Limonium Hybrid Production and Post-Pollination Biology.

A thesis presented in partial fulfilment of the requirements
for the degree
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in Botany at
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Ian N. Watson.
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Abstract.

The aim of this thesis is to determine barriers to interspecific hybridisation in selected *Limonium* species. Production of new commercially viable hybrids is essential to the development of the *Limonium* cut flower market. Very few studies have been published with respect to pollination and post-pollination biology of this genus so this study contributes a better understanding of events leading to fertilization and some of the factors involved in interspecific hybrid production.

The thesis initially introduces the genus and its cultivation. Later chapters involve aspects of post-pollination biology and determination of normal development associated with fertilization, including pollen tube growth, ovary and ovule growth and embryo production. This information is used to judge the success, barriers and problem associated with new hybrid *Limonium* production.

Interspecific hybrids were not obtained, but the information provided serves to highlight difficulties in hybrid production and provides opportunities for more directed breeding programmes.

Acknowledgments.

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Finally, I thank family an friends for their support during this research.

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1.

The Genus *Limonium*.

1. Taxonomy, Distribution, Phylogeny.

The genus *Limonium* (family Plumbaginaceae) contains 150 largely perennial and some annual species (Huxley *et al.*1992). The plant is typified by entire or pinnatifid leaves arranged in basal rosettes or clustered at axils. Inflorescences are corymbose panicles of terminal spikelets. Flowers are 5-merous consisting of a coloured or hyaline calyx, often persistent after anthesis, surrounding a corolla of variable colour. The five stamens are epipetalous. Species are usually dimorphic for pollen and stigmas and homostylous, although heterostyly, pollen dimorphism-stigma monomorphism, stigma and pollen monomorphism, secondary monomorphism and male sterile apomicts do occur in some sections. Ovaries are unilocular and contain a single ovule, the fruit as a single seeded dehiscent capsule.

Present day *Limonium* taxonomy is based largely on Boissier's 1879 system (Baker, 1953). Several changes and additions have been made to Boissiers's 1879 classification. *Statice* was rejected as a *nomen ambiguum* (Baker, 1953 quoting Lawrence, 1940; 1947) and replaced with *Limonium*. Prior to this change Boissier's *Statice* contained both the sea lavenders and the thrifts, these two groups now separated to *Limonium* (Miller) and *Armeria* (Wildenow) respectively. In addition, the sections *Schizopetalum* (Boissier), *Pterolimon* (Hooker), and *Arthrolimon* (von Meuller) have been added to Boissier's original scheme (Baker, 1953) (Table 1.01).

Goniolimon, a genus closely related to *Limonium* (Fig. 1.03) was distinguished from *Limonium* by Boissier by the virtue of capitate stigmas (Baker, 1953) but before the discovery of the capitate condition in several *Limonium* species. *Goniolimon*, in addition to other qualities, can be distinguished from *Limonium* by the presence of free anthers. There is synonymy in several species of *Limonium* and *Goniolimon*.

Section	Subsection	
1. *Pteroclados*	Odontolepidae	*L.sinuatum, L.thouini*
	Nobiles	*L.perezii, L.macrophyllum*
2. *Ctenostachys*	-	*L.mucronatum, L.pectinatum*
3. *Plathymenium*	Rhodantheae	*L.flexuosum, L.congestum*
	Chrysantheae	*L.aureum, L.sinense. L.australe*
4. *Limonium*	Genuinae	*L.vulgare, L.gmelinii*
	Densiflorae	
	Dissitiflorae	
	Steirocladae	*L.cosyrense, L.dregeanum*
	Hyalolepidae	*L.latifolium, L.bellidifolium*
	Sarcophyllae	*L.axillare, L.arabicum*
5. *Sphaerostachys*	-	*L.globuliferum*
6. *Jovibarba*	-	*L.jovibarbum*
7. *Schyzhymenium*	-	*L.echoides*
8. *Circinaria*	-	*L.purpuratum, L.perigrinum*
9. *Polyarthrion*	-	*L.caesium , L.ornatum*
10. *Myriolepis*	-	*L.ferulaceum, L.diffusum*
11. *Syphonantha*	-	*L.tubiflorum*
12. *Psylliostachys*	-	*L.suworowii, L.spicatum*
13. *Schizopetalum*	-	*L.macrorhabdon, L.griffithii*
14. *Pterolimon*	-	*L.plumosum L.peruvianum*
15. *Arthrolimon*	-	*L.salicornaceum*
16. *Goniolimon*	-	*G.tataricum, G.dumosum*

Table 1.01. *Limonium* Sections and Subsections (including *Goniolimon*)with examples (Baker, 1953).

Species inhabit coastal plains , semi arid, desert and saline areas in a broad band from Japan through China, India, the Middle East, and North Africa to Western Europe. *Limonium* also occurs in isolated groups on the South and North American continents, Australia, and the Canary and Cape Verde Islands (see Fig. 1.02). There are correlations between geographic distribution and reproductive method (Baker, 1953 *b*.) Certain subsections contain apomictic species *ie* Densiflorae and Dissitiflorae and these have probably arisen through lack of pollinating agents. Secondary monomorphism of pollen and stigmas has arisen from dimorphy in Genuinae, Chrysantheae and Steirocladae/Hyalolepidae. Secondary monomorphy found in species probably facilitates long distance dispersal, individual isolated genets being able to multiply by self fertilization. Baker (1953) suggests no separation between Steirocladae and Hyalolepidae. Species with continuous distributions are usually dimorphic for pollen and stigmas. Some *Limonium* sections, *Circinaria*, *Pterolimon*, *Schizopetalum* and *Arthrolimon* contain species with capitate stigmas. It is likely that this condition has developed through geographic isolation (Baker, 1953; 1966) rather than by derivation from dimorphism.

It is thought likely that the genus had its origins in the western Old World region, the present day centre of variability being the Mediterranean. During the Eocene, North Africa and South America were in contact and it is suggested by Baker (1953) that migration of the genus occurred from Africa to the American continents. From its new position in South America the genus then spread northwards by long distance dispersal and aided by secondary monomorphism. *L.humile* is thought to have originated in the North American Continent and then spread back to western Europe. The remainder of the genus radiated from the Old World to the Far East. The pattern of migration for the Asian Chrysantheae appears to follow that of Genuinae described above, the long distance dispersal of *L.australe* being aided by secondary monomorphism.

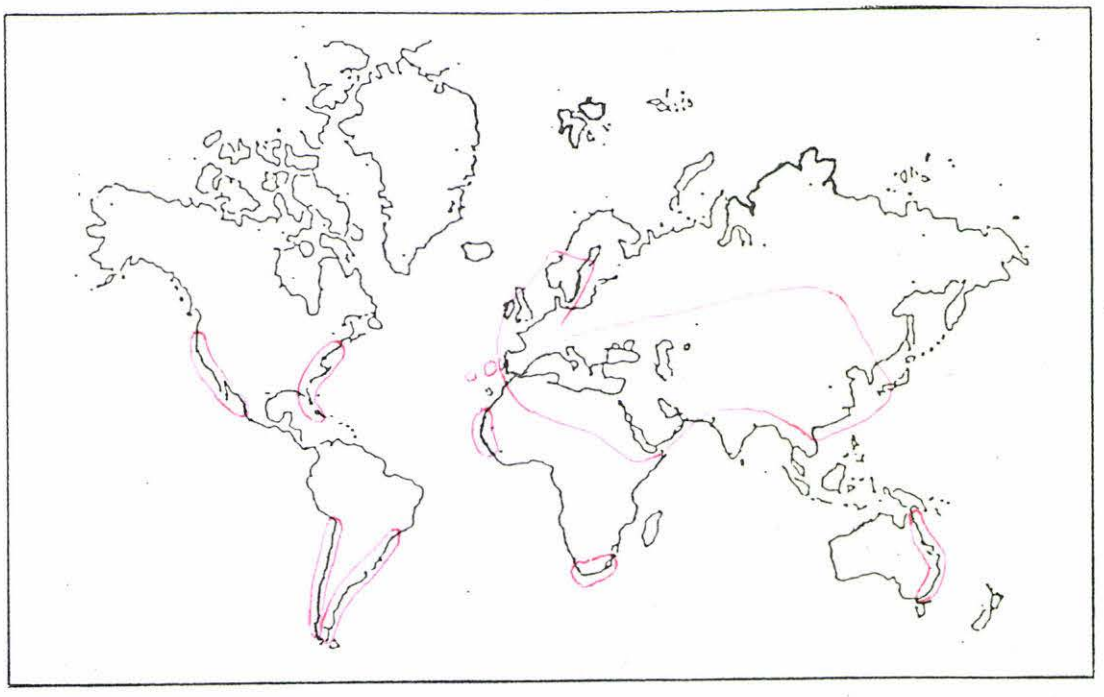


Fig. 1.01. Global distribution of *Limonium* (and *Goniolimon*) and sections. From Baker (1948).

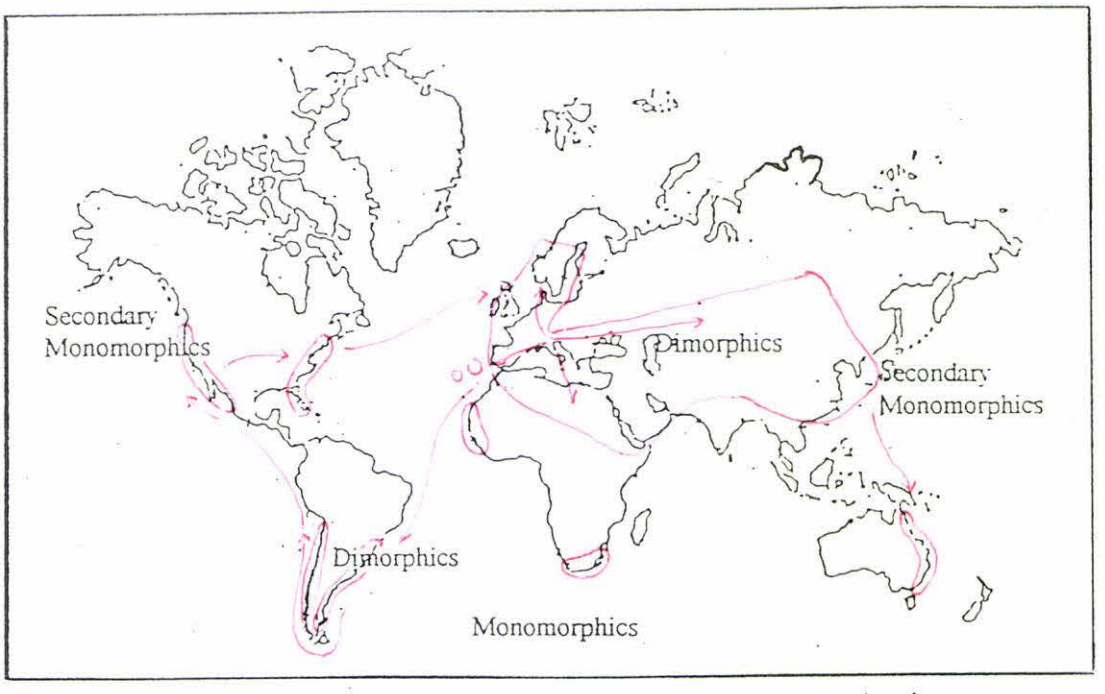


Fig. 1.02. Distribution patterns in *Limonium* with respect to reproductive biology according to Baker (1953 *b*.).

The following figure (Fig. 1.03) illustrates the phylogenetic relationship of *Limonium* and closely related genera (Baker, 1966).

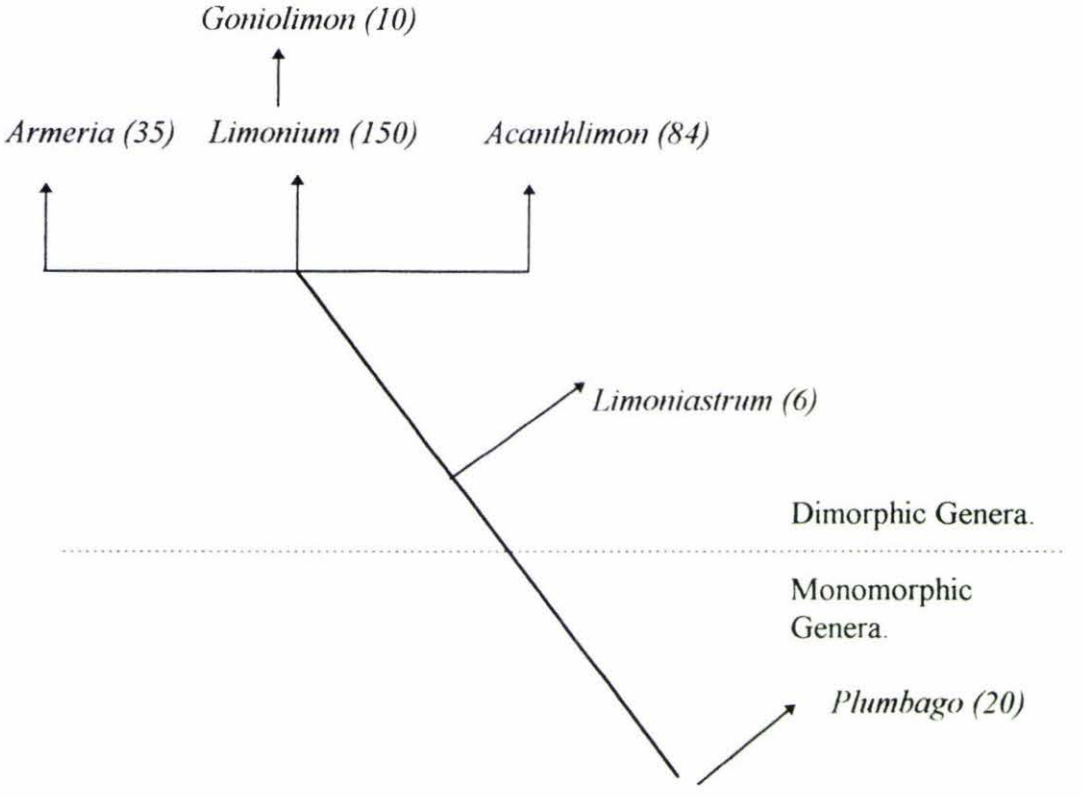


Fig. 1.03 Phylogenetic scheme for Plumbaginaceae (From Baker, 1966). The number of species in each genus is given in brackets.

Within the genus *Limonium*, phylogenetic derivation is postulated to have led through several stages culminating in comparatively rare apomicts and secondary monomorphs (monomorphy from dimorphy), possibly as a result of geographic isolation, and predominantly heterostylous dimorphic species (Baker, 1966)(See Table 1.02).

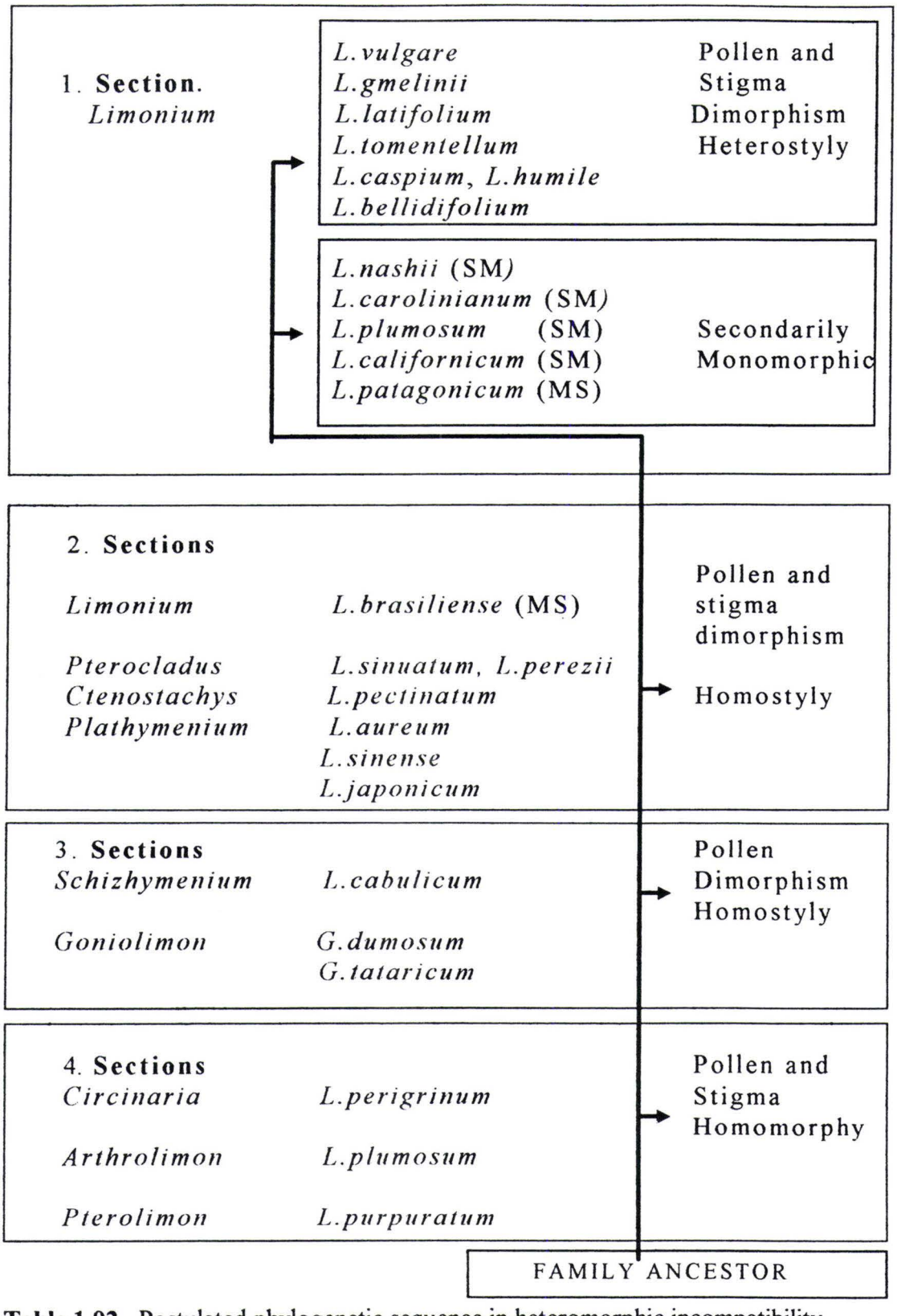


Table 1.02. Postulated phylogenetic sequence in heteromorphic incompatibility systems.(SM= secondary monomorphism MS= male sterile apomicts) (From Baker,1966) (Modified).

2. **Cultivation**.

The wide global distribution of *Limonium* means considerable variation in form, physiology and habitat tolerance occurs between species. According to Harada (1992) *Limonium* tolerates a well drained soil with a pH of 6.5 and E.C (electrical conductance) of 0.5. Plants can be divided into type I and type II, seasonal and free flowering plants respectively. Type I plants must be well established and require a period of winter vernalisation to induce flowering the following summer while type II plants, if given suitable light and temperature (20-25 °C) will flower all year round (Harada, 1992).

The commercial success of *Limonium* species and hybrids as cut flowers is dependent largely on flower colour and form, vase life, and the persistence of calyxes on the inflorescence. Several hybrids and species are in commercial production. Some examples include *L.sinuatum*, a species with a wide variety of colours in compact spikelets, especially valuable when dried and *L.perezii* with corymbs of persistent purple calyxes, *L.perigrinum* produces an open spike of conspicuous rose petals and calyxes; *L.latifolium* and *L.dumosum* (syn. *Goniolimon dumosum*) are valued for their long flowering stems but lack colourful calyxes. *Limonium* hybrids in commercial production include Saint Pierre and Bertlaard (*L.latifolium* X *L.caspium*), Charm Blue (*L.latifolium X L.gmelinii*), the Oceanic series (*L.latifolium* X *L.dumosum*) and Diacean (*L.latifolium* X *L.bellidifolium*).

Interspecific hybridisation has been used to transfer specific traits between species. A long stemmed form of *L.perigrinum* is a product of the cross between *L.perigrinum* and *L.purpuratum* (Morgan *et al.* 1995 (in press)). Embryo rescue is used to salvage often aborted interspecific hybrid embryos. Artificial cultivation and subsequent cloning is used to multiply such individuals. Transformation of *L.perigrinum* has been attempted using *Agrobacterium* but with little success. (E..Morgan *pers. comm.*)

Fig. 1.05. The colourful and persistent calyx of *L.sinuatum* makes this species a valuable cut flower.

Commercially produced *Limonium* plants often originate from tissue culture, having distinct advantages over seed raised plants in that they are more uniform, and have a better stem length, flower colour and vase life (Harada, 1992).

The considerable range of *Limonium* species available for interspecific hybridisation attempts, and advances in embryo rescue techniques, suggest considerable potential for the development of superior and alternative commercially viable forms. Research into barriers to and possibilities for wide crosses within this genus continues.

2.

Intraspecific Crosses.

Introduction

1. Self-Incompatibility.

The large majority of *Limonium* species are self-incompatible (Baker, 1966). Self-incompatibility (S.I) is a mechanism by which self fertilization is prevented. This mechanism normally ensures obligate outbreeding but requires a nearby compatible partner for pollination to occur.

Limonium exhibits sporophytic self-incompatibility (Brewbaker, 1957). This form of S.I involves the action and interaction of multiple alleles at the *S* locus. Sporophytic S.I differs from gametophytic S.I in that it is controlled by interactions between sporophytic domains of the pollen and stigma, rather than by actions and interactions between the pollen gametophyte and the sporophyte tissue of the pistil. In sporophytic systems the S component is determined by the diploid parent; the S gene products are therefore found in the tapetum derived pollen grain wall and in the pistil. Sporophytic S.I systems can be diallelic or multiallelic. Diallelic sporophytic S.I, found in *Limonium*, is expressed as heteromorphy. The cryptic expression of multiallelic S.I such as that shown in the Brassicaceae, contrasts with heteromorphic diallelic systems. So long as alleles encoding the S gene exine products are codominant, and are the same as either of the pistil *S* alleles, inhibition of either germination or elongation of the pollen tube will occur. Pollen will however be able to germinate on stigmas without any of the pollen S genes (Lewis, 1979; Heslop-Harrison, J. 1978; Pandey, 1979; Dzelzkalns *et al.* 1992) (Fig. 2.01). In *Armeria* and *Limonium* the B/papillate morph represents the homozygous recessive form (A/ papillate

combinations are also homozygous), while the A/cob morph is heterozygous (Baker,1966). Inhibition of pollen germination and stigma penetration in an incompatible pollen-stigma combination occurs at the stigmatic surface.

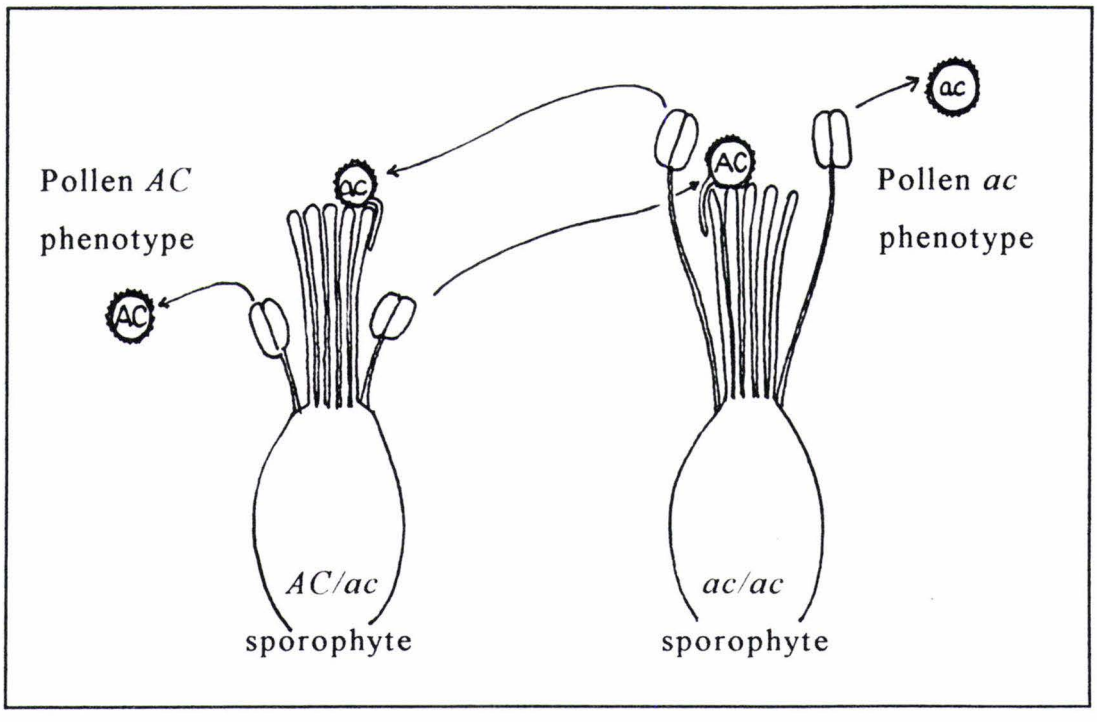


Fig. 2.01. Crossing relationships revealing S supergene activity in heteromorphic incompatibility.

Adhesion of a compatible grain to the stigma surface is an essential prerequisite to pollen hydration and germination. In the Plumbaginaceae, adhesion involves mechanical lodgement of compatible pollen exine processes to the stigma surface (Dulberger, 1975). Mattsson (1983) showed that in *Armeria*, lipids were borne externally on B type pollen relative to lipids sunken deep within the exine processes of A type pollen. Successful adhesion of pollen grains would, according to Mattsson, depend upon contact between the exine lipid and the stigma cuticle (Fig. 2.02). The lipid based 'lock and key' theory proposed by Mattsson (1983) does not explain the adherence of A grains to cob stigmas (Dulberger,1975) so it is likely additional mechanisms of chemical recognition are involved in sporophytic control of S.I at the stigma surface.

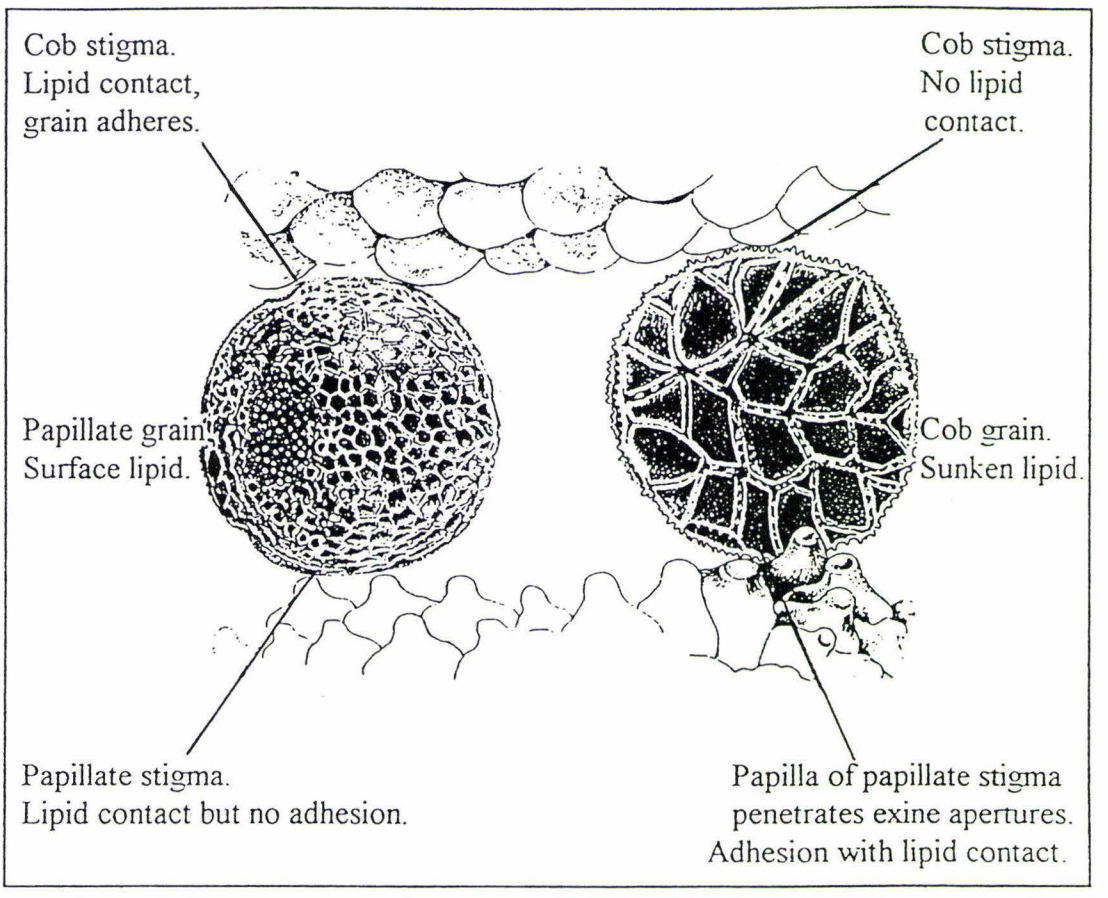


Fig. 2.02 (From Richards, 1986) An illustration of the role of exine processes in the functioning of sporophytic S.I in *Armeria*. (equally applicable to *Limonium*)

Other theories also attempt to explain the link between S.I and control of adhesion. Dumas and Gaude (1983) suggested control of adhesion is maintained by the presence of a membrane like structure on the exine of pollen similar to the membrane-like superficial layer found on the outside of *Brassica* pollen (Elleman and Dickinson,1986). Dumas *et al.* (1984) suggested a lectin mechanism is responsible for specific recognition in S.I reactions.

Following adhesion of a compatible pollen grain a period of pollen grain rehydration occurs. Rehydration requires the flow of water from the stigmatic papillae, the rate of which is primarily determined by the gradient of osmotic potential between the stigma and the pollen grain (see Chapter 5). In cryptic Brassicaceae, changes occur in the

pollen grain coat enabling a hydraulic continuity between the grain and papillar cuticle. (Elleman and Dickinson, 1986). With a compatible pollen-stigma interaction, viable pollen will germinate and pollen tube production will occur on the stigma surface. Penetration of the stigma and growth of the pollen tube down the style to the ovule may follow.

2. *Limonium* Floral Morphology.

The majority of naturally occurring *Limonium* populations depend on insect vectors for pollination (Richards, 1986 quoting Woodell, 1978). The floral morphology of this genus strongly reflects this relationship. Flowers are generally tube-like and contain nectar, and in the majority of *Limonium* species, heteromorphic.

a. Heteromorphy.

Heteromorphic *Limonium* species usually present flowers in two forms; A/cob and B/papillate. These forms have strikingly different pollen and stigmas. The A/cob morph will produce pollen with a coarse reticulation and a cob type stigma. The other floral form, B/papillate, generates pollen with fine reticulation and a stigma described as papillate (Fig. 2.03). For pollen germination and seed set within heteromorphic species, a compatible (legitimate) cross would involve a pollen-stigma combination of either A/papillate or B/cob (Fig. 2.04). The laws of legitimacy apply equally to homostylous and distylous heteromorphs. It is a matter of contention as to whether distyly represents a relict or recent condition within *Limonium* (Baker, 1966) (see Table 1.02), but it is in addition to heteromorphy, likely to have contributed, or contributes to, the promotion of outcrossing.

