Pollen tube - pistil interaction and fertilization in *Lilium longiflorum*.

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'Pollen tube - pistil interaction and fertilization in *Lilium longiflorum*'
door Juliette Janson te verdedigen op 18 september 1992 te Wageningen.

Bij de interactie tussen de pollen buis en de stamper van *Lilium longiflorum* dient men te realiseren dat het hier om een indirect contact gaat, via het exudaat.

Dit proefschrift.

De verwijdering van de lelie stijl, ter verkrijging van interspecifieke hybriden, leidt zelfs tot een onvolkomen functioneren van het compatibele interactie systeem.

Dit proefschrift.

De embryozak van *Lilium longiflorum* vormt geen filiform apparaat en lijkt tot na de fusie van de gameten op een laag niveau van metabolische activiteit te verkeren.

Dit proefschrift.

De gevolgen van bestuivingsprikkels komen in *Lilium longiflorum* niet tot uiting in structurele veranderingen van de stamper en in eiwitpatronen van de zaadbeginsels.

Dit proefschrift.

De 'enucleate cytoplasmic bodies' in de embryozak zijn de overblijfselen van de zaadcellen.

(Russell, 1983)

De hoge prijzen in de tax-free shops op Schiphol in vergelijking met de prijzen in de Nederlandse winkels kunnen buitenlandse bezoekers doen vermoeden dat in Nederland nauwelijks belasting wordt betaald.

Door de gedeeltelijke vervanging van vast wetenschappelijk personeel door goedkopere aio's en vervolgens veel te weinig post doc plaatsen te creëren, verdwijnt kennis en kunde bij de universiteit.

De invoering van een scheidsrechter in ultimate frisbee zal het verantwoordelijkheidsgevoel van de spelers, zoals in een aantal sporten het geval is, sterk reduceren.

De waardering voor een aantal taken en beroepen die nu hoofdzakelijk door vrouwen worden uitgevoerd zal stijgen als ook daar de emancipatie door zal dringen.

De aanwezigheid van een aantal verschillende typen microscopen kan, bij juist gebruik, een extra rendement hebben.

(Van Aelst en Janson, een theepauze in 1991)

Het zal nog jaren duren voordat wetenschappelijk is aangetoond of bloemen werkelijk van mensen houden.

Dankwoord

Dank U, dank je, bestemd voor alle mensen die op enige wijze hebben bijgedragen aan de totstandkoming van dit proefschrift. Enkelen van hen wil ik hierbij nogmaals in de schijnwerpers zetten.
Bijvoorbeeld Siep Massalt. De foto's in dit proefschrift zijn slechts een selectie uit de vele die er tijdens het onderzoek gemaakt zijn. Uren heb jij hiervoor in het donker staan ontwikkelen en afdrukken, met een gedenkwaardige gezamelijke eindsprint.
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And last but certainly not least dank ik Olav.

Contents

Abbreviations

A - antipode
B - bundle
C - cuticle
CC - central cell
CB - cytoplasmic body
CSER - cisternal smooth endoplasmic reticulum
CW - cell wall
D - dictyosome
E - exudate
EC - egg cell
ER - endoplasmic reticulum
ES - embryo sac
FC - fibrillar component
II - inner integument
L - lipid droplet
LER - lamellar endoplasmic reticulum
N - nucleus
NC - nucellar cell
NE - nuclear envelope
NP - nuclear pore
NU - nucleolus
M - mitochondrion
O - ovule
OI - osmiophilic island
P - plastid
PA - placenta
PB - paramural body
PG - pollen grain
PL - plasmalemma
PS - persistent synergid
PT - pollen tube
S - starch
SC - secretory cells
SER - smooth endoplasmic reticulum
SN - sperm nucleus
SP - stigmatic papillae
V - vacuole
W - wall of the ovarian cavity
Z - zygote

General introduction

1

In this thesis the interaction between pollen tubes and the pistil with the subsequent fertilization is studied in *Lilium longiflorum*. This was carried out in intact pistils using electron microscopy (chapters 2 and 3), light microscopy and electrophoresis (chapter 2). A reproductive calendar with on a time scale an overview of different processes in the flower is presented in chapter 6.

The interaction was also studied after flower manipulations (chapter 4) and placental pollination in vitro (chapter 5). The reason for this is explained in the following review about compatible, incompatible and interspecific pollen tube growth in *Lilium*, the guidance of the pollen tube and other aspects of the interactions between the pollen tubes and the pistil.

Secretory activity of the pistil and pollen tube growth in *Lilium*

- Secretory activity of the pistil -

The cells covering the stigma and hollow style of *Lilium longiflorum* are secretory (Rosen, 1971) and also the pathway to the ovules of *L. leucanthum var. centifolium* and *L. regale* is lined with secretory cells (Welk et al., 1965). In *L. longiflorum* the ultrastructure of the stigmatic papillae and secretory cells in the style differs. The latter have a transfer wall; the ingrowth of the cell wall lacks in the papillar cells (Miki-Hirosige et al., 1987). With the increase of age of the pistil, from 1 - 2 days before anthesis till 3 - 4 days after, an increase in thickness and complexity of the secretory surface of the canal cells seems to take place (Rosen, 1971).

The parenchyma cells of the style might be involved in the exudate production as well. Yamada (1965) observed an increase of colloidal bodies in the cytoplasm of the parenchyma cells, especially of those in the proximity of the stigma, and a decrease of starch grains in these cells during the development of the pistil. The bodies were not observed in the secretory cells. The stylar exudate has the same affinity for the used stains as the colloidal bodies, which then have largely disappeared from the parenchyme tissue. The colloidal material is rich in proteins, polysaccharides, reduced sugars and lipids (Yamada, 1965). The parenchyma cells might thus contribute to the exudate in the style.

The exudates at the stigma and in the style of *L. longiflorum* differ in their composition and the time of emerging. The protein pattern after electrophoresis of the stylar exudate showed more bands when compared with stigmatic exudate. Already two days before anthesis drops of exudate gather between the papillar cells at the stigma. During ten days after anthesis about 67 mg of stigmatic exudate with a pH of 4.3 is produced. In the style exudate production is rarely observed before anthesis. Two days after anthesis the stylar exudate contains polysaccharides and lipid particles (pH 5.0) (Miki-Hirosige et al., 1987). Labarca and Loewus (1973) observed however an appearance of exudate on the stigmatic surface soon after anthesis. Stylar exudate accumulated till about five days after anthesis and at the stigma the amount of exudate increased at least till seven days after anthesis, both determined via the amount of carbohydrates produced (Labarca and Loewus, 1973).

- Pollination and pollen tube growth -

Pollination of *L. longiflorum* influenced the appearance of the stigma. The difference in appearance after compatible and incompatible pollination differed strong between cultivars (Labarca and Loewus, 1973). Pollination is not correlated with marked changes in the ultrastructure of the secretory cells of the style (Rosen and Thomas, 1970), neither after compatible nor after incompatible pollination (Rosen, 1971). Crang (1969) did observe necrotic processes taking place in the cells adjacent to the secretory cells in the style within four days after pollination, but not in an unpollinated style. This seems to be not plainly the result of the disappearance of the colloidal bodies from those cells as described above. Campbell and Linskens (1984a) observed an accelerated pistil and sometimes tepal senescence after intraspecific pollination. Senescence of the flower will be given more attention in a later instant.

The developmental stages of the pistil and of the pollen grains influence the pollen tube growth. In flower buds the growth of compatible pollen tubes is retarded when compared with pollen tube growth in flowers (Ascher and Peloquin, 1966a). Pollen grains harvested 1 to 6 days before anthesis had a low germination percentage in vitro. Drying pollen grains of 1 to 2 days before anthesis to a low moisture content greatly improved germination (Lin and Dickinson, 1984).

The speed of pollen tube growth is also influenced by temperature. After anthesis the length of compatible pollen tubes, reached 48 hours after pollination, increased in a temperature range from 11°C till 30°C. In the latter the pollen tubes reached the end of the style (Ascher and Peloquin, 1966b).

The pollen tubes probably take up substances from the exudate while growing through the style. After incubation of detached flowers or isolated pistils of *L. longiflorum* in a myo-inositol (^{14}C and ^{3}H) solution, radioactivity was traced in stigmatic and stylar exudate (^{14}C) and in cell walls of the pistil (^{3}H, autoradiography). Pollination did not influence the labelling pattern in the different flower or pistil parts (Kroh et al., 1970a). Labelling pistils with myo-inositol-U-^{14}C resulted in radioactivity in both compatible and

incompatible pollen tubes. With autoradiography (^{3}H) it was determined that the label appeared especially in the wall of the pollen tubes (Kroh et al., 1970b). After labelling detached pistils with D-glucose-U-^{14}C radioactivity was traced in the exudate and in both the cytoplasm and the wall of the pollen tubes (Labarca and Loewus, 1973). Pollen tubes take up a polysaccharide component, which was present in labeled stigmatic exudate and was subsequently injected into the style (Labarca and Loewus, 1972). The products of the secretory cells of *L. longiflorum* at the stigma and in the style create a medium through which the pollen tubes grow and of which substances are used. The study of the pistil-pollen interaction is in fact a study of the interaction between the secretory products and the pollen tubes (Rosen, 1971).

Self-incompatibility in lily

- The age of the pistil and the self-incompatibility reaction -

The presence of the incompatibility reaction of *L. longiflorum* is dependent upon the age of the flower. Till 4 days after anthesis incompatible pollen tubes reach, 48 hours after pollination at room temperature, only half the length of compatible pollen tubes. In older flowers this difference decreases. The same length is reached ten days after anthesis, when the growth of both compatible and incompatible pollen tubes is reduced. Seed set did then not appear. Till nine days after anthesis, compatible pollination resulted in seed set. After incompatible pollination seed set only occurred when carried out between six and nine days after anthesis. Incompatible bud pollination did not result in seed set (Ascher and Peloquin, 1966a).

- The temperature and the self-incompatibility reaction -

Also the temperature influences both compatible and incompatible pollen tube growth in *L. longiflorum*. In 48 hours at 11 or 39°C incompatible pollen tubes grow as far as compatible pollen tubes. In between those temperatures the length of the incompatible pollen tubes is smaller compared with compatible ones, but also increases with rising temperatures (Ascher and Peloquin, 1966b). At 19°C the difference between compatible and incompatible pollen tube length is significant from 3 days after pollination. In styles incubated at 12.5°C there was no significant difference until 6 days after pollination (Ascher and Peloquin, 1970).

- The place of the self-incompatibility reaction -

The incompatibility reaction of *L. longiflorum* is not taking place at the stigma. The initiation of pollen tube formation and germination at the stigma is independent upon the compatibility (Hiratsuka and Tezuka, 1979). Also in an in vitro test comparing stigmatic exudate from compatible and incompatible stigmas the pollen tube growth was not

influenced by the compatibility (Rosen, 1971). Fett et al. (1976) concluded that the incompatibility reaction is located in the lower half of the style as was determined by a partial heat treatment of the styles (see below). In styles incubated at 24°C the speed of compatible pollen tube growth doubled between 12 and 24 hours after pollination, whereas the rate of incompatible pollen tube growth did not (Ascher and Peloquin, 1970). This reaction takes place before the pollen tubes reach the lower end of the style, which is thus not the place the incompatibility reaction is initiated. The self-incompatibility reaction appears not to be due to localized stylar barriers, which was also concluded for interspecific-incompatibility reactions. Compatible or incompatible pollination of style sections without a stigma at the ovarian or at the stigmatic end shows the reduced pollen tube length of incompatible pollen tubes when pollinated at either end (Ascher, 1977). In *L. henryi* the stigmatic papillae and other stigmatic cells play no role in the self-incompatibility system. The message of incompatibility is given in the upper quarter of the style and is not reversible when pollen tubes enter a grafted compatible style (Lawson and Dickinson, 1975).

Stylar treatment of *L. longiflorum* with RNA inhibitors suggested that RNA synthesis in styles is necessary for the incompatibility reaction. It also suggested that RNA synthesis in pollen tubes is required for compatible pollen tube growth and that lily pollen grains carry sufficient RNA for growth as an incompatible tube (Ascher, 1971). In the growing pollen tube tip two types of vesicles are observed. The larger ones arise from the golgi apparatus and contribute to tube wall and plasmalemma formation. The origin and function of the smaller vesicles is unknown (Rosen et al., 1964). The small vesicles or the membranous reticulum, their appearance is depending upon the fixation procedure, in the pollen tube tip are likely to contain RNA (Dashek and Rosen, 1966). Isolation of nucleic acids from styles of the same cultivar as the pollen tubes and injecting the extract into a compatible style prior to pollination resulted in shorter pollen tubes (Campbell and Ascher, 1974).

- Overcoming self-incompatibility -

Many different efforts were made to overcome self-incompatibility. The best results are likely achieved with temperature treatments. The self-incompatibility reaction can be inactivated by a heat treatment (6 minutes at 50°C) of pistils of *L. longiflorum*, leaving the stigma out of the warm water bath (Hopper et al., 1967), when carried out from anthesis till 4 days after anthesis (Ascher, 1975). Campbell and Linskens (1984c) observed the removal of the incompatibility already after 1-2 minutes submersion in 50°C, while compatible pollen tube growth was retarded after 4-5 minutes of treatment. In styles incubated at 31 and 39°C the incompatible pollen tube length reached after both 24 and 48 hours is similar to the length reached by compatible pollen tubes (Ascher and Peloquin, 1970). Van Tuyl et al. (1982) observed seed set after self-pollination and incubation at 26°C and to a lesser extend at 23°C.

Flushing the hollow style with distilled water did not inactivate the incompatibility

reaction (Ascher, 1975). Not all the exudate present in the style is however removed after a single flush with water (Campbell and Linskens, 1984b). Stylar exudate collected from cross-pollinated pistils promoted in some cases, depending upon cultivar and flushing time after pollination, incompatible pollen tube growth (Amaki and Higuchi, 1991).

A treatment of the flower with the auxin naphthalene acetamide (NAA) applied at the basis of the pistil at the time of incompatible pollination, delayed the senescence of the embryo sacs and resulted in more seeds compared with untreated flowers (Emsweller et al., 1960). NAA increased the functional activity of transfer cells of the placenta, the appearance of starch grains in the adjacent tissues and the differentiation of ovules in the sterile *Lilium* hybrid 'Black Beauty' (Herich and Herichova, 1991). Seeds were also obtained after applying NAA or the cytokinin N(6)-benzyladenine mixed in lanolin to the style following self-pollination (Matsubara, 1973). Several flower organ extracts applied to the stigma prior to self-pollination and heat treatments of pollen also resulted in seeds after self-pollination of *L. longiflorum*, although not as many as obtained after cross-pollination (Matsubara, 1981). Van Tuyl et al. (1982) did however not observe any influence of pollen heat treatment on overcoming self-incompatibility. The use of mentor pollen, irradiated compatible pollen applied together with incompatible pollen, did overcome self-incompatibility (Van Tuyl et al., 1982).

Interspecific crosses in lily

Interspecific crosses are valuable for lily breeding purposes. In that way properties of different species can be combined in one plant. In interspecific hybridisation programs of CPRO-DLO *L. longiflorum* is used for its forcing ability and growth vigour; *L. henryi* for resistance to viral diseases and *Fusarium oxysporum* causing bulb rot; *L. candidum* for pure whiteness, fragance, low light and temperature tolerance; *L. concolor* and *L. dauricum* both for earliness, flower form and colour (Van Tuyl et al., 1988). Problems arise when attempts are made to achieve these interspecific crosses.

- The place of pollen tube inhibition after interspecific pollination -

The pollen tube length reached in the style after interspecific pollination does not show any relationship with the length of the style of the male parent (Asano, 1985a). Reciprocal differences in pollen tube length reached in the style and capsule set occur (Ascher and Drewlow, 1971).

Ascher and Peloquin (1968) distinguish two kinds of interspecific incompatibility in *Lilium*. In one kind the pollen tube growth stops upon reaching the stylar canal and the tubes show an abnormal morphology. In the other combinations the pollen tube growth continues in the style and can, depending on the combination of parents, even reach the length of intraspecific incompatible pollen tubes and have a normal morphology (Ascher and Peloquin, 1968). A model with barrier and promotion genes present in the pistil and penetration and reaction genes present in pollen was developed to explain this pattern of

upper and lower inhibition after interspecific crosses (Asano, 1985b). Asano (1980c) proposed a stylar factor accelerating compatible pollen tube growth, which is thus absent when in vitro growth on stigmatic exudate is concerned. In interspecific crosses the poor pollen tube growth was proposed to be the result of the impossibility to use this accelerating factor (Asano, 1980c).

In addition to Ascher (1977) who concluded the absence of a localized barrier as mentioned above, Asano (1981) observed that the pollen tube growth was equally inhibited after pollination with *L. regale* at different heights of the style of *L. longiflorum* (at the stigma and at one and two thirds of the style).

- Circumventing stylar barriers in interspecific crosses -

Incubation of styles at 39°C, which inactivates the self-incompatibility reaction, had no effect on the interspecific incompatibility (Ascher and Peloquin, 1968). Injection of the style of *L. longiflorum* with exudate of *L. longiflorum* prior to pollination can increase the pollen tube growth otherwise inhibited by interspecific, short growth incompatibility (Ascher and Drewlow, 1975). Injection of stigmatic and stylar exudate of the same species as the pollen grains into the pistil before interspecific pollination at respectively the cut-style (see below) or the stigma, did promote pollen tube growth in the combination Asiatic hybrid 'Connecticut King' with *L. longiflorum*, but the pollen tubes only reached the ovules in case of cut-style pollination (Amaki et al., 1990).

Myodo and Asano (1977) obtained embryos from crosses between widely related species after cut-style pollination, a technique in which the style is cut off just above the ovary and pollen is applied at the cut surface. Stylar barriers are so circumvented. A longitudinal cut in the part of the style left at the ovary was made in some investigations, but although the authors refer to this method as interstylar pollination, in this thesis the term of cut-style pollination or method is used. This is done to discriminate between the cut-style method as explained above and the interstylar pollination as carried out in our experiments, in which the style remains at the ovary and pollen is applied through a slit.

The embryos obtained after interspecific cut-style pollination did not develop as well as those obtained from a compatible stigmatic pollination. They remained small and immature. Also the interspecific endosperm is present in a small quantity or sometimes almost absent in stead of the endosperm normally formed (Myodo and Asano, 1977). Embryo culture is applied to avoid abortion (Asano and Myodo, 1977b and Myodo and Asano, 1977). When cut-style pollination was used to overcome self-incompatibility a number of normal seeds were obtained (Asano and Myodo, 1977a). The abnormal development of the endosperm after interspecific crosses seems thus not to be the consequence of the method and might point towards an incompatibility of genomes, as does the sterility of the obtained hybrids. After crosses between distantly related species carried out in 15 combinations, the hybrids generally show a high pollen sterility (Asano, 1978). The fertility of interspecific F1 hybrids can be restored by colchicine treatment leading to tetraploid plants (Van Tuyl et al., 1986a). Sometimes allotriploids were obtained

in cut-style crosses with *L. longiflorum* as the female parent (Asano, 1978).

Embryos obtained after interspecific crosses with *L. nepalense* as the female parent aborted in a late stage during their incubation in embryo culture (Van Tuyl et al., 1988), which was carried out in the absence of endosperm. Light intensity and temperature influence the number of embryos obtained after cut-style pollination of *L. longiflorum* with *L. candidum* and embryo culture (Van Tuyl et al., 1986a). The use of endosperm acquired from intraspecific crosses as nurse tissue improved the culture of interspecific embryos of 0.3 - 0.4 mm (30 - 35 days after pollination), which would resume their embryonic growth similar to the normal pattern as observed in vivo (Asano, 1980a).

Ovary slice culture was already applied at 20 days after self-pollination of *L. formosanum*, when the embryos are about 0.1 mm long, and resulted in seeds which germinated (Hayashi et al., 1986). Different methods were also combined: cut-style pollination followed by ovary slice culture already at 5 days after pollination (DAP), ovule culture at 42 DAP and embryo culture at 70 DAP (Straathof et al., 1987) or in vitro grafted-style method or in vitro cut-style pollination both followed by ovary, ovule and embryo culture (Van Tuyl et al., 1991).

Some seeds were obtained after stigmatic pollination of the cross *Lilium* hybrid 'Enchantment' with *L. longiflorum* 'White Europe' with the help of mentor pollen and embryo culture. In the reciprocal cross both the mentor and the pioneer (irradiated pollen applied before the interspecific male parent) pollination technique followed by embryo culture resulted in some seeds (Van Tuyl et al., 1982).

In all the above mentioned methods the numbers of embryos obtained were low.

- Identification of interspecific hybrids -

Apart from morphological characteristics (Asano, 1978; Asano, 1980b and Myodo and Asano, 1977) interspecific hybrids can be identified by microscopical chromosome analysis (Myodo and Asano, 1977) and the peroxidase-iso-enzyme pattern obtained after isoelectric focusing (Van Tuyl et al., 1986b and 1988).

Pollen tube guidance

After interspecific pollination of *L. longiflorum* with the *Lilium* hybrid 'Mont Blanc' the pollen tubes show a winding pattern at the stigma and stop in the upper half of the style. In case of cut-style pollination with the same combination of species the pollen tubes generally stop in front of the micropyle. Different stimuli are supposed to lead to a pollen tube growth toward and into the micropyle respectively (Van Roggen et al., 1988).

Brink (1924) postulated three possible explanations of how pollen tubes find their way to the micropyle: 1. diffusion of substances having a chemotropic effect, 2. anatomical features of the tissues traversed or 3. at random growth over free surfaces and finding the micropyle by chance. Iwanami (1953) also mentions gravity as a pollen tube guide. Heslop-Harrison and Heslop-Harrison (1986) concluded that the pollen tube

growth in the pistil is directed by especially anatomical features and is linked with the ability of the pollen tubes to respond to them. But there is still some place for chemotropic control in locating the transmitting tissue and on entering the ovule.

- Chemotropical guidance of the pollen tube -

To gain more insight in the chemotropic activity of tissues, in vitro experiments were carried out. Rosen (1964) compared different in vitro tests concerning the determination of chemotropic activity and discussed their ability to distinguish between stimulation and chemotropism. Different tests have been carried out with lily. Rosen (1959, 1961) observed positive chemotropic activity of the stigma and substigma of *L. longiflorum*, weak or no activity in the lower style and no activity in stigmatic exudate and the ovary. Stigmatic exudate did enhance germination and growth. In 1971 Rosen did however report chemotropic activity of stigmatic exudate as well. Cells at the stigma surface and secretory cells in the style are sources of chemotropic activity, which is found in the secretion products of these cells (Rosen, 1971). Miki-Hirosige (1964) observed activity of the stigma, style and ovary of *L. longiflorum* but not of the ovules. Using the depression test, which involves placing pollen in a centre well in an agar plate in between two other wells with on one side the material to be tested and on the other the control respectively, Welk et al. (1965) concluded activity of the ovules of *L. leucanthum var. centifolium* and *L. regale*, occurring at the micropylar side after testing ovule halves. When testing both sides of the micropylar half, the micropylar side showed activity. A secretion of a chemotropic substance emerging from the micropyle or an absence of diffusion of a factor from the wounded integuments, nucellus and embryo sac were concluded.

Especially the chemotropic activity of the ovules is of interest because of the lack of a stimulus to enter the micropyle after interspecific cut-style pollination. Chemotropic activity is observed between different species of a genus: a stigma of *L. longiflorum* with pollen of *L. auratum* (Iwanami, 1953) and pollen of *L. regale* (Rosen, 1961). Comparisons between different species of *Lilium* show little variation in chemotropic activity within the species in the different parts of the pistil (Miki-Hirosige, 1964). But the species used by Welk et al. (1965) are not discussed in this article. The difference in chemotropic activity of the ovule as discussed in these two articles could be due to the difference in species, although the results are reasonable constant within the genus. Also a variation in time of sampling, presumably implying differences in the developmental stages of the flowers, was suggested by Welk et al. (1965). Other causes can be differences in testing methods and media, in growing and testing conditions and in the timing of the experiment, e.g. incubation of the pistil material before applying the pollen.

Identification of the chemotropically active substance in *L. longiflorum* is still limited to terms like heat-liable, dialysable, not precipitable by acetone (Rosen, 1961) or meta-stabile to heat and able to pass through a collodium or cellulose membrane (Miki, 1954 and Miki-Hirosige, 1964). A list of tested compounds without chemotropic activity was published by Rosen (1961). Some of these substances did stimulate pollen tube

growth. *Antirrhinum majus* pollen is attracted to calcium. There is a calcium gradient in the pistil reaching its maximum in the ovules (Mascarenhas and Machlis, 1962a). High levels of calcium were present in the synergids of *Pennisetum glaucum* and less in the cytoplasm of the egg cell, central cell and antipodal cells. After antimonate treatment calcium was also precipitated in the micropyle and appressed to the surface of the outer integument (Chaubal and Reger, 1992). A range of other substances were tested for their chemotropic activity to *Antirrhinum majus* pollen and apart from coconut milk, no positive chemotropic attraction has been found (Mascarenhas and Machlis, 1962b). In lily neither coconut milk (Rosen, 1961) nor calcium (Rosen, 1964) are chemotropically active. In the pollen tube tip of *L. longiflorum* a calcium accumulation was observed after culturing on medium containing ^{45}Ca and subsequent tracing with autoradiography (Jaffe et al., 1975). In pollen tubes a calcium gradient is observed with chlorotetracycline (CTC), which binds to membrane bound calcium. The fluorescence was emerging especially in the tip of the pollen tube (Reiss and Herth, 1978). Hydrated pollen grains show already an increased CTC fluorescence intensity gradient towards the germination colpus (Reiss et al., 1985). If calcium had been chemotropically active it would result in the orientation of the growing part of the pollen tube towards the source of this substance which is already present in relatively large amounts in the pollen tube tip.

There are different theories about the mechanism causing the behaviour of the pollen tubes growing towards chemotropic substances. As a reaction to a chemotropic substance the bending of the pollen tube is the result of an unequal growth of the wall of the two sides of the pollen tube, probably caused by a substance directly or indirectly inhibiting the extensibility of the wall material (Tsao, 1949). Rosen (1961) postulated that the chemotropic factor might inhibit the synthesis of extensible compounds of the pollen tube wall. The ratio rigid - extensible is then influenced by the chemotropic factor, resulting in pollen wall extension towards the increasing concentration. Occurrence of chemotropic activity is so depending upon the concentration of the chemotropic factor and the distance of the pollen tube to the material.

Chemotropic inactivity was explained to be caused by inhibitors. Placing a membrane between the gynoecial tissues and pollen of *Antirrhinum majus* prevented the inhibitor to pass through while the chemotropic substance could diffuse and cause directional growth of the pollen tubes towards the material (Mascarenhas and Machlis, 1962b). According to Miki-Hirosige (1964) positive and negative chemotropism are explained by a positive and a negative factor, respectively. These factors may have a different sensibility towards heat treatments.

Whatever theory is correct, according to Rosen (1964) there is no universal chemotropic substance active in all plant species. It might be that there are also different mechanisms occurring in different plant species, active on itself or different ones in combination. Another problem is the translation of the in vitro experiments to in vivo situations. After applying pollen in the style of *Lilium* the pollen tubes grow preferably towards the stigma and the ovary. There is no preference for any of those directions, which points, according to Iwanami (1953), towards an absence of a chemotropic

gradient in the style. But also in vivo some phenomena point towards an existence of chemotropic activity. Localized chemotropic agents leading to ovule penetration are likely present in *Salpiglossis sinuata*, because of the high efficiency of fertilization after pollination with low pollen densities and the radical change in growth direction needed for micropyle penetration in absence of an anatomical guide (Hepher and Boulter, 1987). In *Paspalum* ovules a substance present at the edges of the outer integument and in the micropyle is related to the directional growth of the pollen tube into the embryo sac (Chao, 1971). The micropylar nucellus of *Beta vulgaris* presumably has a secretory activity probably for the guiding of the pollen tube towards the embryo sac (Bruun and Olesen, 1989).

- Guidance of the pollen tube by the structure of the pistil -

Apart from chemotropical substances the structure of the pistil can guide pollen tube growth. The trichome structure of *Pennisetum typhoides* guides the pollen tubes towards the ovary (Heslop-Harrison and Reger, 1988). The pollen tube pathway is in *Strelitzia reginae* (Kronestedt et al., 1986) and *Phaseolus acutifolius* (Lord and Kohorn, 1986) restricted to the presence of exudate in respectively the ovary and in the (partly) hollow style.

Interactions between pollen tubes and the pistil

After interspecific cut-style pollination the interaction between the pollen tubes and the pistil is at least partly disrupted. Pollination at the stigma or pollen tube growth triggers processes in the ovary. In the *Lilium* hybrid 'Enchantment' stigmatic pollination with irradiated pollen can stimulate (parthenocarpic) fruit set. This phenomenon was also observed in *L. longiflorum* although to a lesser extend (Van Tuyl et al., 1982). A hormone treatment (NAA) stimulated the weight per fruit of *L. longiflorum*, till at least 20 days after the treatment (Emsweller and Stuart, 1948).

In *Strelitzia reginae* a thickening of the cell walls of the subepidermal cells towards the secretory cells of the hollow style is established within four days after pollination. This thickening was not observed in unpollinated flowers (Kronstedt et al., 1986). The germination of pollen grains of *Talinum mengesii* is regulated by the maternal parent (Murdy and Brown Carter, 1987). The pistil of peach ripens from the top to the basis. Pollination enhances the degeneration of the stigmatic papillae, what is cocominant with the production of stigmatic exudate. During the passage of pollen tubes in the style the amount of starch decreases in the cells of the transmitting tissue. The pollen tube growth stops at the end of the style and the growth is resumed when exudate is observed at the obturator (Herrero and Arbeloa, 1989). This secretion is independent of pollination, because it takes place in both pollinated and unpollinated flowers (Arbeloa and Herrero, 1987). At the entrance of the micropyle a circling of the pollen tube is observed. The embryo sac might not be mature when the tube arrives at the ovule. At the time that one

of the two ovules in the ovary is fertilized, the other ovule is degenerating (Herrero and Arbeloa, 1989).

Another phenomenon that points towards an interaction between the ovary and pollen tube growth is described in avocado, in which one ovule is present per ovary. Of the large number of pollen tubes at the stigma often only one pollen tube reaches the end of the style, sometimes two but in that case two embryo sacs with nucelli are present in the ovules. In the latter it was also observed that only one pollen tube reaches the end of the style (Sedgley, 1976). It is possible that in this case a phenomenon is introduced during the development of the style which only allows one pollen tube to pass. Sedgley (1976) mentions nutrition and inhibition as the responsible factors.

The above described interactions took place while the pollen tube was present. One step further are processes taking place in the mature pistil prior to the arrival of the pollen tubes, but in answer to their presence. Herefore a communication within the pistil is needed, a signal which travels ahead of the pollen tube tips. A signal which is induced by e.g. pollination or pollen tube growth and transfers to other plant organs. It is a preparation of those organs for the things to come. Some signals are discriminant in that they can transfer a message in the kind of the inducing factor. The receiving and target organs have to be in a stage in which they can respond to these signals, e.g. maturity of the flower. The result of the signal can be traced by applying the inducing factor and measure the changes in the plant before the inducing factor has arrived. It might often happen that the changes occur once the inducing factor has arrived. An interaction as observed in peach might be the result. Examples of activation of plants before the inducing factor has arrived are rare.

Amaki and Yamamoto (1988) observed a difference in compatible and incompatible pollen tube growth in compatible activated styles in comparison with incompatible and not activated styles. The stigmas were pollinated and removed after 12 hours followed by grafting a stigma with pollen grains onto the style. The pollen tube promoting factor has a velocity of 0.5 cm/hour (Amaki and Yamamoto, 1988). Six hours after cross-pollination of *L. longiflorum* the activities of several enzymes in the style at 30-15 mm from the stigma was higher than those in the stylar segments of non- or self-pollinated pistils (Amaki et al., 1989).

Also in *Petunia hybrida* pollination and pollen tube growth trigger processes in the style. In the neck of the style, the zone just underneath the stigma, an increase of the number of polyribosomes and an appearance of 'embayments' (single-membraned cytoplasmic inclusions associated with the plasma membrane) are observed before the pollen tubes, both compatible and incompatible, have arrived at that point (Herrero and Dickinson, 1979). This is not taking place in bud pollinated (Herrero and Dickinson, 1980a) or in unpollinated styles (Herrero and Dickinson, 1979). Prepollinating *P. hybrida* with pollen of *Nicotiana tabacum* significantly enhanced *Petunia* pollen tube growth applied 24 hours later (Hoekstra and Van Roekel, 1988). Both compatible and incompatible pollen tubes increase their growth speed on entering the style and the compatible pollen tubes then reach a higher speed than incompatible ones, which eventually stop. After grafting

experiments with *P. hybrida* the importance of the top 1 mm of the pistil in the incompatibility reaction was confirmed. An incompatible stigma grafted to a compatible pistil resulted in the reduction of the number of pollen tubes measured at one quarter of the style (Herrero and Dickinson, 1980b). In styles pollinated with compatible or incompatible pollen an increase in the amount of starch is observed in front of the pollen tube tips. After the incompatible pollen tubes have past, no change is observed in the transmitting tissue, in contrast with the passage of compatible pollen tubes after which the quantities of starch, lipids and microbodies are reduced (Herrero and Dickinson, 1979). An increase in activity of two enzymes was observed in a part of the style before it was reached by the pollen tubes, so creating a wave preceding the growth of compatible pollen tubes. Incompatible pollen was unfortunately not tested (Roggen, 1967).

