *Dryandra formosa* was the only species studied to have dominant lips covered with a cuticle and it had the least transmitting tissue in the groove region compared with other species possessing a groove with enclosed papillae. An observation while hand pollinating showed that no matter how much pollen was applied to the groove region, the pollen failed to stick. This contrasted with *D. quercifolia* in which pollen readily adhered to the pollen presenter. It is unlikely that pollen type is responsible for this as both species had similar grains (Ladd et al., 1996). This species may require extensive manipulation of the pistil in order to push the groove open for successful pollen deposition.

Regardless of individual species characteristics, there appeared to be three features which restricted access to the stigma and the subsequent growth of pollen tubes. The first restriction was located at the cavity, the dimensions of which influenced the number of pollen grains that access the stigma and in turn their potential to germinate. The second restriction was the significant narrowing of the transmitting tissue tract below the pollen presenter, and the third, the subsequent and further narrowing of the transmitting tissue tract in the lower part of the style. These combined characteristics could potentially

reduce the total number of pollen tubes in the style and the ultimate number reaching the ovary.

When considering the importance of the potential controlling influence of the stigmatic cavity to reproductive success one must also consider whether pollen grain contact with the stigma is necessary for germination, especially when exudate is present. In *Protea* hybrids whose pollen was larger than the cavity opening, pollen germination was successful on the outside of the cavity (van der Walt and Littlejohn, 1996b). This contrasted with *B. menziesii*, a species with a dry stigma where pollen must be close to the groove to germinate (Fuss and Sedgley, 1991a). Pollen grains were observed to germinate on the outside of the cavity in both *D. quercifolia* and *D. formosa* (personal observation).

This study has further demonstrated that pistil structure has the potential to influence species fertility and may contribute to the low fruit to flower ratio observed in the family. In particular, the confinement of the stigma to a cavity, potentially limits pollen grain access to the stigma. This location may be an adaptation to an arid environment, its enclosure potentially reducing water loss from the stigmatic surface. Supporting this view are the protruding papillae of the subtropical *M. integrifolia* compared with the enclosed stigma of the Western Australian species. An investigation of tropical species may help to further elucidate this view.

Chapter Five

The relationship between ovule number, transmitting tissue abundance and pollen tube number at the stylar base: An exploratory study across ten angiosperm families

Abstract

Transmitting tissue is essential for the passage of the pollen tube to the ovary, yet within some genera of the Australian Proteaceae the amount of transmitting tissue at the base of the style is severely reduced. How much transmitting tissue is needed for the passage of one pollen tube is unknown, however it may vary amongst angiosperms. To determine whether reduced transmitting tissue in the Proteaceous pistil is unusual and whether it affects pollen tube passage and thus seed set, the anatomy of the lower style of eight proteaceous species, and seventeen species from nine other angiosperm families, was examined. Pistils were sectioned and the area of transmitting tissue measured using image analysis software. Overall stylar structure and tissue areas were generally consistent within families, but differed significantly between families. The proportion of transmitting tissue to the rest of the style was very low in the Proteaceae compared to other families. Pollen tube number at the base of the style correlated to transmitting tissue area, and transmitting tissue cell number was correlated to ovule number. Species with multiple ovules (≥12) had the largest quantities of transmitting tissue area and highest numbers of pollen tubes. The ratio of transmitting tissue cells to pollen tubes was approximately 1:1 for these species, a ratio much lower than for species with few (≤2) ovules. All species examined except *Triticum aestivum* showed pollen tube number close to ovule number, suggesting that ovule number had a strong influence.

Introduction

The ultimate role of the flower is to facilitate fertilisation of the ovule, production of the seed, and thus the survival of the species. The flower comprises non-reproductive organs; calyx and corolla or the perianth, and reproductive organs; stamen and carpel. The carpel comprises three parts: (1) stigma; a region which catches and receives pollen released from the anther sacs during pollination (2) style; which elevates the stigma and contains the transmitting tissue through which the germinated pollen grows as a tube carrying the male sperm cells to the (3) ovary; which encloses the ovules. The internal and external structure of the carpel facilitates the processes of pollination and fertilisation, and in particular the transmitting tissue within the style plays a significant role.

Transmitting tissue is a region of highly cytoplasmic secretory parenchyma cells arranged in files. Between the cells is a secretion which serves as the specialised substrate through which pollen tubes grow intercellularly towards the ovary. Pollen tubes of most species absorb nutrients from this carbohydrate rich secretion (Jensen and Fisher, 1969; Cresti et al., 1976; Herrero and Dickinson, 1979; Herrero, 1992). In addition, the transmitting tissue functions as the site of recognition and control of the passage of incompatible pollen tubes (Linskens, 1975; Cresti et al., 1976; Knox, 1984; Cheung, 1995). The physiological importance of transmitting tissue is well established.

However, as competition for nutrients is responsible for pollen tube attrition in some compatible pollinations (Winsor and Stephenson, 1995), the question then arises regarding the minimum quantity of transmitting tissue required to support pollen tube passage. This can be considered as having a structural basis.

When examining the minimum structural requirements for successful fertilisation the number of ovules present must be considered, as the ovary can be no more successful than the maximum number of ovules it contains. In Chapter Four, the transmitting tissue of proteaceous species narrowed significantly from the pollen presenter to the base of the style, where it consisted of less than twenty files of cells in some cases. In addition, the

Proteaceae show very low seed set. Is it possible that narrowing of the transmitting tissue tract at the base of the style contributes to this low seed set? Or alternatively, is the quantity of transmitting tissue present sufficient for the routine passage of the two pollen tubes required to achieve maximum fertilisation of the flower? If so, why do so many flowers lack pollen tubes at the ovary?

Many proteaceous floral features are unusual amongst the angiosperms. To investigate whether the Proteaceae differ from other angiosperm families regarding the quantity of transmitting tissue at the base of the style, eight proteaceous species, and seventeen species from nine other angiosperm families, including one monocotyledon, were investigated. Pistils were transversely sectioned at the base of the style above the ovary. The transmitting tissue was quantified using image analysis software associated with a light microscope. The area of transmitting tissue was compared to the number of pollen tubes observed at the base of the style and to the number of ovules. The anatomy of the style and transmitting tissue was also considered.

Materials and Methods

Study species

Current taxonomic positions according to Cronquist (1981):

Dicotyledonae

Lauraceae:	*Persea americana* (avocado)
Cucurbitaceae:	*Citrullus lanatus* (watermelon); *Cucumis sativus* (cucumber)
Rosaceae:	*Malus pumila* (apple), *Prunus avium* (cherry), *P. dulcis* (almond); *Pyrus communis* (pear)
Leguminosae:	*Acacia baileyana*; *A. karroo*

Proteaceae:	*Banksia coccinea, B. ericifolia, Dryandra formosa, D. nana, D. quercifolia, Hakea bucculenta, Isopogon cuneatus; Macadamia integrifolia*
Myrtaceae:	*Eucalyptus leucoxylon, E. spathulata; E. woodwardii*
Santalaceae:	*Santalum acuminatum* (quandong)
Anacardiaceae:	*Pistacia vera* (pistachio)
Solanaceae:	*Lycopersicon esculentum* (tomato); *Petunia hybrida* (petunia)

Monocotyledonae

Graminae:	*Triticum aestivum* (wheat)

Study material was grown from seed in the glasshouse or collected from the Waite Orchard (Plate 5.1), Waite Arboretum, a commercial cut flower property and a private garden, both in Blewitt Springs, South Australia (35°10'S, 138°34' E).

Preparation and sectioning of material

Pistils were collected fresh from each of three plants per species and the lower portion of the style at the junction with the ovary, cut and retained. This tissue was fixed for two to five days in 2.5 % glutaraldehyde in 0.025 M sodium phosphate buffer, pH 7.0, and stored in buffer at 4 °C. The tissue was dehydrated through an alcohol series, embedded in glycol methacrylate and positioned vertically, aided by folded paper within a gelatine capsule. Serial transverse sections of the style were made at the point of origin of the style above the ovary. At least ten (3 µm) sections per flower, at every tenth section, were collected. Sectioned material was stained with Periodic acid-Schiff's reagent and Toluidine Blue O (TBO) (O'Brien and McCully, 1981), and viewed using a Zeiss Axiophot photomicroscope (D-7802).

Quantification of transmitting tissue at base of style

Programs were written to quantify features of the transmitting tissue and style for each transverse section taken using the image analysis software, VideoPro 32 (Appendix). Features measured at the base of the style included total stylar area, transmitting tissue area, area of an individual transmitting tissue cell and the number of transmitting tissue cells. These measurements were extrapolated to calculate the area of intercellular space and cell wall in the transmitting tissue. This calculation used the transmitting tissue cell size and number, coupled with the total transmitting tissue area. Photographs were routinely taken for each of the species and used in combination with image analysis measurements to determine the packing constraints of the transmitting tissue cells.

Determination of pollen tube numbers at the base of the style

In cases where pollen tube data had not been published, hand pollinations were performed. The following species were hand pollinated: *Banksia ericifolia, Hakea bucculenta, Isopogon cuneatus* (Proteaceae), *Acacia baileyana* (Leguminosae), *Prunus dulcis* (Rosaceae) and *Petunia hybrida* (Solanaceae). Pistils were fixed in Carnoy's solution and prepared for fluorescence microscopy as for *Dryandra* pistils (pages 10-11). Between 20 and 100 pistils were viewed and the number of pollen tubes present at the base of the style recorded. The average number of pollen tubes was calculated from these results and only pistils containing pollen tube(s) were included in this calculation.

Statistical Analysis

All analyses were performed using programs from the Biomedical (BMDP) statistical software package (Dixon, 1993).

A Repeated Measures Analysis of Variance was used to test for significant differences between species for measurements of the transmitting tissue. The overall effect of species was tested using Wald's test for significance. Where a significant effect was determined a model was fitted to produce estimated mean values and standard error for each species. A

pairwise comparison was made using the difference in estimated means relative to the estimated error of this difference for species within a family. The result was recorded as a z score which was assumed to be a standard normal deviate. Due to the number of potential pair wise comparisons, a z value of $z>2$, or $z>3$ was assigned as the critical level used to judge for significant differences between species. This procedure followed the logic of the Bonferroni Principal of adjusting for multiple comparisons. The program 5V (Unbalanced Repeated Measures) was used (Dixon 1993).

Plate 5.1

Prunus dulcis (almond) in flower in the Waite Orchard


ALMOND
Prunus amygdalus
var. THOMPSON

Results

Species studied had styles with a core of transmitting tissue surrounded by ground tissue, vascular bundles, an epidermis and cuticle (Plates 5.2-5.13). The majority of species had eusyncarpous styles although there were exceptions. The Cucurbitaceae species had lobed transmitting tissue (Plate 5.3), and *Malus pumila* and *Pyrus communis* (Rosaceae) had multiple unfused styles, each with a single, centrally positioned column of transmitting tissue (Plate 5.4). In contrast, the *Lycopersicon esculentum* style was pseudosyncarpous, a single style containing multiple columns of transmitting tissue (Plate 5.12).

The complexity of stylar tissues varied between families. Species of the Cucurbitaceae and Solanaceae had simple styles which consisted primarily of transmitting tissue, parenchyma and vascular bundles (Plates 5.3, 5.12). The composition of the *Triticum aestivum* style was also simple, but its plumose stigma with receptive cells arranged in multiseriate branches down the length of the style gave it a characteristic shape (Plate 5.13). Species of the Leguminosae, Proteaceae, Myrtaceae, Santalaceae and Anacardiaceae had styles with polyphenol containing cells and included all the Australian species studied (Plates 5.6-5.11). Styles of myrtaceous species also contained oil glands (Plate 5.9). Transmitting tissue of *Persea americana* was partly exposed and was associated with a groove-type structure (Plate 5.2). The transmitting tissue of the *Pistacia vera* pistil had a profusion of large cortical cells on one side fanning out in a 'v' shape (Plate 5.11).

The number of transmitting tissue cells and area of transmitting tissue varied significantly ($P<0.001$) between species, but were similar within each family, although there were exceptions (Table 5.1). In the Myrtaceae and Cucurbitaceae, the number of transmitting tissue cells varied significantly between species. Myrtaceous species also varied with regard to area of transmitting tissue and total stylar area, as did the Rosaceae. There were significant differences between species of the Proteaceae regarding total stylar area. Styles of the Solanaceae and Rosaceae were significantly different regarding the proportion of transmitting tissue within the style.

Cucumis sativus had the greatest number of transmitting tissue cells and the largest transmitting tissue area, *Isopogon cuneatus* and *Acacia baileyana* had the least. There was a difference of almost seven hundred cell files between these species. The size of an individual transmitting tissue cell varied significantly overall, but not between species of the same family (Table 5.1).

The intercellular space and cell wall area of the transmitting tissue varied significantly between species, however this area was small compared to the cellular area. Myrtaceous species had the largest area of intercellular space and cell wall which was reflected by the loose arrangement of transmitting tissue cells (Plate 5.9). The intercellular space was also large for solanaceous species, in particular *Lycopersicon esculentum* (Plate 5.12). The arrangement of *Petunia hybrida* transmitting tissue cells was similar (Plate 5.12). In all cases the intercellular space was filled with secretion.

Regardless of the size of the style or the area of transmitting tissue, the percentage of style which was transmitting tissue was greater than 10 % in all species except those of the Proteaceae and *Acacia baileyana.* In some species such as *Persea americana, Petunia hybrida* and two Rosaceae species, *Malus pumila* and *Pyrus communis*, there was at least 40% transmitting tissue in the style.