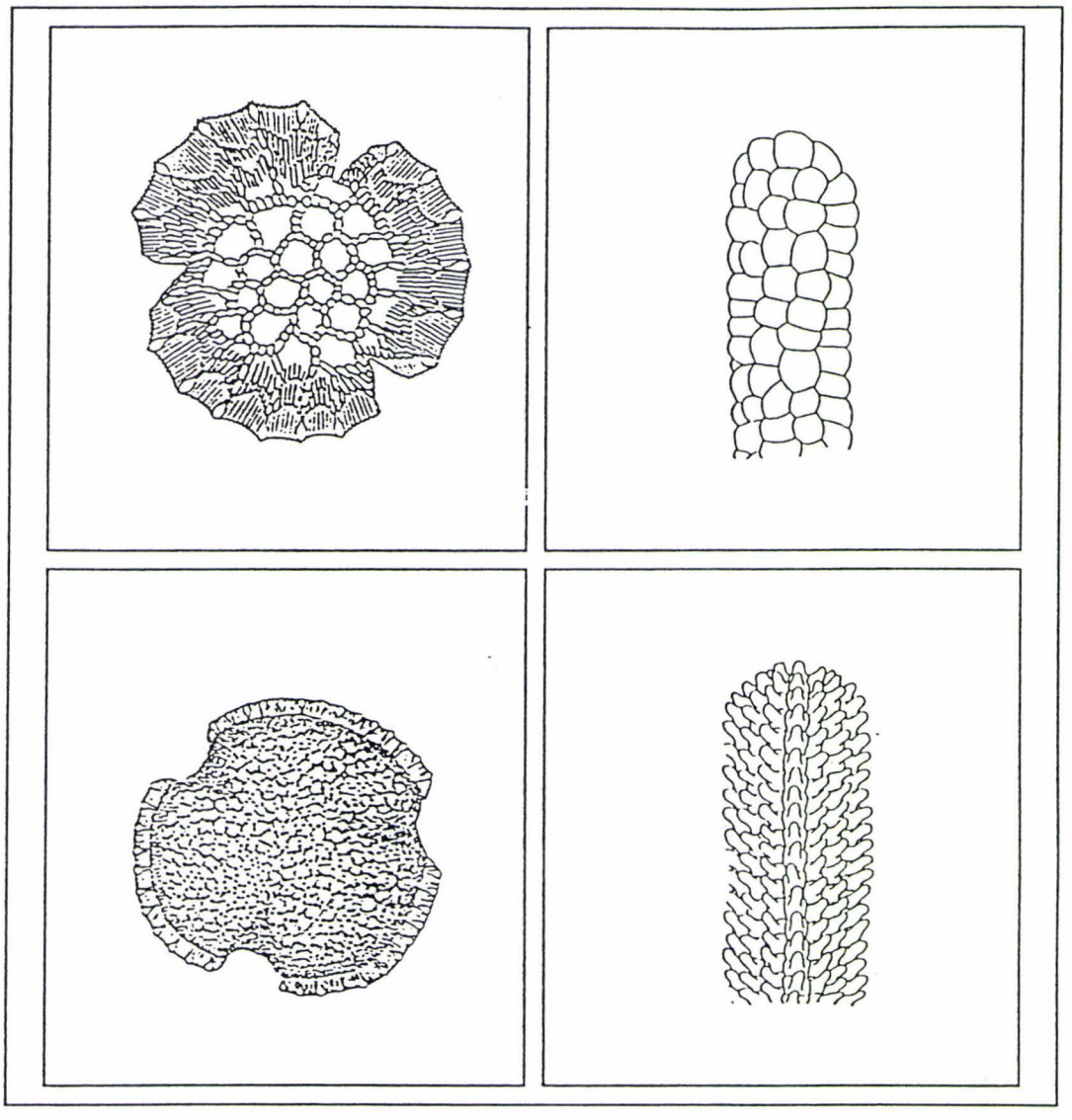


Fig. 2.03. Pollen and respective stigma types: A/cob and B/papillate found in heteromorphic *Limonium* species

Distylous pollen stigma combinations are identical to that of homostylous heteromorphic species.

b. Monomorphy.

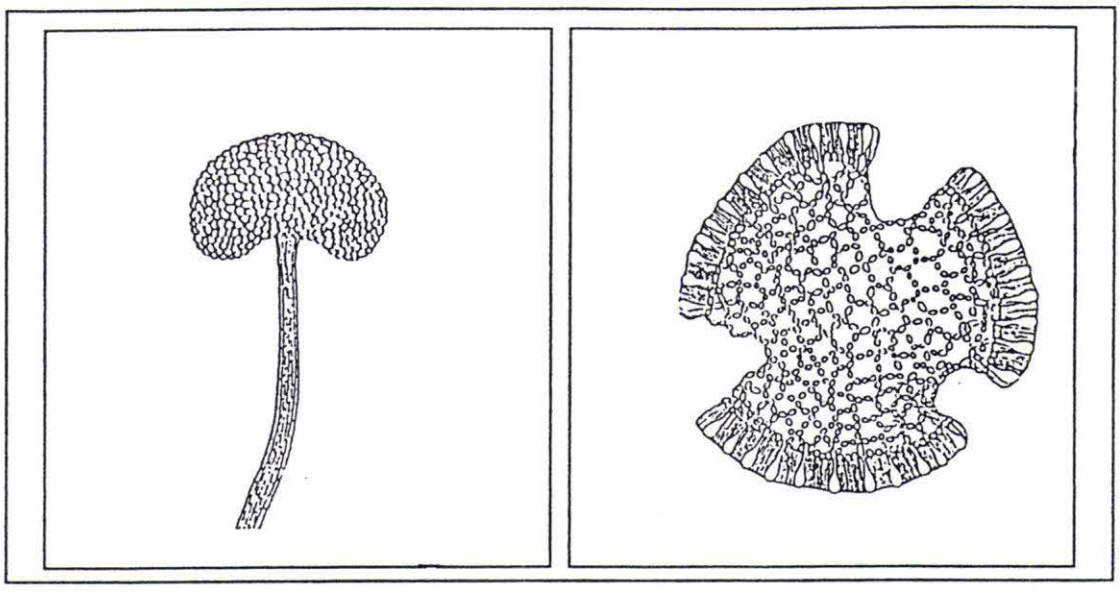


Fig. 2.04. Capitate stigma and close meshed type A type pollen found in most monomorphic *Limonium* species.

Rarer, monomorphic species (apart from secondary monomorphs) possess capitate stigmas and B, but more commonly, A type pollen (Fig. 2.02). Monomorphic species are self compatible *eg L.perigrinum* (Burge and Morgan, 1993). It is possible the relatively open dish-like flowers of these species encourages self pollination by presenting a less effective means of reducing indiscriminate pollen deposition compared with tube-shaped self incompatible flowers.

Secondary monomorphs, derived from dimorphic ancestors, have an A/papillate pollen-stigma combination with an exception being B/papillate *L.echioides*. All secondary monomorphs produce within flower self-compatible pollen-stigma combinations. Some secondary monomorphs have stigma/pollen combinations of either A/cob or B/papillate and in many cases these species have proven to be male sterile and agamospermic. Experiments showed that pollen of secondary (derived) monomorphic *Armeria* obeyed the laws of legitimacy, however the stigmas of derived monomorphic *Armeria* accept and germinate A and B pollen indicating a breakdown of heteromorphic incompatibility (Baker,1966) and it is suggested similar results should be found in *Limonium* and that monomorphism in fertile species serves to

overcome difficulties associated with long distance dispersal so that isolated plants can set seed by self pollination. Apomicts are likely to have arisen where there is a significant lack of pollinating agents (Baker 1953, *b*). The styles of apomictic species studied are still functional and obey the laws of legitimacy (Dulberger,1975).

Fig. 2.05. A 'bee fly' visiting Blue Fantasia 88 (B/pap). Note the position of the anthers above the stigmas.

The following diagram summarises compatible crossing relationships in *Limonium* and *Armeria*.

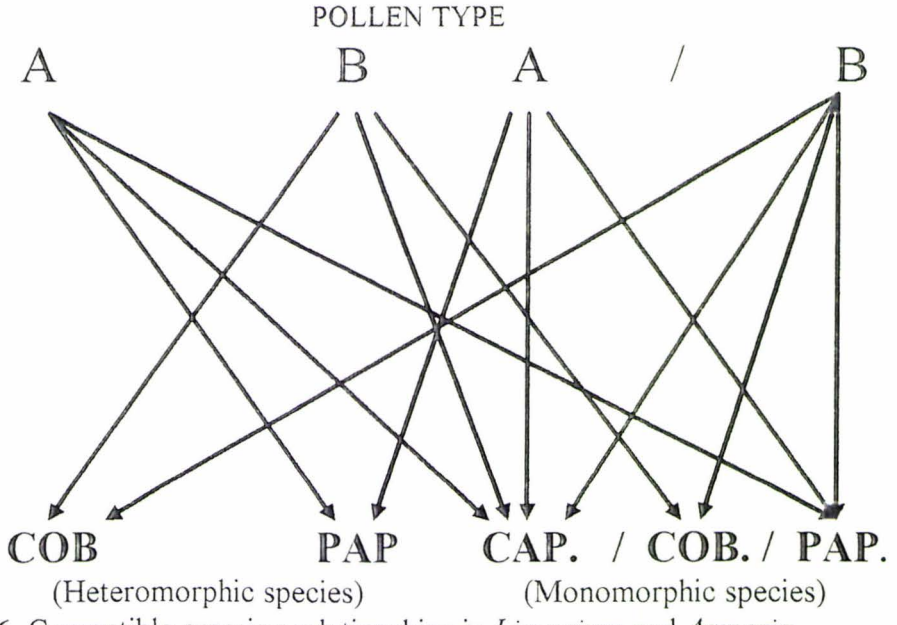


Fig. 2.06. Compatible crossing relationships in *Limonium* and *Armeria*.

Previous studies have been largely limited (with the exception of Dulberger, 1975) to pollen grain adherence to the stigma surface. This chapter aims to provide a better understanding of the pollination biology and aspects of fertilization biology within three selected *Limonium* species. It is hoped results will provide information helpful to future crossing attempts.

Materials and Methods.

1. Materials.

The following methods involved material from the *Limonium* plants:

1. *Limonium perigrinum* (Bergius) c.v "Ballerina rose".
2. *Limonium sinuatum* (L.) Pacific Strain. Seed from King's Seeds.
3. *Limonium aureum* (L.)

All plants except *L.sinuatum* were glasshouse grown, minimum temperature 15 °C and ventilation temperature 25 °C. *L.sinuatum* was initially grown outdoors until maturity, then brought into the glasshouse to prolong flowering and to conduct experiments.

2. Methods.

Intraspecific pollinations.

Intramorph pollinations were made on one plant of *L.perigrinum*. Intramorph and intermorph pollinations were made in heteromorphic plants of *L.aureum* and *L.sinuatum*. In all species, anthers were removed from flowers pre-dehiscence. Anthers were collected and allowed to dehisce in ambient conditions. To ensure maximum pollen viability, the aforementioned pollinations were made immediately after dehiscence, using collected anthers. No pollination post emasculation was the control.

Emasculated flowers from pollinated and control plants were collected eight to ten hours after pollination. Pollen grain adhesion to stigmas was established by examination under a binocular microscope at the time of flower dissection.

Penetration of the stigma and the extent of pollen tube growth within pistils was determined by fluorescence microscopy (Shivanna and Rangaswamy,1992) and recorded. After hydrolysis in concentrated NaOH, pistils dissected from flowers were washed in distilled water and then placed in 0.1% aniline blue (water soluble) in 0.1M phosphate buffer. The NaOH treatment and washing in distilled water removes pollen grains that have adhered and/or germinated on the stigma but failed to penetrate its surface. Pollen tubes in pistils were detected by viewing the treated pistils under a Zeiss microscope with epifluorescence condenser IVFI and U.V filter. Further examination of pollinated pistils to detect embryo presence was carried out under a binocular microscope *c.* two weeks after the initial pollination in both species.

In addition, *L.aureum* (A /cob) ovary, ovule growth and general embryo appearance was measured using an eyepiece micrometer at 0, 3, 6, 9 and 15 days following intermorph pollinations. Unpollinated pistils provided the control. The number of flowers collected at each time period (pollinated/control) were as follows: Day zero: 15/ N.A*, Day three: 10/10, Day six: 22/15, Day nine: 24/10, Day 15: 10/5.
A previous investigation showed that in pollinated pistils, ovules less than 0.5 mm in length at six days did not contain embryos. Occurrence of ovaries and ovules without embryos were recorded up to nine days post-pollination but the dimensions considered to be 'outliers' and so not included in data expressing change in ovary and ovule size in the six, nine and 15 day samples. The change in mean length and width of ovaries and ovules is presented in Fig. 5.12*a* and *b*. Error bars represent standard errors of the means.

* N.A= not applicable.

Results.

Floral examination showed *L.sinuatum* and *L.aureum* were heteromorphic for both pollen and stigmas. *L.perigrinum* was monomorphic (capitate) with B pollen. *L.aureum* and *L.sinuatum* intraspecific crosses showed A pollen adhered to both cob and papillate stigmas while B pollen adhered to cob stigmas but not to papillate stigmas. A type pollen did not germinate on cob stigmas. In *L.perigrinum* selfed pollen adhered to the capitate stigma. In *L.sinuatum* and *L.aureum* intraspecific crosses, only pollen stigma combinations A/papillate (Fig. 2.07) and B/cob resulted in penetration of the stigma surface by pollen tubes. Selfed pollen in monomorphic *L.perigrinum* also produced pollen tubes that penetrated the stigma and grew to the ovule.

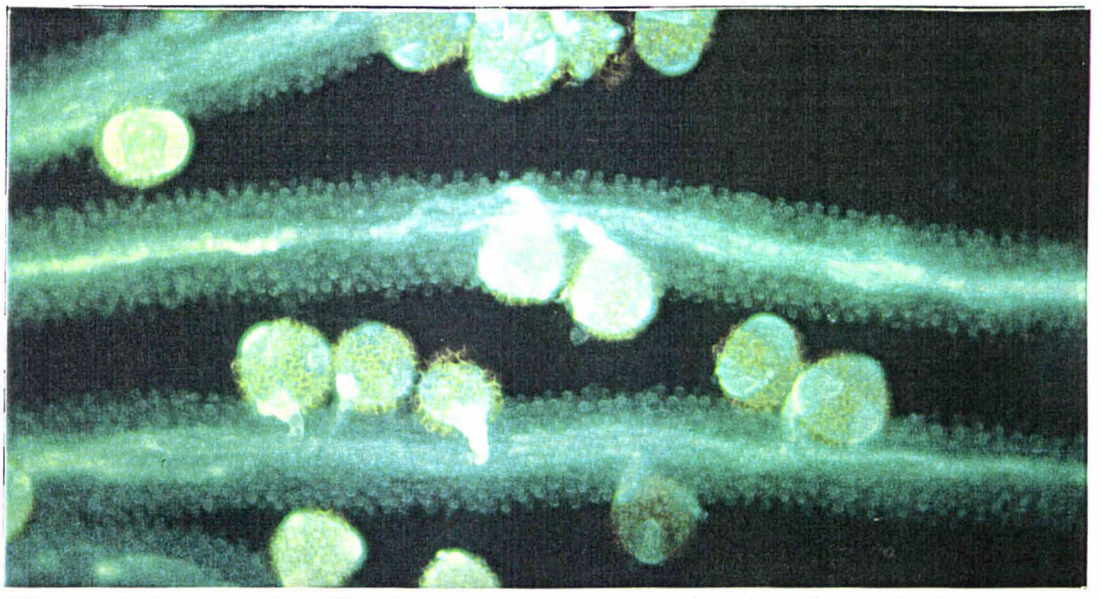


Fig. 2.07. Intermorph pollination of *L.sinuatum* showing A pollen and tubes on papillate stigma (150X)

Pollen tubes entered stigmas between papillae then grew through the stylar transmitting tissue to the base of the style. Numerous pollen grains were seen to have penetrated the stigmas, especially in *L.aureum* and *L.sinuatum* where the stigmatic surface area is relatively large to that of capitate stigmas of *L.perigrinum*. Most pollen tubes did not progress further than the top of the style and usually one pollen tube per style (five) entered each ovary. It was noted that styles containing pollen tubes were curled around each other within the flower prior to staining.

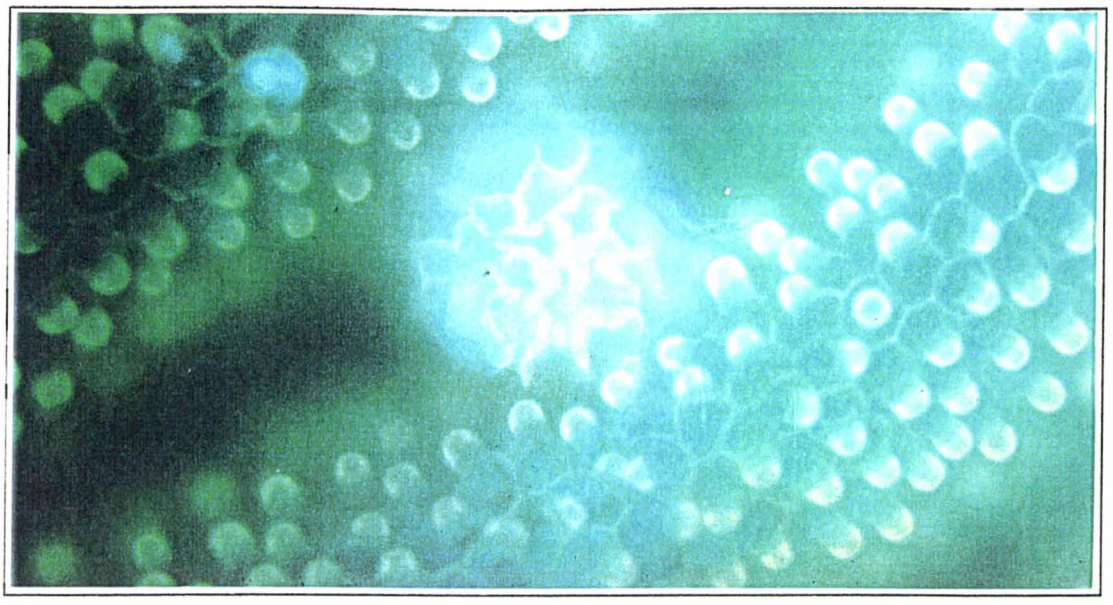


Fig. 2.08. Type A pollen of *L.sinuatum* germinating on a papillate stigma of the same species (400X).

Once pollen tubes reached the top of the ovary they grew inwards towards the central axis of the pistil entering the obiturator. Tubes then grew toward the micropyle of the single orthotropous ovule *via* the obiturator if numerous, congregating within the micropylar region , becoming distorted and apparently disorientated.

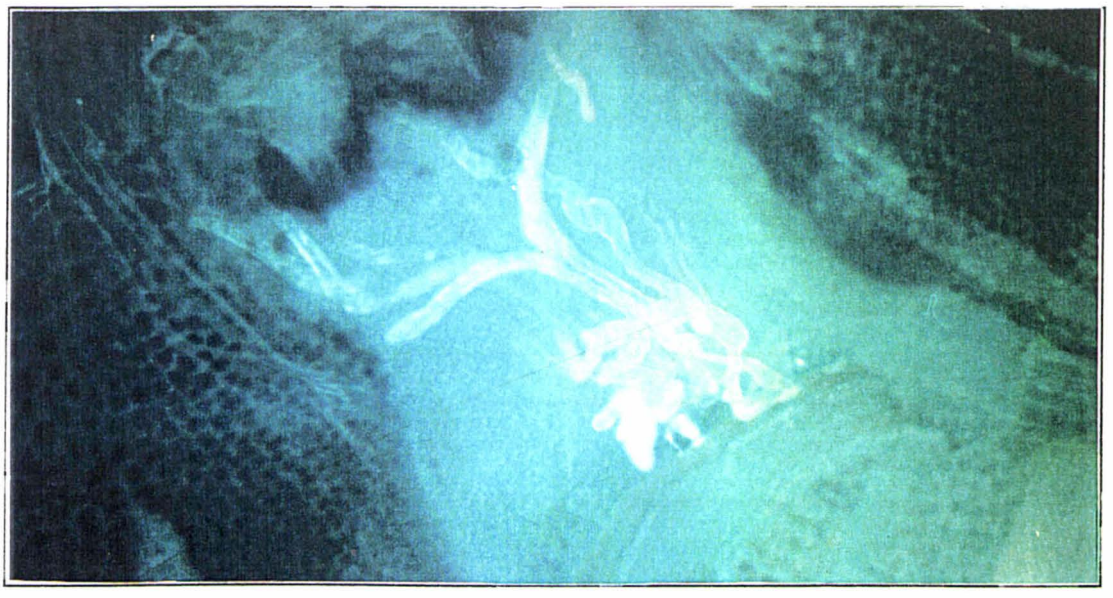


Fig. 2.09. *L.sinuatum* intermorph pollinated pistil .Pollen tubes in obiturator(150X)

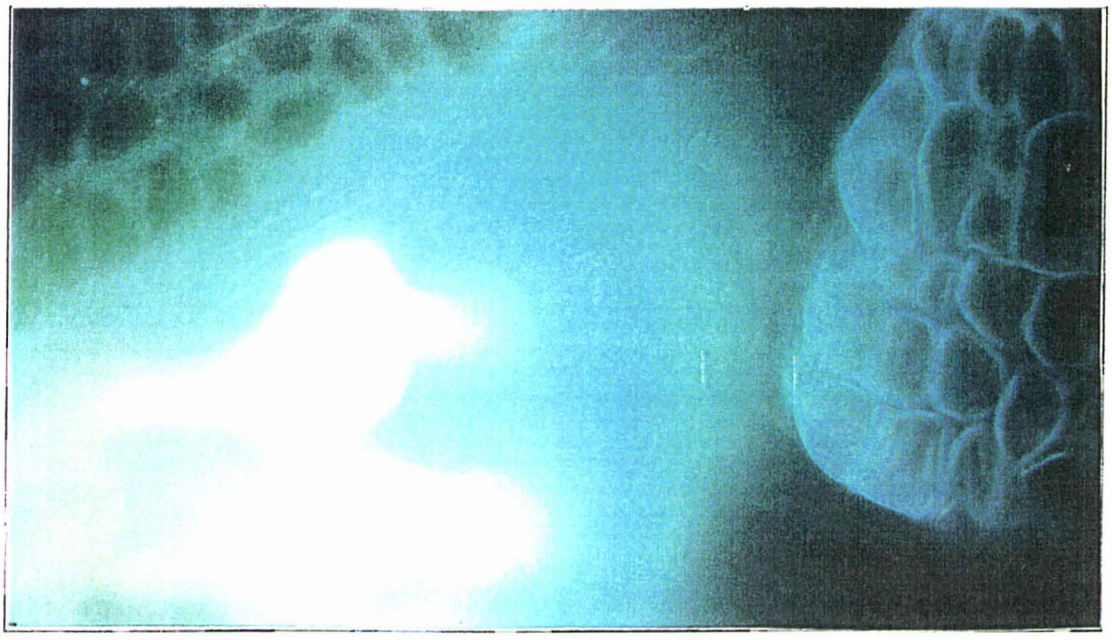


Fig. 2.10. Branching and distortion of pollen tubes in the micropylar region of the obiturator. *L.sinuatum* intermorph pollination (400X).

Examination of *L.sinuatum* and *L.perigrinum* pistils ten days after intermorphic and self pollination respectively, revealed embryos in many ovules.

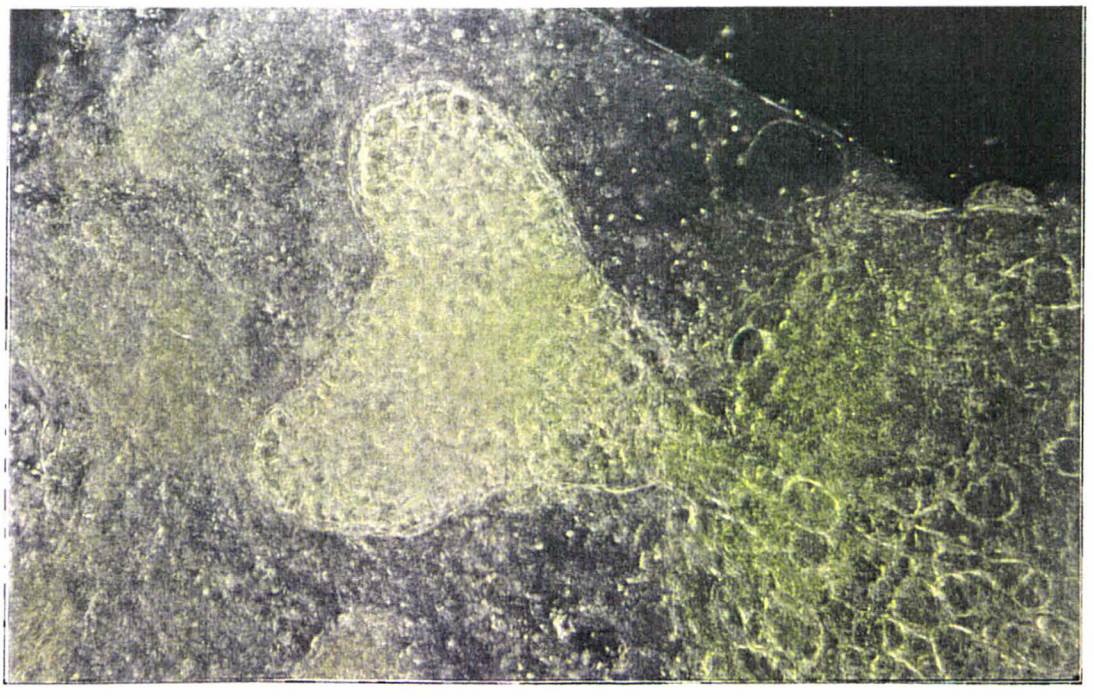


Fig. 2.11. An embryo of *L.perigrinum c.* two weeks following self pollination. (400X)

Changes in length and width of ovaries and ovules of *L.aureum* cob/A at specified intervals following intermorph pollination and no pollination (control) are shown in Figs 2.12*a* and *b* and described as follows. Unpollinated ovules became excessively shrivelled after nine days and so were not measured beyond this point. It can be seen that ovary length and width increased more rapidly in the pollinated pistils than in the unpollinated pistils. Ovaries of pollinated pistils increased in length from *c.* 0.75 mm. (0 days) to *c.* 2.7mm (15 days) and in width from *c.* 0.4mm to 0.8 mm over the same 15 day period. The maximum size attained by ovaries of unpollinated pistils was *c.* 0.8 mm long and 0.6 mm wide at nine days, considerably smaller than in pollinated pistils. Ovaries increased more rapidly in length than width. The rate of increase in ovary length in pollinated pistils was higher between 9 -15 days than during the previous nine days. Unpollinated pistil ovary length and width increase was slower than in pollinated pistils and theses began to shrivel at nine days. In pollinated pistils, ovule length and width increased relatively constantly for the initial nine days following pollination. The length of the ovule, as seen with ovary length, increased most rapidly between 9-15 days. Ovule length in pollinated pistils increased from *c.* 0.3 mm to 1.22 mm over 15 days while the width increased from *c.* 0.25 mm to 0.7 mm. Both width and length of the ovules in control pistils decreased over the 15 day period, both falling about 0.1 mm over nine days. Ovaries of unpollinated pistils remained pale cream in colour through the 15 day trial, whilst pollinated pistils were had become green by six days. At six days post-pollination, ovules contained globular embryos, heart stage embryos from nine days and torpedo to early hook stage embryos at 15 days. Embryos were undetectable by dissection from ovules at three days. Of the total pollinated pistils examined from 6-15 days (54), seven ovaries were pale, had grown relatively little, and did not contain embryos. These seven pistils was not included in data for pollinated pistils in Figs 2.12 *a* and *b*. Error bars are twice the standard errors of the means.

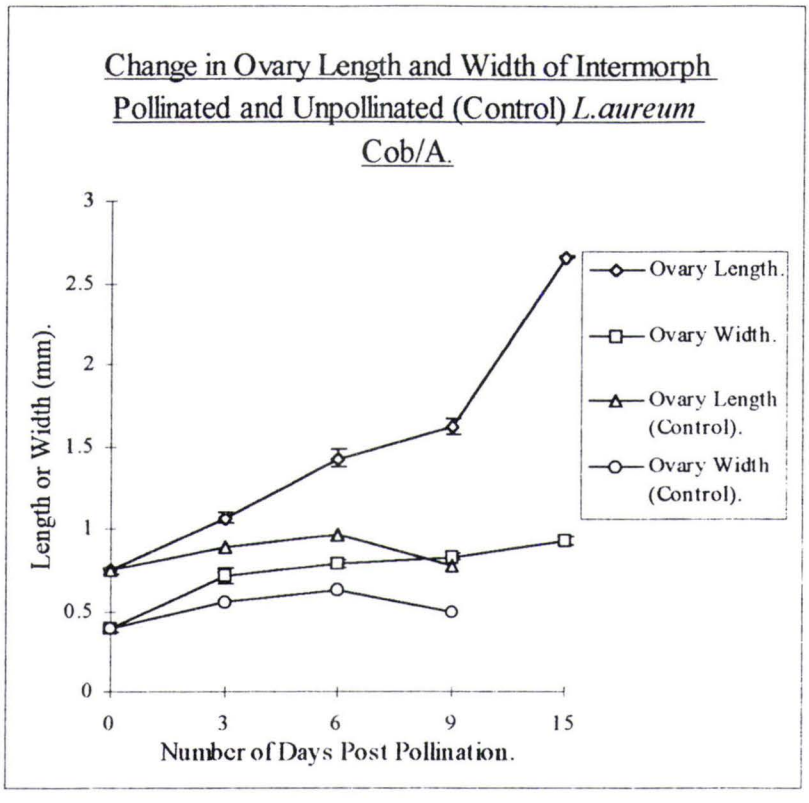


Fig. 2.12 *a*. Change in ovary size in pollinated and unpollinated *L.aureum* Cob/A

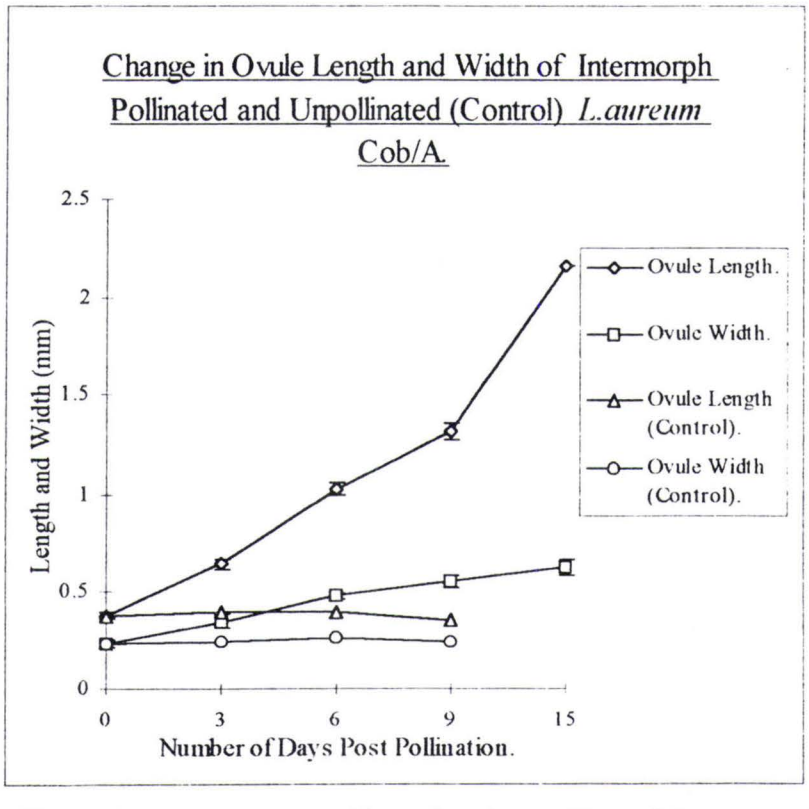


Fig. 2.12 *b*. Change in ovule size in pollinated and unpollinated *L.aureum* Cob/A.

Discussion.

It was shown that pollen and stigmas of heteromorphic *L.aureum* and *L.sinuatum* followed the laws of legitimacy. Pollen grains failed to germinate and penetrate stigmas in incompatible pollen stigma combinations. This failure may have been attributable to insufficient hydration of incompatible pollen grains. In an assessment of pollen hydration, the contribution of atmospheric water should be considered in addition to stigmatic water (Richards, 1986). By examination of pollen tube growth in intramorph pollinations, it appears that in these two species, within morph incompatibility operates at the stigma surface. However it has been demonstrated in *Primula*, a genus with a sporophytic S.I system comparable to *Limonium*, that within morph incompatibility can occur for different *Primula* species, at a number of different stages (Shivanna *et al*. 1981), namely:

(a) Lodgement, adhesion and germination of pollen on the stigma.
(b) Penetration of the stigma papilla by the pollen tube.
(c) Growth of the pollen tube in the stigma.
(d) Growth of the pollen tube in the style.

Incompatibility is rarely complete in *Primula* but results from an accumulation of effects at each of these stages (Richards and Ibrahim, 1982). It is possible that species of *Limonium* yet to be studied, may show variation in degree and spatial control of S.I for intraspecific and interspecific intramorph pollinations.

The fraction of pollen tubes reaching the ovary grew largely without branching until they reached the micropylar region of the obiturator. At this point, if several tubes have reached the ovule, pollen tubes become branched and distorted. This has also been observed in *Spinacia* (Wilms, 1974). It is possible that once one tube has entered the ovule (and possibly effected fertilization) a chemotropic signal attracting other pollen tubes to enter the micropyle, is no longer necessary, production is halted, and the following tubes become disoriented.

Observed curvature of styles in response to pollen tube growth has also been established in *Linum* (Lewis, 1943). This response is useful in determining the presence of pollen tubes in the style without staining procedures such as fluorescence microscopy.

