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EXPLORATION OF *LIMONIUM* INTERSPECIFIC BREEDING POSSIBILITY

A thesis presented in partial fulfilment of
the requirement for the degree of Master
of science in plant science
at Massey University
Palmerston North
New Zealand

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1995

EXPLORATION OF *LIMONIUM* INTERSPECIFIC BREEDING POSSIBILITY

Abstract

Interspecific crossability was investigated in the genus *Limonium* (Plumbaginaceae). Six *Limonium* species were chosen for this study, five of which are dimorphic and *L.perigrinum* which is monomorphic. Ovary, ovule and embryo development was investigated, as were *in vitro* pollen germination and pollen tube growth. Unilateral incompatibility was observed in 8 interspecific combinations. A high frequency of interspecific crossability was observed between *L.perezii* X *L.sinuatum* and *L.sinense* X *L.aureum*. Pollen tubes were frequently observed penetrating the ovules in these crosses. Pollen tube growth that terminated in the styles or was restricted to the stigmas was found in some *Limonium* interspecific crosses. Abnormalities of pollen tube growth in the interspecific crosses included heavy callose deposits at the tips of pollen tubes; pollen tube branching and pollen tube growing in the wrong direction.

Embryo, ovule and ovary development was studied with *L.perezii* plants following conspecific pollination. Three distinct groupings of florets can be recognised at the basis of their post-pollination growth and development. Twenty-six percent of conspecific pollinated florets showed no ovary and ovule growth. No embryo was found in this group. In eleven percent of florets, ovaries and ovules grew up to Day 12 after pollination and then shrivelled. No embryo was ever found in this group. Sixty-three percent florets produced embryos following conspecific pollination and developed normally.

The viability of *Limonium* pollen was assessed with Alexander's stain and fluorochromatic reaction (FCR) stain.

Optimal conditions for *in vitro L.perezii* pollen germination and tube growth were established. Poly-ethylene glycol and filter paper supports were of particular significance. *In vitro* pollen germination rate of about 40% was achieved. Plant growth regulators (IAA, GA_3 and ethylene), some minerals (manganese sulphate, copper sulphate) and prehydration treatment were used in experiments to improve pollen germination and tube growth. None of these factors, however, had positive effect on

either pollen germination or tube growth. It was found that while *L.perezii* pollen tube growth tolerates a wide range of temperature, there is an optimum between 20°C-25°C.

ACKNOWLEDGEMENTS

I am most grateful to my supervisor, Associate Professor D.W. Fountain for his excellent guidance, constructive criticism and patience while I was carrying out experiments and writing my thesis.

I wish also to offer my special thanks to Ed Morgan for his advice and extensive help with experimental work. I appreciate the suggestions he has made in the course of the writing of this thesis.

My sincere thanks are also extended to all the staff in the Plant Biology and Biotechnology Department for their help throughout the years of my study and to the staff in the Plant Growth Unit of Massey University for the work they performed in the glasshouse to maintain the necessary conditions for my work.

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GENERAL INTRODUCTION

The genus *Limonium* belongs to the family Plumbaginaceae. According to Baker(1953b), there are more than 150 *Limonium* species. *Limonium* species are believed to originate from several regions of the northern hemisphere. The overall distribution of the genus embraces all five continents (Baker 1953b). *Limonium* was formerly called *Statice*, but in 1947 Lawrence' proposal was approved at the International Botany Conference and the generic name was changed to *Limonium* (Tsurushima 1992a).

Most *Limonium* species are perennial herbs or shrubs, sometimes woody at the stem base, although there are also some annuals (Huxley *et al.* 1992). Apart from species which become upright with an elongate stem, most *Limonium* species produce a circular cluster of leaves and exhibit a rosette form. The inflorescences of *Limonium* are mostly corymbose panicles or spikes (Huxley *et al.* 1992). *Limonium* species can be divided into two types: seasonal and free flowering types on the basis of their flowering patterns. Seasonally flowering species mainly flower during summer, while the flowering of free flowering species is influenced by temperature. The free flowering species will keep producing flowers throughout the year if temperature and light are favourable (Harada 1992). Flowers of *Limonium* possess a tubular calyx and five petals united only at the base. Both sepals and corolla are small, most flowers are only 3-4mm in diameter. The flowers of *L.perigrinum* are considered the largest, being 15-16mm in diameter (Tsurushima 1992a). The sepals of *Limonium* flowers often have as bright a colour as the corolla has and often persist to fruiting after the petals have senesced, so, when flower colour is described, it often means the colour of the sepal. Many *Limonium* species are presently grown commercially around the world for use as cut flowers. The unique branching inflorescences of *Limonium* are perfect as fillers for bouquets, corsages, baskets and other flower arrangements.

Limonium crops have a fast growing market in many countries. In Japan in 1985 the area under cultivation nationwide was 188 hectares. This had increased to 280 hectares by 1988 and it is now estimated to be approaching 400 hectares (Tsurushima 1992a). It should be pointed out that the most widely grown *Limonium* species for cut flowers is *L.sinuatum*. It makes up about 80% of the Limonium under cultivation in the world.

The remaining 20% is made up of other species(Tsurushima 1992a). To cope with a rapid increase in market demand for new and novel flowers, generation of interspecific hybrids has become a more popular strategy for producing new forms and colours. The first marketed interspecific *Limonium* hybrid was "Misty Blue" which was released by Dainchi Engei company in 1984. This hybrid was from a cross between *L.latifolium* and *L.caspia* (Tsurushima 1992b). "Lemon Star" is a yellow interspecific hybrid of *L.aureum* and *L.sinense.* " Charm Blue" is a hybrid grown from seedlings obtained from a cross between *L.latifolium* and *L.gmelinii* (Harada 1992). The significance of interspecific breeding is that it provides a way to combine the respective merits possessed by different species into a hybrid. Some objectives in breeding programs may include increasing the range of flower colours, increasing flower size, increasing stem length long stems and abundant flower production. In addition, features including year round production, heat resistance of plants, cold resistance, disease resistance and good water uptake are also the targets for breeders (Tsurushima 1992a).

Interspecific hybridization often relies on techniques such: *in vitro* techniques as embryo rescue, ovule and ovary culture, isolation and fusion of protoplasts. "Blue Star" was obtained by culturing embryos produced by hybridization between *L.perezii* and *L.sinense* (Tsurushima 1992b). Recently a long stemmed form of *L.perigrinum* was produced by hybridization with *L.purpuratum* (Morgan *et al.* in press).

A successful interspecific hybridization program not only relies on biotechnological techniques but also on the knowledge of morphological features of the sexual reproductive organ, flowers; the understanding of physiological features of the reproduction process; and the understanding of pollen behaviour in interspecific crosses. The main purpose of this study is to explore the possibilities of producing hybrids between several *Limonium* species. Six *Limonium* species were chosen for this present study. They were

L. perigrinum (Bergius)

L. sinuatum (L.) Mill

L. perezii (Stapt)

L. aureum (L.) Hill

L. sinense (Girard) Kuntze

L. caspia (*L.bellidifolium*)

The objectives of this work were:

(1). To obtain knowledge about pollen behaviour on the stigmas
and in the styles of related species.

(2). To determine the barriers to interspecific crosses

(3). To collect data for ovary, ovule and embryo growth in intraspecific crosses

(4). To assess techniques for testing *Limonium* pollen "viability"

(5). To investigate *Limonium* pollen germination *in vitro*

(6). To obtain knowledge relevant to taxonomic relationships and mechanisms
of breeding incongruities

CHAPTER ONE INTERSPECIFIC CROSSABILITIES IN *LIMONIUM*

1.1 INTRODUCTION

Interspecific hybridization has been widely used in plant breeding to combine desirable characteristics, which are not available in only one of the species, into one individual. Various fertility barriers have been observed in attempts at interspecific hybridization, which preclude unions between taxonomically remote species. As more incongruity phenomena confronted in interspecific crosses were discovered and examined in greater detail, new techniques and methods have been found to overcome them. In any interspecific hybridization programme, it is always necessary to investigate the plant species which might be used as parent plants in the crosses in order to find out what merits the species possess before outlining the cross scheme. *Limonium* species as potential cut flower crops have some unique characteristics. In this introduction a brief description of the main botanical and horticultural characteristics of six *Limonium* species is given. This is followed by a review of the literature on interspecific hybridization.

1.1.1 The Description of Six *Limonium* Species

L.perigrinum This is a perennial shrub native to South Africa (Huxley *et al.* 1992). This semi-erect shrub can grow up to 1 meter tall. Its 10 cm long leaves envelop stems at the base and the inflorescence shows dichotomous branching and dense spikes. Unlike other *Limonium* species, *L.perigrinum* produces large flowers (1.5-1.6mm in diameter) with bright pink colour in both sepals and petals. It can be used as the main flower in ornamental arrangements. (Plate 1.1, 1.2).

L.sinuatum. This biennial or perennial *Limonium* species originally comes from the Mediterranean region (Baker 1953a). It is perhaps the only *Limonium* species which has vast number of varieties under cultivation. The leaves are arranged in rosette form; the stiff angular branching flower stalks grow straight up. It produces a corymbose inflorescence. The sepals and petals either are the same colour or different from one another, in a range of pastel colours including white, cream, rose, deep rose, pink,

apricot, yellow, lilac, violet, sky blue and purple (Huxley *et al.* 1992). *L.sinuatum* is cultivated as a cut flower crop for both fresh and dry flower arrangements. (Plate 1.3, 1.4)

L.perezii. This is a perennial *Limonium* species and native to Canary Islands (Baker 1953a). This subshrub can grow up to 70 cm. Its broad ovate leaves form the rosette base. The strong flower stems grow straight up, supporting large heads of small flowers. The calyx is funnelform with purple or blue coloration, the corolla often appears white or yellow (Huxley *et al.* 1992). (Plate 1.5, 1.6)

L.aureum. This perennial herbaceous *Limonium* originates from Siberia and Mongolia (Huxley *et al.* 1992). Before the flower-stems appear, the plant resembles the rosette of a dandelion. *L.aureum* produces profuse flower-bearing tender stems. The calyx of the flowers is bright yellow colour and funnelform. The corolla is also yellow and is longer than the calyx (Huxley *et al.* 1992). (Plate 1.7, 1.8)

L.sinense. This is a biennial *Limonium* which is native to Japan, Korea, China and New Caledonia (Huxley *et al.* 1992). The oblong glabrous leaves grow in the base of the plant, its erect flowering stems can grow up to 50 cm tall. *L.sinense* produces a paniculate inflorescence. The calyx is white and tubular in form, five yellow petals exceed the calyx in length. (Plate 1.9, 1.10)

L.caspia (*Limonium bellidifolium*), It is a perennial herbaceous *Limonium* and originally comes from Europe-Songoria (Baker 1953a). The obovate basal leaves are 2-6cm long forming the base of the plant, dying at anthesis. Its paniculate flowering stems start from near the base. *L.caspia* produces dense, corymbose inflorescences displaying a great number of very small flowers of purple corolla and white-violet calyx (Huxley *et al.*1992). (Plate 1.11, 1.12)

Plate 1.1

Plate 1.2

Plate 1.1 Potted plant of *L.perigrinum* **Plate 1.2 A flower of *L.perigrinum***

L.perigrinum flower has five base-jointed sepals and five petals, both are bright pink in colour. The flower has five segregated styles, five segregated stigmas and five stamens.

Plate 1.3

Plate 1.4

Plate 1.3 Potted plant of *L.sinuatulm* Its leaves are in a rosette arrangement, its flowering-stems can grow up to about 30cm tall.

Plate 1.4 *L.sinuatum* flowers (x3) The calyx is pale pink in colour and is like a funnel, five petals are orange yellow in colour.

Plate 1.5

Plate 1.6

Plate 1.5 Potted plant of *L.perezii* The large leaves grow at the base, a long inflorescence stem bears a big head of small flowers.

Figure 1.6 *L.perezii* flowers (x3) Violet calyx appears funnelform, five white petals exceed calyx in length.

Plate 1.7 Plate 1.8

Plate 1.7 Potted plant of *L.aureum* The basal leaves have senesced, the tender tangling flowering-stems are supported by two sticks.

Figure 1.8 *L.aureum* flowers (x1.5) The sepals and petals both are orange yellow in colour, petals extend beyond the calyx.

Plate 1.9

Plate 1.10

Plate 1.9 Potted plant of *L.sinense* The oblong leaves are arranged in rosette form and its inflorescence is a panicle.

Plate 1.10 *L.sinense* flowers (x3) The calyx is white in colour and a narrow funnelform, the corolla is yellow and extends beyond the calyx.

Plate 1.11 **Plate 1.12**

Plate 1.11 Potted plant of *L. caspia* Some of its basal leaves have senesced. The flowering-stems bear profuse small flowers.

Plate 1.12 *L. caspia* flowers (x1.5) The tiny flowers are only about 2mm long, five purple petals are longer than white-violet calyx.

1.1.2 Dimorphism and Monomorphism in *Limonium*

The *Limonium* flowers have 5 sepals, 5 petals, 5 segregated styles, 5 stigmas, 5 stamens and a unilocular ovary which contains a single ovule (Baker 1948, 1966). Figure 1.1 shows the basic structure of *Limonium* flowers. Of the six *Limonium* species chosen in this study, five are dimorphic, only *L.perigrinum* being monomorphic (Baker 1953a). Dimorphism means two distinctly different types of individual exist within a species. In dimorphic *Limonium* species, dimorphism is expressed in the flowers and hence within the reproductive process. There are two types of pollen, designated as type A and type B; and two types of stigma: cob and papillate. The cob stigma is found in a flower producing type A pollen, while type B pollen occurs in the flowers with papillate stigmas (Baker 1953a). Both types of pollen and both types of stigmas of *Limonium vulgare* have been illustrated in detail by Baker (1948). These two types of pollen and two types of stigmas are found throughout the genus with only slight deviations from the typical appearances (Baker 1953a). The dimorphic *Limonium* species are completely self-incompatible but cross-compatible, which means that type A pollen will only produce pollen tubes which lead to successful fertilization when it is placed on papillate stigmas, and type B pollen is correspondingly successful only when placed on cob stigmas. Self-pollination (i.e. type A on cob, or type B on papillate) results in a complete failure of germination of the pollen grains, the inhibition is on the stigma surface (Baker 1966). The relationships of type A pollen and papillate stigma, type B pollen and cob stigma are defined as legitimate combinations, while type A pollen and cob stigma, type B pollen and papillate stigma are defined as illegitimate combinations (Baker 1966). Figure 1.2 shows legitimate and illegitimate pollinations.

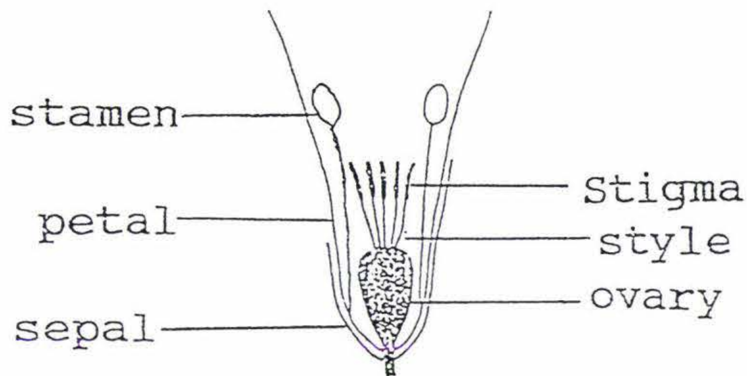

Figure 1.1 Diagrammatic longitudinal section of *Limonium vulgare* (After H.G.Baker 1966)

The typical *Limonium* flower has 5 sepals, 5 petals, 5 segregated styles, 5 stamens and an unilocular ovary which contains a single ovule.

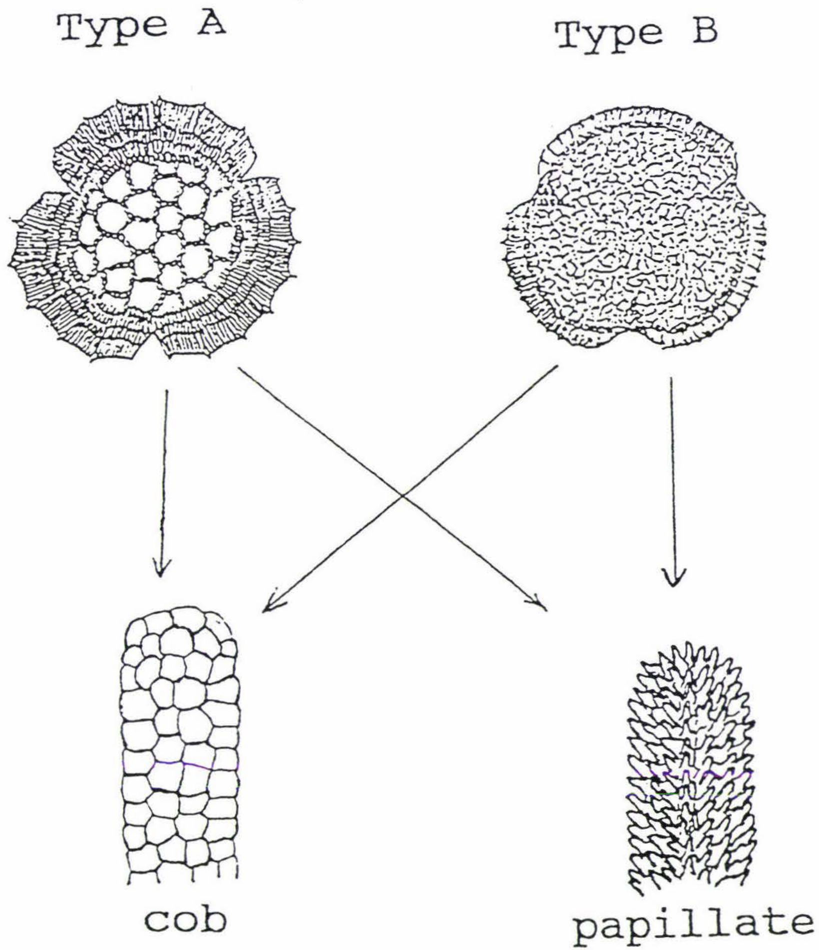

Figure 1.2 Dimorphism of *Limonium* and pollination There are two distinct individuals existing in a dimorphic *Limonium* species, cob stigma plant with type A pollen and papillate stigma plant with type B pollen. Dashed arrows represent illegitimate pollination, solid arrows represent legitimate pollination. This diagraph was adapted from Baker (1953a).

1.1.3 Interspecific crosses

The term incompatibility has been used in the broad sense to refer to all matings in which the reproductive process is not able to be completed, however the term incongruity was suggested by Hogenboom (1975) to describe the failure of the reproductive process in interspecific crosses. Barriers preventing interspecific or intergeneric hybridization can be those occurring prior to fertilization (pre-zygotic), and those occurring after fertilization (post-zygotic) (Zenkteler 1990). Prezygotic incongruity barriers may include: the failure of pollen adherence on stigmas, the failure of pollen germination on the stigmas, abnormal pollen tube growth in the style, the failure of pollen tube penetration of ovary or ovule and the failure of fusion of gametes. The most common postzygotic barrier is embryo abortion which among other things may be caused by absence of endosperm or by abnormal endosperm development (Zenkteler 1990). Williams and Knox (1982) have surveyed large numbers of interspecific crosses in genus *Rhododendron* which comprises some 900 species. They have found that the discrimination against foreign pollen occurred at seven developing stages:

(1) Failure to germinate
(2) Arrest of pollen on the stigma surface shortly after germination
(3) Abortion of tubes at various levels in the stylar canal
(4) Failure to penetrate ovary
(5) Failure to penetrate ovule
(6) Failure to enter embryo sac
(7) Failure to fertilize with female gamete

The site of arrest is a characteristic of species crossed. In interspecific crosses of *Rhododendron* arrested pollen tubes also showed cytological abnormalities including spiralling pollen tubes, coiling pollen tubes, balloon-like tube tips, spiky tube tips, bursting tips, and heavy callose deposition either on the external walls or in plugs.

In interspecific crosses another common manifestation is the occurrence of so-called unilateral incompatibility. This occurs when one parental species is self-incompatible (SI) and the other is self-compatible (SC), the pollen of the SC species is rejected by the pistil of the SI species; in the reciprocal cross, the pollen of the SI species is

accepted by the pistil of the SC species (Heslop-Harrison 1982). This rule has been demonstrated in many interspecific crosses (Hinata *et al.* 1974, Sampson 1961, Harder 1993). For example, *Erythronium albidum* and *americanum* both belong to the Liliaceae. *E.americanum* is a self-incompatible species while *E.albinum* is self-fertile. The cross exhibited characteristic unilateral interspecific incompatibility. *E.americanum* (SI) pollen germinated and the pollen tube grew unimpeded in styles of E.*albidum* (SC), whereas the reverse cross resulted in incompatible reaction (Harder 1993). As for crosses between two self-incompatible species, the results of pollination can be compatible or incompatible. According to Sampson (1961) who made intergeneric crosses with 10 genera of *Cruciferae*, most crosses were incompatible, but cross-compatibility also occurred.

Although some interspecific hybrids have been achieved in genus *Limonium* in the past, there is a lack of knowledge about pollen behaviour in *Limonium* interspecific crosses and a lack of information on the potential of interspecific hybridization in *Limonium*. The present study aims to obtain knowledge about pollen behaviour and to determine the barriers to the production of interspecific hybrids between six *Limonium* species. By analysing the results of crosses made between six potentially useful parent species within the *Limonium* genus, it is possible to determine where incongruity barriers exist and to assess the likelihood of obtaining *Limonium* hybrids.

1.2 MATERIALS AND METHODS

1.2.1 Material:

Mature potted plants of the six *Limonium* species used in this study were supplied by the New Zealand Institute for Crop and Food Research Ltd located at Levin Research Centre. The plants were transported to and continuously grown in a glass house in Plant Growth Unit of Massey University. The minimum temperature of the glasshouse was 20^{0}C. The ventilation temperature was set at 25^{0}C. The plants used in the crosses were the following:

twelve *L.perigrinum* plants
eight *L.sinuatum* plants (4 'cob' , 4 'papillate')
seven *L.perezii* plants (4 'cob' , 3 'papillate')

six *L.aureum* plants (3 'cob', 3 'papillate')

six *L.sinense* plants (3 'cob', 3 'papillate')

five *L.caspia* plants (All of five were 'cob' plants)

Pollinations were carried out between February 1994 and June 1994.

1.2.2 Interspecific crosses:

Reciprocal crosses were made between each pair of six *Limonium* species comprising two types:

Legitimate combinations: type A pollen on 'papi' stigma
type B pollen on 'cob' stigma

Illegitimate combinations: type A pollen on 'cob' stigma
type B pollen on 'papi' stigma

The crosses between dimorphic species and monomorphic *L.perigrinum* comprise those in which *L.perigrinum* as a female receives both types of A and B pollen from dimorphic species and those in which *L.perigrinum* pollen (B pollen only) was pollinated on both 'cob' and 'papi' stigmas of dimorphic species.

1.2.3 Pollination Method:

L.perigrinum is a monomorphic, self-compatible species. The flowers of *L.perigrinum* were emasculated before the anthers dehisced. The other five *Limonium* species chosen in this study are dimorphic, self-incompatible species. Although preliminary experiments had confirmed that in self-incompatible species , self pollen did not germinate on the stigma, or even seldom adhered to the stigma, the stamens of the flowers of dimorphic species were removed before cross-pollination. The flowers of *L.perigrinum*, *L.sinuatum*, *L.perezii* last only for one day, while the flowers of *L.aureum*, *L.sinense*, *L.caspia* last for 3-4 days. The time of flower opening was influenced by the temperature and the time of sunrise. Pollination was always carried out at 10:00-11:00 in the morning and only the freshly opened flowers were used. Pollination were made by applying anthers that had dehisced to the stigmas until the stigmas had a clearly-visible excess load of pollen. Pollen viability was assayed using either Alexander's stain (Alexander 1969) or fluorescein diacetate (Knox 1979).

1.2.4 Pollen adherence examination

One hour after pollination, the pollinated stigmas were taken off the plants with a pair of tweezers and were shaken several times before being mounted on the slides. The cover slip was put on the stigma without using any mounting liquid in order to prevent pollen grains being washed away. The number of pollen grains per stigma was counted. Fifteen stigmas from three flowers were examined for each interspecific cross.

1.2.5 Observation of pollen tube growth

Pollen tube growth was examined following hand pollination as described above. In preliminary experiments, the pistils were taken off the plants at 1, 2, 4, 6 hour and overnight interval after pollination in order to find the optimum time for the observation of pollen tube growth. It was found that the pollen tubes had grown to their final position within 2 hours in all interspecific crosses. A six hour interval which was much longer than necessary for pollen tube growth was chosen in the present study to accommodate any possible deviation from a 2 hour growth limitation period.

To study the pathway and physiology of pollen tube growth in interspecific crosses, a modified aniline blue squash technique (Kho and Baer 1968) was used. Six hours after cross-pollination, the pistils were removed from the plant and softened in 8 M NaOH for 2-4 hours, depending on the size and the hardness of the pistils. After the softening treatment, the pistils were thoroughly washed in distilled water twice and then retained in distilled water for an hour to allow the remnants of NaOH out of the tissue. After that the pistils were stained overnight in 0.15% decolorised aniline blue (water soluble aniline blue was the product of BDH chemical Ltd. Product number: 34003) which was dissolved in 0.1 M K_3PO_4. Before observation, pistils were squashed in stain on a microscope slide by gentle pressure on the coverslip with a pencil. The edges of the coverslip were then sealed with nail polish to prevent dehydration of the pistil tissue. Slides prepared in this way could be stored at 4^0C for months. All preparations were observed using a Zeiss epi-fluorescence microscope with the G 365 excitation filter , chromatic beam splitter FT395 and barrier filter LP420. Pollen tubes gave bright blue fluorescence of the aniline-stained callose polysaccharide component of their walls and plugs under UV light (355-425 nm). The presence of pollen tubes and their pattern of growth in the stigma, style and ovary was recorded and the results were microphotographed with Zeiss camera and Kodak Ektachrome 64T colour film.

1.3 RESULTS

1.3.1 VERIFICATION OF STIGMA AND POLLEN MORPHOLOGY

Pollen grain and stigma morphology was checked by microscopy for all six *Limonium* species used in the study to verify previous description of pollen type and stigma type by Baker (1953a). For *L.sinuatum*, *L.perezii*, *L.aureum*, *L.sinense* and *L.caspia*, dimorphism observed in pollen grain and in stigma morphology was consistent with descriptions given by Baker (1948, 1953a).