The Proteaceae, Leguminosae and Graminae showed some of the smallest areas and lowest numbers of transmitting tissue cell files in the species studied. When compared to the total stylar area, the proportion of transmitting tissue in species of the Leguminosae with the exception of *A. baileyana,* and species of the Graminae was the result of a very small stylar area, and overall these species had a high proportion of transmitting tissue compared to the rest of the style. In contrast, the transmitting tissue of the Proteaceae style was disproportionately small, comprising less than 3.5 % of the total stylar areas.

There were two groups of species based on ovule number. The first group consisted of species with one or two ovules and the second with multiple ovules (≥12). There was no pattern between species with one or two ovules regarding the number of transmitting tissue cells and maximum number of pollen tubes at the base of the style (Table 5.2, Plate

5.14). In this group the amount of transmitting tissue varied from less than ten cells (*Isopogon cuneatus*) to more than 300 (*Prunus avium*). For proteaceous species the maximum number of pollen tubes was one, whilst *Triticum aestivum* 19 pollen tubes were reported. *Triticum aestivum* was however the exception rather than the rule, and most species had three or less pollen tubes.

Species with multiple ovules had large numbers of transmitting tissue cells at the base of the style and high numbers of pollen tubes (Table 5.2). *Acacia baileyana* was the only exception, this species had low numbers of transmitting tissue cells and a high number of pollen tubes.

The ratio of transmitting tissue cells to pollen tubes was generally higher in species containing one or two ovules, with values as high as 77 cells/pollen tube in almond although *Triticum aestivum*, had a ratio of only 1.9 cells/pollen tube (Table 5.2). For species with multiple ovules this ratio ranged from 1.38 cells/pollen tube for *Acacia baileyana* to 7.4 cells/pollen tube for *Petunia hybrida.* Correlations between pollen tube number and other features are summarised in Table 5.3. There were correlations between pollen tube number and transmitting tissue area and between ovule number and number of transmitting tissue cells. Poor correlations were found between pollen tube number and area of intercellular space, ovule number and pollen tube number, and between ovule number and area of transmitting tissue.

Table 5.1: Quantity of transmitting tissue at the base of the style for species from a range of angiosperm families

Family and Species	Common Name	Transmitting tissue cells at base of style	Transverse sectional area at base of style (10^2 µm^2±s.e.) Transmitting tissue cell	Transmitting tissue region (cellular)	Transmitting tissue region (non-cellular)	Total style area	% of style that is transmitting tissue	% transmitting tissue that is cell wall or intercellular space
Proteaceae								
Banksia coccinea	Scarlet Banksia	[a]19.39±45.76	[a]1.00±0.23	[a]23.07±49.39	[a]14.40±27.57	[b]1385.27±306.33	[a]2.68±4.36	37.84
B. ericifolia		[a]25.69±45.76	[a]1.20±0.23	[a]32.40±49.39	[a]7.67±27.57	[a]1596.53±306.33	[a]2.46±4.36	20.51
Dryandra formosa	Golden Dryandra	[a]15.40±45.76	[a]1.00±0.23	[a]17.20±49.39	[a]2.07±27.57	[a]1854.27±306.33	[a]1.04±4.36	10.53
D. nana		[a]54.53±45.76	[a]1.33±0.23	[a]74.20±49.39	[a]4.47±27.57	[c]2235.27±306.33	[a]3.24±4.36	5.56
D. quercifolia	Oak-leaf Dryandra	[a]18.95±45.76	[a]1.26±0.23	[a]24.20±49.39	[a]4.73±27.57	[c]2759.07±306.33	[a]1.33±4.36	17.86
Hakea bucculenta		[a]25.20±45.76	[a]2.13±0.23	[a]51.13±49.39	[a]6.20±27.57	[c]2395.33±306.33	[a]2.40±4.36	10.53
Isopogon cuneatus		[a]8.76±45.76	[a]2.13±0.23	[a]17.73±49.39	[a]2.53±27.57	[a]583.73±306.33	[a]3.34±4.36	10.53
Macadamia integrifolia		[a]15.42±45.76	[a]1.33±0.23	[a]21.27±49.39	[a]6.07±27.57	[a]1708.40±306.33	[a]1.58±4.36	22.22
Anacardiaceae								
Pistacia vera	Pistachio	54.05±45.76	3.53±0.23	200.60±49.39	21.87±27.57	1852.47±306.33	11.07±4.36	1.18
Cucurbitaceae								
Citrullus lanatus	Watermelon	[a]279.90±45.76	[a]3.20±0.23	[a]882.80±49.39	[a]31.67±27.57	[a]4816.20±306.33	[a]16.43±4.36	4.05
Cucumis sativus	Cucumber	[b]708.22±45.76	[a]1.67±0.23	[a]1126.27±49.39	[a]120.73±27.57	[a]4058.20±306.33	[a]31.00±4.36	10.12
Graminae								
Triticum aestivum	Wheat	20.95±45.76	2.00±0.23	35.33±49.39	16.60±27.57	420.60±306.33	22.81±4.36	32.69

Continued next page

Table 5.1: Quantity of transmitting tissue at the base of the style for species from a range of angiosperm families *continued*

Family and Species	Common Name	Transmitting tissue cells at base of style	Transverse sectional area at base of style (10^2 µm^2±s.e.)				% of style that is transmitting tissue	% transmitting tissue that is cell wall or intercellular space
			Transmitting tissue cell	Transmitting tissue region (cellular)	Transmitting tissue region (non-cellular)	Total style area		
Lauraceae								
Persea americana	Avocado	127.35±45.76	3.53±0.23	456.73±49.39	70.13±27.57	972.00±306.33	57.82±4.36	14.17
Leguminosae								
Acacia baileyana	Cootamundra wattle	^{a}14.05±45.76	^{a}1.07±0.23	^{a}12.20±49.39	^{a}3.00±27.57	^{a}496.60±306.33	^{a}4.32±4.36	21.43
A. karroo		^{a}31.20±45.76	^{a}2.67±0.23	^{a}84.40±49.39	^{a}10.73±27.57	^{a}911.47±306.33	^{a}10.25±4.36	11.96
Myrtaceae								
E. leucoxylon		^{a}232.39±45.76	^{a}1.07±0.23	^{a}254.27±49.39	^{b}207.87±27.57	^{b}4163.40±306.33	^{a}11.13±4.36	45.02
E. spathulata		^{a}196.51±45.76	^{a}2.00±0.23	^{a}368.53±49.39	^{a}74.07±27.57	^{a}2126.73±306.33	^{a}20.14±4.36	17.49
E. woodwardii		^{b}468.41±45.76	^{a}1.80±0.23	^{b}861.67±49.39	^{ab}174.13±27.57	^{b}3681.93±306.33	^{a}27.51±4.36	17.28
Rosaceae								
Malus pumila	Apple	^{a}276.59±45.76	^{a}1.00±0.23	^{a}288.00±49.39	^{a}18.67±27.57	^{a}609.93±306.33	^{b}47.95±4.36	6.51
Prunus avium	Cherry	^{a}329.43±45.76	^{a}1.33±0.23	^{a}451.53±49.39	^{a}33.13±27.57	^{b}2404.00±306.33	^{a}19.87±4.36	7.04
P. dulcis	Almond	^{a}263.57±45.76	^{a}1.13±0.23	^{a}365.20±49.39	^{a}31.47±27.57	^{b}2397.33±306.33	^{a}17.10±4.36	8.31
Pyrus communis	Pear	^{a}224.64±45.76	^{a}1.27±0.23	^{a}304.33±49.39	^{a}76.53±27.57	^{a}830.27±306.33	^{b}45.01±4.36	20.64
Santalaceae								
Santalum acuminatum	Quandong	128.61±45.76	2.20±0.23	279.07±49.39	33.67±27.57	962.73±306.33	32.87±4.36	11.00
Solanaceae								
Lycopersicon esculentum	Tomato	^{a}595.68±45.76	^{a}1.00±0.23	^{a}479.47±49.39	^{a}160.20±27.57	^{a}2525.00±306.33	^{a}24.97±4.36	25.00
Petunia hybrida	Petunia	^{a}458.45±45.76	^{a}1.13±0.23	^{a}517.27±49.39	^{a}68.87±27.57	^{a}1248.80±306.33	^{b}45.52±4.36	12.19

Numbers with a different letter are significantly different (z>3). Pairwise comparisons have been made within each family but not between families. Note: the statistical model fitted to the data assumes a constant standard error value

Table 5.2: Mean number and ratio of transmitting tissue cells and pollen tubes at the base of the style and ovule number of species from ten angiosperm families

Family and Species	Common Name	Transmitting tissue cells at base of style (±s.e.)	Pollen tubes at base of style	Ratio of transmitting tissue cells to pollen tube (cells/pollen tube)	Number of ovules	Reference
Proteaceae						
Banksia coccinea	Scarlet Banksia	19.39±45.76	0.85	22.81	2	Fuss and Sedgley (1991b)
B. ericifolia		25.69±45.76	1.17	21.95	2	
Dryandra formosa	Golden Dryandra	15.40±45.76	1.00	15.4	2	
D. nana		54.53±45.76	-	-	2	
D. quercifolia	Oak Leaf Dryandra	18.95±45.76	1.00	18.95	2	
Hakea bucculenta		25.20±45.76	1.13	22.30	2	
Isopogon cuneatus		8.76±45.76	1.0	8.76	1	
Macadamia integrifolia		15.42±45.76	1.14	13.53	2	Sedgley (1982/83)
Anacardiaceae						
Pistacia vera	Pistachio	54.05±45.76	1.45	37.26	1	Shuraki and Sedgley (1994)
Cucurbitaceae						
Citrullus lanatus	Watermelon	279.90±45.76	93.8	2.98	255.5	Sedgley and Buttrose (1978)
Graminae						
Triticum aestivum	Wheat	20.95±45.76	19.1	1.85	1	Saini et al. (1983)

Continued next page

Table 5.2: Mean number and ratio of transmitting tissue cells and pollen tubes at the base of the style and ovule number of species from ten angiosperm families *continued*.

Family and Species	Common Name	Transmitting tissue cells at base of style (±s.e.)	Pollen tubes at base of style	Ratio of transmitting tissue cells to pollen tube (cells/pollen tube)	Number of ovules	Reference
Lauraceae						
Persea americana	Avocado	127.35±45.76	3.05	41.75	1	Sedgley (1977)
Leguminosae						
Acacia baileyana	Cootamundra wattle	14.05±45.76	10.58	1.32	12	
Myrtaceae						
E. spathulata		196.51±45.76	120.9	1.63	39.9	Ellis and Sedgley (1992)
E. woodwardii		468.41±45.76	248.7	1.88	200	Sedgley and Smith (1989)
Rosaceae						
Prunus avium	Cherry	329.43±45.76	10.75	30.64	2	Granger (1995)
P. dulcis	Almond	263.57±45.76	3.44	76.62	2	
Santalaceae						
Santalum acuminatum	Quandong	128.61±45.76	2.4	53.59	2	Sedgley (1982a)
Solanaceae						
Petunia hybrida	Petunia	458.45±45.76	62.4	7.35	200+	

Table 5.3: Linear regressions comparing variables at the base of the style in nineteen species of angiosperms

Correlation	R^2
Pollen tube number and transmitting tissue area	0.687
Pollen tube number and intercellular space and cell wall area	0.289
Pollen tube number and ovule number	0.445
Pollen tube number and transmitting tissue cell number	0.418
Ovule number and transmitting tissue area	0.455
Ovule number and transmitting tissue cell number	0.646

R^2 values obtained from linear regression of data from Table 5.2 for those species where pollen tube and ovule numbers were available

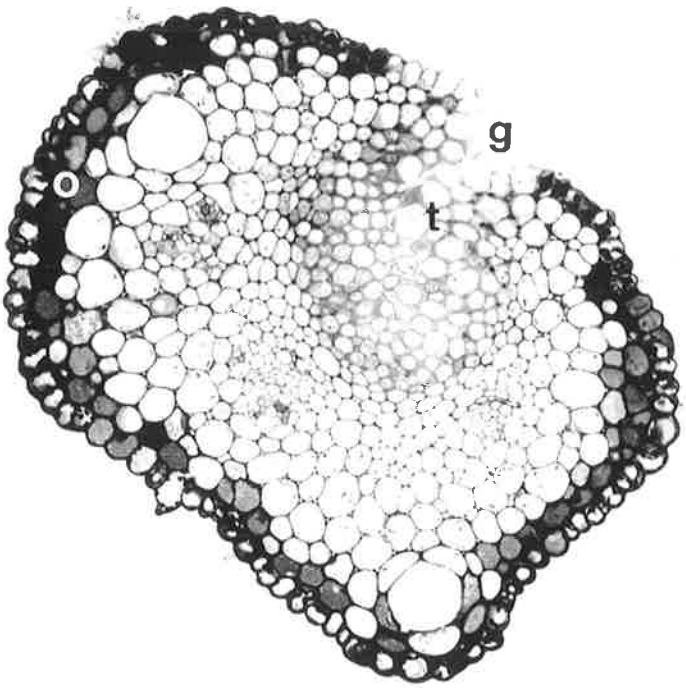
Plate 5.2

Light micrographs of *Persea americana* (Lauraceae) taken at the base of the style above the ovary. Sections stained with PAS and TBO

(a) Style showing polyphenol containing cells (o) associated with the epidermis. Note the skewed position of the transmitting tissue (t), one surface exposed in a groove (g) which runs the length of the style. Bar represents 50 μm.