The presence of embryos in intermorph pollinated *L.aureum* and *L.sinuatum* and self pollinated *L.perigrinum* ovules suggests fertilization is occurring. It is most unlikely these species are apomictic (see Introduction this chapter). Gross morphological and physiological changes such as increase in length and width of ovaries and ovules and increase in ovary chlorophyll content in fertilized plants, provide simple ways to assess fertilization success.

Summary.

This chapter confirms that for the heteromorphic species *L.aureum* and *L.sinuatum* sporophytic self-incompatibility occurs at the stigma surface by lack of adherence (except for A grains on cob stigmas), absence of pollen grain germination and therefore penetration of pollen tubes into the stigma surface. Self-incompatibility for these heteromorphic species is complete at the stigma surface under the defined growing conditions. *L.perigrinum* is self compatible revealing a breakdown in self - incompatibility in this species. Stylar curvature, changes in ovary dimensions, especially length, and accumulation of chlorophyll in ovary walls are obvious indications of pollen tube growth and presence of early embryo growth respectively. These observations will make assessment of crossing attempts and fertilization success easier.

3.

Interspecific Crosses.

Introduction.

The production of new hybrids with desirable attributes is important in the development and expansion of the *Limonium* cut and dried flower industry. The considerable heterogeneity in floral form and colour of *Limonium* presents an opportunity for the development of new interspecific hybrids particularly new combinations of desirable floral form and calyx colour.

This chapter investigates barriers to *Limonium* hybrid production in crosses between selected *Limonium* species and hybrids.

Materials and Methods.

1. Materials

Limonium plants used were:

Limonium perigrinum (Bergius) c.v "Ballerina rose" (monomorphic, B/capitate) Sect. Circinaria.

Limonium aureum (L.) (dimorphic, A/cob and B/papillate) Sect. Limonium

Limonium caspium (Willd) c.v "Blue Cloud" and "Blue Lace". (A/Cob and B*/Pap)

Limonium sinuatum (L) Pacific Series. (dimorphic, A/cob and B/pap) Sect. Pteroclados.

Oceanic Blue and White (hybrid of *L.latifolium* (Sm) and *L. dumosum* (*G.dumosum*)). A*/cob.

Blue Fantasia 88. (Unknown parentage). (B*/pap)

* see results pertaining to pollen viability/production.

L.sinuatum was from seed supplied by Kings Seeds Ltd. (Pacific Series). Oceanic Blue is a sport of Oceanic White. All plants, except *L.sinuatum* and the Oceanics were flowered in a glasshouse with a minimum temperature 15 °C and ventilation temperature 25 °C. *L.sinuatum* was grown to flowering outdoors, then transferred to the glasshouse for experimental work. The Oceanic Series material came from freshly cut flowers grown in a shadehouse at the Levin Research Centre. All plants, except the Oceanic Series, were pot grown.

Fig. 3.01. The rose petals and calyx of *L.perigrinum* (above) and inflorescence (below)

Fig. 3.02. The open trailing form of *L.aureum* (above) is unsuitable for cut flowers but its yellow flower and calyx are desirable (below).

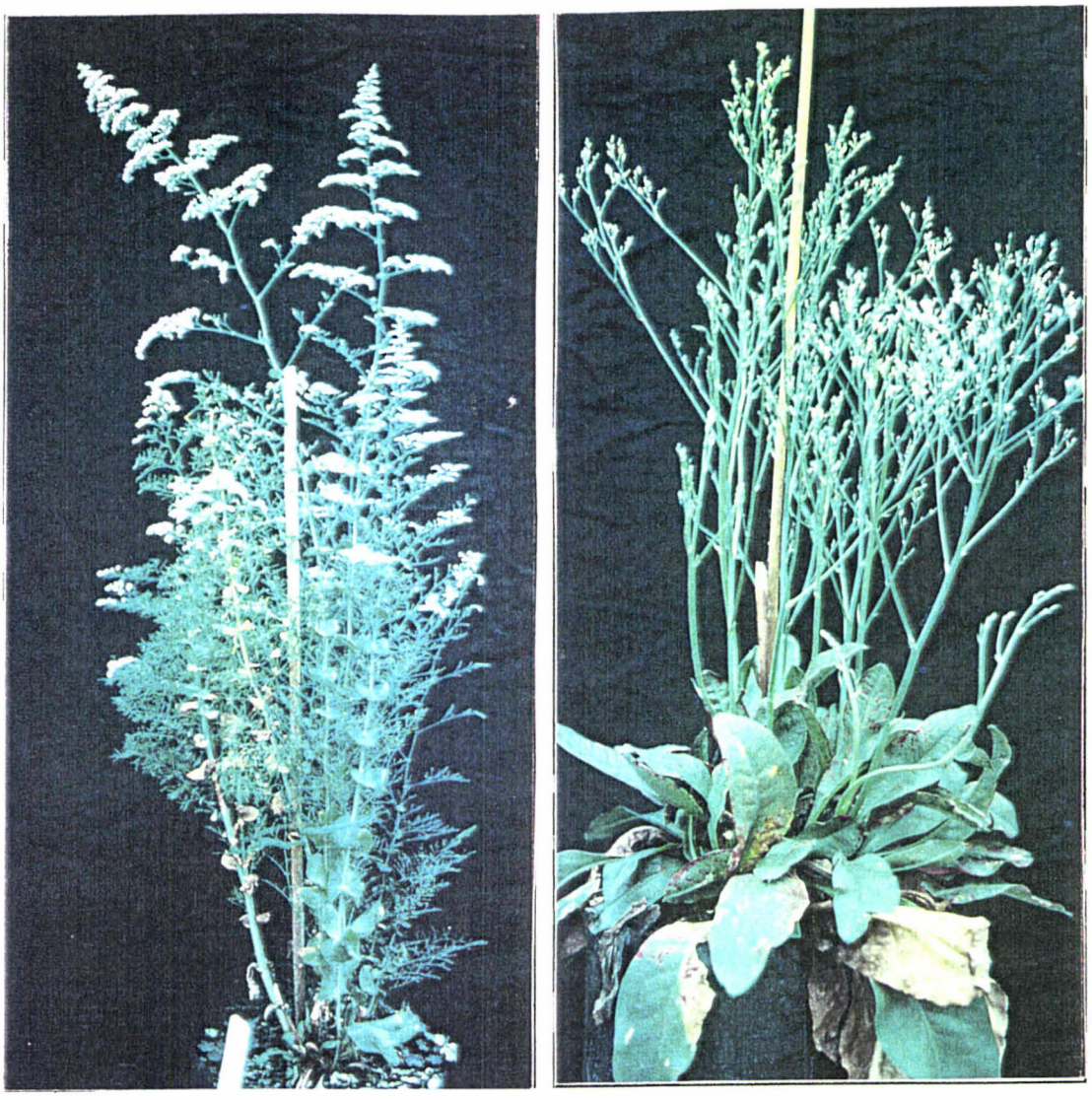


Fig. 3.03. *L.caspium* c.v "Blue Lace" (left top and bottom), c.v "Blue Cloud" (right top and bottom)

Fig. 3.04. Shadehouse grown Oceanic White.

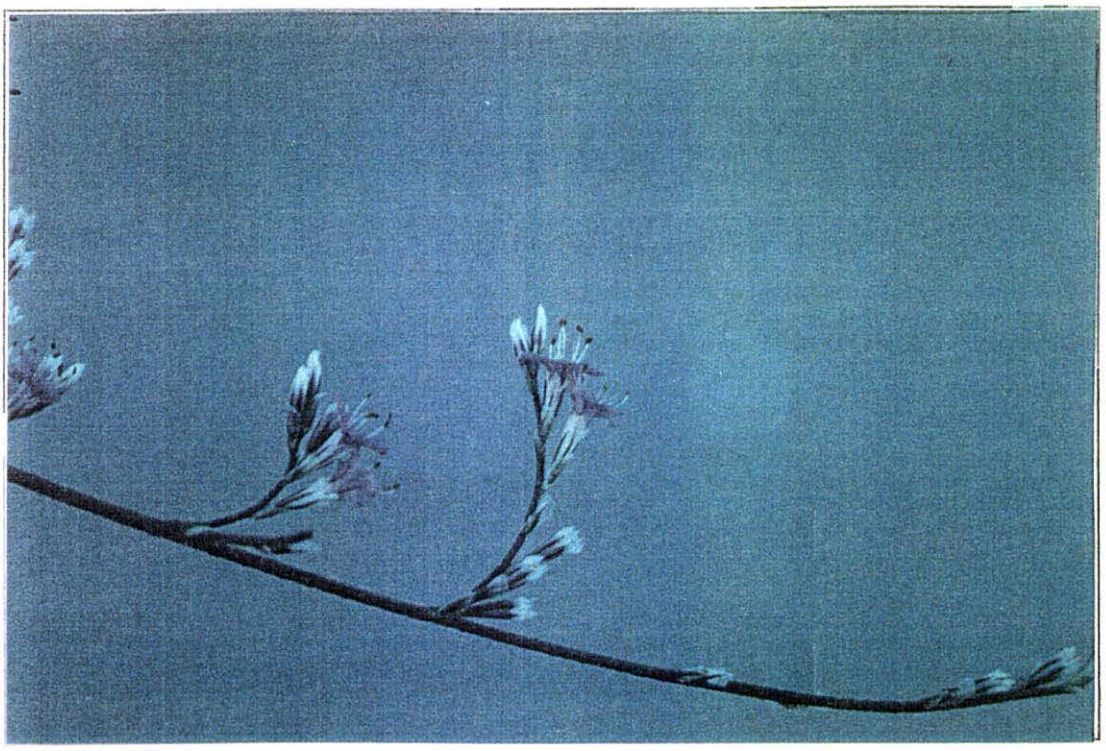


Fig. 3.05. Blue Fantasia 88.

Fig. 3.06. *L.simiatum* plants (above) and showing compact arrangement of calyxes (below)

2. Methods.

Crossing experiments were conducted from April-June 1994. Crosses were restricted to species and cultivars flowering in the same period and reciprocity of crossing attempts was restricted by non viability of pollen in certain species.

(a) Pollen Viability.

Before interspecific crosses were attempted, pollen viability of these species were established by the FCR* reaction. Pollen producing, indehiscent anthers were collected from four or five flowers and then allowed to dehisce under a bench lamp in the laboratory. Pollen from these anthers were tested by 'washing' the anthers in a drop of the following solution on a microscope slide. Fluorescein diacetate in acetone (see Appendix 3.) was prepared and added dropwise 0.3 M sucrose solution (Shivanna and Rangaswamy, 1992). These slides were viewed using a Zeiss microscope with epifluorescence condenser IVFI and U.V filter. Viability of pollen was determined by counts of at least 500 grains at 100X magnification. Where viable pollen was not produced, the general appearance of anthers and pollen were recorded. For species, cultivars or hybrids with anthers indehiscent at anthesis, anthers were placed in Alexander Stain (Alexander, 1969)(Appendix 2.) and if anthers were seen to contain pollen, it was teased from them into the surrounding Alexander stain and examined by conventional light microscopy.

* Fluorochromic reaction.

(b) Interspecific Crosses.

Intermorph and intramorph interspecific crosses were attempted in the following combinations. Restrictions on possible crosses within the plant material provided were determined by pollen viability and availability of flowers (see Results).

Crosses attempted (including morphotypes) were:

1. Oceanics (A/Cob only) X *L.aureum* (A/cob and B/Pap)
2. Oceanics (A/Cob only) X *L.perigrinum* (B/capitate).

3. *L.caspium* c.v "Blue Cloud" (A/cob) X *L.aureum* (A/cob and B/Pap).
4. *L.caspium* c.v "Blue Cloud" (A/cob) X *L.perigrinum* (B/capitate).
5. *L.caspium* c.v "Blue Lace" (B/pap) X *L. aureum* (A/cob and B/pap).
6. Blue Fantasia 88 (B/Pap) X *L.aureum* (A/cob and B/pap).
7. *L.perigrinum* (B/cap) X *L.sinuatum* (A/cob and B/pap).

Flowers in receipt of pollen were emasculated prior to pollination to remove any chance of self-pollination. As anthers are epipetalous, emasculation usually also involved removal of the petals. Anthers containing pollen to be used in crossing attempts were removed from flowers prior to dehiscence and placed in a petri dish. The collected anthers were then allowed to dehisce in ambient conditions (glasshouse conditions) and used to pollinate pistils of emasculated recipient plants. This method allows rapid and relatively simultaneous pollination of numerous flowers and reduces loss and variability in pollen viability over time. Unpollinated ,emasculated flowers (control) were used for comparison to pollinated pistils.

Pollinated and control pistils and the surrounding calyxes were removed 8-10 hours following pollination. Removal of petals before pollination made dissection of pistils from flowers easier at the time of examination, as petals often became entangled with pollinated stigmas. Pistils were separated from calyxes under a binocular microscope and examined for adherence of grains. For examination of pollen tubes in pistils, whole pistils were placed in 1M NaOH for one hour, washed in distilled water to remove NaOH, then stained in a drop of 0.1% aniline blue in phosphate buffer (Appendix 1.) upon a microscope slide and covered with a coverslip (Shivanna and Rangaswamy, 1992). A stronger aniline blue solution appeared to give better results and penetration by aniline blue was achieved after 12 hours. Slides were placed in a refrigerator for this period as aniline blue is light sensitive. Squashed pistils were examined for germination frequency and extent of pollen tube growth using a Zeiss microscope with epifluorescence condenser IVFI and U.V filter. Results were recorded as total number of pollen grains germinated on the pistil, the number of

pollen tubes halted in the style, the number halted in the ovary, from that, the numbers halted in the stigmatic tissue, were derived as the remainder.

For pistils in crosses where pollen tubè growth reached the ovary, additional crosses were made. These pistils and controls were then removed from flowers at six days and examined for embryos. Six days was assumed to be sufficient time for globular to heart stage embryo growth and for the morphological and physiological changes associated with the presence of embryo growth (see Chapter 2.).

Results.

(a) Pollen Viability.

At the time the above crosses were attempted anthers of *L.aureum* A/cob and B/pap, and *L.perigrinum* showed *c.* 90% pollen viability. *L.caspium* c.v "Blue Lace" anthers produced *c.*50% viable pollen while c.v. "Blue Cloud" failed to produce any pollen. Anthers of the Oceanics did not produce viable pollen, and often one or two anthers in each flower did not contain any pollen. All anthers of Blue Fantasia 88 contained pollen but were indehiscent. Both morphs of *L.sinuatum* produced *c.* 75% viable pollen. Examination of pollen exines and cytoplasm by Alexander stain showed that the pollen from the Oceanics and Blue Fantasia 88 was malformed compared with the viable *L.aureum* and *L.perigrinum* pollen. Oceanic pollen had an exceptionally irregular exine and contained a very shrunken cytoplasm. Blue Fantasia 88 also had pollen with a shrunken cytoplasm but a relatively normal exine. Pollen from these two species did not have obvious colpi. Staining of *L.caspium* c.v. "Blue Cloud" anthers in Alexander stain revealed a complete absence of pollen. Many of the grains in *L.caspium* c.v "Blue Lace" were small and devoid of cytoplasm. Viable pollen from *L.aureum* and *L perigrinum* in contrast with the non-viable pollen, were full of cytoplasm, and had obvious colpi and exine ornamentations consistent with A and B pollen (B pollen only for *L.perigrinum*)(see Chapter 2).

(b) Interspecific Crosses.

Examination of pistils showed that with the exception of *L.perigrinum*, A pollen adhered to cob and papillate stigmas but to a greater degree on papillate stigmas. B pollen adhered only to cob stigmas although the occasional B grains could be made to adhere to papillate stigmas. Both A and B pollen adhered readily to *L.perigrinum* capitate stigmas. As indicated by the washing process in preparation for aniline blue staining, only A grains germinated on papillate stigmas and B grains on cob stigmas, regardless of the species involved. Both A and B grains germinated on *L.perigrinum* capitate stigmas. Although all stigmas were heavily loaded with pollen (where pollen adhered to stigmas) there was considerable variation in the numbers of pollen grains germinated on each pistil within any particular cross. Table 3.01 summarises the results from interspecific crosses. Data is expressed as a proportion of the total average number of pollen tubes halted in three particular regions of the pistil.

CROSS	Number of pollinations	Proportion of pollen tubes halted in		
		Stigma	Style	Ovary
Oceanics X *L.perigrinum*	66	0.73	0.14	0.13
Oceanics X *L.aureum.*	83	0.78	0.17	0.05
L.caspium c.v "Blue Cloud" X *L.perigrinum*	6	1.00	0	0
L.caspium c.v "Blue Cloud" X *L.aureum.*	8	0.78	0.22	0
L caspium c.v "Blue Lace" X *L.aureum.*	10	0.92	0.04	0.04
Blue Fantasia 88 X *L.aureum*	7	0.85	0.15	0
L.perigrinum X L.sinuatum	10	1.00	0	0

Table 3.01. Proportional pollen tube distribution in pistils of stated crosses.

Of the 66 crosses of the Oceanics with *L.perigrinum*, multiple pollen tubes entered the ovary in only four pistils. Of the remaining 57 pistils, single pollen tubes were found in the ovaries of 13. In the 88 crosses of the Oceanics with *L.aureum*, multiple pollen tubes entered the ovaries in 12 pistils; single pollen tubes entered ovaries of 25 pistils. Of the cross between *L.caspium* c.v "Blue Lace" X *L.aureum* only one pollen tube entered the top of the ovary in one cross. None of the pollen tubes found in ovaries grew more than half way through the obiturator toward the micropyle and in the majority of cases were halted at the top of the ovary. In the Oceanic crosses with *L.perigrinum* and *L.aureum*, pollen tubes that had entered ovaries often ended in swellings within the obiturator. Pollen tubes failed to reach the ovaries in the remainder of crosses .

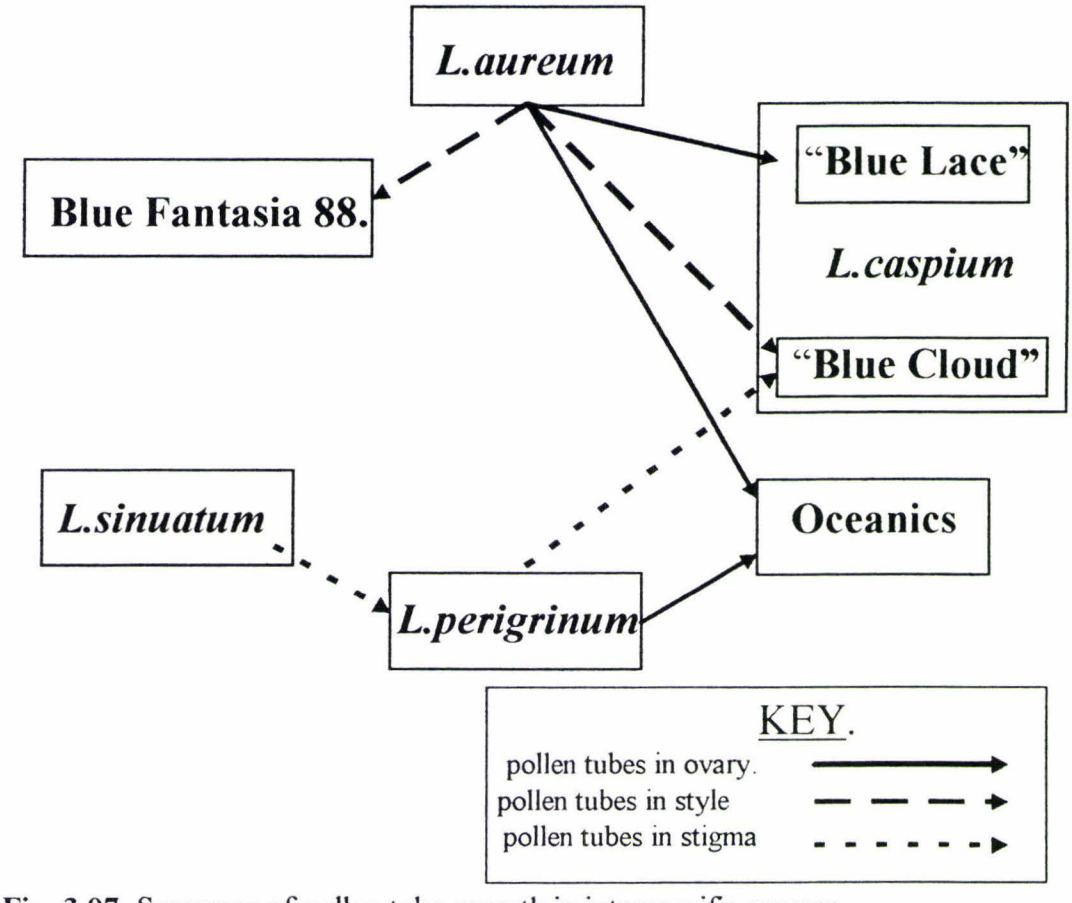


Fig. 3.07. Summary of pollen tube growth in interspecific crosses.

Additional crosses were made for Oceanics X *L.perigrinum* and Oceanics X *L.aureum* for examination of embryo growth. Examination of ovules revealed no embryos at six days post-pollination and no change in colouration of the ovary wall.

No interspecific *Limonium* crosses were achieved.

Discussion.

It was shown in this chapter that pollen will germinate and at least enter the stigma in any cross so long as the pollen stigma combinations are compatible. The germinability of A and B grains on monomorphic *L.perigrinum* stigmas indicates an inability to discern pollen types. The pollen of this species does however obey laws of legitimacy, indicating a relaxation of incompatibility response in only the stigmas of this species. In legitimate crosses, providing fertile ovules and pollen are present, barriers to interspecific pollination exist after stigma penetration by pollen tubes, and at different levels in the pistil by inhibition of pollen tube growth. In such cases, inhibition of pollen tubes in the pistils is most likely a result of genetic differences between species. Of the crosses examined in this chapter, the number of pollen tubes entering the ovaries compared with intraspecific crosses (Chapter 2) was very low. The inability of these pollen tubes to reach the micropyle resulted in failure of fertilization.

Male sterility was observed in several of the *Limonium* plants. This male sterility may have an environmental basis, or possibly a hybrid basis especially in the Oceanics and Blue Fantasia 88. The degree of pollen non-viability found in species, apart from *L.caspium* c.v. "Blue Cloud", suggests hybridity contributes to male sterility in the Oceanics and Blue Fantasia 88.

Female sterility, unless correlated to a gross morphological abnormality, is difficult to assess but if plants are male sterile then the same plants may also be female sterile. Female sterility may have contributed to the failure of pollen tubes to progress to the

micropyle in cases where they had entered the obiturator. It is possible that sterile ovules failed to produce chemotropic substances that would otherwise attract pollen tubes. Alternatively, the obiturator may act as an additional barrier to crosses where genetic differences between species inhibit pollen tube growth.

This chapter provides information on unsuccessful crosses complementing literature on successful crosses to the extent that correlations can be found between geographic ranges of participant species and the likelihood of interspecific crosses. Successful interspecific crosses have been achieved between species with relatively adjacent or overlapping geographic ranges. For example crosses between *L.sinense* and *L.aureum* of the subsection Chrysantheae produced the hybrid Lemon Star. These species have ranges Japan-Korea-China and Siberia-Mongolia respectively. The hybrid Beltlaard is a result of cross between *L.caspium* and *L.latifolium*, these plants were from South Russia-Bulgaria and South East, Central Europe. *L. perigrinum* and *L.purpuratum,* although requiring embryo rescue, have also been successfully crossed (Morgan *et al.* 1995. in press). Both these plants are species from South Africa. The Oceanics are a result of a cross between *L. latifolium* x *L.dumosum*, the latter found in Turkestan. *L. gmelini* from eastern Europe and Siberia also crosses with *L.latifolium* to give the commercial hybrid Charm Blue. A hybrid between *L.sinuatum* (Mediterranean) and *L.perezii* (Canary Is.) has recently be obtained through embryo rescue (E. Morgan *pers. comm.*) The lack of hybrids from very distantly related species (Chapter 1.) supports a direct correlation between phylogenetic proximity of species, therefore to a large degree natural range, and the likelihood of hybridisation.

Crosses attempted between the *Limonium* in this chapter were from widely different ranges and degrees of phylogenetic derivation. *L.perigrinum* originates in South Africa, *L.aureum* from Mongolia, *L.caspium* is from Central Europe to Asia minor and *L.sinuatum* from the Mediterranean. It is likely this was a contributing factor to post-pollination barriers in these interspecific hybridisation attempts.

With the above correlations in mind, future attempts at *Limonium* hybrid production could be more directed. Plants selected would possess between them, attributes that when combined, have a greater potential for a commercially successful hybrid *ie* desirable inflorescence form and colour and genetic similarity by virtue of phylogeny.

Summary.

Interspecific hybridisation was unsuccessful. Pollen tubes will penetrate stigmas in all legitimate crosses irrespective of interspecific genetic differences. Barriers to fertilisation occur by inhibition of pollen tube growth at different levels of the pistil. Hybrid sterility is a possible factor in inhibition of pollen tubes in certain crosses. Suggestions are made to reduce post-pollination barriers and increase the likelihood of developing interspecific hybrids.

4.

Aspects of *Limonium* Pollen Physiology

Introduction.

1. Pollen Viability.

Examination of pollinated stigmas in legitimate crosses (Chapter 2 and 3) showed that although large numbers of pollen were attached to the stigmas, a relatively small proportion of grains germinated and penetrated the stigma surface. It is possible this was due to loss of viability of the grains post anther dehiscence, or low pollen viability due to developmental abnormalities pre-dehiscence. The complete lack of pollen viability recorded in some species (Chapter 3) is examined with respect to developmental anomalies in Chapter 5.

Pollen viability is obviously a principal concern in attempts at crossing plants. Trinucleate pollen grains inherent in sporophytically controlled incompatibility systems (Brewbaker, 1957) have a high rate of metabolism in conditions of high humidity (Hoekstra, 1979; Hoekstra and Bruinsma, 1975; 1979;1980). Consequently, pollen grains lose viability more rapidly under moist conditions.

The high levels of humidity found in glasshouse situations could contribute considerably to the loss of pollen viability. As pollen viability is strongly correlated to pollen germinability (Shivanna and Heslop-Harrison, 1981), maximum pollen viability at pollination is important in increasing the chance of ovule fertilization.

This investigation was made to determine the rate at which pollen from two species of *Limonium* lost viability under known levels of humidity, and to find to what degree

loss of viability post anther dehiscence is likely to have contributed to the low degree of pollen germination observed on stigmas of attempted legitimate crosses (Chapter 2).

2. The Influence of Humidity Upon Pollen Grain Dimensions.

Many authors (Heslop-Harrison and Shivanna, 1977; Gilissen, 1977; Zuberi and Dickinson, 1985) have shown that pollen grains swell in response to atmospheric water, but in *Brassica olearacea* (Stead *et al.* 1979) uptake of water is achieved with little increase in volume. Rehydration of desiccated pollen grains on compatible stigmatic surfaces, an essential prerequisite for germination, is known to be dependent upon the regulated turgidity of the stigma (Heslop-Harrison, 1979). It is possible that the particular osmotic potential of pollen grains also plays a role in self-incompatibility reactions *ie* the successful hydration of a viable pollen grain upon a compatible stigmatic surface may be due at least in some part to the particular water demands of that pollen type. Work by Shivanna *et al.* (1983) on *Primula vulgaris*, a plant with a similar self-incompatibility system to *Limonium* (see Chapter 2) showed that pin and thrum pollen (analogous to type A and B pollen of *Limonium*) responded differently to high humidity levels, in terms of percentage germination upon the stigma surface and concluded that germination of a legitimate grain upon a stigma is a function of the relative humidity in the immediate environment of the grain at the stigma surface. Hydration of the pollen grain and its subsequent germination involves interactions with the water environment of the stigmatic surface and surrounding atmosphere.

The aim of this investigation was to determine any differences in the hydration patterns of A and B pollen grains of two *Limonium* species. Behaviour was measured in terms of changes in pollen grain dimensions and volume during hydration under constant relative humidity *in vitro*.

3. Pollen Grain Germination *In Vitro* and *In Vivo*.

Although the FCR test provides a relatively quick method for determining pollen viability, strictly speaking the viability of a pollen grain is not necessarily correlated with its ability to effect seed set by fertilization of a viable ovule. Consequently, a more accurate method for determining the viability of pollen is achieved by examining the ability of pollen grains to germinate and produce a pollen tubes.

In vitro germination of trinucleate pollen is unfortunately notoriously difficult (Shivanna and Rangaswamy, 1992). Several ions are essential to germination of pollen grains *in vitro*, specifically boron and calcium. The role of boron is enigmatic but it is thought calcium plays a role in pollen tube tip growth (Steere and Steere, 1989). Excessive or insufficient calcium inhibits pollen tube growth while magnesium and potassium are added to the germination media to permit calcium activity (Brewbaker and Kwack, 1963). Of considerable significance to pollen germination is the availability of water in the germination medium. The pollen grain plasma membrane state appears to be a crucial factor in germination (Shivanna and Heslop-Harrison, 1980). Controlled hydration allows reorganisation of the membrane to a continuous lipid bilayer with osmotic properties of the vegetative cell contents promoting the turgidity required for germination (Heslop-Harrison, 1979). If rehydration of pollen is too rapid the plasma membrane fails to undergo adequate phase change and grains burst. Alternatively insufficient water uptake may inhibit germination. The availability of water to pollen grains can be controlled by a variety of means; sucrose is often used both to reduce the osmotic potential of the medium and to provide a carbon source for pollen tube growth. Alternatively polyethylene glycol (PEG), non-toxic to pollen, can be added to the medium to control the concentration of water. Thirdly the concentration of agar in the growth medium will also determine water availability. All media should contain boron, calcium ,

magnesium and potassium while water availability can be controlled through different concentrations of agar and/or sucrose and PEG.

In vivo examination of pollen hydration and germination on compatible stigmas presents technical difficulties. Success in this area has been achieved through rapid fixation and use of electron microscopy following thin sectioning (Dickinson and Lewis 1973a) but such methods were beyond the scope of this work.

In addition to *in vitro* pollen hydration and viability studies, this chapter includes attempts on *in vitro Limonium* pollen germination and a brief examination of *in vivo* legitimate pollen hydration upon a stigma.

Methods and Materials.

1. Pollen Viability.

Stamens were removed from opening flowers of glasshouse grown *L.caspium* c.v. "Blue Lace" and *L. aureum*. The anthers from collected stamens were placed on microscope slides and allowed to dehisce under ambient conditions.

As levels of pollen viability are likely to be variable between anthers of each species, anthers were randomly selected for testing. An initial determination of pollen viability was made for both species at time zero minutes.

Stamens were placed in petri dishes with relative humidities of 35%, 65%, and 95% and a temperature of 25 °C. Relative humidity was controlled by lining each petri dish with filter paper soaked in glycerol water solutions (Dr. H.A. Outred. *pers. comm*. Appendix 4.)..

A random selection of four or five anthers were taken from the three relative humidity treatments after 25, 50, 75, 100, 150, and 200 minutes and viability of the pollen samples determined by the FCR reaction. A solution of fluorescein diacetate in

acetone was prepared (Appendix 3.) and added dropwise to 0.3 M sucrose solution (Shivanna and Rangaswamy, 1992)

A drop of this solution was placed on a microscope slide and anthers of selected stamens were 'washed' in this to remove pollen grains. The slides were viewed using a Zeiss microscope with an epifluorescence condenser IVFI and U.V filter . Counts of viable and non-viable grains (minimum of 100 grains) were made from four non-overlapping fields of view at 100X magnification for both species, in each humidity treatment at the specified time intervals. The proportions of viable and non-viable grains for each field of view were recorded and means presented (Figs 4.03 *a* and *b*. Results). Error bars are standard errors of the means (SEM).

2. The Influence of Humidity On Pollen Grain Dimensions.

This experiment involved placing pollen from dimorphic *L.aureum* and *L.perezii* in conditions of known humidity and recording dimensions of the A and B pollen grains over time.

A small chamber, suitable for placement upon a microscope stage, was constructed from a block of perspex. The chamber walls were lined with filter paper soaked in distilled water/glycerol (Appendix 4.) to provide an approximately 95% R.H environment. A glass coverslip provided an airtight seal. A small stand was constructed within the chamber so the pollen grains could be placed upon it and observed under a light microscope.

Pollen grains were collected from recently dehisced anthers and brushed onto a segment of coverslip. Initial readings of pollen width and lengths were made at zero minutes; the piece of coverslip with relatively dehydrated grains was then placed within the chamber.