Since there is no published information on *L.perigrinum* stigma type and pollen type, an attempt was made in this study to identify them. *L.perigrinum* was found to be a monomorphic species possessing a capitate stigma and type B pollen which was identified under compound microscope at x400 magnification using the criteria set up by Baker (1948). Plate 1.13 shows the capitate nature of the *L.perigrinum* stigma.

1.3.2 POLLEN ADHERENCE

Pollen adherence in the crosses between dimorphic species

In legitimate crosses: The number of pollen grains on a stigma was examined an hour after pollination under the microscope at x100 magnification. In all legitimate interspecific combinations, which mean type A pollen of species (1) was put on the 'papi' stigma of species (2), type B pollen of species (1) was on 'cob' stigma of species (2) and *vice versa*, pollen adhered to the stigma easily. The average number of pollen grains per stigma was in a range of 10 - 44 (Table 1.1)

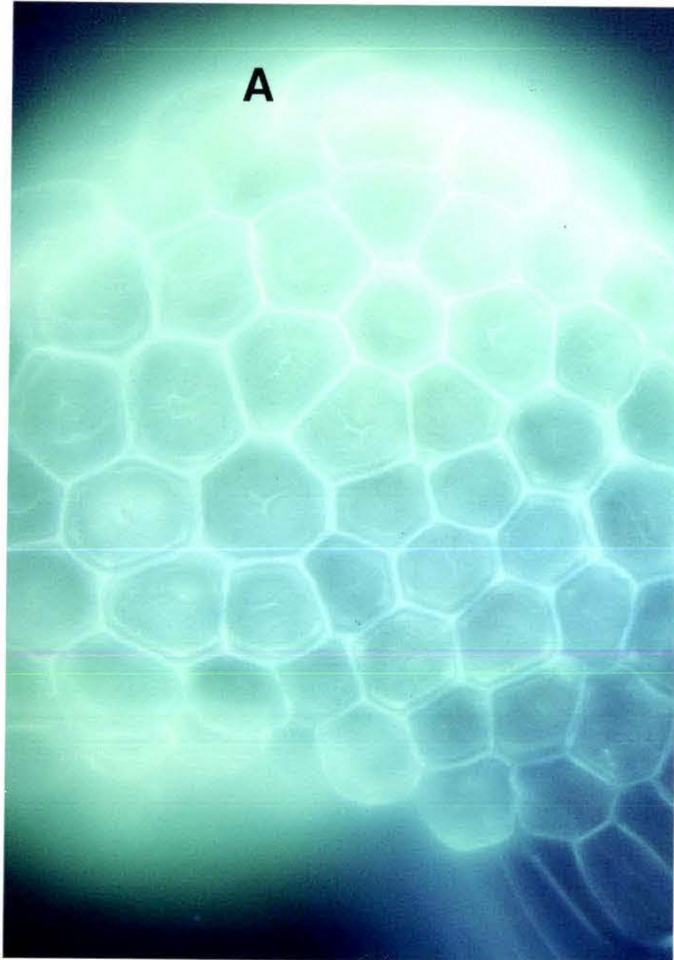

Plate 1.13 ***L.perigrinum* capitate stigma.** Photo A-----U.V. fluorescence photomicrograph of *L.perigrinum* capitate stigma following staining with aniline blue (x400). B-----Diagrammatic drawing of *L.perigrinum* capitate stigma and style.

Table 1.1 Pollen adherence in legitimate *Limonium* interspecific crosses

	L.sinuatum ♀		*L.perezii* ♀		*L.aureum* ♀		*L.sinense* ♀		*L.caspia* ♀	
	A/pap	B/cob	A/pap	B/cob	A/pap	B/cob	A/pap	B/cob	A/pap	B/cob
L.sinuatum ♂	//////	//////	44.5	34.3	20.6	11.3	25.1	41.2	¦¦¦¦¦	15.4
L.perezii ♂	16.2	¦¦¦¦¦	//////	//////	16.1	17.4	20.6	34.2	¦¦¦¦¦	16.7
L.aureum ♂	16.1	¦¦¦¦¦	18.9	23.8	//////	//////	19.2	19.5	¦¦¦¦¦	13.3
L.sinense ♂	14.3	¦¦¦¦¦	17.5	14.4	21.3	18.7	//////	//////	¦¦¦¦¦	13.0
L.caspia ♂	12.9	¦¦¦¦¦	16.2	¦¦¦¦¦	10.0	¦¦¦¦¦	16.2	¦¦¦¦¦	//////	//////

(1) ////// ---- conspecific combination. (2) ¦¦¦¦ ---- The crosses were not conducted. (3) The values in the cells are the mean of the pollen grain numbers per stigma from 15 stigmas of 3 flowers.

Illegitimate crosses: In all but one of the illegitimate interspecific combinations, pollen adherence was poor. Only a few pollen grains (average not more than 3) were found on a stigma and sometimes there was no pollen at all. However, in the illegitimate combination of *L.sinense* (cob)♀ x *L.aureum* A♂, the number of pollen grains per stigma was 18.56($\pm$21.23), which was very close to that (19.58$\pm$7.01) for the legitimate combination of *L.sinense* cob♀ x *L.aureum* B♂.

Pollen adherence in the crosses between *L.perigrinum* (monomorphic) and dimorphic species

Pollen adherence in the crosses between *L.perigrinum* and dimorphic *Limonium* species showed complicated features. When *L.perigrinum* was used as the female parent, both type A and B pollen of dimorphic species adhered to the capitate stigma, though the affinity was different. The number of type B pollen grains per stigma was in the range of 11.5-18.1, while type A pollen grains was in the range of 5.0-13.3 (Table 1.2). When *L.perigrinum* was used as pollen donor and the dimorphic species as a female parent, *L.perigrinum* pollen adhered to 'cob' stigmas much more easily than to 'papi' stigmas (Table 1.3), which further demonstrated *L.perigrinum* possesses type B pollen .

1.3.3 POLLEN GERMINATION AND TUBE GROWTH IN *LIMONIUM* INTERSPECIFIC CROSSES

In all legitimate interspecific pollinations, pollen germinated on the stigmas, but the extent of pollen tube growth varied greatly depending on the species used in crosses. On the contrary, no pollen grain germinated in illegitimate pollination, except for the crosses in which *L.perigrinum* was used as female parent. Both type A and B pollen of dimorphic species germinated on *L.perigrinum* capitate stigma. The pollen tubes of type A pollen and type B pollen of the same species grew to similar levels in the *L.perigrinum* pistil. How far the pollen tubes penetrated was found to be a characteristic of individual species.

The pollen tube growth in legitimate interspecific crosses is summarised in Figure 1.3. The extent of pollen tube growth can be classified into three groups: pollen tube growth was restricted to the stigma; pollen tube was blocked in the style; pollen tube penetrated the ovary. The last group can be further divided into: pollen tube penetrating ovary with

Table 1.2 Pollen adherence in the crosses in which *L.perigrinum* was female parent

	L.sinuatum ♂		*L.perezii* ♂		*L.aureum* ♂		*L.sinense* ♂		*L.caspia* ♂	
	A	B	A	B	A	B	A	B	A	B
L.perigrinum ♀	10.2	14.8	13.3	18.1	5.9	11.5	5.0	13.1	9.47	¦¦¦¦

(1) ¦¦¦¦¦¦ ---- The cross was not conducted. (2) A--type A pollen. B--type B pollen. (3) The values are the mean of pollen grain numbers per stigma from 15 stigmas of 3 *L.perigrinum* flowers.

Table 1.3 Pollen adherence in the crosses in which *L.perigrinum* was pollen donor.

	L.sinuatum ♀		*L.perezii* ♀		*L.aureum* ♀		*L.sinense* ♀		*L.caspia* ♀	
	papi	cob	papi	cob	papi	cob	papi	cob	papi	cob
L.perigrinum ♂	3.0	9.7	1.6	10.9	4.8	12.3	4.0	16.1	¦¦¦¦	6.0

(1) ¦¦¦¦ ---- The cross was not conducted. (2) The values are the mean of pollen grain numbers per stigma from 15 stigmas of 3 flowers.

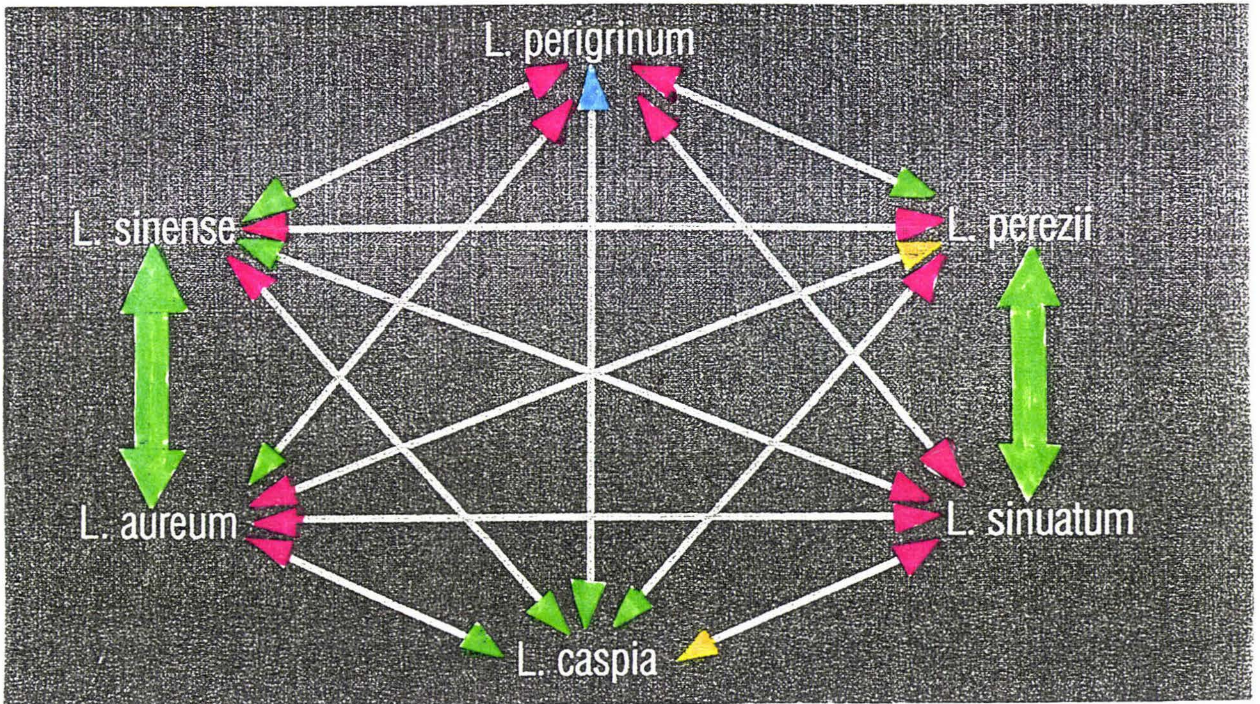

Figure 1.3 Interspecific crosses between 6 *Limonium* species

(1) The arrows show the direction of pollen transfer.

(2) Different colours of arrow represent the different crossabilities.

-----The blue arrow represents the cross in which pollen germinated but the tube growth was restricted in the stigma.

-----The pink arrows represent the crosses in which pollen tubes were arrested in the styles.

-----The small green arrows represent the crosses in which pollen tubes penetrated the ovary at low frequency.

-----The big, thick green arrows represent the crosses in which pollen tubes penetrated the ovary at high frequency.

-----The yellow arrows represent the crosses in which pollen tubes vanished in the style, the site of interspecific inhibition could not be decided.

very low frequency and pollen tube penetrating ovary with high frequency.

Tube growth restricted to stigma

Only in one cross was the pollen tube blocked in the stigma. In the cross between *L.perigrinum* as female parent and *L.caspia* as pollen donor, pollen germinated within a half hour after pollen deposition. Most of the pollen tubes however were arrested immediately after penetrating the surface of the stigma, the pollen tube being only half the diameter of pollen grain and the longest one being no longer than about 2 times the diameter of the pollen grain. Although observations were extended to 48 hours after pollination, no further growth of pollen tubes occurred (Plate 1.14).

Pollen tube blocked in style

In the following groupings of 15 crosses, the pollen tube was found to be blocked in the style. These crosses were:

L.perigrinum ♀ x *L.sinuatum* A (or B) ♂
L.perigrinum ♀ x *L.perezii* A (or B) ♂
L.perigrinum ♀ x *L.sinense* A (or B) ♂
L.perigrinum ♀ x *L.aureum* A (or B) ♂

L.sinuatum ♀ x *L.perigrinum* ♂
L.sinuatum ♀ x *L.aureum* ♂
L.sinuatum ♀ x *L.sinense* ♂
L.sinuatum ♀ x *L.caspia* ♂

L.sinense ♀ x *L.perezii* ♂
L.sinense ♀ x *L.caspia* ♂

L.aureum ♀ x *L.perezii* ♂
L.aureum ♀ x *L.sinuatum* ♂
L.aureum ♀ x *L.caspia* ♂

L.perezii ♀ x *L.sinense* ♂
L.perezii ♀ x *L.caspia* ♂

Plate 1.14 U.V. fluorescence photomicrograph of *L.perigrinum* on which are *L.caspia* pollen and pollen tubes (x160). Pollen tube growth was restricted to the stigma, the pollen tubes were short, the longest one was about 2 times diameter of the pollen grain.

L.perigrinum as female parent In the crosses between *L.perigrinum* as female parent and dimorphic species as male parent, *L.perigrinum* capitate stigmas received both type A pollen and type B pollen. Tube growth of type A and type B pollen was found to reach the same level. The pollen tubes of *L.sinuatum*, *L.sinense* and *L.perezii* grew 1/3 of the way down *L.perigrinum* style. Only *L.aureum* pollen tubes reached the ½ way down the style.

L.sinuatum as female parent *L.perigrinum* pollen did not germinate on the papillate stigma of *L.sinuatum* plant, which was expected since *L.perigrinum* pollen was found here to be B type. When cob *L.sinuatum* was used as female parent, *L.perigrinum* pollen germinated and the longest pollen tube reached the 1/3 of the distance down the style. In the crosses *L.sinuatum* x *L.aureum* and *L.sinuatum* x *L.sinense* many pollen grains (9-25 grains) germinated on the stigma and many pollen tubes grew into the style tissue. The longest tube observed was only one pollen grain diameter (about 49μ) short of reaching the ovary. Many *L.caspia* pollen grains germinated on *L.sinuatum* stigmas, most pollen tubes stopped growth in the stigma and only a few pollen tubes were seen in the top of the style.

L.sinense as female parent There were two crosses in which pollen tubes were blocked in the style when *L.sinense* was used as female parent. Many pollen grains (13-28 pollen grains) germinated on the stigma and the longest tube was half way to the ovary (Plate 1.15).

L.aureum as female parent In the crosses of *L.aureum* x *L.perezii*, *L.aureum* x *L.sinuatum*, and *L.aureum* x *L.caspia*, the site of interspecific inhibition was in the stigma for most pollen tubes, however, a few tubes arrested in the top part of the style.

L.perezii as female parent In the cross of *L.perezii* x *L.caspia*, the longest pollen tubes observed were in the style tissue, about 2/3 way to ovary. In the cross of *L.perezii* ♀ x *L.sinense* ♂, many pollen grains (average more than 20 grains per stigma) germinated on the stigma, most pollen tubes arrested in the style. the longest tube was 2/3 of the way down the style.

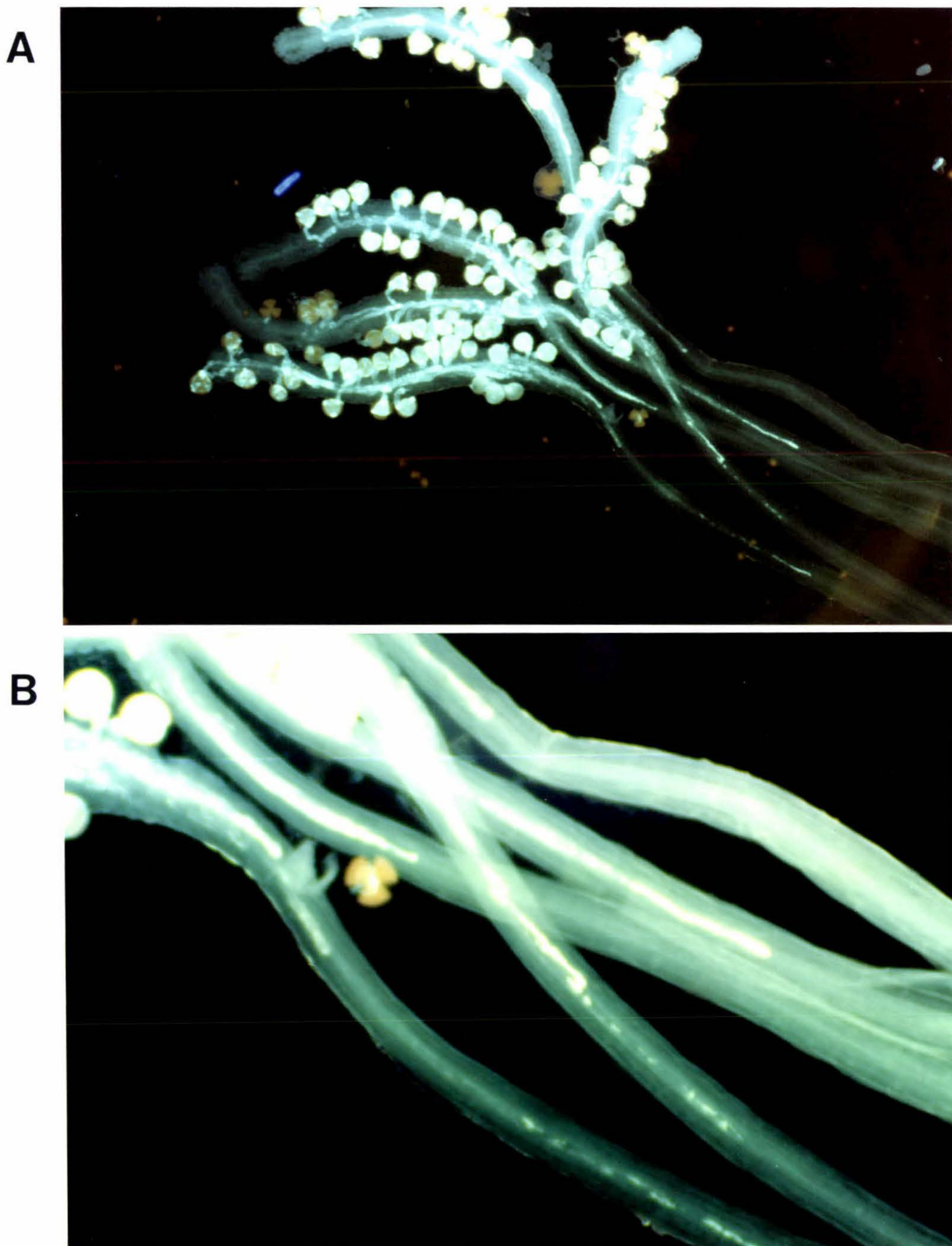

Plate 1.15 U.V. fluorescence photomicrograph of *L.sinense* stigmas and styles showing *L.perezii* pollen tubes which were blocked in the styles. Photo A shows five stigmas and five styles of *L.sinense* with many germinated *L.perezii* pollen grains (x100). Photo B shows the further enlarged styles in which pollen tubes stopped in their growth (x160).

Pollen tube penetrating ovary

For those crosses where pollen tubes were observed to penetrate the ovary two groupings were distinguished:

Pollen tube frequently penetrating the ovary Pollen tube penetrating ovary was observed in the cross combination of *L.perezii* and *L.sinuatum* (in both direction), and in the combination of *L.sinense* and *L.aureum* (in both direction) at high frequency, namely, the tube penetrating the ovary was observed in six flowers out of 20. It is often the case that more than one pollen tube grew through a style to ovary (Plate 1.16, 1.17). The number of tubes in a style sometimes could not be counted, because the tubes grew so closely together. Although many pollen tubes were seen inside the ovary, only in two cases has the pollen tube seen to have actually entered the ovule, one in the cross of *L.perezii* cob x *L.sinuatum* B and another in the cross of *L.sinense* papi x *L.aureum* A. The ovules often shifted their position during slide preparation, which may have reduced the chance of seeing the tube penetrating the ovule.

Pollen tube occasionally penetrating ovary In the following 8 crosses, pollen tubes penetrated the ovary at low frequency.

L.perezii ♀ x *L.perigrinum* ♂
L.sinense ♀ x *L.perigrinum* ♂
L.sinense ♀ x *L.sinuatum* ♂
L.aureum ♀ x *L.perigrinum* ♂
L.caspia ♀ x *L.perigrinum* ♂
L.caspia ♀ x *L.perezii* ♂
L.caspia ♀ x *L.sinense* ♂
L.caspia ♀ x *L.aureum* ♂

The frequency of pollen tube penetration to the ovary was very low, only 1 or 2 or 3 tubes were seen penetrating the ovary in the observation of a total of 20 flowers in each cross above. Most pollen tubes were blocked in the style. No pollen tube was observed reaching the ovary in their reciprocal crosses, which indicates that unilateral incompatibility operated in these eight crosses.

Pollen tube vanished in the style

The site of interspecific inhibition could not be decided in following two crosses:

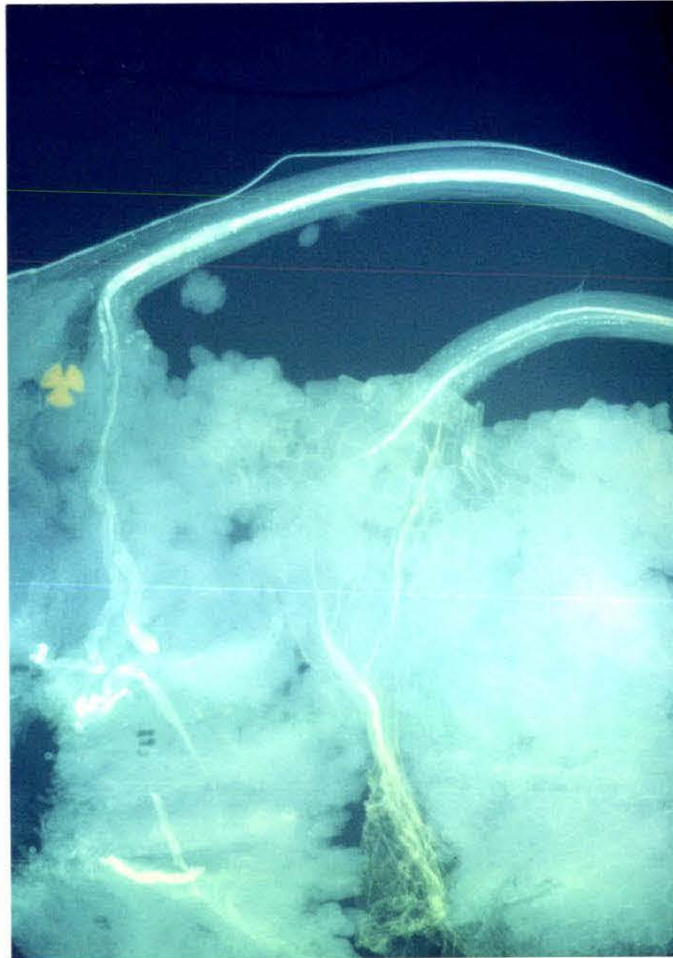

Plate 1.16 U.V. fluorescence photomicrograph of *L.sinuatum* ovary showing *L.perezii* pollen tubes (x100). Two styles connected with the ovary, more than one pollen tubes grew through the styles and entered the ovary. The pollen tubes showed twisted and swollen tips.

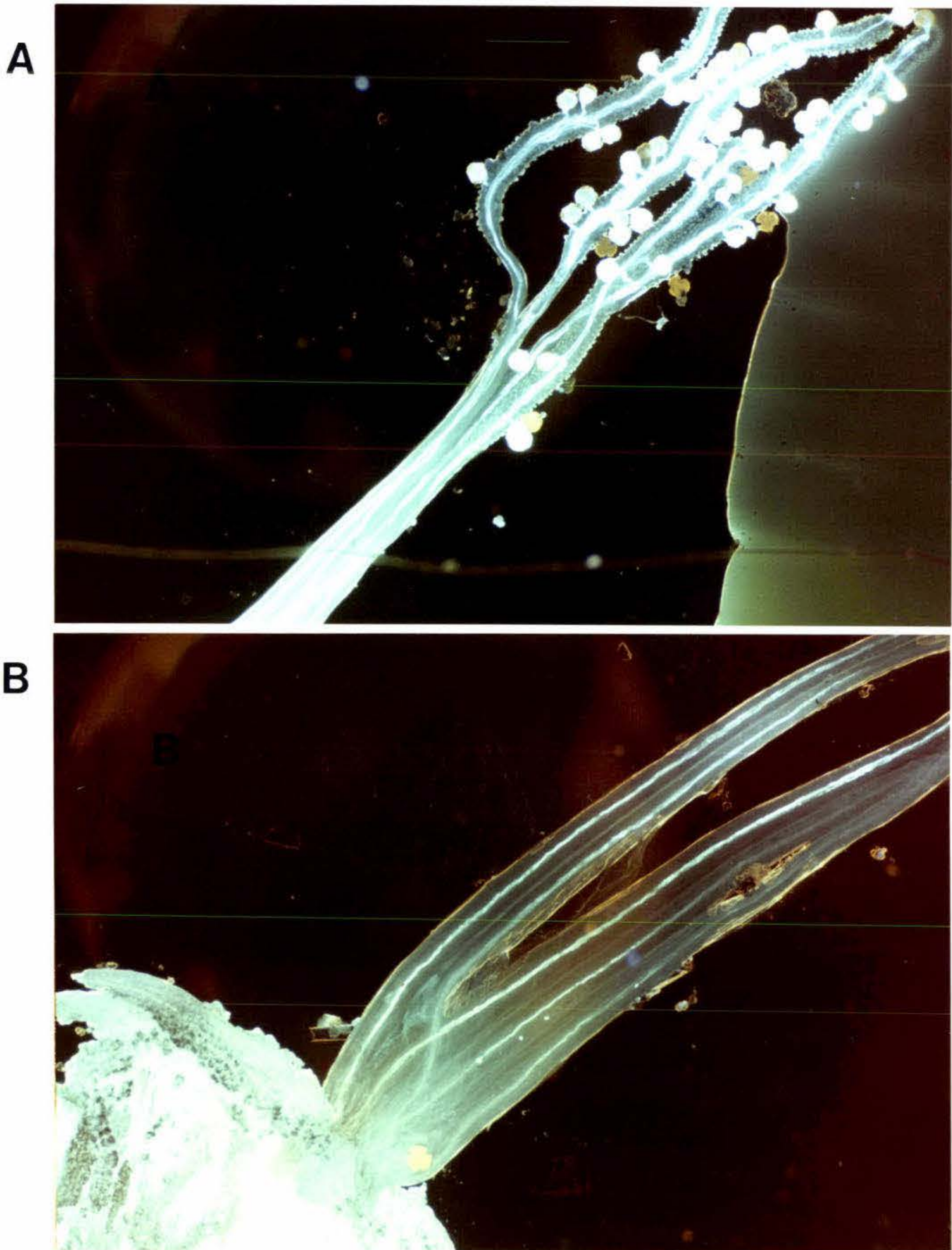

Plate 1.17 U.V. fluorescence photomicrograph of *L.sinense* pistil showing *L.aureum* pollen tube penetrating the ovary with high frequency.