(b) Style showing arrangement of transmitting tissue cells (t). Bar represents 50 μm.

a



b

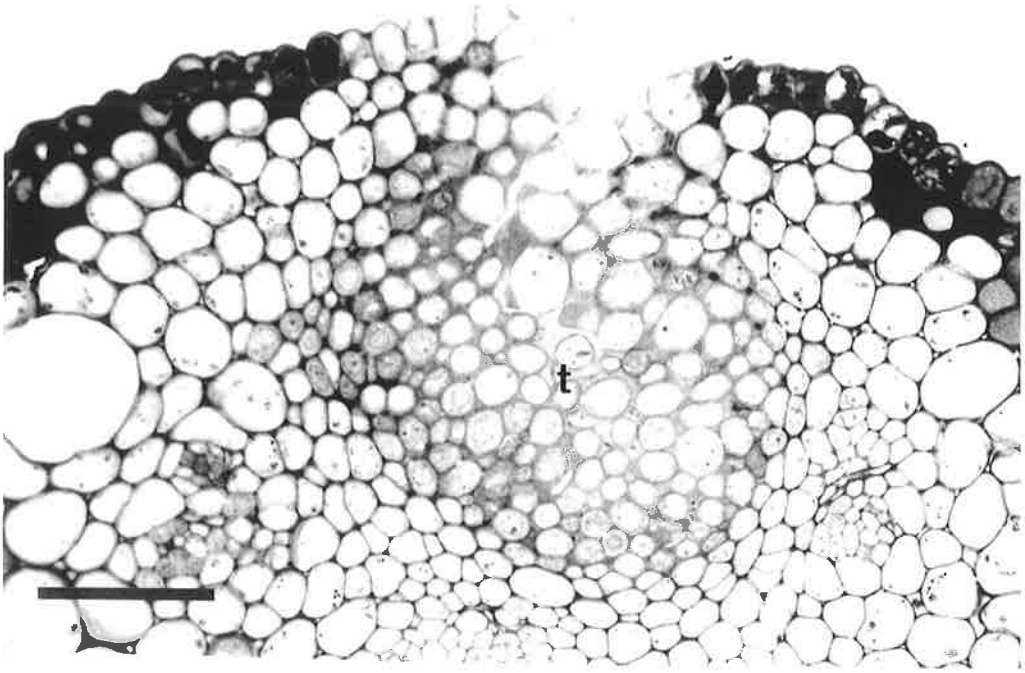


Plate 5.3

Light micrographs of species of the Cucurbitaceae family taken at the base of the style above the ovary. Sections stained with PAS and TBO

(a) Style of *Citrullus lanatus* (watermelon) showing lobed configuration of transmitting tissue (t) and vascular bundles (v). Bar represents 400 µm.

(b) *Citrullus lanatus*. Arrangement of transmitting tissue cells (t) and cortical cells containing starch grains (arrow). Bar represents 50 µm.

(c) Style of *Cucumis sativus* (cucumber) showing lobed configuration of transmitting tissue (t). Bar represents 500 µm.

(d) *C. sativus*. Arrangement of transmitting tissue cells (t). Bar represents 50 µm.

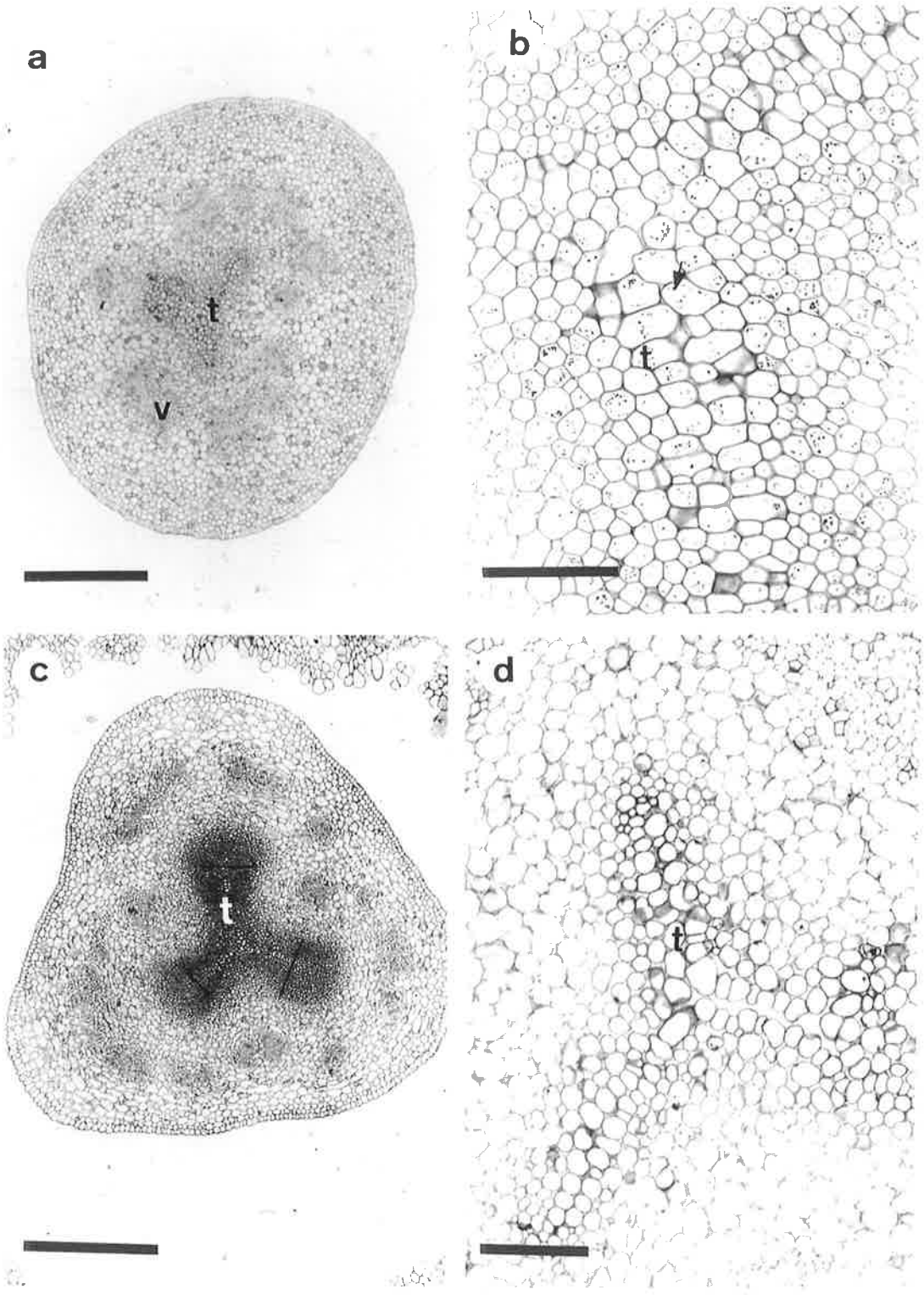

a
t
v
b
t
c
t
d
t

Plate 5.4

Light micrographs of species of the Rosaceae family taken at the base of the style above the ovary. Sections stained with PAS and TBO

(a) Styles of *Malus pumila* (apple). Note central core of transmitting tissue in each style (t) and hairs (arrowhead) associated with the base of the style. Bar represents 200 µm.

(b) Arrangement of transmitting tissue cells (t) within a style of *M. pumila*. Bar represents 50 µm.

(c) Styles of *Pyrus communis* (pear) showing central core of transmitting tissue (t) in each style, three vascular bundles (v) and hairs (arrow) associated with the base of the style. Bar represents 200 µm.

(d) Arrangement of transmitting tissue cells (t) within a style of *P. communis* and vascular bundles (v). Bar represents 50 µm.

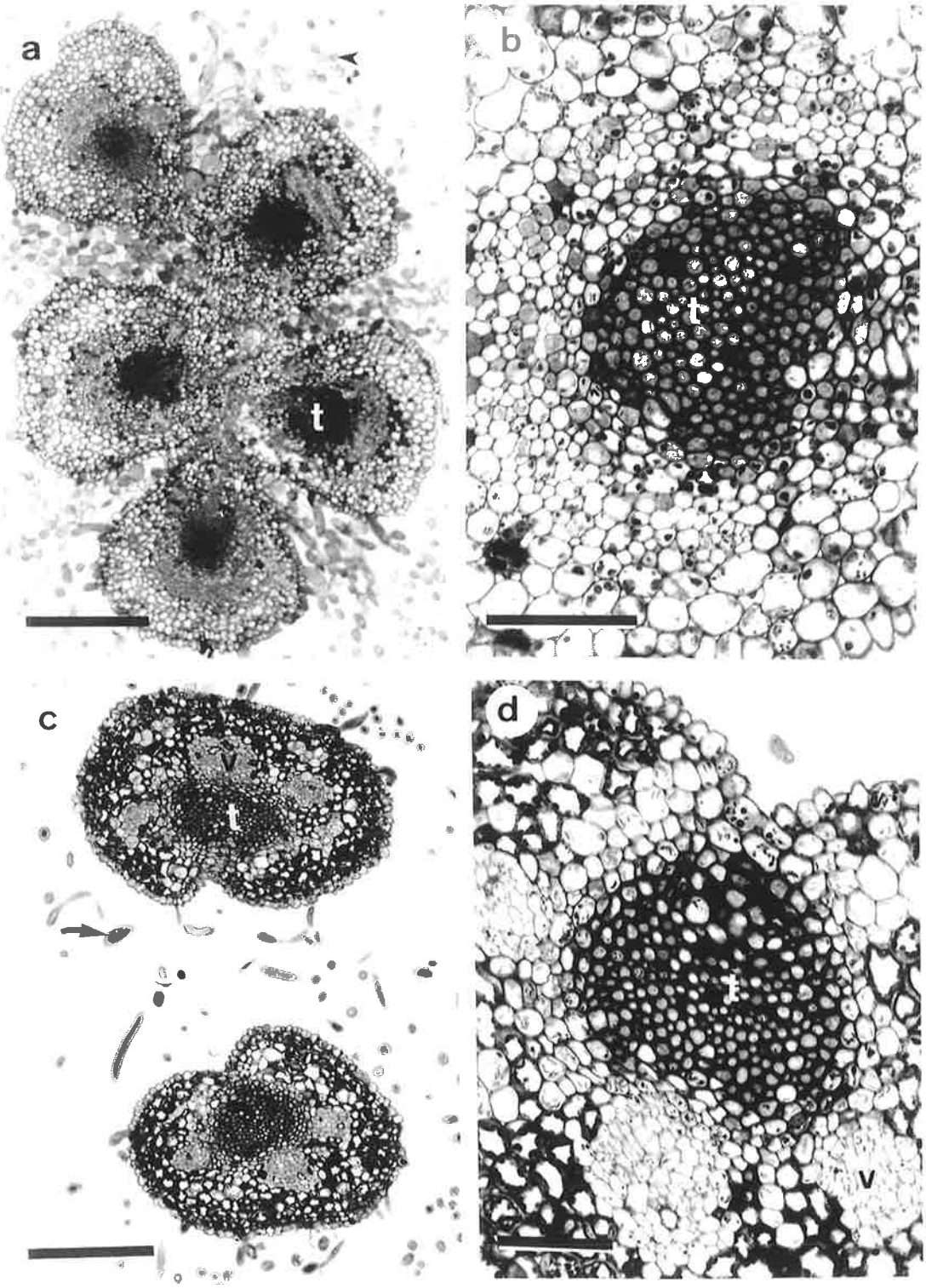

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Plate 5.5

Light micrographs of species of the Rosaceae family taken at the base of the style above the ovary. Sections stained with PAS and TBO

(a) Style of *Prunus avium* (cherry) showing transmitting tissue (t), vascular bundles (v) and cortical cells containing starch grains (arrowhead) and polyphenols (o). Bar represents 100 μm.

(b) *P. avium*. Transmitting tissue cells (t) containing starch grains (arrowhead) and surrounding cortex (c). Bar represents 50 μm.

(c) Style of *P. dulcis* (almond) showing transmitting tissue (t) and five vascular bundles (v). Bar represents 100 μm.

(d) Arrangement of transmitting tissue cells (t) in style of *P. avium*. Bar represents 50 μm.