Different pollen tube populations present in one pistil can interact. After compatible pollination half-way down the style of *Nicotiana alata* the length of the pollen tubes towards the ovary is as large as the length reached towards the stigma. Compatible and incompatible pollination of the stigma resulted in shorter pollen tubes towards the stigma (Mulcahy and Mulcahy, 1987). The style of *Erythronium grandiflorum* has three canals and pollen tube growth in one canal can influence the growth in another (Cruzan, 1990).

Also the ovary can influence the pollen tube growth. In *Nicotiana alata* prepollination before cut-style pollination induced longer pollen tubes when this cut-style pollination was carried out closer to the ovary. Pollen tube growth is thus stimulated by an ovary activated by pollination (Mulcahy and Mulcahy, 1986a). Styles of *Petunia hybrida* were cut just in front of the pollen tubes and the cut surface was submerged in a culture medium. In case pollination was carried out in flowers the semi-vivo pollen tubes protruding from the styles were longer than in case lose styles without an ovary were pollinated (Mulcahy and Mulcahy, 1985). In *Nicotiana sylvestris* and *N. tabacum* the presence of the ovary at the time of pollination and initial pollen tube growth, did not influence the length of the semi-vivo pollen tubes. No difference was observed in in vivo pollen tube growth in styles of *N. sylvestris* with and without ovary (Kandasamy and Kirsten, 1987). Mulcahy and Mulcahy (1986a) did report an increase in average pollen tube length reached in *N. alata* styles with ovary. In detached flowers of *N. alata* the glucose and fructose level in the ovaries decreased before the arrival of the compatible pollen tubes. This decrease was not so pronounced in unpollinated flowers (Tupy, 1961).

Before the pollen tubes have reached the ovary of *Petunia hybrida* an increase in ribosomal RNA content and protein synthesis in the ovary was observed, the latter especially in the carpels (Linskens, 1973). Before the arrival of both compatible and incompatible pollen tubes, Linskens (1974) observed an increase of the amount of label, supplied as radioactive amino acids, incorporated in proteins in the ovary. Also Deurenberg (1976a and 1976b) observed a stimulation of protein synthesis in the ovary before the pollen tubes have arrived. Using an in vitro protein synthesis method a quantitative difference in protein metabolism was observed between self- and cross-pollinated pistils (Deurenberg, 1976a and b). Qualitative differences of in vitro synthesized proteins of the ovaries of self- and cross- and unpollinated flowers appear in the protein patterns obtained

after gel-electrophoresis (Deurenberg, 1977).

A variety of processes is thus taking place in parts of the pistil which are not in direct contact with the inducing pollen tubes. Herefore a communication within the plant is needed, a system that delivers the message that the pollen tubes, and sometimes even what kind of pollen tubes, are arriving.

What brings the message?

The total peroxidase activity increases especially in cross-pollinated *Nicotiana alata* styles and less after self-pollination. During pollen tube growth through the style the activity of different iso-enzymes can increase, decrease or remain unchanged, sometimes depending upon the type of pollination (Bredemeijer, 1974). During aging of the styles an increase of peroxidase-iso-enzyme P10 activity is observed, which is accelerated by pollination, irrespective whether compatible or incompatible. The increase in the activity of P10 is probably the result of auxin or auxin-induced ethylene activity (Bredemeijer, 1982b). Pollination promotes ethylene production, with a sharp increase at the time the pollen tubes penetrate the stigma (Bredemeijer, 1982a). Ethylene production in one cell induces the production in neighbouring cells. In the basal part of a self-pollinated style P10 is induced although the pollen tubes were not present at that part (Bredemeijer, 1982b).

Thirty minutes after pollination of *Dianthus caryophyllus* with pollen of a mini carnation, an increase of the ethylene production of the pistil is observed. The pollen has not germinated yet, so that a candidate for the induction of this increase is a chemical signal from the pollen grains, probably 1-aminocyclopropane-1-carboxylic acid (ACC). Pollination of carnation with sweet pea, which contains more ACC than carnation pollen grains, results first in a strong ethylene production compared with pollination with carnation pollen grains. This is followed by a decrease. The pollen grains germinated but did not grow into the stigma. The increase of the ethylene production as observed after normal pollination, is associated with pollen tube growth in the style. The influence of intraspecific pollination on the perianth is taking place from 12 - 24 hours after pollination (Whitehead et al., 1983). Wilting of carnation flowers was observed after 6 hours of ethylene treatment. Ethylene increased the leakage of chloride ions from vacuoles which coincides with the decline in water uptake by cut flowers which already begins 2 hours prior to visual wilting (Mayak et al., 1977). Application of ACC to stigmas of cut carnation flowers immediately stimulated ethylene production by the pistil and 20 minutes later by the petals. Indoleacetic acid (IAA) had no such effect. Removal of the ethylene produced by the gynoecium had no effect on the time till petal senescence (Reid et al., 1984).

Pollination also accelerates the senescence of the corolla in *Phalaenopsis amabilis* (Trippi, 1971). Pollen tube growth in the style of *Petunia hybrida* accelerated wilting as did removing of the stigma or half the style (Gilissen, 1976). Ethylene production had strongly increased at 3 hours after pollination or wounding, which both also accelerated senescence. The pollen grains also contain a high concentration of ACC. Exogenous application of ethylene to unpollinated flowers accelerated the onset of senescence

(Whitehead et al., 1984). An inhibition of the synthesis, induced by pollination, or the action of ethylene did not influence the pollen tube growth, nor did the application of the ethylene precursor ACC at different intervals prior to pollination (Hoekstra and Van Roekel, 1988). In *Cyclamen persicum* pollination induced an increase in ethylene production and a corolla abscission after some days instead of a slow wilting (Halevy et al., 1984). An increased ethylene production after pollination was also observed in *Triteleia laxa* (Han et al., 1991). In *Digitalis* pollination reduced flower longevity and a weakening of the abscission zone of the corolla was detected from 8 hours after pollination, while the pollen tubes were still in the stigmatic zone. An inducing stimulus has to move at least 4 mm/hours through the style and ovary (Stead and Moore, 1979). The wilting process of several *Lilium* hybrids was not much influenced by exposure to exogenous ethylene (Woltering and Van Doorn, 1988).

One of the two synergids of cotton degenerate before the pollen tubes arrive at the ovules. This degeneration was not observed in ovules of unpollinated flowers. Gibberelic acid (GA), supplemented to a culture medium, induced a similar degeneration as observed after pollination. A diffusion of GA from the pollen tubes towards the embryo sac via the plasmodesmata in the style is supposed. From the degenerated synergid calcium is probably released, attracting the pollen tubes and inducing the opening of the pollen tube into the degenerated synergid. Supplementing the culture medium with IAA resulted in a fusion of the polar nuclei which subsequently divided. In unpollinated flowers the polar nuclei remain unfused (Jensen el al., 1983). The removal of the anthers in a *Petunia hybrida* flower bud, till a length of about 15 mm, reduced the growth and the anthocyanin production of the corolla. Applying GA_3 at the place where the anthers were removed, decreased the reduction of these processes, which are independent from each other (Weiss and Halevy, 1989).

In orchids IAA plays a role in postpollination processes as well (Strauss and Arditti, 1982). Pollination induced prematurely coloration of the lip (Arditti et al., 1973). Applying ACC or ethylene to isolated *Cymbidium* lips also hastened coloration (Woltering, 1989). IAA induced both coloration and stimulated ethylene production (Woltering, 1990). Transcription and translation regulate this anthocyanin accumulation (Woltering and Somhorst, 1990). Self- and cross-pollination of blueberry resulted in a larger ethylene production than that observed in unpollinated flowers. IAA treatment also increased the amount of ethylene produced (Hall and Forsyth, 1967).

On average two hours after incompatible pollination of *L. longiflorum* a variation in electric potential between the pedicle and an area just underneath the stigma is observed. The germination and/or pollen tube penetration seem responsible, because *Petunia hybrida* pollen grain application at the stigma did not induce a variation in potential in at least 8 hours, the duration of the experiment. There is a large variation between the time of pollen grain application and the observation of the change in potential. In these experiments the electrodes were placed between half an hour till even more than two hours prior to pollination (Spanjers, 1978). In Spanjers (1981) the pollination was carried out first followed by the insertion of the electrodes at one and three cm above the ovary

and in the pedicle. The time between the pollination and the variation in the potential has now increased to 5.5 hours, probably due to the place and time of electrode application. Self-pollination induced another change in bioelectric potential compared with cross-pollination. Removal of the stigma or pollinating with killed compatible pollen induced a change in potential like the one recorded after cross-pollination. No pollination, pollination with pollen from another genus or killed incompatible pollen applied at the stigma, did not show a change in bioelectric potential (Spanjers, 1981). Also in *Petunia hybrida* pollination creates a change of the electric potential. The curves of the potential are dependent upon the age of the style and the type of pollination, compatible or incompatible (Linskens and Spanjers, 1973).

Not only the pistil but also the pollen tubes are involved in electric phenomena. Around in vitro growing pollen tubes an electric field is registered. The current emerges from the pollen grain and enters the pollen tube (Weisenseel el al., 1975). Potassium and hydrogen ions play an important role in this phenomenon (Weisenseel and Jaffe, 1976). Sperber and Dransfeld (1981) observed an orientation of the pollen tubes in a strong magnetic field, in which an orientation towards the north and the south pole occurred in an equal frequency. In an electric field *Lilium* pollen had a tendency to grow toward the anode (Nakamura et al., 1991). Depending upon the growth conditions pollen tubes of *Agapanthus umbelatus* grew parallel to an electric field when applied near the electrodes (Malhó et al., 1992).

In *Incarvillea* a mechanical irritation of the stigma with a soft brush resulted in an action potential (AP1) in the stigma. After pollination another action potential (AP2) is observed in stigma and style. In the tissue of the ovary a stimulation of the oxygen consumption was observed 60 - 90 seconds after the arrival of AP2. Also in *Lilium martagon* an action potential similar to AP2 was observed and the respiration changes as well. AP2 was also induced after applying solutions of 2,4-D and IAA to the stigma. Mechanical irritation did not result in an action potential (Sinyukhin and Britikov, 1967). White (1907) observed an increase of the respiratory activity of the pistils of many species at 4 - 6 days after pollination. The CO_2/O_2 ratio in the gas phase is around pollinated pistils usually higher. A longer incubation was needed to measure a difference in *Lilium candidum* when compared with other dicotyledonous species (White, 1907).

Not only after pollination but also after wounding of other organs than the pistil similar processes as described above are observed. Mechanical wounding or heating of the terminal leaflet of leaf 1 of tomato induced the production of a proteinase inhibitor (PI), not only in that leaflet, but also in the second leaf. The heat stimulus produced an electric response, the wounding was not tested in this connection. Aspirin (acetylsalicylic acid) reduced the electric activity and the systemic PI response after a heat stimulus (Wildon et al., 1989). Damaging leaves of several plant species results in an electrical change which may spread throughout the shoot (Van Sambeek and Pickard, 1976). Wounding of mature pea tissue also elicited polysome formation in tissues other than the wounded area (Davies and Schuster, 1981).

The research in this thesis

In the literature review presented above we saw that cut-style pollination is carried out to obtain interspecific hybrids. There is however a poor ovule penetration as was mentioned. Different stimuli were proposed, one for growing towards the ovule and one for penetration of the micropyle. The first, a stimulus to grow towards the ovule, was present after cut-style pollination in an interspecific combination. The latter, the stimulus for penetration, was missing, which might be the consequence of an interspecific barrier. The research presented in this thesis was focused on the second question: whether the poor ovule penetration was due to the absence of an ovule enhancement, which might be induced during pollination at the stigma and pollen tube growth through the style. This is thus missing after cut-style pollination and might result in a poor penetration.

Interesting in this context is the probably chemotropic guidance of the pollen tube into the micropyle. The literature on chemotropic activity of the ovary and the ovules of lily contradict, as was reviewed above. This was the reason to carry out our own experiments. In these, ovules did not show positive chemotropic attraction, both on an agar plate and on a millipore filter. Prepollination of the flower before the ovules are excised, hereby inducing an ovule enhancement which probably lacks after cut-style pollination, did also not influence the behaviour of the pollen tubes.

This led to the question of how the pollen tubes behave in the pistil prior to ovule penetration. This, together with the interaction of the pollen tube with the pistil followed by fertilization, was studied in chapters 2 and 3. It is followed by the study of pollen tube growth after different flower manipulations such as cut-style pollination (chapter 4). Placental pollination in vitro gives more opportunities to manipulate both pollen and ovary and is the topic of chapter 5. In chapter 6 a reproductive calendar is presented.

Pistil exudate production, embryo sac development, receptivity and pollen tube growth in *Lilium longiflorum* Thunb.

2

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Introduction

The development of the micro- and megagametophyte and the pollen tube growth in lily has been a topic of study for many years. The growth and incubation conditions and the plant material can have a large influence on the time scale of those processes and even disturb them completely. The length of the pollen tubes is strongly influenced by the temperature, and increases from 11 till 30°C for compatible and from 11 till 39°C for incompatible pollen tubes (Ascher and Peloquin, 1966b and 1970). The result is that data from the literature are hard to compare when obtained under different circumstances. Before manipulations of a system are carried out, which is the aim of our future work in lily, the main basal processes must be well understood.

An exterior parameter for the developmental phase of different processes inside the flower bud might be desired for practical and fundamental work. Erickson (1948) found that the flower bud growth of *Lilium longiflorum* 'Floridii' is exponential and its length is precisely related with processes of anther development: length, fresh weight, dry weight, meiosis and mitosis. The growth of the anther was studied in more detail by Gould and Lord (1988) and was not equally dispersed over its surface resulting in some regions growing more than others. The relationship between total anther and tepal length is characterized by two phases. Plotted both at a logarithmic scale, the length of the anther and of the flower bud have two connected linear relationships. In the second phase a relative smaller length increase of the anther in relation to the bud length takes place compared with the first phase.

In the *Lilium* hybrid 'Enchantment' the development of the pollen wall and changes in cell organelles of developing pollen were related to the flower bud length (Willemse and Reznickova, 1980). The megasporogenesis of the same cultivar was studied by De Boer-de Jeu (1978), but unfortunately the relation to the bud length was not described. A difficulty hereby might be that the development of the embryo sacs in an ovary is not synchronous as is observed in *L. longiflorum*, in which there is a variation between cultivars as well (Van Went et al., 1985).

In this chapter the exudate production, embryo sac development, stigma and style receptivity and pollen tube growth of *L. longiflorum* are studied as processes of and in

relation to flower bud development.

Material and methods

Plants of *Lilium longiflorum* Thunb. were grown yearround in pots in a greenhouse of CPRO-DLO, Wageningen, with additional illumination in the wintertime. Bulbs were stored at a low temperature (0°C). The day temperature was on average 17 - 20°C, with peaks of 30 - 35°C in the summer, the night temperature had a minimum of 15°C. In the experiments the development of flower buds of *L. longiflorum* 'Gelria' was studied and for pollinations the compatible 'White American' was used. Flower buds of 'Gelria' were emasculated 1 - 2 days before anthesis and the stigma was covered with an aluminium foil cap, which was always done when used after anthesis. Unless otherwise stated, pollination was carried out two days after anthesis. To determine the relationship between the bud length and the time to anthesis, 35 buds were observed. In some experiments flowers were placed in a growth chamber with 16 hours light at 25°C and if so, those cases will be mentioned in the text.

- Embryo sac development -

The developmental stages of the embryo sacs were determined after fixing peeled ovaries in FPA_{50} (37% formaldehyde : 99% propionic acid : 50% ethanol = 5 : 5 : 90) for at least 24 hours (Herr, 1973). The ovary was divided into four sectors each having an equal number of ovules, with sector 1 at the top of the ovary and sector 4 at the basis, according to Van Went et al. (1985). Subsequently the ovary parts were dissected and a placenta with one row of ovules was pre-weakened in 88% lactic acid at 60°C for 45 min. The inner integuments with nucelli and embryo sacs were mechanically isolated and cleared in Herr solution (Herr, 1973). The embryo sac contents was observed using a differential interference contrast microscope.

- Structure of secretory cells in the ovary -

Parts of the placentas from flowers of different ages, both pollinated and unpollinated, were fixed for transmission electron microscopy (TEM) to study their ultrastructure and the production of exudate. The tissues were dissected in 0.1 M phosphate buffer at pH 7.2 and subsequently fixed for 4 hours in 3% glutardialdehyde in the same buffer. After repeated rinses in the buffer, the tissues were post-fixed for 6 hours in 1% osmium tetroxide in the phosphate buffer containing 1.5% potassium hexacyanoferrate (II) ($K_4Fe(CN)_6$; Van Dort et al., 1983). During dehydration in a graded ethanol series the material was stained for 1 hours in 0.5% uranyl acetate in 70% ethanol. The tissues were embedded in low-viscosity resin (Spurr, 1969). Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Philips 301 and a JEOL JEM-1200EX II.

- Pollen tube growth -

The length of the pollen tubes in the style was measured after longitudinal dissection of the style followed by staining with water diluted cotton blue (Asano, 1980c) or by isolating the pollen tube bundle with a preparation needle. In both halves of the style the longest pollen tube was measured. Sperm cell formation was observed using an aqueous solution of 50 μg/l DAPI (4'6-diamidino-2-phenylindole.2HCl) at pH 4.0.

To study the amount of exudate and the pollen tube growth in this substance stigmas, parts of the style and the ovary were frozen in solid nitrogen in an Emscope SP2000A. The tissues were cryo-fractured, followed by sublimation for 3 min. at -80°C and sputtering with gold. The material was observed with a JEOL JSM-35C cryo scanning electron microscope (cryo SEM).

To follow pollen tube growth with the non-cryo SEM, material was fixed 4 hours in 5% glutardialdehyde in 0.1 M phosphate buffer, pH 7.2, with 0.25 M sucrose, rinsed in buffer with sucrose and postfixed for 6 hours in an aqueous solution of 1% osmium tetroxide. After rinsing and dehydration in ethanol the material was critical point dried over CO_2 and sputtered with palladium. The material was observed with a JEOL JSM-5200 SEM.

To determine pollen tube penetration in the micropyle, ovules were cleared in a mixture of lactic acid, glycerol and water (1:2:1) at 80°C for 30 min., stained for two minutes in this solution supplemented with 1% aniline blue at the same temperature and destained again in the clearing solution at 80°C (modification of Gerlach, 1977).

- Electrophoretic analysis -

Electrophoretic protein separation was carried out with the PhastSystem™ of Pharmacia. Ovules or other pistil tissues were homogenised in the presence of a small volume of water or 0.01 M tris HCl buffer, pH 6.8 (usually two rows of ovules in 20 μl) and centrifuged at 16000g for 10 min. Each combination of sample and electrophoretic procedure was at least repeated twice using material of different flowers. Different gels and power procedures were used:

* native polyacrylamide gels in a gradient of 8 - 25% polyacrylamide with samples of unpollinated ovules, 2, 4, 5 and 6 days after anthesis (DAA) and pollinated ovules, 2, 3 and 4 days after pollination (DAP),
* sodium dodecyl sulphate (SDS) polyacrylamide electrophoresis in a gradient of 8-25%. Prior to application the samples were boiled for 3 min. in 10 mM Tris-HCl pH 8.0, 1mM EDTA, 2.5% SDS, 0.01% bromophenol blue with 5% ß-mercapto-ethanol, the concentrations in the final solution. The same range of samples as the above mentioned were used, supplemented with samples of the ovary wall at 2 DAP and compared with an unpollinated control of the same age,
* isoelectric focusing (IEF) in a pH range 3 - 9 with ovules from 1 till 7 DAP or unpollinated ovules from 3 till 9 DAA. Also two dimensional electrophoresis was carried

out with IEF (pH range 3 - 9) with samples of ovules at 7 DAP compared with ovules at 9 DAA, or with IEF (pH range 4 - 6.5) and samples of 2 DAP and 4 DAA in the first dimension and a native gradient gel (8 - 25%) in the second.

The gels were stained in the PhastSystem Development Unit with coomassie brilliant blue according to Olsson at al. (1988) for a SDS-page and with adjustments as described in the 'Development Technique File No. 200' of Pharmacia for native-page and IEF. In the latter the gels were first fixed in 20% trichloroacetic and the preservation solution was omitted. The silver staining was a modification of Olsson et al. (1988) as well. The gels were fixed in trichloroacetic acid as described, but both before and after the 8.3% glutardialdehyde treatment the gel was rinsed twice in 10% ethanol and 5% acetic acid in water. The silver nitrate treatment was preceded and followed by extra Milli-Q water rinses. The subsequent development of the gels took place in a aqueous solution of 2.5% sodium carbonate with 0.015% formaldehyde and was continued by hand till the desired darkness of the gel was achieved.

Results

- Exudate production at the stigma and in the style -

The exudate production was studied in buds from 80 mm to an open flower, 2 days after pollination. A bud reaches 80 mm at about 8 days before anthesis (fig. 1). In this stage the stigma is still almost dry although exudate is erupting locally at the surface of the stigmatic papillae as shown in fig. 2, a cryo SEM micrograph of a part of the stigma. Just underneath the stigma (fig. 3) and halfway down the style a thin layer of fluid is covering the secretory cells, accumulating where two cells meet each other. At the basis of the style the cells are still flattened and also here some fluid is gathering as described above.

At a length of 100 mm, six days before anthesis, and about 1 cm underneath the stigma, the exudate is especially present at the top of the rounded secretory cells. Around this accumulation the layer of fluid is still thin, comparable to that observed in a bud of 80 mm. Some fluid is also gathering where two cells meet each other (fig. 4).

Three to four days before anthesis, at a bud length of 120 mm, more exudate has been secreted at the stigma and in the style. At the stigma some papillae are covered together by one layer of exudate, while others are still solitary (fig. 5). In the style the local accumulation of exudate on the top of each secretory cell has spread out, at a number of places, by flowing together with the exudate on neighbouring cells (fig. 6). At other cells the accumulation has enlarged (fig. 7). Halfway down the style this pattern of secretion is not observed, and the exudate is gathering were the cells border each other (fig. 8). This also occurs at the basis of the style where the secretory cells have rounded up (fig. 9).

Two days after anthesis only the top of the stigma papillae are visible above the thick layer of exudate (fig. 10). In the top of the style an almost continuous layer has been

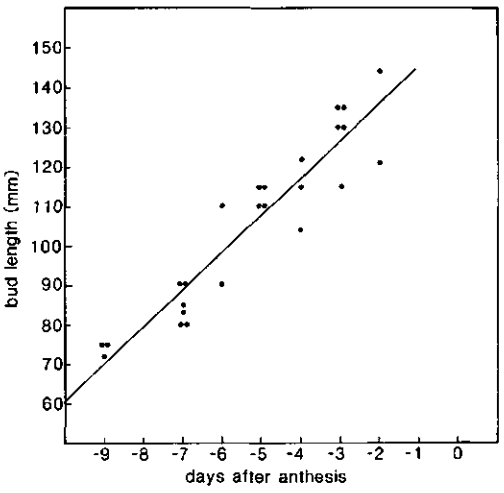

Fig. 1. The growth of the tepals of buds of *Lilium longiflorum* 'Gelria'. The line represents $y = 9.3x + 154$, $r = 0.95$, $n = 33$, experiment carried out in September.

formed by fusion of the accumulations on top of the secretory cells. The stylar canal has three lobes and the exudate is preferentially gathering in the most abaxial parts, having a continuous smooth surface (fig. 11). Where the layer is thin, in between the lobes, the surface of the exudate is irregular: secretory cells protruding into the stylar canal form bumps in the exudate layer. In between the lobes, at the top of a secretory cell, the exudate layer is about 1 μm thick, in the lobes a thickness of 60 μm is reached.

- The structure of the secretory cells in the ovary -

The long secretory cells in the style gradually shift towards shorter, more spherical cells in the ovary, covering the placentas in each of the three cavities. Each placenta has two rows of ovules. As observed by SEM or light microscopy, the inside of the pericarp has a smooth appearance with scattered stomata. When observing the placental cells with the TEM, the wall ingrowths appear to border a more fibrillar cell wall (fig. 12). The size of the wall ingrowths increases when comparing two with six days after anthesis. Figs 12-16 are thus from cells developing their wall ingrowths. Different structures near the plasma membrane at the place of the developing wall ingrowths can be observed. Electron lucent (fig. 13) and electron dense vesicles both possessing a membrane, fuse with the plasmalemma. Both types of vesicles have a comparable size of around 60 nm (fig. 14) and probably originate from dictyosomes (fig. 15). In between the wall ingrowths strands of plasma are present. The result is a whimsical inner granular wall, with paramural bodies, osmiophilic islands and a fibrillar component.

Two days after anthesis the nucleus is surrounded by starch grains. The basal part of the cell, the side opposite the wall ingrowths, is more vacuolate compared to the upper part. The plasma is rich in endoplasmic reticulum, both smooth and rough, mitochondria, mono- and polysomes and dictyosomes which are actively producing vesicles. There are

some lipid bodies. The secretory cells are connected mutually and to the underlying parenchyma cells by plasmodesmata. The latter cells are also rich in starch. The secretory cells are only partly connected with each other and where their radial and transverse walls disjunct an electron dense site in the cell wall is observed (fig. 16). This darkening of the cell wall was not observed in the intercellular spaces in the underlying tissue. The cuticle is usually still present at this point and is vanishing towards the top of the secretory cell. Large fragments of a thin cuticle were observed in connection with the thicker one observed in between the cells. The last cells of the secretory zone sometimes still have their cuticles but it is largely stretched and a contact with the cell wall is only present at the side of the cells.

Six days after anthesis the amount of starch in the placental secretory cells has decreased and the amyloplasts are more osmiophilic. No difference was observed between the placental cells at the micropylar side of the ovules and those situated at the central placenta or those present between two adjacent ovules.

- The development of the megagametophyte -

With the technique of fixing and clearing of ovules, different stages of embryo sac development can be easily determined. The embryo sac development takes place according to the Fritillaria type (Bambacioni-Mezzetti (1928) reviewed by Johri and Ambegaokar, 1984), but during the last cycle of mitotic divisions to form the complete embryo sac, no equatorial plane of the spindle of the chalazal triploid nucleus is formed and the segregation of the chromosomes is disturbed (figs 17 and 18 at p. 29). The result is a 7-nucleate embryo sac, which finally contains 2 antipodals. The polar nuclei do not fuse until fertilization (see also chapter 3). The haploid polar nucleus remains in its position close to the egg apparatus, whereas the triploid polar nucleus stays close to the

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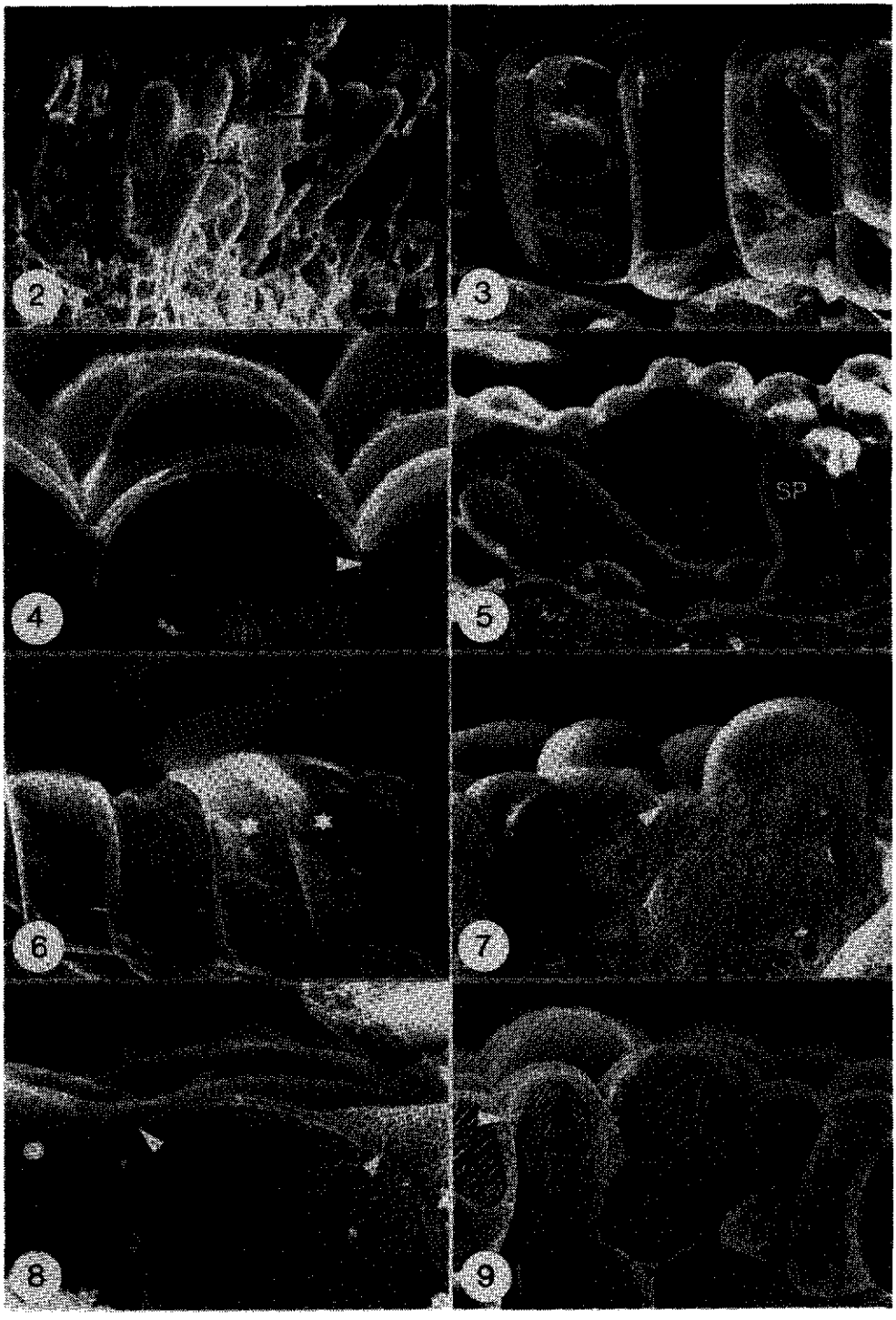
Fig. 2. A cryo SEM micrograph of a stigma of a flower bud of 80 mm. Arrows indicate what might be the first production of exudate. Magn. 518x.

Fig. 3. In a bud of 80 mm, just underneath the stigma, secretory cells are covered by a thin layer (arrow) as observed by cryo SEM. Magn. 2396x.

Fig. 4. Exudate gathers especially at the top of the secretory cell, but also, although to a lesser extend, in between the cells (arrowhead). A cryo SEM observation in the top of the style of a bud of 100 mm. Magn. 5338x.

Figs 5-9. Cryo SEM observations in a bud of 120 mm. Fig. 5. Exudate covering the stigmatic papillae (SP). Magn. 697x. Fig. 6. In the top of the style exudate accumulations are observed to cover more than one secretory cell (*). Magn. 1769x. Fig. 7. Just underneath the stigma at the top of the secretory cell a large accumulation of exudate is present, but also in between the cells the amount of exudate is prominent (arrowhead). Magn. 3382x. Fig. 8. Half-way down the style the exudate gathers in between the secretory cells (arrowheads). Magn. 5406x. Fig. 9. At the basis of the style the secretory cells have rounded themselves and a small amount of exudate has gathered in between them (arrowhead). Magn. 2771x.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antipodals. Just before anthesis a quarter of the embryo sacs are in the seven-nucleate stage, but also the 2-nucleate stage can still be found. One day after anthesis the 7-nucleate stage is present in at least 65% of the embryo sacs, as a total of one row of ovules. Two or three days after anthesis in 77% is mature. These numbers vary considerably among experiments, between seasons and also within flowers. In February for instance no ovule was in the mature stage at 2 days after anthesis. In the same flower in which the 65% was found an exceptional 92% was observed in another row of ovules. These are results from an experiment carried out in September. In all experiments the development of the embryo sacs of sector two of the flower is ahead of the other sectors (fig. 19).

- Pollen tube growth at the stigma and in the style -

After pollinating the stigma, the bicellate pollen grains germinates and the pollen tubes grow over the stigma followed by a passage through the slits in the stigma lobes towards the stylar canal (figs 20 and 21). The growth over the stigma seems to take place into all directions apart from places close to the slits. Arriving at the side of the stigma, which happens because of the undirected pollen tube growth, the pollen tube remains growing in the exudate and because of the three lobed shape of the stigma it finally grows towards one of the three slits. In a growth chamber the first sperm cell formation occurs 13 to 16 hours after pollination, when the pollen tubes have penetrated the style for 15 - 25 mm. At 24 hours after pollination there are still some pollen tubes with an undivided generative cell.

The speed of the pollen tube growth was determined by measuring the length of the pollen tubes reached after different time intervals after pollination and subsequent incubation in the growth chamber. After a slow start, the pollen tubes grow 2.4 mm/hour

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Figs 10-11. Cryo SEM observations in a flower two days after anthesis. Fig. 10. The thick layer of stigmatic exudate (arrows) covers the papillae (SP) almost completely. Magn. 504x. Fig. 11. In the top of the style exudate is gathering in the lobes, hereby creating a smooth surface. Magn. 1108x.