Photo A---*L.sinense* stigmas with many germinated *L.aureum* pollen grains (x100).

Photo B---Many pollen tubes grew through the styles and entered the ovary (x160).

L.perezii ♀ x *L.aureum* ♂

L.caspia ♀ x *L.sinuatum* ♂

In these two cases, many pollen grains germinated on the stigma and many pollen tubes grew through the stigma to the style. The tubes in the style, however, became thinner and fluorescence of the tubes became fainter. The tubes were found to vanish in the lower 2/3 part of the style. No additional experiments were conducted to examine this phenomenon further.

Abnormalities of pollen tube growth

The tips of inhibited pollen tubes sometimes showed a heavy callose deposit, which was observed in nearly all interspecific crosses conducted in the present study. The swollen tips occurred in stigmatic tissue (Plate 1.18), occurred in the style (Plate 1.19) and inside the ovary (Plate 1.16). Non-directional tube growth was another disorder observed in *Limonium* interspecific crosses. In the cross of *L.sinense* (papi) ♀ x *L.caspia* A ♂, a pollen tube grew upwards in the stigma and then turned back to grow in the basipetal direction (Plate 1.20). In the cross of *L.sinense* (papi)♀ x *L.perezii* A ♂, a tube grew laterally, it grew into the tissue outside the transmitting track. The other abnormalities in pollen tube growth which were seen included: branching of the tube (Plate 1.21), and two tubes coming out respectively through two pores of one pollen grain (Plate 1.22). These abnormal pollen tube growth manifestations were neither linked to the arrest sites of pollen tube nor restricted to any particular cross. The abnormalities were observed in the crosses in which pollen tube growth was restricted in the stigma; in the crosses in which pollen tube growth arrested in the style and the crosses in which the longest pollen tube grew to the ovary.

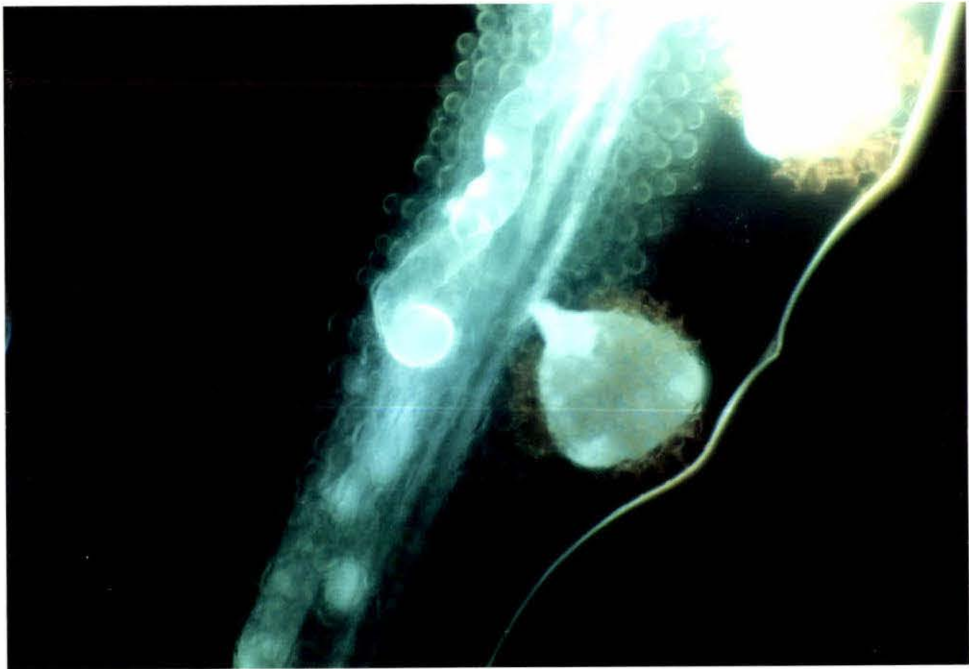

Plate 1.18 U.V. fluorescence photomicrograph of a *L.sinuatum* stigma showing the *L.perezii* pollen tube with a swollen tip (x400).

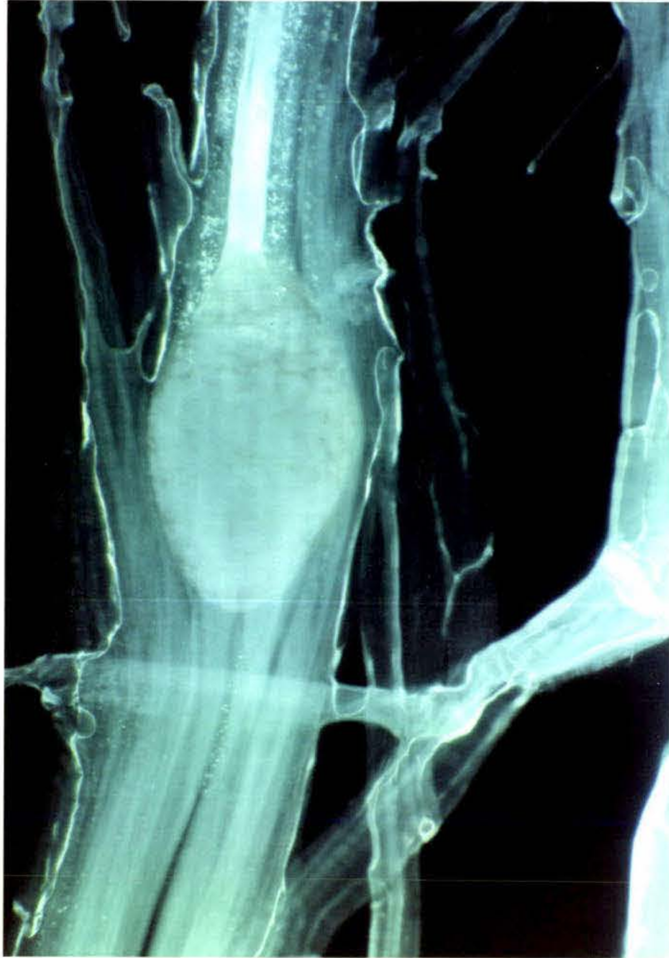

Plate 1.19 U.V. fluorescence photomicrograph of a *L.perigrinum* style with a *L.aureum* pollen tube which had stopped growth and the tube had a big swollen tip (x400).

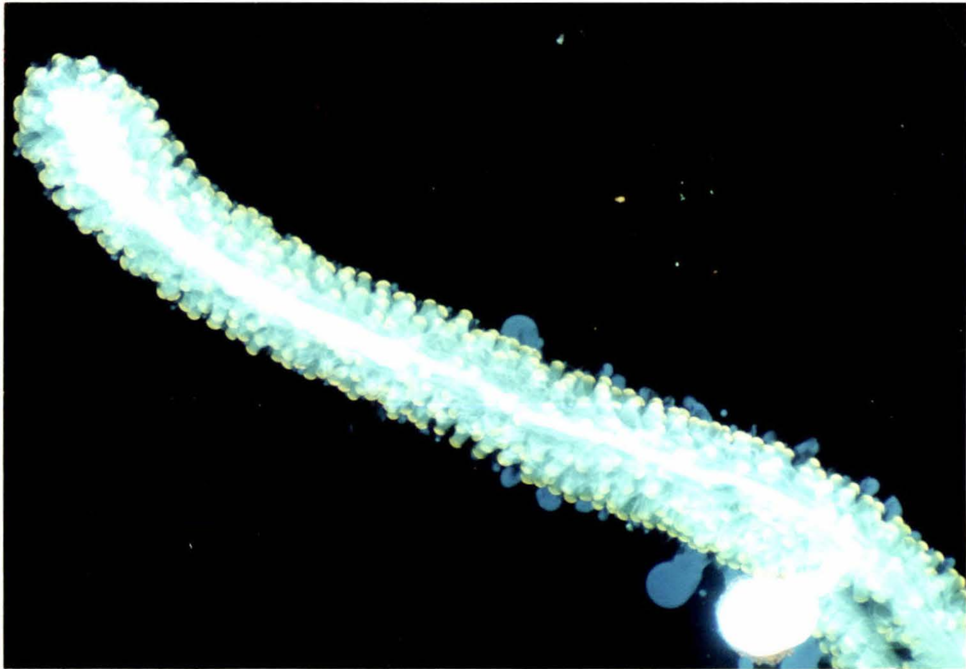

Plate 1.20 U.V. fluorescence photomicrograph of a *L.sinense* stigma with a *L.caspia* pollen tube which grew apically at first and then turned back growing basipetally (x160).

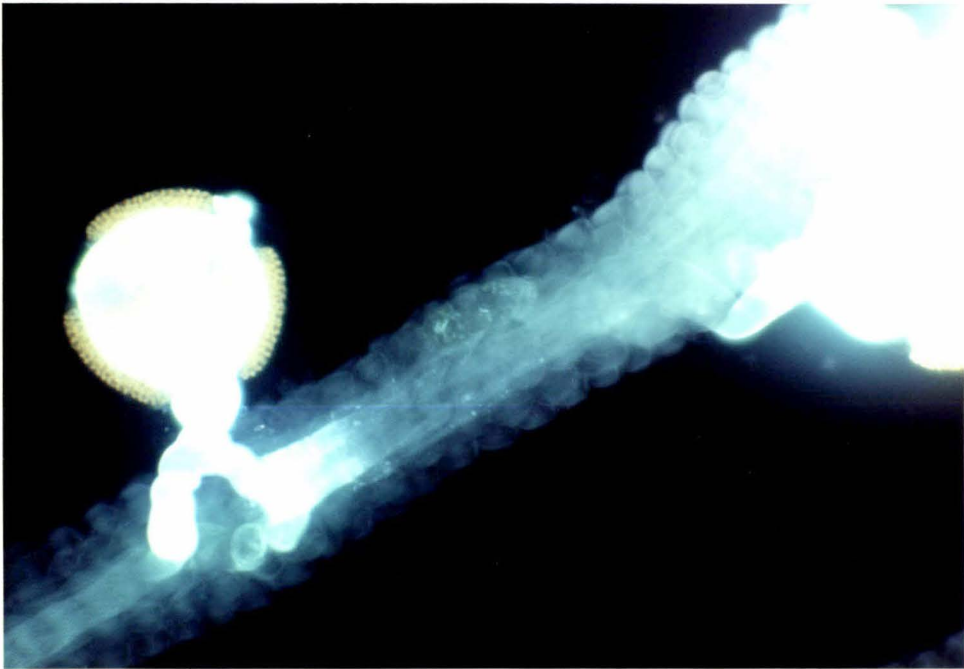

Plate 1.21 U.V. fluorescence photomicrograph of *L.caspia* stigma with a *L.perezii* pollen tube which became branched soon after germination (x400).

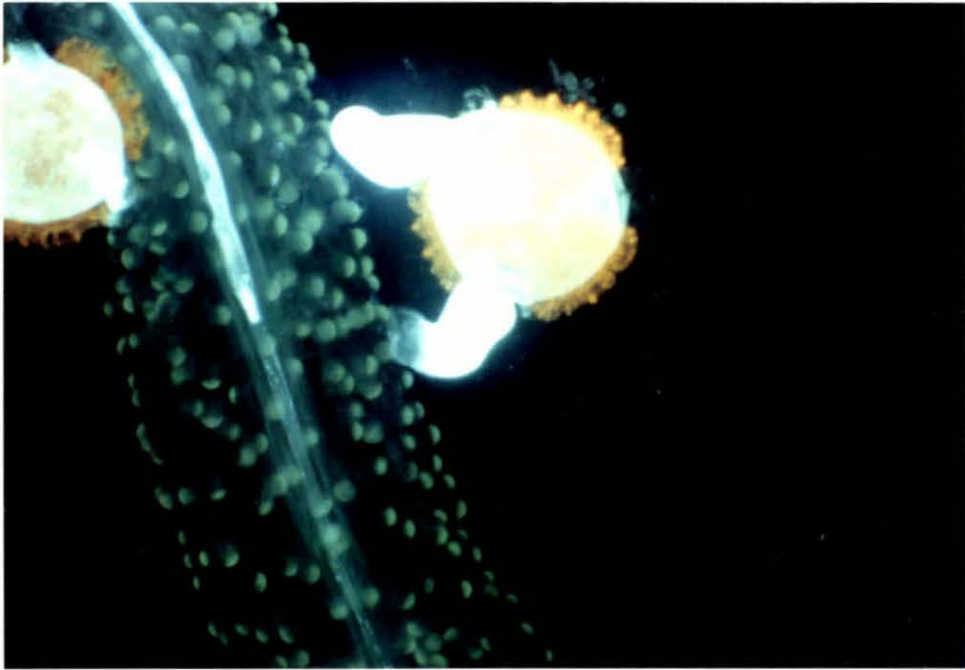

Plate 1.22 U.V. fluorescence photomicrograph of a *L.perezii* stigma with a *L.caspia* pollen grain from which two pollen tubes coming through two pores respectively (x400).

1.3.4 THE MEAN STYLE AND STIGMA LENGTHS OF 6 *LIMONIUM* SPECIES

The mean style length and the mean stigma length of 6 *Limonium* species are shown in Table 1.4.

Table 1.4 Mean stigma and style lengths of 6 *Limonium* species

	stigma (mm)	style (mm)	stigma + style(mm)
L.perigrinum	0.11 (±0.02)	11.76 (±0.41)	11.87 (±0.40)
L.perezii (cob)	2.38 (±0.22)	4.93 (±0.63)	7.31 (±0.66)
L.perezii (papi)	2.4 (±0.21)	4.79 (±0.76)	7.19 (±0.69)
L.aureum (cob)	1.42 (±0.05)	4.42 (±0.491)	5.84 (±0.14)
L.aureum (papi)	1.40 (±0.03)	3.61 (±0.21)	5.01 (±0.23)
L.sinense (cob)	1.17 (±0.11)	4.71 (±0.22)	5.89 (±0.24)
L.sinense (papi)	1.33 (±0.12)	3.98 (±0.15)	5.32 (±0.18)
L.sinuatum (cob)	2.27 (±0.31)	4.49 (±0.49)	6.77 (±0.49)
L.sinuatum (papi)	2.15 (±0.13)	4.50 (±0.23)	6.65 (±0.30)
L.caspia (cob)	0.92 (±0.07)	2.66 (±0.29)	3.52 (±0.37)

(1) Values were the mean of 15 determinations of 3 flowers. (2) Values in parentheses are standard deviation. (3) Measurement was made under the compound microscope with a micrometer at x100 magnification.

Of the six *Limonium* species, *L.perigrinum* is monomorphic and the others are dimorphic species in which the heteromorphism includes dimorphism of the pollen and the dimorphism of stigmas, which has been described above. Investigation of style length revealed that *L.aureum* is heteromorphic not only in terms of pollen type and stigma type but also in terms of the style length and in a complementary variation in the positions of anthers and stigmas. In *L.aureum* flowers the long style is associated with cob stigmas. The cob stigma extended beyond the stamens (containing type A pollen), whereas short styles are linked to papillate stigmas and in this case the stamens (containing type B pollen) extended beyond papillate stigmas. *L.sinuatum*, *L.perezii*, and

L.sinense were found not to be heterostylous species. In those species the lengths of the style linked to cob stigma and the style linked to papillate stigma were observed to be similar. As for *L.caspia*, because only cob plants were used in the present study, papillate plants were not examined. It is not known in this species whether styles with cob stigma and styles with papillate stigma have the same length or not.

1.4 DISCUSSION

1.4.1 Pollen adherence

Pollination in flowering plants is regarded as a sequential series of interactions between pollen and pistils, including pollen contact and attachment to the stigma surface, pollen hydration, germination to produce pollen tubes, pollen tube growth through stigma and styles; pollen tube entry into the ovary, ovule and release of two sperm nuclei into embryo sac, and finally double fertilization. A failure at any one of these points means no seed is produced. Pollen adherence is of physiological importance since it is the first step of interaction between pollen and pistils.

The results of interspecific crosses of dimorphic *Limonium* species revealed substantial differences in pollen adherence in legitimate crosses and illegitimate crosses. With one exception there was almost no adherence in illegitimate crosses. It might be reasonable to assume that the different structure of stigmatic papillae in the two morphs and two pollen types are involved in the control of incongruity confronted in *Limonium* interspecific crosses. Actually this functional morphological description is also given for the pollen stigma interaction within *Limonium* species (Dulbergert 1975). In conspecific legitimate combination, there is a remarkable topographical complementarity between the contour of stigma and exine pattern of pollen grains, such as, the size of the papillae tips and the distances between them approximate the size of the holes and the distances between their centres in the exine reticulum of type A pollen (Dulberget 1975). It is likely that this lock-and-key mechanical relation also exists in interspecific crosses as a control factor in interaction of stigma and pollen, since the stigma structures of different species are similar and the pollen exine patterns of different species also have similarities throughout the *Limonium* genus (Baker 1948).

An exception was found in the cross between *L.sinense* cob♀ x *L.aureum* A♂, the number of pollen grains per stigma in this illegitimate cross (18.5) is very close to that of legitimate cross: *L.sinense* papi ♀ x *L.aureum* A ♂ (19.24) . However, no pollen germinated on the stigma in the illegitimate combination while pollen germinated in legitimate combination, although the similar pollen adherence in both crosses. This result indicates that there must be mechanisms other than initial adherence operating during the interaction between stigma and pollen grains that control pollen germination. Chemical interactions between wall-held substances in the stigmatic papillae and in pollen grains could well play an important role in this case.

1.4.2 Unilateral incompatibility

Unilateral incompatibility was observed in the following 8 crosses:

L.perezii ♀ x *L.perigrinum* ♂

L.aureum ♀ x *L.perigrinum* ♂

L.sinense ♀ x *L.perigrinum* ♂

L.caspia ♀ x *L.perigrinum* ♂

L.sinense ♀ x *L.sinuatum* ♂

L.caspia ♀ x *L.sinense* ♂

L.caspia ♀ x *L.aureum* ♂

L.caspia ♀ x *L.perezii* ♂

Pollen tube penetration to the ovary was observed in these crosses, but in their reciprocal crosses unilateral incongruity was exhibited and pollen tube growth was arrested in the style. *L.perigrinum* is a monomorphic, self-compatible *Limonium* species. Four crosses out of eight are the combinations between *L.perigrinum* and dimorphic, self-incompatible *Limonium* species. Lewis and Crowe (1958) and earlier workers found that pollen tubes of certain self-compatible (SC) species of angiosperms were inhibited in the styles of self-incompatible (SI) species, but the reciprocal crosses were compatible. The behaviour of the SI x SC pollination crosses determined here however is not in agreement with those Lewis and Crowe (1958) observed. The pollen tubes of *L.perigrinum* (SC species) were not inhibited in the styles of four *Limonium* SI species, pollen tubes grew straight to the ovary. In contrary, pollen tubes of SI species were blocked in the styles of *L.perigrinum*. Four other unilateral incompatible crosses

occurred between dimorphic species. When *L.caspia* was the female parent, pollen tubes of *L.sinense*, *L.aureum* and *L.perezii* were occasionally observed penetrating the ovary of *L.caspia*, but not one *L.caspia* tube was seen reaching the ovary in reciprocal crosses. In all eight unilateral incompatible crosses, there is a commonality which is that the style of the female parent was shorter than that of male parent, so the unilateral incompatibility of *Limonium* might be explained by the inability of pollen of short-styled species to traverse the style of a longer-styled species. *L.perigrinum* has a much longer style, about 12 mm than all others, which range from 7.31 mm (cob) and 7.19 mm (papi) to the shortest 3.52 mm (*L.caspia* 'cob').

Unilateral interspecific incompatibility has also been observed in *Rhododendron* crosses. The same explanation relating to pollen tube length compared to style length was given by the authors (Kho and Baer 1973). It might be worthwhile to try style-pollination in unilateral incompatible *Limonium* crosses in future studies. To perform style-pollination, the styles of long-styled species can be cut down to the same length as the style of short-styled species, or even shorter. If pollen from short-styled species can be induced to germinate on the cut style surface and grown into the style of long-styled species, it should be reasonable to expect the pollen tubes of short-styled species to reach the ovary after style pollination, on the condition that the unilateral incompatibility of *Limonium* is only caused by the difference of style lengths between long-styled species and short-styled species.

1.4.3 Interspecific barriers and self-incompatibility

Self-incompatibility has been defined as the inability of a fertile seed plant to produce zygotes after self-pollination. It is based upon the inherited capacity of the flowers of a self-incompatible plant to reject their own pollen and to accept pollen from other self-incompatible plants in the population (Nettancourt 1984). The molecular mechanism of self-incompatibility remains unclear. However it is now widely assumed that the pistil produces an S-genotype-associated glycoprotein that binds to specific receptor sites on pollen tubes of the same S genotype (Knox and Williams 1986), which results in the failure of self-pollen germination or the arrest of self-pollen tube growth. S-glycoproteins which were isolated from style tissue of *Nicotiana alata* were demonstrated to inhibit *in vitro* tube growth of pollen which has the same genotype as

the style tissue (Jahnen *et al.* 1989). Many workers proposed that the S-genes govern non-functioning of the pistil-pollen relationship, not only within but also between species (Pandey 1969; Williams *et al.* 1981). Pandey (1969) has suggested for *Nicotiana* species that the S-gene has two independent functions governing pollen tube rejection at different levels within the pistil. These are: (1) sporophytic specificity controlling rejection of foreign pollen at or close to the stigma, and (2) gametophytic controlling rejection of incompatible intraspecific pollen further down the style. These two functions of the S-gene may represent distinct points of genetic control. Pandey's hypothesis was based on the observation that inhibition of pollen in the stigma is characteristic of *Nicotiana* interspecific incompatibility behaviour. Irrespective of whether S.I. or S.C. species are involved, inhibition in the style is a characteristic of the intraspecific incompatibility of all six S.I. species in *Nicotiana* (Pandey 1969). However, it has been difficult to explain results of many other interspecific crosses in the basis of self-incompatibility (Ascher & Peloquin 1968; Newton *et al.* 1970). Hogenboom (1975) suggested that the mechanism governing non-functioning between species is different from that within species, and incongruity is the major limiting factor to interspecific hybridization. This theory proposes that the reproductive system of each species has diverged through evolution such that co-ordination between pollen and pistil in interspecific crosses is incomplete, leading to malfunctions in pollen tube growth or later after fusion of the nuclei (Ellis *et al.* 1991). Self-incompatible *Limonium* species have a typical sporophytic control system, self-pollination results in complete failure of germination of pollen grains. The site of inhibition is at the stigma surface (Baker 1966). In contrast to this, in *Limonium* interspecific crosses, many stages of the reproductive process were inhibited. The inhibition site was in the stigma in the cross between *L.perigrinum*♀ x *L.caspia*♂. The pollen tubes were blocked in the styles in 15 crosses. Pollen tube penetration of the ovary was observed in 12 crosses. Clearly, the sites of inhibition in *Limonium* interspecific crosses is different from that of the self-incompatible reaction. Even in the cross between *L.perigrinum* and *L.caspia* the pollen behaviour is different from that of self-pollination. In self-pollination the pollen grains do not germinate at all, but in the cross of *L.perigrinum*♀ x *L.caspia*♂ pollen germinates, pollen tubes grow to 2 times the diameter of the pollen grains and then tube growth stopped in the stigma tissue. Results reported here for *Limonium* therefore

support Hogenboom (1975)'s theory that incongruity operates at several levels.
If we apply the theory that incongruity is a by-product of evolutionary divergence, the interspecific pollen-pistil interaction should reflect the evolutionary relationships between species and reflect taxonomic distance. The results of *Limonium* interspecific crosses conducted in this work do fit the theory well. The high crossability was only observed in intra-sectional crosses and intra-subsectional crosses. The pollen tube penetration to the ovary at high frequency was observed in the crosses between *L.perezii* x *L.sinuatum* (in both directions) and in the crosses between *L.sinense* x *L.aureum* (in both directions). *L.perezii* and *L.sinuatum* belong to the same section, *Pteroclados* in the classification of Baker (1953a); *L.sinense* and *L.aureum* belong to the same subsection, *Chrysantheae* in the same classification scheme. In some intersectional crosses, pollen tubes were also seen penetrating the ovary but at very low frequency; in most intersectional crosses pollen tube growth was stopped in the style.