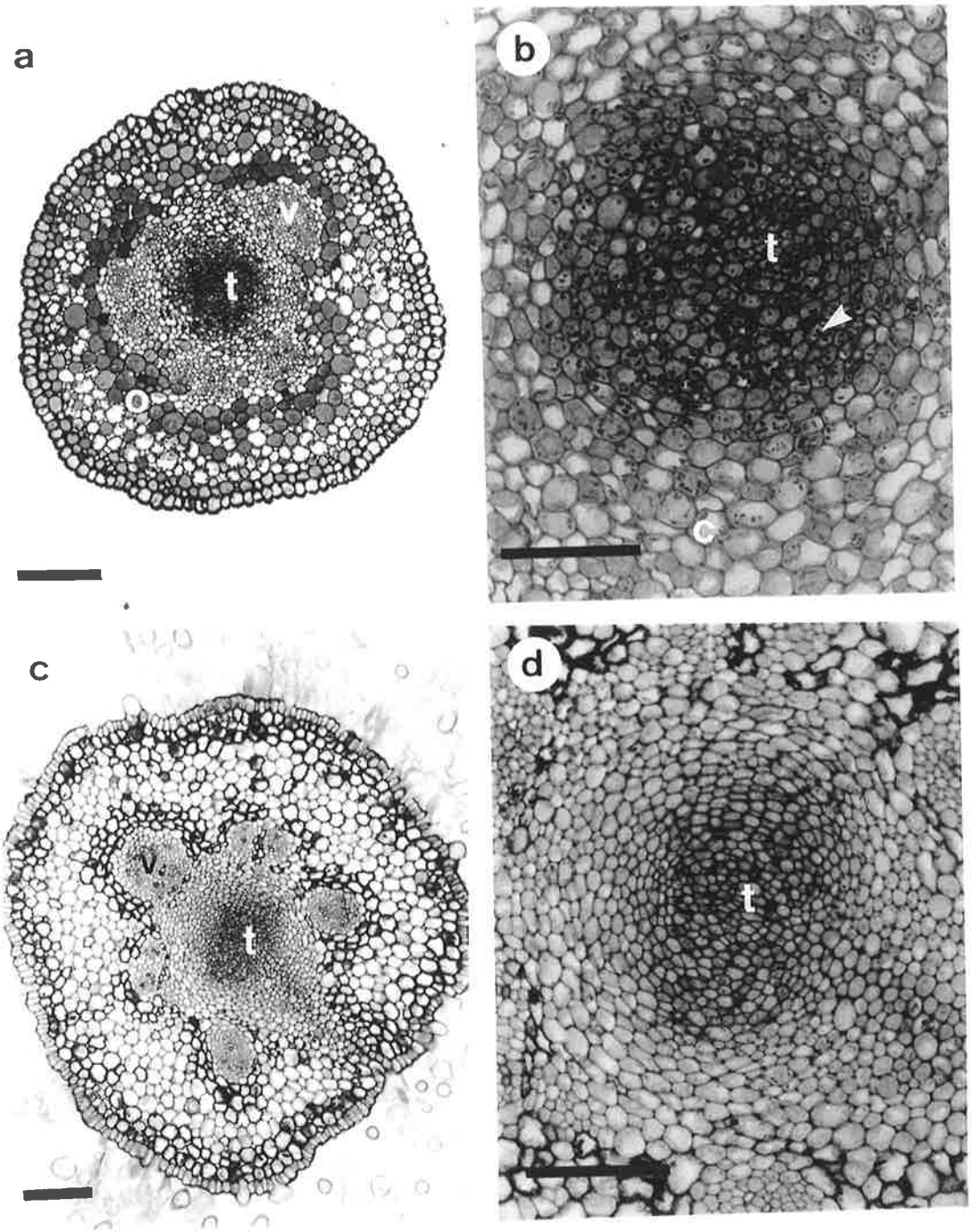
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Plate 5.6

Light micrographs of species of the Leguminosae family taken at the base of the style above the ovary. Sections stained with PAS and TBO

(a) Style of *Acacia baileyana* (Cootamundra Wattle) showing transmitting tissue cells (t) and enlarged epidermal cells containing polyphenols (o). Bar represents 50 µm.

(b) Style of *A. karroo* showing outer rings of polyphenol containing cells (o), cortical cells containing starch grains (arrowhead) and skewed position of transmitting tissue (t). Bar represents 50 µm.

(c) Style of *A. karroo* showing loose arrangement of transmitting tissue cells (t). Bar represents 50 µm.

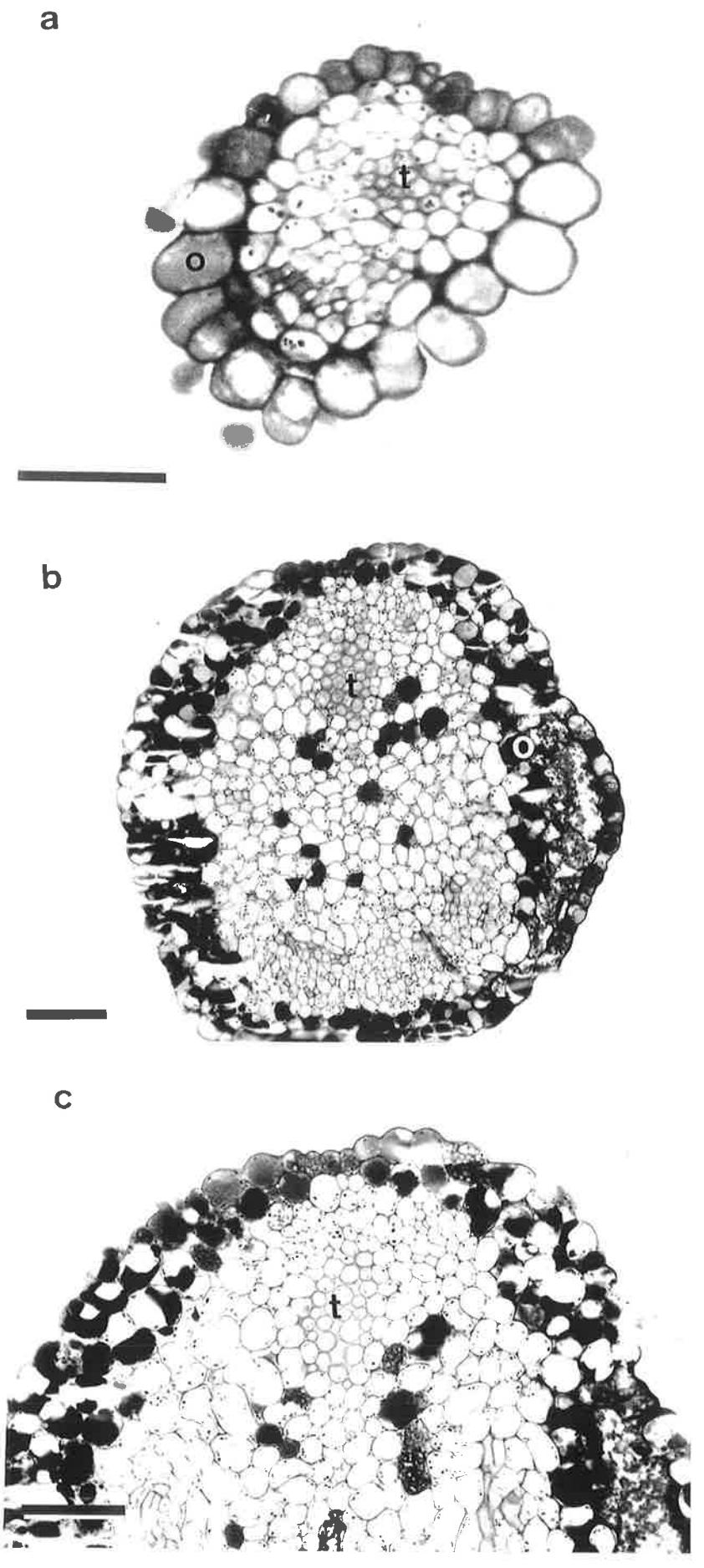
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Plate 5.7

Light micrographs of proteaceous species at the base of the style above the ovary. Sections stained with PAS and TBO

(a) *Banksia coccinea*. Note transmitting tissue (arrowhead). Bar represents 100 µm.

(b) *B. ericifolia*. Note transmitting tissue (arrowhead). Bar represents 200 µm.

(c) *Dryandra formosa*. Note transmitting tissue (arrowhead). Bar represents 100 µm.

(d) *D. nana*. Note transmitting tissue (arrowhead). Bar represents 200 µm.

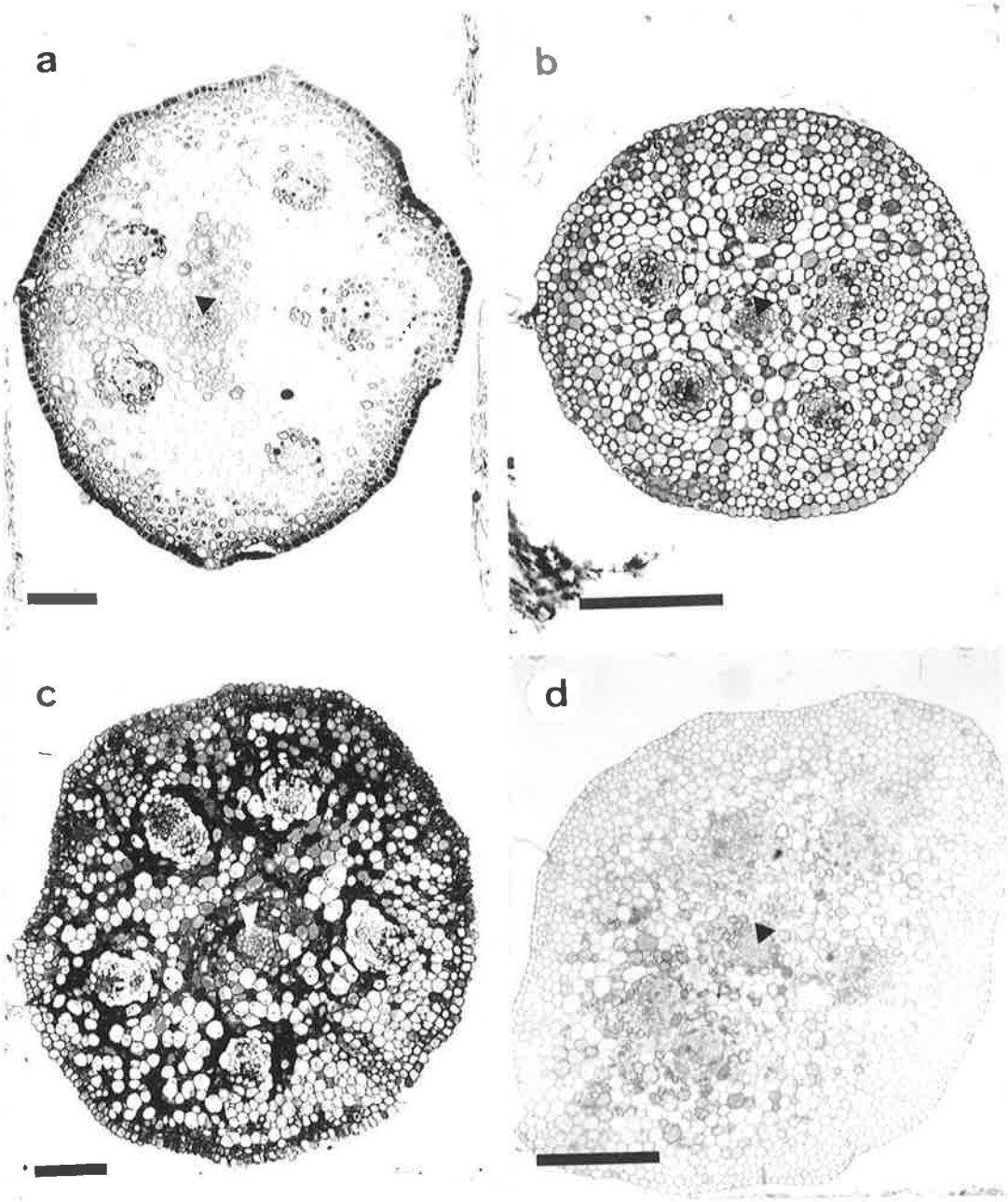
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Plate 5.8

Light micrographs of proteaceous species at the base of the style above the ovary. Sections stained with PAS and TBO

(a) *Dryandra quercifolia*. Note transmitting tissue (arrowhead). Bar represents 200 µm.

(b) *Hakea bucculenta*. Note transmitting tissue (arrowhead). Bar represents 200 µm.

(c) *Isopogon cuneatus*. Note transmitting tissue (arrowhead). Bar represents 50 µm.

(d) *Macadamia integrifolia*. Note transmitting tissue (arrowhead). Bar represents 200 µm.

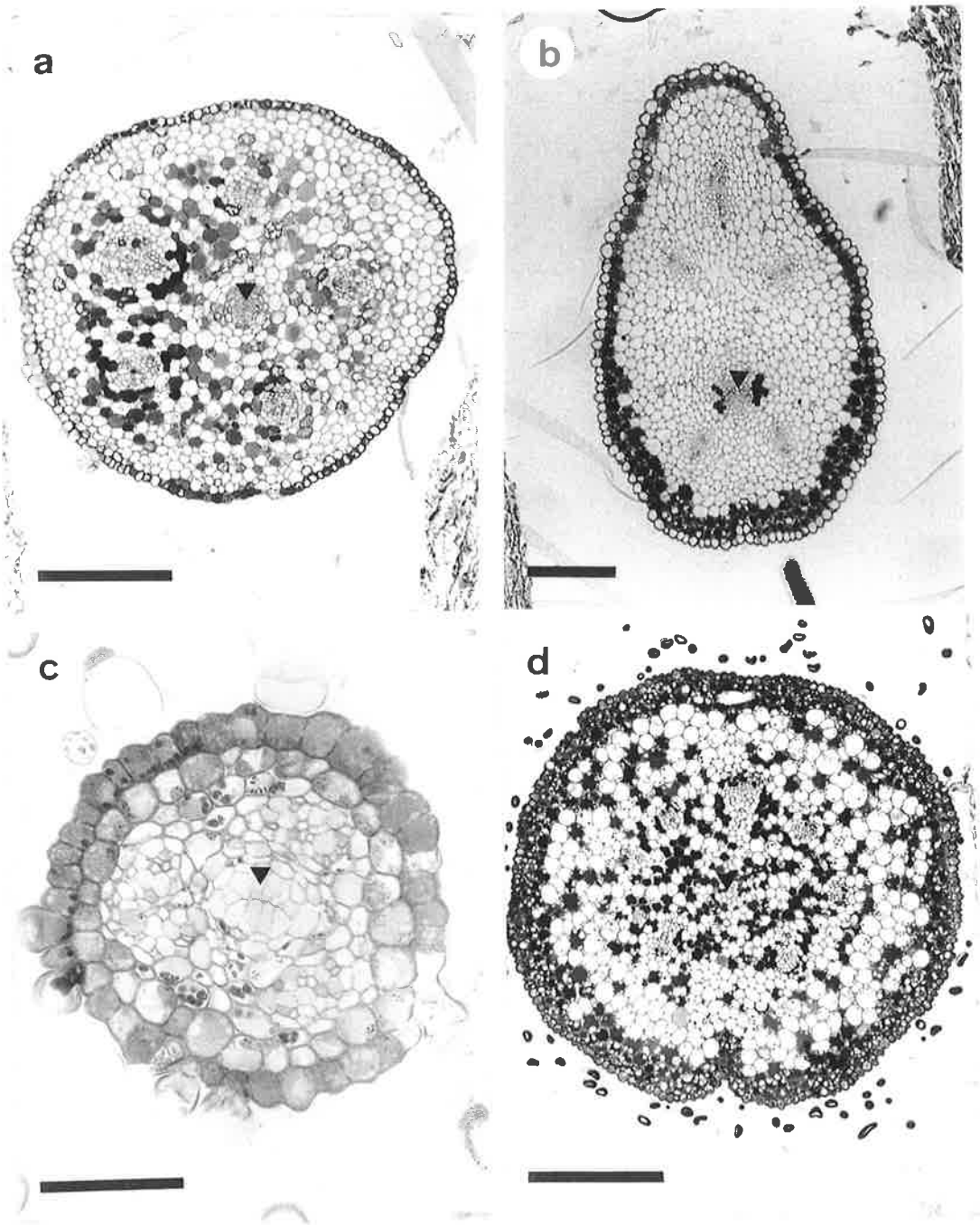
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Plate 5.9

Light micrographs of species of the Myrtaceae family taken at the base of the style above the ovary. Sections stained with PAS and TBO

(a) *Eucalyptus woodwardii* style showing transmitting tissue (t), ten vascular bundles (v) and oil glands (o). Bar represents 200 µm.