Figs 12-16. Observations made with TEM of placental cells two days after anthesis (DAA). Abbreviations: D dictyosome, ER endoplasmic reticulum, FC fibrillar component, OI osmiophilic islands, PL plasmalemma, PB paramural body, S starch. 1: refers to the outer fibrillar layer and 2: to the inner granular, fibrillar layer of the cell wall. Terminology according to Dashek et al. (1971). Fig. 12. The fibrillar cell wall with wall ingrowths of a different structure of a placental cell. Magn. 13.9Kx. Fig. 13. Fusion (arrow) of electron lucent vesicles with the plasmalemma. Note the paramural bodies. Magn. 52.1Kx. Fig. 14. Electron lucent (arrowhead) and osmiophilic vesicles are of comparable size and present in the proximity of the wall of the secretory cell. Magn. 32.0Kx. Fig. 15. A dictyosome with electron lucent (arrowheads) and electron dense (arrows) vesicles in its proximity. Magn. 41.8Kx. Fig. 16. Electron dense site in the cell wall (*) where two placental cells separate. The cuticle (C) is present at this point but vanishes further towards the top of the cell. Magn. 11.2Kx.

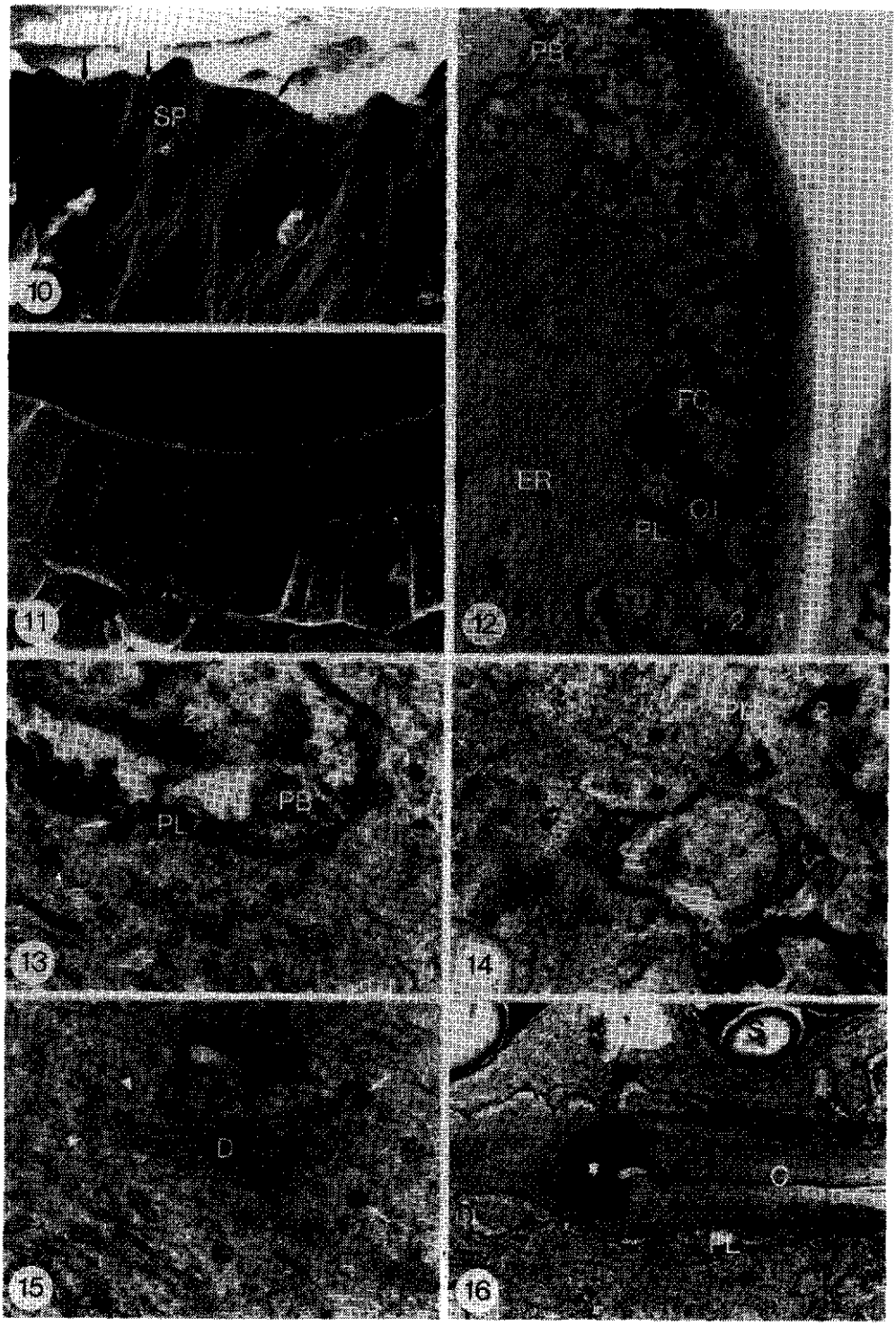
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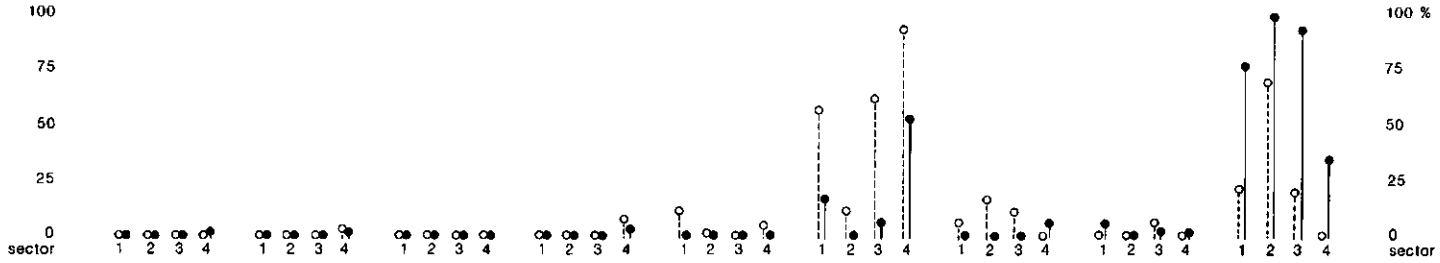
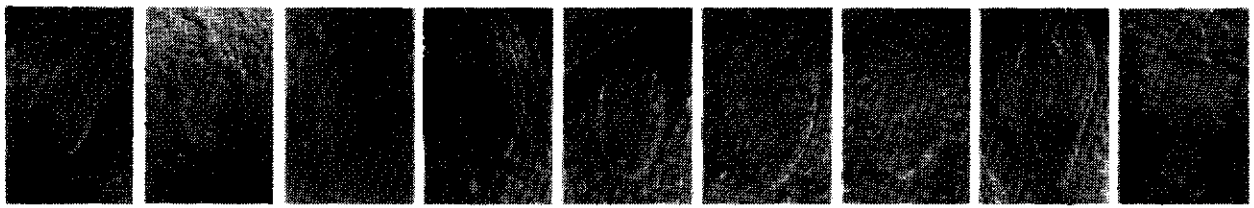
Fig. 19. The percentage of embryo sacs of *Lilium longiflorum* in a developmental stage per sector of the ovary (sector 1 at the top, 4 at the basis of the ovary) at one day before anthesis (o--o) and one day after anthesis (●–●).





developmental stage	division to the 2 nucleate stage	2 nucleate stage	division to the first 4 nucleate stage	first 4 nucleate stage	division to the second 4 nucleate stage	second 4 nucleate stage	division to the 7 nucleate stage	7 nuclei in the embryo sac	ripe embryo sac

from 6 hours till about 48 hours after pollination (n=72 styles, July-September). Then the pollen tubes have reached the end of the style, after which measurements are difficult. The time of the day at which pollination is carried out does not influence the pollen tube length reached after 24 hours, which is on average 47 mm (n=43 styles tested in 7 groups between June-September, standard deviation between the groups $\sigma_{n-1}=3.5$ and between all styles $\sigma_{n-1}=5.4$, growth chamber). After 48 hours the pollen tubes have reached the ovary. In the greenhouse this growth was slower.

Two days after pollination, which is four days after anthesis, an enormous number of pollen tubes can be observed in the exudate in the top of the style. Using cryo SEM only little contact with the secretory cells was observed. The pollen tubes grow sometimes close to the exudate surface, especially at places where the exudate layer is thin. In the stylar lobes the secretory cells are first bordered by an exudate layer, followed by a layer with pollen tubes surrounded by fluid and then again a layer of exudate. Pollen tubes also appear as rows on top of each other (fig. 22). Two days after pollination and incubation in the greenhouse some pollen tubes have arrived halfway down the style. Here as well the stylar canal has three lobes and the exudate layer is of varying thickness. At places were an abundant amount of exudate is present, the pollen tubes seem to grow freely in the fluid, being spherical in cross section. At places where the layer is thinner, the pollen tubes more often have a flattened appearance (fig. 23). Similar as observed near the top, also halfway down the style the exudate layer has a smooth surface.

At the basis of the style the pollen tubes grow from the stylar surface towards one of the three central situated placentas. Pollen tubes hereby seem to follow the secretory cells, although some bend earlier than others, so they spread out.

- Pollen tube growth in the ovary -

Two days after pollination, when the first pollen tubes have reached the end of the style, exudate fills the space between the inner integument and the pericarp (fig. 24). At the outer integument fluid is detected only at the points of contact with the pericarp. This amount has increased four days after pollination. By that time the placenta is covered by an abundant amount of exudate (fig. 25). Seven days after pollination the space between the ovules is sometimes partly filled with exudate but also a situation comparable to that observed two days after pollination can be found. Unfortunately we could not observe whether there is any fluid in the micropyle. Pollen tubes were present in the exudate.

On entering the ovary the first few ovules are usually neglected by the pollen tubes. Especially the first ovule has a different shape, being more spherical compared with the flattened shape of the ovules further down the ovary. Pollen tubes grow in the exudate, over the placenta in between the two rows of ovules and one by one bend 90° to grow in between two adjacent ovules towards their micropylar side (fig. 26). After dissecting flowers at different intervals after pollination later arriving pollen tubes were observed to either grow further down the ovary to penetrate still empty micropyles or bend towards the same or the first ovules. At four days after pollination the central pollen

tube bundle still has not reached the basis of the ovary.

Arriving at the micropylar side the pollen tubes have to bend again about 90° to reach the inner integument. Hereby they usually grow towards the base of the flower, parallel with the pollen tubes in the cental bundle, but an upward growth has also been observed. In this way a small bundle of pollen tubes is formed at the micropylar side of the ovules. This narrow path as well is lined with secretory cells. To penetrate the micropyle again a slight change in direction is necessary (fig. 27). Later arriving pollen tubes fill up the space between the inner integument and the pericarp. Pollen tubes are also observed to bend back towards the central bundle, and even a second return to the micropylar side of the ovules is no exception.

- Penetration of the ovule -

Penetration of the ovules starts three (growth chamber) to four (greenhouse) days after pollination and after 7 days of pollen tube growth the penetration of a maximum percentage of ovules has been reached (table 1). This percentage varies with the time of the year, between and within the flowers, in the latter both between different rows of ovules and between the different sectors. An example of the difference between sectors is the observation that in sector two the percentage of penetration is higher in comparison with the ingrowth in the whole row of ovules (52 vs. 29%, the average of two flowers, 5 DAP, but also confirmed in other experiments). This is a consequent difference in contrast to the differences between the rows of ovules. The axis of the flowers is close to horizontal. When the position of the three ovarian cavities relatively to the main axis of the plant is recorded and the percentage of ingrowth is determined per cavity, there is a variation, but this is, as mentioned above, not consequent. When the axis of the flower is kept vertical with the stigma downward, directly following compatible pollination, the

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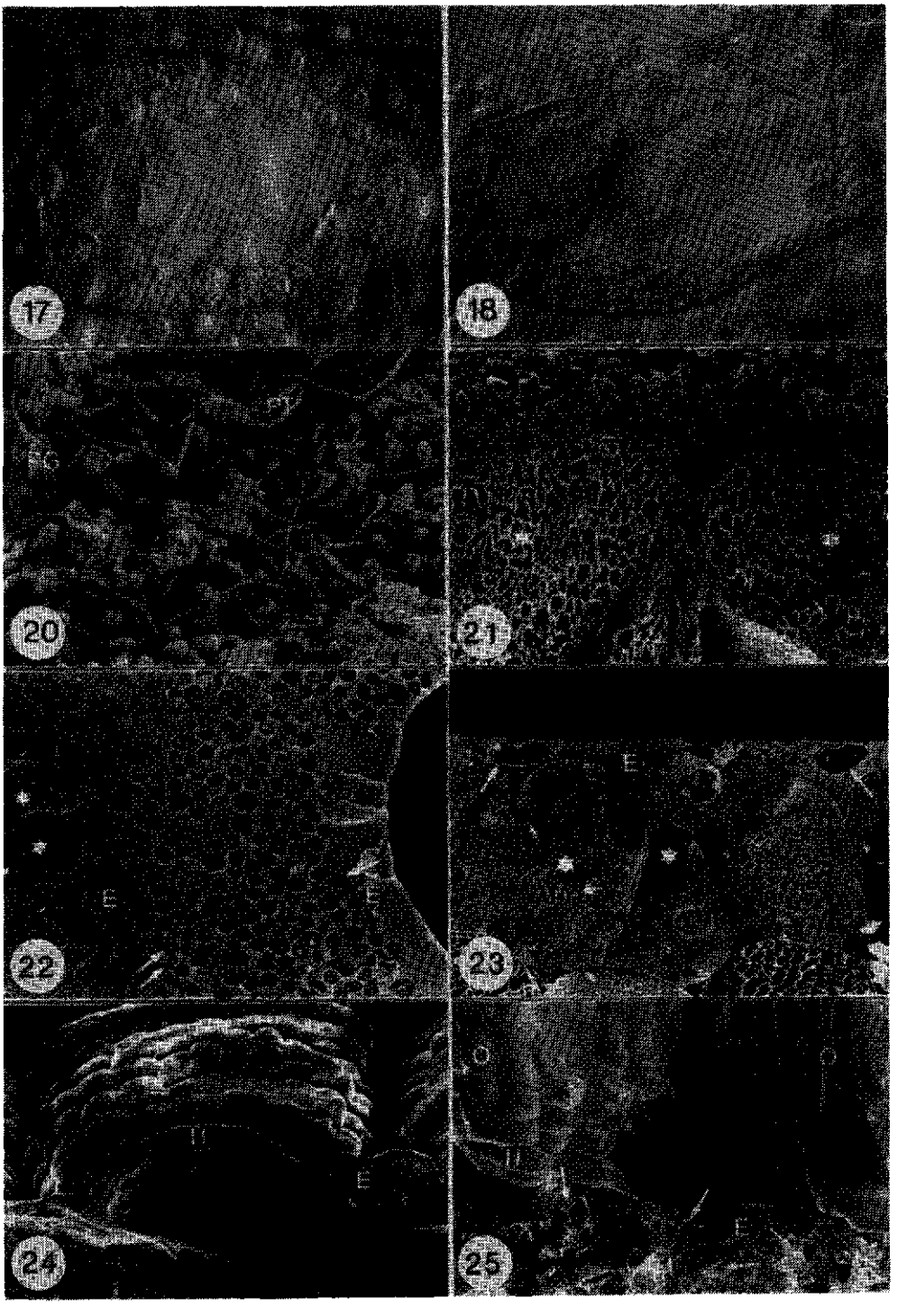
Figs 17-18. During the last mitotic division to form the mature embryo sac the chalazal spindle (arrow) lacks an equatorial plane (fig. 17) and the segregation of the chromosomes is disturbed (fig. 18). Nomarski interference microscopy after clearing, magn. 183x.
Figs 20-21. Compatible pollination of *L. longiflorum* 'Gelria' with 'White American'. Fig. 20. The pollen grains (PG) germinate and the pollen tubes (PT) grow over the stigma as observed with SEM. Magn. 240x. Fig. 21. A SEM micrograph of pollen tubes (PT) growing in between the stigma lobes (*) into the stylar canal. Magn. 96x.
Figs 22-23. Secretory cells (*), exudate (E) and pollen tubes (arrows) two days after compatible pollination as observed with cryo SEM. Fig. 22. In the top of the style, at both sides of the pollen tube bundle a layer of exudate is present. Magn. 1022x. Fig. 23. Half-way down the style a part of the pollen tubes have an irregular shape. Magn. 2556x.
Fig. 24. A cryo SEM micrograph of an ovule after the pericarp was broken away, but leaving its print behind in the exudate (E). The exudate fills the space between the inner integument (II) and the pericarp. Magn. 514x.
Fig. 25. The placenta (arrows) is covered with exudate (E) at four days after pollination. Inner integument (II), ovule (O), magn. 479x.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pollen tubes reach the ovules.

- Electrophoretic analysis of the ovules -

The protein patterns on the gels of ovule samples did in some cases show differences between pollinated and unpollinated ovules of the same age, using the different electrophoretic methods as described in the Materials and Methods section, but the differences could not be repeated. Neither pollination nor aging induced a constant difference in protein patterns in the mentioned intervals. As an example, after two dimensional electrophoresis, IEF (pH 3 - 9) followed by a native gradient gel, of ovules sampled at 9 DAA in comparison with 7 DAP, after which at least a part of the ovules are fertilized, some differences occurred (fig. 28 and 29), but this variation in the protein pattern could also not be repeated.

- Senescence of the flower -

Six days after pollination (8 DAA) the perianth starts to turn brown evenly and after another three days it has died and drops, together with the stamens. The style is wilting synchronously with these organs, but this process starts at the stigma and at the top of the style and proceeds to the basis followed by its dropping. The dropping of the style might be delayed by one day. In unpollinated flowers the change of perianth colour from white to brown is delayed with 4 days. (Experiment with 6 flowers.) Pollinating the

Table 1. Penetration percentages at different days after pollination (DAP), carried out at two days after anthesis, as determined after pollination and incubation in the growth chamber and in the greenhouse. Per flower three rows of ovules (on average 195) were checked after staining with aniline blue for pollen tube penetration. The results of two flowers were averaged apart from growth chamber 5-7 DAP, in which the percentages of 4 flowers were averaged. Experiment carried out in September.

DAP	growth chamber	greenhouse
2	0	0
3	38	0
4	76	29
5	70	63
6	82	80
7	90	92

flower in a growth chamber or in a greenhouse, just before the style drops does not result in ingrowth of the pollen tubes into the ovules. There is a poor germination because the stigma has almost dried out.

- Embryo and seed formation

After pollen tube penetration in the embryo sac the fusion of the two sperm nuclei, one with the nucleus of the egg cell and one with the haploid polar nucleus, starts almost immediately (see chapter 3). The nucleus of the central cell divides at 12 days after pollination (DAP). The first division of the zygote was observed from 15 DAP (figs 30 and 31), and soon the majority of the developing embryos is in a two cellular stage. The development is not synchronous between the different ovules and 17 DAP also four-cellular embryos were observed next to the bicellate ones. The endosperm has then more nuclei than the embryo. 35 DAP the embryo is less than 1 mm of length and egg shaped. 42 DAP it has grown to a length of 1.5 - 2 mm, 48 DAP it starts to bend and is larger than 2 mm. 65 DAP the average length has increased to 3 mm and in cross section the embryos are flattened. The length of the larger embryos from 35 DAP on varies considerably. Around 75 DAP the seed capsule is mature (see also chapter 6).

In case of a good seed set, which implies the complete swelling of the ovary, all ovules of the ovary enlarge, but only a part of them include an embryo. In case of a poor seed set the swelling of both the ovules and the ovary is restricted to just a part of the ovary. Neither the removal of the perianth at the time of pollination, what will be necessary in some manipulation experiments, nor damaging it influences the percentage of seeds with an embryo (table 2). In nine out of ten flowers sectors two or three of the ovary show the highest percentages of seeds with embryos. This percentage is especially low in sector four (table 2). After pollination at different style lengths (see chapter 4) the percentage of ovules with a pollen tube in the micropyle seems to run parallel with the percentage of seeds with an embryo.

- Pollen tube growth in flower buds -

In buds from seven days before anthesis pollen tube growth, as observed 24 hours after pollination, is possible. The length of the pollen tubes is however strongly reduced compared with pollinating at or after anthesis. From anthesis till about seven days later the length of the pollen tubes, reached 24 hours after pollination, is more or less constant at its highest level. In older flowers it drops again. This tendency is also obvious when the pollen tube growth after 40 hours is measured, with a difference that pollen tube growth is also found in even younger flower buds.

With cryo SEM pollen tubes were observed in the top of the style at one and three days after pollinating a flower bud of 120 mm (three to four days before anthesis). One day after pollination the exudate layer has merged to a larger extent than the exudate in a flower bud of 120 mm. The accumulations on the top of the secretory cells were no

longer observed. The pollen tubes grew both over the secretory cells and in between two cells, always covered by at least a thin layer of exudate. On cross section some pollen tubes protrude into the stylar canal at places were only a thin layer of exudate is present (fig. 32). The pollen tubes are at the most stacked in three layers. Three days after pollination, at about anthesis, there are far more pollen tubes growing through the top of the style, compared with one day after pollination (fig. 33). They are packed close together in the exudate and also here the pollen tubes directly bordering the stylar canal protrude into it. Sometimes there is even doubt whether a layer of exudate is covering these pollen tubes (fig. 34).

The development of the embryo sacs is not influenced by pollination or pollen tube growth as was accessed in an experiment in which three different bud lengths were compared: 100, 110 and 120 mm (the length at the start of the experiment). Pollen tube growth was allowed for either five or three days, in the latter case the buds were pollinated two days later, and compared with unpollinated buds. All 9 buds were emasculated at the start of the experiment and per pistil one row of ovules was observed. During the experiments the buds of 110 and 120 mm opened. Also in other experiments (including a total of 12 flower buds) in which two and three days of pollen tube growth was allowed, no influence was detected. In these experiments penetration of the micropyle by a pollen tube only occurred in ovules were the embryo sac was in its mature stage.

- Receptivity -

Pollinating in the interval of four days before anthesis till seven days after anthesis and allowing five days of pollen tube growth, carried out with cut flowers in a growth chamber, results in penetration of ovules (table 3). Outside this time interval no micropyle penetration was observed. Pollination at the two extreme days of this period results in a penetration percentage which is lower than that of the intervening dates. This receptivity

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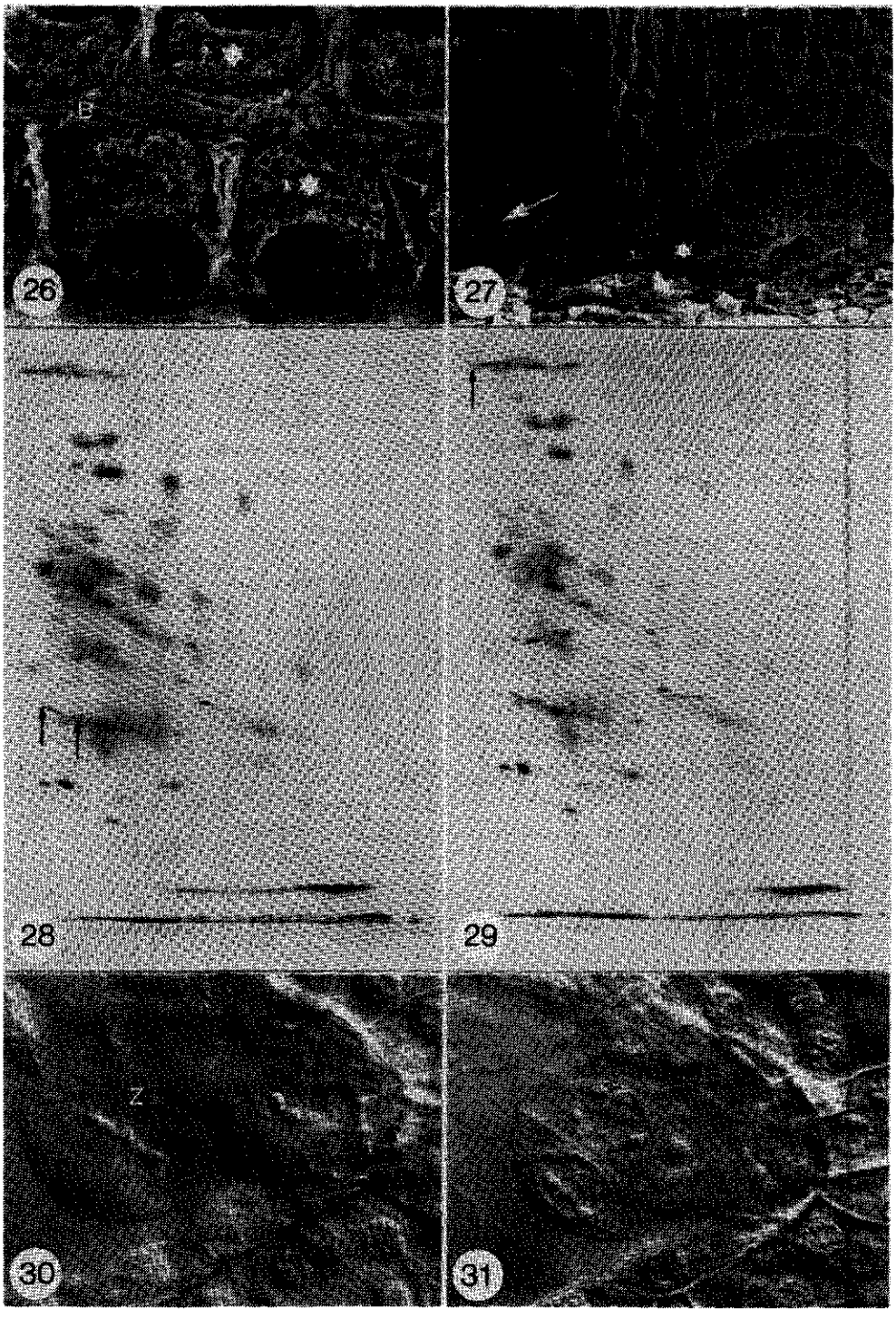
Fig. 26. Once arrived in the ovary the pollen tubes bend 90° from the central bundle (B) and grow over the placenta in between the ovules (here broken away, *) towards the micropylar side. A SEM micrograph, 6 days after pollination (DAP), magn. 192x.

Fig. 27. After growing from the central bundle and arriving at the pericarp, the pollen tubes bend again (*) and form a bundle at the micropylar side of the ovules. To reach the micropyle (arrow) another change of directory from the micropylar bundle is necessary. A SEM micrograph, 7 DAP, magn. 346x.

Figs 28-29. Protein pattern of unpollinated (fig. 28) ovules 9 DAA and pollinated (fig. 29) ones (7 DAP) of the same age after IEF on a pH gradient of 3 - 9, followed by a 8 - 25% polyacrylamide gradient. Arrows indicate some differences between the gels. Those differences were however absent in other experiments. Magn. 2.4x.

Figs 30-31. Division of the zygote (Z) 15 DAP (fig. 30) and the two cellular embryo (fig. 31) as observed with Nomarski. Magn. 300x.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period differs however for flowers left at the plant in the greenhouse.

Discussion

- Flower bud length -

A relationship of certain aspects of flower bud or flower development, whether or not pollinated, to a time scale is desired to serve as a matrix for further experiments. Some experiments are destructive and it is therefore impossible to determine the time for the bud to develop till anthesis. Other interventions might influence the speed of

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Table 2. Percentages of seeds with an embryo per sector of the ovary (sector 1 is the top and sector 4 is the basis of the ovary) and per flower after different treatments of the perianth: undamaged, damaged and removed. Experiment carried out in the greenhouse from May-July, n= the number of flowers, all seed were counted, on average 392 seeds per capsule.

treatment	n	sector	% embryo
perianth intact	4	1	38
		2	36
		3	39
		4	14
		average	32
damaged perianth	4	1	26
		2	34
		3	27
		4	19
		average	27
removed perianth	2	1	26
		2	37
		3	45
		4	27
		average	34

development of the flower. So even if it is possible to monitor the days to anthesis, additional experiments have to be carried out to determine this influence. The bud has to be related to other buds, but this can also be done to a standard. This standard can be e.g. colour, length and width and is preferably observed without disturbing the bud. In the reported experiments the bud length was the standard. Gould and Lord (1988) measured an exponential growth of the tepals of *L. longiflorum* 'Nellie White' on a length interval of 25 to 100 mm. An exponential enlargement until one day before anthesis was observed by Erickson (1948) in *L. longiflorum* 'Floridii'. Erickson suggested that the logarithm of bud length is a more precise indication than any other index which could be derived from chronological data. The interval in this chapter in which the bud growth was studied is smaller compared with the studies of Erickson. In our experiments the linear line fits better than the exponential one. The relation between bud length and days before anthesis results in a cloud of points in a graph, and even if the relation between the two factors is not linear the points used in the experiments still fit well into the cloud. The length of a flower bud in a certain stage can differ between and of course within cultivars or even among flowers of the same plant. Apart from that, in our experiments the bud length was related to days to anthesis, which is in 24 hours time intervals. And although in greenhouses there seems to be a slight preference of flower buds to open in the morning, probably due to the rise in temperature, buds will open at any time of the day. But despite these uncertainties the well fitted linear relation between bud length and time till anthesis is striking. Still care should be taken by relating the flower buds used in the experiments to the standard for the reasons mentioned above.

- Exudate production -

Exudate production was observed in buds of different lengths. A limitation of the used cryo SEM technique is that different layers or composition of fluid and the cuticle are hard to distinguish. E.g. in a bud of 80 mm, at the basis of the style, a little amount of fluid is observed, which could be exudate but also some other extracellular fluid, e.g. water from condensation, or both. The secretory cells have not rounded up at that stage and a transfer wall was not observed with the used method. The same problem emerges when observing the stigma of a bud of 80 mm. The fluid observed might be exudate, in which case it implicates that the production by the papillar cell is not evenly spread out over its surface. This was also observed at the surface of the obturator of *Ornithogalum caudatum*, at which in a later instant the substance merged as well (Tilton and Horner, 1980). It might also be a substance of a different contents than the exudate, because at the stigma of a bud of 120 mm the surface of the exudate is not smooth in appearance, which is expected when the fluids have the same contents. Another explanation is of a mechanical nature. A cuticle covers the papillae in early stages. The production of exudate expands it till it ruptures (Dickinson et al., 1982). Some of the exudate might still be caught in this cuticle debris.

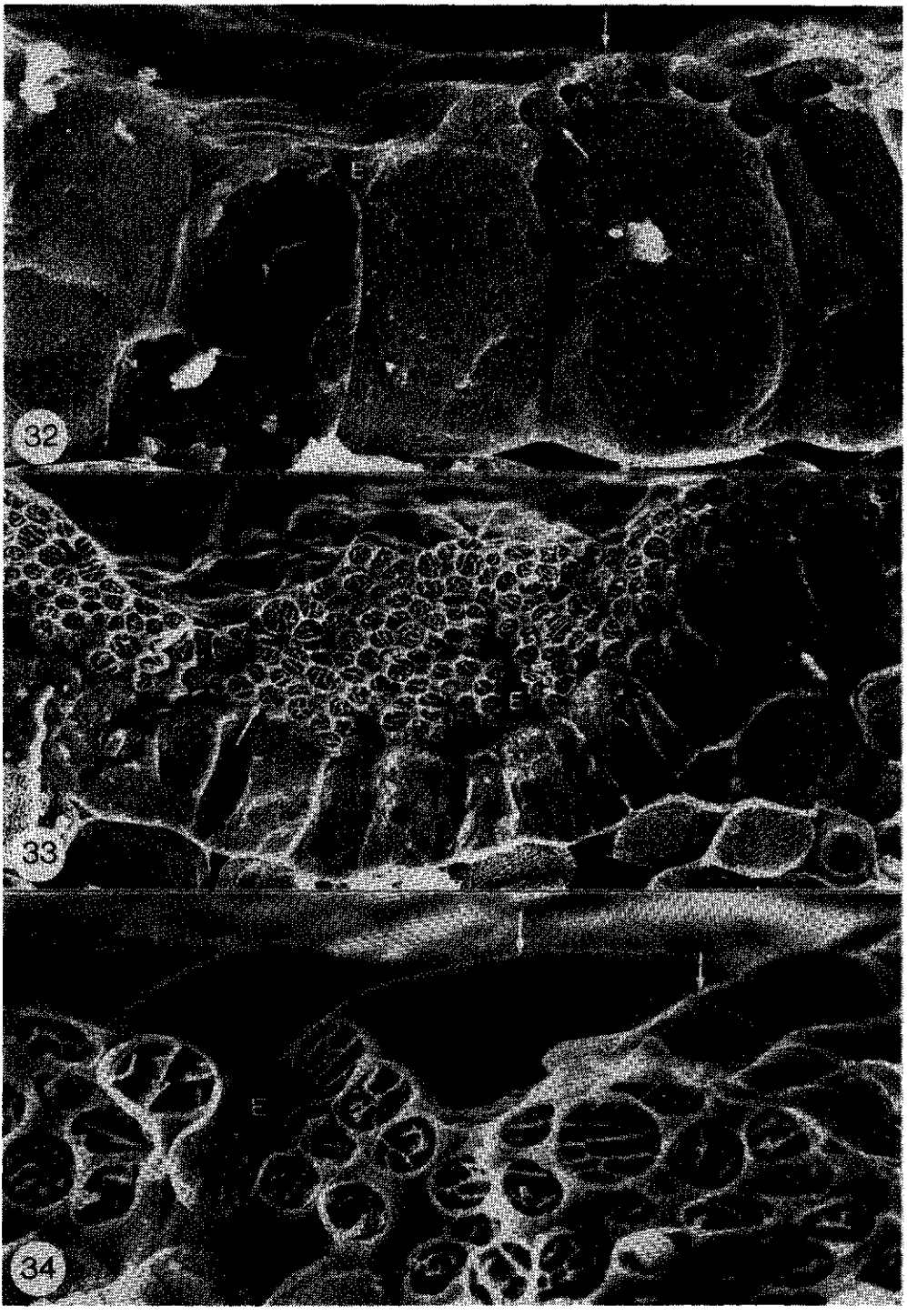
From the pattern of exudate production in the top of the style it is proved again

that the exudate is produced in the style and not flowing down from the stigma, which was indirectly proven by Rosen (1971). The production of exudate at the stigma and in the style starts in the bud stage and is not triggered by pollination. The shape of the exudate accumulations in the top of the style in a bud of 100 or 120 mm points towards a restriction in exudate movement. In between two secretory cells there is a reasonable amount of exudate, being a thinner layer towards the top of the secretory cell at which there is a tremendous accumulation again. In case of a free fluid the exudate would have levelled itself. Because of the absence of this phenomenon it might be that the cuticle is the restricting force, bulging out in case of exudate production. In *L. longiflorum* Rosen and Thomas (1970) did observe the exudate being captured by the cuticle till about the time of anthesis. Also in the study of Miki-Hirosige et al. (1987) it seems that the cuticle is lifted up or a large amount of bladders were formed. Rosen (1971) proposed a dissolving or rupturing, Dickinson et al. (1982) a fragmenting and 'floating off'. It is unknown whether the remains of the cuticle are present in between two secretory cells, which indicates that it is torn, as was observed at the placenta. If not it might be possible that, keeping the fusion of the accumulations in the top of the style in mind, the whole cuticle is floating from the cells.