1.4.4 Abnormalities of pollen tube growth

The arrested pollen tubes in *Limonium* interspecific crosses were seen to be associated with a variety of abnormal tip growth forms. The most wide spread abnormality was the heavy callose deposit at the tips of the inhibited pollen tubes. This is a typical incongruity reaction and was found in many interspecific or intergeneric crosses. In the interspecific cross of *Capsicum pubescens* x *Capsicum chacoense*, the heavy callose deposit was sometimes accompanied by spiral tip growth or an opening at the pollen tube tips (Lelivelt 1993). In interspecific crosses of *Rhododendron* , it was found that the pollen tubes may arrest at any of 7 developing stages (Williams and Knox 1982). The site of tube arrest within the pistil is dependent on the species crossed, but heavy callose deposit was found in nearly all 7 developing stages. The callose deposit reaction was also found in self pollination of self-incompatible species. The callose was produced in self-pollination of rye (*Secale cereale*) which is a self-incompatible species. In incompatible matings rye pollen tubes grew intercellularly for a distance of four or five cells in the multicellular papillae before the growth was arrested. The callose deposit was found to cover about one-third of the pollen grain surface and the basal part of the pollen tube (Vithanage *et al.* 1980). Presumably, it represents modifications of polysaccharide synthesis or deposition (Williams and Knox 1982), but what induces

these modifications is unknown though there is much speculation. According to Heslop-Harrison (1978) the interaction of pollen and stigma is a recognition event mediated by interaction of stigma surface and pollen wall components. Callose formation is not only part of the plant response to an incompatible mating, is also part of the response to other stimuli such as infection by pathogens and wounding (Vithanage *et al.* 1980). It seems the callose deposition response can be induced by different elicitors or at least by elicitors from different sources. It might be the case that there are a number of mechanisms for the callose response.

Non-directional tube growth was found in *Limonium* interspecific crosses. This phenomenon may indicate that the foreign pollen tube growth lacks guidance within the pistil. In flowering plants the pollen tube serves as the vehicle for transporting the sperm to the egg. The research on how pollen tubes growing unerringly to their destination revealed that ovules seem to provide signals for target-directed growth of pollen tubes (Mascarenhas and Machlis 1962, Welk *et al.* 1965, Kandasamy *et al.* 1994, Hülskap *et al.* 1995). Presumably, the non-directional tube growth of *Limonium* interspecific crosses might result from the failure of communication between pollen tubes and the signals emanating from the ovules.

1.4.5 The prospect for interspecific hybridization in *Limonium*

The work reported here has shown great potential for producing *Limonium* interspecific hybrids, though only six species were used in the crosses. There were 12 crosses in which pollen tubes penetrated the ovary. These are shown by green arrows in Figure 1.3. In the crosses between *L.aureum* x *L.sinense* and the crosses between *L.perezii* x *L.sinuatum*, pollen tube penetration to the ovary occurred at high frequency. Although pollen tube penetration to the ovary or ovule does not necessarily mean that the fertilization is actually taking place and that viable seeds are produced, it needs further investigation to check that ovule penetration occurs and that fusion takes place. Work by other workers does provide the evidence for successful fertilization in some *Limonium* interspecific crosses. For example, a *Limonium* hybrid, Lemon star was obtained through interspecific cross between *L.aureum* and *L.sinense* (Harada 1992). Another *Limonium* hybrid has recently been obtained from the cross of *L.perezii* and

L.sinuatum through embryo rescue (Morgan, Burge unpublished).

L.perigrinum is the only *Limonium* species which produces large flowers, the diameter of the flower is around 1.5-1.6 cm, also the flowers of *L.perigrinum* have a desirable bright pink colour. Creation of a hybrid between *L.perigrinum* and other *Limonium* species in order to increase the size of flower or change flower colour would be most desirable, but the results obtained in this work show that if *L.perigrinum* is used as a female parent, the chance to achieve an interspecific hybrid is low. In the crosses of *L.perigrinum* which was the female parent and 5 other *Limonium* species, none of pollen tubes reached the ovary. *L.caspia* pollen tubes were restricted in the stigma; the pollen tubes of *L.perezii*, *L.sinuatum*, *L.sinense* reached the top 1/3 part of *L.perigrinum* style and *L.aureum* pollen tubes, reaching the farthest, grew to the ½ of the style. However it is not so disappointing when *L.perigrinum* was used as pollen donor. In the cross in which *L.perigrinum* was used as a male parent, its pollen tubes occasionally entered the ovary of *L.perezii*, *L.sinense*, *L.aureum* and *L.caspia*, though most pollen tubes were blocked in the styles. Results of this study clearly show that opportunities exist to develop new *Limonium* cultivars through interspecific hybridisation, providing the individual crosses are tested by the kinds of studies represented here, from post-pollination events at the stigma, in the style and beyond at ovary and ovule penetration levels.

CHAPTER TWO OVARY, OVULE AND EMBRYO DEVELOPMENT OF *L.PEREZII* FOLLOWING THE COMPATIBLE CONSPECIFIC POLLINATION

2.1 INTRODUCTION:

Although *Limonium* has recently been increasing in importance as a cut flower crop (Tsurushima 1992a), the embryogenesis of *Limonium* has not been extensively studied. *Limonium perigrinum* is a self-compatible, monomorphic species and its ovule and embryo development after self- and cross-pollination was studied by Burge and Morgan (1993). In their work, cross-pollinations were made between *L.perigrinum* selected from gardens around New Zealand and *L.perigrinum* grown from seeds introduced from South Africa. They found 4 types of ovule growth patterns in terms of ovule size and embryo development in both self- and cross-fertilization. The ovules of type 1 developed normally and visible embryos were found at Day 9 after pollination. The ovules of type 2 never grew after pollination and no embryo was found. The ovules of type 3 stopped growth before Day 18 after pollination and the embryos inside those ovules had reached globular or heart stages but no further growth was seen. The ovules of type 4 grew until Day 18, but despite their increased size, these ovules did not contain embryos. They showed that 56% of cross-pollinated florets but only 18% of self-pollinated florets produced embryos that developed normally (type 1).

L.perigrinum is one of six *Limonium* species chosen for study in this thesis, the other five are dimorphic, self-incompatible species. In order to find out whether the ovule growth patterns described for *L.perigrinum* apply to other *Limonium* species, *L.perezii* was chosen for the investigation. Experiments were designed to provide a description of ovary, ovule and embryo growth and development in conspecific crosses within this species. Knowledge of these processes in individual species may help future attempts at interspecific hybridization using this species. In many crop plants, differences in embryo growth rates among species apparently result in incompatibility, leading to arrested growth of embryos in interspecific hybrids (Ahmad and Slinkard 1991). It seems that successful hybridization may require the two species being crossed to have synchronous embryo development, therefore the objective of this investigation focuses on the growth pattern as well as growth rate of ovule and embryo of *L.perezii* following the compatible, conspecific pollination.

2.2 MATERIALS AND METHODS

Four potted *L.perezii* plants were selected, three were cob plants used as female parents and one was a papillate plant used as the pollen source. The plants were grown in a glasshouse with minimum temperature of 20°C during the night and maximum temperature of 25°C in daytime. The investigation was conducted in August and September 1994. Pollination was carried out at 10:00 - 10:30 in the mornings. Type B pollen from papillate *L.perezii* was hand-pollinated on the cob stigma of *L.perezii*. Excess pollen grains were loaded on the stigmas for successful pollination. Thirty pollinated flowers were collected at 3-day intervals. The day on which pollinations were carried out was defined as Day 0. Thirty non-pollinated flowers were picked at Day 0 for ovary and ovule measurement as a control. The flowers were dissected under stereo microscope and the lengths of ovaries, ovules and embryos were measured under a compound microscope with an eyepiece micrometer. The measurements of ovary, ovule and embryo were recorded from Day 0 to Day 33.

2.3 RESULTS

The *L.perezii* pistil has five segregated styles which are connected to the rim of the top of the ovary. A single ovule is situated in the unilocular ovary. The ovule which possesses two integuments is rather unique in terms of its connection to the ovary. Although the *L.perezii* micropyle points upwards, the bottom of the ovule does not connect with the ovary, which means the hilum does not lie in line with the micropyle. The connection between the ovule and the ovary is implemented by a cord (funicle) which runs from the receptacle-end inner tissue of ovary up to the top of outer integument of the ovule (Figure 2.1). The outer integument encloses the inner integument which protrudes beyond the former to form the micropyle.

Three types of floral development were found following conspecific pollination in *L.perezii* (Table 2.1).

Table 2.1 Developmental groups of *L.perezii* in terms of ovary, ovule and embryo growth following conspecific pollination.

groups	developmental characteristics	tube penetrating ovary & ovule	percent (%)
Type 1	No ovary & ovule growth; no embryo was found. The ovary shrivelled from Day 6.	yes	26%
Type 2	The ovaries & ovules grew up to Day 12 and then shrivelled. No embryo was found.	yes	11%
Type 3	The ovaries, ovules and embryos developed normally and grew up to Day 30. The embryos were detectable by Day 3 and reached torpedo stage at Day 12.	yes	63%

Thirty flowers were assessed at 3-day interval.

In a group designated as Type 1 (about 26% of the florets), the ovary and ovule did not grow at all after pollination. Presumably, the fertilization did not take place, though pollen tube penetration was seen in all flowers. No embryos were ever found in this group. Both ovary and ovule started to shrivel at Day 6 and had dried out by Day 15. Type 2 florets made up 11% of the total. The ovary and ovule started to grow after pollination and growth may continue up to Day 12. The ovules often shrunk before ovaries did. No embryos were found inside these enlarged ovules.

Type 3: around 63% of florets developed normally in terms of ovary, ovule and embryo growth.

Embryo development in *L.perezii* showed varied growth rates between individual plants, even though the pollinations were made on the same day. Specimens collected from different plants at any time, especially during the early stages of development, contained embryos of varying sizes. However, the embryo development was synchronous among ovules from the same individual plant. The following description is based on the data collected from one plant. The average length of the ovary was 1.04 ± 0.07 mm at Day 0 (the day pollination was carried out). The ovary started growing after pollination and had nearly reached the full size by Day 24. The ovary wall

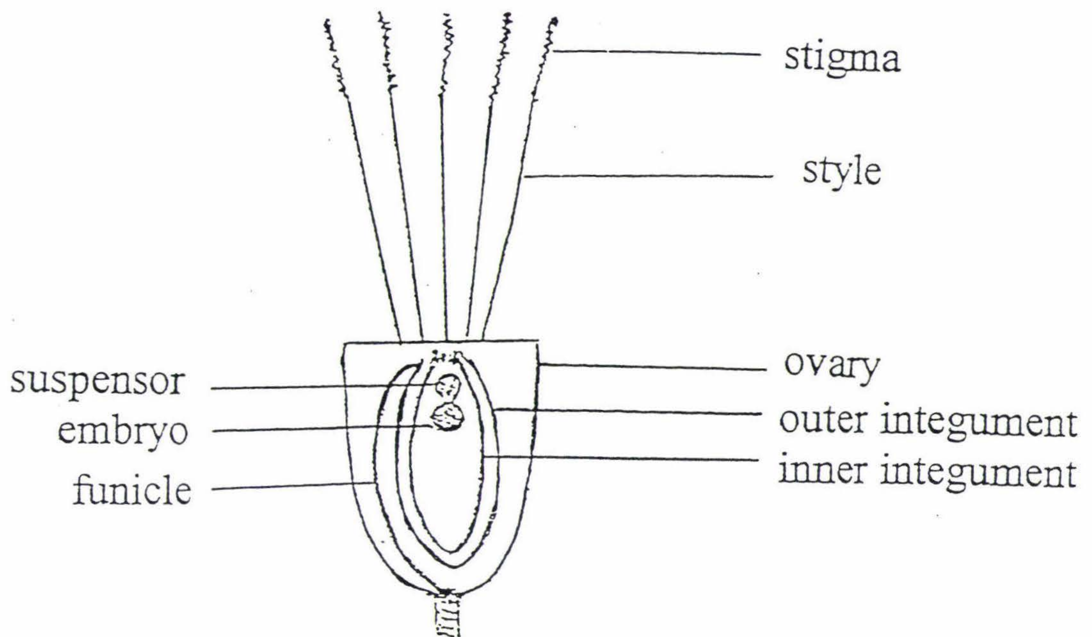

Figure 2.1 Diagrammatic longitudinal section of *L.perezii* pistil following fertilisation. *L.perezii* has 5 segregated stigmas and 5 segregated styles. Only one ovule is situated in the unilocular ovary. Here a successful fertilisation has produced an embryo. The funicle has a appearance of a cord connecting ovary and ovule. The suspensor is found above the embryo inside the ovule.

showed a red appearance from Day 27 and stopped growth by Day 30. The ovary had reached 4.2±0.2 mm by then (Figure 2.2). The appearance of red coloration in the ovary wall seems to be the sign of maturity.

The length of ovules was 0.57±0.05 mm at Day 0 and the growth of ovules followed that of the ovary. By Day 30 the ovules had grown to the maximum size: 3.9±0.08 mm (Figure 2.3).

Embryos were detectable by Day 3. They were in the globular stage which appeared as a spherical mass of cells attached to an enlarged suspensor which also had a spherical appearance (Plate 2.1). The embryos were only 0.04±0.005 mm in diameter and the suspensors were a little bigger than the embryos, being 0.045±0.004 mm in diameter. Both the embryos and the suspensors were colourless at Day 3. By Day 6 the embryos were either in the globular or early heart stage and both the embryos and the suspensors had become green in colour (Plate 2.2). Most embryos were in the heart stage and a few had reached early torpedo stage at Day 9 (Plate 2.3). Torpedo-shape embryos with polarized axes and prominent cotyledons were observed at Day 12 (Plate 2.4). By Day 15 the embryos had grown considerably and completely filled the seed cavity. The embryo continued to grow in length between Day 15 and Day 30, but after Day 30 the length of embryo did not increase any further (Figure 2.4). All embryos under observation showed normal growth except one which ceased growth at the globular stage and had turned pale brown by Day 15, while other healthy embryos had passed the torpedo stage by then.

The suspensor of *L.perezii* consists of a spherical mass of cells in its early development, which kept growing until Day 12. The diameter of the suspensors by Day 12, around 0.2 mm, remained constant from Day 12 to Day 27. The suspensor had the appearance of a shallow cup (Plate 2.4). The suspensor started degenerating from Day 27 and its size had been considerably reduced to about 0.12 mm by Day 33 with the suspensor tissue developing a brown colour by then (Figure 2.5).

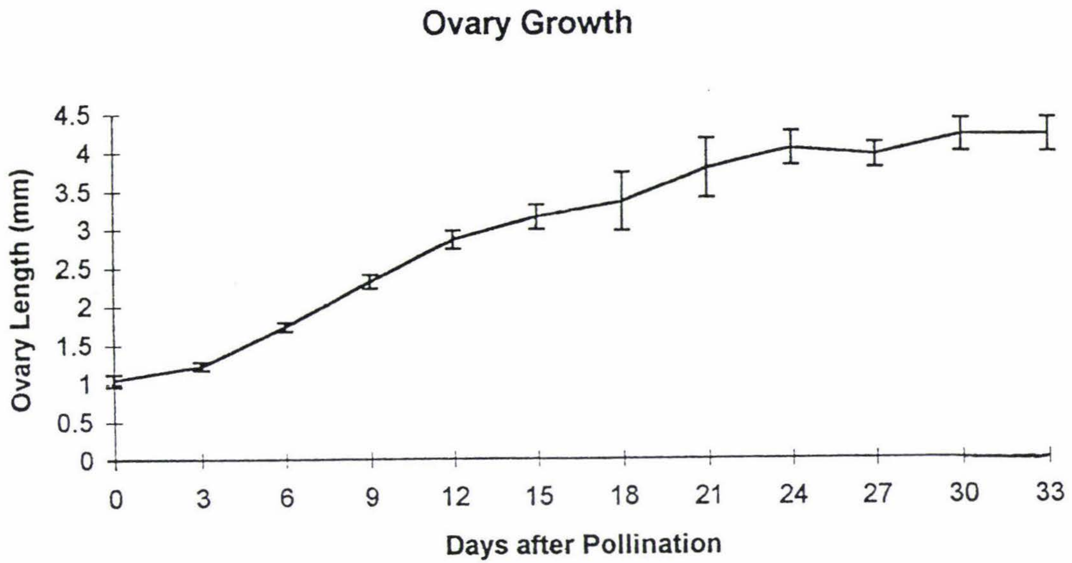

Figure 2.2 *L.perezii* ovary growth following conspecific compatible pollination. Day 0 was the day on which the pollination was carried out. The length of the ovary was about 1.04 mm at Day 0. The ovary stopped growing after Day 30, the length of the ovary was around 4 mm. Data points are mean of 30 measurements, bars are standard deviation.

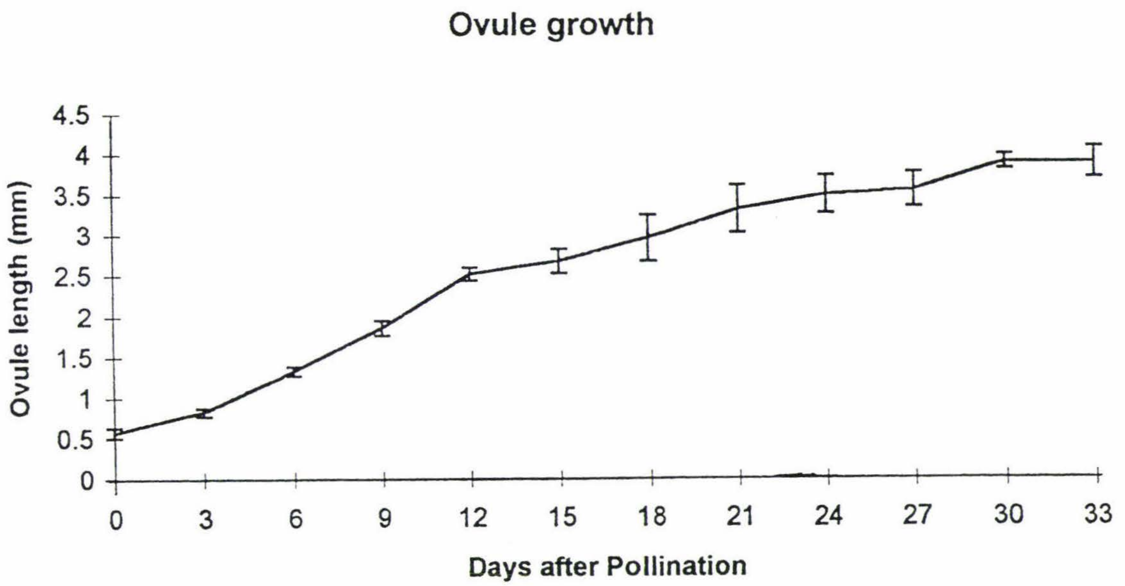

Figure 2.3 ***L.perezii*** **ovule growth after compatible conspecific pollination.** The length of the ovule at Day 0 was about 0.57 mm. The ovules had grown to their maximum size at Day 30, around 4 mm in length, no further growth occurred after that. Data points are mean of 30 measurements, bars are standard deviation.

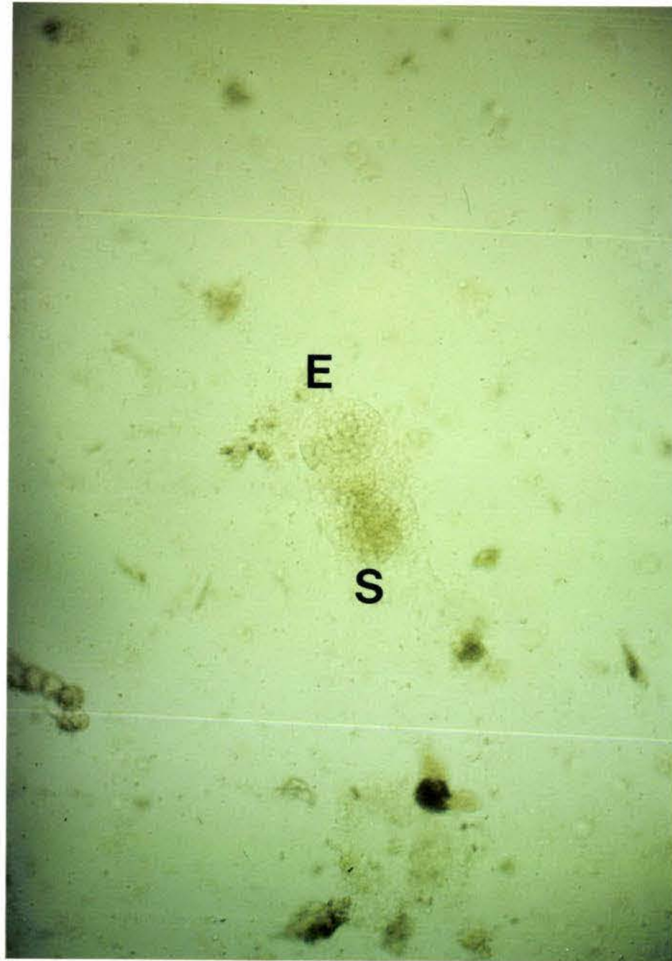

Plate 2.1 A three days old *L.perezii* embryo and a suspensor following compatible conspecific pollination. The embryo (E) was 0.04 mm in diameter and that of the suspensor (S) was 0.045 mm. The photo was microphotographed at x400.

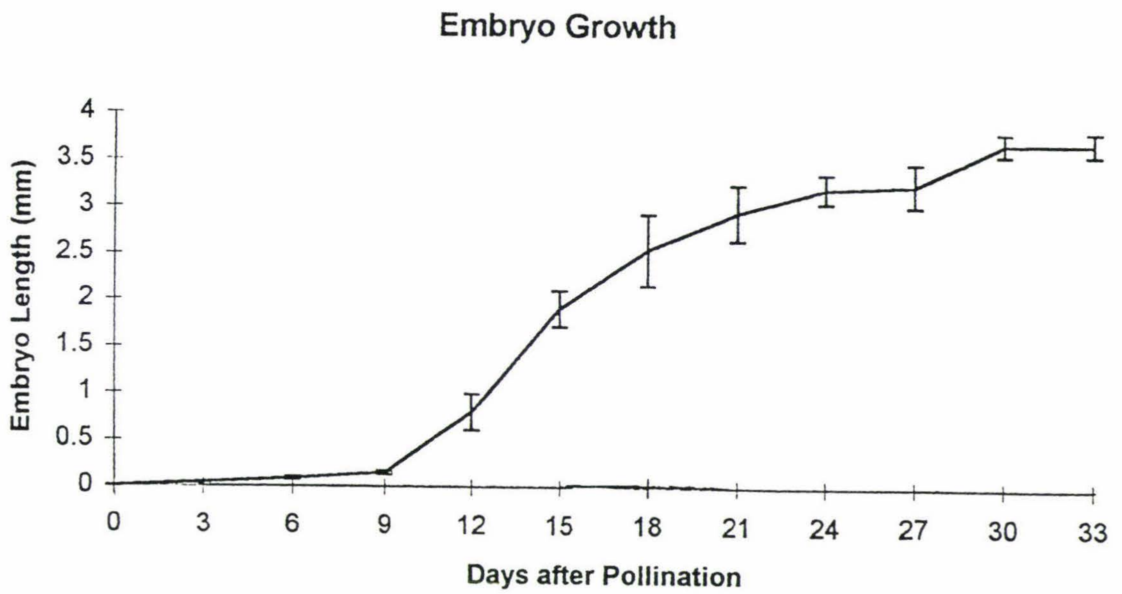

Figure 2.4 ***L.perezii*** **embryo growth after compatible conspecific pollination.** The pollination was carried out at Day 0. The embryos were in globular stage at Day 3, most embryos in heart stage at Day 9 and torpedo stage at Day 12. Data points are mean of 30 measurements and bars are standard deviation.

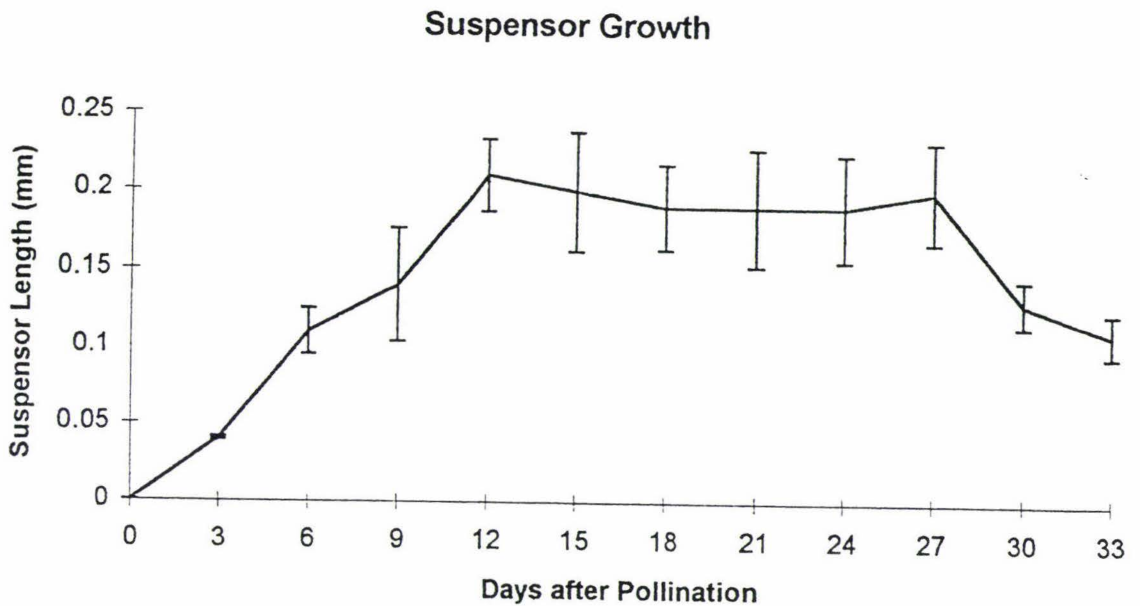

Figure 2.5 ***L.perezii*** **suspensor growth after compatible conspecific pollination.** Spherical suspensors were detectable at Day 3 and kept growing until Day 12. From Day 12 to Day 27 the size of suspensor remained the same and the suspensors showed a shallow cup shape. The suspensors started degenerating from Day 27, the size of suspensor had considerably been reduced by Day 33 and the colour of suspensor had turned into brown. Data points are mean of 30 measurements and bars are standard deviation.