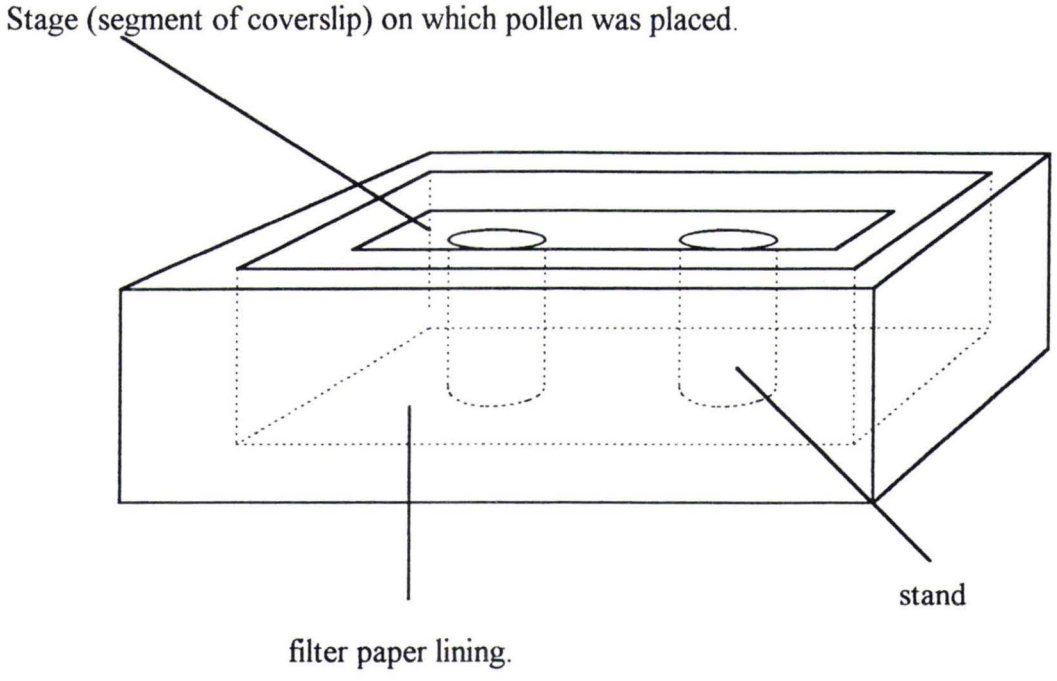


Fig. 4.01. Diagram showing perspex humidity chamber without coverslip seal. The stage needed to be placed at the top of the chamber so pollen on it could be viewed at high magnification.

Widths and lengths of ten pollen grains were measured using a calibrated eyepiece micrometer at 5 minute intervals (400x magnification) to 30 minutes for each of the four pollen grain types. Obviously misshapen or shrunken grains were ignored. Volume of pollen was calculated in using $^4/_3 \Pi abc$ and $^4/_3 \Pi r^3$ (Fig. 4.02).

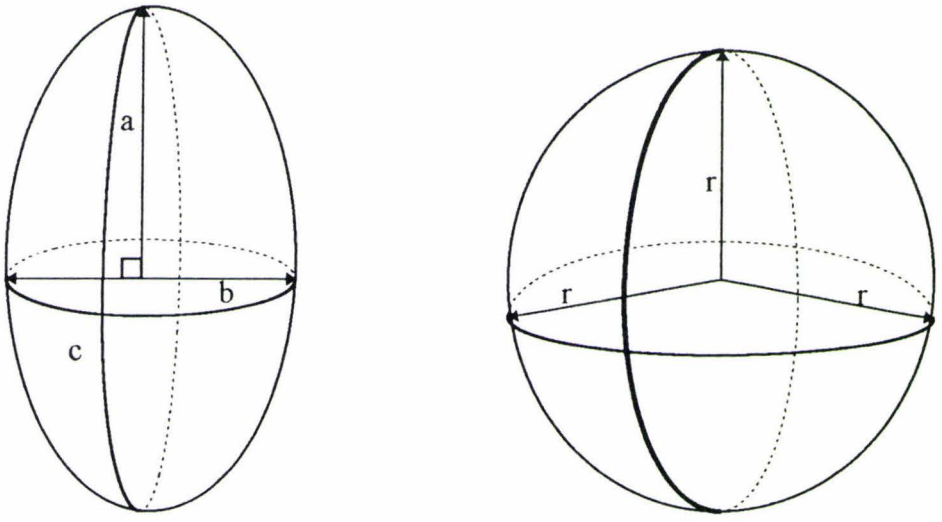


Fig. 4.02. A dehydrated grain (left) and hydrated grain (right). (pollen grain apertures run length wise.)

Changes in length and width and calculated volumes for *L.aureum* and *L.perezii* A and B pollen are given in Table 4.01 and in Figs 4.05-4.06 (Results). Error bars are standard errors of the means.

3. Pollen Grain Germination.

In vitro L.aureum B pollen germination was attempted. Following viability testing by FCR (see section 1 this chapter), attempts were made to find conditions suitable for pollen germination and tube growth pollen using a variety of media and techniques.

Essential minerals were selected as for trinucleate *Brassica* pollen germination media (Shivanna and Rangaswamy, 1992):

$Ca(NO_3)_2$ 300 mg/l
KNO_3 100 mg/l
$MgSO_4$ 200 mg/l
H_3BO_3 100 mg/l

Using this solution, a range of sucrose concentrations were dissolved with 1.5% agar and placed as a drop on a microscope slide and allowed to solidify. Pollen was then placed on the surface of the agar drops and the dimensions and degree of grain bursting recorded for each sucrose condition.

The point at which pollen grain lysis was minimal at minimum sucrose concentration, was recorded. It was assumed this point was optimal in terms of total water uptake into the pollen grain. Control of water uptake rate was then controlled by adding increasing concentrations of agar, to 4.5% ,to the mineral and optimal sucrose solution. Pollen was placed on set drops containing optimal sucrose, minerals, and range of agar concentrations: 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5% and degree of lysis recorded.

An alternative method was used in conjunction with the above combinations in order to prevent 'drowning' of pollen grains. A piece of dialysis membrane was placed over a drop of agar with the lowest degree of pollen grain lysis and *L.aureum* pollen dusted onto it . This is an adaptation of the rather time consuming method employed by Alexander and Ganeshan (1989) facilitating rapid measurement of pollen samples. The overlying membrane allows passage of ions from the underlying solution while preventing drowning of pollen. An obvious drawback to this method is that growth of pollen tubes is at least limited to carbon resources contained within the grain at the time of germination. (see Fig. 4.07 Results).

A simple time lapse photographic record was made of *in vivo* pollen hydration and germination of a *L.sinuatum* A type grain upon a *L.sinuatum* papillate stigma. (see Figs. 4.08 *a-f*).

Results.

1. Pollen Viability.

Maximum fluorescence appeared to be achieved in pollen grains five to ten minutes after exposure to fluorescein diacetate. The pollen viability data was recorded at maximum fluorescence. This investigation showed viability of *L. caspium* c.v "Blue Lace" and *L. aureum* pollen decreased rapidly in a broad range of relative humidities. In all three relative humidity treatments, the pollen viability of both species decreased most rapidly in the first 75 minutes after exposure. At least 50% of the initial pollen viability (t = 0) for both species was lost in this initial 75 minute period. Pollen viability decreased most rapidly in the 95% R.H. treatment. The rate of loss in viability was lowest in the 35% R.H. treatment. Pollen of both species exhibited an apparent decrease in rate of viability loss at 25 minutes in the 65% relative humidity treatment. In the 65% R.H environment, the rate of loss in viability following 25 minutes was approximately intermediate to that recorded in 95% R.H. and 35% R.H. treatments (see Figs. 4.04 *a*.and *b*. over page).

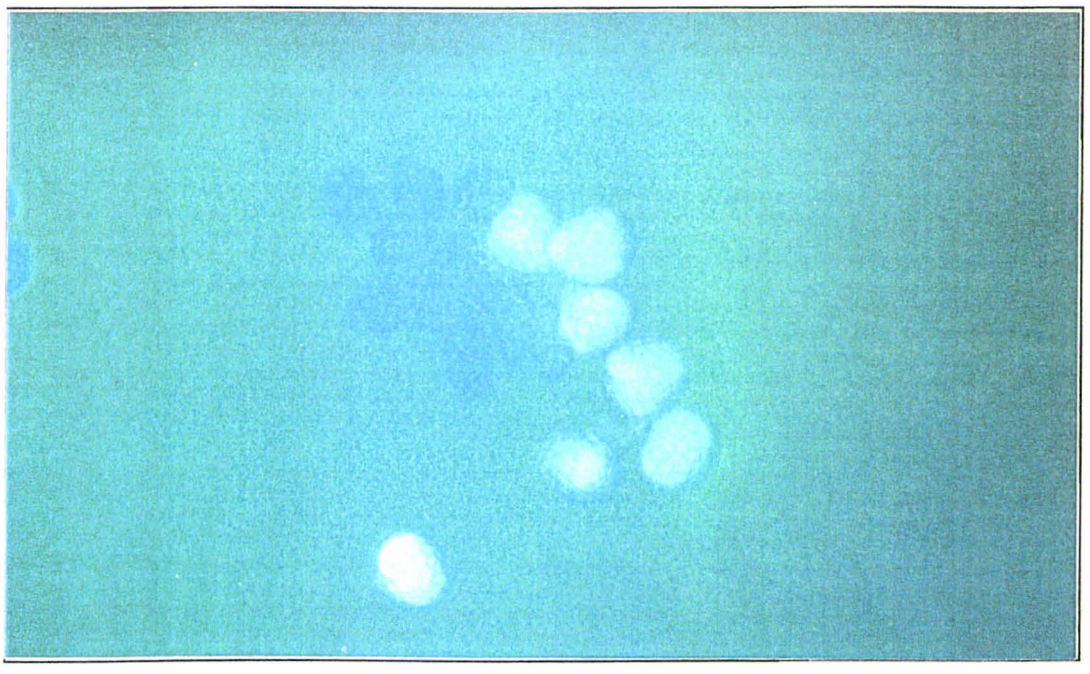
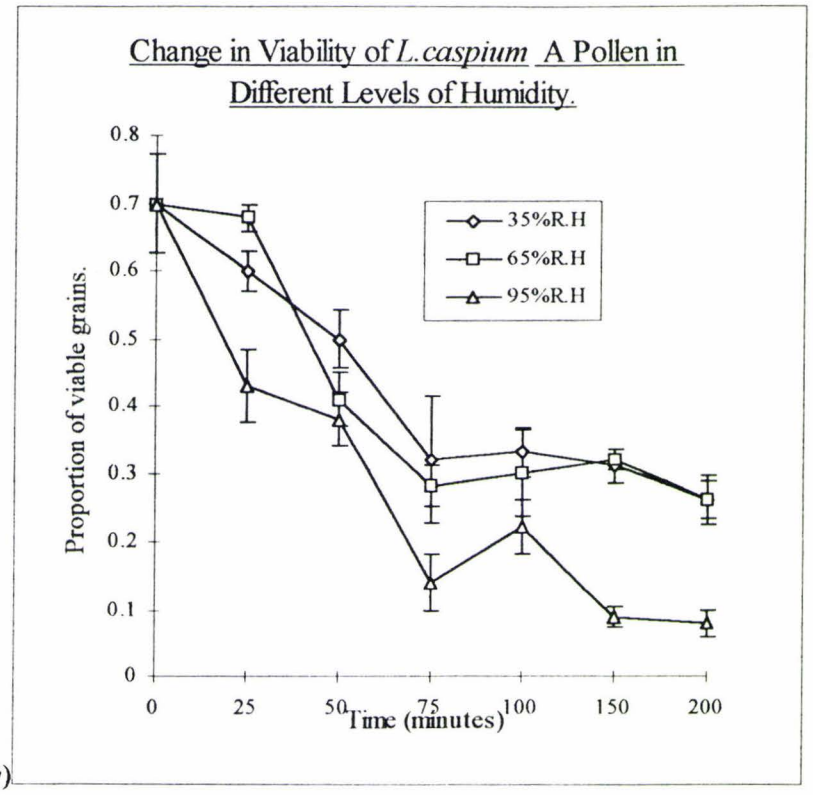
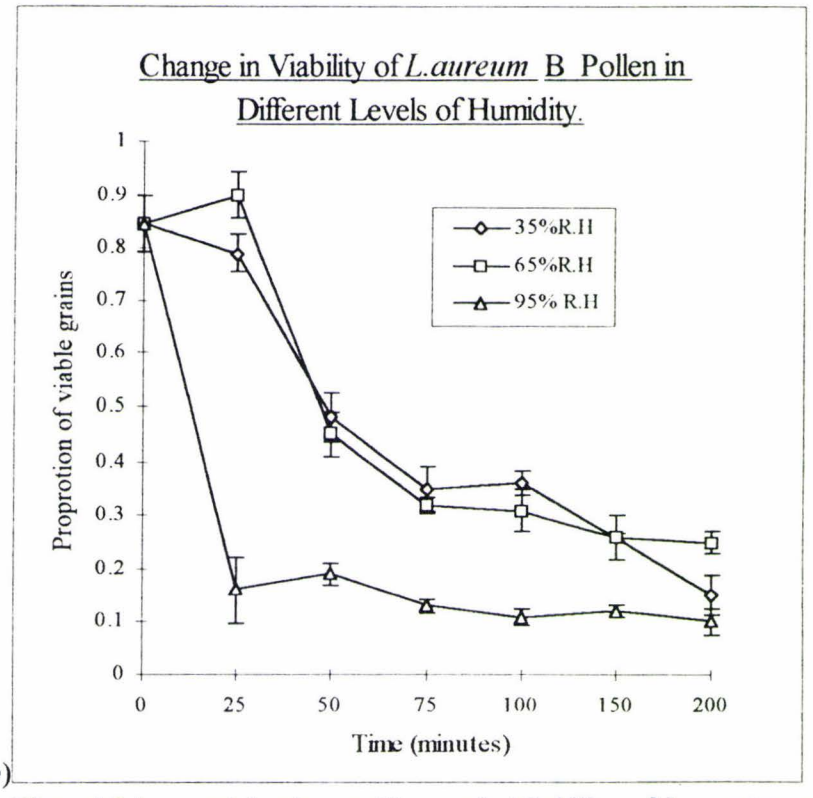


Fig. 4.03 Viable and non-viable pollen grains as indicated by presence and absence of fluorochromasia.



(*a*)



(*b*)

Figs. 4.04. ***a* and *b*. above.** Change in Viability of *L.caspium* and *L.aureum* pollen under three relative humidity treatments.

2. Influence of Relative Humidity on Pollen Grain Dimensions.

Both A and B pollen from *L.aureum* and *L.perezii* showed a general decrease in length and increase in diameter upon hydration. In both species, the type B pollen was larger than the type A pollen but rates of change in dimensions between the two grain types in each species did not appear to differ significantly (see Figs. 4.05 *a.* and *b.*) In *L.aureum* the volume of type B pollen appeared to undergo little increase while type A pollen of this species increased in volume with hydration (Fig. 4.06 *a.*). In *L.perezii* type A pollen increased in volume while type B pollen, after an initial increase, seemed to decrease in volume (Fig. 4.06 *b.*).

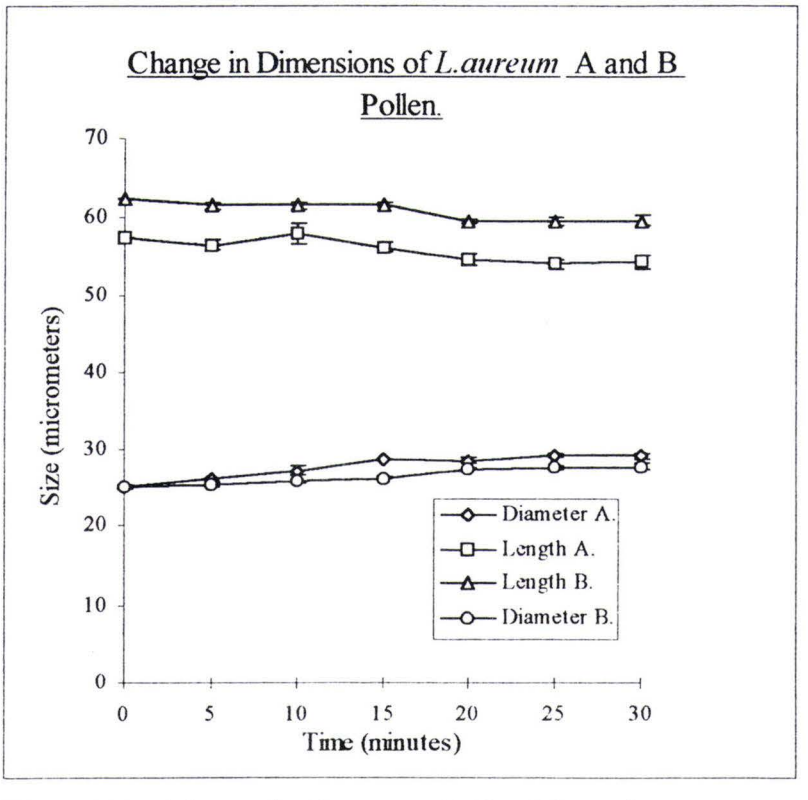


Fig. 4.05 *a.* Change in *L.aureum* Pollen Dimensions.

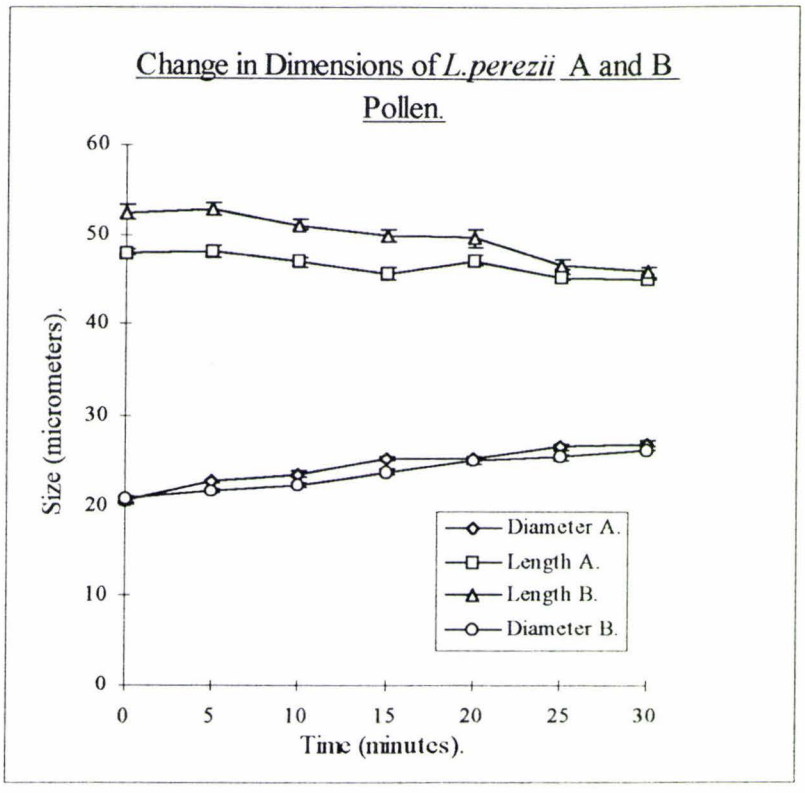


Fig. 4.05 *b*. Change in *L.perezii* pollen dimensions.

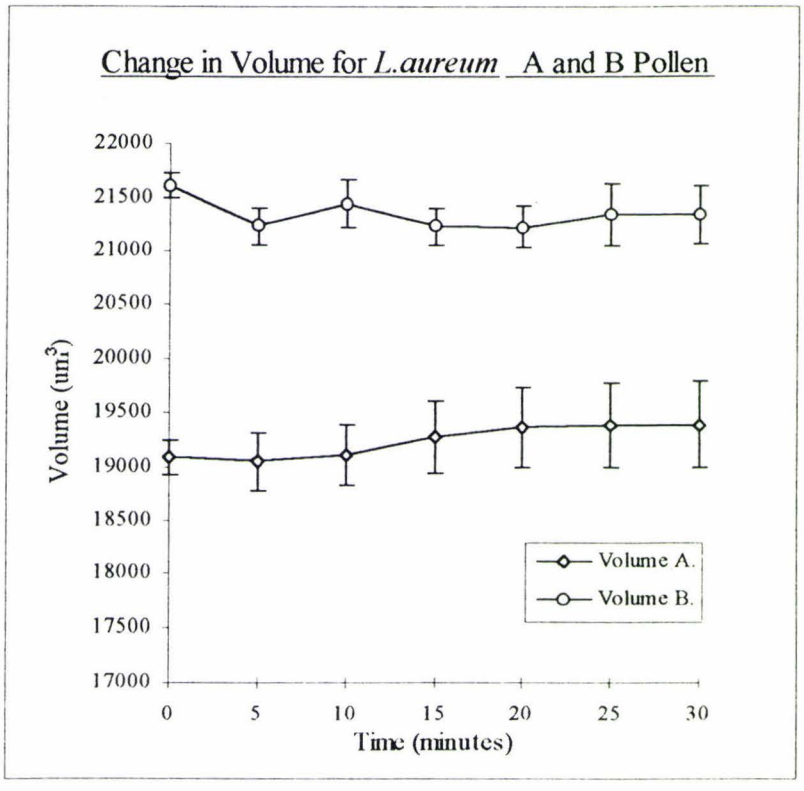


Fig. 4.06 *a*. Change in *L.aureum* Pollen Volume.

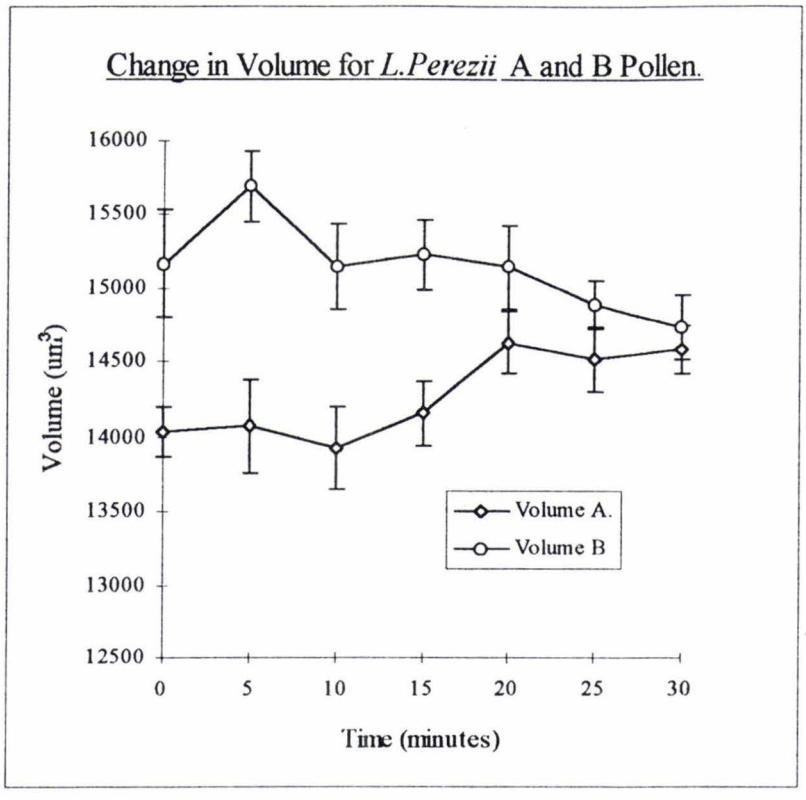


Fig. 4.06 *b*. Change in *L.perezii* Pollen Volume.

Table 4.01 Change in Volume for *L.perezii* and *L.aureum* upon hydration.

	dehydrated (t=0) volume (um^3)	hydrated (t=30) volume (um^3)
L. aureum	**A.** 19085 +/- 81 **B.** 21602 +/- 70	**A.** 19395 +/- 190 **B.** 21338 +/- 138
L. perezii	**A.** 14037 +/- 150 **B**. 15166 +/- 182	**A.** 14580 +/- 128 **B.** 14739 +/- 82

3. Pollen Germination

Pollen failed to germinate on liquid media. Pollen germinated best on a dialysis membrane overlying the aforementioned mineral combination with 4.5% agar and

25% sucrose. An excessive rate of hydration led to bursting of pollen grains before germination. A water deficit resulted in failure of the grains to germinate although some degree of hydration was achieved. In the grains that did germinate pollen tube growth rarely exceeded two to three times the diameter of the pollen grain and bursting of the pollen grain soon ensued.

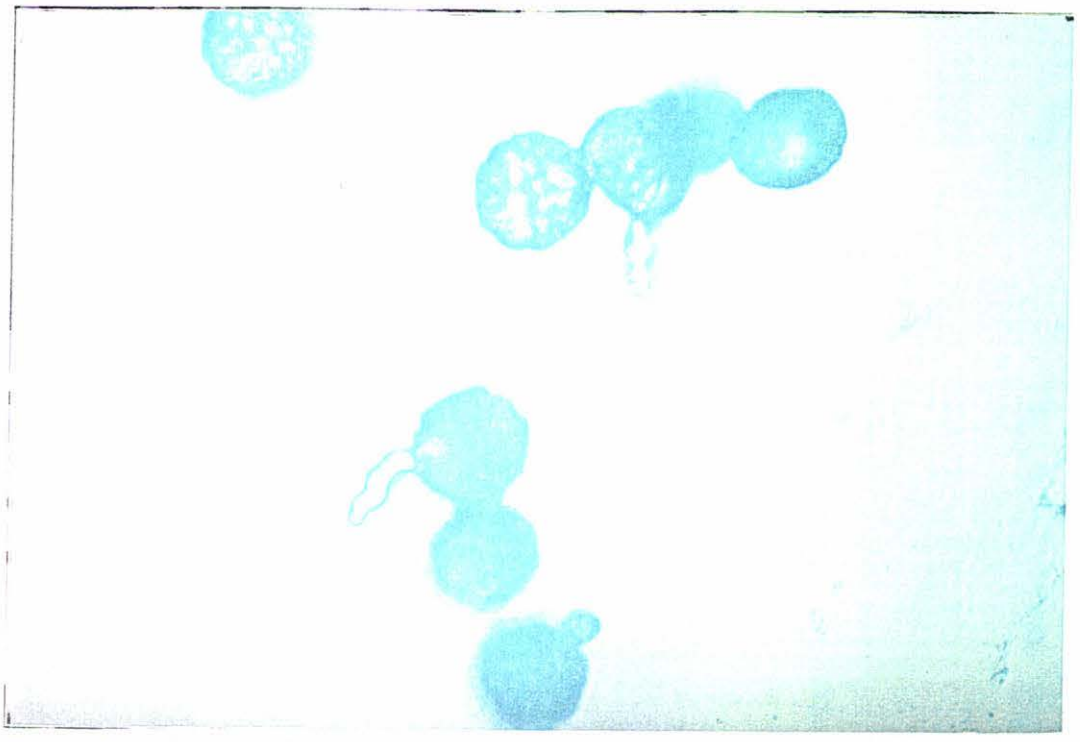


Fig. 4.07. Pollen germinating on a dialysis membrane.

In the brief *in vivo* examination of pollen grain hydration and germination on a compatible stigma, the grain gradually hydrated and changed in shape. There also appeared to be a change in the optical properties of the grain as it became increasingly hydrated. Germination of the grain and penetration of the stigmatic surface occurred 10-12 minutes after pollination as evidenced by the appearance pollen tube (see Figs. 4.08 *a-f* over page)

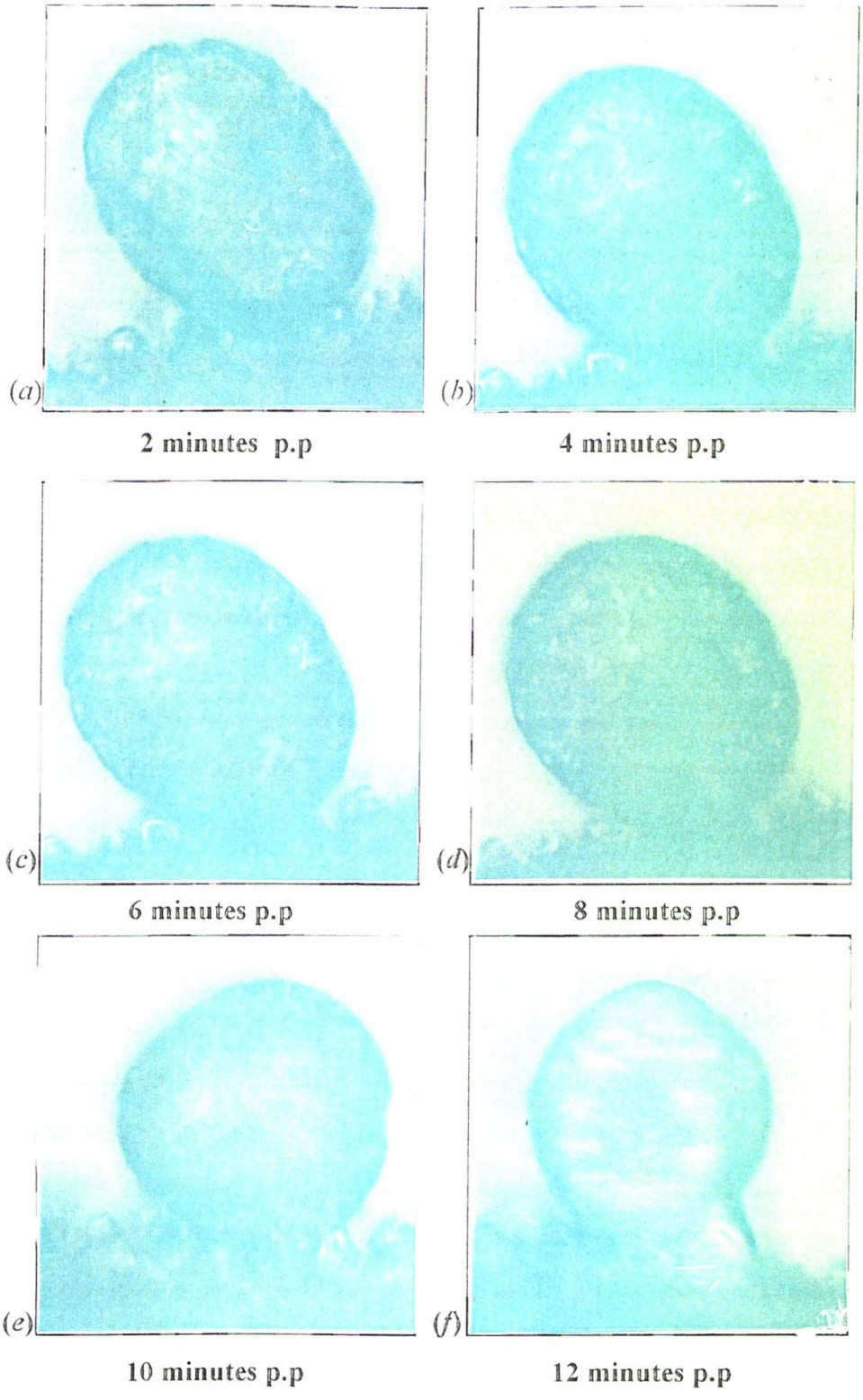


Fig. 4.08 ***a-f.*** Progressive hydration of *L.sinuatum* A pollen grain on a papillate stigma of the same species over a twelve minute period. (Mag 400X) p.p = post-pollination.

Discussion.

The rapid loss of viability in trinucleate pollen grains in humid conditions has been documented in several instances (Hoekstra and Bruinsma, 1975;1980). Pollen of Graminae is an exception (Heslop-Harrison, 1979*a*). Here low humidity is more deleterious to viability than higher humidity.

The initially relatively small decrease in viability in the 65% R.H treatment at 25 minutes (Figs. 4.04 *a* and *b*), suggested pollen grains of the two species increased their ability either to process fluorescein diacetate and/or retain fluorescein to a level higher than at zero minutes, and also to a level higher than seen in the 35% R.H and 95% R.H treatments. Pollen is shed from anthers in a dehydrated and relatively inactive state. Rehydration accelerates cell activity and induces a phase change in the vegetative cell plasma membrane (Shivanna and Heslop-Harrison, 1980). It has been observed in *in vitro* pollen germination studies, that pollen grains will burst if rehydrated too rapidly (Kubo, 1960). It is suggested that the plasma membrane becomes elastic with phase change and that if rehydration is too rapid, the comparative rigidity of the dehydrated membrane state prevents expansion of the vegetative cell and bursting of the grain occurs (Coster *et al.* 1976, Crowe *et al.* 1989). The 65% R.H condition may have induced a phase change sufficient to accommodate expansion of the cell membrane, so that on rehydration in the staining procedure, grains were less likely to burst than if pre-treated in 35% or 95% R.H. The highest rate of pollen viability loss in the 95% R.H. treatment and lowest rate in the 35% R.H. treatment suggests the degree of pollen grain hydration determines the rate of cellular metabolism hence the life expectancy and germinability of *Limonium* pollen.