In the disappearance of the cuticle from the placenta cutinase produced by the pollen tubes is not involved, at least not initially since, as in pollinated flowers, in unpollinated flowers the cuticle is lost except for the fragments found in between the cells. Because of the observation of thin fragments connected with the thicker ones covering the walls in between the secretory cells, the cuticle is stretched at first and ruptures. The pollen tubes have to grow in an exudate in which fragments of cuticles are present.

The cryo SEM method was also chosen to detect whether there is any fluid in the micropyle. Because of the breaking properties, the plane of fracture was situated along the inner integument, and never followed a cross section through the micropyle. A suitable way to achieve the latter may be the use of a cryo-fraise. But even if there is any fluid detected, it might not solve the problem of the origin of it. Due to the damage caused by ice crystal formation the ultrastructure of the cells can not be observed after the used cryo SEM method. If any fluid is observed in the micropyle can still originate from the placenta. This all is of interest to find the combination and sequence of processes which lead to the entrance of the pollen tube into the micropyle and subsequently into the synergid.

Figs 32-34. Cryo SEM observations in the top of the style after pollination of a bud of 120 mm. Fig. 32. One day after pollination only a few pollen tubes are detected and a straight growth over the complete surface of the secretory cells is observed. The pollen tubes (arrows) are covered by a thin layer of exudate (E) hereby protruding into the stylar canal. Magn. 3394x. Fig. 33. Three days after pollination more pollen tubes (arrows) have invaded the style and a larger amount of exudate (E) has been formed. Magn. 1300x. Fig. 34. The pollen tubes (arrows) being closest to the stylar canal seem not to be covered by exudate (E). Three days after pollination, magn. 4333x.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- Structure of the secretory cells -

The structure of the secretory cells in the style after cryo SEM preparation of large tissues is not clear due to ice crystal formation, as discussed above. But at two days after anthesis a distinct layer can be observed in the secretory cells where a transfer wall is expected. In younger stages this layer is not evident. It might be missing but also of a different chemical composition or rigidity. In *Lilium regale* a transfer wall was not present at four days before anthesis (Vasil'ev, 1970). In *L. longiflorum* the maximum development of this wall is achieved from three days after anthesis and the appearance does not change when the flower is pollinated (Rosen and Thomas, 1970).

The ultrastructure of the secretory cells covering the placenta is similar to the

Table 3. Penetration percentages at 5 days after pollination of pistils of different ages (DAA = days after anthesis) when pollinated. Flower buds and flowers were either left at the plant in the greenhouse or cut and placed in water in the growth chamber. Per ovary three rows of ovules were observed after staining with aniline blue. Each percentage is the average of two pistils, experiment carried out in September.

DAA	growth chamber	greenhouse
-8	0	1
-4 - -5	13	0
-3 - -4	34	1
-3	74	1
0	56	6
1	44	5
2	69	36
3	47	29
4	84	44
5	70	38
6	53	30
7	17	-
8	0	-
9	0	1

secretory cells in the style of *L. longiflorum* or *L. regale* as described by several authors (Dashek et al., 1971; Dickinson et al., 1982; Miki-Hirosige et al., 1987; Rosen, 1971; Rosen and Thomas, 1970; Vasil'ev, 1970). Both cell types have a transfer wall and a comparable composition of the cytoplasm. The outer layer of the transfer wall might be of a cellulosic nature associated with the fibrillar component in the inner wall, which is more of a pectic nature (Dashek et al.,1971). A sign of the fibrillar component in the inner wall is shown in fig. 12. The inner wall might be built up from the contents of vesicles with a diameter of 70 nm, seen elsewhere in the cytoplasm close to the dictyosomes (Dickinson et al., 1982). This size is comparable to the 60 nm vesicles found in our experiments. Next to the predominant role of Golgi bodies, endoplasmic reticulum is involved as well in the production of the cell wall of secretory cells in the style (Miki-Hirosige et al., 1987). If the origin of the exudate is in the parenchyma cells underneath the secretory cells, as was discussed by Yamada (1965), the latter are at least partly altering the exudate concerning the activity in these cells (Rosen and Thomas, 1970). Miki-Hirosige et al. (1987) launched the idea that the exudate is first stored in the cell wall before secretion. Crystals as observed by Gawlik (1984) in *Lilium leucanthum* were not found in our study.

- Pollen tube growth -

The cryo SEM method provides an impression of the exudate production and pollen tube growth. Preparation for traditional SEM largely washes the exudate away. In the pistil the pollen tubes are situated in the exudate and using the cryo SEM the pollen tubes can only be observed in fractures. For an overview of the pollen tube growth across a surface, for example at the stigma or in the ovary, the traditional SEM is more suitable. But using this method the pollen tubes collapse at places and their position might have been somewhat altered because of removing this exudate.

Pollen tubes at the stigma observed with non-cryo SEM do not have a directed growth towards the slits between the lobes apart from those being in close range of this slits. The pollen tubes grow over and in between the stigmatic papillae but are not observed entering the stigmatic surface apart from through the slits. During their growth they might bend down into one of the slits because the edges of the lobes are rounded and the pollen tubes, while following the surface, bend down as well. At that place there might also be a mixing of exudates from the stigma and the style leading to a directing of the pollen tube growth. While continuing this growth direction the pollen tubes enter the style.

Germination and the first six hours of pollen tube growth at the stigma of *L. longiflorum* was studied by Hiratsuka and Tezuka (1979). About 50% of the pollen grains in their experiments did not germinate and the variation in the pollen tube length is enormous. The longest pollen tube after six hours is 1 mm, so comparable to our results the initial growth is much slower than the 2.4 mm/h measured in the style. This linear growth of 2.4 mm/h was detected from 6 hours after pollination.

In *Petunia hybrida* Mulcahy and Mulcahy (1982) related two phases of pollen tube growth, differing in speed of growth and the appearance of callose plugs, to a shift from autotrophic to heterotrophic metabolism. Rosen and Gawlik (1966) observed a change in the ultrastructure of the pollen tubes in compatible pistils of *L. longiflorum*, but not in incompatible ones and related it to a relatively autotrophic first phase of nutrition and a more heterotrophic second phase. The self-incompatibility reaction occurs however between 12 and 24 hours after pollination as was measured by the pollen tube length in styles incubated at 24°C (Ascher and Peloquin, 1970). That should be the time interval in which the shift occurs from a growth on its own reserves to an uptake of substances from the pistil. In our experiments however the change in growth speed occurred after six hours. It its therefore unlikely that the change of speed is related to the shift. A slow germination and initial growth in the stigmatic exudate, which differs from stylar exudate (Dickinson et al., 1982 and Miki-Hirosige et al., 1987), is more likely the cause of the slow start. The complex nature of the stigmatic exudate is a good medium for acceptance and germination of pollen grains, but not of the right composition and/or concentration for a fast pollen tube growth. The concentration of solutes will vary due to evaporation in the greenhouse and the water condition of the plant. In the style probably a more constant medium with a pollen tube growth promoting constitution can be offered. To explain the acceleration of pollen tube growth in the style after stigmatic pollination compared with pollen tube growth during the same period in just stigmatic exudate, Asano (1980c) proposed a stylar factor enhancing the pollen tube growth within 12 hours.

The influence of pollination on stigmatic exudate was not studied with cryo SEM. Such an influence has been studied by Campbell and Linskens (1984a) reporting a decrease in the stigmatic exudate of *L. longiflorum* after self or cross pollination, after observing the general appearance and a touch test, i.e. much simpler techniques.

Sperm cell formation was not synchronous between different pollen tubes and was taking place at different pollen tube lengths. After interspecific pollination sperm cells were even observed in some ungerminated pollen grains (in the combination *L. longiflorum* x *L. henri*, unpublished). In the studied compatible combination this was not observed. After different manipulations (see chapters 4 and 5) sperm cell formation appeared to take place after a certain time interval, rather than after achieving a certain pollen tube length. Sperm cell formation is a process triggered by the activation of the pollen grain, although the pollen tube presence is important as well.

The pollen tubes in the top of the style are not in contact with the secretory cells. There is a separation caused by an exudate layer. Initially there might have been a contact but because of the continuous production of exudate the pollen tubes might have 'floated off'. Close to the surface of the exudate the pollen tubes lack as well. If the first pollen tubes grew in the proximity of the secretory cells, they might already have been covered by an exudate layer. This layer was also pushed towards the centre by the continuous exudate production. Later arriving pollen tubes preferred growing in between the secretory cells and the older pollen tubes or mix with the latter, resulting in a zone deprived of pollen tubes.

In the top of the style there is hardly any contact between the pollen tubes and the secretory cells. Guidance of those pollen tubes by the shape of the secretory cells as observed in *Lilium auratum* (Iwanami, 1953) does not seem to be likely, although the exudate layer between the pollen tubes and the secretory cells can be the result of constant exudate production by which the bundle is pushed away. A guidance by earlier pollen tubes is probably more likely. The first pollen tubes to arrive obviously do not have this guidance. In *L. auratum* a chemotropic gradient was not found (Iwanami, 1953). Also in the exudate in the style of *L. longiflorum* no cytochemical difference was detected along this route (Dickinson et al., 1982). Pollen tubes can however induce a gradient by consuming substances from the exudate. After labelling flowers with myoinositol-U-^{14}C and -2-^{3}H the radioactivity was traced in stigmatic and stylar exudate (Kroh et al., 1970a). Both compatible and incompatible pollen tubes take up label and use it for wall synthesis (Kroh et al., 1970b). Also in the cytoplasm of the pollen tubes radioactivity was traced after injection of labelled stigma exudate in the style (Labarca and Loewus, 1972). In experiments using D-glucose-U-^{14}C the radioactivity was traced both in exudate and in the cytoplasm and the cell wall of the pollen tube (Labarca and Loewus, 1973).

Chemotropy has been suggested in other species. Chao (1971) observed in *Paspalum orbiculare* a water soluble periodic acid-Schiff's substance, originating from degenerating inner and outer integument cells which hereby formed the micropyle and some intercellular spaces, and related it to directing the growth to the filiform apparatus. In *Strelitzia reginae* the pollen tubes grow through a stylar canal as well. In the ovary the growth is restricted to the transmitting tissue, probably due to a chemotropic agent or feeding substances (Kronestedt et al., 1986). The pollen tubes of *Beta vulgaris* might be directed towards the embryo sac by a presumed secretory activity of the micropylar nucellus (Bruun and Olesen, 1989). A secretory function of the nucellar cells has also been suggested for *Gasteria verrucosa* (Franssen-Verheijen and Willemse, 1990). In *Ornithogalum caudatum* a micropylar exudate is secreted by the nucellar cap and the inner integument (Tilton, 1980).

Also the structure of the pistil can direct the pollen tube growth. Because of the trichome structure of the stigma of *Pennisetum typhoides* most of the pollen tubes grow towards the ovary. After studying normal and mutant stigmas there seems no reason to suggest a chemotropic guidance in this species (Heslop-Harrison and Reger, 1988). In spinach the pathway of the pollen tubes through the style is determined by the structure of the cell walls and the morphology and distribution of the central core of the transmitting tissue (Wilms, 1980)

Another explanation which is very attractive for the pattern of pollen tube growth in *L. longiflorum*, was launched by Mascarenhas (1975), that straight pollen tube growth might occur when no external stimuli are changing the longitudinal growth axis. So translated to the situation in *L. longiflorum* it implies that once arrived in the style, the pollen tubes remain their straight growth, possibly helped by their external induced

gradient. But in the ovary some abrupt bends have to be made. The top of the pollen tube bundle exists of pollen tubes bending one by one, indicating an autonomous reaction, and not grouped for one ovule. This indicates that the stimulus to bend vanishes once used by one tube. But because later arriving pollen tubes bend to grow in between the same ovules again the stimulus should recover. Arriving at the micropylar side the pollen tubes bend again because of their tendency to grow in the proximity of the secretory cells. This zone ends at this side of the ovules to be bordered by the pericarp with the smooth surface and the scattered stomata. Because of the lack of space the pollen tubes were unable to pierce themselves in between the outer integument and the pericarp although some exudate is present there as well.

Arriving at the micropylar side of the ovule and growing along the secretory cells, the pollen tube entering the micropyle has to adjust its growth-direction again. The first pollen tube to arrive probably grows directly towards the micropyle when there is exudate produced to cover that pathway. Later arriving pollen tubes have to find their way around this penetrating pollen tube and at first mainly grow close to the funiculus, but later the pollen tubes fill up the space between the inner integument and the pericarp.

- Pollen tube growth in the ovary -

Two days after pollination the pollen tubes reach the end of the style. After another two days they have not yet reached the basis of the ovary. The length of the style is about 9 cm, the length of the ovary between 4 and 5 cm. The speed of pollen tube growth in the style was determined by measuring the longest pollen tube. In the ovary the pollen tubes bend to reach the micropyle. So the longest pollen tubes do not determine the extension of the pollen tube bundle into the ovary, unless they do not penetrate an ovule. Still not reaching the basis of the ovary in two days does point towards a slower growth of the pollen tubes in the ovary. This could be caused by a difference in exudate composition.

The pollen tube growth in the ovary shows a pattern. The first arriving pollen tubes neglect the first ovules. A stimulus to bend lacks, a straight growth is the result. Pollen tubes arriving later have several possibilities: either to bend to the initially neglected ovules, or to the ovules that have already been visited or to other ones further down to the basis of the ovary. The result is a pattern of the top of the pollen tube bundle where per ovule only one or none pollen tube bends to grow towards the micropylar side of those ovules. There was no difference in ultrastructure between the placental cells at the central placenta, in between two ovules and close to the micropyle, which gives no reason to assume a secretory gradient to be built up by these cells. Also in the ovule a large secretory activity, which is able to maintain a gradient towards the central placenta, seems absent (chapter 3). There is a possibility that a gradient is absent at this point and that the pollen tubes take some of the stylar exudate with them into the ovary. When it is used they start curving but remain growing, which results in the bending in between the ovules.

- Electrophoretic analysis of the ovules -

Pollination and aging did not influence the protein pattern of ovules of *L. longiflorum* as far as can be determined with native or sodium dodecyl sulphate polyacrylamide gradient electrophoresis or isoelectric focusing in the tested ranges. Even after fertilization no repeatable differences were observed. It might be that there are some differences but too small to detect by the used methods. Pollination induced a quantitative difference in protein metabolism in the ovary of *Petunia hybrida*, even before the pollen tubes had arrived. This was determined after polysome isolation and in vitro protein synthesis (Deurenberg, 1976b). These proteins of cross-, self- and unpollinated ovules also showed a different electrophoretic protein pattern before the arrival of the pollen tubes (Deurenberg, 1977).

The absence of a clear difference in protein pattern even after fertilization again points towards a slow development of the ovules as is the first division of the zygote which was taking place not until 15 DAP.

- Senescence of the flower -

After compatible pollination the wilting of both the tepals and the pistil accelerates. Campbell and Linskens (1984a) observed an influence of pollination of *L. longiflorum* on the pistil and only occasionally on tepals. In unpollinated pistils the tepals collapse first during which little changes were observed in the appearance of the pistil (Campbell, 1983). In our experiments a more synchronous wilting took place. In *L. philippinense* Crang (1969) observed a necrotic reaction of the stylar cells, exclusive the secretory cells, to pollen tube growth, being absent when unpollinated. Vasil'ev (1970) reported a disappearance of starch in the secretory cells after pollination. There is no significant influence of pollination on uptake and distribution or on incorporation of label, applied as myoinositol, into insoluble material (Kroh et al., 1970a). In *Petunia hybrida* the pollen tube growth in the style accelerated wilting (Gilissen, 1976). Wilting occurred fastest after cross-pollination and slowest when the flower was left unpollinated. Self-pollination had an intermediate effect (Gilissen, 1977). Pollination did increase ethylene production and exogenous application of ethylene to unpollinated flowers stimulated senescence (Whitehead et al., 1984). Flowers of several *Lilium* hybrids had however a low sensitivity for exogenous ethylene in their wilting process (Woltering and Van Doorn, 1988).

Also in other species a reaction of the pistil to pollination or pollen tube growth was observed. In *Strelitzia reginae* a thickening of the cell walls of the subepidermal cells towards the secretory cells of the style is realized within four days after pollination. In unpollinated flowers this was not observed (Kronestedt et al., 1986). The pistil of peach is ripening from the top to the basis. Pollination stimulates the degeneration of the stigmatic papillae and coincidently a stigmatic secretion is produced. The passage of pollen tubes through the style is accompanied by the reduction of starch by the cells of the transmitting tissue (Herrero and Arbeloa, 1989).

When observed with the cryo SEM, the ultrastructure of pollen tubes can hardly be interpreted. One day after pollination of a bud of 120 mm, the pollen tubes are observed in the top of the style. They are not in a large quantity, being the first to invade. The plasma of those pollen tubes, broken close to the tip and subsequently slightly dehydrated by etching, appears as a complicated network of ice crystals. Three days after pollination a majority of the cross sections of the pollen tubes in the top of the style has a different appearance. Most likely the tubes were broken at a larger distance from their tip, i.e. in the region with vacuoles. These two groups mix but there is a preference of the pollen tubes with the network, presumably the ones broken closer to their tips, for growing close to the secretory cells. This preference is not so pronounced in a flower two days after pollination, which can be caused by the abundancy of exudate. In a bud of 120 mm the amount of exudate is restricted but still increasing. Pollen tubes might prefer to grow close to the exudate source. When there is enough exudate the need to grow close to the source might be not so evident. Using light microscopy, Crang (1969) observed in flowers of *L. philippinense* that younger pollen tubes grew closer to the secretory cells than older ones as judged by their cytoplasmic contents. No cytoplasmic or wall connections between the pollen tubes and the secretory cells were found.

In abundancy of exudate the cross section of the pollen tubes is mostly spherical. Half way down the style of a flower two days after pollination areas with less exudate and oval, oblate or irregular shaped pollen tubes are present. The exudate has levelled itself in this stage and is continuous with areas in which larger amounts of exudate are present, which is in the stylar lobes. In the latter areas the pollen tubes are more spherical. If differences in the quality of the exudate produced did exist in this stage, they will diminish due to diffusion. A difference in the osmotic pressure of the exudate between the areas, causing the difference in appearance of the pollen tubes, thus seems less likely. The internal pressure of the pollen tube is confronted with the surface tension of the exudate. This might interfere with the wall synthesis at the pollen tube tip. We could not observe whether the wall is thinner when facing the surface of the exudate using this technique. An inability of the pollen tube to grow in the gas phase might also cause this phenomenon.

Three days after pollination of a bud of 120 mm it seems that not all pollen tubes are covered by exudate, which is still not present in abundancy. The older pollen tubes, situated furthest away from the secretory cells might have been lifted up by the younger pollen tubes. The rigidity of the pollen tube wall and the osmotic pressure in the vacuolate part of the pollen tube must be rather high since it does not collapse when protruding out of the exudate.

Pollination or pollen tube growth does not influence the development of the embryo sac. When penetration of the micropyle occurred the embryo sac had already reached its seventh nucleate stage. In peach the embryo sac is not mature at the time the pollen tube arrives and it first circles in front of the micropyle (Herrero and Arbeloa, 1989). In

L. longiflorum the production of exudate starts before the bud opens. Pollen tube growth is possible but retarded. Arriving in the ovary the first ovules are neglected, the stimulus to bend is absent, and the pollen tubes grow toward ovules further down the ovary. In sector two the development of the embryo sacs is ahead of the rest of the ovary (see also Van Went et al., 1985) and therefore the invaded ovules happen to be mature although others in the ovary might not be. Later arriving pollen tubes grow further down the ovary and meanwhile those ovules are mature as well. The relation between the pollen tubes and the ovules at this stage might thus be explained by exudate production at the stigma and in the style, leading to a slower pollen tube growth in early bud stages during which the ovules mature, and the growth pattern of the pollen tubes in the ovary, with first invading the zone with embryo sacs in the most developed stage. A relation between pollen tube growth and exudate is also be present in peach, where the growth stops at the end of the style and resumes after a secrete has been formed (Herrero and Arbeloa, 1989), although this happens in flower and not in a flower bud as described above.

- Receptivity -

Ascher and Peloquin (1966a) pollinated different stages of flower buds, starting two days before anthesis, and flowers of *L. longiflorum* and also observed a long period of receptivity, as monitored after 48 hours of pollen tube growth at room temperature and by seed set. From one day before till nine days after anthesis cross pollination resulted in seed set, which is a shorter interval compared with the interval in which pollen tube growth is possible. This was also observed in our experiments when the penetration of ovules is compared with the period in which pollen tube growth is possible. Compared with our data, the buds of Ascher and Peloquin seem to be receptive in a later stadium and flowers allow pollen tube growth for a longer period.

The ultrastructure of the ovule of *Lilium longiflorum* Thunb. before and after pollination.

3

J. Janson

Introduction

After cut-style pollination just above the ovary the percentage of ovules of *Lilium longiflorum* with a pollen tube in the micropyle is low. The pollen tubes do find the inner integuments, sometimes react to the micropyles but only a part penetrates the ovules (chapter 4). The communication between the pollen tube and the ovule seems disturbed. An ovule enhancement leading to penetration is probably absent after cut-style pollination (Van Roggen et al., 1988). In cotton a synergid degenerates in a pollinated flower, hereby probably producing pollen tube attracting substances, but when the flower is left unpollinated this does not occur (Jensen and Fisher, 1968; Jensen et al., 1983).

In the synergids a filiform apparatus is usually present and their cytoplasms differs from that of the egg cell. This cytoplasm can differ in the polarity of the cells as observed in *Ornithogalum caudatum* (Tilton, 1981), *Agave parryi* (Tilton and Mogensen, 1979), *Saintpaulia ionantha* (Mogensen, 1981), cotton (Jensen, 1965) and *Petunia hybrida* (Van Went, 1970a and b). In *Gasteria verrucosa* the egg cell showed polarity (Franssen-Verheijen and Willemse, 1990). Also the metabolic activity of the synergids may be obvious as in *Ornithogalum caudatum* (Tilton, 1981), *Agave parryi* (Tilton and Mogensen, 1979), *Capsella bursa-pastoris* (Schulz and Jensen, 1968) or increases sharply during anthesis of *Petunia hybrida* (Van Went, 1970a).

Because of the structure of the filiform apparatus and the metabolic activity of the synergids a secretory function is supposed for these cells. This might be a secretion of chemotropic substances (Van Went, 1970a; Mogensen, 1972; Mogensen, 1981; Tilton and Mogensen, 1979). In *Spinacea oleracea* enzymes are secreted by the synergid to dissolve the middle lamellae of the nucellus tissue to create a micropylar channel between the embryo sac and the micropyle (Wilms, 1981).

In this chapter ovules of *L. longiflorum* are observed using transmission electron microscopy, with first attention for unfertilized ovules of both unpollinated and pollinated flowers, followed by a study of ovules during and after fertilization. An attempt is made to relate structure to the communication between the pollen tube and the ovule and whether the structure of the ovule changes due to pollination or pollen tube growth.

Materials and methods

Plants of *Lilium longiflorum* Thunb. 'Gelria' and 'White American' were grown in pots in the greenhouse of CPRO-DLO, Wageningen. The minimum night-temperature was 15°C and the day-temperature was on average 22°C. Flower buds were emasculated one or two days before anthesis and the stigma was covered by an aluminium foil cap to avoid cross-pollination. The day of anthesis was recorded. At two days after anthesis half of the flowers were pollinated with pollen of 'White American'. The pollen tube growth in the style was measured with water diluted cotton blue (see chapter 2). The end of the style was reached three days after pollination and after four days the first ovules were penetrated. This were not the first ovules in the top of the ovary, because these are neglected by the pollen tubes at first (see chapter 2). At different intervals the ovules of sector two (see chapter 2) were decapitated at the chalazal end in 0.1 M phosphate buffer at pH 7.2, to promote penetration of fixative and other embedding fluids, before separation from the placenta. This was followed by a fixation for 4 hours in 3% glutardialdehyde in the same buffer. After a rinse in this buffer the material was post-fixed for 6 hours in 1% osmium tetroxide in the phosphate buffer containing 1.5% potassium hexacyanoferrate (II) ($K_4Fe(CN)_6$; Van Dort et al., 1983). During dehydration in a graded ethanol series the material was stained for 1 hour in 0.5% uranyl acetate in 70% ethanol. The ovules were embedded in low-viscosity resin (Spurr, 1969). Ovules were sectioned longitudinally and transversely and ultrathin sections were alternated with sections of 1 to 2 μm for observation with respectively the transmission electron microscope (TEM) or the phase contrast microscope. Ultrathin sections were stained with uranyl acetate and lead citrate before observation with a Philips 301 or a JEOL JEM-1200EX II. A list of abbreviations used in figures is present at the page facing the general introduction of this thesis.

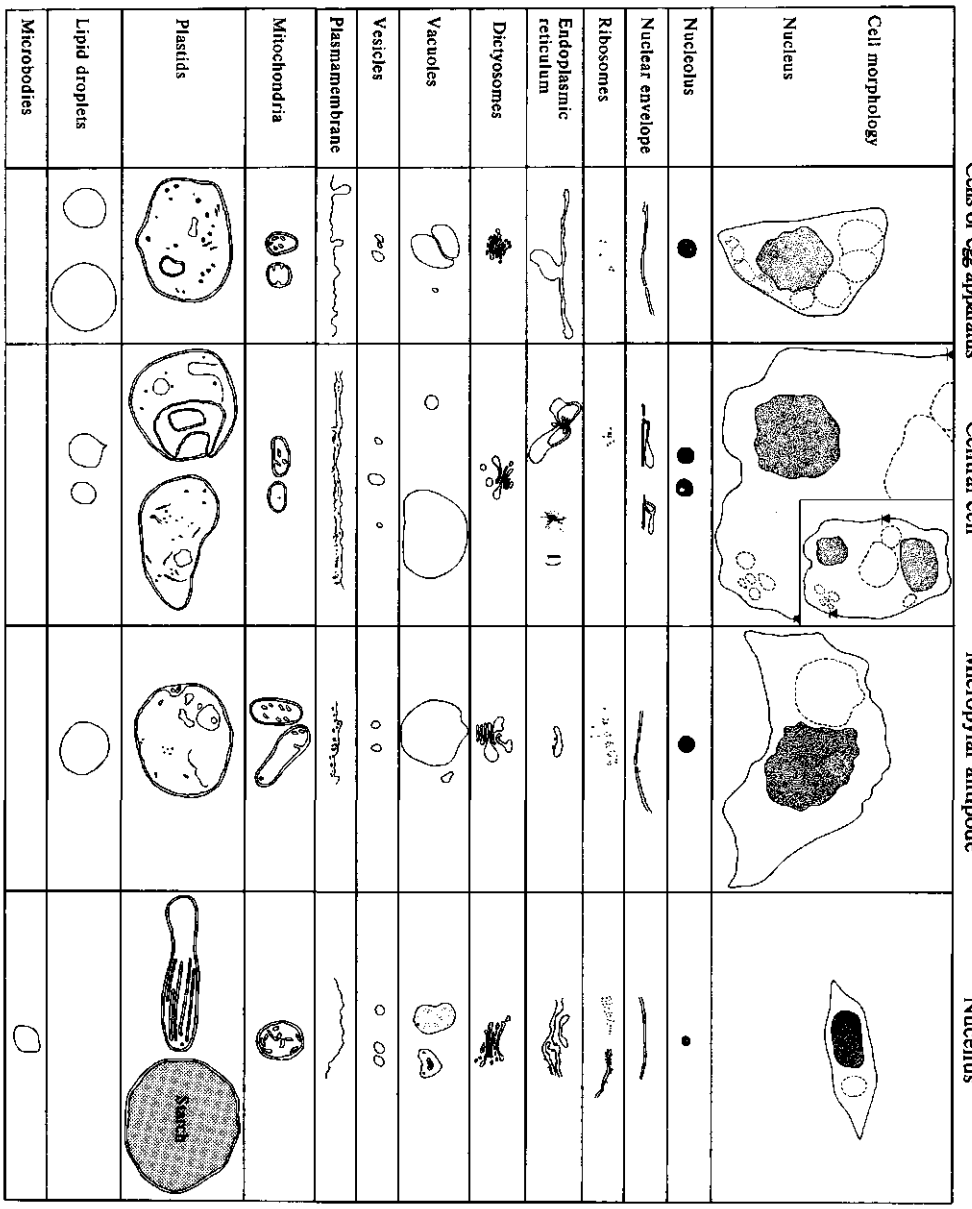
To check the seed set flowers were also pollinated and kept in the greenhouse till mature pods were produced.

Results

Before fertilization

Fifteen ovules were studied before fertilization. Their age differed from two till six days after anthesis. Eight of these ovules originated from pollinated flowers from one till four days after pollination in which pollen tube penetration had not taken place. These ovules were compared with those from unpollinated flowers of the same age. Also ovules of different ages were compared. A survey of the ultrastructural analysis of the cells of the egg apparatus, the central cell, the micropylar antipode and the nucellar cells is shown schematically in table 1.

Table 1. A survey of the ultrastructural analysis of the cells of the egg apparatus, the central cell, the micropylar antipode and the nucellus of *Lilium longiflorum* Thunb. The central cell is relatively large compared with the other cells studied, and the cell morphology is presented at a smaller magnification in the upper right hand corner of the column. In the cell morphology the dotted lines represent the vacuoles, the grey symbols represent the nuclei, in which a darker structure indicates a higher degree of condensation of the chromosomes.
1) see figs 12 and 13 of this chapter.



- The egg apparatus -

No ultrastructural differences could be found between the contents of the three cells of the egg apparatus, the egg cell and the two synergids. No filiform apparatus is detected. Paramural bodies were observed along the cell wall between the embryo sac and the nucellus. Sometimes there was a difference in electron density of these bodies between the different cells of the egg apparatus but these differences were not consequent. No degeneration of a synergid occurs before the arrival of a pollen tube. In all 15 observed embryo sacs the three nuclei within one egg apparatus have the same degree of chromosome condensation, but also between the embryo sacs there is hardly any difference. They have nuclear pores of a comparable size and density and the two nucleoli appear similar as well (fig. 1). In some ovules micronucleoli (Jensen, 1965) are observed next to the large ones.

An association of the outer membrane of the nuclear envelope with smooth endoplasmic reticulum (SER) occurs and is limited in number and extend when compared with the nuclear envelopes of the polar nuclei in the central cell. The cells of the egg apparatus contain vacuoles throughout their full length, but at the micropylar end the size of the vacuoles is smaller. At the micropylar end a varying appearance of the cells of the egg apparatus can be observed. In some cells the number of lipid droplets is large (fig. 2), in other cells plastids set the scene (fig. 3). But in just the majority of the embryo sacs there is no cell observed in which one organelle type is dominating or clustering at the micropylar end. This variation between the cells within an egg apparatus is sometimes also occurring in the middle of the cell or at the more chalazal end. Although the presence of the vacuoles is obvious the contents of the other organelles can vary, eg. fig. 4 in which in one cell a relative large amount of mitochondria is found. These differences are also not consistent over the various observed ovules and do not seem to interfere with the fact whether those ovules were originating from pollinated or unpollinated flowers.