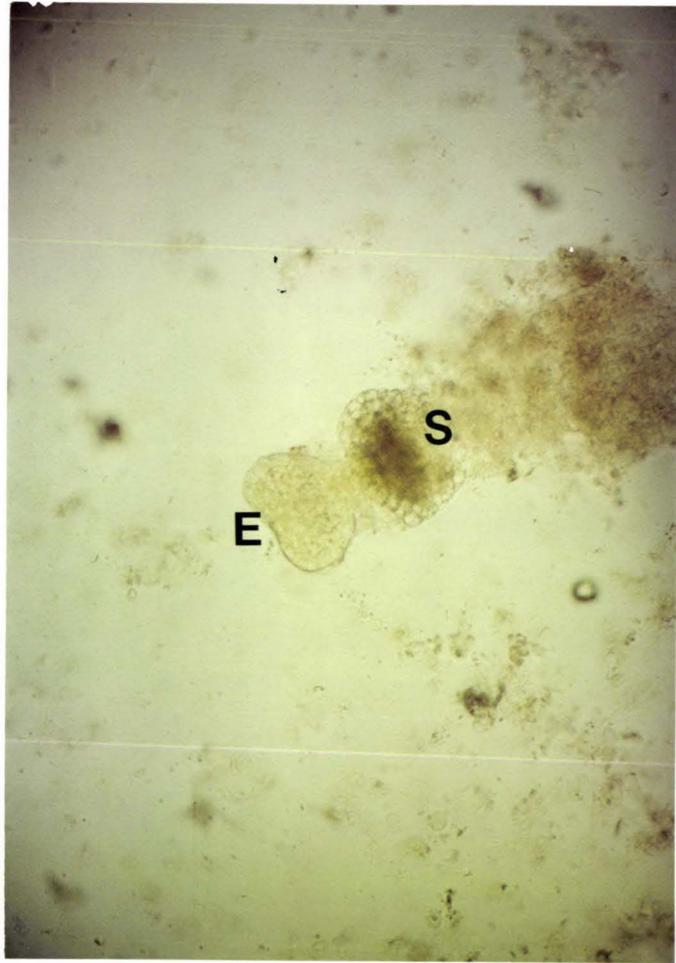

Plate 2.2 ***L.perezii* embryo and suspensor at Day 6 following conspecific pollination.** The embryo (E) was in its early heart stage, being about 1 mm in length. The suspensor (S) was spherical shape with a diameter about 0.1 mm and connected with the embryo by a short stalk. The photo was microphotographed at X100.

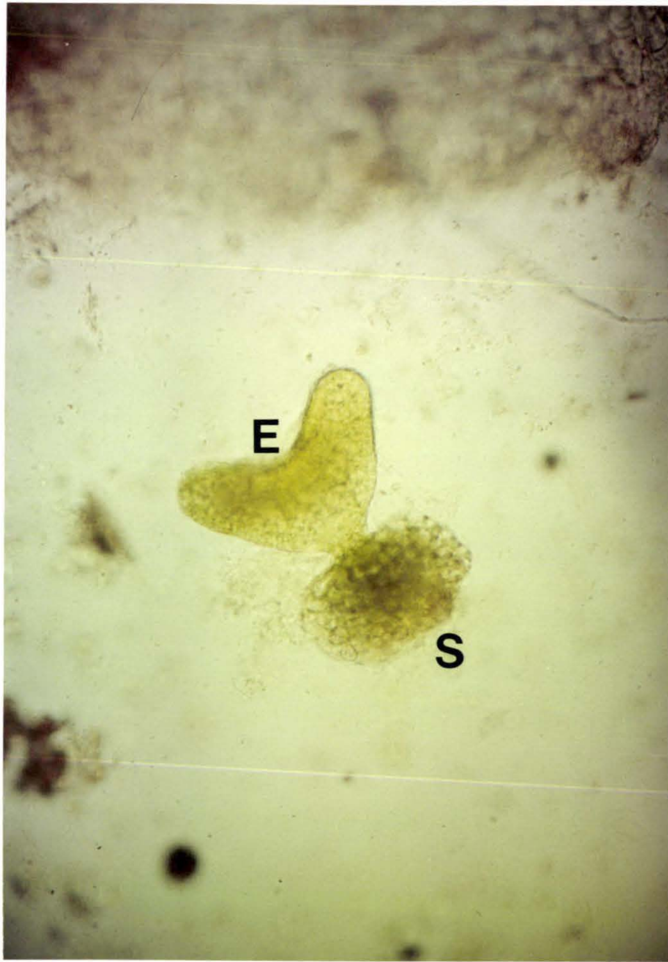

Plate 2.3 ***L.perezii*** **embryo and suspensor at Day 9 following conspecific pollination.** The embryo (E) was in heart stage, about 0.15 mm in length. The suspensor (S) was about 0.14 mm in length. The photo was microphotographed at X100.

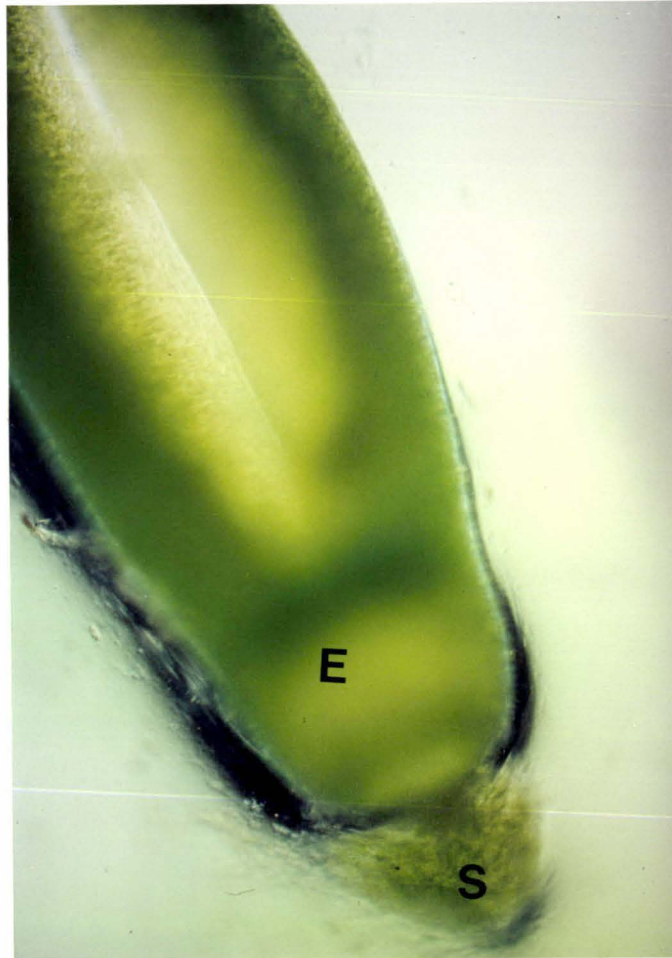

Plate 2.4 ***L.perezii* embryo and suspensor at Day 12 following conspecific pollination.** The embryo (E) was in torpedo stage about 0.8 mm in length. The suspensor (S) has a shallow cup shape. The photo was microphotographed at X100.

In *L.perezii* it appears that the development of the ovary, ovule, embryo and suspensor coordinate with each other. Figure 2.6 shows related growth of the ovary and the ovule assuming that "length" is a useful indicator of the state of growth and development of each structure.

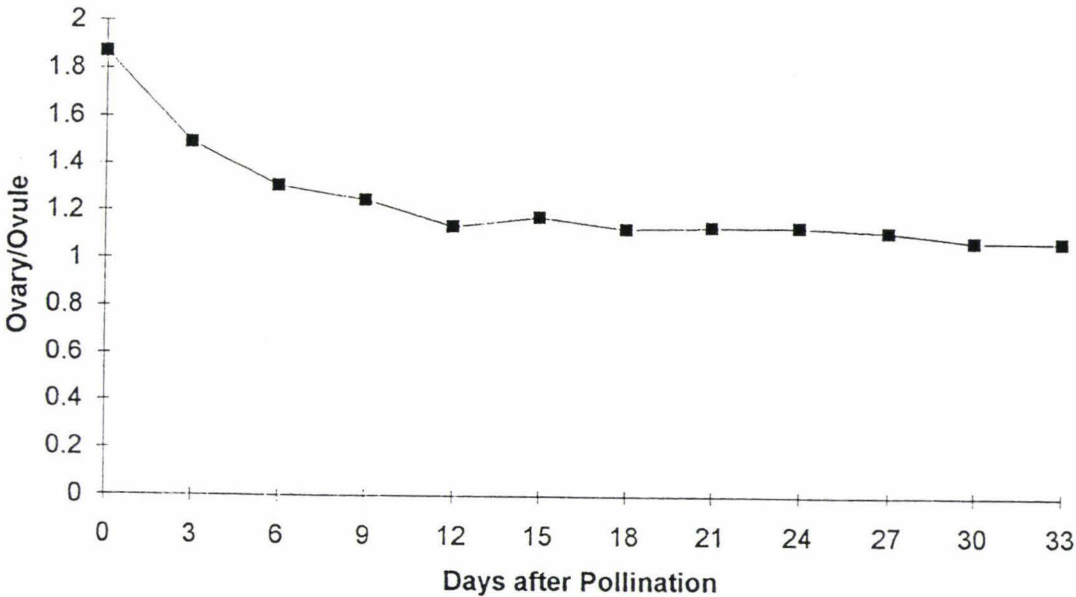

Figure 2.6 Concurrent growth of the ovary and ovule of *L.perezii*. The ovary / ovule ratio was calculated from means shown in Figure 2.2 and Figure 2.3.

The length of the ovary was about 1.9 times of that of the ovule at Day 0 (the day pollination was carried out). From Day 0 to Day 6 the growth of the ovule was faster than that of the ovary following pollination. By Day 6 the length of the ovary was about 1.3 times of the length of the ovule. After Day 9 growth rates of both ovary and ovule were similar and the ratio of ovary length to ovule length was constant, at around 1.2. Three developmental periods were recognised when growth rates of the ovule and the embryo were compared (Figure 2.7). Again, the length dimension is assumed to be

representative of developmental stage.

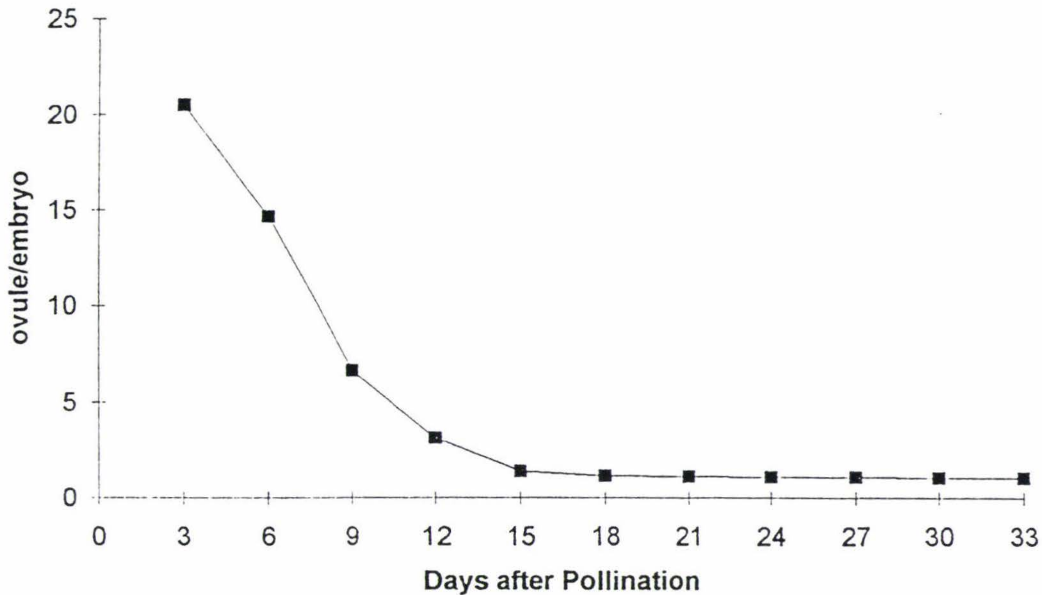

Figure 2.7 Concurrent growth of the ovule and embryo of *L.perezii*. The ovule / embryo ratio was calculated from means shown in Figure 2.3 and Figure 2.4.

The embryos were not detectable until Day 3 (First period). At Day 3 the length of the ovules was about 20 times of the embryo length. From Day 3 to Day 12 (second period) the growth rate of the embryo was greater than that of the ovule. By Day 15 the ratio of the ovule length to embryo length had reached 1.4. From Day 15 to Day 30 (Third period) this ratio of 1.4 remained constant until both reached the maximum size by Day 30. Figure 2.8 shows correlation growth of embryo and suspensor.

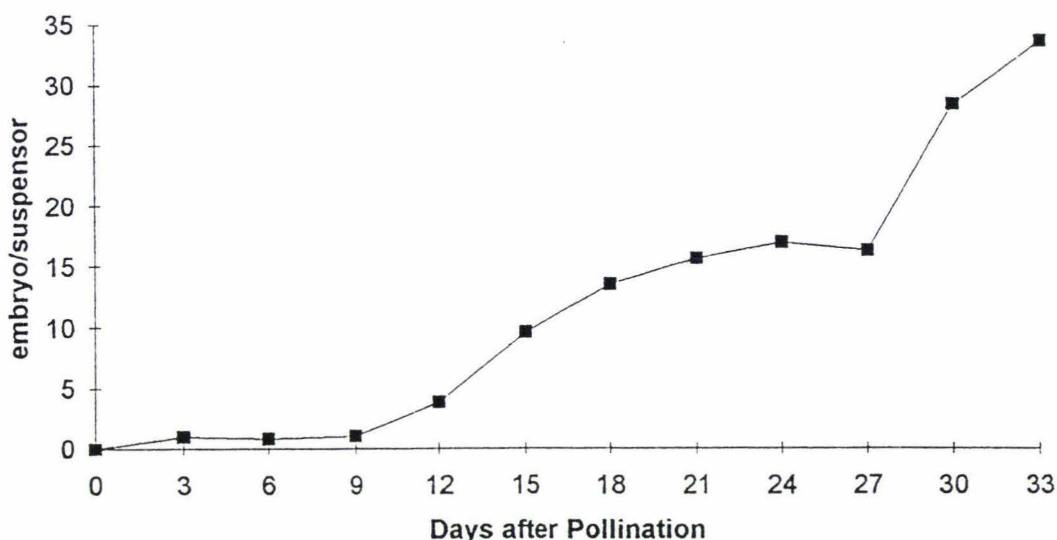

Figure 2.8 Concurrent growth of the embryo and suspensor of *L.perezii*. The embryo / suspensor ratio was calculated from means shown in Figure 2.4 and Figure 2.5.

There are three developmental phrases. From Day 0 to Day 9 (stage 1), the embryo and the suspensor grow at a similar pace; the ratio of the embryo length to the suspensor length is about one. During this time the embryo goes through the globular stage to the heart stage. In the period from Day 12 to Day 27 (stage 2), the embryo keeps growing while the suspensor does not grow at all. The embryo develops from the torpedo stage to being nearly mature over this period. After Day 27 (stage 3) the ratio of embryo size to suspensor size rises, which results mainly from the decreased size of the suspensor because the suspensor shrinks after day 27.

2.4 DISCUSSION

L.perezii ovule was found to be a peculiar type of megasporangium which is only seen in some members of Plumbaginaceae and Opuntia families (Maheshwari 1950). The micropyle of the ovule points upwards and the funicle is parallel to it. According to Maheshwari (1950) this peculiar type of ovule is formed as follows: the nucellar protuberance is at first in the same line as the axis, but the rapid growth on one side causes it to become anatropous. The curvature does not stop but continues until the

ovule has turned over completely so that the micropylar end once again points upwards. A term, circinotropous was given to this peculiar megasporogenesis (Maheshwari 1950). It is unknown whether fertilization occurred or not in the ovules of type 1 and type 2. Neither ovary nor ovule of type 1 grew after pollination. The ovaries and ovules of type 2 florets started to grow following pollination, but the growth terminated at different stages, which corresponded to the sizes of fertilized ovules between 3 and 12 days after pollination, but no embryos were found inside these enlarged ovules. It may be that fertilization did occur (ovary and ovule growth being induced by fertilization) and was followed by embryo abortion which must have happened before Day 3 since visible embryo was seen at Day 3 under stereo microscope. The growth of ovary and ovule of type 2 may also occur through parthenogenesis whether pollination occurs or not.
The normal development of the ovary, ovule and embryo of *L.perezii* showed correlated growth in terms of space and time (Figure 2.6, Figure 2.7 and Figure 2.8). The ovary, ovule and embryo sac are a complex integrated system (Batygina *et al.* 1992). Regulatory mechanisms must function during the development of ovary, ovule and embryo of *L.perezii* to ensure the coordination between them. Although the morphogenetic correlations between the embryo and the maternal tissues have been emphasized by many investigators, the mechanisms underlying it has not been clarified (Batygina *et al.* 1992, Vasilyeva *et al.* 1987, Vasilyeva *et al.* 1988).

After the first mitotic division of the zygote, the embryo differentiates into the embryo proper and the suspensor. The suspensor of *L.perezii* possesses a spherical appearance from Day 3 to Day 12 and then gradually becomes a shallow cup in shape. While a filamentous suspensor is one of the most familiar images of embryogenesis in angiosperms (Raghavan 1986), diversity does exist in terms of suspensor morphology, and the spherical suspensor described here for *L.perezii* is not necessarily surprising. The spherical structure of suspensor was also found in *Genisteae monosperma* (Orchidaceae family). In *Genisteae monosperma*, the suspensor appears as an enormous spherical structure virtually engulfing the small proembryo (Raghavan 1986). The function of the suspensor is often thought to be to push the growing embryo into the cavity of the embryo sac. However, an exclusive role for suspensors in the transportation of nutrients to embryo propers is often put forward (Murray 1988).

Although the data obtained in this study does not allow assessment of the nutritive role of the suspensor in the embryo development, it may be reasonable to suppose that the suspensor of *L.perezii* might play a role in nutrition transportation from maternal tissue to the embryo, or even provide some growth substances for the embryo development, since the suspensor of *L.perezii* accompanied the embryo for most of its development. The suspensor started to shrink as late as Day 27. The embryo stopped growing after Day 30.

Comparison of floral development of two species, *L.perezii* (as reported here) and *L.perigrinum* (as reported by Burge & Morgan 1993) after pollination revealed a number of differences. In *L.perigrinum,* although 74% if self-pollinated florets produced embryos, about 33% of embryos ceased development at the globular to heart stage of development, whereas only one of *L.perezii* embryo ceased growth at the globular stage and turned brown colour in all observations of this work. *L.perezii* therefore has a much higher percentage (63%) of embryos which developed normally than that of *L.perigrinum*. The present investigation did not extend to seed maturity, but it can be reasonably assumed that *L.perezii* would have higher seed set than *L.perigrinum*.

CHAPTER THREE THE DETERMINATION OF *L.PEREZII* POLLEN VIABILITY BY STAINING TECHNIQUES

3.1 INTRODUCTION

Pollen viability is important in agriculture and for plant breeders since pollen must be viable at the time of pollination for seed or fruit set to occur (Heslop-Harrison 1992). Heslop-Harrison and Heslop-Harrison (1984) have defined pollen viability as the competence of pollen to deliver two male gametes to the embryo sac. According to this criterion, a viable pollen grain must go through following events to deliver the gametes (Figure 3).

The pollen grain must:

----- attach to a stigma (a)

----- hydrate on the stigma (a)

----- germinate and produce a pollen tube (b)

A pollen tube must:

----- penetrate the stigma (b)

----- grow through the transmitting tract of the style (c)

----- penetrate the ovary and ovule (d)

----- enter the embryo sac and release the gametes

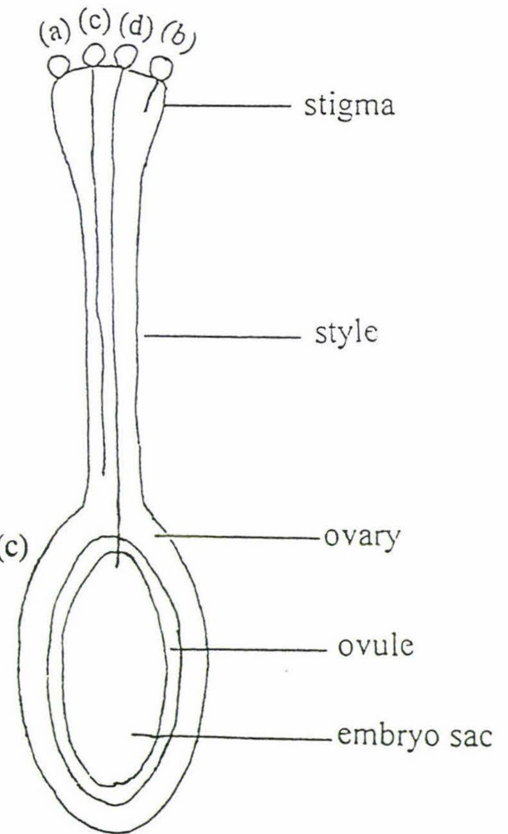

Figure 3

It is very difficult to assess all the stages above; nevertheless, the following basic methods are currently available for testing pollen quality.

----- determination of seed set or ovule development after pollination

----- determination of pollen germination and tube growth in living pistils (using decolorised aniline blue to detect callose in pollen tubes)

----- pollen germination *in vitro*

----- staining of pollen

(Knox and Williams 1986)

The first two methods appear to be the most reliable in terms of viability determination but may be complicated for some species by the effects of pistil screening mechanisms, such as self-compatibility or competition of pollen grains. The results of investigations on *Limonium* using the first two experimental approaches are described in chapter 1 and chapter 2. Pollen staining techniques and pollen culture *in vitro* are used as simple and fast methods to assess pollen viability (Knox and Williams 1986).

Most of the available staining techniques measure particular cellular features that are considered characteristics of a living cell. Alexander's stain was demonstrated to stain both aborted and non-aborted pollen of most of the angiosperms and the spores of gymnosperms differentially (Alexander 1969). The stain mixture contains malachite green which stains the pollen wall green, acid fuchsin which stains the cytoplasm red, and orange G, a background stain for nuclear dyes. The aborted pollen should appear green, since it only has a cell wall, while non-aborted pollen appear red with a thin green layer outside.

Fluorochromatic reaction (FCR) is the most widely used test of pollen viability. It actually depends on pollen enzyme activity and the integrity of the pollen vegetative cell membrane (Heslop-Harrison and Heslop-Harrison 1970). FCR was first proposed by Rotman and Papermaster (1966). They found that certain non-fluorescent fatty acid esters of fluorescein were hydrolysed in living mammalian cells to give fluorescein. Fluorescein diacetate is commonly used for assessing pollen viability. The non-polar fluorescein diacetate is non-fluorescing and readily penetrates the membrane of the pollen vegetative cell where endogenous pollen esterase enzymes cleave fluorescein diacetate and releasing fluorescein. Fluorescein traverses membranes with great difficulty and so accumulates intracellularly, where it can be detected by fluorescence microscopy. In this study, Alexander's stain and the FCR test were used to assess *L.perezii* pollen quality.

3.2 MATERIALS AND METHODS

Limonium perezii pollen was used in the staining experiments in this study. *Limonium perezii* plants were grown in a glasshouse at the Plant Growth Unit of Massey University. The temperature of the glasshouse was controlled within the range of 20^0C-25^0C. Freshly opened *L.perezii* flowers were collected at 10.00 in the morning. The flowers were put in an open Petri dish until the pollen was needed.

3.2.1 Alexander's Stain

The Alexander's stain (Alexander 1969) was made by mixing the following components in the order given:

95% alcohol	10 ml
Malachite green	10 mg (produced by George, T. Gurr Ltd. product No: 18924)
Distilled water	50 ml
Phenol	5 g
Chloral hydrate	5 g
Acid fuchsin	50 mg (BDH chemical Ltd. Product No:34319 2E)
Orange G	5 mg (George T.Gurr Ltd. Product No: 18458)
Glacial Acetic acid	1 ml

The amount of glacial acetic acid depends upon the thickness of pollen wall. *L.perezii* pollen requires 1 ml glacial acetic acid in the mixture to give the best staining effect (unpublished).

3.2.2 Fluorescein Diacetate Medium

Fluorescein diacetate was dissolved in acetone, 10 mg in 5 ml. This stock solution was added dropwise to 100 ml medium which contained 55% sucrose, 1.6 mM $Ca(NO_3)_2$, 2 mM H_3BO_3, until the first persistent milkiness appeared.

3.2.3 Observations

Both A and B types of *L.perezii* pollen were used in this study. A drop of Alexander's stain and fluorescein diacetate medium were mounted on the slides respectively. Pollen was dispersed into the stain drop directly by holding the anther stalk, and then covered

with a coverslip a few minutes later. The pollen in Alexander's stain was observed using a compound microscope at X400 magnification while pollen in Fluorescein diacetate medium was observed under the fluorescence microscope using UV light with a ultraviolet filter (ZEISS fluorescence microscope. UV light 300-400 nm, Exciter filter G365).

The slides were scanned in a number of random traverses and at least 200 grains were scored for each preparation.

3.3. RESULTS

3.3.1 Alexander's stain

L.perezii pollen grains are spheroidal shape with three germ pores. With Alexander's stain, aborted and non-aborted pollen grains were readily distinguished under the compound microscope (Plate 3). Aborted pollen grains appeared green indicating no cytoplasm inside the pollen wall. The red colour was dominant in non-aborted pollen grains indicating the existence of cytoplasm. The sizes of aborted and non-aborted pollen were seen to be different. Thirty non-aborted and 25 aborted pollen grains were randomly chosen and measured. Non-aborted pollen was larger, being $58.8 \pm 2.6\mu$ in diameter than aborted pollen, being $38.3 \pm 5.8\mu$ in diameter.

Pollen from different *L.perezii* plants were found to show different proportions of non-aborted pollen (Table 3.1).

Table 3.1 *L.perezii* pollen viability % of four plants with Alexander's stain.

	Plant 1	Plant 2	Plant 3	Plant 4
Total	301	300	298	301
Non-aborted	298	291	260	296
%	99%	97%	87%	98.3%

Plant 1-4 are cob plants with A pollen. Values shown are the mean of three replicates. pollen was collected at 10.00 am at the same day from similarly aged inflorescences which belonged to four different plants. Pollen was scored under a compound microscope at x100 magnification.