(b) *E. woodwardii*. Loosely packed cells of transmitting tissue (t) surrounded by polyphenol containing cells (o). Bar represents 100 µm.

(c) *E. leucoxylon* style showing transmitting tissue (t) and polyphenol containing cells of cortex (c). Bar represents 200 µm.

(d) *E. leucoxylon.* Note loosely packed cells of transmitting tissue (t). Bar represents 50 µm.

(e) *E. spathulata* style showing transmitting tissue (t) surrounded by cortex of polyphenol containing cells (c). Bar represents 100 µm.

(f) *E. spathulata* showing transmitting tissue (t). Bar represents 50 µm.

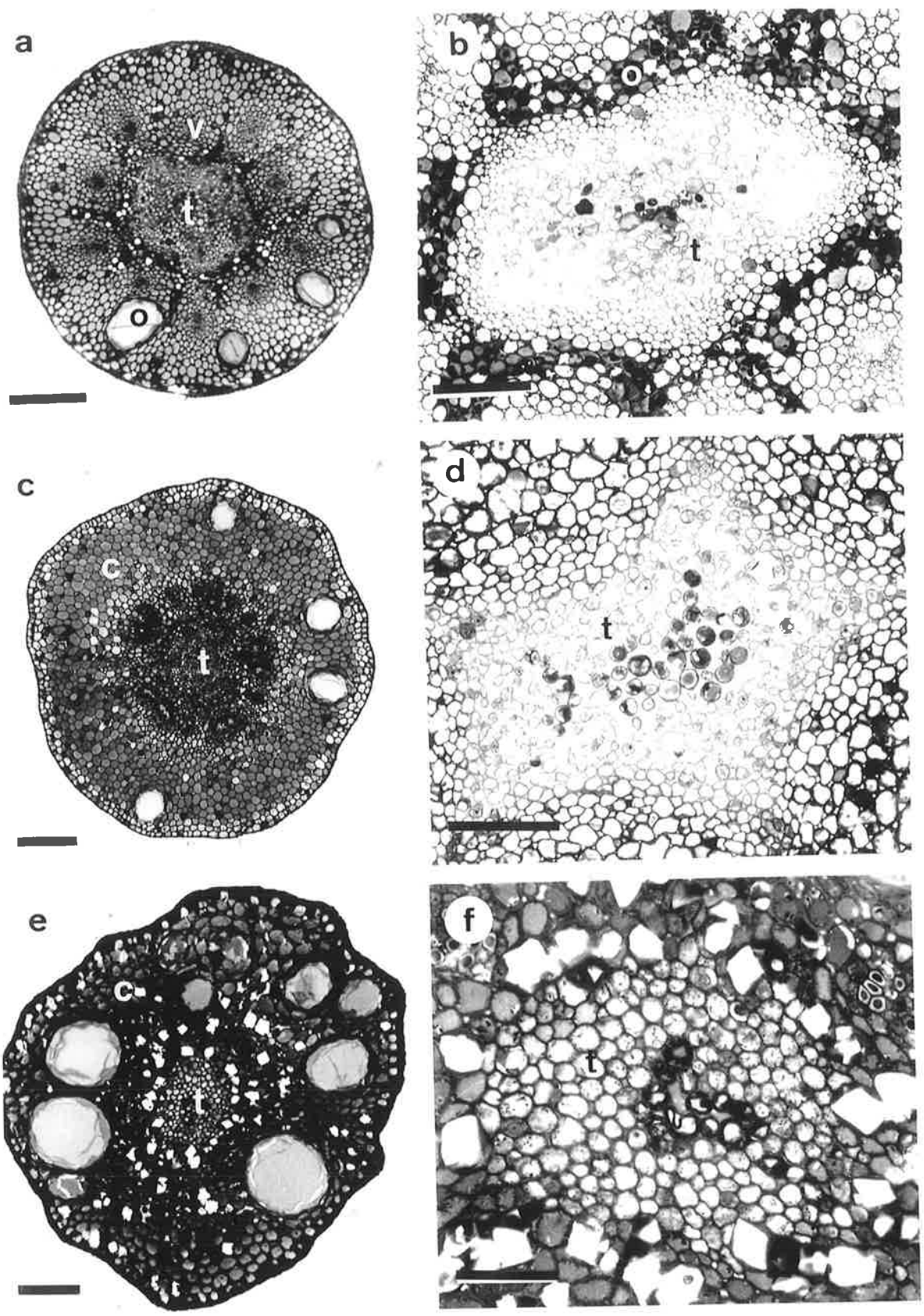
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Plate 5.10

Light micrograph of *Santalum acuminatum* (Santalaceae) taken at the base of the style above the ovary. Sections stained with PAS and TBO. Note thickened walls of transmitting tissue cells (arrowhead) surrounded by cortex of polyphenol containing cells (o). Bar represents 50 μm.

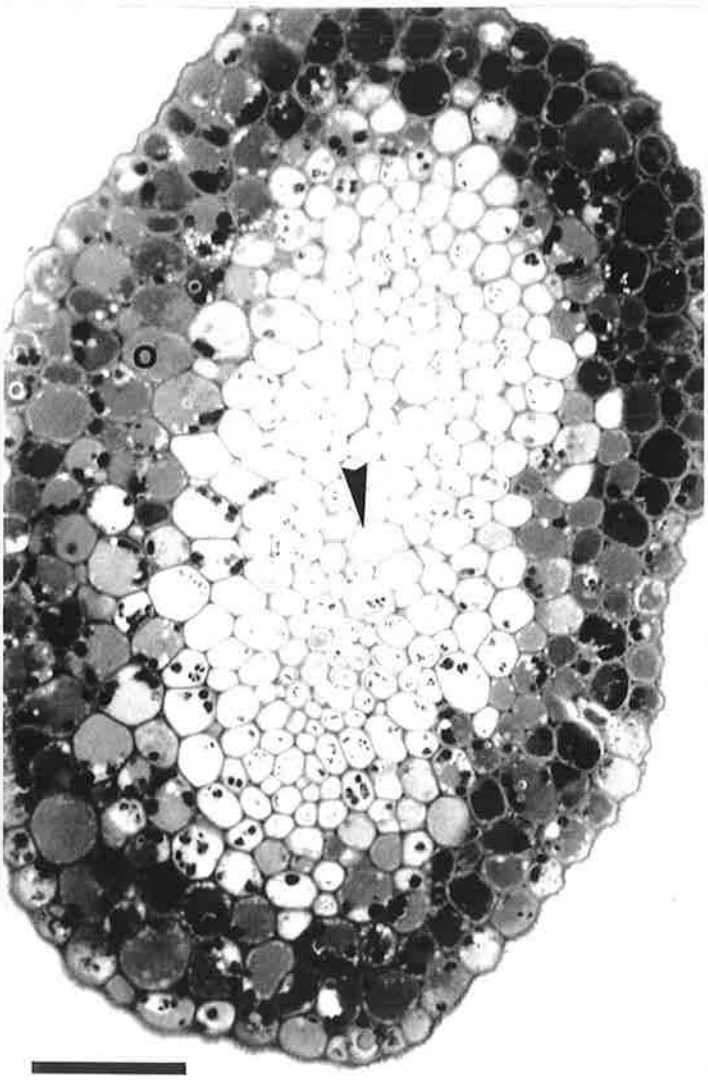

Plate 5.11

Light micrographs of *Pistacia vera* (Anacardiaceae) taken at the base of the style above the ovary. Sections stained with PAS and TBO

(a) Style showing columns of transmitting tissue cells (arrow) and region of enlarged cortical cells (e). Bar represents 100 μm.

(b) Transmitting tissue (t). Bar represents 50 μm.

(c) Enlarged cortical cells (e). Bar represents 50 μm.

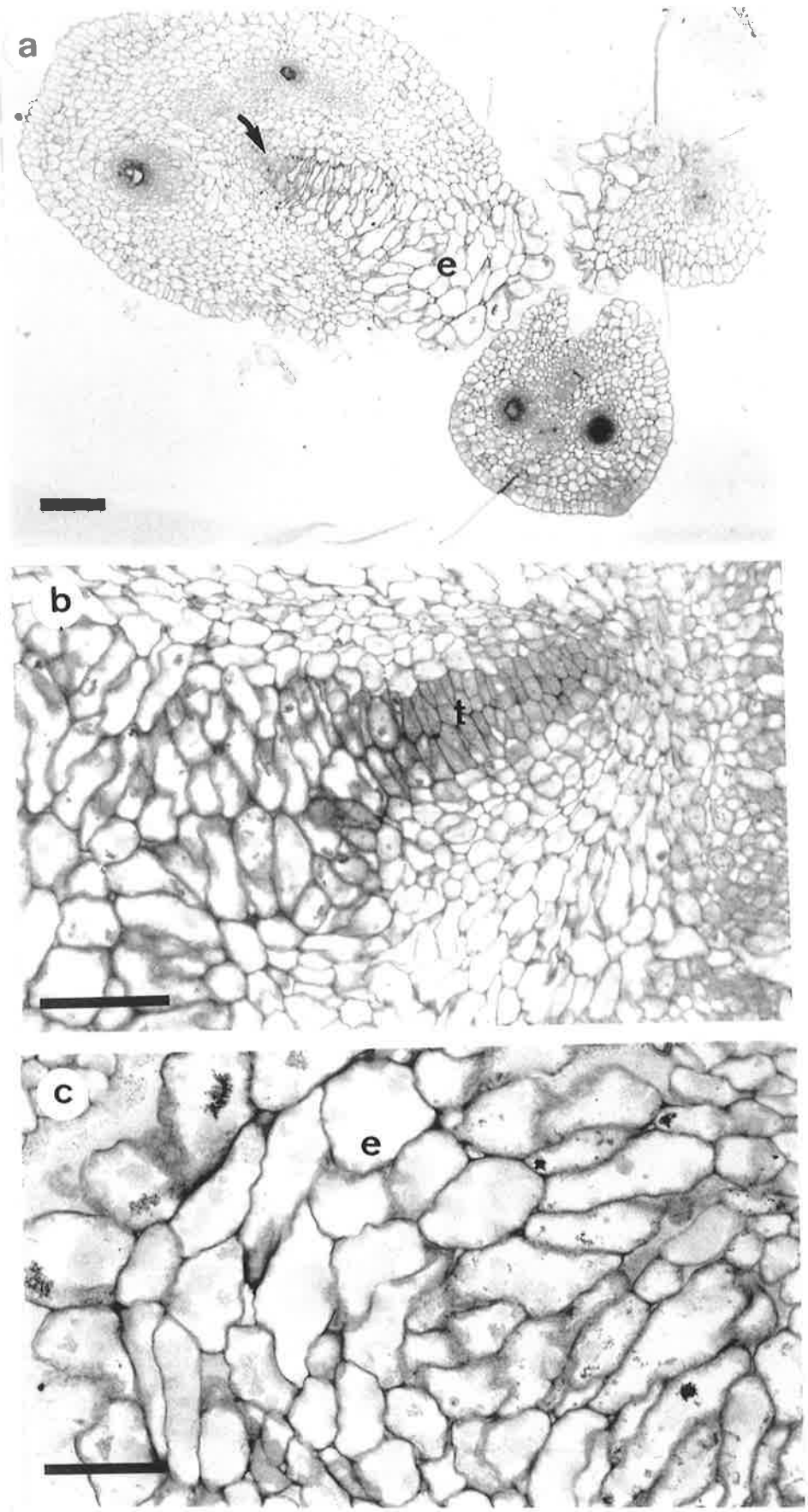
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Plate 5.12

Light micrographs of species of the Solanaceae family taken at the base of the style above the ovary. Sections stained with PAS and TBO

(a) Style of *Lycopersicon esculentum* showing multiple columns of transmitting tissue (t) surrounded by cells of the cortex (c). Bar represents 200 μm.

(b) Transmitting tissue (t) of *L. esculentum*. Note arrangement of cells and large intercellular spaces (i) filled with secretion. Bar represents 50 μm.

(c) Style of *Petunia hybrida* showing central column of transmitting tissue (t). Bar represents 100 μm.

(d) Transmitting tissue of *P. hybrida*. Note arrangement of cells and large intercellular space (i) filled with secretion. Bar represents 50 μm.