In the youngest observed stage of the ovules, two days after anthesis, the cells of the egg apparatus vary even more in outlook between each other when compared with the older embryo sacs. One of the cells of the egg apparatus contains far more ribosomes, both mono- and polysomes, than in either cell of the egg apparatus in mature ovules. Some egg apparatus contain a cell with lamellar endoplasmic reticulum (LER, fig. 5) and others one or more cells with bundles of microtubules (fig. 6). Such phenomena are absent

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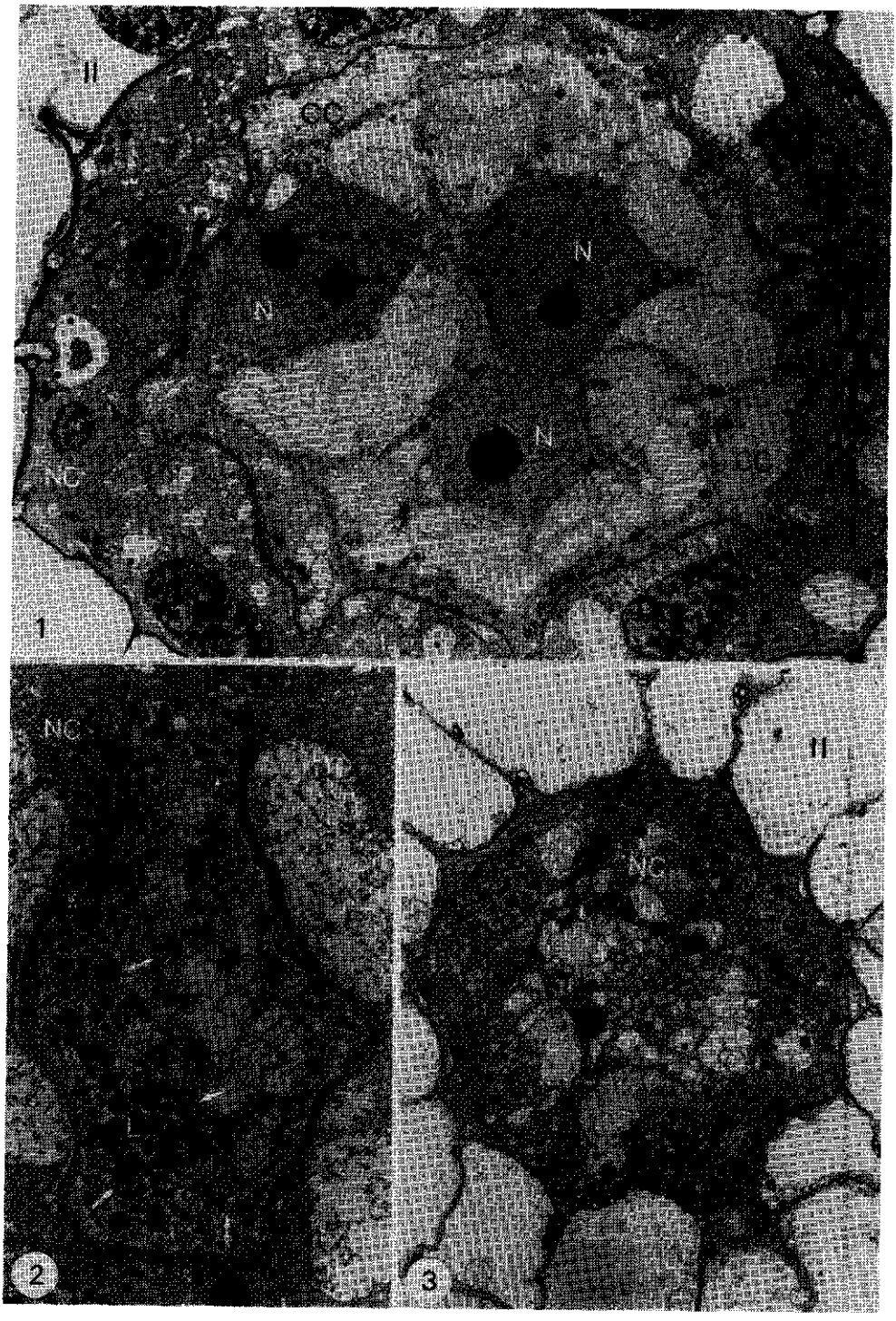
Figs 1-3. The egg apparatus of *L. longiflorum*. Fig. 1. Transverse section through an embryo sac at two days after pollination (DAP), with the three nuclei of the egg apparatus at one level and having the same degree of condensation. Magn. 1336x. Fig. 2. One of the three cells of this egg apparatus contains far more lipid droplets compared with the other two at transverse section close to the micropylar end of the embryo sac. Arrows indicate the cell walls between the three cells of the egg apparatus, fixed at four days after anthesis (DAA). Magn. 297x. Fig. 3. A concentration of plastids at the micropylar end of one of the cells of an egg apparatus, observed at 4 DAP in a transverse section. Magn. 1024x.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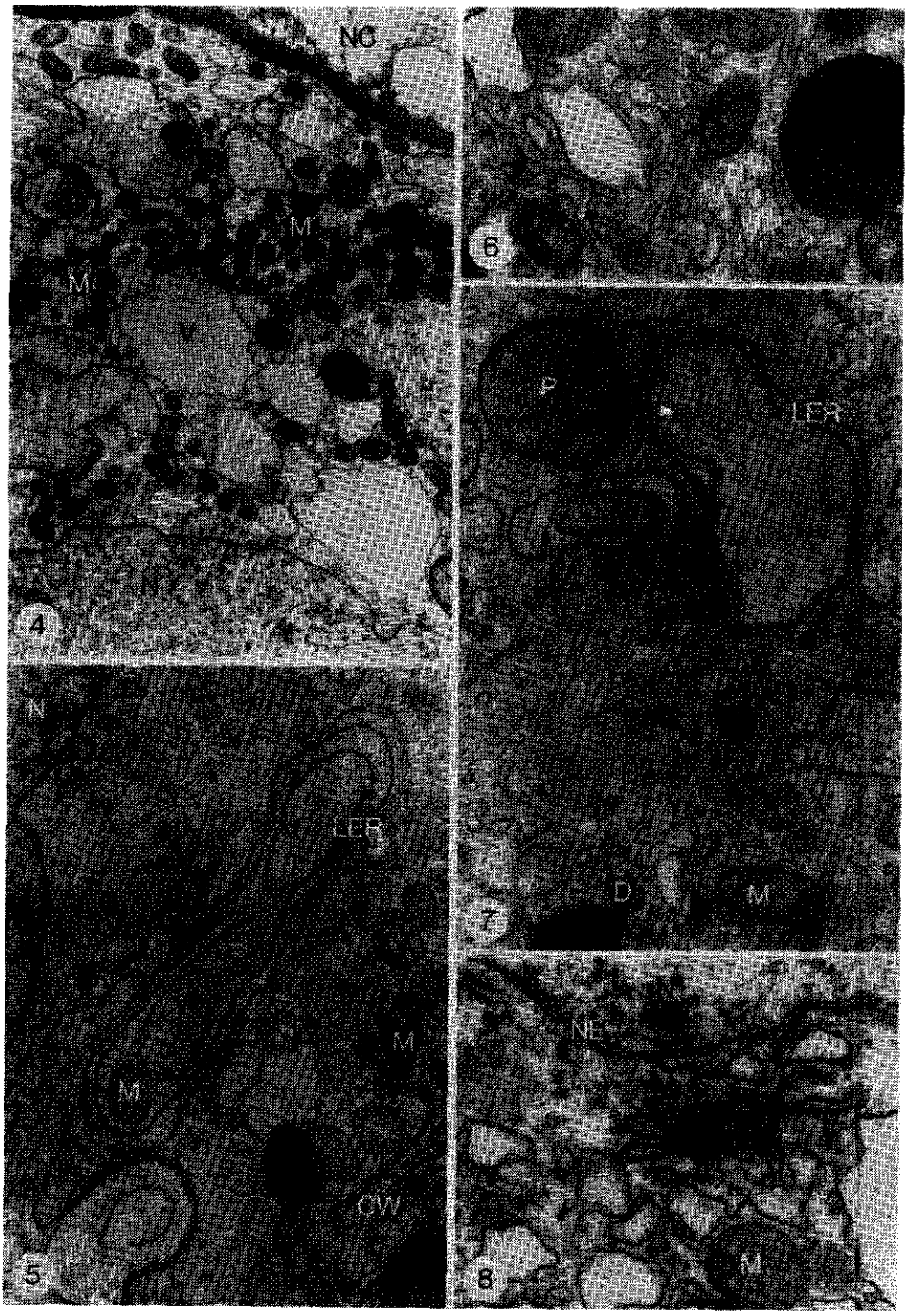
in the egg apparatus of mature ovules. When compared with later stages structures which were referred to as paracrystalline protein by Dickinson and Potter (1978) and membranous fragments by De Boer-De Jeu (1978) are more common (fig. 7). Although LER disappears in the egg apparatus during the development, their junctions of endoplasmic reticulum (ER) do not disappear. In later stages they can get continuous with the outer membrane of the nuclear envelope (fig. 8). The extensions of these junctions, the ER, are not as long as observed in the central cell.

The plastids lack starch grains but during the development of the ovules an increase of electron dense particles is found in these organelles. There is a slight gradual difference in the appearance of these particles between the three cells of the egg apparatus, which is in some ovules more obvious than in others. In the egg cell and the persistent synergid as observed just after fertilization, these particles have enlarged.

The cells of the embryo sac are separated from each other by plasma membranes and a cell wall. In this wall tubular structures are observed, sometimes connected with the plasmalemma. The wall has a more electron dense centre, sandwiched between more translucent regions (fig. 9). In between the cells of the egg apparatus and between these cells and the central cell the thickness of the cell wall is very irregular but the average thickness hardly changes with its position, apart from the places where it meets the nucellus. In some ovules one of the walls was branched, leaving an end wall into one of the cells (fig. 9). Plasmodesmata crossing the cell walls between the cells of the egg apparatus themselves and towards the central cell were not observed.

Because there is no obvious difference between the structure of the three cells of the egg apparatus, the orientation of those cells was determined after both transverse and longitudinal sectioning, in an attempt to make a distinction between them. In the youngest observed embryo sacs the nuclei are more often in one plain of transverse sectioning compared with the older stages. But even after 4 days of pollination, when the first ovules are fertilized, there are still some ovules with their nuclei in this one plain. Between ovules from pollinated and unpollinated flowers, before the arrival of the pollen tubes in the ovary, no difference in this orientation was observed. There also seems to be no preference of the orientation of the three nuclei of the egg apparatus when taking the vascular bundle of the funiculus as a point of reference. The position of the egg cell, detected by its fertilization, differs also to this reference.

Figs 4-8. The egg apparatus of *L. longiflorum*. Fig. 4. A relatively large number of mitochondria is populating this cell at the level of the nucleus. Transverse section through an ovule at 4 DAA. Magn. 6680x. Fig. 5. Circles of LER are present in some cells of the egg apparatus at a young stage, 2 DAA. The cytoplasm is, relatively to the older stages, rich in ribosomes. Magn. 13.4Kx. Fig. 6. Microtubules (arrowheads) are still present in some cells of the egg apparatus at 2 DAA, longitudinal section, magn. 20.0Kx. Fig. 7. Paracrystalline protein or membranous fragments (arrowheads) present inside and outside LER. 2 DAA, magn. 16.6Kx. Fig. 8. A junction with tails (arrows) of ER associated with the outer membrane of the nuclear envelope, observed in the egg apparatus at 4 DAA. Magn. 32.1Kx.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At the micropylar side the cells of the egg apparatus border the nucellus cells. In transverse sections from the micropyle towards the chalazal end of the embryo sac, the central cell appears for the first time at the periphery of the embryo sac in sections in which the nuclei of the egg apparatus are still present. There was no consistent difference between the three cells of the egg apparatus in the extent to which they were in contact with the nucellus, this relatively to the position of the vascular bundle.

- The central cell -

The length of the embryo sac of *L. longiflorum* is about 0.3 mm of which the majority is occupied by the central cell. The polar nuclei do not fuse until after fertilization. The haploid nucleus is situated close to the egg apparatus. The triploid nucleus is positioned at the chalazal pole. The position of the micropylar polar nucleus varies compared to the position of the vascular bundle of the ovule, the reference point. Its place also seems to be independent of the position of the egg cell, as was observed in fertilized embryo sacs.

Both nuclei have a comparable degree of chromosome condensation, which equals or is less diffuse than the nuclei of the egg apparatus. In 20% of the observed embryo sacs, the appearance of the nucleoli in both nuclei differ in structure. In that case the nucleoli of the triploid nucleus have only small vacuoles, the ones of the haploid nucleus have a large vacuole next to the smaller ones which were observed in the triploid nucleus. In case there is no difference observed both nucleoli have small vacuoles. The outer membrane of the nuclear envelope of both nuclei show an association with SER (fig. 10). Sometimes the inner membrane bulges out as well (fig. 11).

In the proximity of both nuclei LER (fig. 12) is observed, in which lipid droplets, mitochondria, small vacuoles and plastids can be found. Cisternal smooth endoplasmic reticulum (CSER) as shown in fig. 13 only appeared at the chalazal pole. Paracrystalline structures, storage sites of lipoproteins of LER (De Boer-De Jeu, 1978) were not found. Instead a probably more diffuse structure in which separate membranes can be

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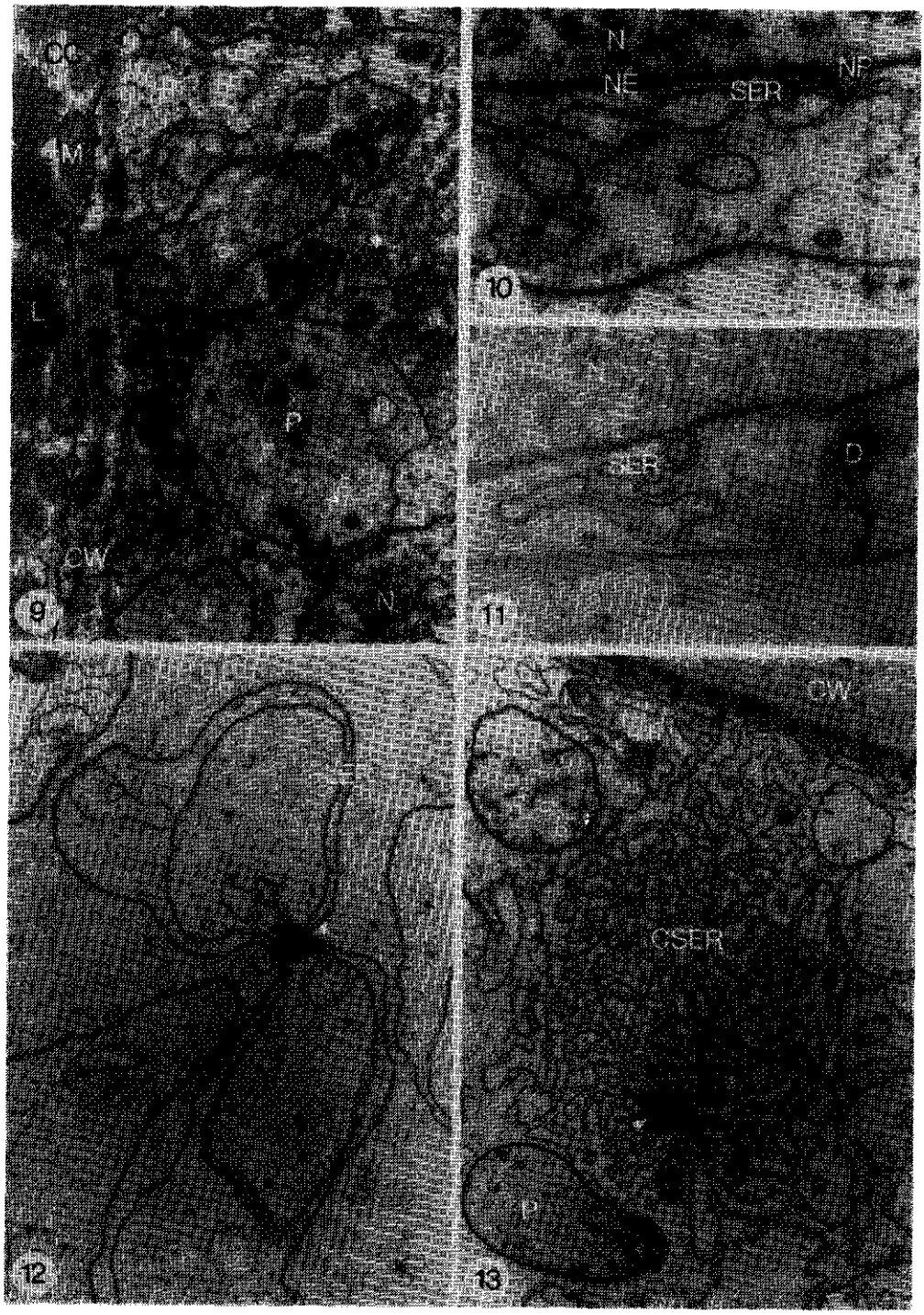
Fig. 9. A detail of the cell wall between the central cell and one of the cells of the egg apparatus. Note the tubular structures in the cell wall and the branch (*). 2 DAP, transverse section, magn. 14.0x.

Fig. 10. An association of the outer membrane of the nuclear envelope of the triploid polar nucleus with SER. 2 DAP, magn. 34.1x.

Fig. 11. The inner membrane of the nuclear envelope of the triploid polar nucleus bulges out and the outer membrane is associated with SER. The ovule originated from an unpollinated flower. 5 DAA, magn. 32.1x.

Fig. 12. LER present in the central cell and associated with a junction (arrowhead). 4 DAA, magn. 11.0Kx.

Fig. 13. CSER as observed in the central cell in the proximity of the triploid polar nucleus and associated with a junction (arrowhead). 4 DAA, magn. 12.1K.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distinguished is regularly observed and is referred to as junctions (figs 12 and 13). Membranous fragments or paracrystalline protein remain present throughout the development of the ovules from at least 0 to 4 days after pollination or 2 to 6 days after anthesis in case the flower was left unpollinated.

In between the two nuclei large vacuoles are present. Dictyosomes are scattered throughout the plasma at the poles of the central cell. Lipid droplets and mitochondria are predominantly situated along the plasma membrane, especially where the central cell borders the nucellus (fig. 14). Preferentially they appear in groups in which the centre contains mainly mitochondria with at the sides lipid droplets. Other groups appear more mixed. Plastids can be present in those groups as well, but more often they are spread out predominantly along the plasma membrane. The density of organelles in the groups is comparable to the density found in the egg apparatus at places lacking vacuoles. No difference in cell organelle composition was observed between ovules from unpollinated and pollinated flowers.

- The antipodals -

The embryo sac has usually two antipodals (fig. 15) with in each a triploid or perhaps hexaploid nucleus. In at least three of the 15 observed embryo sacs however an extra cell wall is formed at the chalazal end of the embryo sac, excluding an extra cell, small in size and without a nucleus. In other embryo sacs only a part of this wall was realized, so that the wall ends in the chalazal antipode without forming an extra compartment.

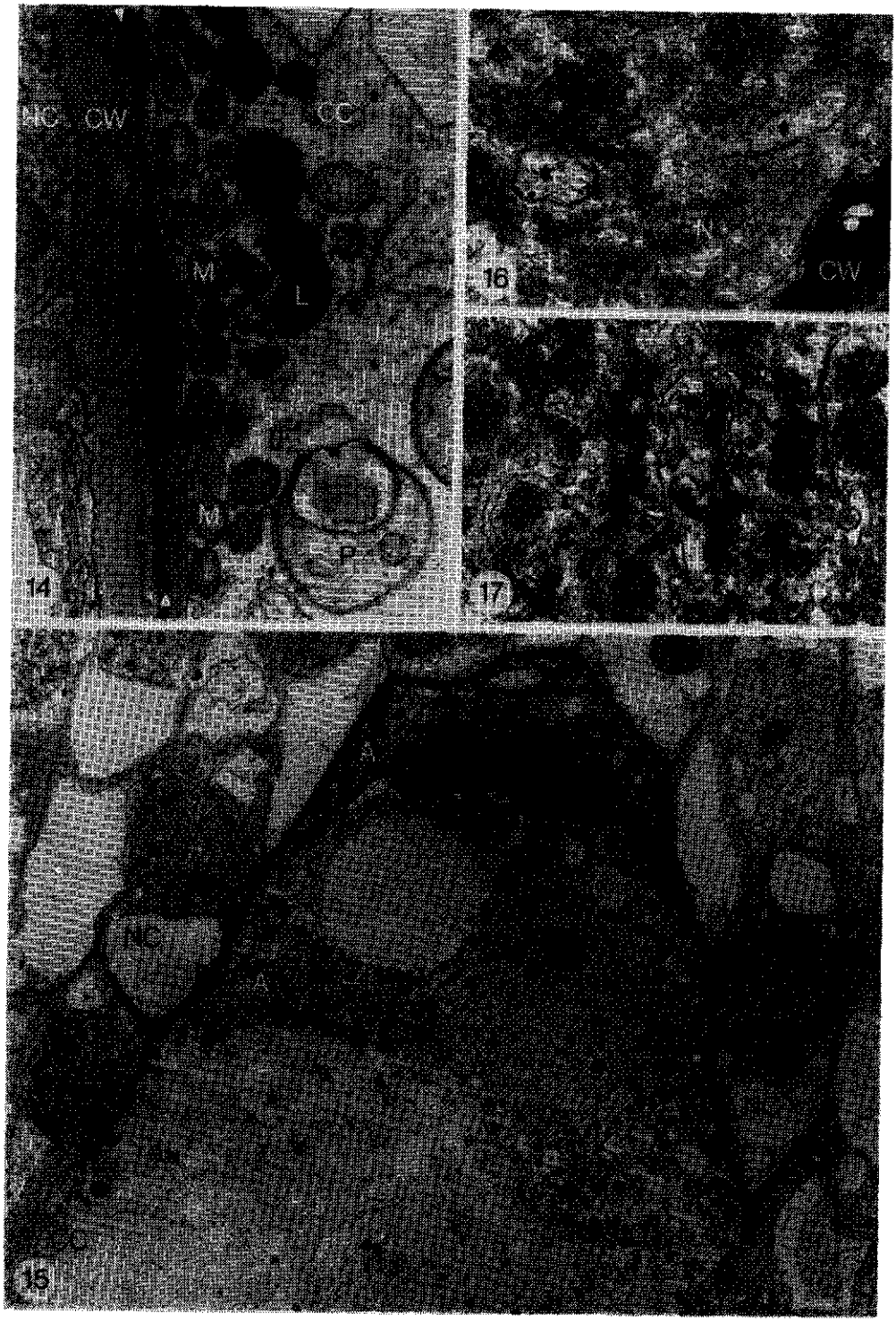
The chromatin in the micropylar antipode is more condensed than in the nuclei of the egg apparatus and the central cell, but not as much as in the chalazal antipode. In the latter nucleus inclusions or lobes of plasma (fig. 16) and fragments of membrane-like structures (fig. 17) are found. The plasma also appears more electron dense (see fig. 15), due to a darker ground plasma, the presence of more ribosomes and more closely packed organelles compared with the micropylar antipode. In some ovules the nucleus is partly surrounded by one layer of ER. In those cells plastids are observed with even more ER layers, which are strikingly electron dense (fig. 18).

Fig. 14. In the central cell groups of mitochondria and lipid droplets are situated predominantly at the periphery. In the cell wall between the nucellus and the central cell at the height of the triploid polar nucleus a dark discontinuous layer (arrowheads) is observed. 2 DAP, transverse section, magn. 15.0Kx.

Fig. 15. An overview of the two antipodals of *L. longiflorum* at 2 DAA. Magn. 1336x.

Fig. 16. Cytoplasmic inclusions (*) in the lobed nucleus of the chalazal antipode. 2 DAP, transverse section, magn. 17.4Kx.

Fig. 17. Fragments of membranes (arrowheads) are observed in the nucleus of the chalazal antipode. 4 DAA, transverse section, magn. 21.0Kx.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The nucleus of the micropylar antipode has nuclear pores and the nucleoli contain small vacuoles. SER association with the outer membrane of the nuclear envelope was observed in all the embryo sacs. In the cytoplasm only little ER is present. In some cells ER junctions are observed. On average dictyosomes are scarce and if their number is higher they only contain few cisternae. The cytoplasm contains much lipid droplets and mitochondria. The plastids lack starch grains. The size of the vacuoles is varying enormously between the different micropylar antipodals, from the size of a plastid or even smaller to about one quarter of the nucleus. A survey of the cell contents is given in table 1. Pollination did not influence the described pattern.

- The nucellus and the micropyle -

The number of cell layers of the nucellus increases from the micropylar side, where it is only one cell layer thick, towards the chalaza, where it has increased to three to five layers. Apart from variation in the dispersal of organelles within a cell the contents of two adjacent cells can vary considerably as shown in fig. 19 where two cells differ in the number of ribosomes. There are however similarities. The cells are on average rich in ribosomes compared with the egg apparatus and the central cell of the embryo sac. Also much SER, rough endoplasmic reticulum (RER) and vesicles are present in the cytoplasm. The mitochondria have on average more cristae compared with the embryo sac. Dictyosomes are actively producing predominantly small vesicles, not only in the proximity of the micropyle but also at the chalazal side (fig. 20). The vacuoles have an electron dense debris. At the micropylar side plastids resemble not fully differentiated chloroplasts and contain some thylakoids (fig. 21). When proceeding towards the chalaza large starch grains are found in the plastids (fig. 22). Lipid droplets are scarce, microbodies are more often present. Microtubules are observed as well. An overview of several organelles in the nucellus is given in table 1. Pollination did not induce a difference.

Plasmodesmata are present between the cells of the nucellus, at the chalazal pole equally or more frequent than at the micropylar pole. At the chalazal pole some cells of the nucellus are still dividing.

There are no plasmodesmata from the nucellus towards the embryo sac. The cell wall between the nucellus and the embryo sac changes in appearance and thickness, from the egg apparatus towards the antipodals. At first the wall is on cross section less

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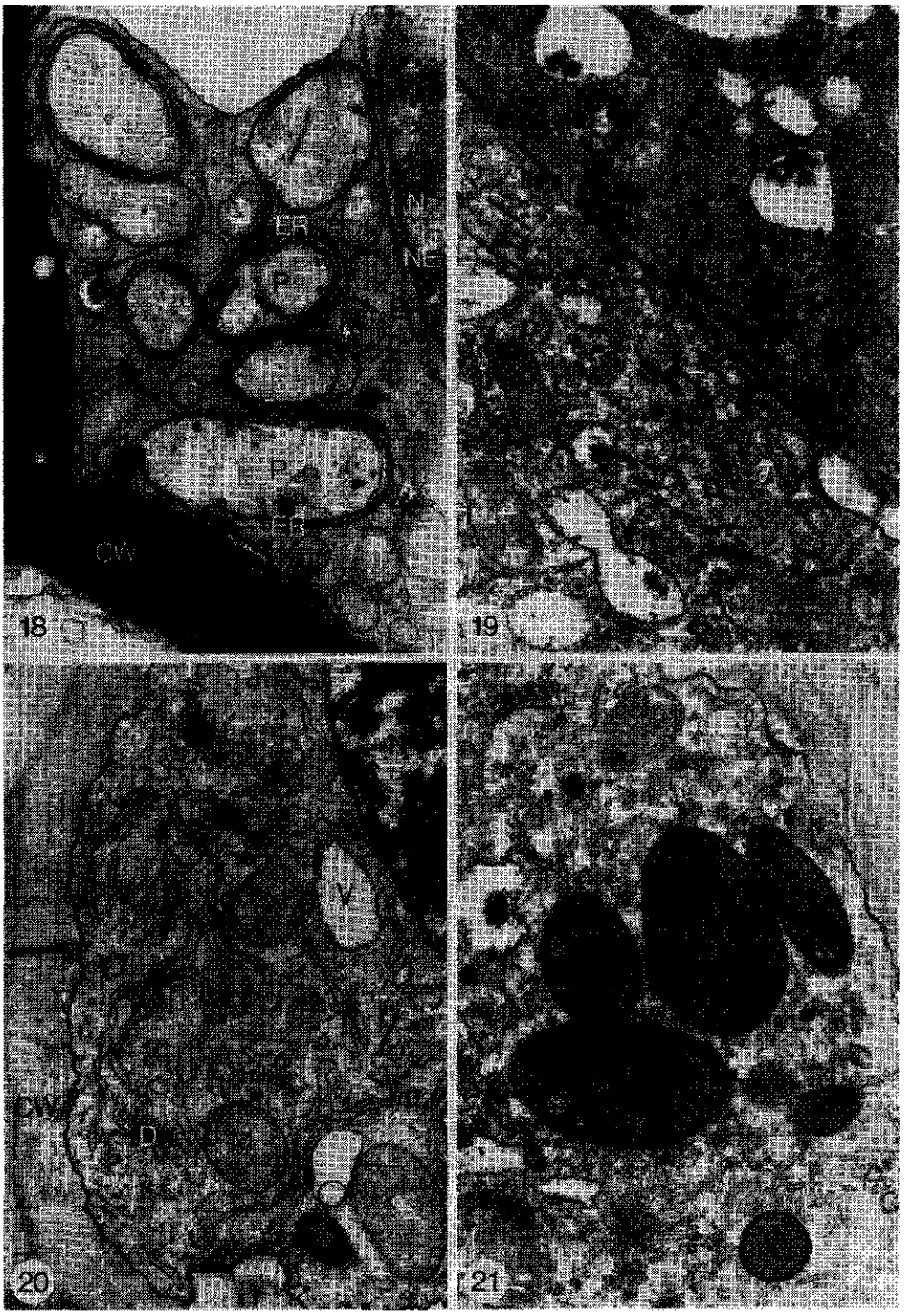
Fig. 18. Plastids surrounded by ER in the chalazal antipode of an ovule at 2 DAP. Transverse section, magn. 19.3Kx.

Fig. 19. Two adjacent nucellar cells close to the micropyle, differ in ribosome population. 2 DAA, longitudinal section, magn. 15.4Kx.

Fig. 20. Dictyosomes producing small vesicles in a nucellar cell at the height of the triploid polar nucleus in the embryo sac. 4 DAA, transverse section, magn. 15.4Kx.

Fig. 21. Thylakoids in the plastids of the nucellar cells close to the micropyle. 2 DAP, magn. 20.0Kx.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electron dense at the nucellar side, which gradually changes to a denser wall at the side of the egg apparatus at which also some more darker debris and tubular like structures are found (fig. 23). In the proximity of the triploid nucleus of the central cell, the wall is at the nucellar side electron lucent, changing into a more electron dense wall towards the embryo sac. In this dense part of the wall a darker layer is formed which is at first discontinuous, both in transverse (fig. 14) and in longitudinal section. This layer seems to originate from the embryo sac. The wall also increases in thickness. Arriving at the antipodals that darker layer has completely occupied the more electron dense layer in the cell wall and at the nucellar side a darker layer is observed as well (fig. 24).

There is no opening in the nucellus from the micropyle to the embryo sac to serve as a pollen tube pathway. Also no degeneration of nucellus cells is observed at that place. Towards the inner integument the nucellus is covered with a cuticle. This cuticle is locally stretched or ruptured and substances are present in the intercellular spaces around the nucellus which are likely to be a part of the micropyle (fig. 25).

The micropyle is formed by the inner integument, has three lobes near the outside of the ovule where the cells are covered by a cuticle. These three lobes change into a canal in which again a cuticle is found which does not border the cell walls at all places. Often an excrete in between the cuticle and the cell walls of the cells of the inner integument is present. In these highly vacuolate cells dictyosomes are observed which actively produce vesicles (fig. 26).

Pollen tube penetration and fertilization

In the ovules pollen tube penetration was observed from 4 days after pollination. A total number of 10 ovules from 4, 5, 7 and 10 days after pollination (DAP) were studied after pollen tube penetration. In sector two all ovules swell but only 50% of those seeds had a at a light box detectable embryo (average of 8 flowers).

In all ovules the plasma of the pollen tube was degenerated. After the three lobed micropyle had changed into a canal, the micropyle is on cross section filled with one pollen tube. Next to this pollen tube a discontinuous debris of the cuticle is present (fig. 27). A penetration by more than one pollen tube was never observed. In one ovule the pollen tube penetrated the micropyle but did not grow through the nucellus. In that embryo sac non of the three cells of the egg apparatus were degenerated, whereas the plasma of the pollen tube was.

In the observed ovules the penetration of the nucellus is never in line with the micropyle. The pollen tube makes a curve, lifts up (fig. 28) or pushes the cuticle apart and grows in between two nucellus cells. Here the pollen tube grows through the walls of these cells and was also observed in between the wall and the plasma membrane of the nucellus cells. After penetration through the nucellus there are still places left at which the cuticle around the nucellus is discontinuous. Degeneration of a nucellus cell close to the micropyle is taking place in some ovules. In the majority of the ovules the ultrastructure of the nucellus has not altered, in comparison with before pollen tube penetration.

However when time proceeds the nucellar cells next to the pollen tube develop a thickening of the bordering cell walls (fig. 29). Their plastids still lack starch. At 10 DAP the cell wall of the pollen tube is not closed by this thickening of the nucellar cell wall. A thickening of the cell walls of the inner integument bordering the pollen tube is also observed.

- The pollen tube discharge in the embryo sac -

After arrival of the pollen tube in the embryo sac, which usually takes place at the side of this embryo sac but always at the place of the egg apparatus, the pollen tube grows towards the micropylar basis of this embryo sac. This happens in between the nucellar cells and the plasmalemma of the cells of the egg apparatus (fig. 30). Finally it opens into one of the three cells of the egg apparatus (fig. 31).