The very limited life expectancy of *L.caspium* and *L.aureum* pollen grains highlights the importance of performing pollinations immediately after anther dehiscence, especially in situations where plants are glasshouse grown.

Results from the hydration study on the comparative behaviour of *L.aureum* and *L.perezii* A and B pollen grains indicated that although the two types of pollen respond to hydrating conditions similarly in terms of decreasing length and increasing width, the volume of type A grains shows an increase in volume over that of B grains. It appeared that A type grains compared to B grains were more responsive to high humidities as shown by a relatively rapid hydration mediated increase in volume.

Heslop-Harrison (1979) suggested that if the stigmatic surface remains turgid then it acts as an infinite reservoir of water for the pollen grain and therefore has a constant water potential (Ψ); water flows from the stigma toward the grain so long as $\Psi_{stigma} > \Psi_{pollen}$. Flow of water resulting in hydration and germination of a grain is therefore governed by the water potential of the pollen grain, $\Psi_{poll} = \Psi_m + \Psi_s + \Psi_p$ where Ψ_s is attributable to solutes of the pollen grain, Ψ_m representing the matric potential and Ψ_p the equitable wall pressure. Heslop-Harrison divided the hydration of a pollen grain into three stages: the initial flow of water from the stigma hydrating and dilating the grain followed by a second phase where solutes are lost by exudation from apertures and thirdly a stage where the plasma membrane has become organised and the pollen grain vegetative cell behaves as an ideal plant cell. The system by which water enters the pollen grain from the stigma can be presented as a simple electrical analogue *eg* flow of water is determined by resistances.

The observed change in shape seen in *Limonium* pollen (Figs. 4.05 *a* and *b*) means successively more of the intine is exposed as the grain becomes hydrated. The initial high water resistance provided by the exine is bypassed as more of the intine becomes exposed by shortening of grain length. The rate of water uptake by the grain becomes

successively faster as the grain changes shape and aperture openings widen. The plasma membrane is now able to cope with the rapid increase in volume by virtue of phase change. The change in pollen dimensions is multifunctional, allowing increasing, but not damaging amounts of water to enter the vegetative cell, and also affecting a widening of the germinal apertures to allow emergence of the growing pollen tube.

With respect to the hydrodynamics of pollen described by Heslop-Harrison (1979) one might be able to explain the initially anomalous readings manifested especially in *L.perezii* type B pollen, by a decrease in volume upon hydration. It is possible B type grains are more sensitive to high humidity than A type grains. Rapid hydration of B grains in high humidity without adequate phase change may have caused excessive solute loss and hence a lowering of matric potential and therefore reduced ability for subsequent water uptake. Alternatively, the apparent decrease in volume following the initial sharp peak in *L.perezii* B pollen grain volume (see Fig. 4.06) could be explained in terms of damage to the plasma membrane by excessive water uptake and hence high turgor pressure, prior to adequate phase change. The outcome of such a scenario could be a leaky membrane losing water that had entered the grain by virtue of its matric potential. The latter theory could explain the rapid decrease in pollen grain volume seen after five minutes in *L.perezii* B pollen. Type B pollen of *L.aureum* did not exhibit a rapid loss in volume such as that seen in *L.perezii*, but nevertheless showed a decrease in volume. The results indicate A grains of both species are able to tolerate hydration in high humidity significantly better than their B type counterparts. This suggests the alternative gametophytic physiologies between A and B grains going beyond sporophytically determined qualities such as exine sculpturing and size differences.

It is likely the physiology of the vegetative cell by differential response to environmental water between A and B grains, plays a role in pollen stigma

interactions and has some part in the action of self- incompatibility in addition to the primary action of the pollen grain sporophytic domain.

The results from the experiment on pollen grain volume changes suggesting differential sensitivity to humidity is in agreement with results obtained by Shivanna *et al.* (1983), where desiccated pin grains of *Primula vulgaris* germinate well , but thrum grains require careful hydration before germination can be achieved on stigmas.

The pollen germination trials accentuate the sensitivity of *Limonium* pollen grains to water economy. Trinucleate grains require exact conditions for germination and those of *Limonium* are no exception.

Summary.

Limonium pollen rapidly loses viability especially in high humidity. Upon hydration, A and B pollen dimensions change similarly in terms of decreasing length and increasing width, but differ in terms of volume change. *In vitro* pollen germination was achieved through a correct balance of nutrients and control of water availability.

5.

Anther and Ovule Development in *Limonium.*

Introduction.

Oceanic White and Blue Fantasia 88 showed obvious male sterility manifested in non-viable malformed pollen (Chapter 3). It is possible these plants were also female sterile. Examination of micro and megasporogenesis in these two plants in comparison with fertile species, may reveal information about the timing and symptoms of male sterility and reveal gross morphological and/or histochemical characteristics correlated with possible female sterility.

1. Microsporogenesis.

Anther development in tobacco has been reviewed by Goldberg *et al.* (1993). Pollen development is intimate and dependent on the anther and remains so until the grain is removed from the anther by a potential pollinating agent. In microsporangia producing fertile pollen, diploid sporophytic cells of the anther undergo meiotic divisions to form haploid microspore mother cells ($2n \rightarrow 4 \times n$). Subsequent mitotic divisions lead to formation of mature pollen. A large number of studies have focused upon tobacco anther development *eg* Kultunow *et al.* (1990); Esau, (1977). Goldberg *et al.* (1993) divided anther development into two phases. The first involves cell and tissue differentiation and formation of pollen tetrads. Phase two involves enlargement of the anther followed by tissue degeneration, culminating in anther dehiscence and production of mature pollen grains.

Male sterile mutant plants have been used in the examination of anther development (Koltunow *et al.* 1990; Chaudhury, 1993; Laser and Lersten, 1972) and it has been shown that male sterility affects most anther cell types and tissues at various stages of

development. Interspecific hybrid sterility in particular, can have its origins from meiosis I -meiosis II (Edwardson, 1970).

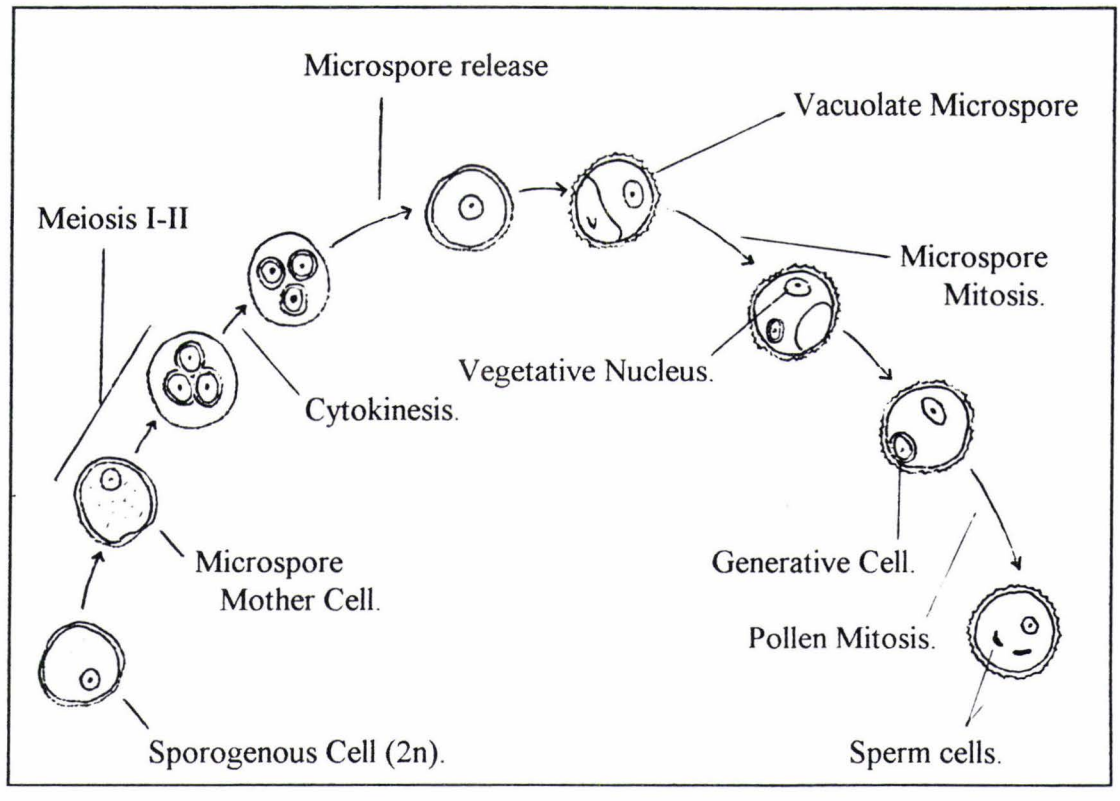


Fig. 5.01 Normal Microsporogenesis (trinucleate pollen) in Dicotyledons. (Adapted and simplified from Laser and Lersten (1972)).

2. Megasporogenesis.

The *Limonium* ovary is unilocular with basal placentation. The ovule is orthotropic and bitegmic. *Limonium* has two phasic tetrasporic *Fritillaria* type embryo sac development (Haig, 1990). *Penaea* type development was recorded for an unknown *Limonium*. The *Fritillaria* type development differs from the more common *Drusa* type development by fusion of the three chalazal nuclei following meiosis. In *Fritillaria* type development, cytokinesis is absent after meiosis II; therefore four nuclei, rather than eight, lie within a coenomegaspore. Following two meioses the embryo sac can be classified further by the number of divisions of the micropylar megaspore nucleus. In two phasic development the micropylar megaspore nucleus divides twice to form two synergids, an egg and a polar nucleus. In addition, the somatic megaspore nucleus derivative divides twice (two phasic) to form four non-

functional nuclei. Three of the four somatic megaspore derivative nuclei often combine to form a triploid chalazal nucleus. The number of nuclei in a mature embryo sac is not constant. In some *Fritillaria* types triploid nuclei degenerate so chalazal nuclei are lacking; all functional nuclei are derivatives of the micropylar megaspore nucleus (Fig. 5.02).

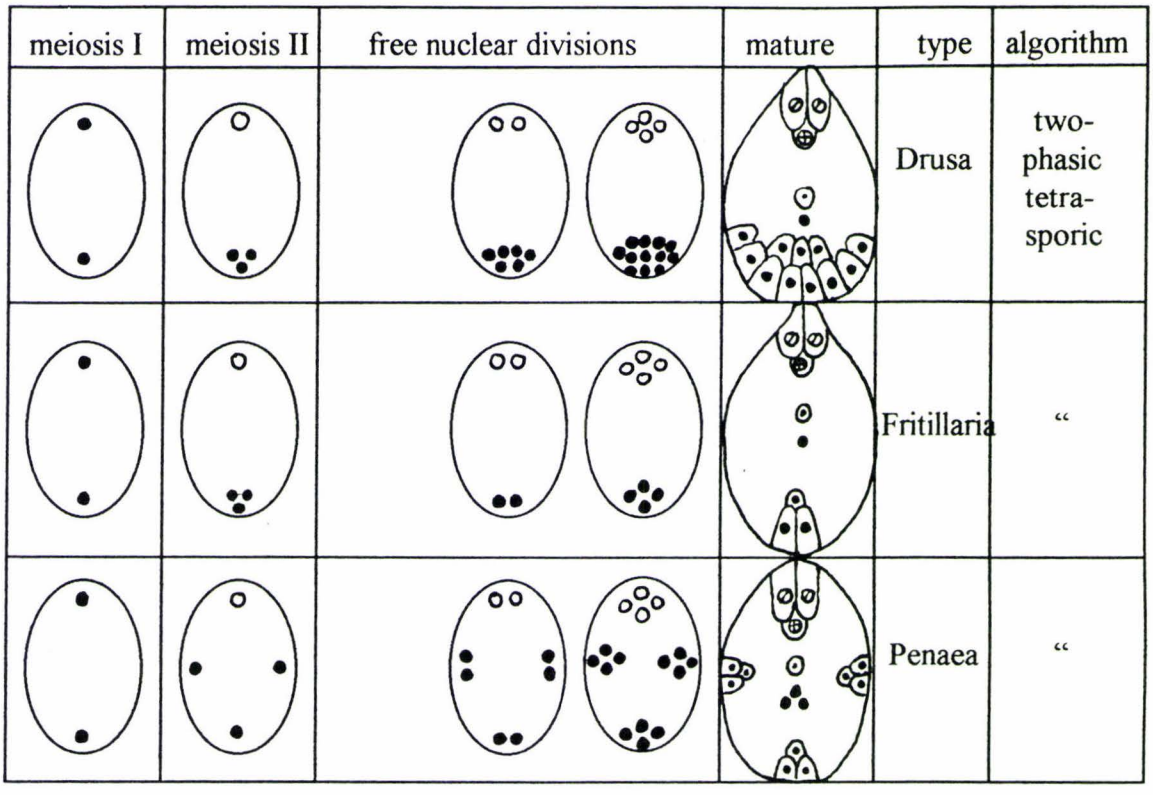


Fig. 5.02.Two phasic tetrasporic embryo sac development.

Key: Somatic megaspore nucleus (or derivative) • ; Fusion of somatic megaspore nuclei ⁂ ; Germinal megaspore nucleus o ;Synergid nuclei ⌀ ; Egg nucleus ⊕ ; Polar nucleus ⊙ . (From Haig (1990)).

This chapter investigates microsporogenesis and megasporogenesis in male sterile and fertile species of *Limonium*. It was hoped the timing and some symptoms of male sterility observed in chapter 3 could be determined. The possibility of female sterility in male sterile species was investigated and an attempt made to show correlates between cytological and morphological characteristics in female sterile ovules.

Materials and Methods.

Materials.

Four *Limonium* lines were selected:

Limonium perigrinum c.v "Ballerina rose". - Male and female fertile.

Limonium sinuatum. Pacific Series.- Male and female fertile.

Oceanic White. (*L.dumosum* X *L.latifolium*) -Male sterile.

Blue Fantasia 88.(unknown parentage) - Male sterile.

L.perigrinum and Blue Fantasia 88 were glasshouse grown with minimum temperature 15°C and ventilation temperature of 25° C. *L.sinuatum* was initially grown outdoors from seeds supplied by Kings Seeds Ltd, then transferred to glasshouse conditions at the onset of flowering. Oceanic White was shadehouse grown. A description of fertility status has been given in chapters 2 and 3.

Methods.

Entire flower spikes, containing a range of floral stages from mature to immature, were removed from inflorescences of the four plants and immediately placed in FAA for at least 48 hours. Open, unpollinated, flowers were treated separately. Following fixation, spikes were removed from FAA and with the aid of a binocular microscope, individual flowers were dissected from spikelets. Flowers of each plant were divided into groups based on relative flower size and length of the calyx relative to the outer floral bract. This provided separate collections of flowers at different relative stages of development for each plant. Each plant contributed at least six collections of flowers at different stages, each collection consisting of at least five flowers. Groups of immature flowers were then placed in 70% ethanol. Open flowers were treated similarly.

Each group of flowers was dehydrated through the ethanol tertiary-butyl alcohol series, starting at 70% ethanol. Prior to the dehydration series, where size allowed, immature flowers were separated from the floral bract to allow better penetration of chemicals. In very immature flowers, where the floral bracts completely enclosed the calyx, flowers were treated in entirety. The pistils of many open flowers became separated from the petals during the FAA treatment and were therefore treated in isolation. Epipetalous anthers of open flowers were treated in isolation for that reason.

Following dehydration, specimens were wax imbedded and thin sectioned using a Reichert ultramicrotome at 10um thickness. Sectioned material was stained with safranin O and fast green (Johansen, 1940) and mounted in DPX.

Longitudinal sections of anthers and ovules for each plant at different stages of development were examined. Complete sequences of embryo sac sections were used to present a 400 X scale model of the embryo sac constituents and surrounding ovule, for each of the species at four different stages. Unclear, incomplete, or oblique serial sections of ovules were not used for diagrammatic representations. Unfortunately such exclusion reduced measurable ovules to one or two per group. Particular recorded morphologies are not necessarily representative of other group members. Anthers corresponding to these embryo sac developmental stages were also recorded. Measurements were obtained using an eyepiece micrometer at 400 X magnification.

Results.

Gametogenesis in ovules and anthers has been recorded. Microspore and megaspore and respective sporangia development are presented separately. As megaspore development is of principal interest in this investigation, observations of microsporogenesis were covered to a lesser degree.

1. Anther and pollen development.

Examination of male fertile plants, *L.perigrinum* and *L.sinuatum* combined, showed early anthers consisted of five layers of cells: the outer epidermis, endothecium, middle layer, tapetum and innermost sporogenous cells. Anther cell layers, with the exception of the middle layer and degenerating tapetum, increased in size by expansion through development of the anther. Pollen had regular B and A type exines at the uninucleate stage for *L.perigrinum* and *L.sinuatum* respectively while the middle layer had become crushed and difficult to distinguish and the tapetal cell had enlarged more than other cell layers. Following the first pollen mitosis the tapetum had begun to degenerate and the endothecial cells became enlarged. At maturity, anthers of *L.sinuatum* contained trinucleate pollen. Pollen had become detached from anthers in *L.perigrinum* during the wax imbedding process. At maturity, the tapetum of both species had completely degenerated, and the enlarged endothecial cells had developed radial wall thickenings.

In Blue Fantasia 88 and Oceanic White, meiosis and release of pollen from the tetrad occurred. Grains developed an exine, contained a small amount of cytoplasm but did not become multinucleate. The tapetal layer degenerated during development and the endothecium developed radial thickenings as found in the fertile anthers of *L.perigrinum* and *L.sinuatum*. The following photographs are a selection of stages in anther development for fertile *L.sinuatum* and *L.perigrinum*, and male sterile Blue Fantasia 88 and Oceanic White.

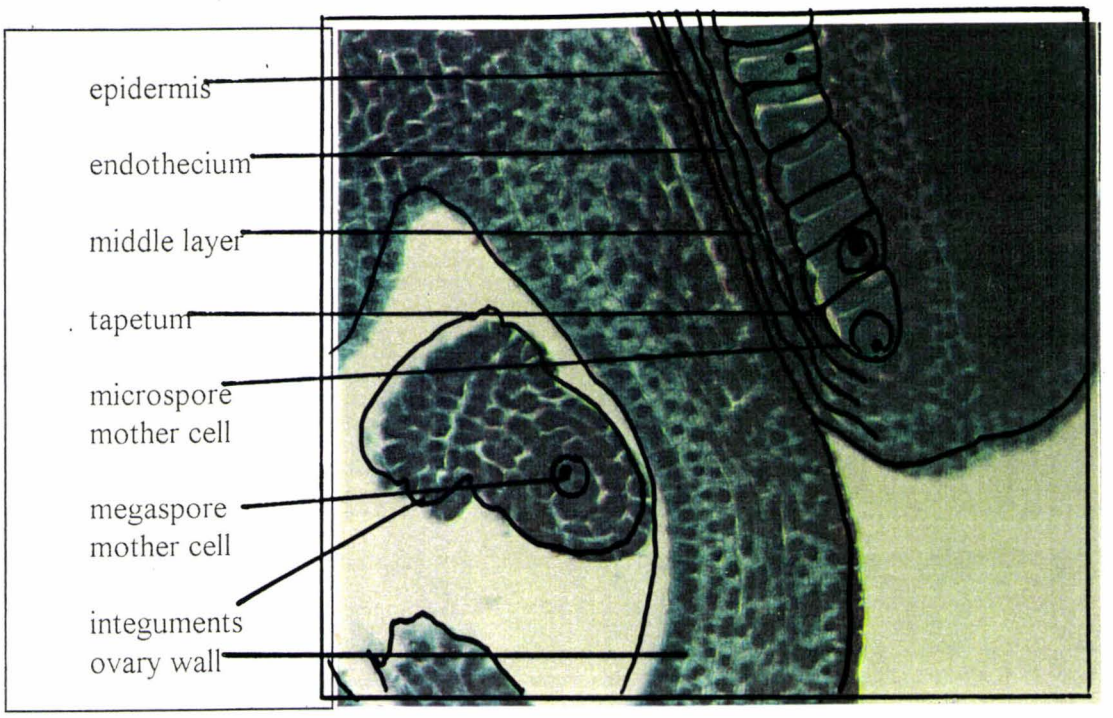


Fig. 5.03. Very early stage of *L.perigrinum* anther and ovule development. The ovule contains a megaspore mother cell but the integuments have yet to develop. The anthers consist of five layers of cells, the innermost are microspore mother cells. (400X)

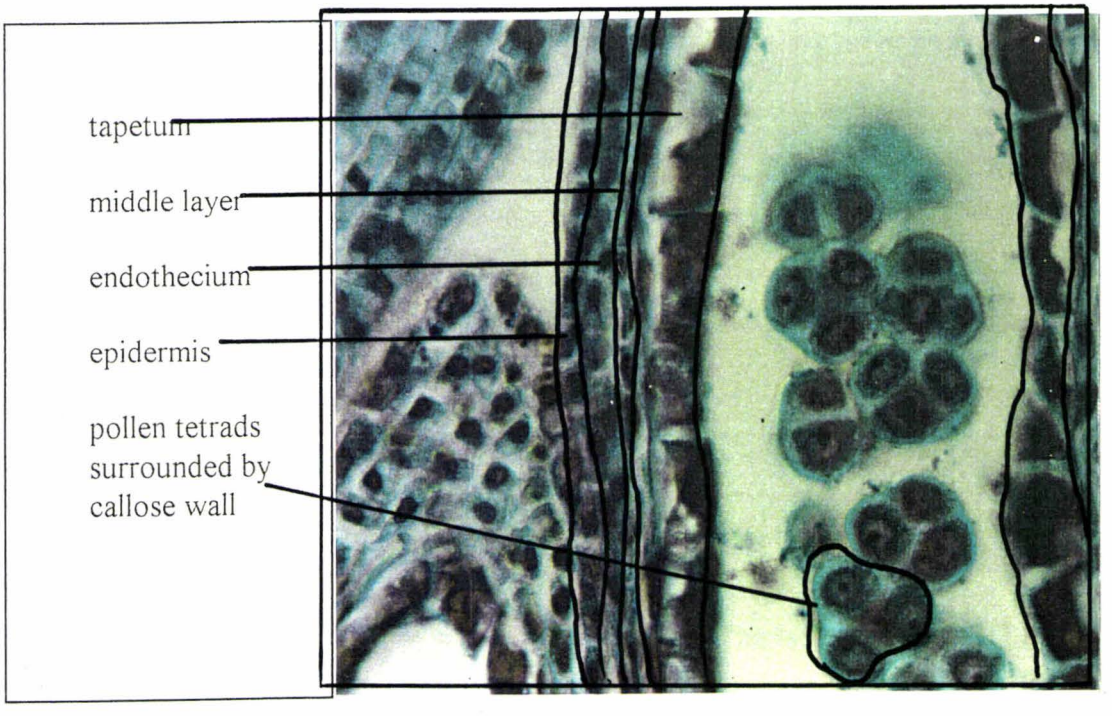


Fig. 5.04. Final stages of meiosis in anthers of *L.sinuatum*. (400X).

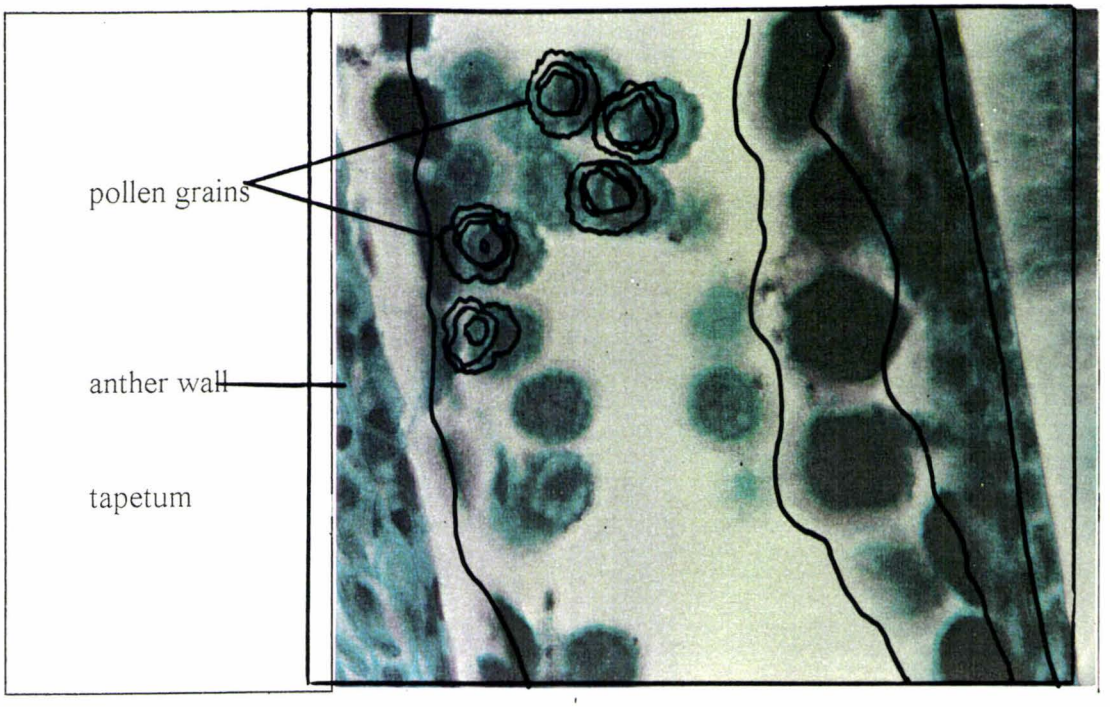


Fig. 5.05. Release of pollen grains from the tetrad (*L.sinuatum*). (400X).

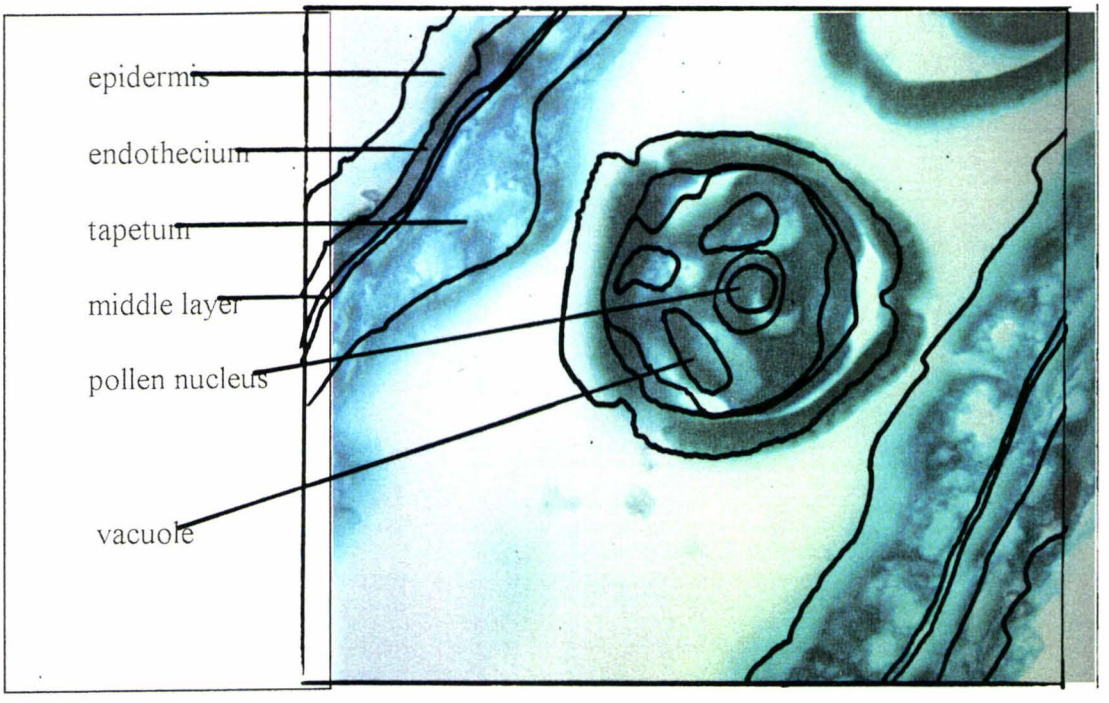


Fig. 5.06. Uninucleate, vacuolate *L.perigrinum* pollen. The tapetal cells have enlarged disproportionately compared with cells of the endothecium and epidermis, these cells having remained a similar size. The grains are relatively full of cytoplasm and a regular exine has formed (400X).

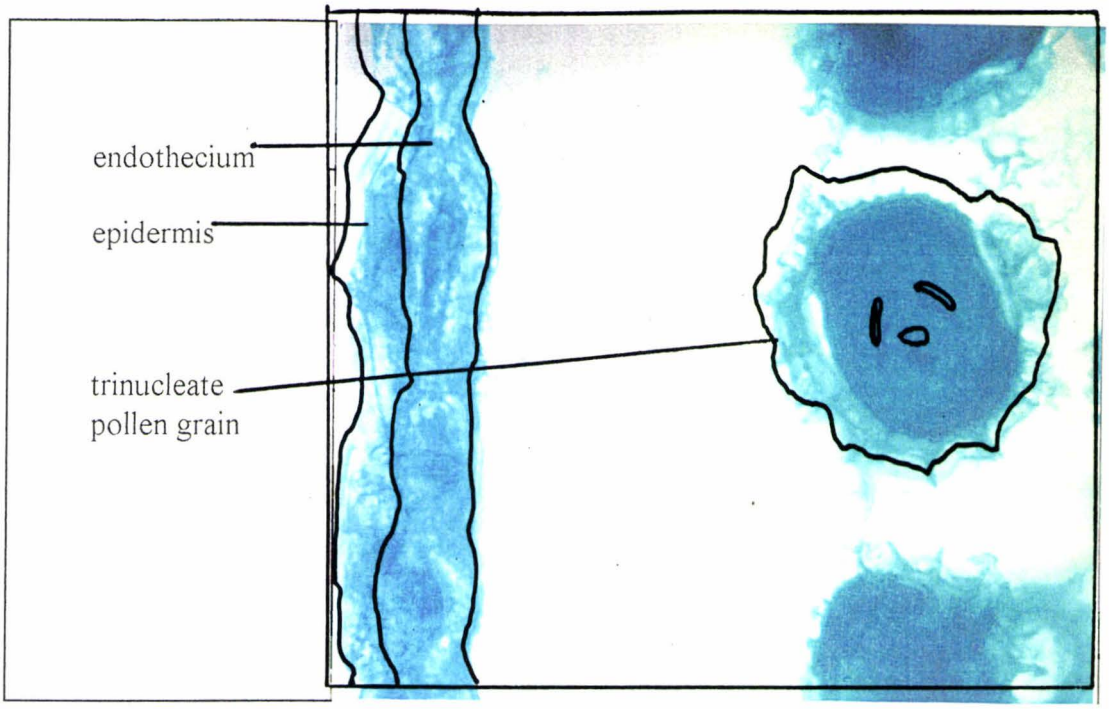


Fig. 5.07. Mature *L.sinuatum* anther with trinucleate pollen grains. The tapetum had completely degenerated. Thickenings had developed on the enlarged endothecial cells (400X).

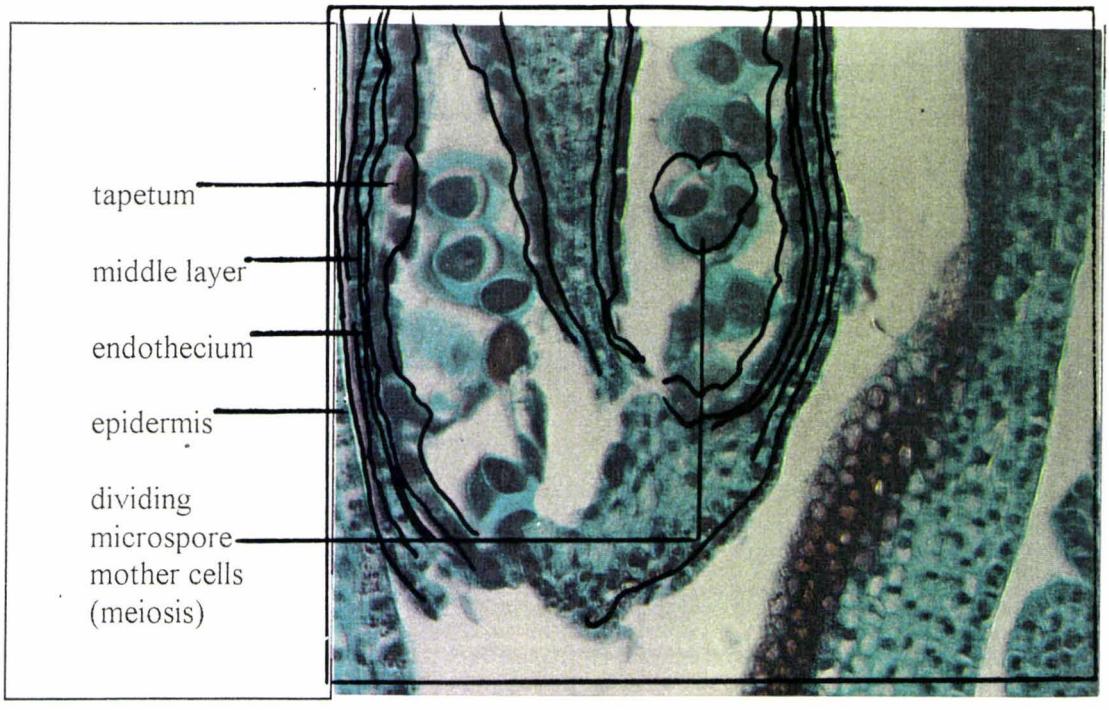


Fig. 5.08. Meiosis in Blue Fantasia 88 (400X).