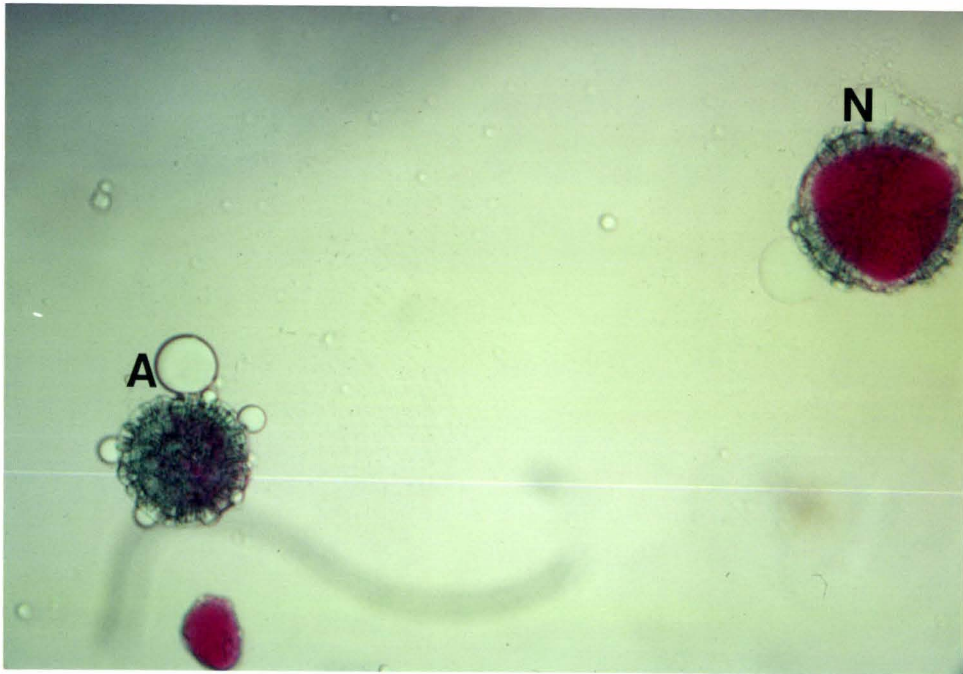

Plate 3 ***L.perezii* pollen grains stained with Alexander's stain.** Non-aborted pollen (N) has red cytoplasm and a thin green layer of cell wall. Aborted pollen (A) is completely green in colour, since it only has a cell wall and it is smaller. The photo was microphotographed at X160 magnification.

It was found in this study that the percentage of non-aborted pollen was influenced by the age of the inflorescence. *L.perezii* plants may produce one or two inflorescences at a time. The freshly opened flowers last only one day. They open at early morning (The time of flowering is influenced by temperature, humidity and the time of sunrise) and then the white petals curl up at the following morning, but the purple sepals remain open for months. Normally, an inflorescence produces abundant flowers for about 2-3 weeks from the initiation of flowering, and then it produces fewer and fewer flowers.

One inflorescence was monitored to study the effect of inflorescence age on pollen viability. Results are shown in Table 3.2. Pollen grains taken from the inflorescence in its first week of flowering showed 97% non-aborted pollen with Alexander's stain. At the 6th week counted from the first day of flowering, the percentage of non-aborted pollen fell down to 78%.

Table 3.2 *L.perezii* non-aborted pollen percentage with Alexander's stain at first week and sixth week from initiation of flowering.

	First Week	Sixth Week
Non-aborted %	97%	78%
4 pores pollen / total non-aborted	0	4.2%

L.perezii type A pollen was used. The results shown here came from the single experiment in which approximately 50 pollen grains were assessed at each time.

The pollen collected from the 6-week old inflorescence not only showed decreased non-aborted pollen percentage but also showed diversity in pollen grain morphology in terms of numbers of pollen germ pores. *L.perezii* pollen grains normally have 3 germ pores but in the pollen from the aged inflorescence (in this case, six-week old), a low frequency of abnormal pollen grains with 4 germ pores or even 5 germ pores was found. In a total of 332 non-aborted pollen grains which appeared red with Alexander's stain, 4.2% pollen grains had 4 pores (Table 3.2) and one had 5 pores. This

phenomenon was not found in the observation of pollen grains collected from the inflorescence in its first week flowering.

3.3.2 FCR TEST

In fluorescein diacetate, the *L.perezii* pollen grains can be classified into three groups: pollen with strong fluorescence, the pollen with much fainter fluorescence and the pollen with no fluorescence at all. If the pollen grains with strong fluorescence were classed as viable ones, the pollen grains with no fluorescence were clearly able to be confidently classed as nonviable. It is hard to decide however whether to count faint fluorescing pollen into viable or non-viable groups. In this study the faint fluorescing pollen was counted into non-viable group.

The sucrose concentration in fluorescein diacetate medium must be adjusted to suit the water potential of the pollen to minimize the bursting of pollen. A series of sucrose concentrations of between 30% and 60% was used to assess water potential requirement of the *L.perezii* pollen. Table 3.3 shows the *L.perezii* pollen rupture percentage in the sucrose media.

Table 3.3 *L.perezii* pollen rupture % in a series of concentrations of sucrose media.

sucrose	rupture %
30%	35.2%
35%	29.4%
40%	22.2%
45%	18.5%
50%	10.1%
55%	1.5%
60%	0%

Sucrose was the only constituent in the liquid medium. *L.perezii* type A pollen was used.

L.perezii pollen rupture percentage decreased as sucrose concentration increased. The pollen rupture percentage was 35.2% in 30% sucrose liquid medium and that dropped

to 1.5% in 55% sucrose medium. Therefore it is necessary to use relatively high sucrose concentration in FCR staining procedure for assessing *L.perezii* pollen viability. In this study 55% sucrose was chosen, although there was still 1.5% pollen rupture. When 60% sucrose was used for the staining procedure, pollen grains did not show fluorescence at all.

The rupture percentage was found to be influenced by the way the cover glass was mounted on the slide. Pressing the coverslip was found to accelerate pollen rupture.

When Alexander's stain was compared with FCR, Alexander's stain tended to give a higher percentage of non-aborted pollen than FCR (Table 3.4).

Table 3.4 Comparison of Alexander's stain with FCR in estimating *L.perezii* pollen viability (%).

	Alexander's stain	FCR
Plant 1	96.7%	76%
Plant 2	99.3%	83.6%

L.perezii type A pollen was used. The values shown here are the average of 3 replicates.

3.4 DISCUSSION

Generally, *L.perezii* plants have a very high percentage of non-aborted pollen when assessed with Alexander's stain. The term "viable pollen" is not used here because this stain does not give information on whether the contents of the pollen grain are living on not. Although Alexander's stain and FCR gave different results for the same pollen, there was a correlation between the two results (Table 3.4). Plant (1) showed higher viability percentage than plant (2) with Alexander's stain, this was also shown by the FCR result. It may indicate that both stains do reflect the viability to some extent. However, Alexander's stain only tells whether the pollen is empty or not, since it stains both pollen cell wall and protoplasm differentially. FCR, on the other hand, tests two properties of the pollen vegetative cell, the esterase activity and the possession of an intact plasmalemma capable of retaining the released fluorescein. FCR should provide better correlation of pollen stainability with pollen viability, since it is widely accepted that the state of the membrane of the vegetative cell is one of the principal determinants

of germination capacity (Shivanna and Heslop-Harrison 1981).

For most plant species the outer wall of pollen contains one or more apertures which are designated areas for pollen germination and tube growth. These apertures are called germ pores. Although *L.perezii* pollen grains normally have 3 germ pores, the 4 pores or 5 pores pollen were found in flowers produced by aged inflorescence. It seems that the appearance of 4 pores or 5 pores pollen is correlated with aging processes of the inflorescence, since 4 pores or 5 pores pollen were not found in flowers produced by first-week inflorescece. There appear to be no reports in the literature concerning this phenomenon. The phenomenon was not investigated further in this study.

FCR test requires integrity of pollen cell membrane (Heslop-Harrison and Heslop-Harrison 1970), which means the osmotic value of FCR staining medium must match with water potential of pollen grains in order to prevent pollen rupture. It was found that 15% sucrose provided a satisfactory match for most plant species, though some plants, such as grass pollen need 30% sucrose in the medium (Knox 1979), but even at such sugar concentration *L.perezii* pollen still bursts (Table 3.3). There were still 1.5% *L.perezii* pollen grains ruptured in 55% sucrose medium. When sucrose concentration was raised to 60% *L.perezii* pollen did not rupture. However 60% sucrose concentration can not be used in FCR staining test, because the pollen grains did not show fluorescence at all. This may be because the pollen could not take up fluorescein diacetate molecules in 60% sucrose medium. It is also possible that such high sucrose concentration may somehow interfere pollen esterase enzyme activity, therefore fluorescein diacetate molecules stay intact and no fluorescein is released.

CHAPTER FOUR *L. PEREZII* POLLEN GERMINATION AND POLLEN TUBE GROWTH *IN VITRO*

4.1 INTRODUCTION

In vitro germination of pollen is widely used for viability testing, under the general assumption that pollen that germinates and produces a tube *in vitro* is likely to do so *in vivo*, and to fertilize the egg (Heslop-Harrison 1992).

The study of pollen germination and tube growth *in vitro* has developed rapidly. The pollen grains from many species have been demonstrated to germinate successfully in *in vitro* systems (Potts and Marsden-Smedley 1989; Sedgley 1974; Cheng and McComb 1991).

Pollen of angiosperms is bi- or trinucleate at the time of dehiscence. Binucleate grains contain one generative and one vegetative nucleus; the generative one undergoes a mitotic division during pollen tube growth. The trinucleate pollen grains contain one vegetative and two generative nuclei, the second mitosis having occurred already prior to anthesis (Hoekstra and Bruinsma 1975). Brewbaker (1967) surveyed 2,000 species: 30% shed their pollen as trinucleate cells. According to Brewbaker (1967), trinucleate species tend to have "dry" stigmas and binucleate species often have "wet" stigmas. The "dry" type and "wet" type are differentiated by whether or not the stigma surface carries an exudate. The "dry" stigma surface has little or no secretion at maturation, while the "wet" stigma secretes exudate which is continuous with the secretion produced by the cells of the stylar canal or transmitting tissue. *Limonium* species belong to trinucleate taxa possessing a "dry" stigma surface (Brewbaker 1967).

Generally, binucleate pollen grains germinate easily *in vitro* and show a high percentage of germination, while trinucleate pollen grains are difficult to germinate *in vitro*. If they do so, normally the percentage is low and the pollen tubes are short (Bar-Shalom and Mattson 1977; Johri and Shivanna 1977; Vasil 1960).

The reasons for poor germination of trinucleate pollen grains *in vitro* are often attributed to two factors: a special mode of hydration or a deficiency of some specific chemical factors (Mulcahy and Mulcahy 1988; Bar-Shalom and Mattson 1977). The hydration of trinucleate pollen often requires water-restricting conditions. In liquid medium trinucleate pollen grains may either burst or leak severely leading to failure of

germination (Bar-Shalom and Mattson 1977). The pollen of *America maritima*, for example, tends to burst in solutions having less than 70% sucrose but germinates in solid medium (Bar-Shalom and Mattson 1977).

Some trinucleate pollen needs not only solid medium, but also a high osmotic potential in the medium. Agar and high concentration of sucrose have been used to provide ideal culture conditions and to increase osmotic value. However a high concentration of sucrose, such as 70%, is a very unlikely situation on the stigma (Bar-Shalom and Mattson 1977). Besides, a high concentration of sucrose might interfere with the utilization of other substrates in an *in vitro* system for pollen germination (Zhang and Croes 1982).

Polyethylene glycol (PEG) has proved to be a good substitute as an osmoticum. It is not metabolized in pollen and can provide water potentials over a wide range (Zhang and Croes 1982; Subbaiah 1984; Read *et al.* 1993). It was reported that *Petunia hybrida* pollen germination and tube growth were much better in medium with PEG than the medium with sucrose as the major component (Zhang and Croes 1982). Cashew (*Anacardium occidentale* L.) pollen produced the maximum germination and tube growth in medium containing 30% PEG and 20% sucrose (Subbaiah 1984).

The cellophane method is another way to cope with recalcitrant pollen germination. La Cour and Faberge (1943) were the first people to use small squares of non-water-proof cellophane floating on a drop of sugar solution to overcome pollen sinking and poor germination. Narasimhan (1963) combined cellophane with filter paper to support sugarcane pollen germination. Alexander and Ganeshan (1989) further developed the cellophane method. They inserted cellophane between the top and the second sheets of a booklet made of filter papers which were soaked in nutrient medium. The top filter was then removed to expose cellophane on which pollen was dusted. Pollen from various horticultural species was reported to germinate well with this method.

It has been known for many years that boron is an essential ingredient in media for pollen germination *in vitro* of most plant species. Cashew (*Anacardium occidentale* L.) pollen does not germinate in the medium without boric acid, though sucrose and calcium both exist in the medium (Subbaiah 1984). More than 50% of the pollen germinated with the addition of boric acid to the medium (Subbaiah 1984). The effect of boron is unique and far surpasses the effect of any known hormone or other chemical substance

on pollen germination and tube growth (Vasil 1960). The effect of boric acid in the medium for *L.perezii* pollen germination was examined in this study.

Calcium is widespread and abundant in most flowering plant tissues, averaging around 1.5% of the dry weights of leaves, 1.2% in shoots and 0.2% in seed. Pollen grains are low in calcium content, averaging about 0.03% (Brewbaker and Kwack 1963). Exogenous calcium is necessary for pollen germination and tube growth. The essential role of the calcium ion in pollen germination and tube growth *in vitro* was confirmed in 86 species representing 39 plant families (Brewbaker and Kwack 1963). Calcium is required for the germination of corn pollen (Cook and Walden 1967). The optimum calcium level for growth of pollen tubes of *Tradescantia* was found to be about 5×10^{-4} M (Steer and Steer 1989). The optimum concentration may differ for germination of pollen from different genera or species.

Trinucleate pollen germination usually needs not only boron and calcium as mineral nutrients in the medium but also some organic nitrogenous supplements as well. For example, casein hydrolysate was reported to stimulate many plant species' pollen germination, such as *Silene dioica* and *Zea mays* (Mulcahy and Mulcahy 1988).

Some plant growth substances have been demonstrated to influence *in vitro* pollen germination and tube growth. Indole-3-acetic acid (IAA) and gibberellic acid (GA_3) increased Date Palm (*Phoenix dactylifera* L.) pollen germination rate as well as tube elongation to 3-4 times that of control (Asif *et al.* 1983). Search and Stanley (1970) demonstrated that ethylene gas at the concentration of 10-1000 ppm enhanced *Pyrus communis var* "Winter nelis" pollen germination rate and tube elongation.

It was found that the modes of the hydration of binucleate pollen and trinucleate pollen were different. Prehydration treatment was found to be necessary for pollen germination of some trinucleate species. For example, *Zea mays* pollen required prehydration in a saturated atmosphere for 60 min at 25°C for pollen germination (Mulcahy and Mulcahy 1988).

Temperature is another factor which influences pollen germination and tube growth both *in vitro* and *in vivo*. The influence of temperature on germination was demonstrated with pecan (*Carya illinoensis*) pollen. A high temperature (75°C) was shown to reduce *Brassica juncea* pollen viability completely. The pollen grains failed to germinate on the stigma (Rao *et al.* 1992).

Limonium vulgare is the only *Limoniun* species for which germination data *in vitro* is available (Bar-Shalom and Mattson 1977). *L.vulgare* pollen germinated on a medium with 35% sucrose, 2% agar, 100 ppm boric acid, 100 ppm potassium nitrate, 200 ppm magnesium sulphate, 300 ppm calcium nitrate. The percentage germination was 24%. In the paper, the author surveyed 58 trinucleate species for pollen germination. Unfortunately, much detailed information about *L.vulgare* pollen germination and tube growth was not given. *L.perezii* pollen does not germinate on the medium which was used for *L.vulgare* pollen by Bar-Shalom and Mattson (1977) (unpublished data).

The aims of this study were to develop a suitable medium for *L.perezii* pollen germination and tube growth, and to explore physiological features of *L.perezii* pollen germination. In this study, various medium components commonly used in pollen germination media were tested. The effects of IAA, GA_3 and ethylene were also examined. As humidity and water uptake are of upmost importance for pollen germination *in vitro*, the mode of hydration of *L.perezii* pollen was studied. The effect of temperature on *L.perezii* pollen germination and tube growth was also tested.

4.2 MATERIALS AND METHODS

4.2.1 Plant Material

Mature *L.perezii* pot plants provided by New Zealand Institute for Crop and Research, Levin, grew in a glasshouse at the Plant Growth Unit of Massey University from February 1994 to January 1995. The day temperature of the glasshouse was controlled to not exceed 25°C or to go lower than 20°C at night. The plants were watered once a day in the early morning. Freshly opened *L.perezii* flowers were collected at 10.00 am on fine days. The flowers were kept in an open Petri dishes until the pollen was needed. Normally, the time period for flowers sitting in the Petri dishes did not exceed one hour.

4.2.2 The Basic Germination Medium

As none of the existing media for pollen germination of other plant species was suitable for *L.perezii*, a new medium was developed. This medium contained 20 mM boric acid,

6 mM calcium nitrate ($Ca[NO_3]_2 4 H_2O$ BDH chemicals Ltd. product No: 10305), 0.1% casein hydrolysate, 7% sucrose and 23% polyethylene glycol-20,000 (PEG-20,000 BDH chemicals Ltd. product No: 29864) . The fresh medium was prepared with distilled water and always made just before use.

4.2.3 Semi-solid Agar Medium

L.perezii pollen did not germinate on this semi-solid medium which was used for exploring the osmotic requirements for *L.perezii* pollen germination. The agar medium was prepared with 2% agar, 100 ppm boric acid, 100 ppm calcium nitrate and sucrose from 10%, 20%, 30%, 40%, 50%, 60%, 70%. The agar medium was heated in microwave oven and then rolled on to the slide with a glass rod. When the medium had set, the pollen was dusted over it. The slide was put in an open Petri dish and incubated at 25°C.

4.2.4 Dialysis-tubing & Filter-paper Supporter

Dialysis tubing and filter paper were used for *L.perezii* pollen germination. The method is similar to the booklet procedure of Alexander and Ganeshan (1989) described above. No.1 Whatman filter paper was cut into 40-mm squares and SERVAPOR dialysis tubing (made by SERVA company, Product No: 44145 purchased from Scientific Supplies Limited, Panmure, Auckland) was cut into approximately 40-mm square pieces. Seven filter-paper sheets were put on a pile, the dialysis tubing sheet was inserted between the top and second filter-paper sheets and then the whole pile was stapled together to form a "booklet" which was placed in the basic germination medium (see section 4.2.2) for 15 minutes to allow the dialysis tubing sheet to absorb the medium thoroughly. After 15 minutes, the top filter-paper sheet was removed to expose the dialysis-tubing sheet. The dialysis-tubing was blotted dry with a piece of filter paper, and then the pollen was dusted on to the dialysis-tubing sheet with a brush. After that the booklet was placed in a Petri dish and then the Petri dish with the cover on was put in an incubator at 25°C.

4.2.5 Pollen tube growth rate

The pollen tube growth was assessed at 10, 20, 30, 40, 50 and 60 minutes after the pollen culture started. *L.perezii* pollen was supported by dialysis-tubing and filter-paper

system and cultured in the basic medium.

4.2.6 Treatments

Chemical treatments: For increasing pollen germination rate and stimulating tube growth, the effect of two mineral salts: manganese sulphate, copper sulphate and three plant hormones: IAA (BDH chemicals Ltd. product No: 28558), GA_3 (Sigma Chemical Company, product No: G3250) and ethylene (NZIG Special Gases, gas code: 160) were tested. The concentrations used are listed below.

$MnSO_4$	1, 0.1, 0.01, 0.001, 0.0001	(mM)
$CuSO_4$	100, 50, 30, 10	(mM)
IAA	1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6},	(mM)
GA_3	1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4},	(mM)
Ethylene	100, 10, 1, 0.1	(ppm)

$MnSO_4$, $CuSO_4$, IAA and GA_3 were added into the basic medium respectively at these concentrations. Because IAA and GA_3 are barely soluble in water, the medium was prepared in the following sequence. Firstly, boric acid, calcium nitrate and casein hydrolysate were dissolved in distilled water, then sucrose and polyethylene glycol were added. The solution was stirred well until it was clear, then IAA or GA_3 was added into this solution.

For the ethylene treatment, ethylene gas and 600 ml air-tight containers were used. *L.perezii* pollen supported by dialysis-tubing and the filter-paper booklet which had soaked in the basic medium was placed inside the ethylene gas containers. The ethylene concentrations of 100, 10, 1, 0.1 ppm were obtained in the following procedure. Six ml pure (100%) ethylene gas was injected into a 600 ml air-tight container with a 10 ml syringe after 6 ml air was removed, making up 10,000 ppm ethylene gas. It was then diluted in following way. Ethylene gas volumes of 6 ml and 0.6 ml were taken from the 10,000 ppm ethylene gas container and injected into two 600 ml containers making up 100 ppm and 10 ppm ethylene respectively, and then 6 ml and 0.6 ml gas were removed from the 100 ppm ethylene container and injected into another two 600

ml containers making up 1 ppm and 0.1 ppm ethylene. The same amount of air was removed from the containers before ethylene gas was injected.

Prehydration: Three relative humidity conditions, 100%, 90% and 36% were used in the prehydration treatment. 90% RH was prepared by 32:68 (V/V) glycerine and water, 36% RH by 84:16 (V/V) glycerine and water (Sakunnarak 1992) and 100% RH was assumed in a saturated atmosphere.

The pollen grains were dusted onto a piece of non-sticky paper which was placed in a small beaker. The beaker was then put in the humidity box. Pollen was prehydrated in a 36% RH and 90% RH at 25°C for 0.5 hours and in 100% RH, for 1, 2, 4, hours. The basic medium was used for assessing prehydration treatment. After the required incubation period in humidity boxes, the pollen was transferred onto dialysis-tubing sheet supported by filter-paper soaked in basic medium, to allow germination.

Temperature: To find out the effect of temperature on germination of *L.perezii* pollen in the same medium, the pollen was incubated at different temperature conditions : 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C. The basic medium and dialysis-tubing and filter-paper system were also used in this treatment. The only variate was temperature.

4.2.7 Data Collection

For each treatment pollen was incubated for 2 hours (Two hours was found to be sufficient for *L.perezii* pollen germination and maximal tube growth in preliminary experiments.) and then the dialysis-tubing sheet was cut off and mounted on the slide. The pollen on the dialysis-tubing was then stained with Alexander's stain for easy pollen tube counting. For scoring percentage germination, the stained dialysis-tubing sheet was scanned from side to side. Counts of more than 200 pollen grains were made using a compound microscope at a magnification of x100, except for pollen culture on semi-solid agar medium in which fewer than 200 pollen grains were counted. A pollen grain with a tube length that was equal to or longer than the diameter of the pollen grain was considered germinated. For measurement of tube length, 30 pollen tubes were selected randomly and measured under the compound microscope at a magnification of X400. The experiments were run in three replicates and the results were averaged. Pollen was photographed under a compound microscope after staining with Alexander's stain.

4.3 RESULTS

Because *L.perezii* pollen did not germinate on the medium which was used by Bar-Shalom and Mattson (1977) for *L.vulgare* pollen germination, Murashige and Skoog medium (DE Fossard 1983) and Nitche's medium (DE Eossard 1983) were tried next for *L.perezii* pollen, but none of them was suitable for *L.perezii* pollen. The common phenomenon of *L.perezii* culture on these media was pollen rupture and leakage. This suggests that the osmotic value of the media may not be suitable for *L.perezii* pollen.

The semi-solid agar medium with a series of sucrose concentrations was used to explore osmotic requirements of *L.perezii* pollen germination. Table 4.1 shows the pollen rupture rates in the agar medium.

Table 4.1 *L.perezii* pollen rupture rates in agar media

Sucrose	10%	20%	30%	40%	50%	60%	70%
Total	105	96	80	77	97	90	88
Burst	105	96	23	8	3	0	0
Burst %	100%	100%	28.7%	10.3%	3%	0%	0%

L.perezii type A pollen was used. The medium contains 2% agar, 100 ppm boric acid, 100 ppm calcium nitrate and sucrose. Pollen culture was incubated at 25^{0}C. Pollen culture was observed using compound microscope with X400 magnification. The values in the table were the results of a single experiment.

The proportion of pollen grain rupturing was inversely related to osmotic concentration of the medium. As sucrose concentrations of 10% and 20%, all the pollen ruptured. As the sucrose concentration was increased, pollen rupture reduced, but even at 50% sucrose, there was still 3% pollen rupture. At 60% and 70% sucrose, pollen did not burst, but it was still leaking as shown by exudate around the grains in the medium. Not one *L.perezii* pollen grain germinated in this medium, so the semi-solid agar medium is not suitable for *L.perezii* pollen germination, no matter what sucrose concentration. For *L.perezii* pollen germination dialysis-tubing & filter-paper supporter was developed to overcome pollen bursting and leakage. *L.perezii* pollen grains were dusted onto

dialysis-tubing sheet which was supported by filter-papers which had been soaked in the basic medium for 15 minutes. *L.perezii* pollen germinated on dialysis-tubing sheet in the basic medium.

Before using dialysis-tubing sheet, cellophane was assessed in this study. Cellophane wrappers were purchased in a book shop, made by two companies: Hallmark and Murfett Regency. The use of cellophane sheets was exactly the same as dialysis-tubing. The cellophane sheet was inserted between the top and the second sheet of seven filter-paper to make a "booklet" which was then soaked in basic medium. After that the top filter-paper was removed and the cellophane sheet was exposed. Pollen was dusted onto the cellophane sheet after it had been blotted dry with a piece of filter paper. *L.perezii* pollen did not germinate on cellophane sheet of Hallmark company, but germinated on the cellophane of Murfett Regency. The pollen tube growth was quite uniform. The tube length was about 2-3 times diameter of the pollen grain. Nearly all the pollen tubes observed had swollen tips. Because cellophane wrapper is not produced as a biochemical product, the pore size and water permeability of cellophane are unknown. *L.perezii* pollen clearly responds to cellophane made by two companies differentially since the pollen grains only germinated on cellophane of Murfett Reagency. The reasons for the difference are unknown. Another interesting phenomenon was that all the pollen tubes cultured on cellophane of Murfett Regency grew upwards and away from the cellophane sheet surface. This was unexpected from the observations of *Solanum melongena* and *Capsicum annum* pollen by M.P. Alexander and S. Ganeshan (1989), where pollen tubes were found to adhere firmly to the cellophane.