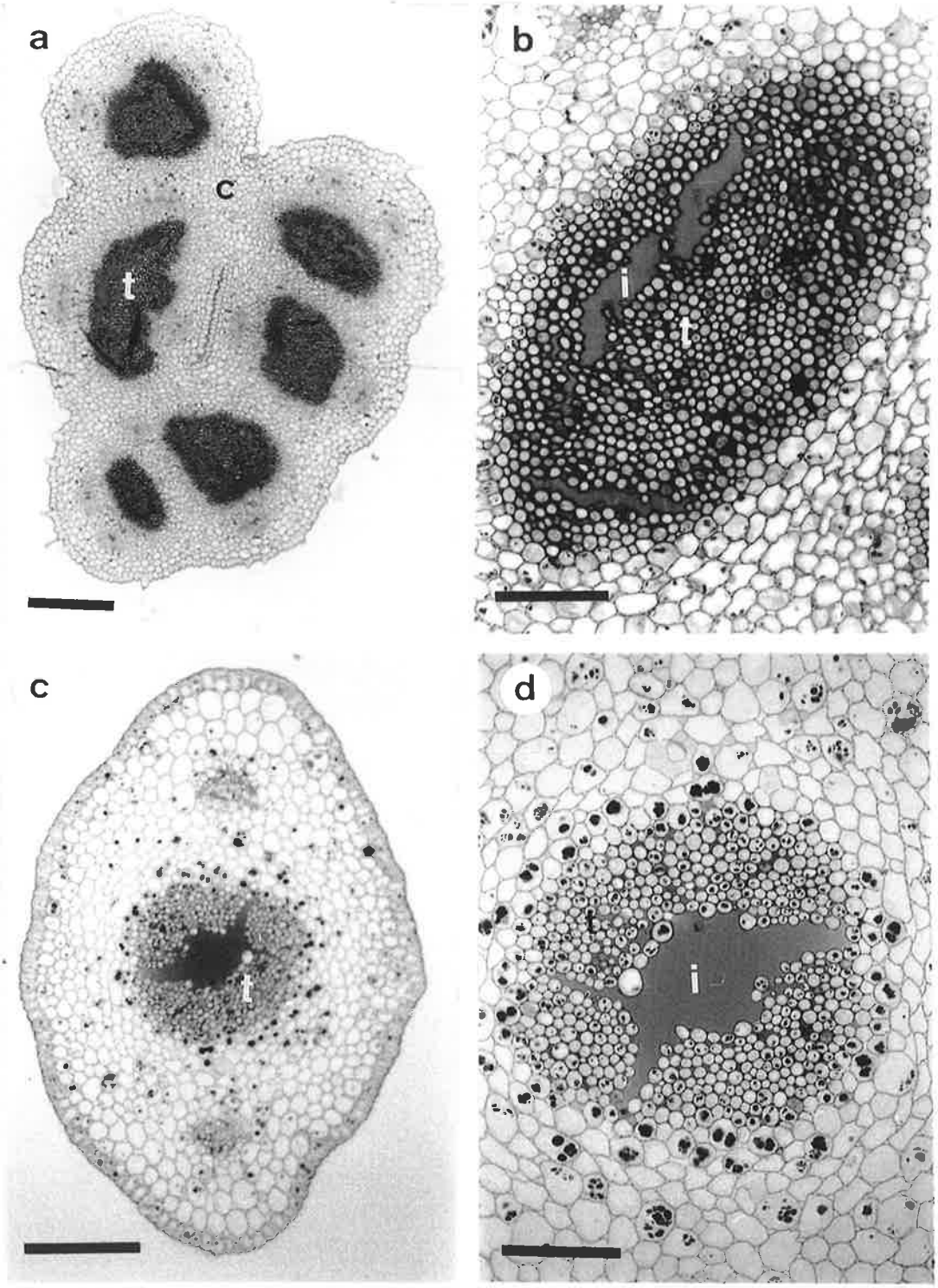
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Plate 5.13

Light micrographs of *Triticum aestivum* (Graminae) taken at the base of the style above the ovary. Sections stained with PAS and TBO

(a) Multiseriate branches of stigma showing enlarged cortical cells (e) and central column of transmitting tissue (t). Bar represents 50 μm.

(b) Arrangement of transmitting tissue cells (t). Bar represents 20 μm.

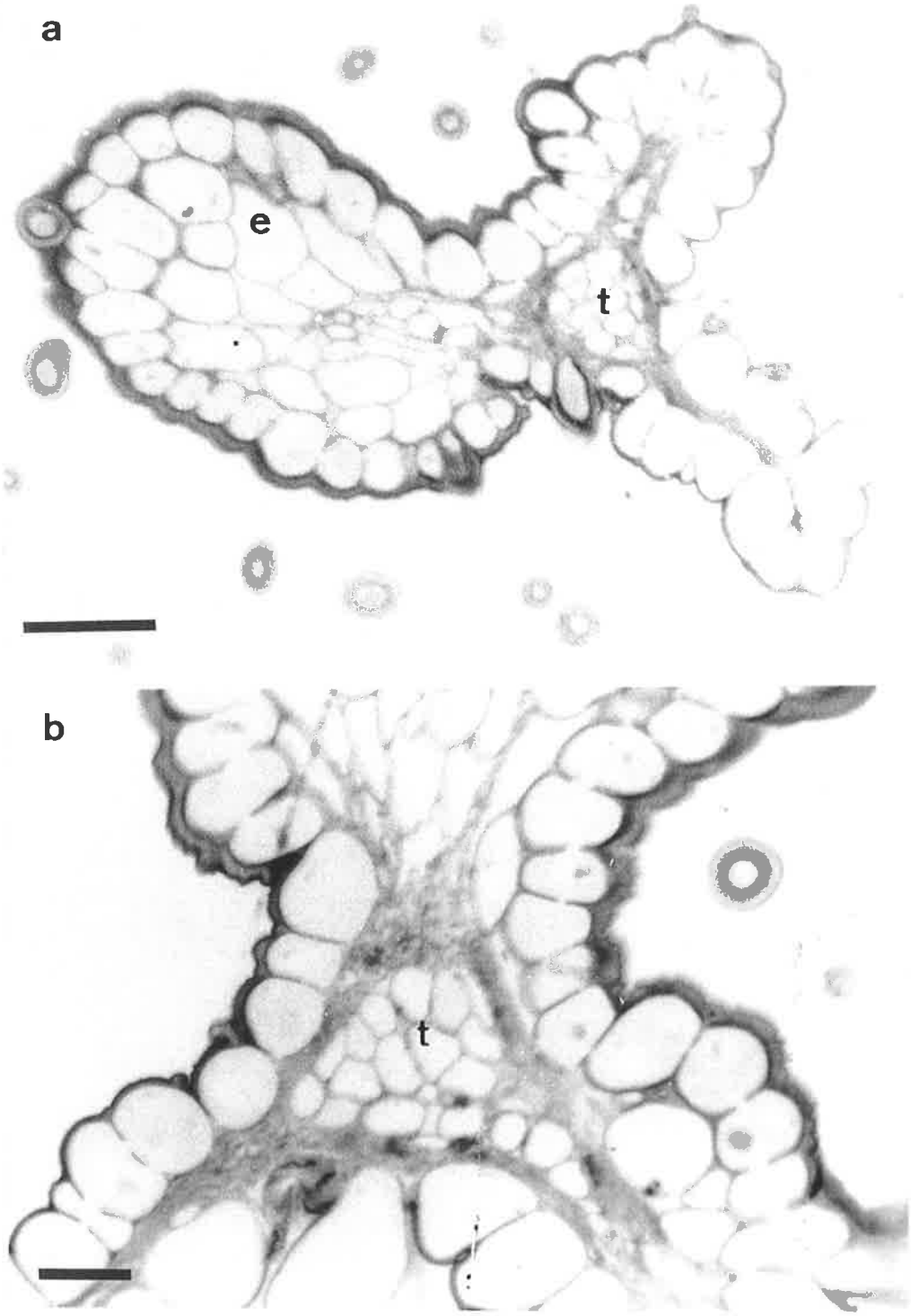
a
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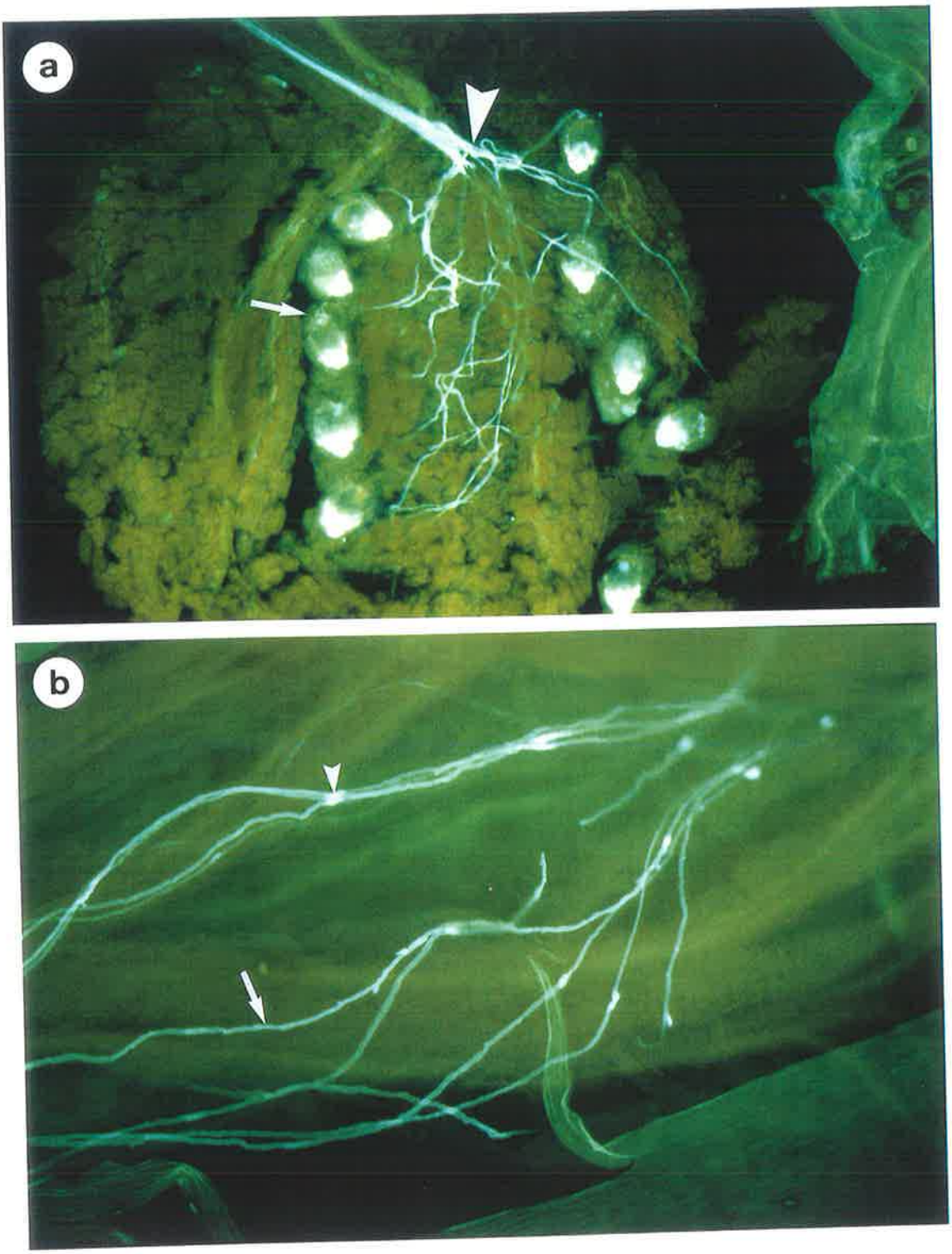
Plate 5.14

Fluorescence micrographs showing pollen tubes at the base of the style. Material stained with aniline blue.

(a) *Acacia baileyana* showing multiple pollen tubes at the base of the style (arrowhead) growing towards ovules (arrow)

(b) *Prunus dulcis* (almond) showing multiple pollen tubes (arrowhead) at the base of the style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Discussion

There was an overall consistency across species regarding the tissues of the style, although the structure, complexity and arrangement of cells varied. The presence in all species of vascular and transmitting tissue highlighted the importance of these tissues within angiosperm floral structure. Similar constituents have been reported many times and across a broad range of species (Cresti et al., 1976; Bell and Hicks, 1976; Nakanishi et al., 1991; Ciampolini et al., 1996).

Four arrangements of transmitting tissue were observed; a single, circular in cross-section column, a lobed column, multiple columns within a single style and multiple styles. *Malus pumila* and *Pyrus communis* both had multiple free styles, each supporting a single column of transmitting tissue which fused into a single ovary of multiple locules. The single style of *Lycopersicon esculentum* had multiple columns of transmitting tissue suggesting that possibly style number had been reduced over species evolution. Styles of the Cucurbitaceae had a single lobed column of tissue which implied a carry over of structure from a time when pistils were separate. *Eucalyptus* species had a single solid column of transmitting tissue which lead to a multi-loculed ovary. It is believed that multiple locules represents the fusion of carpels (Esau, 1965).