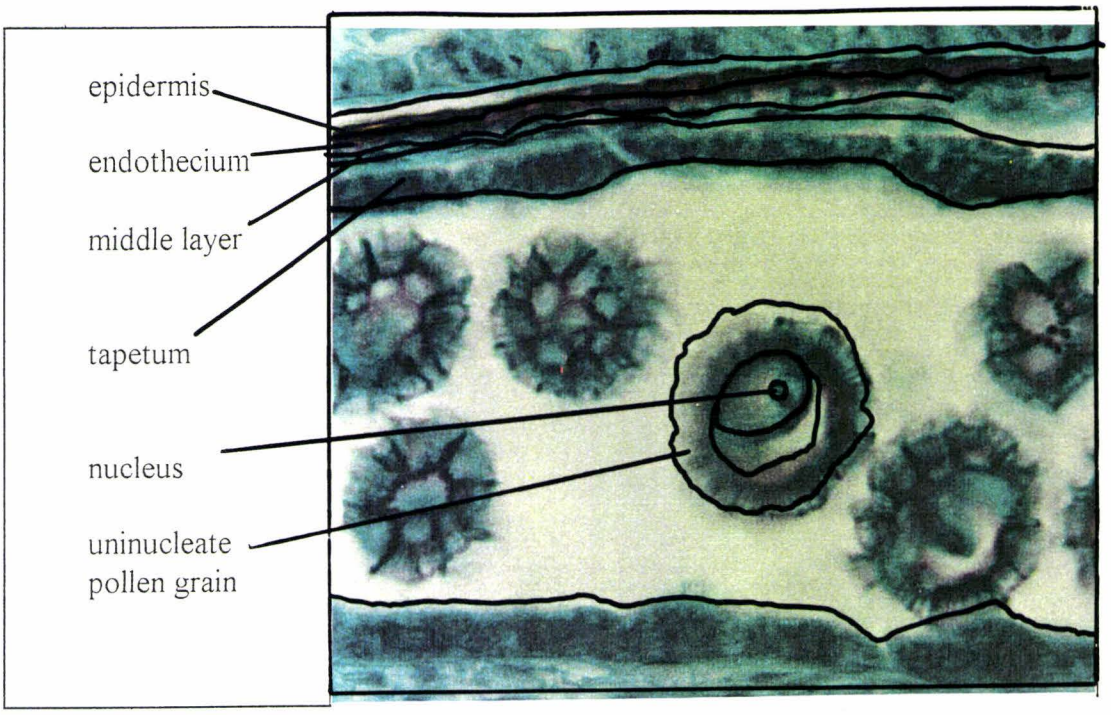


Fig. 5.09. Uninucleate Oceanic White pollen and anther (400X).

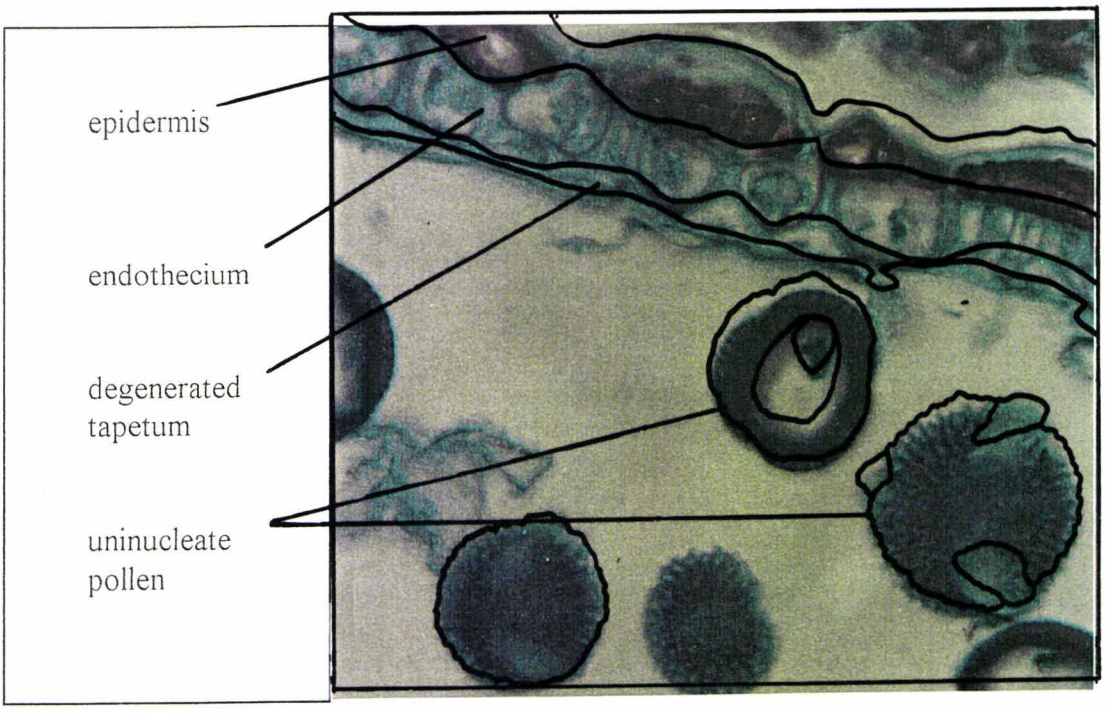


Fig. 5.10. Mature anther and uninucleate pollen in Blue Fantasia 88. The tapetum has degenerated and the endothecium has prominent wall thickenings (400X).

2. Ovule and embryo sac development.

Observations of embryo sac and ovule development are presented separately for each plant. Fertile *L.perigrinum* and *L.sinuatum* are presented first, followed by male sterile Oceanic White and Blue Fantasia 88 ovules. Often, not all embryo sac nuclei could be seen in any one section and so the sums of serial sections are presented as 400:1 scale diagrams. Diagrams show relative positions and numbers of nuclei within the ovule.

Only nuclei between divisions were seen, dividing nuclei may be present, but were difficult to distinguish due to their relatively diffuse nature.

(a) ***L.perigrinum***.

Fig. 5.03 shows an immature anatropous *L.perigrinum* ovule containing a single megaspore mother cell. The integuments had begun to form and the nucellus cells were relatively undivided.

The following embryo sacs and ovules represent later developmental stages.

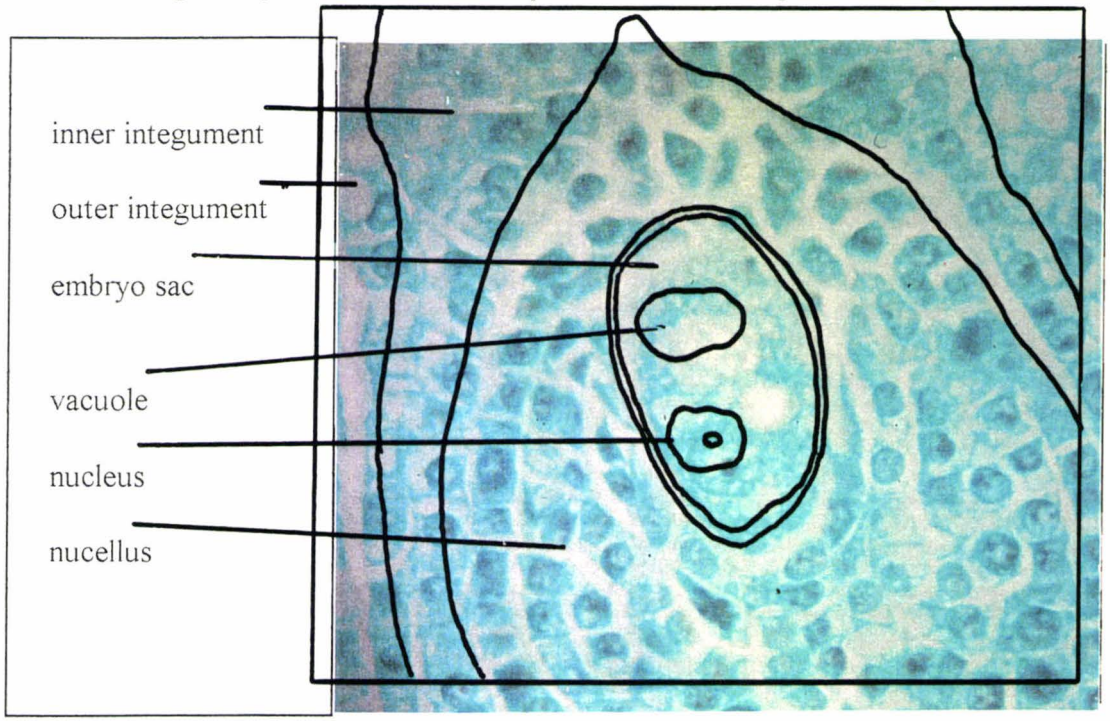


Fig. 5.11*a*. One of five nuclei visible in this *L.perigrinum* embryo sac (400X). Micropylar end at top.

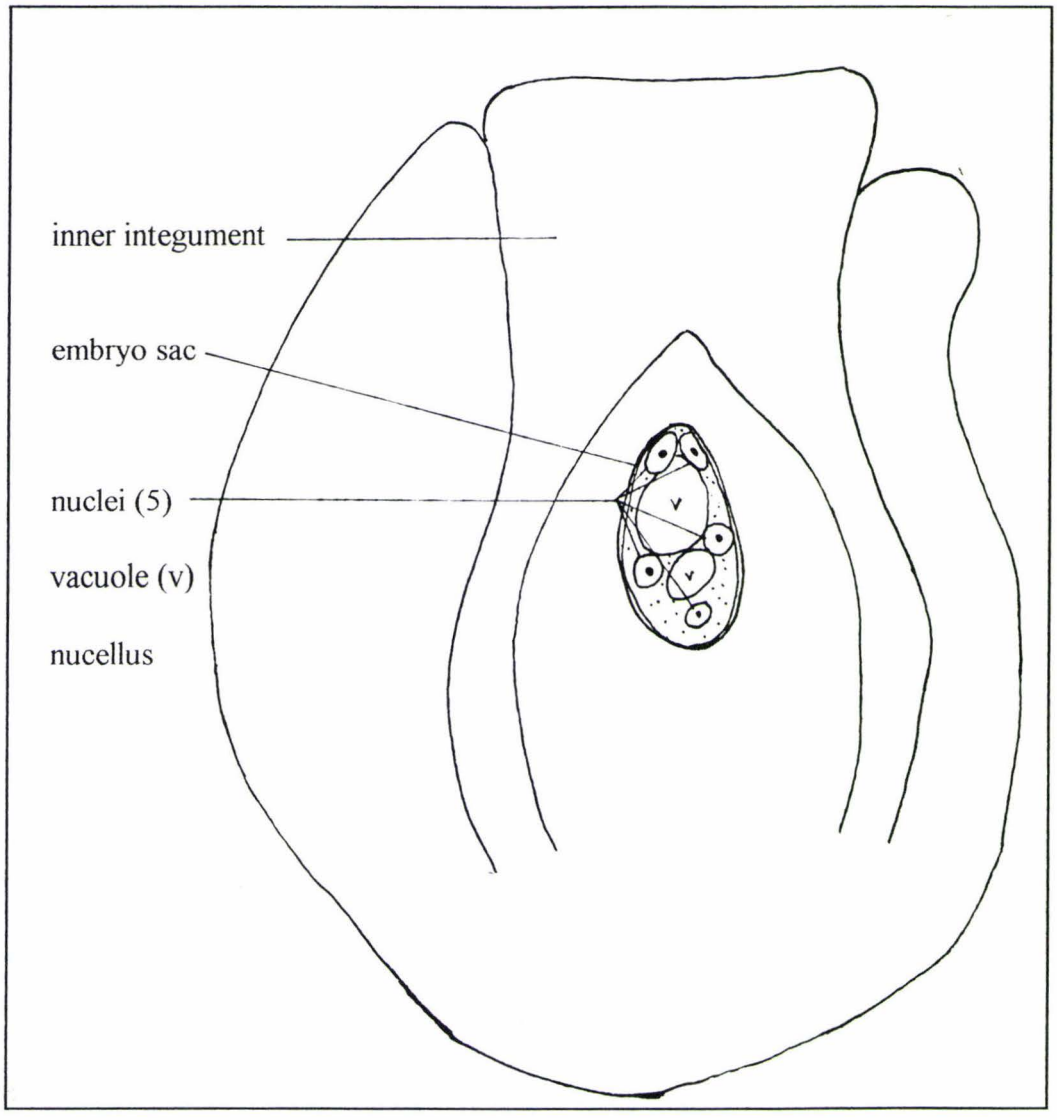


Fig. 5.11*b*. Diagrammatic representation of the five nucleate embryo sac (5.11a). (400X life)

The above diagram was constructed from serial sections of the ovule. Measurements from the medial most section of the ovule were used to construct the position and relative sizes of the ovular tissues and embryo sac position. The relative positions of embryo sac nuclei and vacuoles were determined by their distance from the top centre of the inner integument and edge of the embryo sac. For embryo sac serial sections, the inner integument was assumed to be the most constant feature within each ovule. This method was used for all ovule representations.

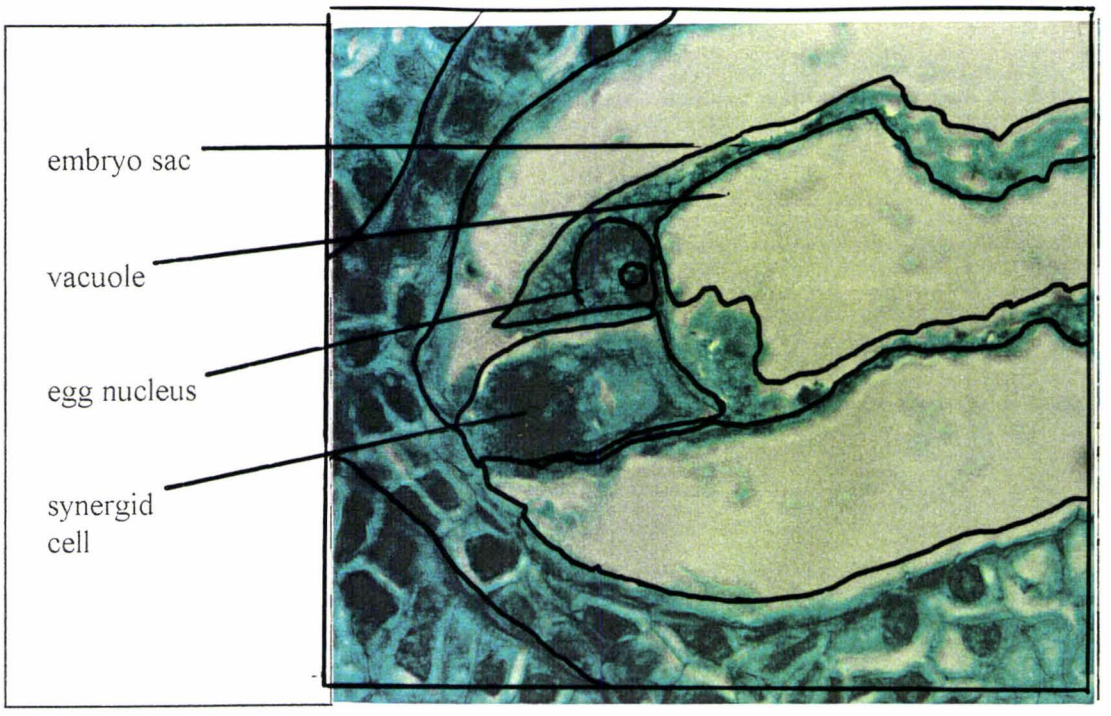


Fig. 5.12*a*. Section through a mature *L.perigrinum* embryo sac showing an egg nucleus and a synergid (400X). The micropyle is towards the left.

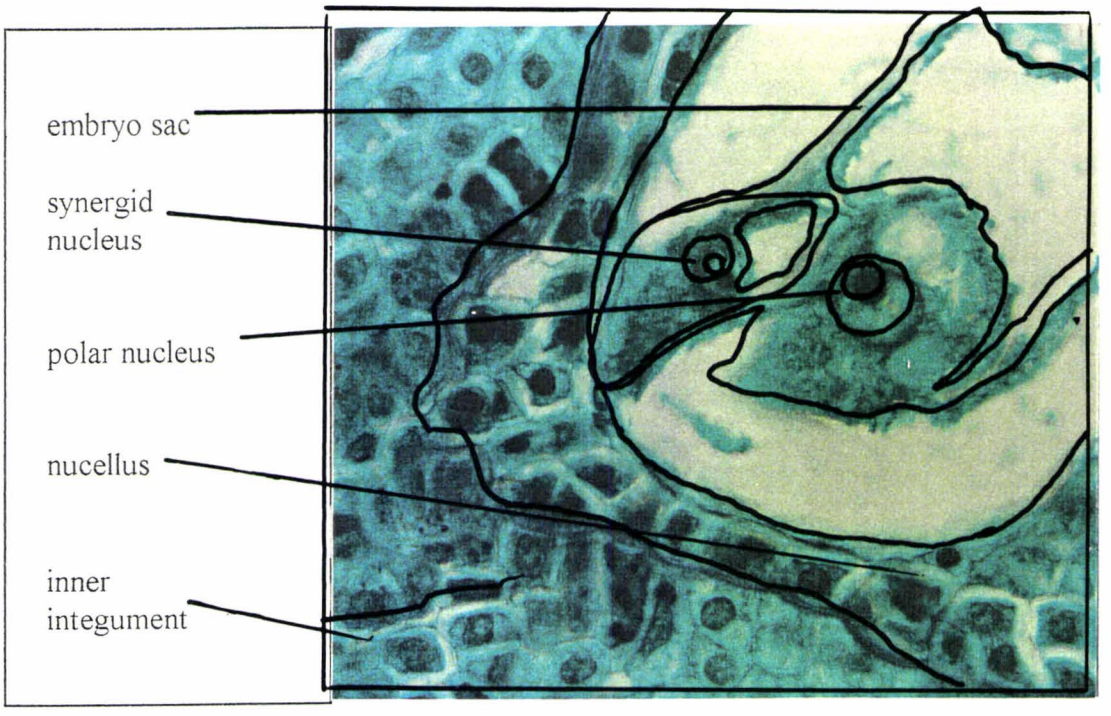


Fig. 5.12*b*. Section through the same embryo sac showing the second synergid and its nucleus, and the polar nucleus (400X). The micropylar end is to the left.

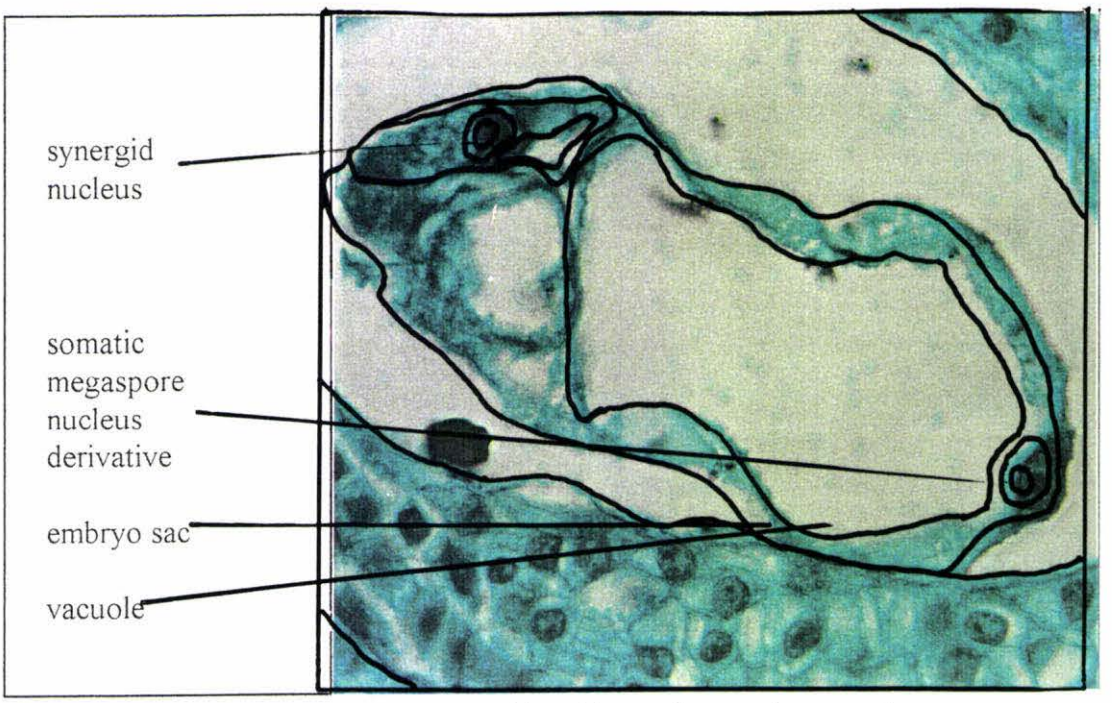


Fig. 5.12*c*. This section shows a synergid and its nucleus and a somatic megaspore nucleus derivative (400X). The micropyle is towards the left.

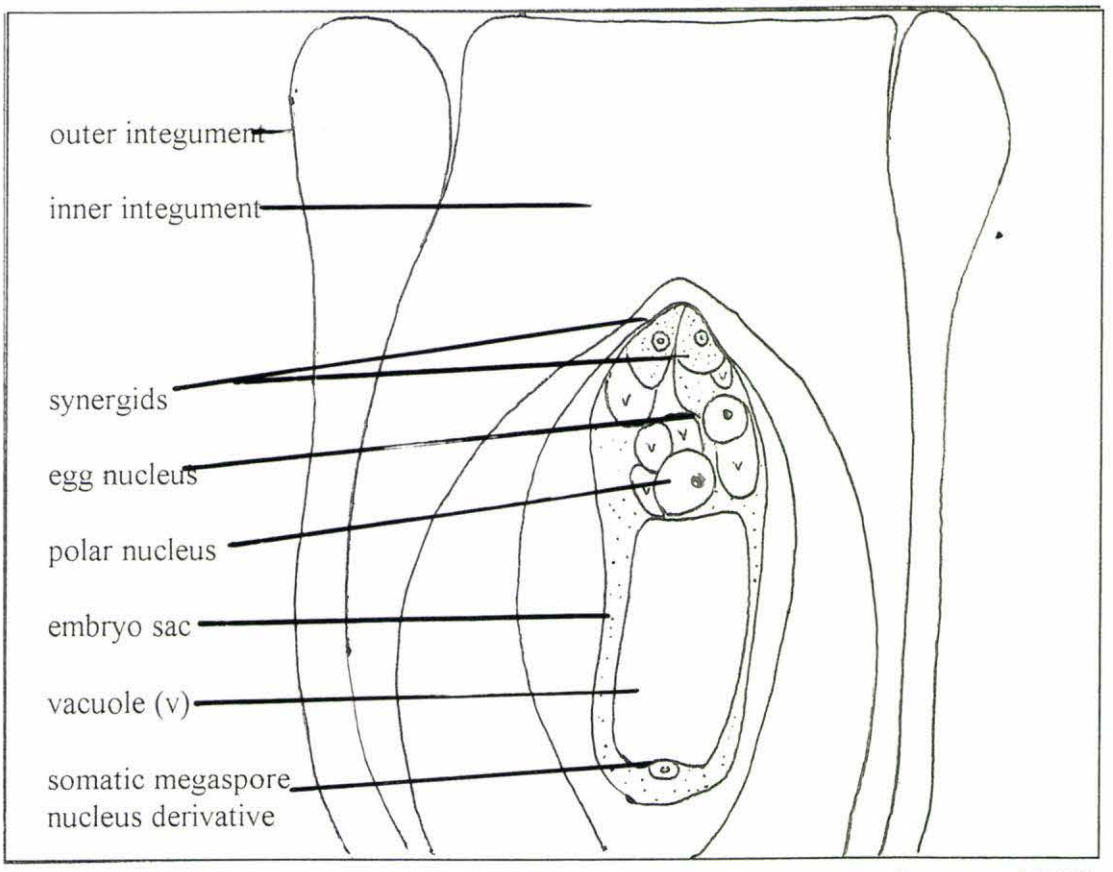


Fig. 5.12*d*. Diagrammatic representation of a mature *L.perigrinum* embryo sac (400X life).

(*b*) ***L.sinuatum***.

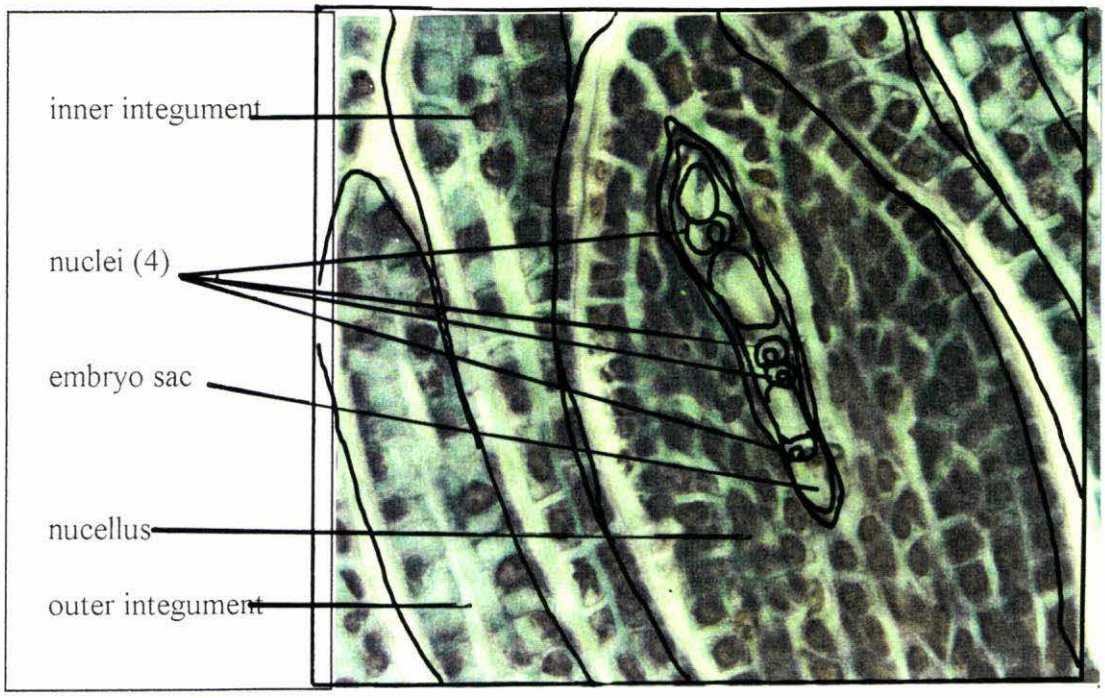


Fig. 5.13*a*. Section through immature *L.sinuatum* embryo sac. Four nuclei were seen in this embryo sac (400X).

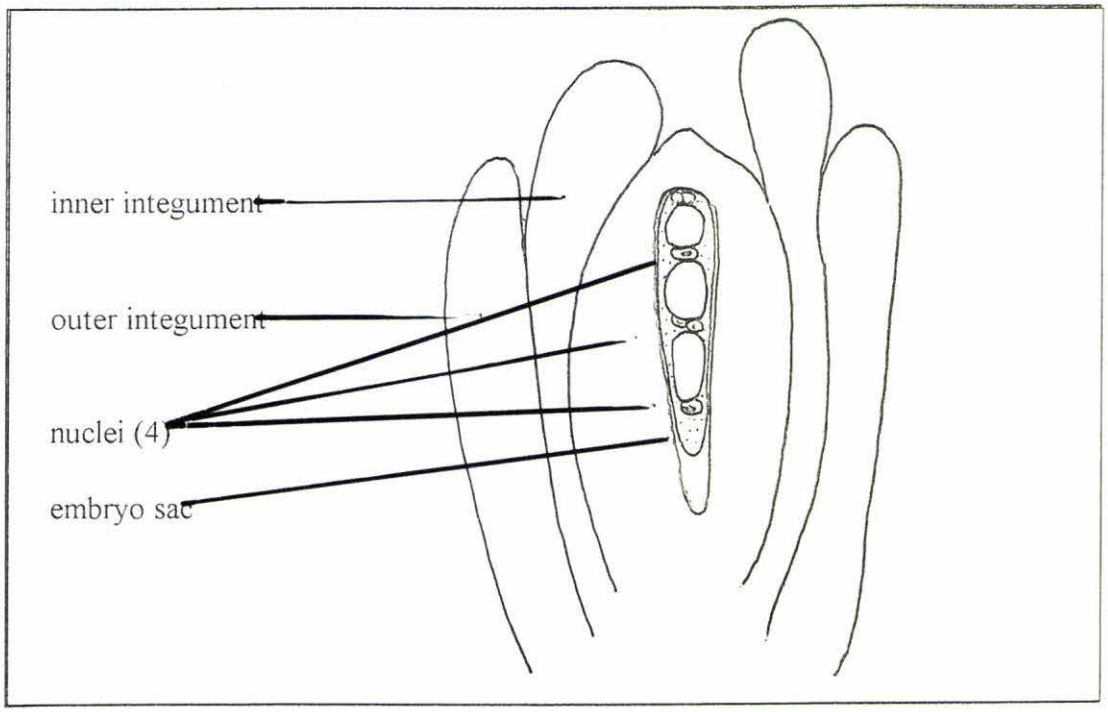


Fig. 5.13*b*. Diagrammatic representation of *L.sinuatum* four-nucleate embryo sac and ovule (400X life).

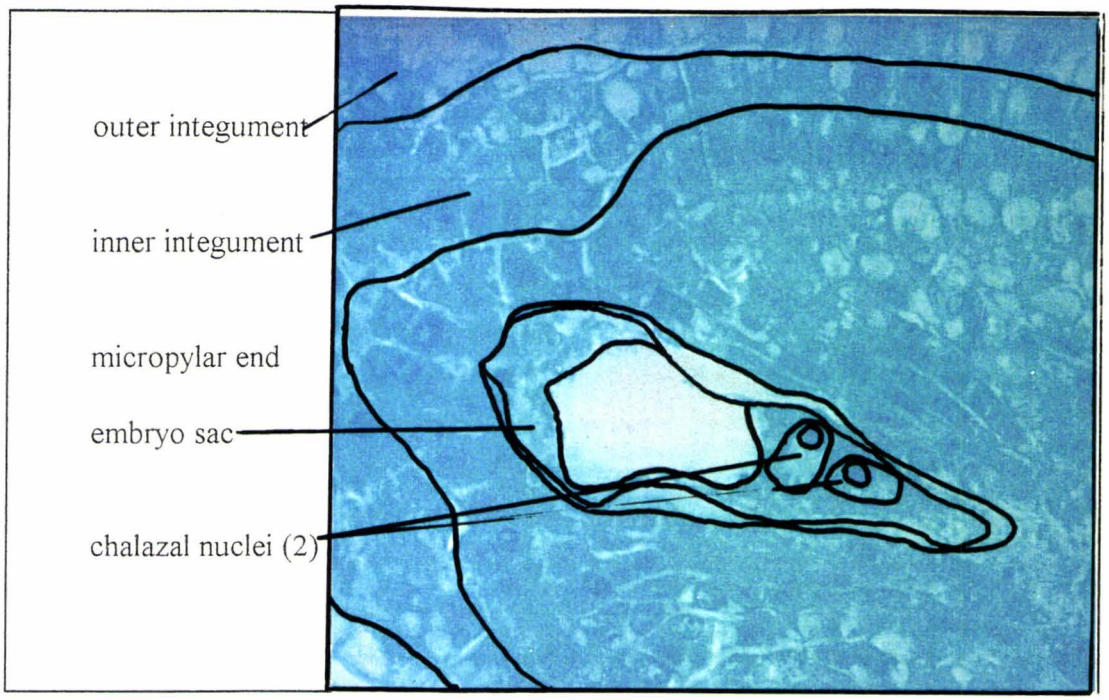


Fig. 5.14*a*. Two chalazal nuclei of a six-nucleate *L.sinuatum* embryo sac (400X).