From that time the pollen tube wall with its opening is embedded in the degenerated plasma of both the synergid and the pollen tube. Large amounts of starch grains but also a small zone without starch are observed in this degenerated plasma mixture. The synergid did not have starch grains before degeneration. In the pollen tube starch grains were however present. In the degenerated plasma remnants of two nuclei, the one of the synergid and the vegetative nucleus of the pollen tube, are observed (fig. 32). They differ in electron density. The degenerated plasma directly borders the plasmalemma of the egg cell, of the persistent synergid and of the central cell, apart from places where the plasmalemma approaches the nucellus. Close to the nucellus a cell wall between the degenerated plasma and the other cells of the embryo sac is still present but the membrane bordering the degenerated plasma seems to be degenerated. The cell wall between the two persistent cells of the egg apparatus themselves but also towards the central cell has at places disappeared, resulting in two closely packed plasma membranes, alternated with places where degenerated plasma has accumulated in between the membranes (fig. 33). Also a local gathering of vesicles in an electron lucent matrix is observed between the two plasma membranes (fig. 34).

- Enucleated cytoplasmic body -

In the degenerated plasma mixture of the penetrated synergid a long but slender membrane bounded area without a nucleus is bordering both the central cell and the egg cell. This enucleated cytoplasmic body is likely a remainder of the cytoplasm of a sperm cell. This body contains vacuoles, mitochondria, lipid droplets, vesicles, dictyosomes and has a higher ribosome density compared with the cells of the egg apparatus and the central cell (fig. 35). At the places where the membrane of this cell borders the membranes of the egg and the central cell they are pressed firmly together, closer than the shortest distance between the other cells of the egg apparatus and the central cell after fertilization. There is no connection between the cytoplasms of this body and its neighbours. It is separated from the degenerated plasma mostly by only one cell

membrane, at some places two membranes were present, and was still present at 7 DAP.

In one ovule two of those nucleus free cells were present. They differ in ribosome number but both have this lining up of membranes towards both the egg cell and the central cell but also towards each other (figs 36 and 37).

- The sperm nuclei -

After pollen tube penetration in the embryo sac at four days after pollination the sperm nuclei are observed in the egg cell and the central cell. They are more condensed and are long and slender, compared with the more rounded nuclei of the egg cell and the central cell. They have nuclear pores (fig. 38) and sometimes small nucleoli (fig. 42) were observed. In a part of the ovules there is a difference in chromatin condensation between the two sperm nuclei as well. The sperm nucleus in the egg cell is more condensed compared with the one in the central cell and also its volume is smaller.

In the central cell the sperm nucleus is not associated with organelles. The density of the organelles in the egg cell is larger when compared with the surrounding cytoplasm of the sperm nucleus in the central cell. The absence of associated parental organelles in the egg cell can not be assured and aberrant organelles were not observed.

- The egg cell -

After the penetration of the pollen tube and the presence of sperm nuclei in the egg cells, the latter are very variable in outlook. The egg cell is in some ovules obviously more

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Fig. 22. Plastids contain starch at the chalazal side of the nucellus. Plasmodesmata are connecting the cells (arrowheads). 2 DAA, magn. 1336x.

Fig. 23. A longitudinal section of the cell wall between the egg apparatus and the nucellus. Tubular structure (arrowhead), 2 DAA, magn. 26.7Kx.

Fig. 24. A longitudinal section through the cell wall between the antipode and the nucellus. 2 DAA, magn. 26.6Kx.

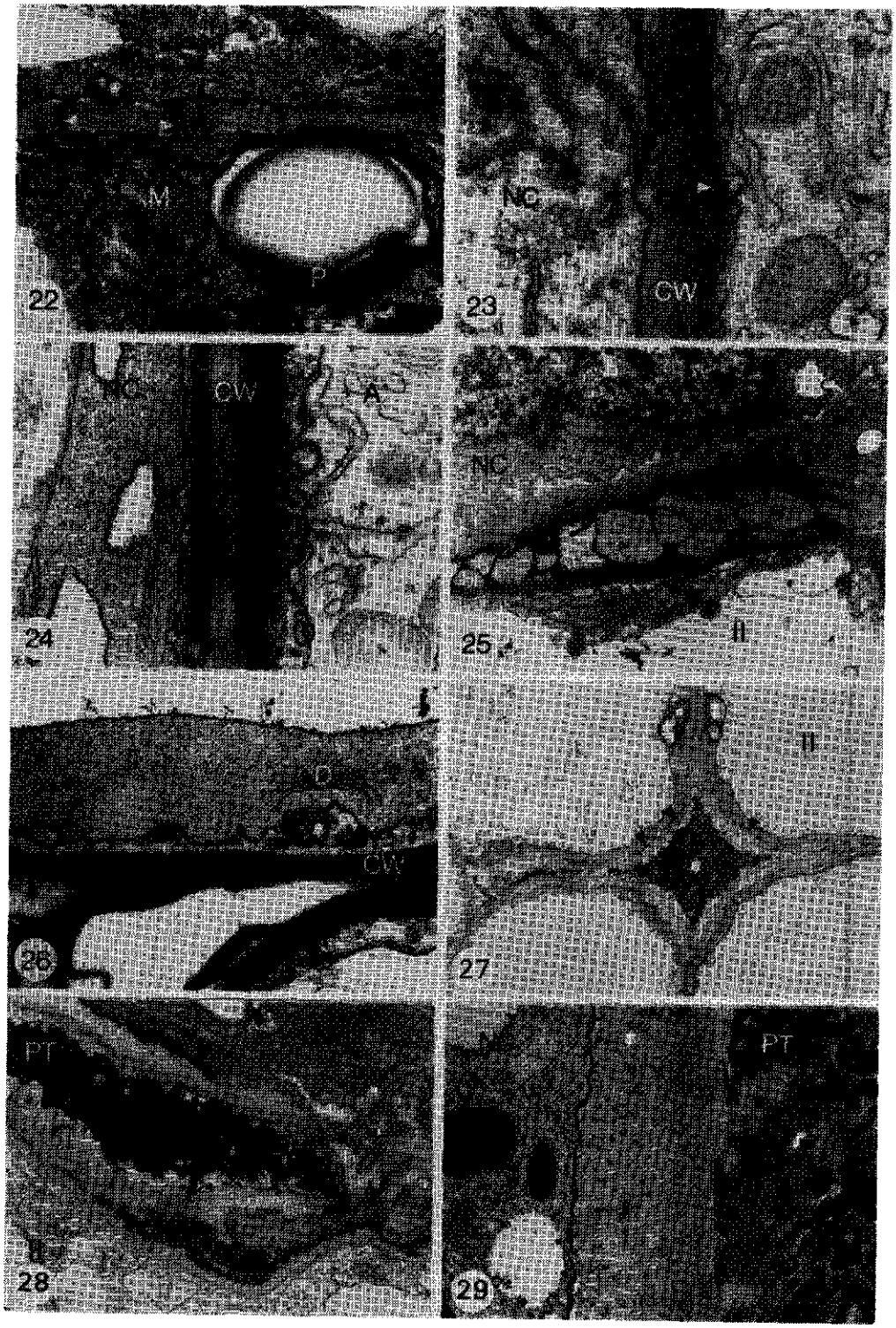
Fig. 25. An intercellular space, probably part of the micropyle, between the inner integument and the nucellus. The cuticle is locally lifted up from the cells walls of both the inner integument (arrowhead) and the nucellus (arrow), and filled with a substance. 3 DAP, longitudinal section, magn. 708x.

Fig. 26. A transverse section through the micropyle. A dictyosome actively produces vesicles. At places a substance (*) is present in between the plasmalemma and the cell wall and in between the cell wall and the cuticle. 2 DAP, magn. 25.1Kx.

Fig. 27. A transverse section through a micropyle with a pollen tube (*). Arrowheads indicate parts of a cuticle. 4 DAP, magn. 3250x.

Fig. 28. A pollen tube in between the inner integument and the nucellus passing the cuticle (arrowheads). 4 DAP, longitudinal section, magn. 7150x.

Fig. 29. A thickening of the cell wall (*) of a nucellus cell adjacent to the penetrated pollen tube, of which the contents has degenerated. 7 DAP, longitudinal section, magn. 14.7Kx.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vacuolated compared with each of the three cells of the egg apparatus before pollen tube penetration. In the majority of the embryo sacs the debris in the plastids of the egg cell has enlarged (fig. 30), forming small starch grains. There is also an increase in vacuoles in the plastids. The number of polysomes increases, RER is formed and the ground plasma is more electron dense. Large lipid droplets appear in some egg cells. After the nuclei line up (fig. 39), fusion starts at one point, but soon more places of contact are formed, also when the nuclei do not have the same degree of chromosome condensation (fig. 40). It seems that even between different sites of the same sperm nuclear membrane places of contact are formed (fig. 41). These points of contact enlarge and because of the numerous places of contact cytoplasm is caught in between the two nuclei. Ten days after pollination there are still arms or islands of cytoplasm present in the diploid nucleus of the zygote.

The nucleoli of the egg cell remain present during the fusion and have small vacuoles at first, but at 7 DAP large vacuoles are observed. While still fusing a large nucleolus with large vacuoles is observed in the former sperm nucleus as well. A possible difference in chromosome condensation, with the sperm nucleus being more condensed, was observed till 7 days after pollination, i.e. 3 days after pollen tube penetration.

- The persistent synergid -

The cytoplasm of the persistent synergid, i.e. the cell of the egg apparatus which does not contain the pollen tube or the sperm nucleus, is similar to that of the egg cell at least until 10 DAP. The debris in the plastids increases as well and at 10 DAP appears as small starch grains. Those starch grains were observed to a lesser extend in the egg cell of the same age. An enlargement of the lipid droplets is observed in some persistent synergids. There is an increase in the electron density of the ground plasma and the number of ribosomes. RER is formed and also the vacuoles in the nucleoli gain in size during the development.

- The central cell -

Before the nuclear fusion, but with the sperm nucleus in the central cell, the outer membranes of the nuclear envelopes of the polar nuclei are associated with SER and of both the triploid and the haploid nucleus the inner membrane is sometimes bulging out. The sperm nucleus has a SER association as well (fig. 38). Just after the sperm nucleus is present in the central cell, bundles of tubular ER are observed from the egg apparatus towards the sperm nucleus (fig. 42). At the basis of the embryo sac, close to the egg apparatus, a horse shoe shaped concentration of cisternal SER is present.

The sperm nucleus makes first contact with the haploid polar nucleus, which in the majority of the embryo sacs still has large vacuoles in its large nucleoli. The small nucleoli lack vacuoles. At 4 and 5 DAP there is only contact between the haploid polar nucleus and the sperm nucleus. A complex of nuclei including the triploid polar nucleus is observed

at 7 DAP. The fusion is taking place at numerous points and the SER seems involved (figs 43 and 44). After 10 days the fusion is still not completed.

In the central cell organelles are still predominantly concentrated along the plasma membrane bordering the nucellus. In the plastids an osmiophilic debris is present in some ovules from 4 DAP and at 10 DAP starch is observed. This development of starch is retarded when compared with the plastids in the persistent cells of the egg apparatus. Gradually the vacuoles enlarge, the ground plasma increases in electron density as does

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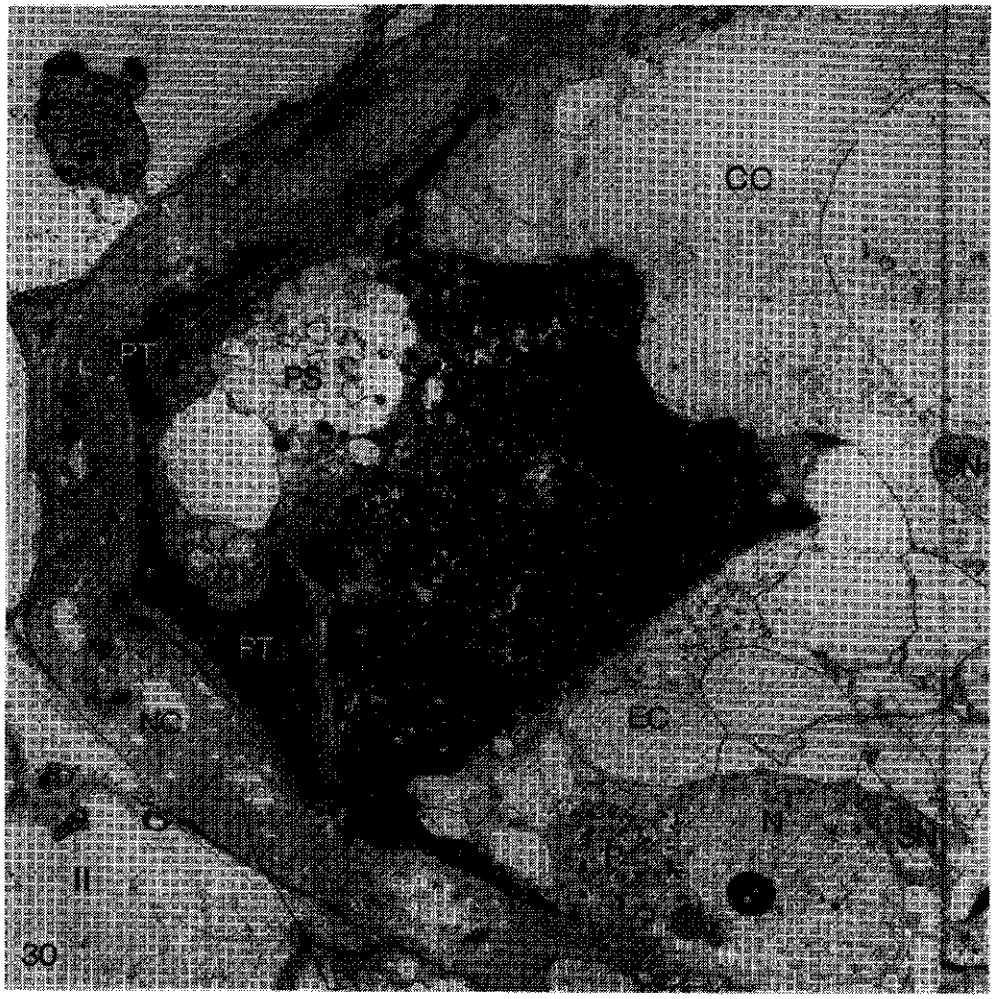
Fig. 30. Longitudinal section through the same ovule as fig. 31. Pollen tube growth at the micropylar side (left) of the embryo sac in between the nucellus and the egg apparatus. 4 DAP, magn. 2160x.



the density of ribosomes. Till 5 DAP circling LER and paracrystalline protein or membranous fragments are still present in the central cell. Subsequently large quantities of RER are observed. ER junctions with only short arms of ER remain present.

- The antipodals -

The cell walls between the central cell and the micropylar antipode and between the two antipodals turn electron dense, starting in some ovules at 4 DAP, but more obvious after 5 DAP. Also the ground plasma of the micropylar antipode darkens and there is an increased density of ribosomes, forming a large population of polysomes. At 4 DAP in a part of the ovules a dark debris is formed in the plastids.

Ten days after pollination the antipodals are still present. In the micropylar antipode the plastids are cup-shaped and contain starch.

The chalazal antipode hardly changes during the observed period.

Discussion

Before fertilization

Parts of the development of the archespore mother cell of *Lilium* till the stage of mature embryo sac have been studied by several authors (Eymé, 1965; Dickinson and Andrews, 1977; De Boer-De Jeu, 1978; Dickinson and Potter, 1978; Plyushch, 1987; Rodkiewicz and Mikulska, 1967). The conspicuous double or multiple membrane inclusions emerging during the development of the archespore mother cell (Dickinson and Andrews, 1977) are present in a relative small amount in the mature embryo sac of *Lilium longiflorum*. Starch disappears from the plastids during meiosis. This is followed by an appearance of electron-opaque particles within the plastids, which by the end of meiosis have enlarged to form starch grains again. Also the ribosome population decreases drastically in this period (Dickinson and Potter, 1978). The mature embryo sac lacks starch

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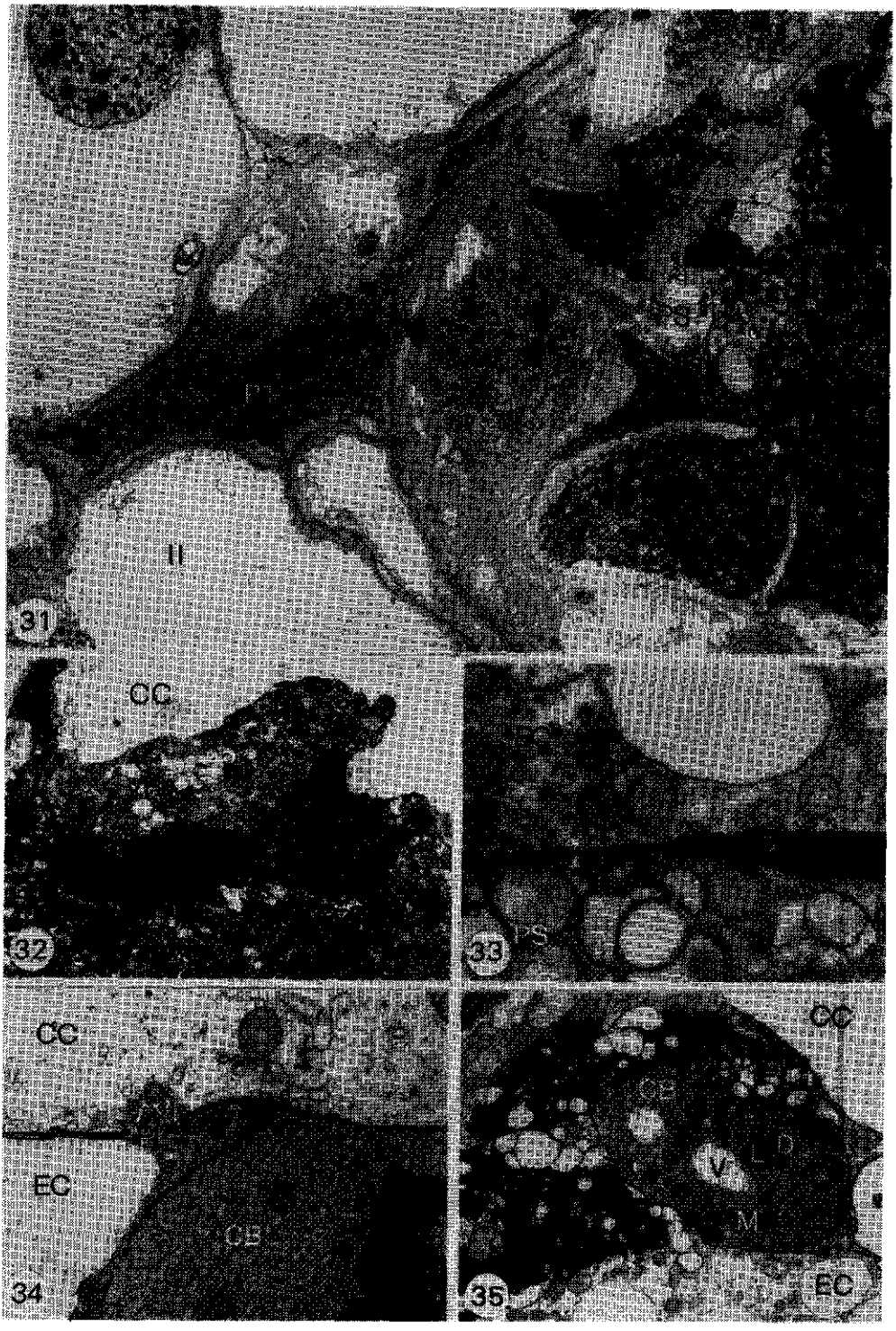
Fig. 31. A pollen tube in the micropyle turns left in front of the nucellus after which it penetrates. After a growth as observed in fig. 30, another section through the same ovule, the pollen tube opens (*). 4 DAP, Magn. 2430x.

Fig. 32. In the degenerated plasma of the synergid and the pollen tube remnants of two nuclei, the former vegetative and synergid nucleus, are present. 4 DAP, magn. 3333x.

Fig. 33. The cell wall between the egg cell and the persistent synergid is at places filled with a degenerated substance and at other places the plasmalemmas are pressed closely together (arrowheads). 4 DAP, magn. 20.0Kx.

Fig. 34. A gathering of vesicles between the plasmalemmas of the egg cell and the central cell. 4 DAP, magn. 10.0Kx.

Fig. 35. A cytoplasmic body in contact with both the central cell and the egg cell. 4 DAP, transverse section, magn. 7000x.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and the ribosome population is small as well. This population increases after fertilization. However, cytoplasmic nucleoloids, apparently aggregations of complete ribosomes which at their disintegration fill the cytoplasm with fresh ribosomes (Dickinson and Potter, 1978), were not observed in the mature embryo sac. In the *Lilium* hybrid 'Enchantment' those nucleolus-like bodies in the cytoplasm were at least present till the stage of the four-nucleate embryo sac and then decrease in number (De Boer-De Jeu, 1978). The ribosome population after fertilization has to have another origin. The dedifferentiation at meiosis was supposed to eliminate the information carrying molecules which are characteristic of the diploid phase of growth (Dickinson and Potter, 1978). The differentiation as observed after fertilization can not be the entrance back into diploid and pentaploid phase, because in the haploid synergid the ribosome population increased as well. Together with the absence of cytoplasmic nucleoloids a different process is taking place. This is a process of activation of the embryo sac cells, resulting in the new synthesis of ribosomes, instead of the dispersal of ribosomes stored in structures in the cytoplasm. The plastids develop another function, which is the storage of starch.

In the four-nucleate embryo sac stage of the *Lilium* hybrid 'Enchantment' plastids, mitochondria and lipids are situated along the embryo sac wall, with a concentration at the poles. A central vacuole is formed, around which LER is gathered. Endoplasmic reticulum plays an important role in this polar distribution (De Boer-De Jeu, 1978). As observed in this chapter much of this distribution of organelles is still present in the mature embryo sac of *L. longiflorum*. ER is predominantly found in the central cell, next to large vacuoles. This distribution is thus likely to originate during megasporogenesis (De Boer-De Jeu, 1978), further back than just the last division to form the mature embryo sac.

Fig. 36. Two cytoplasmic bodies in this section are each in contact with the egg cell and with each other. 4 DAP, transverse section, magn. 1336x.

Fig. 37. The same two cytoplasmic bodies as in fig. 36. Plasmalemmas of both the cytoplasmic bodies and the egg cell and between the bodies are pressed together. Magn. 6123x.

Fig. 38. The nuclear envelope of a sperm nucleus in the central cell before fusion. The outer membrane is associated with SER. 4 DAP, magn. 37.9Kx.

Fig. 39. A sperm nucleus is lined up with the nucleus of the egg cell. No points of contact were observed. 4 DAP, longitudinal section, magn. 10.3Kx.

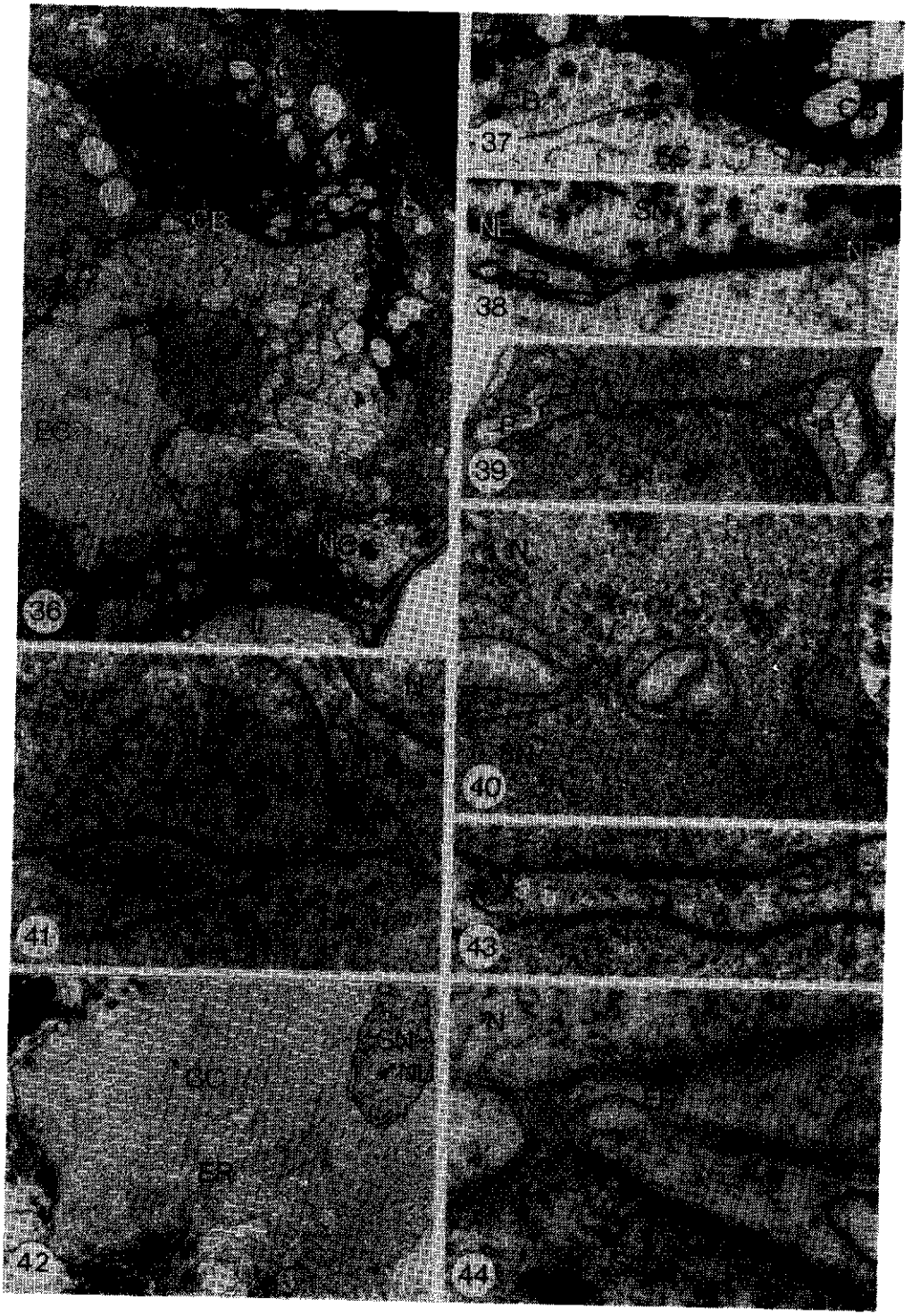
Fig. 40. Several points of fusion in the fertilized egg cell, 7 DAP. Note the different degrees of chromatin condensation of the two partners. Magn. 17.5Kx.

Fig. 41. A fusion between different sites of the same sperm nuclear envelope present in an egg cell at 5 DAP. Magn. 24.3Kx.

Fig. 42. Bundles of ER stretch from the egg apparatus towards the sperm nucleus in the central cell. 4 DAP, magn. 1908x.

Fig. 43. The outer membrane of the haploid polar nucleus (top) and the sperm nucleus (bottom) are both associated with ER. The inner membrane of the polar nucleus makes an extrusion. 5 DAP, magn. 21.6Kx.

Fig. 44. A fusion between the sperm nucleus and the haploid polar nucleus. ER is associated with the outer membrane of the nuclear envelope at the place of fusion. 4 DAP, magn. 33.4Kx.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ER
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SG

Plyushch (1987) compared the ultrastructure of the embryo sacs of two lily species, *L. regale* and *L. candidum*. They differed both from each other as from the species discussed in this chapter. In the egg apparatus of *L. candidum* starch was found and a difference was observed between the egg cell and the synergids in nucleus structure, cytoplasm and cell wall structure. Mitochondria and plastids were more developed and the ribosome number was higher in the synergids. The central cell looked like the one observed in *L. regale* and in both species the golgi apparatus and cisternoid ER in the antipodals were very well developed. Protein crystals were present in the embryo sac of *L. regale*, but absent in *L. candidum*.

In *L. regale* plasmodesmata were found between the three antipodals and the cells surrounding the embryo sac, in contrast with *L. candidum* (Plyushch, 1987) and *L. longiflorum*. In the latter species the plasmodesmata between the megagametophyte and the surrounding nucellus are lost during the pachytene of the first meiotic division (Dickinson and Potter, 1978) and do not reappear.

No consistent difference between the three cells of the egg apparatus of *L. longiflorum* was observed before fertilization. Neither a filiform apparatus, nor a difference in polarity, nor a higher metabolic activity were observed in two of the three cells of the egg apparatus. The larger differences observed between the three cells of the egg apparatus at young stages is either the result of a difference in the speed of development, because in the older stages the cells look more alike, or the consequence of an uneven distribution of organelles before cellularisation. The development of the cells might be different, but the result is more average. The LER and CSER structures disappear from the cells of the egg apparatus, whereas they were present in the megaspore and remain present, although to a lesser extend, in the central cell.

The cells of the egg apparatus probably differ in the amount of starch formed in the plastids of the mature embryo sac, but it is still not clear whether the cell with relatively the least starch is penetrated by the pollen tube and is therefore one of the synergids. There might also be a difference in the extension of the paramural bodies. No consequent ultrastructural differences were found between the cells of the egg apparatus. The development of the embryo sac of *L. longiflorum* stops before the formation of a filiform apparatus. Pollination or pollen tube growth does not trigger a continuation of this development. The cell walls in the embryo sac also do not seem to be well developed. Vesicles, microtubules and ER fragments are present in what might be a diffuse middle lamella, a structure ready to disappear after pollen tube penetration. Although being in this premature stage compared with other species, the embryo sac is ready to receive a pollen tube.

The position of the egg cell in the embryo sac relatively to the vascular bundle, which was taken as a reference point and as observed after fertilization, varied. In *Linum usitatissimum* no consistent orientation of the embryo sac cells relatively to the external sporophytic tissue was found (Russell and Mao, 1990). In sunflower the majority of the degenerated synergids is positioned towards the vascular bundle, the same side where most of the pollen tubes grow through the micropyle. This degeneration starts before the

penetration of the pollen tube (Yan et al., 1991).

The sizes of the cells of the egg apparatus of *L. longiflorum* did also not differ mutually. In *Ornithogalum caudatum* the size of the egg cell is smaller than of the synergids (Tilton, 1981). At the micropylar side of the embryo sac the three cells of the egg apparatus can have different dimensions (see fig. 2). But because of the pattern of pollen tube penetration, with first a turn in front of the nucellus, followed by a penetration and a bend back towards the basis of the embryo sac, hereby lifting up the cells of the egg apparatus, it seems unlikely that the cell with the largest dimension at the basis of the embryo sac gets preferably penetrated. The cells of the egg apparatus are often situated in one transverse plane.

Before the arrival of the pollen tube in the ovule no degeneration of a synergid is observed in *L. longiflorum*. Degeneration is dependent upon penetration of the pollen tube into the micropyle or into the embryo sac. In cotton a chemical communication between the pollen tube and the embryo sac is possible after synergid degeneration, which is triggered by pollination (Jensen et al., 1983). In *Petunia hybrida* the synergid does not degenerate before the arrival of the pollen tube (Van Went, 1970c). Both synergids of *Brassica campestris* degenerate independent upon pollination (Van Went and Cresti, 1988).

In relation to pollen tube penetration, the integumentary and nucellar cells, composing the micropyle may be active. The nucellar cells of *Gasteria verrucosa* might have a secretory function although the dictyosomes are not active in producing vesicles (Franssen-Verheijen and Willemse, 1990). In *Beta vulgaris* they are and a secretory character of the micropylar nucellus is presumed (Bruun and Olesen, 1989). In *Ornithogalum caudatum* most of the micropylar exudate, supposed to contain chemotropic substances, is secreted by the nucellar cap and the inner integument (Tilton, 1980).

Into the micropyle of lily substances are secreted resulting in a lifting up or a disruption of the cuticle as is observed at the nucellus and the inner integument. This might indicate that the secretion originates from these cells. The cells of the latter are however highly vacuolated and lack wall ingrowths, so the amount of substances secreted is likely to be small. At the entrance of the micropyle the cuticle is still bordering the cells of the inner integument, so it seems unlikely that substances are produced by those cells. At this entrance an external fluid is covering the cells of the inner integument as was observed with cryo SEM in chapter 2, but during the fixation and embedding for TEM this fluid is rinsed away. This fluid is continuous with that produced by the placenta. The substances produced by both the nucellus and the inner integument might mix with the placental fluid and so create a directing gradient growth for the pollen tube. The egg apparatus is not likely to be involved in this attraction because of (1) the absence of a filiform apparatus, although some paramural bodies were present in the egg apparatus, (2) the low metabolic activity of the cells of the egg apparatus, (3) the persistence of both synergids before the arrival of a pollen tube and (4) the absence of differences between and the absence of changes occurring in the three cells of the egg apparatus before pollen tube penetration. The nucellus and the inner integument are candidates for the secretion

of chemotropic substances to direct pollen tube growth into the micropyle. No difference in the production of micropylar secrete could be observed between pollinated and unpollinated ovules.

Two antipodals are present in the embryo sac of *L. longiflorum*. The chalazal one has a nucleus with irregularities. Strands of cytoplasm and membranous fragments were observed in this nucleus, probably the result of a disturbed last division (see chapter 2). The darkening of the cell wall around the embryo sac towards the chalaza might be involved in rigidity or in the pathway of nutrients from the nucellus to the embryo sac.