The progenitor of the carpel is proposed to be a curved sporophyll that has fused at the margins, enclosing the ovules (Bailey and Swamy, 1951; Hickey and Winship Taylor, 1996; Winship Taylor and Kirchner, 1996). The pistil of *Persea americana* had a single unlobed column of transmitting tissue and an ovary with one locule. Sedgley and Buttrose (1978) comment upon the evolutionary nature of the *Persea americana* style, which was neither open nor solid. Instead it had a groove that traversed the entire length of the style exposing the transmitting tissue. These authors hypothesise that due to the primitive nature of the Lauraceae family to which this species belongs, *Persea americana* may represent an intermediate stage between the conduplicate carpel of *Drimys* which is

thought to represent the primitive carpel, and the solid style of most dicotyledons *piperita* (Bailey and Swamy, 1951).

Regardless of the evolutionary nature of the styles observed, transmitting tissue was a consistent feature between species, however its quantity varied. Minimum areas were recorded for species of the Proteaceae, Leguminosae and Graminae, and very large areas recorded for the Cucurbitaceae, Solanaceae, Myrtaceae and Rosaceae. Upon closer inspection, differences in transmitting tissue area were the result of differences in cell number and not cell size. Cell size was relatively constant among species. Uwate et al. (1982) observed that during development of *Prunus avium* transmitting tissue, cells elongated at the expense of cell width. The authors believed that this occurrence was related to transmitting tissue function and possibly produced greater intercellular areas and exudate for pollen tube passage. The small area of cells in cross-section observed in this study also suggested a narrowing, rather than a broadening of cells. Smaller cells have a greater surface area than large cells and this may contribute to increased production of intercellular exudates for pollen tube growth.

Families could be divided into two groups based upon ovule number; multiple ovules (≥12) and one or two ovules. A comparison of the number of pollen tubes and number of transmitting tissue cells showed that in general terms, species with multiple ovules had a large area of transmitting tissue and correspondingly high numbers of pollen tubes. However, when transmitting tissue cell number to pollen tube ratio were compared, species with multiple ovules tended to have fewer cells per pollen tube than species with few ovules, many species having less than two cells per pollen tube. This suggests that the minimum number of transmitting tissue cells required to support one pollen tube for species with multiple ovules was closer to 1:1. Additionally, if structural limitations in the lower style are to be considered, they are more likely to occur in these species.

Pistacia vera, Persea americana and *Santalum acuminatum* had a high proportion of transmitting tissue but very few pollen tubes, with the number of pollen tubes loosely corresponding to the number of ovules. Could the number of ovules influence pollen tube

number? Sedgley (1976) observed that in *Persea americana* ovule number in fact appeared to influence pollen tube number. In this species the common ovule number was one and pollen tube attrition occurred until only one pollen tube reached the base of the style. In contrast, for embryos that contained two embryo sacs, eighteen percent of styles contained two pollen tubes.

The potential influence of ovule number over pollen tube number was apparent in *Acacia baileyana* which had multiple ovules, multiple pollen tubes and very few transmitting tissue cells at the base of the style. Newman (1934) observed more pollen tubes than ovules for this species, and in some cases more than one pollen tube was observed to penetrate a single ovule. In this species pollen grains formed a polyad of sixteen grains which produced up to sixteen pollen tubes (Newman, 1934). Individual pollen grain size was small, approximately 17μm in diameter, and the overall polyad size was correspondingly small (48.5-54.7 μm diameter, Wodehouse, 1935). Pollination of this species was very specific, one polyad fitting on the stigmatic cup of one style. Pollen tubes were thereby produced from a single compact source. The nature and size of the polyad and resultant pollen tubes may have allowed for the necessary passage of multiple pollen tubes down the style to fertilise the multiple ovules. Alternatively the production of a post-pollination exudate (Marginson et al., 1985) may have contributed additional nutrients for the growth of multiple pollen tubes.

Very little literature exists regarding pollen tube diameter and the space available for its passage. Scribailo and Barrett (1991a, 1991b) investigated the pollen tube pathway of *Pontederia sagittata*, a heterostylous monocotyledon with a hollow stigmatic canal. These authors calculated the number of pollen tubes that occupied the stylar canal of each floralmorph and found different packing constraints between floralmorphs. Although not directly comparable to species in this study, their results demonstrated that pollen tube width and available stylar area may vary.

Like *Acacia baileyana*, the monocotyledon *Triticum aestivum* had very few transmitting tissue cells and a high number of pollen tubes in the lower style, but unlike *Acacia*

baileyana, it had a single ovule. The pollen-pistil interaction of the Graminae is fundamentally distinct from other monocotyledons and dicotyledons. The primary distinguishing features are rapid germination and growth of the pollen tube (Heslop-Harrison, 1979a; Heslop-Harrison, 1979b) fuelled by its own nutrient source; an abundant supply of polysaccharide particles (P-particles) (Heslop-Harrison and Heslop-Harrison, 1982). The distribution, ultrastructure and development of the P-particles are distinct from the dictyosomes that develop in the pollen tubes of non-grasses. The P-particles often lack continuous bound membranes and their distribution is ubiquitous throughout the growing tube rather than concentrated at its tip (Knox, 1984), a phenomenon observed in other plants such as *Lycopersicon esculentum* (Cresti et al., 1977) and *Nicotiana elata* (Derksen et al., 1995). In addition, production activity of P-particles occurs in the later stages of pollen maturation in the anther, rather than after pollen germination (Heslop-Harrison and Heslop-Harrison, 1982). The transmitting tissue of the Graminae also differed from most angiosperms as it contains very little polysaccharide-rich secretion in its intercellular spaces (Heslop-Harrison, 1979a). The rapid autotrophic growth of pollen tubes could explain the observed high pollen tube numbers and low transmitting tissue numbers in the style of *Triticum aestivum.* They do not however explain why so many pollen tubes were present to fertilise one ovule, especially when reports have shown other Graminae species to have high pollen tube number in the style, but only one pollen tube reaching the ovary (Heslop-Harrison et al., 1985; Rudramuniyappa and Panchaksharappa, 1974). In *Zea mays* a number of structural features in the pistil contribute to the progressive reduction of pollen tube number towards the ovary. These features include competition between pollen tubes at the stigmatic trichomes and within transmitting tissue, elimination of late-entering tubes at the abscission zone of the stigma, and a constricted zone of transmitting tracts in the upper ovary wall and in the vicinity of the micropyle (Helsop-Harrison et al., 1985). It is possible that for *Triticum aestivum* these constrictions occur closer to the ovary, pollen tube number remaining high at the base of the style.

Ovule number did not appear to relate to pollen tube number for three eucalypt species, researchers observing more pollen tubes in the lower style than the number of ovules

present (Ellis & Sedgley, 1992). These authors suggested that the control of offspring numbers (mortality) may be a post-zygotic event. They also noted that the reduced number of pollen tubes in the lower style was partially due to reduced carrying capacity of the transmitting tissue in this region.

The main feature that distinguished the Proteaceae from other species studied was the proportion of transmitting tissue relative to the rest of the style. Most species had high proportions of transmitting tissue (up to 58%), whereas for the Proteaceae, transmitting tissue was a very small component of the whole stylar structure, less than 3.5%. The dual nature of the proteaceous pistil for pollen tube passage and pollen presentation may be responsible for this.

The low proportion of transmitting tissue in the Proteaceae observed in this study was consistent with observations of other proteaceous species, such as *Macadamia* spp. (Sedgley et al., 1985) and *Grevillea banksii* (Herscovitch and Martin, 1989; 1990). Clifford and Sedgley (1993) suggested that the narrowing of transmitting tissue acted as a physical pollen tube filter. Very little specific research has been performed regarding the minimum transmitting tissue cell number required to support the growth of a single pollen tube. For *Banksia menziesii* the ratio of transmitting tissue cells to pollen tubes was suggested to be 11:1 (Clifford and Sedgley, 1993). For the proteaceous species studied, cell number was higher overall than *B. menziesii* and varied from 8.8 cells for *Isopogon cuneatus* to 54.5 cells for *Dryandra nana*. The passage of two pollen tubes is known to be possible for *Dryandra quercifolia* and *D. formosa* (Chapter Two), as both species produced follicles containing two seeds, although the most frequent observation was one seed per follicle. *Banksia* is also known to form one (Vaughton, 1988) or two seeds (Scott, 1982). Production of two seeds per ovary has been observed for *Grevillea banksii* (Herscovitch and Martin, 1989). In the case of *Macadamia*, the development of one ovule suppressed the development of the second and resulted in the production of one seed only (Sedgley, 1982/83b).

In the previous chapter the transmitting tissue of proteaceous species was shown to be reduced from the pollen presenter to the style. Herscovitch and Martin (1989) hypothesised that pollen tube number was limited by the carrying capacity of the transmitting tissue and that the narrowing of this tissue, as seen for instance in *Grevillea banksii,* may provide a simple mechanism serving to reduce pollen tubes to a number equivalent to ovule number. Reduced transmitting tissue was believed to influence pollen tube number in other studies (Marginson et al., 1985; Clifford and Sedgley, 1993; Winsor and Stephenson, 1995). However in comparison to other families the Proteaceae overall appear to have a high ratio of transmitting tissue cells to pollen tubes, particularly compared to species with multiple ovules.

In dicotyledons the size of the pollen grain and its endogenous nutrients may affect the distance the pollen tube can travel fuelled by its own resources, and the degree to which it must rely upon the transmitting tissue for a supply of nutrients. The Proteaceae have very long styles in comparison to other families studied. For example, the style of *Dryandra nana* was up to seven cm long (Chapter Four). It is possible that the length of the style and the resultant distance pollen tubes must travel to reach the ovary influenced the number of transmitting tissue cells required to support the growth of one pollen tube. Cruden and Lyon (1985) found that for species of the Umbelliferae and Cruciferae, and for species of *Solanum,* pollen grain size and stigma depth were correlated, the pollen tube growing autotrophically through the stigma until reaching the transmitting tissue where often it changed to heterotrophic growth. Correspondingly, these authors found that pollen grain size and style length did not correlate. Style length however was found to effect interspecific crosses in *Rhododendron*; the pollen tube unable to reach the ovary when a short-styled male parent was crossed with a long-styled female (Williams and Rouse, 1988). These authors suggested that limited nutritional reserves within the pollen grain, combined with limited access to stylar reserves and pre-programming to a finite pollen tube length correlating with style length, may have limited pollen tube growth. In these cases, style length prevented interspecific fertilisation and maintained species isolation. Although not directly related to the current study, these results indicate that

pollen tube growth was affected by style length. This may help to explain the high transmitting tissue cell ratio observed for the species of the Proteaceae, the pollen tubes relying more heavily on multiple transmitting tissue cells for nutrients to grow the extended distance. Thus the ratio of transmitting tissue cells to pollen tubes suggested by Clifford and Sedgley (1993) for *B. menziesii* may in fact reflect the number of cells required for this species.

This study has shown that great diversity exists between families in stylar structure, quantity of transmitting tissue and complexity of the tissue. The consistent presence of vascular and transmitting tissues reflected the importance of these tissues in angiosperm floral structure and function. Two groups were formed based on ovule number. Species with multiple ovules were potentially limited by their transmitting tissue, many species with a transmitting tissue cell to pollen tube ratio of 1:1. However, no conclusions could be drawn regarding structural limitation, as pollen tube number was generally sufficient to fertilise the number of ovules present. Pollen tube number appeared to relate to ovule number in many cases. Often species had pollen tube numbers equivalent to the number of ovules present, regardless of the abundance of transmitting tissue. The ratio of transmitting tissue cells to pollen tubes was much higher for species with few ovules. *Triticum aestivum* was the only exception, with many pollen tubes, very few transmitting tissue cells and a single ovule. The distinct characteristics of the pollen-pistil interaction of the Graminae may be responsible for these differences. The Proteaceae were similar to other species at the stylar level, however they had a reduced proportion of transmitting tissue relative to the whole style. The length of their styles, a feature setting them apart from the other families studied, may be responsible for the high proportion of transmitting tissue cells to pollen tubes, and for the low numbers of pollen tubes observed in the lower style.

Chapter Six

General Discussion

The applied and fundamental research conducted in this study has contributed to our understanding of certain features of the breeding systems of the Australian Proteaceae, whilst the comparison to other angiosperms has provided some perspective on these features. Combined, these results improve our understanding of the Proteaceae, a family of both economic and environmental importance.

The Proteaceae have a range of breeding strategies, and species vary in the degree of self-compatibility or -incompatibility they exhibit. For example, species such as *Banksia ericifolia* (Goldingay et al., 1991; Carthew et al., 1996), *B. prionotes* (Collins and Spice, 1986), *B. spinulosa* (Carthew et al., 1996), *Grevillea robusta* (Kalinganire et al., 1996) and *Macadamia* spp. (Sedgley et al., 1985) are strongly self-incompatible. In contrast, *G. barklyana* (Harriss and Whelan, 1993), *B. spinulosa* var. *neoanglica* (Vaughton, 1988) and *B. brownii* (Sampson et al., 1994) are largely self-compatible.

The *Dryandra* species studied; *D. quercifolia* and *D. formosa,* share a mixed breeding system, one where both self- and cross-pollen successfully produce pollen tubes and set seed (Chapter Two). The primary incompatibility mechanism that discriminated self- from cross-pollinations was post-zygotic abortion of at least one seed. This syndrome is relatively common in hermaphroditic plants, often serving to increase progeny fitness (Latta, 1995; Burbridge and James, 1991). Stylar discrimination of self-pollen tubes was also observed for *D. quercifolia.* Previously researchers have reported such an occurrence in *Macadamia* spp. and *Banksia coccinea* and suggested that a gametophytic self-incompatibility mechanism was responsible (Sedgley et al., 1985; Fuss and Sedgley, 1991b). Results from this study suggest another possible control which will be discussed in relation to pistil structure.