Pollen tube growth in an upwards direction may not be ideal *in vitro* in terms of nutrient uptake, because the tip of a growing pollen tube is the region that undergoes growth. It might be difficult for *L.perezii* pollen tubes to absorb mineral nutrients since *L.perezii* pollen tubes grew upwards on cellophane. Dialysis tubing surpasses cellophane for the germination of *L.perezii* pollen, as most of the pollen tubes grew on the surface of dialysis-tubing sheet. Therefore, in subsequent studies dialysis-tubing was used and cellophane was discarded.

4.3.1 Pollen Germination on Basic Medium

In this study, pollen germination in basic medium was carried out with the use of

dialysis-tubing & filter-paper supporters. The best pollen germination rate achieved in basic medium was around 40% and the longest tube was about 4 times (235μ) the diameter of the pollen grain.

Boric acid was found to be crucial for *L.perezii* pollen germination. In the basic medium without boric acid, more than 50% of the pollen grains produced a bulge at the pollen germ pores. Only a few pollen grains produced short tubes, which were only 0.5 times diameter of the pollen grain. It was found that *L.perezii* pollen grains tolerate quite a wide range of concentrations of boric acid: from 10-20 mM. In this range of concentration, *L.perezii* pollen had similar germination rates and tube growth.

Calcium nitrate was found to be another crucial constituent in the medium for *L.perezii* pollen germination. In the basic medium without calcium nitrate, more than 90% of the pollen grains leaked severely, though pollen grains did not burst. A few pollen tubes were seen in a whole view of the slide and the pollen tubes were also short, only 0.5 times diameter of the pollen grain. Because only the pollen tubes at least equal to or longer than the diameter of pollen grain were considered germinated, *L.perezii* pollen was considered as showing no germination in medium without either boric acid or calcium nitrate. A specificity in calcium salts was found to be a requirement of *L.perezii* pollen. The pollen only germinated in the basic medium with $Ca(No_3)_2 4H_2O$ (M.W. 236.15) and did not germinate in the basic medium with $Ca(No_3)_2$ (M.W. 164). The pollen germination and tube growth equally well in the medium with 2.45-8 mM calcium nitrate.

Casein hydrolysate (C.H.) was also found to be necessary for *L.perezii* pollen germination. In the basic medium without C.H. a few pollen tubes appeared within 10 minutes, but the tube growth ceased when the tube length reached 0.5 times the pollen grain diameter.

The effect of different molecular weight, 4000, 6000 and 20,000 polyethylene glycol (PEG) on pollen germination was examined in this study. As PEG molecular weight increased, the viscosity of the medium increased as well. PEG-20,000 gave the most viscous medium and was the best for *L.perezii* pollen germination.

In the dialysis-tubing and filter-paper system, PEG was found to be more important than sucrose for *L.perezii* pollen germination (Table 4.2).

Table 4.2 *L.perezii* pollen germination rates on the basic medium and the basic medium without sucrose.

	In Basic Medium	In Basic Medium without Sucrose
Pollen Germination %	39.5%	2%

Basic medium contains: 20 mM boric acid, 6 mM calcium nitrate, 0.1% C.H., 7% sucrose and 23% PEG. *L.perezii* type B pollen was used. Pollen was cultured at 25°C and the results came from single experiment.

In the basic medium without PEG, pollen did not germinate, in the basic medium without sucrose, pollen germinated although with lower germination percentage (2%) (Plate 4.1) compared with that (31.5%) in basic medium with sucrose (Plate 4.2).

For *L.perezii* pollen germination at least 7% sucrose and 23% PEG were required to give the maximum pollen germination percentage and the best tube growth. However, the highest percentage obtained was only around 40% and the tubes were short, the longest being about 235 μ (about 4 times diameter of the pollen grain). Most pollen tubes have swollen tips (Plate 4.2). Some (10-20%) pollen tube tips burst (Plate 4.3). About 3% pollen tubes branched (Plate 4.4), and in around 1.5% pollen, two tubes were produced by one pollen through two pores (Plate 4.5).

It was found that *L.perezii* pollen germination rate and tube length vary greatly between plants (Table 4.3).

Table 4.3 Seven *L.perezii* plants' pollen germination rates (%) and tube growth on the basic medium.

Plant	1(A)	2(A)	3(A)	4(A)	1(B)	2(B)	3(B)
Germ	11.9%	22.3%	32.8%	23.2%	34.6%	32.5%	21.7%
Tube (μ)	70.5±6	70±4	96.3±6	70.1±5	235±16	70.2±3	70±6.5

In the table 1(A), 2(A), 3(A), 4(A) were four *L.perezii* plants with type A pollen and 1(B), 2(B), 3(B) were three *L.perezii* plants with type B pollen. The pollen grains were supported by dialysis-tubing and filter-papers and cultured in basic medium. The germination percentages shown are the average of three replicates. The value for tube length is the average of 30 pollen grains.

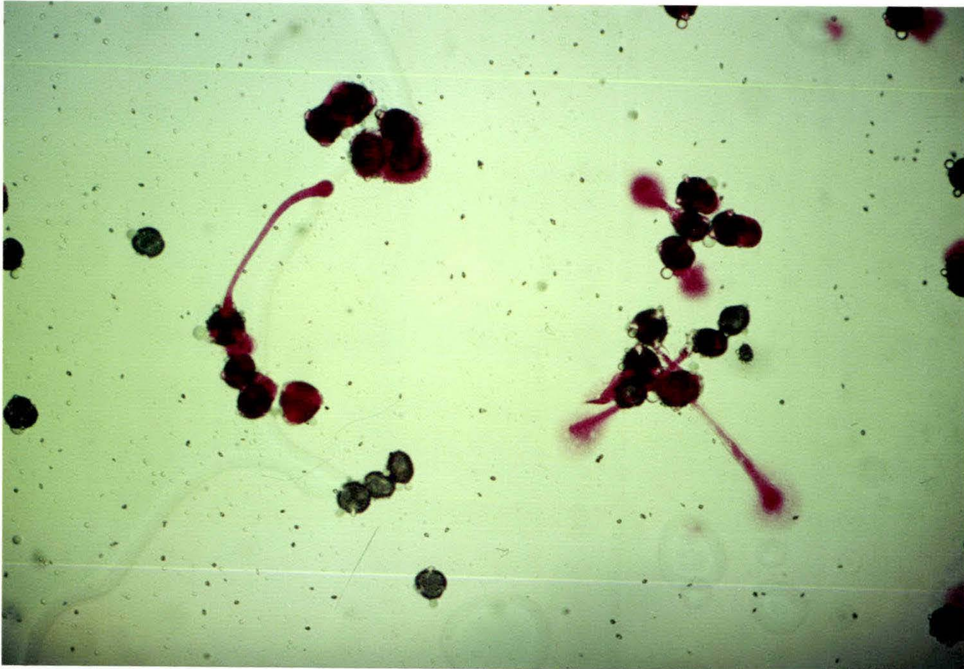

Plate 4.1 *L.perezii* pollen germination in the basic medium without sucrose. *L.perezii* type B pollen was supported by dialysis-tubing & filter-papers which soaked in the medium without sucrose. Pollen germination rate was greatly reduced, being only 2%. Pollen was microphotographed at X100 magnification after being stained with Alexander's stain.

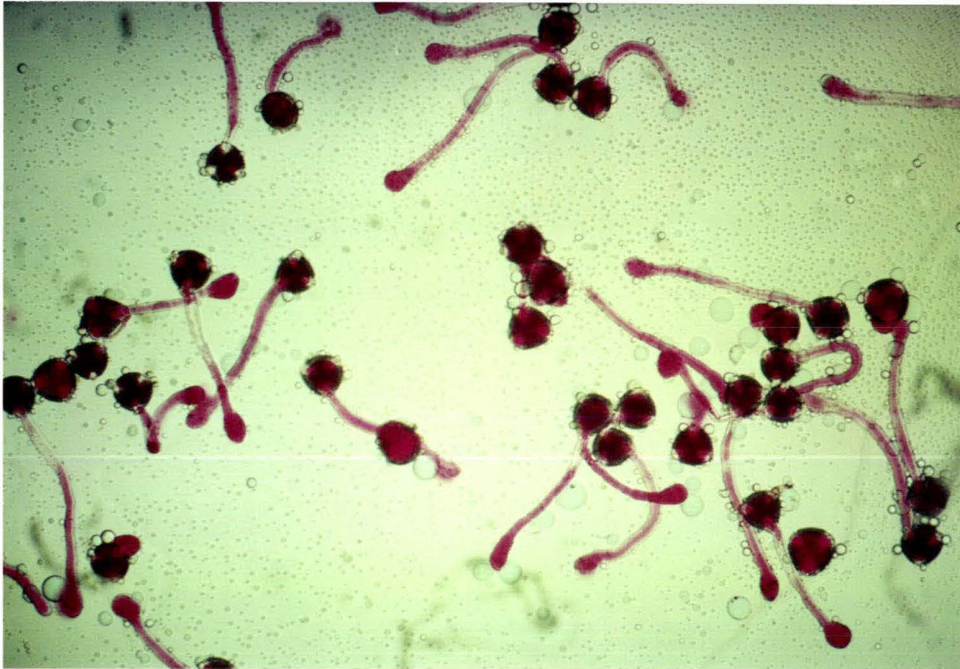

Plate 4.2 ***L.perezii* pollen germination in the basic medium.** *L.perezii* type B pollen was supported by dialysis-tubing & filter-papers. The pollen germination rate was 31.5%. The basic medium contains: 20 mM boric acid; 6 mM calcium nitrate; 0.1% casein hydrolysate; 7% sucrose and 23% polyethylene glycol-20,000. Pollen was microphotographed at X100 magnification after being stained with Alexander's stain.

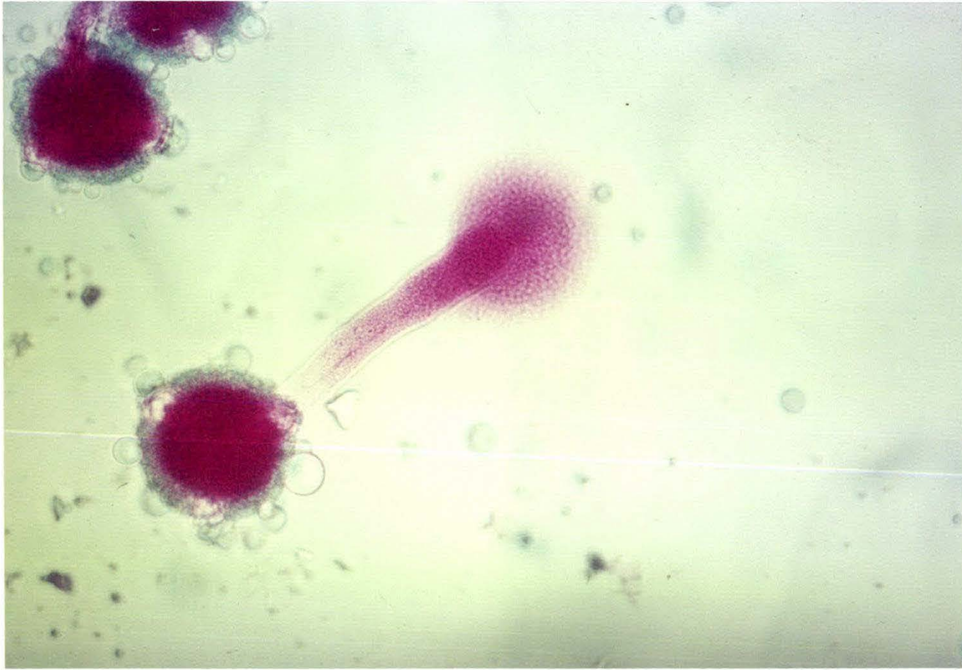

Plate 4.3 *L.perezii* pollen germination in the basic medium showing a pollen tube bursting at the tip. *L.perezii* type A pollen was supported by dialysis-tubing & filter-papers and cultured in the basic medium. Pollen was microphotographed at X160 magnification after being stained with Alexander's stain.

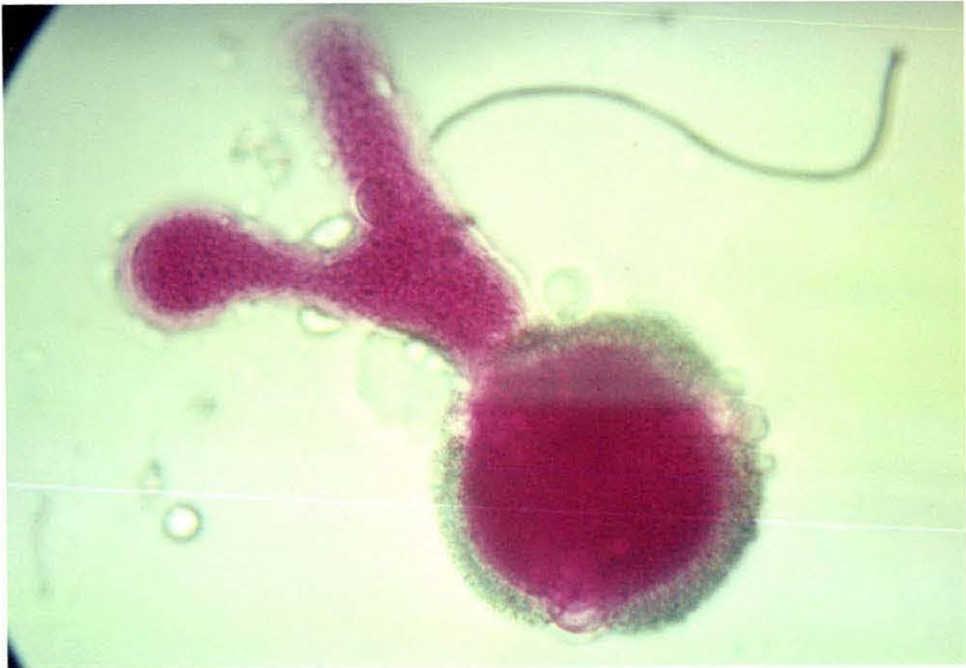

Plate 4.4 ***L.perezii* pollen germination in the basic medium showing pollen tube branching.** Pollen was supported by dialysis-tubing & filter-papers and cultured in the basic medium. About 3% pollen produced branched tubes. Pollen was microphotographed at X400 magnification after being stained with Alexander's stain.

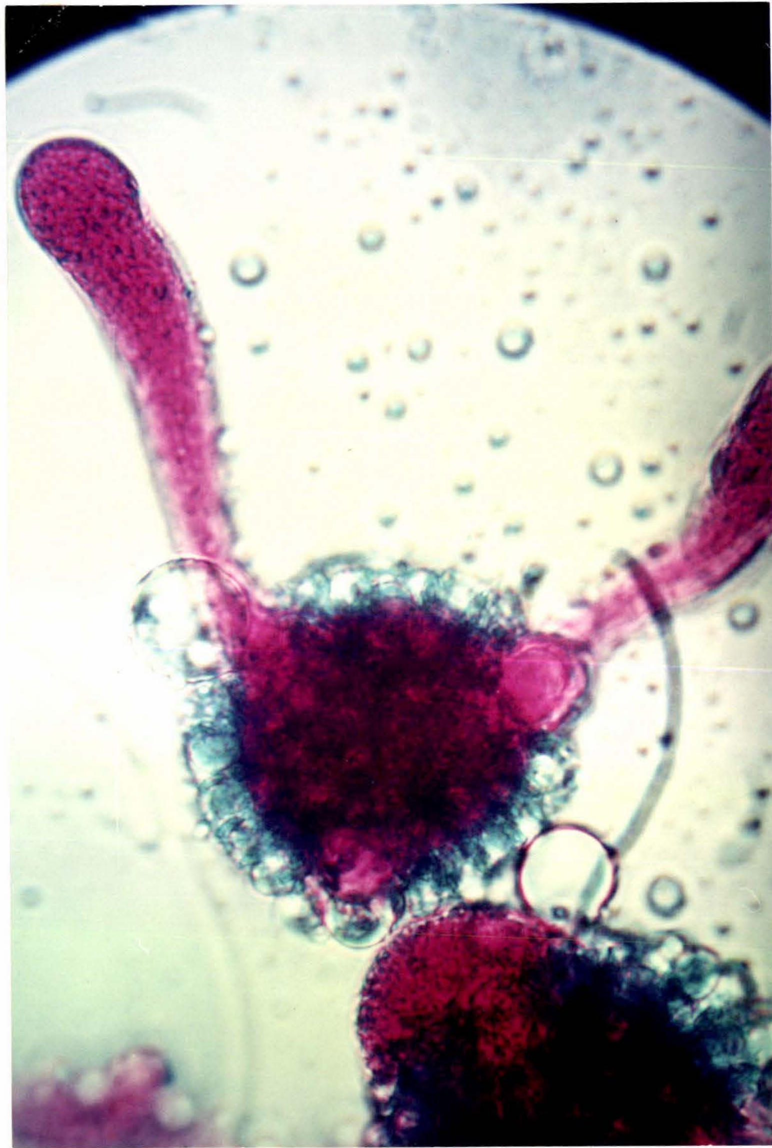

Plate 4.5 ***L.perezii* pollen germination in the basic medium showing two tubes from one pollen.** Pollen was supported by dialysis-tubing & filter-papers and cultured in the basic medium. In about 1.5% pollen, two tubes were produced by one pollen through two pores. The pollen was microphotographed at X400 magnification after being stained with Alexander's stain.

Data in table 4.3 indicates the difference in pollen viability between plants. It was found that the age of inflorescence influences pollen germination percentage and tube length. One inflorescence of a *L.perezii* plant with type A pollen was monitored. Pollen from the flowers opened on the first day showed a very low germination rate (1.5%), tube length being only one pollen diameter ($58.8 \pm 2.9\mu$), but the pollen from the flowers of the same inflorescence on the second day gave nearly the peak germination percentage of 38%, tube length being longest ($201 \pm 18.9\mu$). After about two weeks, the inflorescence was producing fewer flowers and the germination rate of the pollen from these flowers was reduced to about 4%, the tube length being ($70.1\mu \pm 4.6\mu$).

It was found that *L.perezii* pollen requires only a short time for germination *in vitro*. The pollen tube reached its maximum and tube growth ceased within 10 minutes at 25°C.

4.3.2 Experiments For Improving *L.perezii* Pollen Germination And Tube Length

Since maximum germination percentage of *L.perezii* achieved in the basic medium was only around 40% and the tube length was short (4 times diameter of the pollen grain being longest), a number of other chemical and physical factors which have been found to enhance pollen germination and tube growth in other species were examined as potential candidates to improve *L.perezii* pollen germination.

Indole-3-acetic acid

In this study IAA concentrations between 10^{-6} mM and 1 mM was added in the basic medium and pollen germination was carried out on the dialysis-tubing & filter-paper supporter. The results are shown in Table 4.4.

Table 4.4 *L.perezii* pollen germination and tube growth in the basic medium supplemented with IAA.

mM	1x10^{0}	1x10^{-1}	1x10^{-2}	1x10^{-3}	1x10^{-4}	1x10^{-5}	1x10^{-6}	Control
Germ%	37.8%	32.9%	33.7%	39.3%	30.0%	28.4%	30.3%	31.9%
Tube μ	88±6	89±7	76±2	77±3	79±4	77±2	70±2	82±4

L.perezii type B pollen was supported by dialysis-tubing & filter-papers and cultured in IAA supplemented basic medium at 25°C. The germination % was the average of three replicates. The tube length was the average of 30 pollen tubes.

L.perezii pollen germination and tube growth was not influenced by the addition of IAA into the basic medium. The germination percentage and tube growth of IAA treatments were similar to those of the control.

Gibberellic acid (GA_3) The effect of GA_3 on *L.perezii* pollen germination and tube growth was studied by supplementing the basic medium with 10^{-4} mM - 1 mM of GA_3. The results are summarised in Table 4.5.

Table 4.5 The effect of GA_3 on *L.perezii* pollen germination and tube growth.

mM	1x10^{0}	1x10^{-1}	1x10^{-2}	1x10^{-3}	1x10^{-4}	Control
Germ %	20.8%	36.3%	33.6%	36.0%	32.3%	36.2%
Tube μ	64.6±1.5	94±3	88.2±2.9	94±3.1	88.2±3	95.1±7.1

L.perezii type B pollen was supported by dialysis-tubing filter-papers and cultured GA_3 supplemented basic medium at 25°C. The germination% were the average of three replicates. The tube length was the average of 30 pollen tubes.

GA_3 did not have a stimulatory effect on *L.perezii* at any concentration tested. It seems that when GA_3 concentration was high (1 mM), both pollen germination percentage and tube length were reduced. When GA_3 concentration was reduced, from

10^{-1} - 10^{-4} mM, the results were similar to that of the control.

Ethylene

To investigate the possibility of ethylene in improving *L.perezii* pollen germination, ethylene gas at concentrations of 0.1, 1, 10, 100 ppm was used. *L.perezii* pollen grains were supported by dialysis-tubing & filter-paper booklets which were soaked in the basic medium. Ethylene gas was injected into a 600 ml container in which pollen was incubated at 25°C. The results are shown in Table 4.6.

Table 4.6 The effect of ethylene on *L.perezii* pollen germination and tube growth.

ppm	100	10	1	0.1	Control
Germ %	17.8%	20.5%	21.8%	22.0%	24.6%
Tube μ	87.2±3	83.1±2.5	88.9±3.2	78.2±4	89.9±4.1

L.perezii type A pollen was used and supported by dialysis-tubing & filter-papers which were soaked in basic medium. Pollen was cultured in ethylene gas at 25°C. The germination % was the average of three replicates. The tube length was the average of 30 pollen tubes.

Ethylene did not have a stimulatory effect on *L.perezii* pollen germination and tube growth at any concentration tested. The results of all ethylene treatments showed a slightly reduced germination percentage, the control having the highest germination rate, although the difference was not significant.

Manganese Sulphate

Manganese sulphate of concentrations from 10^{-4} mM to 1 mM was added into the basic medium for assessing its effect on *L.perezii* pollen germination . The results are shown in Table 4.7.

Table 4.7 The *L.perezii* pollen germination and tube growth in the basic medium supplemented with $MnSO_4$.

mM	$1x10^0$	$1x10^{-1}$	$1x10^{-2}$	$1x10^{-3}$	$1x10^{-4}$	Control
Germ %	14.4%	15.2%	18.8%	10.0%	16.2%	16.3%
Tube μ	79.1±2	70.5±3.2	76.4±3.9	69.7±2.8	77.5±4.1	76.4±2

L.perezii type A pollen was supported by dialysis-tubing & filter-paper system and cultured at 25°C. The germination % were the average of three replicates. The tube length was the average of 30 pollen tubes.

There was no correlation between pollen germination rates and manganese sulphate concentration. The results of manganese sulphate treatments were similar to that of the control. Manganese sulphate did not influence *L.perezii* pollen germination and tube growth.

Copper Sulphate: The concentrations from 10 mM to 100 mM $CuSO_4$ was added into the basic medium. The results shows $CuSO_4$ does not influence either *L.perezii* pollen germination or tube growth (Table 4.8).

Table 4.8 *L.perezii* pollen germination and tube growth on the basic medium supplemented with $CuSO_4$.

mM	100	50	30	10	Control
Germ %	38.4%	40.5%	43.3%	37.3%	38.7%
Tube μ	117±7	123.6±6	124±5.5	111±5.5	119±5.8

L.perezii type B pollen was supported by dialysis-tubing & filter-papers and cultured in $CuSO_4$ supplemented basic medium. The germination % were the average of three replicates. Tube length was the average of 30 pollen tubes.

Prehydration

L.perezii pollen was hydrated in 90% RH and 36% RH for 0.5 hour before pollen was transferred onto dialysis-tubing sheet to germinate the in basic medium. The germination rate of pollen treated in 90%RH was 16.1% and in 36%RH, 18.0%. Both were lower than control (28.7%) (Figure 4.1).

After pollen was hydrated in 100% RH for one hour, the pollen germination percentage dropped down to 4.9% from 26.6% of the control. When the prehydration time was prolonged to 2, 4 and 6 hours, the pollen did not germinate at all. Clearly, prehydration treatment somehow reduced *L.perezii* pollen germination ability in *in vitro* conditions.

Temperature

To explore whether *L.perezii* pollen germination and tube growth were influenced by temperature, pollen which was supported by dialysis-tubing & filter-papers was cultured in basic medium at seven different temperatures.

The effect of temperature on germination percentage is shown in Figure 4.2. Optimum temperature was in the range of 15°C-35°C. Germination percentage was reduced above 35°C and below 15°C. The temperature was also found to influence tube growth. The pollen tube length was the longest at the range from 20°C-25°C. Beyond this temperature range, the tube length was reduced (Figure 4.3).

It has been mentioned above that some pollen tubes became branched growing on basic medium. An attempt was made to identify whether the branching phenomenon correlates with any particular chemicals or any physical treatments. Observations of the frequency of branching in the chemical treatments described above showed no correlation between branching frequency and concentrations. It was found in this study however that temperature influences pollen tube branching greatly. Pollen tube branching rate was lower at lower temperature. As the temperature was raised, the branching rate increased. Only 3% of pollen had branched tubes at 10°C, while 16.3% at 40°C. (Figure 4.4).

In the observation, branching always occurred at the tip of tubes and then two branches continued to grow from their own tips. The longest branching tube observed was 3 times of diameter of pollen grain.