Penetration by the pollen tube

As discussed in chapter 2 the pollen tube grows in between the stigmatic lobes and through the secretions produced by the secretory cells of both the stylar canal and the placenta. In the ovarian exudate the pollen tube makes several turns before entering the micropyle. In the micropyle the pollen tube meets the nucellus. The route along the nucellus which could be followed by the pollen tube was not predictable. There is more than one intercellular space along the nucellus at which the cuticle was ruptured, leading to holes through which the pollen tube can penetrate. The pollen tube is not showing cutinase activity because the cuticle is pushed apart rather than dissolved. Enzyme activity is however needed for the penetration of the cell walls of the here one cell layer thick nucellus. In cotton a column of nucellar cells disintegrates in the region of the micropyle (Jensen, 1965). No signs of degeneration prior to pollen tube penetration were observed in the nucellus of *L. longiflorum*. After growing through the stigmatic lobes, into the style and the ovary the nucellus seems to be the only place where wall dissolution has to occur. The dissolution of the walls of the nucellus is dependent upon the presence of a pollen tube, which is a good candidate for the production of enzymes for it. The cell wall thickenings developed after a few days by the nucellar cells and the cells of the inner integument, both bordering the pollen tube, is a reaction of those cells to this pollen tube, probably to its enzymatic activity. Wall material is deposited in between the plasmalemma and the cell wall of those ovular cells. The pollen tube was not closed by this thickenings at 10 DAP. In cotton a plug is formed in the pollen tube at the subterminal pore (Jensen and Fisher, 1968).

In the majority of species the pollen tube grows through the filiform apparatus which may act as a guide for the pollen tube (Jensen, 1965) leading the pollen tube directly into the (degenerated) synergid. In *L. longiflorum* the filiform apparatus is absent and the pollen tube seems to search for a cell to penetrate because after entering the embryo sac the pollen tube remains growing along the nucellus. As discussed above there is hardly any difference observed at the ultrastructural level between the three cells of the egg apparatus. Non of the cells seem to be secretory active, hereby creating a gradient. Probably the pollen tube grows without preference into one of the three cells which then degenerates, and is now called the degenerated synergid. In case one of the cells is different it implies that the pollen tube recognizes a difference in the plasma membrane

composition of the cells of the egg apparatus, leading to an interaction and finally the discharge of its contents into the right synergid.

The walls between the egg cell, the persistent synergid and the central cell have disappeared after pollen tube entrance of the embryo sac. The two plasmalemmas are bordering each other, at some places separated by degenerated plasma. The plasmalemma of the degenerated synergid deteriorates. It is likely that none of the cell walls in the egg apparatus had a rigid nature and to effect fertilization no cell wall and in case of the degenerated synergid also no plasmalemma, is then present. The local gathering of vesicles observed between the plasmalemmas is probably one of the remainders of the cell wall. It looks as if the persistent wall material is concentrated there. The discharge of the contents of the pollen tube probably results in a pressure which pushes the wall material away.

In other species the cell wall lacks at the chalazal end of the synergids before fertilization as in *Proboscidea louisianica* (Mogensen, 1978). Before fertilization the cell wall was hardly present at the chalazal pole of the synergids of *Petunia hybrida* (Van Went, 1970a) and at places absent between the central cell and the synergids of *Capsella bursa-pastoris* (Schulz and Jensen, 1968). A cell wall was completely absent at the chalazal pole of the egg cell of *P. hybrida* (Van Went, 1970b) and *Quercus gambelii* (Mogensen, 1972). The formerly continuous chalazal egg cell wall of *Plumbago zeylanica* is locally disrupted near the tip of the pollen tube (Russell, 1982). Material of a degenerated synergid is found in between the plasma membrane of the central cell on one hand and the membranes of the egg cell of *Brassica campestris* (Van Went and Cresti, 1988) and of the chalazal part of the synergid of *Petunia* on the other (Van Went, 1970c).

The enucleate cytoplasmic body is supposed to originate from (one of the) the sperm cells and makes contact with both the egg cell and the central cell (Russell, 1985). Both in this cytoplasmic body and in the cytoplasm of the generative cells of *L. longiflorum* no plastids were observed (Tanaka et al., 1989). Most likely in the beginning there are two cytoplasmic bodies in the degenerated plasma of the synergid and the pollen tube in the fertilized embryo sac of *L. longiflorum*. Both are in contact with the egg cell and the central cell and with each other. No plasmodesmata were present between the two. Those were observed between the sperms of *Plumbago zeylanica* (Russell and Cass, 1981). The cytoplasmic bodies are bordered by only one membrane but at some places remnants of the membrane of the vegetative cell are observed. Also in the embryo sac of barley the male gametes are bordered by a single plasma membrane (Mogensen, 1982) as are the remains of the sperm cytoplasm in cotton (Jensen and Fisher, 1968). The cytoplasmic bodies of lily differ in ribosome density and the dense one only remains even till 7 DAP. So probably the other one, whose cytoplasm is similar to that observed in the two cells of the egg apparatus and the central cell, fuses with one of those cells of the embryo sac shortly after losing its nucleus. There must be a regulation before the discharge of the sperm nuclei or a good communication between the two sperm cells to avoid that the two nuclei are delivered into one cell. The fusion of one of the sperm cells with one of the cells of the female gametophyte is delayed, but two

cytoplasmic bodies were only observed shortly after the discharge of the sperm nuclei. In barley Mogensen (1988) observed only one cytoplasmic body in the degenerated plasma and suggested different fusing mechanisms for the two sperm cells, a complete and an incomplete fusion. Sperm cytoplasm is transmitted into the egg cell and central cell in *Plumbago zeylanica* (Russell, 1980).

Preferential fertilization, i.e. one of the different sperm cells predominantly fusing with either the central or the egg cell, which implies a recognition at the gametic level, is present in *Plumbago zeylanica* (Russell, 1985). There is no indication for this process taking place in *L. longiflorum*. There is however a difference in ribosome number and in the persistence between the enucleate cytoplasmic bodies. Both bodies are at first in contact with both the egg and the central cell. The membrane of the degenerated synergid has disappeared. Recognition might take some time, during which the nuclei of the sperm cells are transferred into the cells of the female gametophyte, followed by a fusion of one of the cytoplasmic bodies with either the central or the egg cell. This model was not included in the gametic fusion models in Russell et al. (1990).

Fertilization

The central cell of *L. longiflorum* is rather large. The polar nuclei are not fusing before pollen tube penetration and the metabolic activity seems low. The nuclei are associated with ER, as is the sperm nucleus when transported in the central cell. The central cell itself is relatively rich in ER. Because microtubules were rare, a network of ER is proposed to move the nuclei and to achieve a contact between them. Jensen (1964) also observed an important role of ER in the fusion of polar nuclei of cotton. In *Petunia hybrida* the polar nuclei are situated close to each other and the outer membranes of the nuclear envelopes are sometimes connected via ER (Van Went, 1970b). But in the fusion between the male and female nuclei only the outer and inner membranes of the nuclei are involved, not the ER (Van Went, 1970c). In *L. longiflorum* the sperm nucleus in the central cell first contacts the haploid polar nucleus. In barley the male nucleus fuses with one of the partly fused polar nuclei (Mogensen, 1982).

In the egg cell of the observed species little ER is present. The nucleus is hardly associated with ER. No strands of ER were observed towards the sperm nucleus. It seems that fusion here is more the result of the lining up of the two nuclei after which the outer membranes bulge out, probably with some help of ER.

The sperm nuclei in the egg and the central cell can differ in condensation. The nuclei are probably first contracted before the transfer through the plasmalemmas, because of their enormous size. In the central cell the nucleus than gets more retracted.

In both the central and the egg cell the fusion is taking place very slowly; the pollen tubes enter from 4 DAP and the fusion has still not been concluded at 10 DAP. During this fusion the cells increase their metabolic activity. The ribosome density enlarges and in the central cell the vacuoles gain in size. Whether the number of ribosomes in the central cell increases is therefore unknown. Before fertilization the mitochondria and lipid

droplets probably form energy units in the central cell, becoming effective when the whole embryo sac is activated.

The circling LER structures found in *L. longiflorum* were also present in the central cells of *L. regale* (Rodkiewicz and Mikulska, 1967; Plyushch, 1987) and *L. candidum* (Plyushch, 1987), so it is supposed to be common in *Lilium* species. In the observed species they disappeared after fertilization and abundant RER was subsequently observed. Also the membranous fragments or paracrystalline proteins vanish. LER is probably converted into RER. The observed LER structures before fertilization are already in a less complex and the junctions in a less condensed form compared with earlier stages of embryo sac development of the *Lilium* hybrid 'Enchantment' (De Boer-De Jeu, 1978). The membranes are used by the ever enlarging embryo sac.

Ten days after pollination the antipodals are still present. In both *L. candidum* (Eymé, 1965) as in *L. longiflorum* two antipodals are observed. In the latter no plasmodesmata towards the central cell were observed. After fertilization the wall between the central cell and the antipodals darkens. The contents of black layers in the cell wall surrounding the embryo sac at the chalazal end is unknown. In *Agave parryi* the vascular tissue of the ovule ends in the chalaza, which is bordered by a hypostase, which in its turn is followed by the apical antipode, together forming a major pathway of metabolites into the embryo sac (Tilton and Mogensen, 1979). In cotton the synergids which have a filiform apparatus are probably involved in the absorption, storage and transport of metabolites (Jensen, 1965). A transfer wall lacks in lily. The antipodals are not rich in dictyosomes, but because of the presence of much lipid droplets and mitochondria they might be a storage place.

Pollen tube growth in *Lilium longiflorum* Thunb. following different pollination techniques and flower manipulations.

4

J. Janson and M.C. Reinders

Introduction

In chapter 2 and 3 the development of the flower bud, the pollen tube growth and fertilization after compatible pollination at the stigma was described. In the genus *Lilium* interspecific pollen tubes formed after pollination at the stigma will either stop while reaching the stylar canal or grow further down, sometimes as far as the length reached by incompatible pollen tubes (Ascher and Peloquin, 1968). After intercrossings of a limited number of species a stop of pollen tube growth on reaching the style occurred after pollination with species closely related to *Lilium longiflorum*, the maternal parent used in these experiments. A stop nearer to the basal part of the style occurs in more distantly related combinations (Ascher and Peloquin, 1968). Reciprocal differences in the absolute and relative length of the pollen tubes in the style were found (Ascher and Drewlow, 1971). The length of the pollen tubes inhibited at the base of the stigma can be enhanced by injecting stigmatic exudate of *L. longiflorum* into its style before pollination with pollen of another species (Ascher and Drewlow, 1975). To realize interspecific crosses cut-style pollination was successfully used in a number of combinations (Asano and Myodo, 1977a; Van Tuyl et al., 1986a and 1991). Hybrid embryos could be obtained in small numbers, but their development is retarded when compared with intraspecific embryos obtained after pollinating the stigma. The endosperm remained liquid or was almost absent and a transfer to tissue culture was necessary to continue the growth of the embryos (Myodo and Asano, 1977).

To overcome self-incompatibility the use of the cut-style method is not necessary because a heat treatment of the style (Hopper et al., 1967; Campbell and Linskens, 1984c) or an application of certain flower extracts to the stigma before (Matsubara, 1981) or growth regulators mixed in lanolin to the style (Matsubara, 1973) just after pollination is sufficient. Also a treatment with naphthalene acetamide at the basis of the flower had effect on fruit development resulting in some seeds after self-pollination (Emsweller and Stuart, 1948).

The poor ovule penetration as observed after interspecific cut-style pollination in lily might be the result of an interspecific barrier in front of or inside the micropyle or an absence of an ovule enhancement due to the cut-style pollination (Van Roggen et al.,

1988). When cut-style pollination was carried out with intraspecific compatible pollen the percentages of ovules with a pollen tube in the micropyle was very low as well (Janson, 1988), indicating an incapability of the pollen tubes to penetrate or the absence of an ovule enhancement.

This chapter reports on manipulations carried out with flowers before or during compatible pollination in order to get more insight in the combined action of both the pollen tube and the ovary.

Material and methods

Plants of *Lilium longiflorum* Thunb. were grown in pots in the greenhouse of CPRO-DLO, Wageningen, with a night temperature not lower than 15°C and a day temperature sometimes being as high as 35°C in the summer. Additional illumination was given in wintertime. Compatible pollinations were carried out with 'Gelria' as the female parent and 'White American' as the male parent. Flower buds were emasculated and the stigma was covered with an aluminium foil cap one or two days before anthesis. When using cut-style pollination the style with the stigma was cut off, stigmatic exudate was applied at the cut surface, followed by the pollen grains and an aluminium foil cap to prevent cross pollination and to avoid evaporation. The length of the style left at the ovary is either expressed in mm, the term just above the ovary implies 3 mm of style left at the ovary, or in percentage of the complete length of the style. In the latter case no part of the style or the stigma was removed at 100%, which is in fact a stigmatic pollination, whereas 95% results in a removal of mainly the stigma. Interstylar pollination was carried out through a slit made in the style just above the ovary after which stigmatic exudate was applied, followed by pollen grains. In chapter 2 flowers were pollinated two days after anthesis and because it took at least two days for the pollen tubes to reach the end of the style, cut-style pollination just above the ovary was carried out at four days after anthesis. In that way the ovules, when reached by the pollen tubes, would be of a comparable age. Pollination half-way down the style was carried out three days after anthesis. After pollination the flowers were either left at the plant or put in a vase filled with water and transferred to a growth chamber with 16 hours of illumination at 24°C.

Pollen tube growth in the style and in the ovary was monitored using a binocular microscope. After dissecting the pistil the pollen tubes were stained with water diluted cotton blue (Asano, 1980c). The length of the longest pollen tube in each of the two longitudinal stylar halves was measured and the two figures were averaged. Pollen tube entry in the micropyle was studied after destaining ovules while still attached to the placenta in a mixture of water, glycerol and lactic acid (1:2:1) for one hour at 80°C. Then the material was stained for two minutes at 80°C in a solution of 1% aniline blue in the same mixture. Subsequently the material was destained shortly at 80°C again in the same mixture (modification of Gerlach, 1977).

To determine sperm cell formation the pollen tubes were dissected from the style or the ovary and stained with an aqueous solution of 50 μg/l DAPI (4'6-diamidino-2-

phenylindole.2HCl) at pH 4.0.

Pollen tube growth in the ovary was studied using scanning electron microscopy (SEM). Therefore the material from the greenhouse was fixed for 4 hours in 5% glutardialdehyde in 0.1 M phosphate buffer, pH 7.2, supplemented with 0.25 M sucrose. This was followed by several rinses in phosphate buffer with sucrose and postfixation for 6 hours in 1% osmium tetroxide in water. Rinsing and dehydration in a graded ethanol series was followed by critical point drying over CO_2, applying the specimen to a preparation holder and sputtering it with palladium/gold. The material was observed with a JEOL JSM-5200 SEM.

In experiments using grafted styles the junction was fastened with a drinking straw of which the ends were afterwards filled with vaseline to avoid evaporation. Junctions were also secured with cling foil. The flowers were incubated in a growth chamber.

To achieve an ovule enhancement pollen tubes were blocked in the style or in the ovary by injecting Mowilith (Hoechst) or cyano-acrylate glue at the desired place. The obstruction was always checked at the end of the experiment using diluted cotton blue as described above to localize the pollen tubes. Another way of activating the ovary in combination with cut-style pollination is allowing pollen tube growth in the style and cut it beyond the pollen tube tips after which cut-style pollination can be carried out.

To asses any stimulation from fertilized ovules to ovules in another cavity with the aim to induce pollen tube penetration of pollen grains applied at or in the ovary, flowers were placental pollinated. Pollen tubes originating from the stigma were blocked in the top of one of the three cavities by Mowilith or cyano-acrylate glue, allowing access to the two other cavities. The cavity downward from the barrier, which was deprived from pollen tubes, was opened and here placental pollination was carried out. One row of ovules was removed resulting in an exposure of the other row of ovules together with the placenta at which pollen grains with or without stigmatic exudate were applied. As a control unpollinated flowers were used. The flowers were incubated in the growth chamber.

Semi-vivo experiments were carried out in a large petri dish with moistened filter paper and closed with parafilm. The dishes were incubated in a stove at 25°C and 16 hours light.

Results

After compatible pollination at the stigma on average 70 - 85% of the ovules (table 1) is penetrated by a pollen tube (fig. 1). This percentage is influenced by the condition of the pistil, eg. age (see chapter 2), time of the year and the condition of the pollen grains, especially important during winter time. After cut-style pollination just above the ovary (fig. 2) the percentage of ovules with a pollen tube in the micropyle is reduced, with a highest average percentage of 9 (table 1, $n=10$, $\sigma_{n-1}=8.5$). After germination at the cut surface the pollen tubes grow through the few millimetres of style and enter the ovary. They hereby grow over a zone with secretory cells at which the pollen tubes spread out when growing towards the placentas of the three cavities of the ovary (fig. 3). If a

pollen tube tends to grow from the secretary zone a bending back can be observed (fig. 4). At the placenta an exudate is produced (see chapter 2) by the secretory cells which cover the surface even far into the slit in between the two rows of ovules. They hereby contact each other and have a more pointed rather than a spherical shape (fig. 5). From the central bundle formed in each of the three cavities the pollen tubes bend and grow in between the ovules towards the micropylar side (fig. 6). After passing the ovule they bend again to grow over the placenta along the funiculi and the inner integuments (fig. 7) of the anatropous ovules. The pericarp is only partly covered with secretory cells. In a later stage when probably more exudate has been formed (see chapter 2), the bending of the pollen tubes at the micropylar side happens farther away from the exudate producing placenta but are still in contact with other pollen tubes (fig. 8). At this side

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Table 1. A summary of different experiments, I till VI. Average percentages of ovules with a pollen tube in the micropyle (per flower two rows of ovules were counted leading to the percentage per flower, values of different flowers were averaged) after compatible pollination at different places in flowers of *L. longiflorum*:

* pollination at the stigma of an intact pistil and of a pistil of which the style was damaged by some longitudinal cuts which were made before pollination.

* cut-style pollination just above (3 mm) and 15 mm above the ovary. The ovary was activated by pollinating the stigma and removing the upper part of the style with the pollen tubes after either 1 or 2 days before cut-style pollination at 3 mm, but not later than the passage of the pollen tubes at that point.

* interstylar pollination just above the ovary. Pollen was applied through a slit in the style. In case of interstylar + activation the pollen tubes from pollen applied at the stigma were blocked in the style by injecting a drop of Mowilith or glue just above the basis of the style beyond which interstylar pollination was carried out. + indicates a simultaneous pollination of stigma and style, 'after' indicates interstylar pollination carried out 2 days later than pollination of the stigma.

experiment		I	II	III	IV	V	VI
pollinated at stigma		78	73	70			
style damaged			85				
cut-style, unactivated	3 mm	9	1		2	5	
	15 mm	11					
activated	1 day	4			3	5	
	2 days	5	10		3		
interstylar pollination			6	2			4
interstylar + activation			8	4			2
interstylar after stigmatic							1
total no. of flowers		50	24	20	10	12	16

pollen tubes grow both towards the basis and towards the top of the ovary and they can even pass a few ovules without reacting to the micropyles (fig. 9). To reach the micropyle, the pollen tube has to leave the placenta and to grow in between the inner integument and the ovary wall. Frequently pollen tubes are observed to grow over the inner integument hereby flattened and in close contact with the underlying cells (fig. 10). Penetration can only be confirmed by the staining procedure used in fig. 1. A part of the pollen tubes being in close contact with the inner integument were not entering the micropyle and stopped their growth. Also a growth towards the micropyle without a penetration followed by a return to the small bundle formed at the micropylar side of the ovules occurs. A return of the pollen tubes from the micropylar side of the ovules to the central pollen tube bundle is also observed. Pollen tube growth in between the outer integument and the pericarp did not occur.

- Manipulation of the style -

Both after cross-pollination at the stigma as after cut-style pollination sperm cells are formed. This is not only occurring when cut-style pollination is carried out just above the ovary, but also when the style is cut off at 25, 50 or 75% of its length. Sperm cell formation takes place from 18 hours after cut-style pollination and this does not differ between the various style lengths. After cross-pollinating the stigma the first sperm cell formation is observed about 2 hours earlier compared with cut-style pollination at the lengths mentioned above. After 20 hours of incubation the pollen tubes derived from stigmatic pollination are longer.

The length of the style in case of cut-style pollination is influencing the percentage of ovules penetrated by a pollen tube, which increases when the style is kept longer (fig. 11). Also the percentage of seeds with an embryo is increasing simultaneously, although at a lower level than the percentage of penetrated micropyles. Removing just the stigma and applying stigmatic exudate before pollination did not influence the percentage of ovule penetration if compared with pollination of the intact pistil. Also removing the stigma one day after pollination, after which the pollen tubes had grown on average 47 mm into the style, did not influence the percentage of penetration, nor did damaging the style by a longitudinal cut (table 1). In a vertical positioned pistil with the stigma downward, the pollen tubes still reach the ovules.

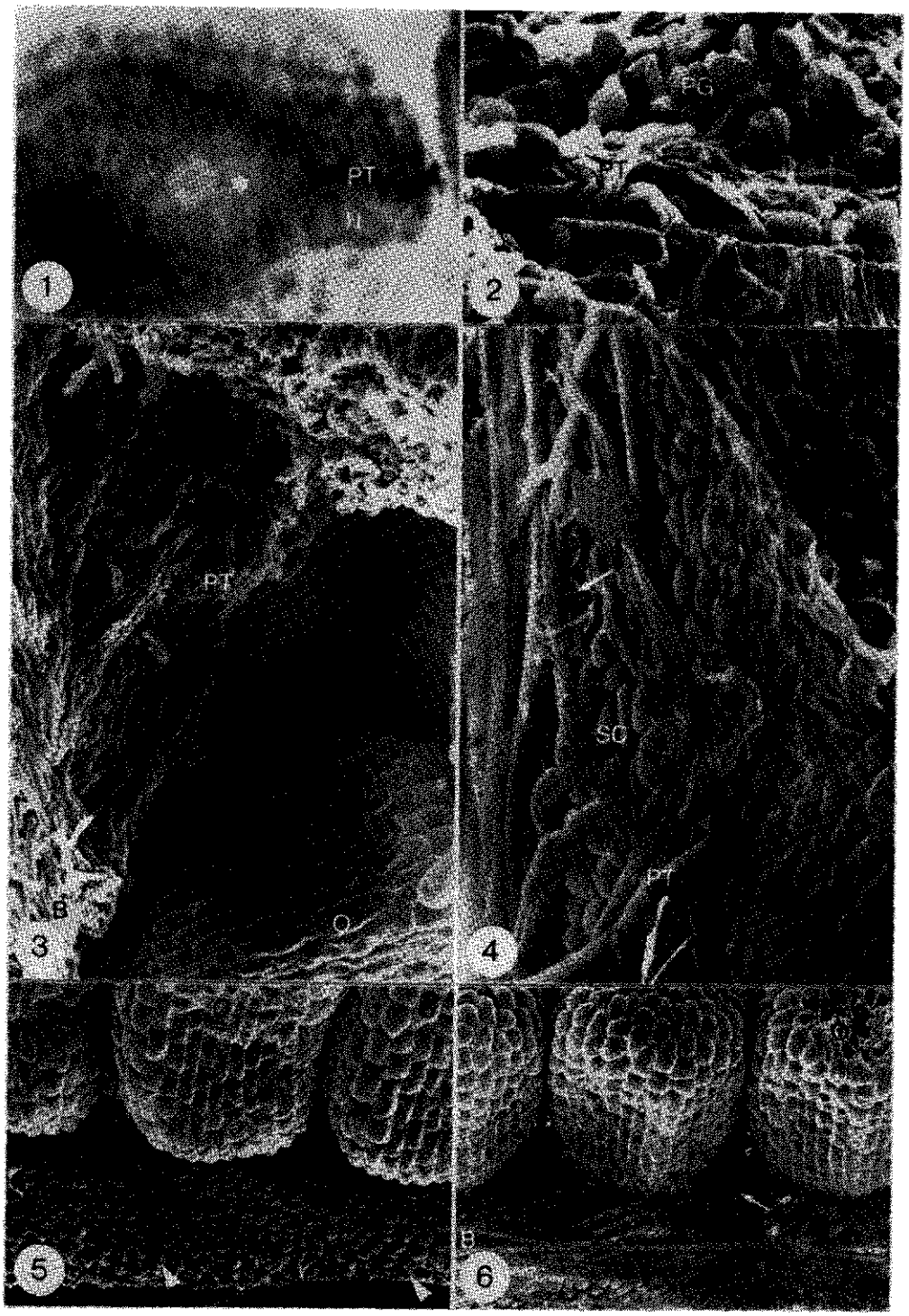
Grafting a stigma with a few mm of style just above the ovary and applying pollen, hardly raised the percentage of penetration when compared with cut-style pollination just above the ovary. When these few mm were raised to a total of 25% of the normal style, including the stigma, the percentage increased to 20. A cut-style pollination carried out leaving a quarter of the style at the ovary gave a micropyle penetration of 9% (average of 5 flowers), which is lower, but not significantly different from the 20% mentioned above, due to the large variation.

- Manipulation of the ovules -

To overcome the absence of any pollination-signal in the cut-style method, ovaries were pretreated by pollination and pollen tube growth in the style after which the latter was cut off before the pollen tubes had reached the ovary. Subsequently cut-style pollination was carried out. It is also possible to block the pollen tubes at about 1 cm above the ovary, by injecting a drop of Mowilith or glue, beyond which interstylar pollination is carried out in between the place of blocking and the ovary. This allows the presence of the pollen tubes in the style during which interstylar pollination was carried out. Both treatments with various incubation times did not influence the low percentage of ovule penetration by the pollen tubes after cut-style or interstylar pollination (table 1).

Also fertilized ovules did not induce a higher percentage of penetration in the placental pollinated cavity of the flowers. After germination at the placenta pollen tubes do grow but only a few micropyles were penetrated, resulting in a penetration percentage of the ovules between 1 and 2% after 5 or 6 days incubation of the placental applied pollen. This percentage did not increase when the stigma was pollinated simultaneously with the placenta or when stigmatic pollination was carried out for up to eight days before pollinating the placenta. The pollen tubes from the style can and do penetrate the other cavities and an ovule penetration of 68% was found. The barrier did block the pollen tubes in the placental pollinated cavity in 77 flowers, which was the total number used in these experiments.

Fig. 1. A pollen tube (PT) in the micropyle observed with light microscopy at nine days after pollination at the stigma. Inner integument (II), nucellus with embryo sac (*). Magn. 392x.
Fig. 2. Germinated pollen grains (PG) at the cut surface (arrowheads) after cut-style pollination as observed by SEM. The stigmatic exudate which was applied before pollination was washed away during the preparation procedure. Pollen tubes (PT), five days after cut-style pollination (DACP), magn. 220x.
Fig. 3. A SEM micrograph of pollen tubes (PT) growing from the style (upper side of the picture), into one of the three cavities, spread out and form a central bundle (B) just before the first ovule (O). The bundle continues in between the two rows of ovules in one cavity. 3 DACP, magn. 307x.
Fig. 4. While entering the ovary (the style is at the top and the basis of the flower is beyond the bottom of the picture) pollen tube growth is restricted to the area with the secretory cells (SC) and a return to this zone is observed if a pollen tube (PT) happens to grow from it. Note some exudate covering a pollen tube and secretory cells (arrow). SEM, 1 DACP, magn. 1124x.
Fig. 5. At the place where the two sides of the placenta are firmly pressed together, the secretory cells are more pointed (arrowheads) rather than spherical as observed at places closer to the ovules (O). SEM, 7 days after anthesis, magn. 467x.
Fig. 6. One day after cut-style pollination a central bundle (B) is formed at the placenta in the ovarian cavity and tubes bend (arrow) to grow in between the ovules (O) towards the micropylar side. A SEM observation, magn. 355x.


PT
1
PG
PT
2
PT
3
O
SC
PT
4
5
B
6

- Pollen tube growth in the style -

In the interactions between pistil and pollen, the presence of the ovary or stigmatic pollination might influence pollen tube growth in the style. Both isolated styles and styles with an ovary were pollinated and incubated in a petri dish. The pollen tube length, per flower averaged over the two style sides of the longitudinal opened style, reached after 44 hours was on average 81 mm (standard deviation 5.9) in styles with an ovary and 79 mm (σ_{n-1} is 4.7) in isolated styles (11 flowers each).

After interstylar pollination carried out at half-way down the style, the pollen tubes grew towards both the stigma and the ovary. Pollinating the stigma did not induce a faster growth towards the ovary nor a reduction of growth towards the stigma (table 2). The pollen tubes reaching an unpollinated stigma stopped their growth in between the stigmatic lobes and formed a thicker walled swollen tip.

Discussion

- Pollen tube pathway -

The pathway of the pollen tubes in pistils is predetermined because of the stigma position or, in case of incomplete pistils, the place of application of stigmatic exudate, i.e. a source of nutrition and a medium for germination and growth. After pollination at the stigma and cut-style pollination the pollen tubes follow a pathway which is lined with secretory cells. The pathway followed is thus dependent on the surrounding nutrition and growth medium. The pattern of pollen tube growth in the ovary after cut-style pollination also does not seem to differ much from that observed after stigmatic pollination as described in chapter 2. In both cases the pollen tubes entering the ovary follow the zone with secretory cells and a central bundle is formed in each of the three cavities. Pollen

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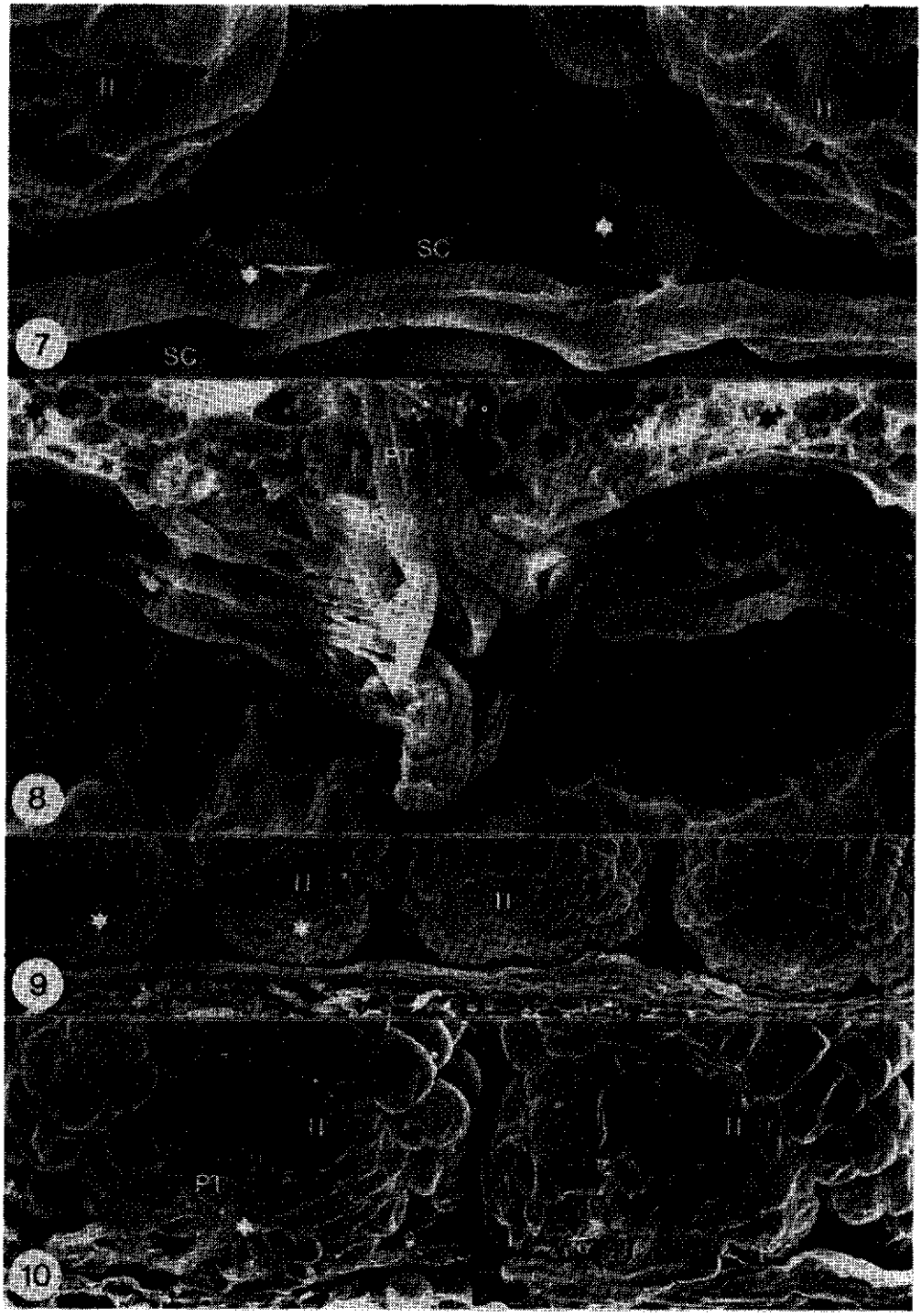
Fig. 7. At the micropylar side of the placenta the pollen tubes bend again (*) to stay in the proximity of the secretory cells (SC) and grow along the inner integuments (II). The pericarp was broken away through the last non-secretive cells. SEM, 3 DACP, magn. 1806x.

Fig. 8. Five days after pollination more pollen tubes have grown in between the ovules, which have been broken away during preparation (*), and the tubes (PT) bend to grow along the inner integuments in a later stage (compare with fig. 7) hereby leaving the zone with secretory cells. SEM, magn. 722x.

Fig. 9. A SEM micrograph of pollen tubes growing along the inner integuments (II) of several ovules at 3 DACP without responding to the micropyles (*). Magn. 271x.