Critical to the process of pollination, particularly for successful commercial plant breeding and seed production, is knowledge of the timing and process of stigma receptivity. In the Proteaceae, the complexity of stigma receptivity should not be underestimated, as it is a combination of both morphological and physiological changes. These changes include groove opening, exudate production and pollen germination. In contrast to previous studies, this investigation combined each of these aspects to determine the time of stigma receptivity (Chapter Two). For *Dryandra quercifolia* and *D. formosa*, there were both similarities and differences with regard to stigma receptivity. Both species were protandrous, and maximum pollen tube germination occurred two to six days after anthesis. However, their pattern of groove opening and exudate production was very different. Also, *D. formosa* had very low pollen tube numbers in comparison to *D. quercifolia*. The groove of *D. quercifolia* opened progressively and remained open, almost gaping for the entire study period. Exudate was produced during this time. In contrast, the *D. formosa* groove displayed precise opening and closing and exudate production four days after anthesis. Such stigmatic behaviour may have potential implications upon the fertility of this species.

The differences in stigmatic behaviour, very low pollen tube numbers, and seeds set relative to the number of flowers produced, prompted further investigation of the pollen presenter (Chapter Four). In particular, the stigmatic cavity appeared to have the potential to limit pollen grain access to the stigma and may have contributed to the observed low fertility. Cavity size and type, and pollen grain size influenced the theoretical holding capacity of the cavity. For example, genera such as *Banksia* and *Dryandra* were particularly limited by their cavity size and configuration, whereas the tubular and protruding papillae configuration of *Isopogon* and *Macadamia* supported a much higher number of pollen grains. Although for the species studied the theoretical pollen holding capacity of the cavity was greater than the number of ovules possessed by that species, its potential influence on the fertilisation process should not be overlooked.

The *Dryandra formosa* pollen presenter was a good example of the influence of the cavity on fertility. Anatomical and morphological investigations of the pollen presenter provided

a potential explanation for the low pollen tube number observed for *D. formosa*. This species has pronounced cuticularised lips around the cavity, a small cavity diameter and a large pollen grain size relative to the cavity. Combined with reduced exudate production and cavity opening for a restricted period of time, these features would severely limit pollen grain access to the stigma. The cuticularised lips appeared to interfere with the ability of the pollen grain to "stick" to the cavity region.

The general morphology and anatomy of the pollen presenters of the eight species studied reflected their taxonomic relationships (Chapter Four). The most closely related genera, *Banksia* and *Dryandra,* more closely resembled each other in gross morphology and form and were similar anatomically, within the pollen presenter.

The species of *Banksia* and *Dryandra* had high quantities of sclerenchyma tissue within the pollen presenter and style which gave rigidity to the pistil. This tissue was absent in the pistils of *Isopogon, Hakea* and *Macadamia* and may reflect the different pollinator regimes. *Dryandra* and *Banksia* are generally pollinated by small marsupials and birds, and the others by birds and insects. Investigation of the pollination ecology in relation to pistil structure would provide further insight into this feature.

A major part of this study focussed upon the quantity and distribution of transmitting tissue within the style (Chapter Four) and differences between proteaceous species and other angiosperms (Chapter Five). Previous investigations of *Macadamia* (Sedgley et al., 1985), *Grevillea banksii* (Herscovitch and Martin, 1989, 1990) and *Banksia menziesii* (Clifford and Sedgley, 1993) showed that the quantity of transmitting tissue decreased significantly from the pollen presenter to the base of the style. These studies hypothesised that this decrease structurally limited fertility by physically reducing the number of pollen tubes reaching the ovary and fertilising the ovules. The low seed set observed in *Dryandra quercifolia* and *D. formosa* prompted further investigation of these species and other proteaceous species of current or potential economic importance. The results obtained from this study support the observed reduction of transmitting tissue reported in previous studies. The greatest reductions occurring from the tip of the pollen presenter to its base

and from this point to the upper style. From the upper style to the base of the style, transmitting tissue cell number decreased, however this decrease was less severe than the decreases observed in the pollen presenter and upper region of the style.

Another notable feature that distinguished species was the presence or absence of transfer tissue. Transfer tissue was present in the pistils of all species except *Hakea bucculenta*. The invaginated wall of transfer cells serves their primary function in the secretion or absorption of nutrients. Transfer tissue was consistently associated with the transmitting tissue. Knowledge of the importance of transmitting tissue to the nutrition of pollen tubes is well established (Jensen and Fisher, 1969; Cresti et al., 1976; Herrero and Dickinson, 1979; Herrero, 1992), and the association of transfer tissue with transmitting tissue suggests some role in the provision of nutrients to the growing pollen tube. In particular, transfer tissue was found in the pollen presenters of species that had reduced amounts of transmitting tissue compared to *H. bucculenta*. It is suggested that transfer tissue assists in the secretion of nutrients in those species with smaller quantities of transmitting tissue. In the case of *H. bucculenta*, such supplementation was not required. In addition, the combined effect of reduced transmitting tissue cells and transfer tissue from the pollen presenter to upper style may have influenced pollen tube growth and number, particularly of less competitive tubes such as self-pollen tubes in a largely self-incompatible plant. Although firm conclusions could not be drawn from this study, these changes may influence available nutrients for pollen tube growth, and spatially limit pollen tube passage.

Collins and Rebelo (1987) and Herscovitch and Martin (1989) suggested spatial limitations as a contributor to the low fertility observed in the Proteaceae. In particular Herscovitch and Martin (1989) focussed their study on *Grevillea banksii* pistils. These authors suggested that regardless of how many pollen grains germinated on the stigma, pollen tube number was reduced to the maximum carrying capacity of the transmitting tissue tract. With only two ovules to fertilise, the narrowing of the transmitting tissue tract would provide a simple mechanism selecting the fastest growing, and most vigorous, pollen tubes to effect fertilisation. Their field experiments consistently showed only two pollen tubes at the base of the style. This observation supports the effect of narrowing of transmitting

tissue tract in the upper style but also implicates the number of ovules as a factor influencing the number of pollen tubes. The reproductive capacity of the flower is governed by the number of ovules it possesses. In a later study, Clifford and Sedgley (1993), suggested that the ratio of transmitting tissue cells to pollen tubes for *Banksia menziesii* was 11:1. In combination, these observations raise two questions: how many transmitting tissue cells are required to support the growth of one pollen tube? Does the number of ovules influence the ultimate number of pollen tubes reaching the ovary? Sedgley (1976) suggested that this was so for *Persea americana* (avocado).

Results from the study of transmitting tissue of proteaceous and other angiosperm species (Chapter Five) showed that species with multiple ovules (≥12) also had large quantities of transmitting tissue, and a correspondingly large number of pollen tubes. When the ratio of transmitting tissue cells to pollen tubes was determined, species with multiple ovules had a ratio of approximately 1:1, yet generally sufficient pollen tubes reached the base of the style to fertilise the ovules. In line with this, the number of pollen tubes reaching the base of the style in species with few ovules (≤2) was also in the range of the number of ovules that were present. However, the ratio of transmitting tissue cells to pollen tubes was significantly higher for these species. Therefore to imply structural limitations to fertility based upon transmitting tissue cell number at the stylar base, those species with multiple ovules are more likely to be structurally limited than those species with few ovules, such as the Proteaceae. However, species may differ in the number of transmitting tissue cells required for successful pollen tube growth. Reduced pollen tube numbers in the styles of proteaceous species suggests that these species require a high ratio of transmitting tissue cells to pollen tubes in comparison to other families. This may be a result of the low proportion of transmitting tissue within the style, and the length of the style.

The evidence acquired by this study (Chapter Five) supports the influence of ovule number upon pollen tube number at the base of the style. *Triticum aestivum* was the only exception, multiple pollen tubes travelling down a very narrow style to fertilise one ovule. The reduced reliance upon transmitting tissue for nutrition to sustain pollen tube growth in the Graminae is most likely responsible for this observation.

The results from the seed germination experiments (Chapter Three) have confirmed a simple technique to increase germination and propagation of species. The exposure of seeds to an incubation temperature of 15°C markedly improved germination and germination rate. This temperature, combined with the recent discovery of the promotional effect of smoke upon germination of Australian species, may help to revolutionise propagation of many Australian species that in the past have been difficult to germinate. Further investigation of a broader range of species would help to increase our knowledge of the complex interactions that influence seed germination.

We can conclude from this study that both diversity and similarity of breeding systems exists within the Proteaceae. In addition, that the proteaceous breeding system has unusual features when compared to other angiosperms. These features include the anatomical complexity of the pollen presenter and the low proportion of transmitting tissue relative to the total style. The structure of the pistil potentially influences the fertility of a given species by limiting pollen grain access to the stigma, a result of the stigmatic cavity which opens and closes, and has finite holding capacity. Internally, the quantity of transmitting tissue provided a further filter to the passage of the pollen tube by its marked reduction from the pollen presenter to the style. Features of the pollen presenter and style, such as the quantity of transmitting tissue cells and also the presence of transfer tissue, may play an important role in the selection of pollen tubes for passage down the style. The size of the inflorescence and presence of so many flowers in one inflorescence may have arisen because of the restricted access of pollen grains to individual stigmas. Additionally, the presence of large floral displays provides greater ability to attract pollinators. The enclosure of the stigma within a groove may be an adaptation to an arid environment, protecting the stigma from desiccation.

This study has raised important questions about the role, influence and evolution of the stigmatic cavity namely, are there differences in cavity type in tropical and arid adapted proteaceous species? Very little research has been conducted on tropical species. Also, what influences the number of pollen tubes from self pollen, a specific recognition system such as that governed by S-alleles, or a simple function of inadequate nutrient supply

contributed by the reduction in transmitting tissue cell number? Furthermore, what is the specific role of the transfer cells in the Proteaceae? Further investigation of *Hakea bucculenta* and other species lacking these cells would contribute to our understanding. This study confirms the complexity of this large ancient family and provides leads for further investigation such as a comparison of the reproductive strategies of relict genera found in the wet tropics of North Queensland with the derived genera of south-west Western Australia. This comparison would provide insight to the evolution of the breeding system of the Australian Proteaceae and may help to explain some of the features observed in derived taxa studied in this thesis.

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Appendix

Example of the VideoPro program used to determine the quantity of transmitting tissue at the base of the style for proteaceous and other angiosperm species. Program one sets up the data file, defining each data column and also performs specified calculations based on the data. The second program is the measurement program which takes the user through a sequence of measurements of the tissue. These measurements are fed back into the set up program (program one).

Similar programs were written for measurement of the tissues within the pollen presenter and for measurement of pollen grain volume.

1. Set-up program

```
'
data close
data units pixel
data scale 1
data aspect 1.000
data column clear
data column 1 identifier    'image number
data column 2 code          'scale factor
data column 3 Area          'whole transmitting tissue area
data column 4 Area          'area of tt where tt cells counted
data column 5 Count         'count of ~ half of the transmitting tissue cells
data column 6 Area          'individual tt cells area
data column 7 Count         'count number of outlined cells
data column 8 Area          'total stylar area
data column 9 Code          'scale factor
'
data formula c2*c3/1000 transmitting tissue area (um2)
data column 10 formula
data formula c2*c6/1000 ttc area (um2)
data column  11 formula
data formula c10*(c5/c4) total number of tt cells
data column 12 formula
```

```
data formula c11/c7 average ttcell area (um2)
data column 13 formula
data formula c9*c8/1000 total stylar area (um2)
data column 14 formula
data formula 100*c10/c14 %transmtiss
data column 15 formula
data formula c12*c13 total tt cell area in transmitting tissue
data column 16 formula
data formula c10-c16    intercellular space and cell wall area
data column 17 formula
data column * record
'
program comment "Press OK to append to datafile.  PAUSE if newfile"
data append
program run tt4meas.prg
'
' end of set-up
```

2. Measurement program

```
'measurement of transmitting tissue at base of style for a range of species and families
'
program control close
measure write disable
measure view disable
program comment "Press PAUSE and select new section"
image freeze
'image name
'image number increment
data identifier     'image file number
'image name identifier
data code           'enter scale factor
program comment "Press OK to trace transmitting tissue"
edit cover
edit size 3
edit line     'trace tissues
edit cover
edit fill
measure field
data column 3 protect 'transmitting tissue area
image store load
edit clear
image unfreeze
'
program comment "Change magnification to see transmitting cells and trace outline"
image freeze
program comment "Divide trans. tissue in half and measure area"
edit size 1
edit line  'draw line across
edit cover
edit fill
```

```
measure field
binary process outline
image store load
data column 4 protect ' area of selected area for tt cell count
'
program comment "Get rid of tissue outlines"
edit size 20
edit mark
edit line
program comment "Put a dot in each cell"
edit size 7
edit cover
edit line   'put dots in tt cells
program pause
program comment "Count total transmitting tissue cells"
measure field
data column 5 protect 'total transmitting tissue cell count for selected area
edit clear
'
program comment "Press ok to draw line and trace cells that cross line"
edit cover
edit size 2
edit line 'trace transmitting tissue cells
program comment "Make gaps between cells for cell count"
edit mark
edit size 3
edit line
'
program comment "Measure transmitting tissue cells area and cell count"
edit cover
edit fill
measure field
measure field
data column 6 protect 'transmitting tissue cells area
data column 7 protect 'transmitting tissue cell count
edit clear
'
image unfreeze
program pause
program comment "Press OK to change magnification"
image freeze
data code           'enter scale factor
program comment  "Trace outline of total tissue"
edit size 3
edit cover
edit line   'trace outline
'
program comment "Measure total tissue"
edit cover
edit fill
measure write enable
measure view enable
measure field
'data column 8 protect   'total tissue area
```

```
'
data column * record
program comment "Press OK to store binary and continue to next image"
binary retrieve
binary write
image write .jpg
image unfreeze
program run tt4meas.prg
'
'end of tt4meas.prg
```