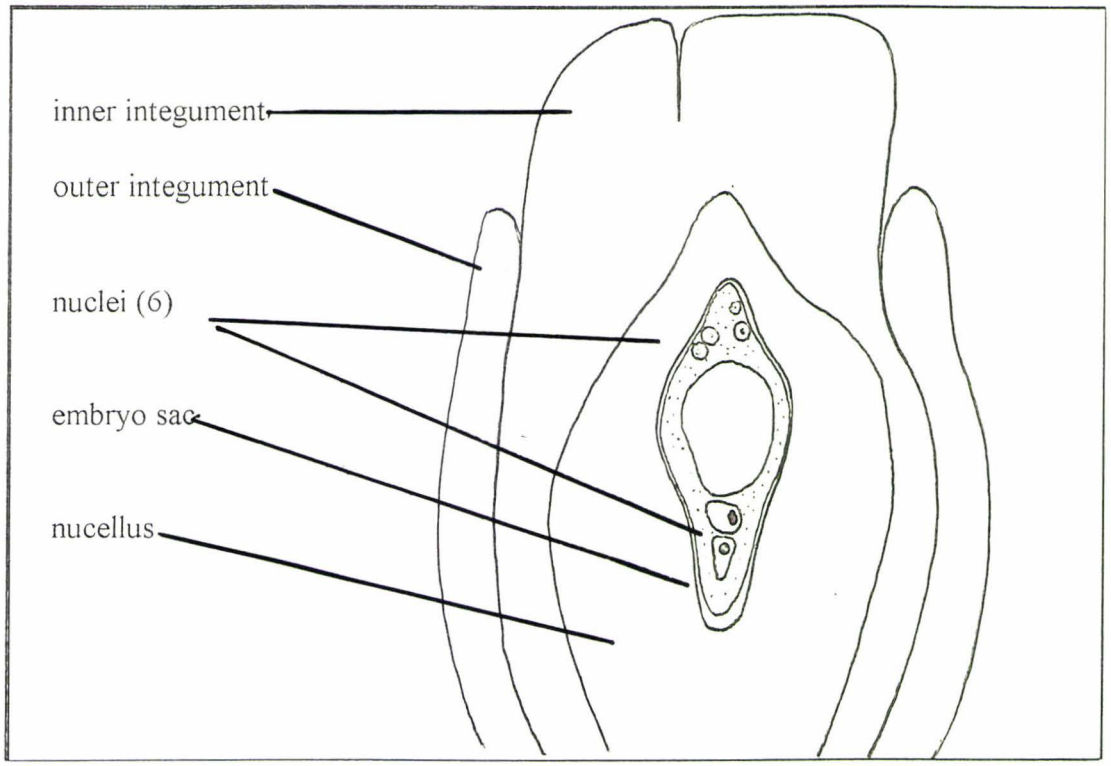


Fig. 5.14*b*. Diagrammatic representation of the six-nucleate *L.sinuatum* embryo sac (400X life).

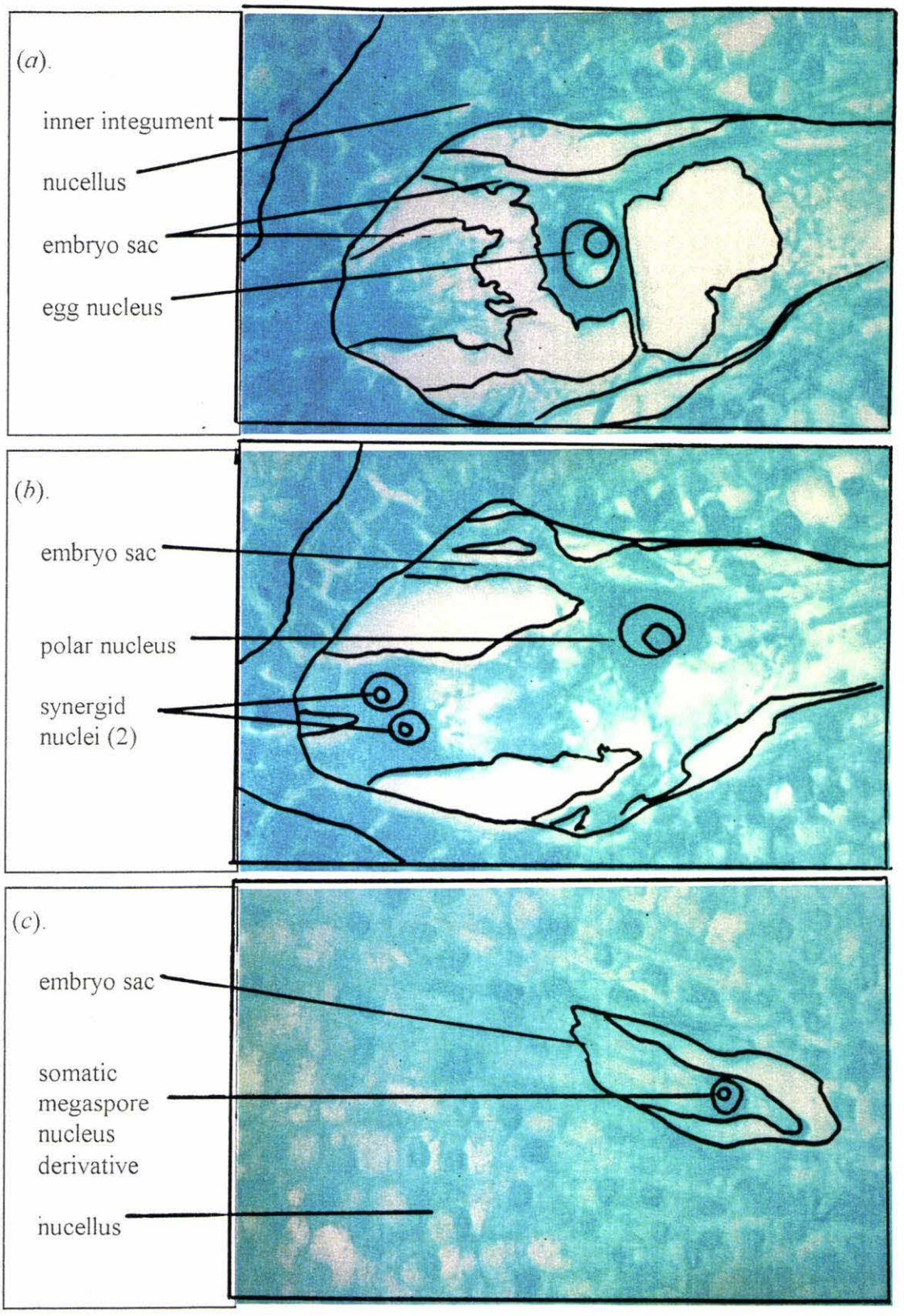


Fig. 5.15 ***a-c***. Three serial sections through the same mature *L.simuatum* ovule showing arrangement of nuclei (400X). The micropylar end is to the left.

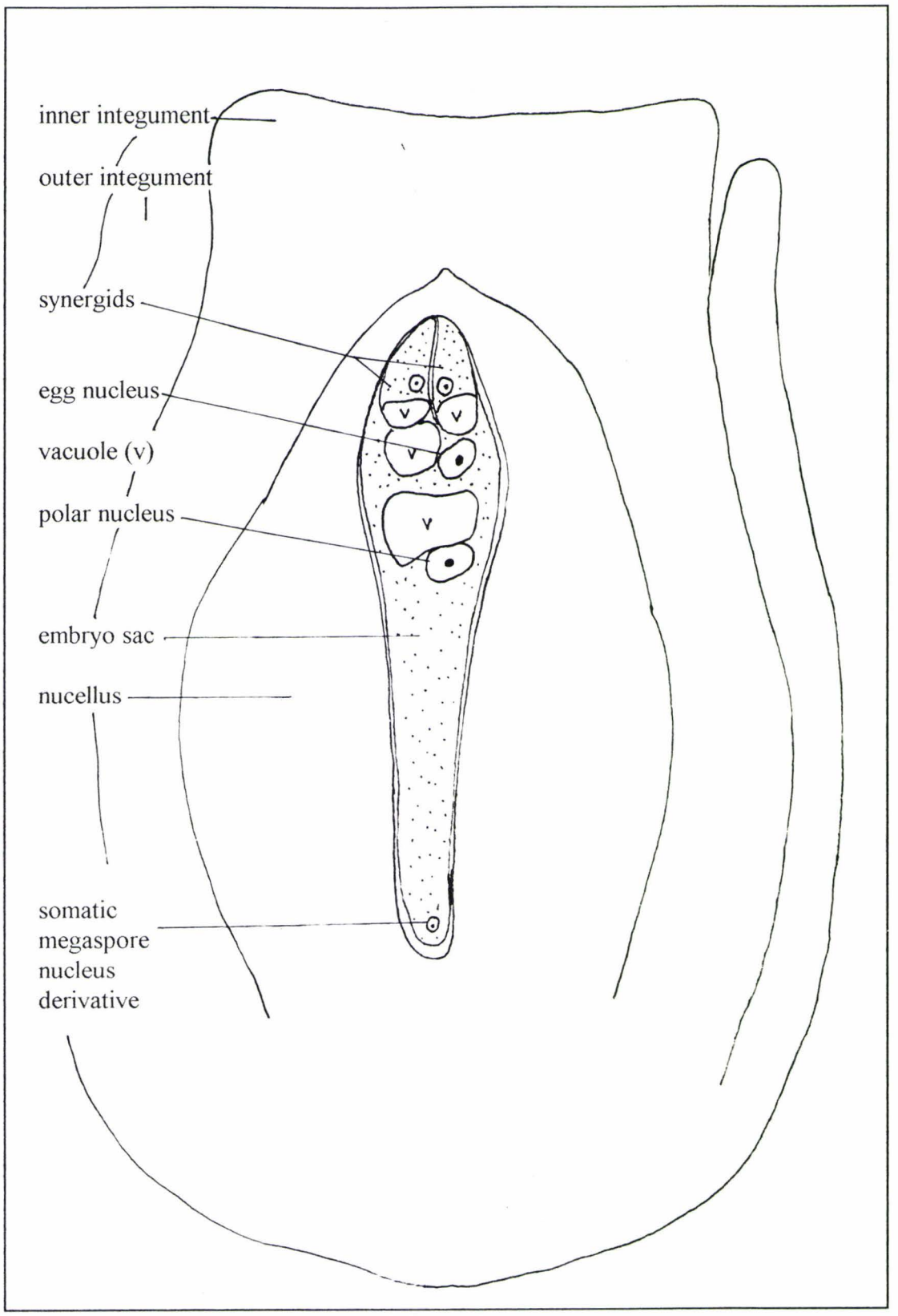


Fig. 5.15*d*. Diagrammatic representation of mature *L.simuatum* embryo sac and ovule (400X life).

(c) **Oceanic White.**

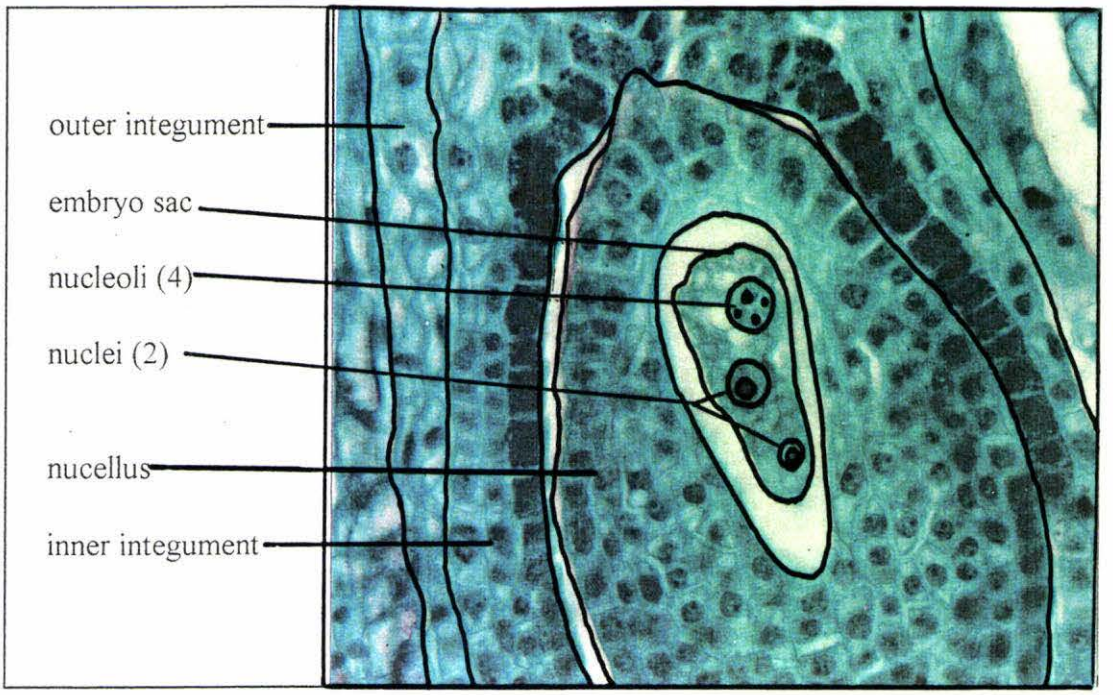


Fig. 5.16*a*. Oceanic White embryo sac containing four nucleoli and two nuclei. Two nuclei are within separate nuclear envelopes while four nucleoli are seen within a single nuclear envelope at the micropylar end (top) (400X).

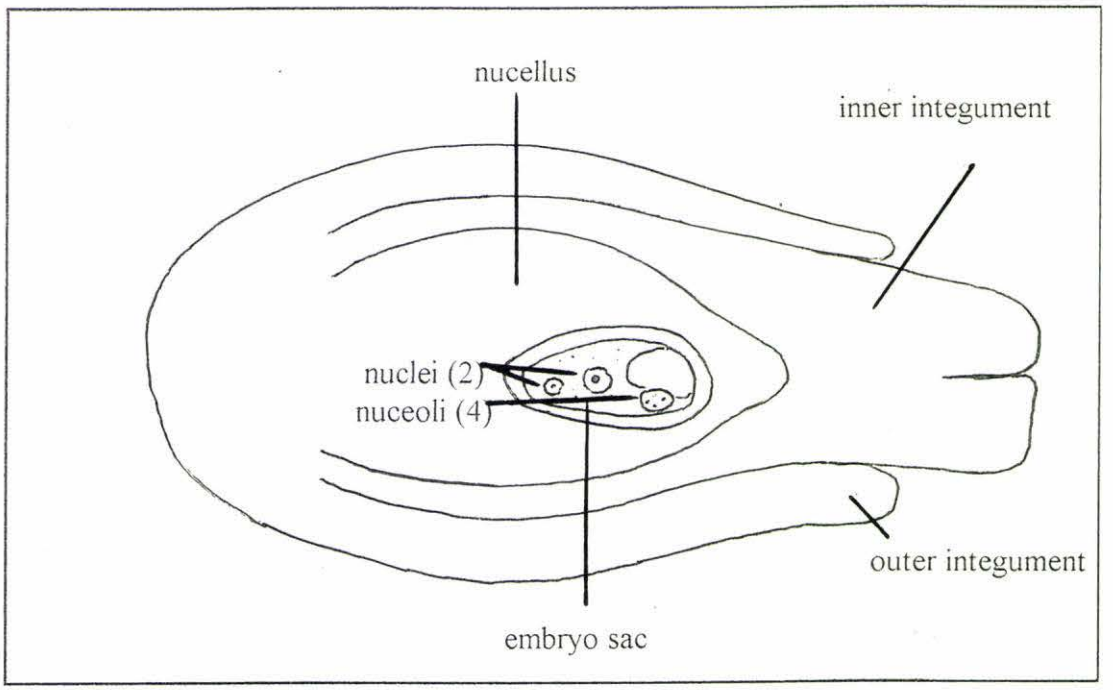


Fig. 5.16*b*. Diagrammatic representation of Oceanic White embryo sac (Fig. 5.16*a*) (400X life).

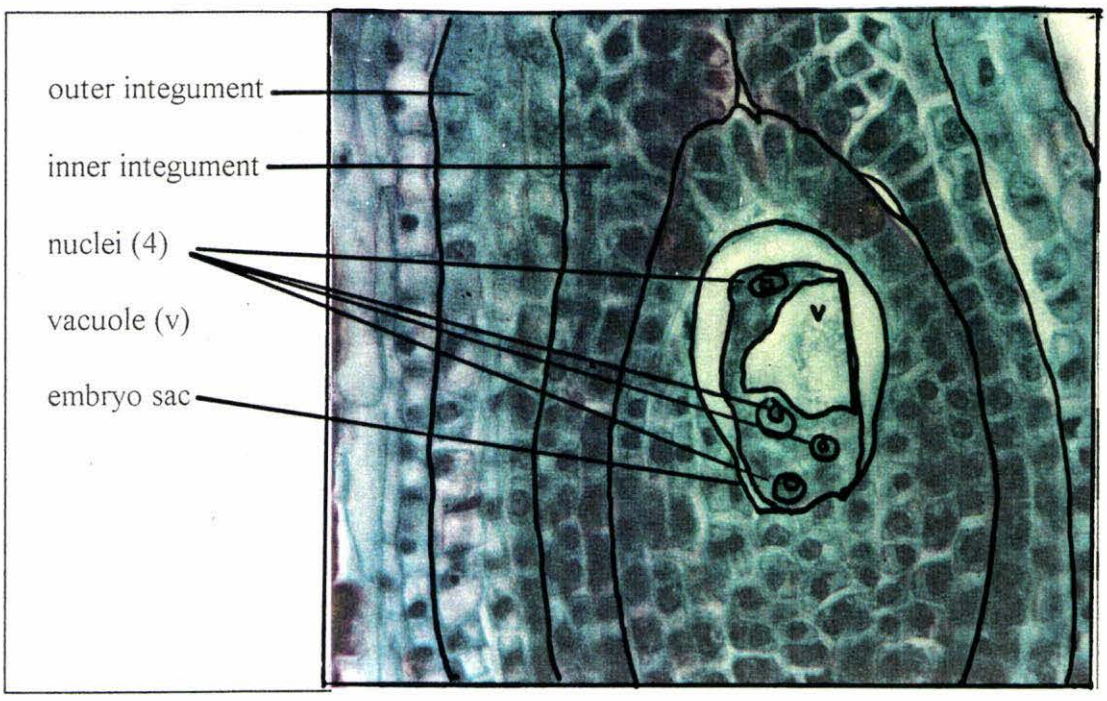


Fig. 5.17*a*. An embryo sac of Oceanic White showing four of six separate nuclei (400X). Micropyle at the top.

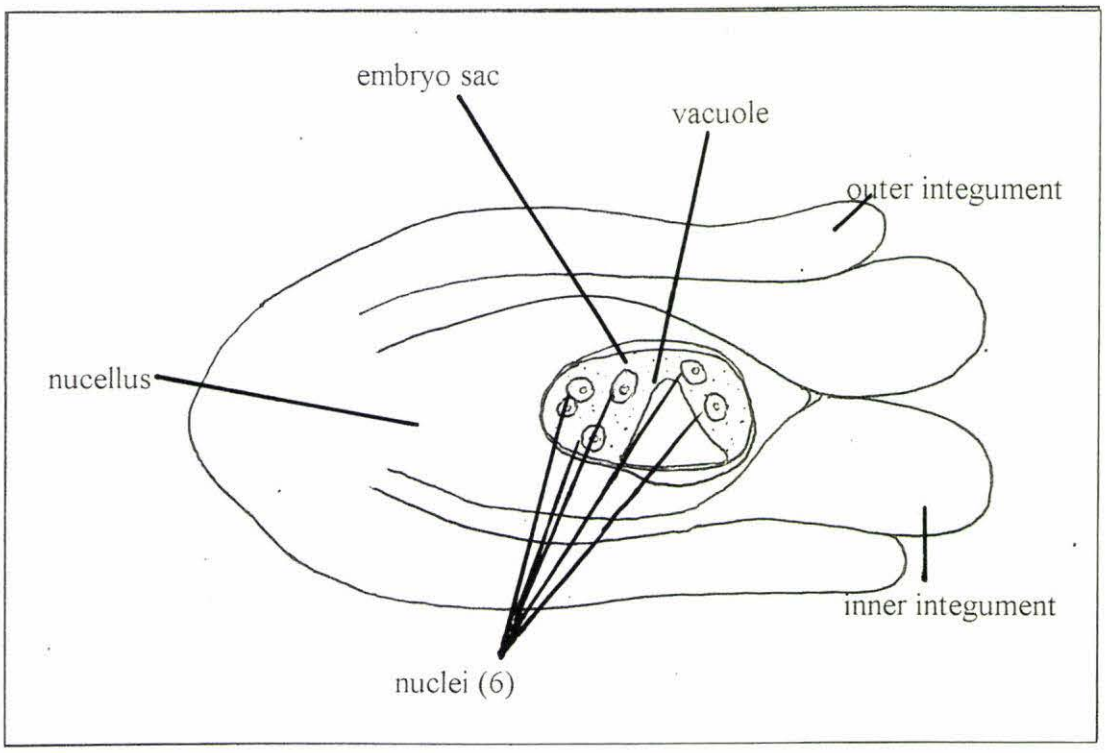


Fig. 5.17*b*. Diagrammatic representation of six-nucleate Oceanic White embryo sac (400X life).

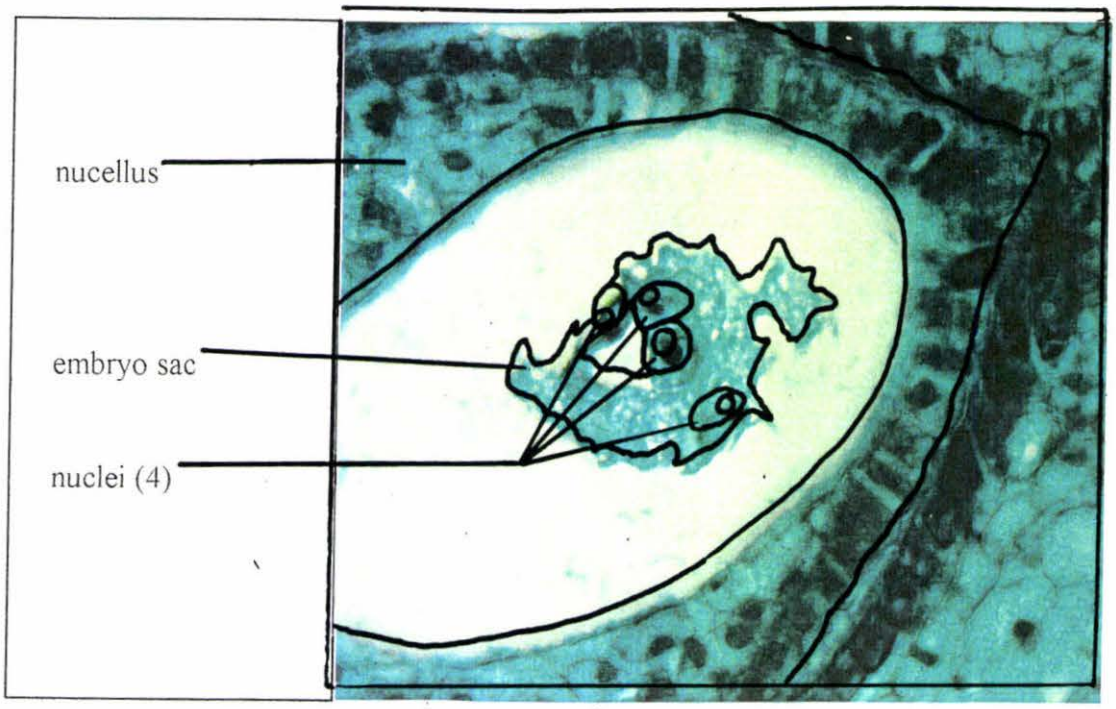


Fig. 5.18*a*. Mature Oceanic White embryo sac with four of ten nuclei (400X).Micropyle on right side.

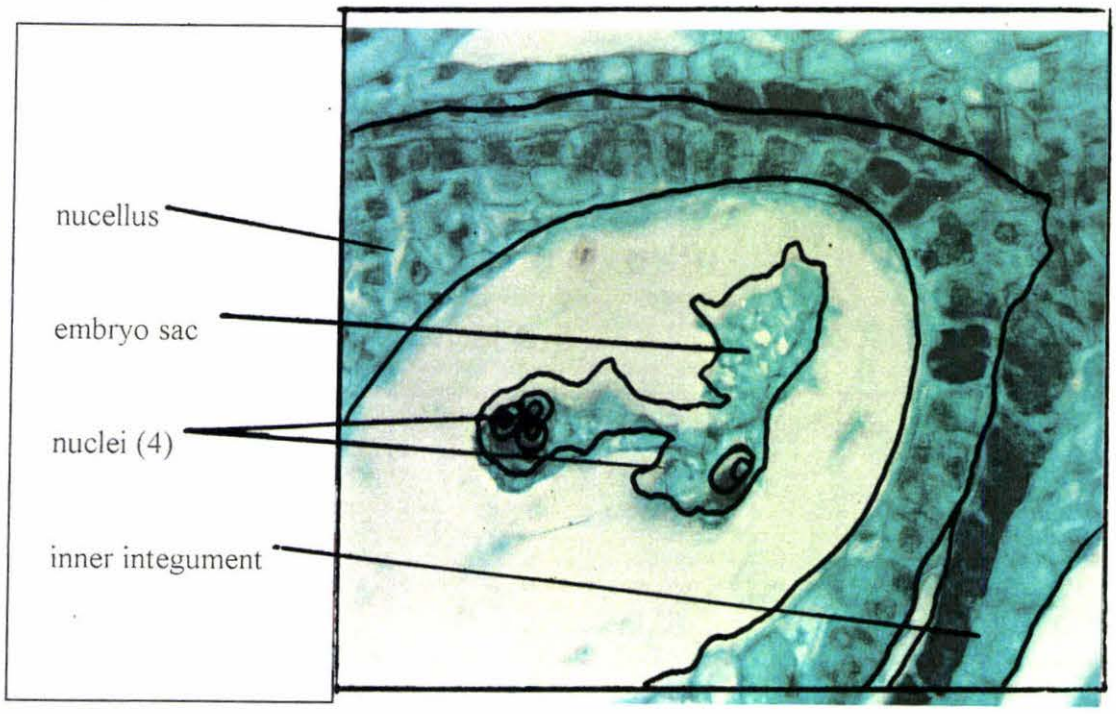


Fig. 5.18*b*. Arrangement of four other nuclei in the same ovule as 5.18*a*. (Oceanic White) (400X).Micropyle on right.

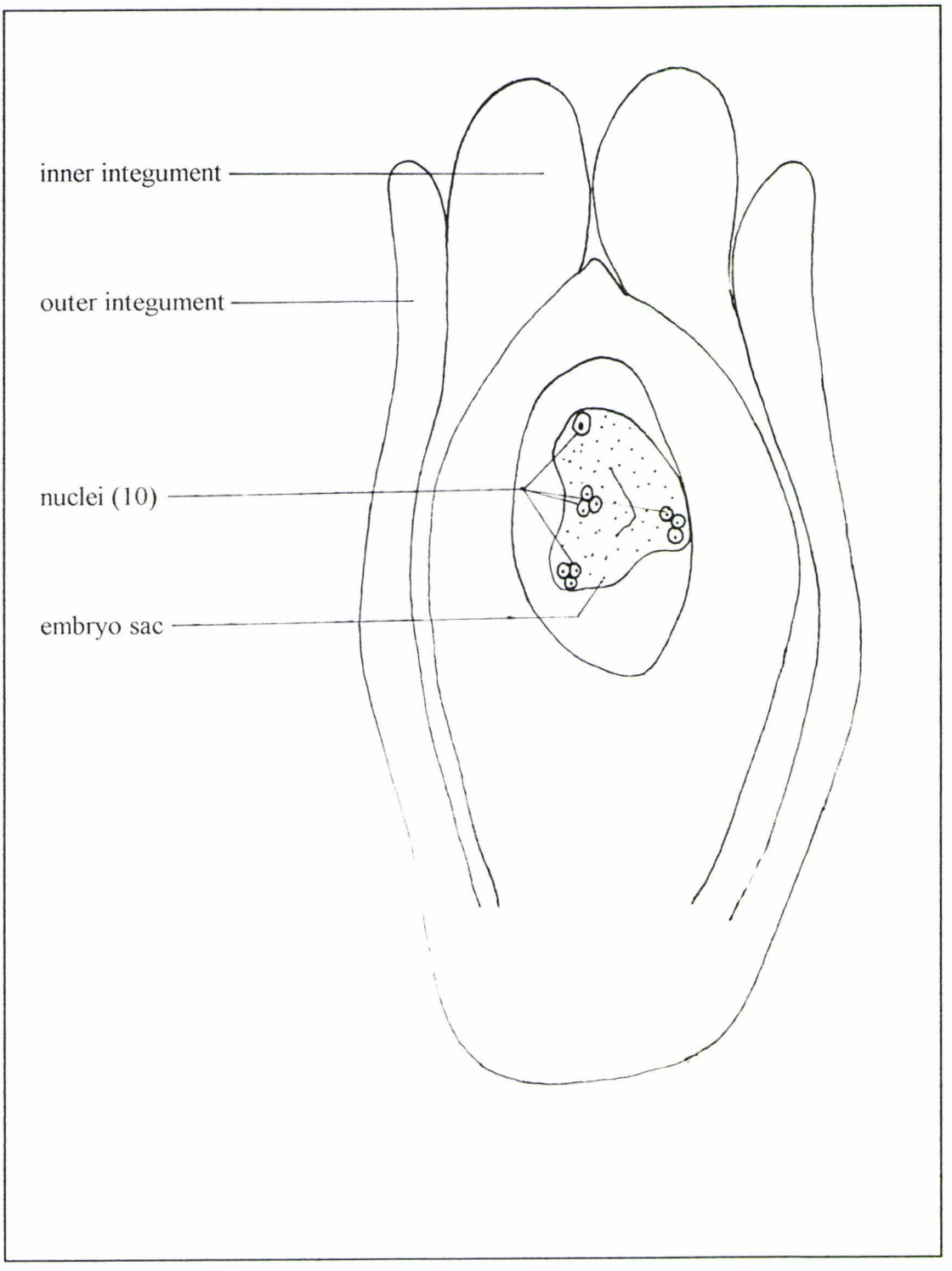


Fig. 5.18*c*. Diagrammatic representation of mature Oceanic White ovule and embryo sac (400X life).

(d) **Blue Fantasia 88**.

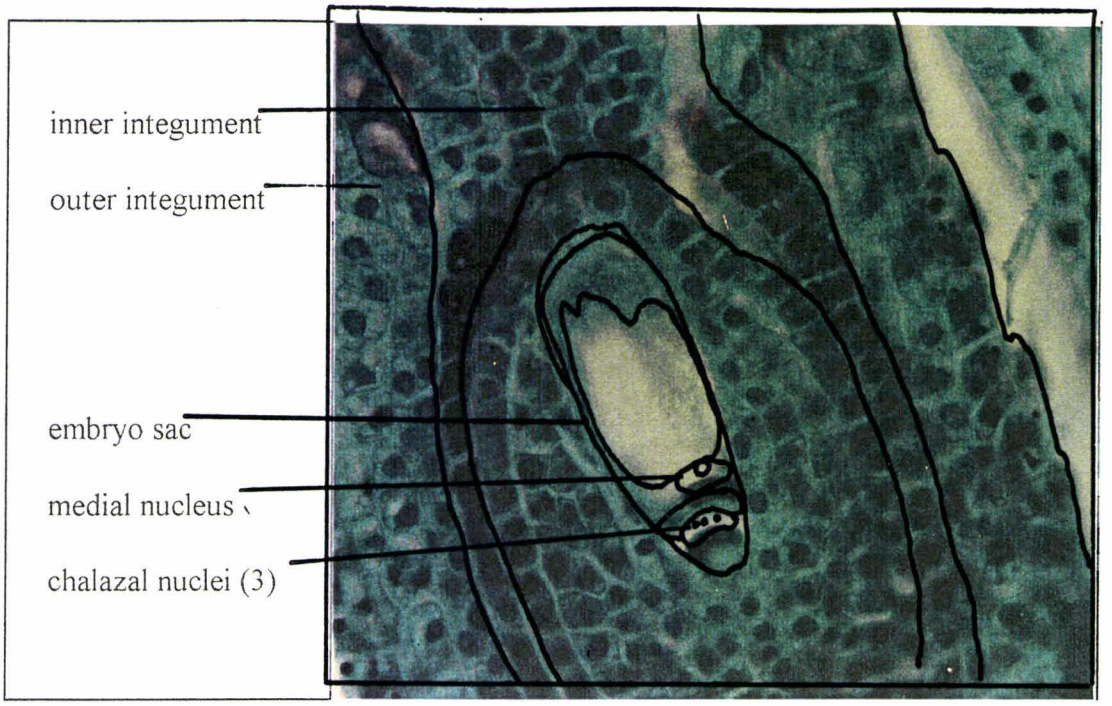


Fig. 5.19*a*. A section an immature Blue Fantasia 88 embryo sac showing a medially situated nucleus and three chalazal nuclei (four of seven nuclei) within a single nuclear membrane (400X). Micropyle at the top.