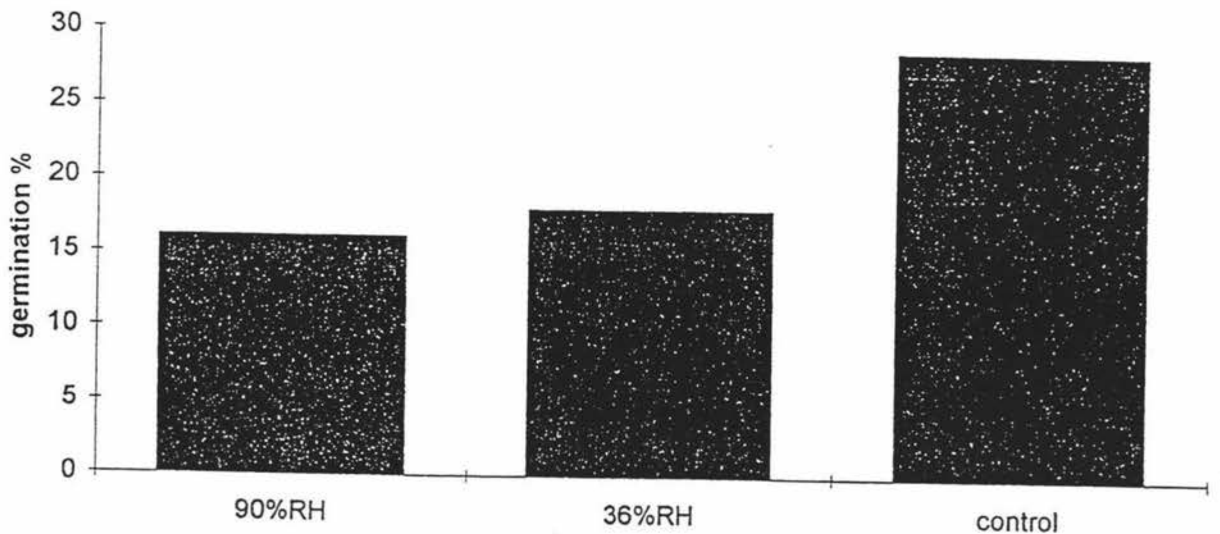

Figure 4.1 The effect of prehydration on *L.perezii* pollen germination. *L.perezii* type B pollen was prehydrated in either 90%RH or 36%RH for 30 minutes. after prehydration pollen was transferred to dialysis-tubing & filter-paper supporters and cultured in the basic medium for two hours at 25°C. Pollen was scored under the microscope at X100 magnification. The germination rate was 16.1% after prehydration in 90%RH, 18% after prehydration in 36%RH and 28.7% of the control. The results came from a single experiment.

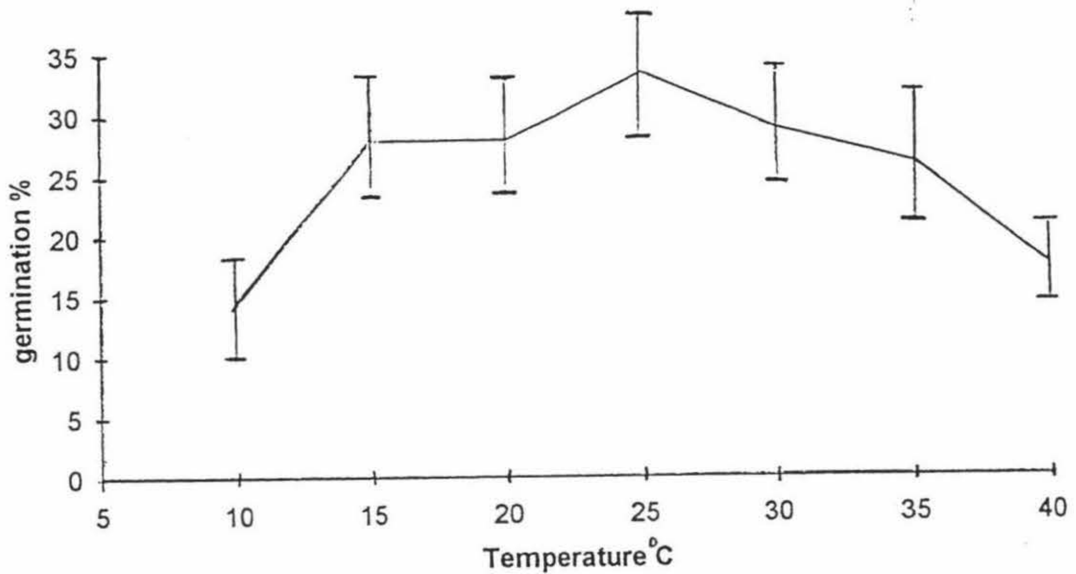

Figure 4.2 The effect of temperature on *L.perezii* pollen germination. *L.perezii* type B pollen was supported by dialysis-tubing & filter-papers and cultured in the basic medium at seven different temperatures. The pollen germination rate was effected by temperature, 35% was the highest at 25°C. The data values are the averages of three replicates and the bars on the graph are standard errors of the mean.

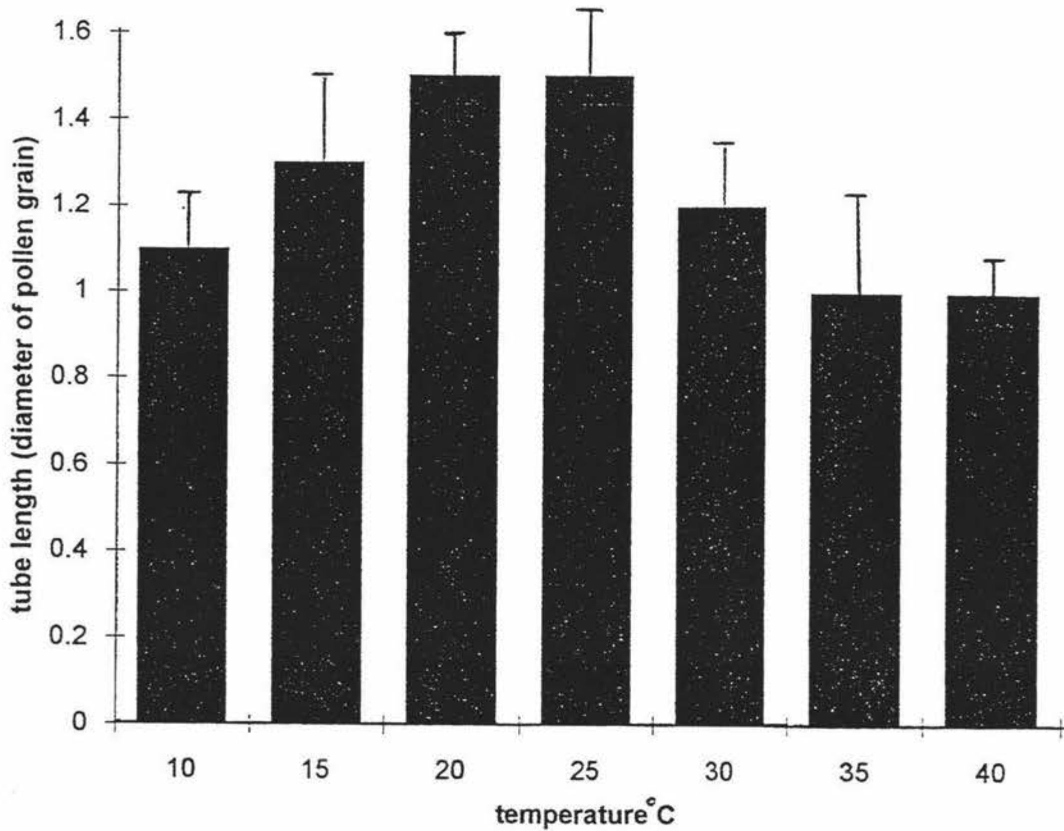

Figure 4.3 The effect of temperature on *L.perezii* pollen tube growth. *L.perezii* type B pollen was supported by dialysis-tubing & filter-papers and cultured in the basic medium at seven different temperatures. Pollen tubes were measured by the times diameter of the pollen grain. The pollen tube growth was influenced by temperature, the tubes being longest at 20°C -25°C. The data values are the averages of 30 measurements and the bars on the graph are standard errors of the mean.

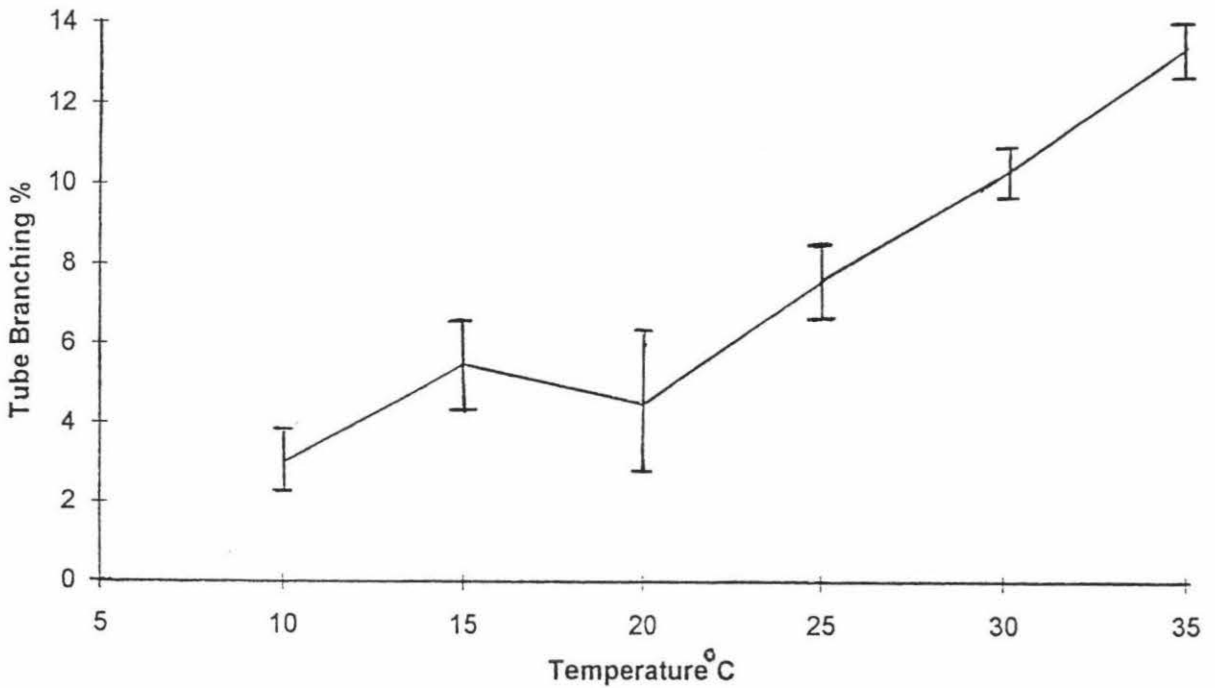

Figure 4.4 The effect of temperature on *L.perezii* pollen tube branching. *L.perezii* type A pollen was supported by dialysis-tubing & filter-papers and cultured in the basic medium at seven different temperatures. The pollen tube branching rate was influenced by temperature, the branching rate increased as the temperature rose. The data values are the mean of three replicates and the bars on the graph are standard errors of the mean.

4.4 DISCUSSION

The results reported above indicated an absolute requirement of *L.perezii* pollen for water-restricting conditions supplied by PEG, sucrose and a dialysis-tubing & filter-paper system. *L.perezii* pollen did not germinate on the agar solidified medium in despite of different sucrose concentrations used in the medium. The pollen grains either ruptured or leaked severely. If the sucrose concentration series is reflecting an effect of osmotic potential on pollen water uptake, the data suggests that pollen absorbed water from agar medium too fast, leading to bursting and severe leaking, although the osmotic values in 40%, 50%, 60% and 70% sucrose medium are relatively high. This phenomenon is not surprising when considering the different hydration modes of bi-and trinucleate pollen on their stigmas. In species with wet stigmas, the exudate provides binucleate pollen with a medium in which to hydrate, supplying not only a source of water at controlled osmotic pressure, but also other materials for metabolism by the grain (Elleman *et al.* 1992). Dry stigmas apparently present the pollen grain with a less receptive surface, the cuticle being coated only with a thin proteinaceous pellicle (Mattson *et al.* 1974). In *Arabidopsis thaliana* when pollen landed on the stigma surface, the pollen exine coating flows out to form a foot through which the pollen tube grows into a space between an inner and outer element of the stigma cell wall (Elleman *et al.* 1992). In this case the cuticle of the stigma cells must be permeable for water to pass into the germinating pollen grains, however the hydration of pollen grains must be controlled in a quite different way than that of wet stigma. The use of a dialysis tubing sheet between pollen and medium which was transferred by filter-paper, simulated the "dry" stigma surface and effectively stopped pollen bursting.

A special requirement exists for sucrose as an energy source. The germination percentage of *L.perezii* pollen which was supported by dialysis-tubing & filter-papers dropped to 2% in the basic medium without sucrose. The poor pollen germination in the medium without sucrose may be explained by the pollen respiration requirement for sucrose.

L.perezii pollen was not considered germinated in the basic medium without boron although a few pollen grains produced short tubes (less than half diameter of the pollen grain). The exact function of boron in the germination of pollen is not yet understood,

however, possible roles of boron may be 3-fold: (1) it promotes absorption of sugar by the pollen for metabolic processes by forming sugar-borate complexes; (2) it increases oxygen uptake; and (3) it is involved in the synthesis of pectic materials for the wall of the actively elongating pollen tube (Vasil 1960).

L.perezii pollen did not germinate and leaked severely in the medium without calcium. The requirement of calcium may be explained by two roles which calcium may play in pollen germination and pollen tube growth: calcium binds to pectate carboxyl groups along the pollen wall (Brewbaker and kwack 1963) and calcium binds to pollen tube membrane (Steer and Steer 1989).
It has been demonstrated that calcium is incorporated into pollen tube membrane by chlorotetracycline (CTC) fluorescence technique (Reiss and Herth 1979). Chlorotetracycline penetrates into the pollen tube when applied to the pollen culture medium. Fluorescence is enhanced when CTC is bound by calcium in a membrane environment, so the enhanced fluorescence acts as a probe for membrane bound calcium. With CTC fluorescence, calcium was shown to incorporated into pollen tube membrane of *Lilium longiflorum* (Reiss and Herth 1979).
The leakage of *L.perezii* pollen in the medium without calcium may due to the imperfect membrane structure synthesized during pollen germination.

Casein hydrolysate (C.H.) is absolutely essential for *L.perezii* pollen germination and tube growth *in vitro*. It has been known for a long time that C.H. has stimulatory effect on pollen germination, especially for trinucleate species (for reference see below). Binucleate pollen germination and tube growth can be described as two distinct developmental periods in terms of nutrient requirement. The first phase is autotrophic and the growth of pollen apparently largely depends upon nutrients reserved in the pollen grain itself. The second phase is heterotrophic and the nutrients required are provided by the stylar tissue. The higher growth rates and callose plug formation is coordinated with the heterotrophic phase. However, trinucleate pollen seems to enter heterotrophic phase immediately upon germination and relies entirely on the growth environment completely (Mulcahy and Mulcahy 1988). It has been demonstrated that the addition of C.H. did not have stimulatory effect on pollen germination percentage

of *Petunia Hybrida*, a binucleate species for at least the first 11 hours of culture, while for *Silene dioica*, (a trinucleate species) both percentage of germination and the tube length were increased significantly 30 min after pollen in a suspension culture (Mulcahy and Mulcahy 1988). The C.H. requirement of *L.perezii* pollen germination and tube growth further provided evidence for the two different developmental patterns existing in bi- and trinucleate species in terms of nutrient dependence. The necessity of casein hydrolysate in the medium for *L.perezii* pollen germination is consistent with other work showing trinucleate species often require more complicated media than binucleate species (Brewbaker and Kwack 1963, Vasil 1960).

L.perezii pollen only needs a short time to germinate *in vitro*. The pollen tube reached its maximum within 10 minutes at 25°C in the current study. This result is in agreement with other rapidly-germinating trinucleate pollen. For example, tube growth of *Aster tripolium* (a trinucleate species) started within 3 min (Hoekstra and Bruinsma 1979). The rapid germination of trinucleate pollen may be explained by high respiration rates and fully-developed mitochondria in the pollen grains (Hoekstra 1979). In general, the phylogenetically advanced trinucleate pollen species respire at much higher rates than the binucleate species (Hoekstra and Bruinsma 1975). The differences in the rate of respiration between bi-and trinucleate pollen can be largely explained by the condition of mitochondria. Trinucleate pollen contains fully developed mitochondria at dehiscence, allowing for a rapid germination. In contrast, many binucleate pollen species have far less developed mitochondria and much lower respiration which leads to slow germination (Hoekstra and Bruinsma 1975, 1979).

Auxins were the first class of plant hormone to be discovered. The discovery of auxin was an outcome of experiments on phototropism. IAA has been reported to increase germination percentage and stimulate tube growth of many plant species: *Antirrhinum majus, Nicotiana tabacum, Thea sinensis* and *Allium cepa* (Loo and Hwang 1944). However *L.perezii* pollen germination and tube growth were not influenced by the addition of IAA into the basic medium. This phenomenon may indicate that *L.perezii* pollen grains are not sensitive to the exogenous IAA ,or the pollen may already have an adequate level of IAA. Any additional supply of IAA to the pollen in the nutrient

medium does not appreciably affect germination or the elongation of the pollen tubes.

Gibberellic acid (GA_3) is another plant hormone which was reported to stimulate both pollen germination and tube growth of some species. For example Date Palm (*Phoenix dactylifera* L) pollen germination percentage increased three times and tube length increased 4 times by addition of 100 ppm GA_3 into the medium (Asif *et al.* 1983). Gibberellic acid (GA_3) did not have a stimulatory effect on *L.perezii* pollen germination at low concentrations and had a prohibitory effect at high concentration (1 mM). Generally, plant physiological responses induced by plant hormones require extremely small concentrations, such as 10^{-9} mM (Moore 1979). For pollen germination *in vitro,* concentration of hormone is also important, relatively high concentration often has an inhibitory rather than a stimulatory effect (Loo and Hwang 1944). The inhibitory effect of GA_3 (1 mM) on *L.perezii* pollen germination may result from a too high concentration. *L.perezii* pollen grains themselves have already sufficient GA_3 or gibberellin family compounds for processes requiring these hormones. The application of excess exogenous GA_3 may have increased gibberellins to inhibitory levels and pollen germination percentage and tube growth were reduced.

Ethylene is important in many plant biochemical processes. Ethylene has been shown to be involved in pollen tube growth of some plant species under *in vitro* conditions. Pollen tube growth was stimulated in proportion to the amount of ethylene applied up to 1000 ppm in *Pyrus communis* (Search and Stanley 1970), however ethylene did not stimulate either *L.perezii* pollen germination nor pollen tube growth at any concentrations tested in the current study.

Manganese sulphate was found to stimulate both pollen germination percentage and tube length of some plant species, for example, pollen germination percentage of *Antirrhinum majus* was increased two fold by addition of 10^{-2} manganese sulphate into the medium and tube length was also increased two times (Loo and Hwang 1944), but it did not influence *L.perezii* pollen germination nor the pollen tube growth.

Copper sulphate was reported to improve tube growth of *Nicotiana tabacum* (Read *et*

al. 1993). *Nicotiana tabacum* germination of 80-90% and straight pollen tubes were achieved with the addition of Copper Sulphate and PEG into the liquid medium. Without both of PEG and $CuSO_4$ in the medium, up to 50% of the hydrated *N.tabacum* pollen grain did not develop further, and the germinated tubes were slowing-growing and abnormal, with thickened walls, kinked growth and fragile, swollen tips. Addition of PEG-6000 increased germination to 80-90% and prevented the bursting of pollen grains and tube tips, tube growth was still slow and kinked and tips remained swollen. Addition of 30 μM $CuSO_4$ produced straight-growing tubes with smooth-sided tips resembling the tips of tubes growing through stylar tissue (Read *et al.* 1993).

Because *L.perezii* pollen growing on basic medium also produced swollen tips, $CuSO_4$ was assessed to investigate whether it was beneficial to *L.perezii* pollen tube growth. However, $CuSO_4$ did not prevent *L.perezii* pollen producing swollen tips as it did to *Nicotiana tabacum* pollen.

It has been known for a long time that better pollen germination of some plant species can be achieved by a prehydration *in vitro* system. *Zea mays* pollen for example requires prehydration in a saturated atmosphere for 60 minutes at 25°C (Mulcahy and Mulcahy 1988). On the contrary, prehydration reduced *L.perezii* pollen germination percentage in all three treatments (100%RH, 90%RH and 36%RH). The question that arises is what function does prehydration play in pollen germination? There are some explanations available from some studies of hydration. The pollen of angiosperms is always desiccated at the time of dispersal. The organization of the plasmalemma is radically altered during the period of dehydration. The membrane has lost its bi-layer structure (Shivanna and Heslop-Harrison 1981). Recovery takes place on prehydration which starts when pollen grains land on stigmas. The establishment of a functioning membrane of the pollen vegetative cell is one of the principal determinants of germination capacity (Heslop-Harrison 1979). It is proposed that controlled prehydration in a humid capacity gives time for the reorganization of the plasmalemma from its largely dissociated state in the partly dehydrated grain (Shivanna and Heslop-Harrison 1981). This hypothesis explains the prehydration effect observed for *Zea mays* very well, but not for *L.perezii* as studied here.

When considering hydration of pollen grains on stigma surfaces, the hydration process

is expected to be a mutual reaction between stigma cells and pollen grains. The water uptake of pollen from stigmatic cells should be associated with some communication between the two. It is likely to be not merely an osmotic adjustment. Pollen grains are believed to emit some signalling chemicals which stimulate the response of stigma cells. An increase in stigmatic secretion after pollen recognition, for example, has been reported for a number of species (Knox and Williams 1986). Prehydration treatment is an artificial environment which separates water uptake from other process in pollen germination. It may satisfy some plant species for pollen germination, but apparently not all.

Pollen tube branching was frequently observed in *L.perezii* pollen tube growth. It was found that percentage of the pollen tube branching was influenced by temperature and branching always occurred at the tube tips. The nature of pollen tube growth is different from that of most other plant cells, in which growth takes place over the entire surface of the cell. Pollen tube growth, in contrast, is restricted to the tip region (Mascarenhas 1993). Pollen tube wall growth was reported to proceed by the continued addition of new wall material to one end (the tip), followed by the generation of a distinctive lining to the elongating cylinder (Steer and Steer 1989). Microfilaments play an important part in the movements of the wall-precursor, organelle, nucleus and gamete in the tube (Heslop-Harrison 1987). The increase in temperature may somehow influence the alignment of microfilaments and change the pattern of deposition of new cell wall materials leading to tube branching.

CONCLUSION

Some species of *Limonium* plants as newly cultivated cut flower crops have a great potential for further development. The work reported in Chapter 1 has shown that interspecific hybridization can be used in aiming to either improve the existing characteristics of the plants or to create new hybrids. High frequency of interspecific crossability was found in the crosses between the species which belong to the same section or the same subsection in plant classification. Pollen tube penetration to the ovary at high frequency was observed in the crosses *L.perezii* X *L.sinuatum* (in both directions) and *L.sinense* X *L.aureum* (in both directions). *L.perezii* and *L.sinuatum* belong to the section *Pteroclados* (Baker 1953a). *L.sinense* and *L.aureum* belong to the subsection *Chrysantheae* (Baker 1953a). In some intersectional crosses, pollen tubes were also seen penetrating the ovaries but at very low frequency; in most intersectonal crosses pollen tube growth was stopped in the styles. *L.perigrinum* is the only *Limonium* species which produces large flowers and its bright pink colour is also desirable for cut flowers. This study shows there is a good chance to produce new cultivars through hybridization between *L.perigrinum* and other *Limonium* species when *L.perigrinum* is used as a male parent. *L.perigrinum* pollen tubes entered the ovaries of *L.perezii*, *L.sinense*, *L.aureum* and *L.caspia*, though the frequency was low.

Various incongruities were observed in *Limonium* interspecific breeding, which operated at several levels. In the cross between *L.perigrinum* as a female parent and *L.caspia* as the pollen donor, the pollen tube growth was restricted to the stigmas. In 15 crosses the pollen tube was found to be blocked in the style. In the cases of pollen tube penetrating the ovaries, fertilization barriers or post-fertilizaton barriers could also exist.

Unilateral incompatibility was observed in the following 8 crosses:

L.perezii ♀ X *L.perigrinum* ♂

L.aureum♀ X *L.perigrinum* ♂

L.sinense♀ X *L.perigrinum* ♂

L.caspia ♀ X *L.perigrinum* ♂

L.sinense ♀ X *L.sinuatum* ♂

L.caspia ♀ X *L.sinense* ♂

L.caspia ♀ X *L.aureum* ♂

L.caspia ♀ X *L.perezii* ♂

Pollen tube penetration to the ovaries was observed in these crosses, but in their reciprocal crosses unilateral incongruity was exhibited and pollen tube growth was arrested in the style.

Abnormalities of pollen tube growth were found in *Limonium* interspecific crosses including heavy callose deposits in the tips of pollen tubes; pollen tube branching and disorder in pollen tube growth direction.

Post-pollination flower development of *L.perezii* can be classified into three groups according to growth patterns of the ovaries, ovules and the embryos. Twenty-six percent of florets showed no ovary and ovule growth at all after intraspecific pollination. No embryo was found in this group. In 11% of florets, ovaries and ovules grew up to Day 12 after pollination and then shrivelled. No embryo was found in this group either. Sixty-three percent florets produced embryos following intraspecific pollination and developed normally. According to this observation, *L.perezii* should have good seed set since in 63% of florets the embryos developed normally, although the present investigation did not extend to seed maturity.

The *L.perezii* ovule belongs to a very peculiar type of megasporangium. The micropyle of the ovule points upwards and the funicle is parallel to it. The *L.perezii* suspensor has a spherical appearance when the embryo is in the globular stage. The suspensor changed into shallow cup shape when the embryos are in the late heart and torpedo stages. This spherical or shallow cup suspensor is rarely found in embryogenesis of angiosperms.

The viability of *Limonium* pollen was assessed with Alexander's stain and fluorochromatic reaction (FCR) stain. The results showed that Alexander's stain tends to give a higher viability percentage than FCR. The FCR test should provide better correlation of pollen stainability with pollen viability, since Alexander's stain only tells whether the pollen has a cytoplasm or not, FCR, however, tests both esterase activity

and an integrity of membrane of the pollen vegetative cell.

Conditions for *L.perezii* pollen germination culture were established in this study. Absolute requirements of boric acid, calcium nitrate, casein hydrolysate were found. The water restricting pollen culture environment was found to be extremely important and *in vitro* techniques including the uses of sucrose, PEG and a dialysis-tubing & filter-paper supporter were designed to allow a hydration system giving optimal germination and initial tube growth. The highest *L.perezii* pollen germination rate achieved in these studies was 30-40%, which is a little higher than 24% for *L.vulgare* reported by Bar-Shalom and Mattson in 1977. The length of pollen tubes was short, the longest attained was 4 times diameter of the pollen grain. A number of chemical and physical factors which have been shown to be essential in stimulating pollen germination of other trinucleate species were tested, but none showed promotive effects on *L.perezii* pollen germination *in vitro*.

These studies further proved the difficulties of germination with trinucleate pollen and that we are not yet able to control the factors for sustained *L.perezii* pollen growth. The environment provided by the style tissue for pollen growth is far more complex than one of artificial media made of available nutrients and inorganic ions which are believed to support pollen growth.

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