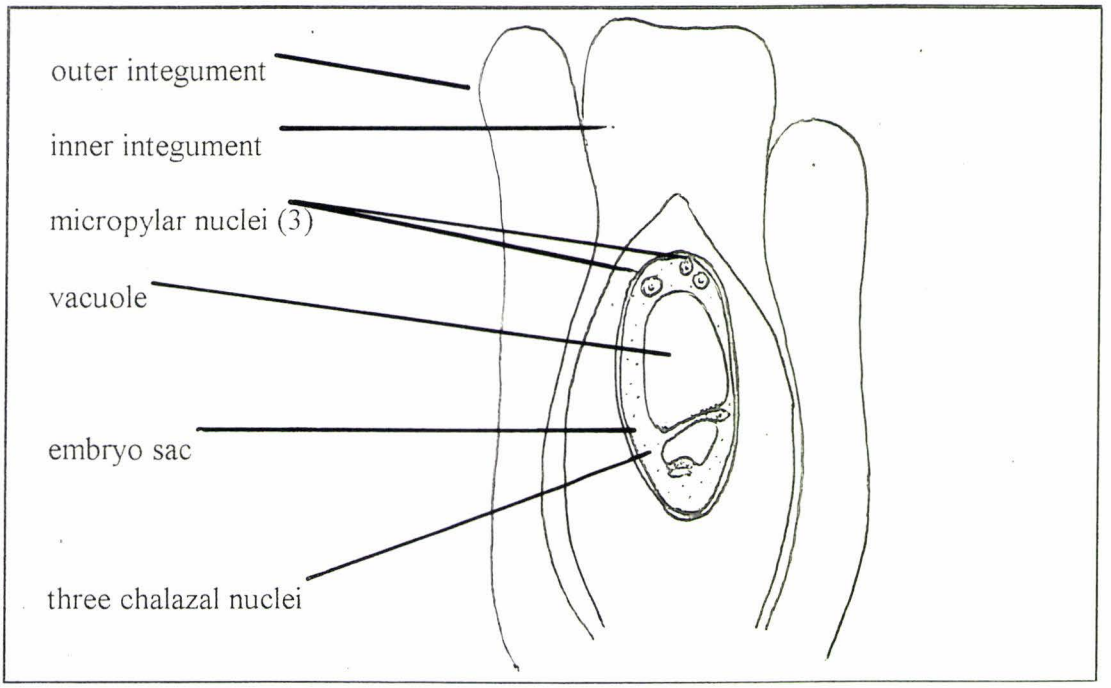


Fig. 5.19*b*. Diagrammatic representation of immature Blue Fantasia 88 embryo sac showing in addition, three nuclei at the micropylar end of the embryo sac (400X life).

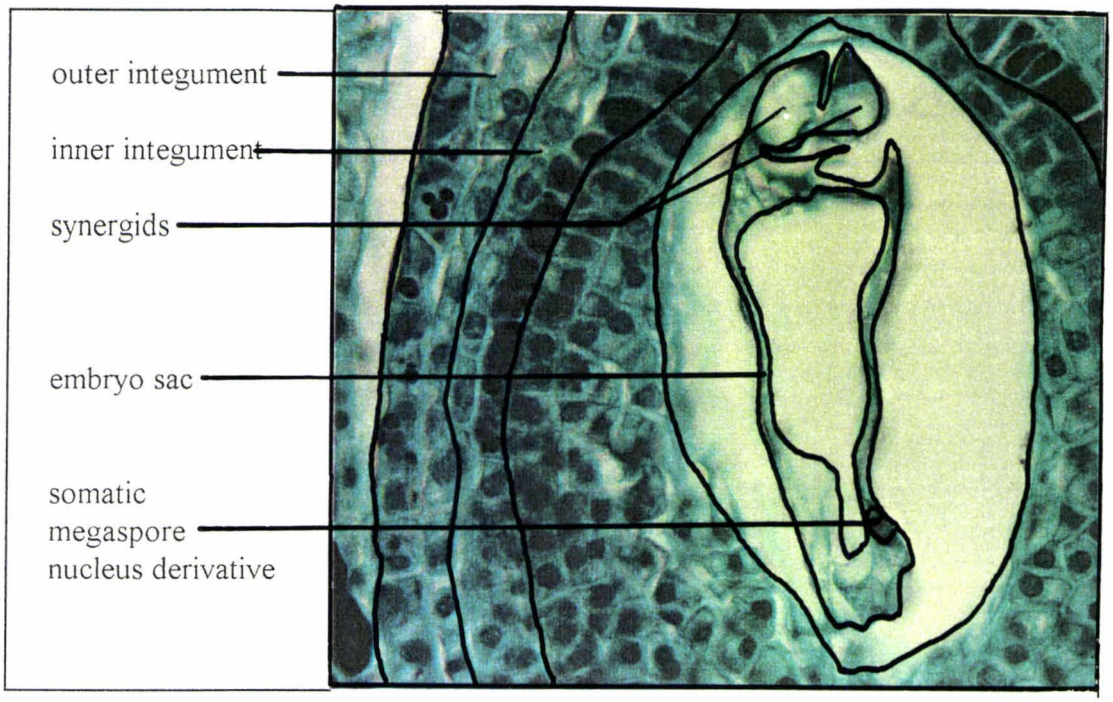


Fig. 5.20 *a.* A section through a more developed Blue Fantasia 88 embryo sac showing the somatic megaspore nucleus derivative position and synergids (400X).

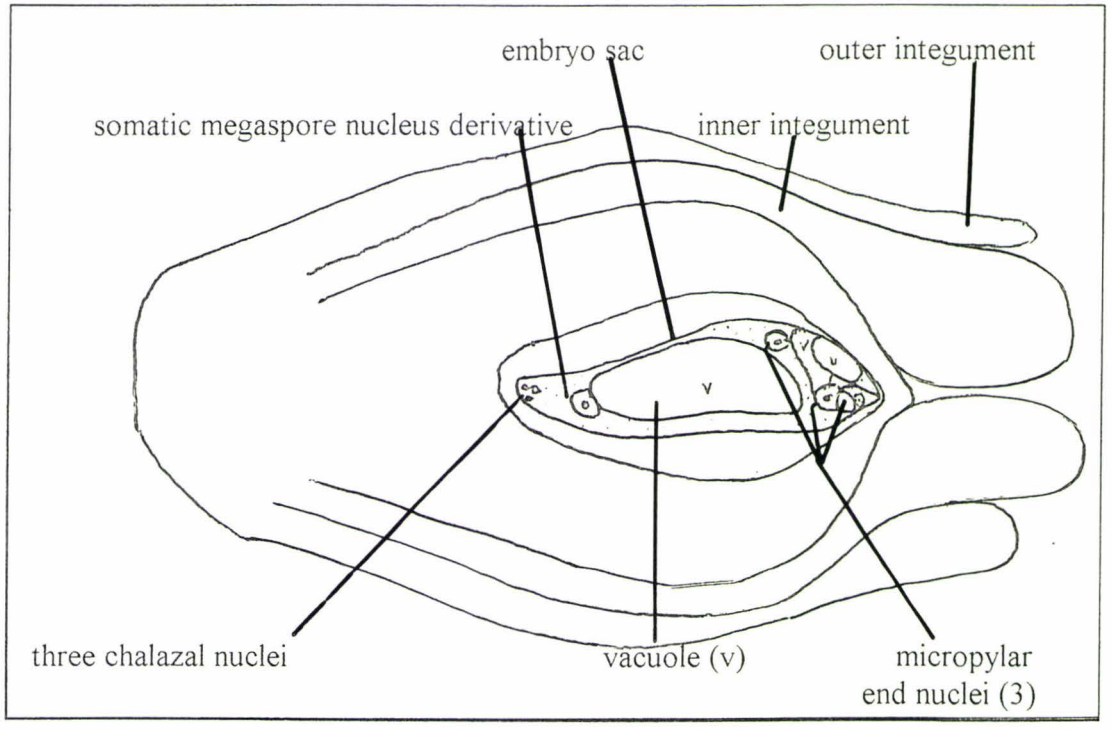


Fig. 5.20*b*. Diagrammatic representation of the above Blue Fantasia 88 embryo sac and ovule showing position of all seven nuclei (400X life).

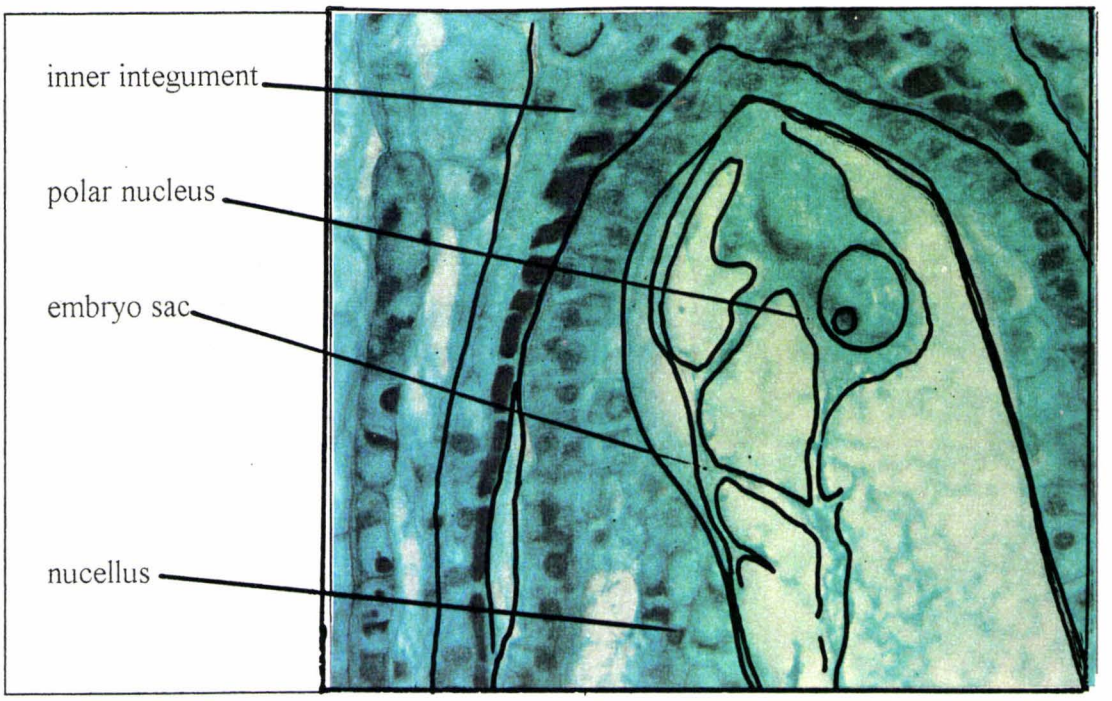


Fig. 5.21*a*. Section through a mature Blue Fantasia 88 embryo sac showing a polar nucleus (400X).Micropyle at top.

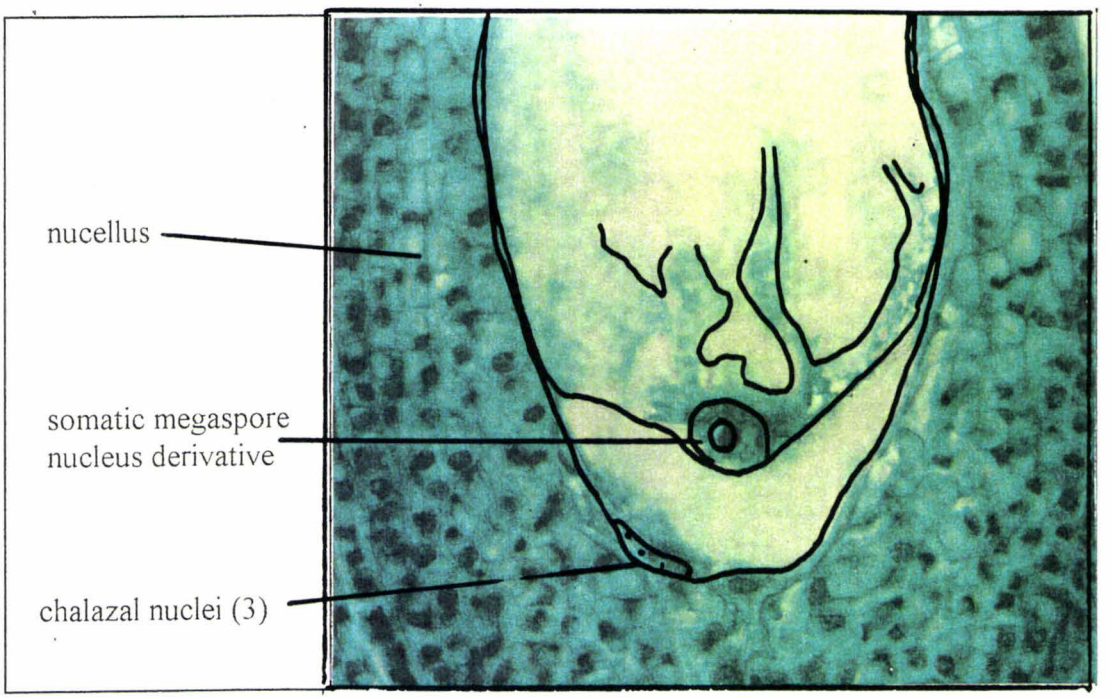


Fig. 5.21*b*. A section through the same embryo sac as 5.21*a* showing positions of the somatic megaspore nucleus derivative and three chalazal nuclei (400X). Micropylar end at top.

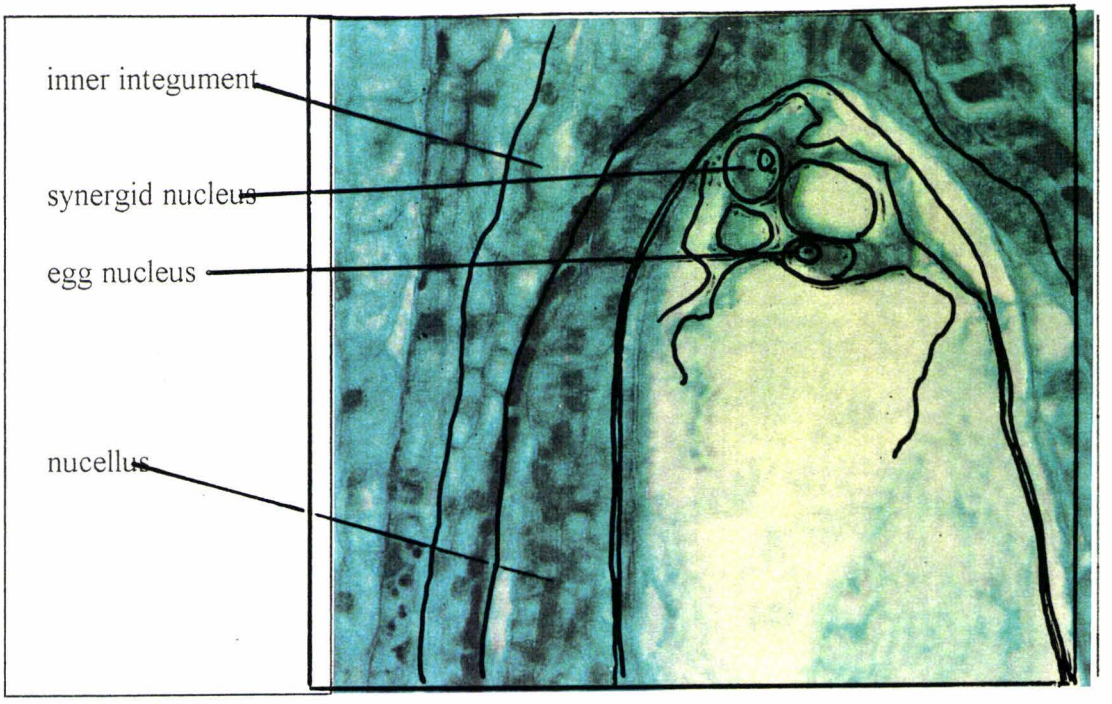


Fig. 5.21*c*. Section through the same embryo sac as 5.21*a*. and *b*. showing position of a synergid and egg nuclei (400X).

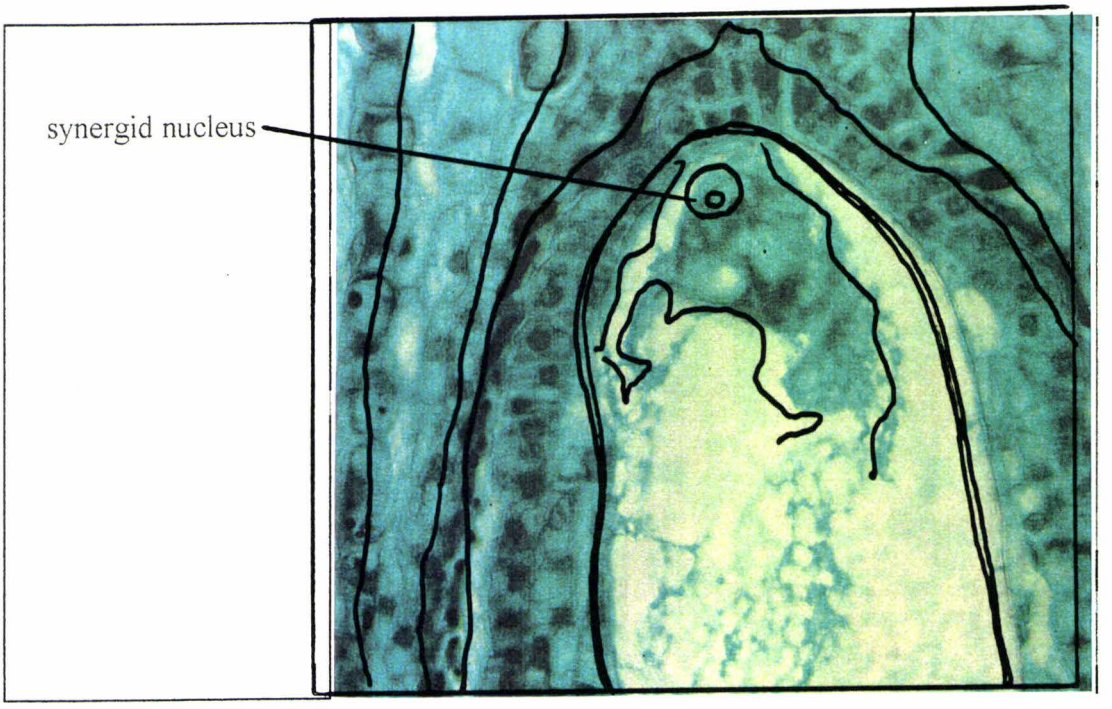


Fig. 5.21*d*. This section through the same Blue Fantasia 88 embryo sac as 5.21*a.-c*. shows the position of the second synergid nucleus (400X).Micropylar end at top.

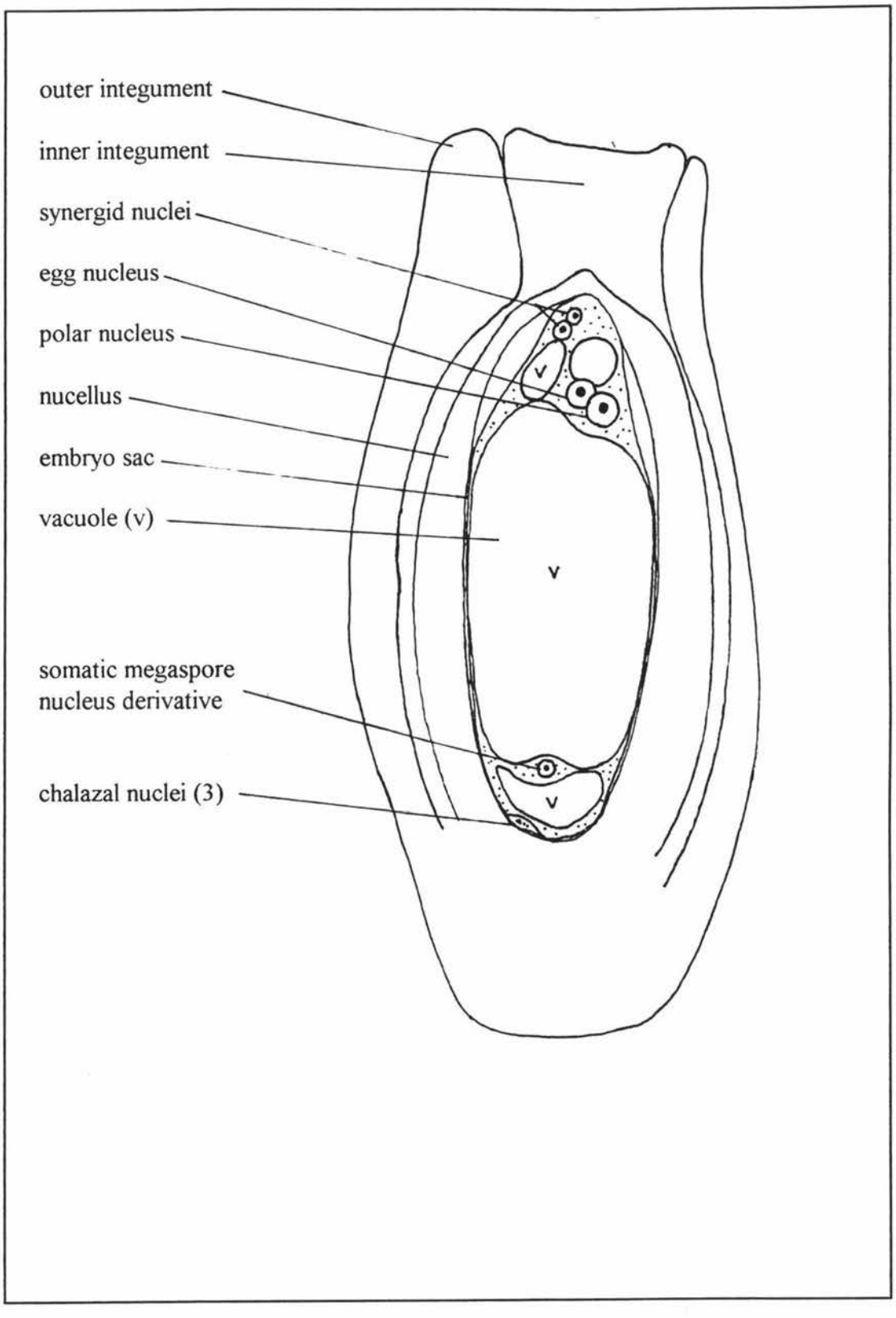


Fig. 5.21*e*. Diagrammatic representation of mature Blue Fantasia 88 embryo sac and ovule (5.21*a-d*.) (400X life).

All mature embryo sacs of fertile *L.perigrinum* and *L.sinuatum* contained synergids, an egg and polar nucleus and a somatic megaspore nucleus derivative. The general appearance of mature *L.sinuatum* embryo sacs differed from *L.perigrinum* in that the *L.sinuatum* embryo sacs were carrot shaped rather than ovoid. Several stages of embryo sac development were identified in these species: figs. 5.11*b*. (*L.perigrinum*) and fig. 5.13*b*. (*L.sinuatum*) are probably of embryo sacs at the second and first free nuclear division respectively. Fig. 5.14*b* shows an embryo sac of *L.sinuatum* between the first and second free nuclear division. As with mature embryo sacs, the shape of immature embryo sacs in these two species at this stage differed in the same way. By virtue of its more developed integuments and number of nuclei, the *L.perigrinum* ovule (Fig. 5.11*b*.) is likely to be more developed than the *L.sinuatum* ovule (Fig. 5.13*b*.). It appeared in *L.perigrinum*, that both integuments were well formed by the second free nuclear division but this was not the case in *L.sinuatum* where the outer integument had yet to enclose the inner integument (Fig. 5.14*b*.). Expansion of the ovule in these two species, especially elongation in *L.perigrinum*, and nuclear division, resulted in a mature embryo sac with five nuclei. The megasporangium was surrounded by fully developed integuments. In both these species, three chalazal somatic nuclei derived from the megaspore nucleus degenerated during development of the embryo sac.

Examination of Oceanic White ovules showed the embryo sac had a shrivelled appearance through development. At no stage could the arrangement of nuclei be attributed to an identifiable stage in expected *Fritillaria* type embryo sac development. The mature embryo sac contained ten nuclei and appeared non-functional. Both integuments failed to develop normally, the outer integument being well short of enclosing the inner integument at ovule maturity.

The mature embryo sac of Blue Fantasia 88 (Fig. 5.21*e*.) appeared to contain nuclei comparable to those found in embryo sacs of mature fertile *L.perigrinum* and *L.sinuatum*. In addition to these five nuclei, the embryo sac retained three derived

from the somatic megaspore nucleus (Fig. 5.19*a.* and *b.*), These could be seen at the chalazal end of the embryo sac. At ovule maturity, the integuments appeared to be well developed and the embryo sac was not shrivelled as in Oceanic White. The embryo sac contained two synergids, an egg nucleus, polar nucleus, and four somatic megaspore derivative nuclei.

Discussion.

Natural populations of *Limonium* are known to contain hermaphroditic, male sterile (gynodioecious) individuals, for example *L. sinuatum, L.vulgare and L.latifolium* (Kaul, 1988). Male sterility in *Limonium* does not necessarily imply female sterility or agamospermy.

Male sterility can be induced by a multitude of factors and be expressed as a variety of phenotypes. Phenotypic expression of male sterility regardless of genetic constitution *ie* genic or cytoplasmic origin, can be categorised into three groups (Laser and Lersten, 1972) : structural, sporogenous and functional male sterility. Male sterility may be non-genetic, occurring spontaneously in response to environmental factors, or genetic. The categories are not mutually exclusive. Sporogenous male sterility is the most common category and in this case the sporogenous tissues may be deformed, or apparently normal but premature abortion of microspores is symptomatic. Such abortion is usually a result of an incomplete pollen genome and due to aberrant meiosis. It appears male sterility in Blue Fantasia 88 and Oceanic White is a result of some developmental malfunction in microsporogenesis prior to the first pollen mitosis. This is evidenced by the halt in development of pollen grains beyond the uninucleate stage. It is most likely that dehiscent anthers are exhibiting largely sporogenous male sterility with sporogenous tissues appearing normal. Further examination of Oceanic White (outdoors) and Blue Fantasia 88 (glasshouse) anthers during winter months revealed that the anthers of both plants, at maturity, were non-dehiscent, contained uninucleate pollen grains with a shrunken cytoplasm, had an irregular exine and lacked obvious colpi. The greater

irregularity of the exine in indehiscent anthers of Oceanic White and Blue Fantasia 88 during shorter day lengths compared with summer, suggests a possible tapetal malfunction. It was observed in glasshouse grown *L. caspium* c.v "Blue Lace" (see Chapter 3) that pollen viability dropped from nearly 50% to complete sterility of pollen in one month. Male sterility in anthers of *Limonium* therefore appears variable in its expression. Male fertility in *Limonium* is sensitive to environmental conditions and it is possible this represents preferential resource allocation to the megaspore over the microspore. The seasonal difference suggests the operation of functional and sporogenous male sterility together. The constancy of complete male sterility in Oceanic White and Blue Fantasia suggests an aberration in development at meiosis. This breakdown of meiosis at least in Oceanic White, may be a result of the plant's hybrid condition. When assessing *Limonium* pollen fertility, it is, in addition to sporogenous sources of male sterility, important to consider possible environmental effects *ie* day length, on functional male sterility.

Embryo sac development in *L.sinuatum* and *L.perigrinum* culminated in the formation of *Fritillaria* type embryo sacs. The mature Blue Fantasia 88 embryo sac, although somewhat different from *L.sinuatum* and *L.perigrinum* by virtue of its persistent chalazal nuclei, still falls within the parameters of normal two phasic tetrasporic development and appears fertilisable. Oceanic White exhibits considerable differences in embryo sac formation compared with the two fertile species. The mature embryo sac lacks the organisation expected in two-phasic tetrasporic development. It is possible the mature embryo sac of Oceanic White represents an agamospermous condition. Richards (1990) classifies the mechanism of agamospermy in *Limonium* spp. as meiotic diplospory (*Taraxacum* type) and hence one could expect restitutional female meiosis and parthenogenesis of the egg cell in Oceanic White. This was not found. All apomictic *Limonium* species exist in self compatible pollen stigma combinations not A/cob as found in Oceanic White. It is most unlikely Oceanic White exhibits agamospermy. The small size of the Oceanic White embryo sac relative to its surrounding sporophytic tissues, apparent lack of

vacuoles, and incomplete outer integument formation, and number of nuclei suggests that a breakdown in normal embryo sac development occurs in meiosis II and therefore disturbance in the following mitoses. A symptom of meiotic breakdown in Oceanic White is an unfertilizable, non-agamospermous embryo sac.

A gross morphological contrast between mature sterile and fertile ovules is the incomplete formation of especially the outer integument in sterile ovules. It is possible the growth of the integument is halted after some aberrant stage in embryo sac development *ie* meiosis II. Robinson-Beers *et al.* (1992) showed both integument development and megasporogenesis were affected in two female sterile mutants of *Arabidopsis*, indicating these two processes were interconnected or interdependent. It is possible the same applies to *Limonium* ovule development. The small size of the embryo sac relative to the size of the surrounding ovule could also be suggestive of an infertile embryo sac. An investigation into ploidy levels in plants may yield additional information as to the source of infertility.

Summary.

This chapter describes some morphological and developmental aspects of megasporogenesis and microsporogenesis in fertile and sterile species and hybrids of *Limonium*. Importantly, a correlation between sterile embryo sac cytology and gross ovule morphology is suggested. This correlation will allow future assessment of female fertility without lengthy analysis of embryo sac cytology.

Summary.

Several aspects of *Limonium* hybrid production and post-pollination biology have been examined.

Firstly, the genus was introduced, its phylogeny, geographic distribution, cultivation were reviewed and significance of further hybrid production to the future prospects of this plant within the cut flower market were discussed.

Intraspecific intermorphic and intramorphic crosses were made within three species of *Limonium*. Aspects of self-incompatibility and post-pollination biology were reviewed and confirmed. Pollen-stigma interactions and other post-pollination events such as pollen tube growth, ovary and ovule development and embryo formation were presented. The results of this study provided a basis by which the success of later interspecific crossing attempts could be gauged.

In compatible interspecific crosses pollen tube growth was halted in different regions of the recipient pistil. The barriers to interspecific crosses were present before fertilization and likely to have resulted from excessive genetic differences between species. No interspecific hybrids were obtained. Future prospects for the development of new interspecific hybrids were discussed.

Several aspects of *Limonium* pollen physiology were also investigated. These included changes in pollen viability under certain enviromental conditions, examination of pollen grain hydration *in vitro* and *in vivo* and pollen grain germination *in vitro*.

Finally, an examination of ovule, embryo sac cytology and anther structure and formation was made by staining of thin sectioned materials. Anther and ovule development in two male sterile species was compared with that of two known male and female fertile species. A relationship between ovule development, its embryo sac condition, and the possible timing of sterility were suggested.

References.

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Appendix.

1. Preparation of aniline blue.

A **0.1** % solution of **aniline blue** (water soluble) was dissolved in **0.1 M KH_2PO_4** (buffer) placed in the dark and allowed to decolourise over 24 hours. This solution is ready for use when straw coloured, and should be stored in a refrigerator as it is light sensitive.

2. Preparation of Alexander stain.

- 95% ethanol 10ml.
- Malachite green 10mg.
- Distilled water 50ml.
- Glycerol 25ml.
- Phenol 5gm.
- Chloral hydrate 5gm.
- Acid fuchsin 50mg.
- Orange G. 5mg.
- Glacial acetic acid 1-4 ml.

3. Preparation of fluorescein diacetate.

A **2mg/ml** solution of **fluorescein diacetate** (FDA) was prepared in **acetone**. When testing pollen viability it is important to determine a concentration of **sucrose** that will inhibit bursting of pollen grains. FDA is added dropwise to a small volume of this sucrose solution until a definite milkiness is achieved. This solution of sucrose and FDA is now ready for use. A fresh solution of sucrose and FDA should be prepared each time as FDA precipitates from the solution over time.

4. Control of humidity by glycerol/water combinations.