Fig. 10. After cut-style pollination just above the ovary an intensive contact between the inner integument (II) and the pollen tube (PT) can be observed. From this image it is impossible to state whether the pollen tube enters the micropyle. A later arriving pollen tube (*) grows towards the micropyle as well but turns and grows back towards the bundle formed at the side of the inner integument. A SEM micrograph, 3 DACP, magn. 542x.


SC
SC
PT
PT
II
II
II
II
II
II
7
8
9
10

tubes bend from this bundle towards the micropylar side of the ovules and make a second curve to grow along the inner integuments. Thereafter a difference occurs between the pollination methods. In case of cut-style pollination the micropyles are penetrated in a low number, in case of stigmatic pollination the majority of the ovules are penetrated. Because of the use of compatible instead of interspecific pollen a barrier was not expected.

Once arriving at the micropyle the stigma derived pollen tubes enter, grow through the micropyle probably on their own reserves or might also be helped by an excreted substance from the nucellus (see chapter 3). In contrast with the pollen grains of the *Lilium* hybrid 'Enchantment' in which starch disappears in a late stage of pollen development (Willemse and Reznickova, 1980) the pollen grains of *L. longiflorum* still have a small amount of starch at the time of anthesis (Janson, unpublished). A pollen tube two days after stigmatic pollination (Janson, unpublished) and the degenerated plasma of the pollen tube and the synergid in the embryo sac (see chapter 3) contain however a large amount of starch grains. An increase of starch is observed in in vitro germinating *L. longiflorum* pollen grains (Dickinson, 1968). During pollen tube growth in the style of *L. longiflorum* reserves as starch, proteins, short chain polysaccharides and the few lipid droplets in the stylar cells are depleted (Dickinson et al., 1982). Pollen tubes take up substances from the stylar exudate (Labarca and Loewus, 1973). In the pollen grain or tube starch and probably other substances will be formed after pollination and during its growth through the style. It might be that the exudate in the ovary is not as rich as that in the style because pollen tubes from stigmatic pollination entering the ovary remain growing but not as fast as in the style, as was observed in chapter 2.

When cut-style pollination is carried out just above the ovary there is hardly an opportunity to take up substances from the style. The pollen tubes might have less reserves and an entrance of the pollen tubes into the micropyle is less satisfying than

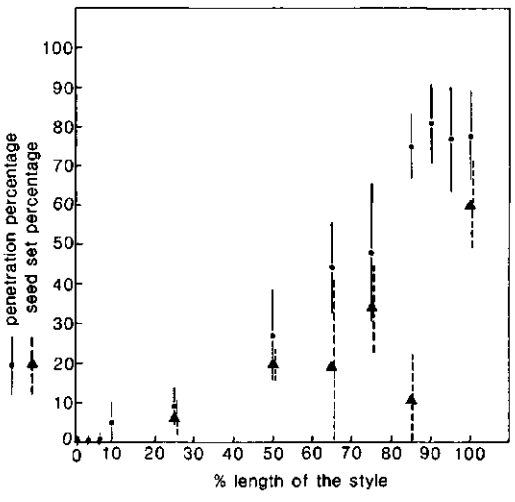


Fig. 11. The percentage of ovules with a pollen tube in the micropyle (●, experiment with 69 flowers, lines represent the standard deviation, σ_{n-1}) and the percentage of seeds with an embryo (▲, experiment with 30 flowers, dotted lines represent σ_{n-1}) achieved after cut-style pollination carried out with compatible pollen and at different style lengths, with only at 100% a stigma present.

growing along a path with more nutrients, being the ovarian exudate. It might also be that the communication between the pollen tube and the pistil is even more delicate than appeared in chapter 2 and is not built up during the short growth after cut-style pollination. A circling of the pollen tubes over the inner integument without penetration and in general a stop of the pollen tube growth or the growth along more than one ovule without responding to the micropyles is the result. Some pollen tubes penetrate probably the micropyle by chance while growing over the inner integument.

So there is the possibility that the ovules are receptive but that the pollen tubes formed after pollinations carried out just above or in the ovary, are not prepared to penetrate the micropyles. An argument to support this idea is the observation that the longer the style is left prior to cut-style pollination the higher the percentage of penetration. So during the growth through the style the pollen tube is prepared to penetrate. When the style is left longer, the pollen tubes can take up more substances from the stylar exudate, which are used in the assembly of the pollen tube, but also contribute to the preparation of the pollen tube. There is no sudden rise in the penetration percentage when increasing the stylar length which leads to the idea that there is no critical length. When pollination was carried out just above the ovary, at least a part of the pollen tubes were able to form sperm cells, but similar to the presence of a pollen tube this also does not mean that the pollen tubes are ready for penetration. The pollen tubes react solitary to this 'ageing' with a large variation between the different pollen grains and tubes.

- Manipulations of the ovules -

The attempts to activate the ovules by allowing pollen tubes to grow in the style or even in the ovary preceding cut-style, interstylar or placental pollination, did not result in a higher percentage of ovules with a pollen tube in the micropyle.

In literature several observations were published which point towards a communication between different flower organs. In cotton pollination or pollen tube growth can induce processes in the ovules like synergid degeneration which does not occur in unpollinated flowers. This degeneration can also be induced in culture by gibberellic acid (Jensen et al., 1983). In *L. longiflorum* the degeneration of the synergid is not observed before pollen tubes enter the embryo sac (see chapter 3). After different stigmatic treatments different bioelectric potentials were registered in the style (Spanjers, 1981), so a communication through the style is possible. After pollination but before the arrival of the pollen tubes in the ovary the carpels of *Petunia hybrida* are stimulated to protein synthesis and an increase in rRNA is observed (Linskens, 1973). Also in the ovary of *Petunia* and before the arrival of the pollen tubes a difference between cross- and self-pollination is reported in protein metabolism (Deurenberg, 1976b) and in influx of organic constituents (Linskens, 1974). In *Nicotiana alata* flowers separated from the plants, the glucose and fructose level decreased in the ovaries before the pollen tubes had reached it. This decrease did not occur when the flowers had remained on the plant (Tupy, 1961).

Our different kinds of experiments, in which different ways of pollination were carried out, we failed to demonstrate any activation of ovules due to pollination or pollen tube growth. Although there is this bioelectric potential in the style (Spanjers, 1981), our experiments do not point to such type or condition of activation. It might be that an activation of the ovules in *L. longiflorum* is not triggering processes to induce pollen tube entrance into the micropyle. After using the cut-style method in interspecific crosses between different lily species a high percentage of seed pods aborted very early. With the mentor pollen technique, in which non-functional (by gamma irradiation) intraspecific compatible pollen was applied to the stigma in a mixture with the pollen of another species, the seed pods aborted less (Van Tuyl et al., 1988). This points to a post-fertilization role of intraspecific pollination or pollen tube growth.

In *Erythronium grandiflorum* the length of the pollen tubes of a pollen donor in one canal was influenced by the presence of other donors in another canal of the same style (Cruzan, 1990). This is an interaction between different pollen tube populations. An interaction of pollen tube growth between the different cavities within the placental pollinated flower was not observed. It might be that the difference in the place of pollen grain application, the placenta or the stigma, prevents any interaction. Also the parameter in our experiments was not the pollen tube length as was in Cruzan (1990), but pollen tube penetration. Probably the interaction has to be intermediated by the ovules to induce pollen tube penetration. Penetration was not taking place in larger numbers compared with non-stigmatic pollinated pistils and thus penetration inducing activation might be absent. Another activation might however still exist but trigger post-fertilization processes. In other words, the chances of embryo development in the placental pollinated part of the flower are larger, because of a post-fertilization stimulus from the normal fertilized ovules

Table 2. Average length in mm with the standard deviation in parentheses of compatible pollen tubes of *L. longiflorum* reached in the style both towards the basis as towards the stigma, after inserting stigmatic exudate and compatible pollen half-way down the style, followed by an incubation of 14 hours. Half of the number of stigmas were pollinated 4 hours before interstylar pollination was carried out. n is the number of styles per experiment, carried out in November-December.

	pollen tube growth towards basis	stigma
exp. 1, n=6		
pollinated stigma	9 (6.3)	10 (4.3)
unpollinated stigma	15 (0.3)	13 (1.8)
exp. 2, n=14		
pollinated stigma	11 (3.6)	10 (4.2)
unpollinated stigma	12 (6.4)	11 (5.6)

in the other cavities.

- Adjustments of the pollen tube pathway -

Our experiments show that the stigma can be omitted for pollination as long as the pollen tubes can reach a certain length in the style. Prior to cut-style pollination at different style lengths stigmatic exudate was applied at the cut end as germination medium and as extra fluid for hydratation. It led to a better and more repeatable pollen tube growth in the style. Without this exudate pollen grains can still germinate in the style (Iwanami, 1953). Gladding and Paxton (1975) pollinated *L. longiflorum* flowers after stigma removal without applying stigmatic exudate and had a fluctuating seed set, as determined by the swelling of the ovule. The pod set was comparable with pollinating a cut-style 1 cm above the ovary. In our experiments there was a large difference in ovule penetration between the two lengths: just stigma removal and cut-style pollination carried out at 1 cm above the ovary, indicating a loss of interaction between the pollen tube and the micropyle. Omitting the exudate before cut-style pollination as was done by Gladding and Paxton (1975), probably led to a fluctuating pollen tube growth. On top of this the two classes of observation with either an ovary with one or many seeds or an ovary with no seeds are very crude. Differences in pollen tube behaviour as was observed in our experiments were therefore unnoticed.

Grafting a style with stigma in a way that the length of the style is reduced to 25% did not have a significant better result than cut-style pollination at the same height, although there was a slight tendency towards an improvement of the penetration. An explanation might be that if the stigma is present the surface for germination is larger as is the amount of exudate. More pollen grains are germinating and, in contrast with cut-style pollination where not all micropyles were covered by a pollen tube, an extra invasion is possible.

- Pollen tube growth interaction -

As shown before (Iwanami, 1953) in lily a growth of the pollen tubes in the style both towards the stigma and towards the ovary is possible. After 48 hours of incubation, pollen tubes reach the same lengths when pollinated at the ovarian or at the stigmatic end of the style (Ascher, 1977). When pollen tubes from the style arrive at the stigma, the thickening of the pollen tube tip might point towards a difference in osmolarity of the exudates. If that is present the slow initial pollen tube growth after pollination at the stigma (see chapter 2) might be caused by this phenomenon.

Pollination of the stigma did not have an influence on the length of the pollen tubes, which grew from pollen applied half-way down the style, in one or both directions. This implies that there is no interaction as observed in *N. alata* in which compatible stigmatic pollination reduces the growth of mid-style applied pollen towards the stigma (Mulcahy and Mulcahy, 1986a) and enhances the growth towards the ovary (Bergamini-

Mulcahy and Mulcahy, 1988).

According to Amaki and Yamamoto (1988) pollination itself can influence the growth medium for pollen tubes. Compatible pollen tube growth in the top of the style of *L. longiflorum* can influence the properties of the style further down to the basis and enhance pollen tube growth without the pollen tubes having reached this area. The inducing factor probably moves with a velocity of 5 mm/h (Amaki and Yamamoto, 1988). Cross-pollination induced a higher activity of several enzymes in the style, when compared with non- or self-pollinated pistils, in the examined area between 15 and 45 mm from the stigma at six hours after pollination (Amaki et al., 1989). According to our pollen tube speed (chapter 2) this is before the pollen tubes could have reached that area. It also fits into the above calculated velocity of 5 mm/h. The velocity of the bioelectric potential measured in the style at 10 - 30 mm from the ovary by Spanjers (1981) was faster, varying between 12 and 54 mm/h, but emerged from 6.5 hours after compatible pollination. Because of the difference in place and time interval it is not possible to fit this signal in the timescale of the above mentioned processes. Also the direction of the translocation of the potential change was in some cases towards the ovary and in others in the opposite direction (Spanjers, 1981). Its speed might however point towards an inducing-role for triggering processes before the arrival of the pollen tubes. In *N. alata* styles Bredemeijer and Blaas (1975) showed an increasing activity of a peroxidase as the result of pollination or penetration of the pollen tubes in the stigma rather than pollen tube growth itself in the style.

In contrast with results in *P. hybrida* (Mulcahy and Mulcahy, 1985 and 1986b), the ovary of *L. longiflorum* had no influence on the length of the pollen tubes in the style. The pollen tube growth in *Petunia* styles detached from the ovary stops at the latest at three third of the style, whereas in intact pistils the ovary is entered. In our experiments the pollen tubes reached the end of the style also in absence of an ovary. In *Persea americana* the embryo sac seems to influence the number of pollen tubes in the basal part of the style or a least there is a relation between the number of pollen tubes in that part of the style and the number of embryo sacs and nucelli in the one ovule (Sedgley, 1976). Although Mulcahy and Mulcahy (1986a) observed an ovarian influence on the pollen tube growth in *N. alata*, Kandasamy and Kristen (1987) did not find one in *N. sylvestris* and *N. tabacum*. In *N. alata* it was also observed that pollinating the stigma, removing the surface of the stigma after ten minutes and pollinating the cut end raised the seed set in comparison with just pollinating the cut surface (Bergamini-Mulcahy and Mulcahy, 1988). After all the attempts in our experiments an activation of the style or the ovules of *L. longiflorum* was not found although there certainly is the impression from literature that also here an activation is taking place.

Placental pollination in *Lilium longiflorum* Thunb.

J. Janson

5

Introduction

To achieve interspecific crosses in lily the cut-style method, in which pollen grains are applied at the cut surface resulting from largely or completely removing the style (Myodo, 1975; Asano and Myodo, 1977a), is used. But the percentage of ovules with a pollen tube in the micropyle is low, even when this technique is carried out with compatible intraspecific pollen of *Lilium longiflorum* (9%), compared with 78% after compatible intraspecific pollination at the stigma (chapter 4).

In the past different in vitro pollination methods have been developed: pollination at the stigma of a cultured pistil, injection of a pollen suspension into the ovary (Kanta et al., 1962) or pollination of the ovule or the placenta (Kanta and Maheswari, 1963; Olson and Cass, 1981). These methods were developed for several aims, e.g. overcoming incompatibility as reported for *Petunia axillaris* (Rangaswamy and Shivanna, 1967) or making interspecific crosses (Zenkteler, 1967 and 1970; DeVerna et al., 1987).

Raquin (1985, 1986) produced haploid and hybrid plants after pollination with irradiated pollen before culturing the ovary of *Petunia hybrida*. A low doses of irradiation given to the pollen grains gave only diploid plants whereas no pollination led to no plants at all. Also in *Cucumis melo* (Sauton and Dumas de Vaulx, 1987) and in *Daucus carota* (Rode and Dumas de Vaulx, 1987) haploids have been produced after stigmatic pollination with irradiated pollen. Although the numbers of obtained plants are small, the results do point towards an activation of the female tissue by pollination or pollen tube growth. Activation of the ovary of *P. hybrida* before it has been reached by the pollen tubes was detected by Linskens (1973, 1974) and Deurenberg (1976b).

Apart from pollination or pollen tube growth the activation of the ovules can also be carried out artificially. Jensen et al. (1983) induced a degeneration of the synergid and a fusion of the polar nuclei in cultured ovules of cotton (*Gossypium hirsutum* L.) by adding gibberellin (GA) and indole acetic acid (IAA) to the medium. The same events took place in in vivo pollinated flowers, but not in unpollinated ones.

To examine the problem of the poor ovule penetration after cut-style pollination of *Lilium longiflorum*, which is a study of the interaction between pollen tubes and ovules, an in vitro system was used. This system gives an alternative range of possible

manipulations of ovules and pollen tubes before or during culturing in vitro, next to the in vivo manipulations as discussed in chapter 4. Placental pollination within the species *L. longiflorum* was carried out with compatible pollen to avoid interspecific or incompatibility barriers. Different media, hormones and pretreatments of the ovules and the pollen grains were examined to realize placental pollination.

Materials and methods

- Plant material -

Plants of *Lilium longiflorum* Thunb. 'Gelria', 'Flevo' and 'White American' and of the Asiatic hybrid 'Whilito' were grown in pots in a greenhouse of CPRO-DLO, Wageningen, with a minimum temperature of 15°C at night and a day temperature of 17 - 20°C, with peaks of 35°C in the summer. Flower buds were emasculated and a cap of aluminium foil was placed over the stigma to prevent cross pollination.

Two series of experiments were carried out: (A) placental pollination in vitro under sterile conditions on a culture medium or (B) grafted style experiments in which a style was placed at a placenta. Preceding the dissection of the placentas of 'Gelria' and 'Flevo' for both types of experiments the following different pretreatments were given to the flowers. 1. Pollinating the stigma and using the ovary before the pollen tubes reached the end of the style, which takes at least two days. 2. Pollinating the stigma and blocking the pollen tubes by injecting Mowilith (Hoechst) or cyano-acrylate glue in the basis of the hollow style or in the top of the ovarian cavities to allow an even longer pretreatment without the pollen tubes entering the micropyles. Apart from these pretreatments, also unpollinated pistils were used.

- Pollen grains and tubes -

L. longiflorum 'White American' was used as a male parent for compatible intraspecific pollination of 'Gelria'. In grafting experiments the combination of pollen of 'Gelria' with 'Flevo' pistils was made as well. In the winter period stored pollen grains were used. Storing was carried out on silicagel at room temperature or at -18°C, both followed by rehydration at 100% RH for at least one hour at room temperature directly before pollination.

To obtain sterilized pollen grains for placental pollination anthers were collected after surface sterilising flower buds just before anthesis with 70% ethanol. After incubation at room temperature for at least 16 hours in a sterile petri dish, the anthers dehisced after opening the petri dish in the laminar flow cabinet. Sterilized pollen grains could be stored for some period in a sealed petri dish on silicagel at room temperature, but not as long as pollen originating from in vivo opened flowers under non-sterile conditions. Placental pollination was carried out using a small spatula. Also pre-germinated pollen, incubated in liquid BK-medium (I, see below) or after a growth through stigma and style

for up to 24 hours was applied at the placenta.

A. Placental pollination

Ovaries were sterilised for a few seconds in 70% ethanol followed by 30 minutes in a 1% aqueous solution of Na-hypochlorite with a few drops of Tween 80. The material was rinsed three times in sterilized millipore-filtered water.

The ovaries were cut longitudinally into 6 sectors, each containing a placenta with a row of ovules and a part of the ovary wall. A few top and basal ovules were omitted because they were not fully developed and generally fail to form seed in the in vivo situation. In some experiments other explants, eg. transversely cut pieces of ovary, were used in an effort to improve the results.

Three sectors of the ovary were placed in a 6 cm petri dish, with either I. BK-medium: Brewbaker and Kwack (1963) salts with 12% sucrose and 0.6% Difco agar noble or Imperial laboratories purified agar, or II. GAM-medium: a modification as described by Prakash and Giles (1986) of the medium developed by Gamborg et al. (1968), or III. MS-medium: a modified Murashige and Skoog (1962) medium, (MS salts and vitamins, 2.0 mg/l glycine, 100 mg/l myo-inositol, 0.001 mg/l naphthalene acetic acid, according to Asano and Myodo (1977b), and supplemented with 10% sucrose and 0.7% of the above mentioned agars, pH 6.0). Also BK-medium supplemented with GA_3 (Sigma G3250) and IAA and an agar plate with just 6% mannitol was used. The latter was carried out to determine whether the nutritious media used, distracted the pollen tubes from penetration.

In case of extended experiments to allow embryo formation, flower buds of a length between 4.5 and 5.0 cm were surface sterilized using 70% ethanol and their anthers with microspores were placed on the agar close to the explant to induce swelling of the ovules (Straathof et al., 1987). Per sector one third of an anther was applied.

The pollinated placentas were incubated at 25°C, either in the dark, or in a 16/8 day/night rhythm, with 150 lux at daytime.

- Pollen tube growth and embryos -

Pollen tube growth in the style and at the placenta was traced by staining with a 5% aqueous cotton-blue solution (Asano, 1980c). One week after placental pollination the presence of a pollen tube in the micropyle was determined with light microscopy after clearing the ovules in a mixture of lactic acid, glycerol and water (1:2:1) for approximately 30 minutes at 60°C, and subsequent staining in 1% aniline blue in the clearing solution for 1 min. at the same temperature followed by destaining the material again in the mentioned clearing solution at 60°C for 5 minutes (modification of Gerlach, 1977). If desired placentas and ovules can be stored in the clearing solution after which the clearing is taking place faster. The results were statistically analyzed using the Fisher exact probability test.

Embryos could be traced by staining the ovules for pollen tubes as mentioned above, but at a lower temperature and with longer incubations in the clearing and staining solutions, selecting the ovules with a penetrated micropyle and examining them with an interference contrast microscope. Also clearing with a saturated aqueous solution of chloral hydrate, without further staining was used to detect embryos, since for this technique no heating is needed.

For scanning electron microscopy (SEM), to determine the pollen tube pathway, specimens were fixed for 16 hours in 5% glutardialdehyde in a 0.1 M phosphate buffer (pH 7.2) containing 0.25 M sucrose, rinsed in the buffer for 2 hours, post fixed for 6 hours in an aqueous solution of 1% OsO_4. After rinsing in water and dehydration in ethanol the pistil material was critical point dried over CO_2 and coated with palladium/gold. A JEOL JSM-35C and a ISI Super-IIIA SEM were used to observe the specimens.

For tracing sperm cells during pollen tube growth and fertilization, pollen grains were incubated for one to two hours in liquid BK-medium with 12% sucrose and 50 μg/l DAPI (4'6-diamidino-2-phenylindole.2HCl), rinsed 4 - 8 times in medium without DAPI and used for placental pollination. To remove excessive fluid the pollen solution can be poured over filterpaper. As a control, flowers were pollinated on the stigma with or without dissolving DAPI in the stigmatic exudate, according to Keijzer et al. (1988) and pollen grains were grown in BK-medium with DAPI.

B. Grafted style pollination

Flowers placed in a vase with water were pollinated and incubated in a growth chamber with 16 hours light at 24°C. After two days of pollen tube growth, when the first pollen tubes had reached the end of the style, the styles were grafted onto a placenta. In experiments using shorter styles this preincubation was shorter to cut the style just in front of the pollen tube tips. Placentas were cut the same way as was done for placental pollination in vitro but only one third of the length of the row of ovules was used. The presence of the BK- or MS-medium disrupted the growth of the pollen tubes from the style to the placenta, which was circumvented by incubation in just a humid test tube without medium and a firm contact between the stylar base and the placenta. The placenta was attached perpendicular to the style using cling or aluminium foil. A barrier to avoid fluids or signals from the style to reach the placenta was made by covering the cut end of the style with a cap of one of the foils in which a hole was pierced with a dissecting needle to allow pollen tubes to reach the placenta.

The grafted style experiments were carried out in test tubes containing a few drops of water to reduce the evaporation of the tissues and sealed with cotton wool and cling foil or silicon plugs. They were incubated for 5 or 6 days in the growth chamber which was also used for placental pollination. To check the pollen tube passage from the style to the placenta the water diluted cotton blue solution as mentioned above was used. The number of ovules with a pollen tube in the micropyle was determined per placenta. Statistical analysis was carried out using an analysis of variance (ANOVA).

In another series of experiments the pollen tubes were blocked in the style with Mowilith or glue as described above and placental pollination of placentas of unpollinated pistils was carried out after which the style was grafted. Those grafts were compared with grafts in which unpollinated styles with a barrier were again grafted to a pollinated placenta. These experiments are thus a combination between the grafted style and placental pollination. *Fluids or a signal from the style, but not from the stylar canal, could* transfer to the placenta while the pollen tubes could not.

Results

A. Placental pollination in vitro

Pollen placed on the placenta germinates and a part of the pollen tubes grow in between the ovules towards the inner integument and the micropyle (fig. 1). The pollen tubes preferentially grow through this pathway in between the ovules, as can be seen in figs 2 and 3, where pollen tubes return to the place of pollination, the placenta, turn and grow back sometimes even in between the same two ovules towards the micropylar side. When the pollen tubes arrive at the micropylar side of the ovules they bend again to either side to stay in the proximity of the secretory cells (fig. 4). Sometimes the inner integument and the micropyle are ignored and the pollen tube grows back towards the placenta where the pollen was applied (fig. 5) or resumes its growth along adjacent ovules. More often the pollen tubes change their growth direction as a reaction to the inner integument or the micropyle. Pollen tubes sometimes follow ridges, e.g. the border of the inner and outer integument or grow over the outer integument (fig. 6). Also circling and turning of one or several pollen tubes over the micropyle occurs (figs 7 and 8). Sometimes penetration of the micropyle can be clearly observed using SEM (fig. 9).

In 25 experiments with a total number of 193 flowers the percentage of ovules with a pollen tube in the micropyle (fig. 10) was determined using a light microscope and the clearing/aniline blue method. Each treatment was repeated at least twice in different experiments. The extend of ovule penetration fluctuated significantly throughout the year. In the winter the penetration of the ovules can be as low as 0% and rise up to an unusual 27% (BK-medium, a total of 208 ovules) in spring or 29% (GAM-medium, a total of 1417 ovules) in autumn. Incubation of flowers for 2 days at 25°C and 16 hours light before using them for placental pollination improved the results in the winter.

Pollen tubes have entered 3% of the ovules 24 hours after placental pollination. A significant higher percentage of penetration (8%) is reached after 2 - 4 days, after which no further increase of the percentage was detected. No difference occurred between BK- and MS-medium, GAM-medium was not tested in combination with the course of ingrowth.

- Influence of explant position on pollen tube penetration -

Different positions and shapes of explants were used to modify in vitro pollination. Cross sections of the ovary of various thickness were placed with one cut end at the medium after which at the other end pollen was applied at the ovules. Also whole ovaries were dissected from their ovary wall and the now exposed ovules were pollinated. To decrease the amount of fluid in the explant, placentas with ovules and a part of the ovary wall were put upright in the culture medium after which pollen was applied at that part of the placenta which emerged the medium surface. As a final variation two rows of ovules remained attached to each other at the former centre of the ovary, the ovary wall

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Fig. 1. Compatible intraspecific placental pollination of *Lilium longiflorum* after which pollen grains (PG) germinate and tubes grow in between the ovules (O) towards the inner integument on the other side. Placenta (PA). A SEM micrograph at 3 days after placental pollination (DAPP) at GAM-medium of a pistil without pretreatment (see text). Magn. 181x.

Fig. 2. Returning from the micropylar side of the ovules to the placenta (PA) the pollen tubes (PT) turn again to grow in between the same or adjacent ovules. Pollen tubes are stained with diluted cotton blue and observed with light microscopy after 1 DAPP at BK-medium of a pistil in which 2 days of pollen tube growth had been allowed preceding placental pollination. Magn. 149x.

Fig. 3. A SEM micrograph of pollen tubes (PT) growing in between the ovules (O). The tube which happens to return to the placenta (PA) grows back to the micropylar side along an adjacent pathway. 2 DAPP of a pistil in which two days of pollen tube growth has been allowed prior to placental pollination, BK-medium, magn. 231x.

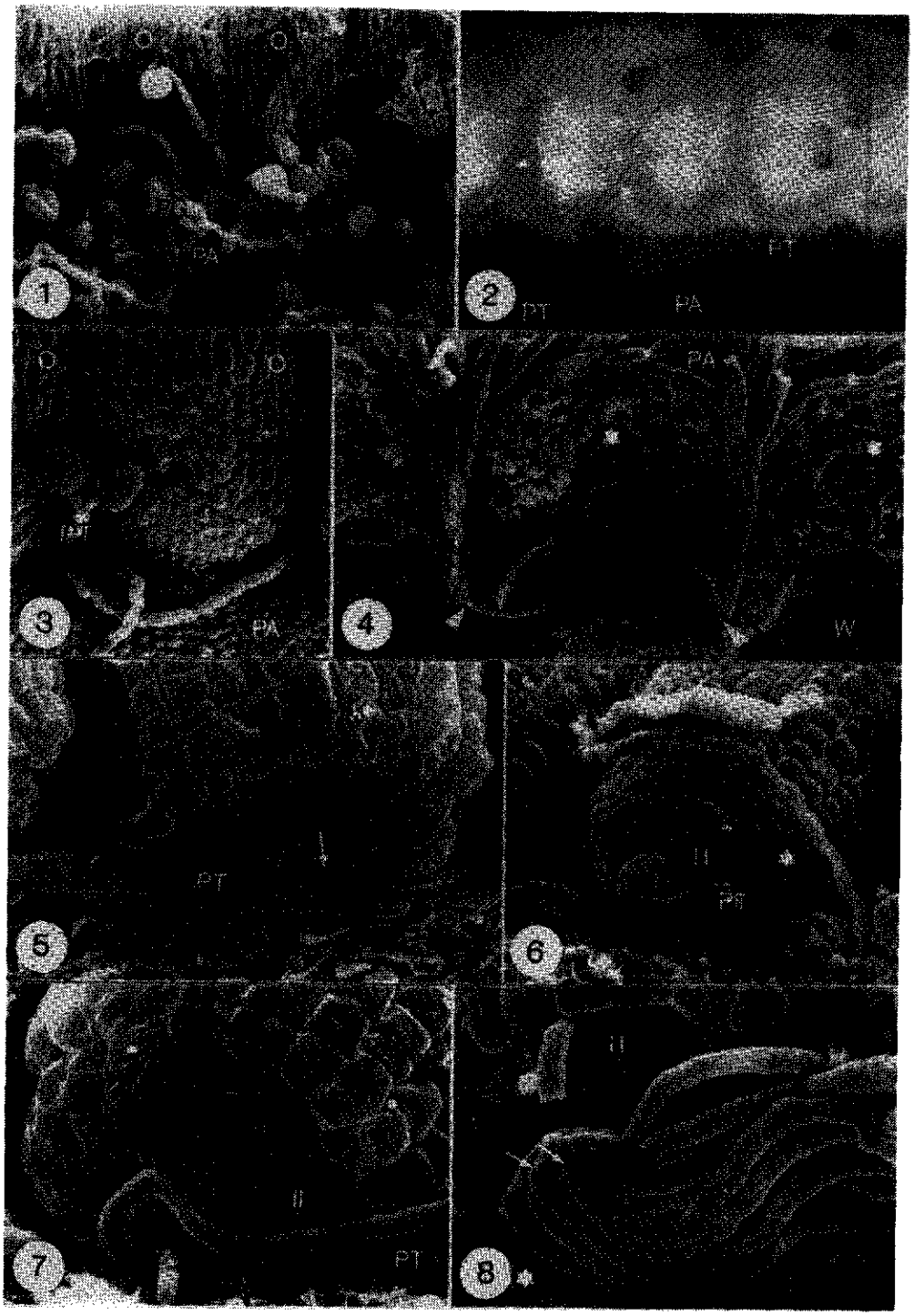
Fig. 4. A SEM overview of the pollen tube pathway after placental pollination after removal of the ovules (*). Germinated at the placenta (PA), pollen tubes grow towards the micropylar side of the ovules, meeting the cuticle over the wall of the ovarian cavity (W), which lacks secretory cells, where they bend to both sides (arrowheads) to grow along the inner integuments. 2 DAPP, BK-medium, pistil was not pretreated, magn. 364x.

Fig. 5. A pollen tube (PT) grows along the inner integument without responding to the micropyle (arrow) and returns to the place of pollination. A SEM micrograph, 1 DAPP, pistil was not pretreated, BK-medium, magn. 462x.

Fig. 6. After placental pollination the pollen tubes (PT) not only circle over the inner integument (II) probably reacting to the micropyle, but also grow over the inner integument along the distal end of the outer integument (*) or even over the outer integument itself. A SEM micrograph, 3 DAPP, pollen tube growth in the style has been allowed for two days prior to placental pollination, GAM-medium, magn. 440x.

Fig. 7. A pollen tube (PT) which reacted to the micropyle by growing to it and even over it, has not penetrated the micropyle and resumes its growth along the inner integument (II). A SEM micrograph, 3 DAPP, the ovary was pretreated by two days of pollen tube growth in the pistil, GAM-medium, magn. 746x.

Fig. 8. Masses of pollen tubes grow over the inner integument (II), some remain growing in the same direction (*), others turn around abruptly (arrows). A SEM micrograph, 1 DAPP, two days of pollen tube growth has been allowed in the style before using the ovary for placental pollination, BK-medium, magn. 847x.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was cut open, and pollen was applied at the placentas. All these different explants did not have a penetration percentage of the ovules as high as achieved generally with the explant described in material and methods. Removing the ovary wall of this explant prior to placing it on its cut surface, did have a significant positive effect on the penetration percentage after placental pollination (4% with and 10% without ovary wall). More damage is however caused to the ovules with the result that a longer incubation for embryo - formation is impossible.

- Influence of the medium -

BK- and GAM-medium seem to have better results than MS-medium, but there are exceptions in which the penetration percentage is higher after culturing on MS-medium. Placental pollination carried out at an agar plate with just 6% mannitol led to a very low penetration (2%), compared with pollination carried out at BK-medium (20%). BK-medium enhances pollen tube germination and growth, but is unsuitable for further ovary culture. A transfer after placental pollination and incubation to e.g. GAM or MS is needed to allow the ovules to remain developing. Supplementing BK-medium with 0.5 or 1.0 μM GA_3 and/or 5.0 μM IAA did not increase the percentage of penetrated ovules, neither did applying small (one pollen grain per ovule) or extra large (an entire coverage of the placenta) quantities of pollen nor did repeatedly applying pollen on the placenta.

After applying the placentas at the medium a fluid forms around the explant, which was caused by pressing the pistil material into the culture medium, by diffusion and by the humid environment. The presence of much fluid is undesired because the pollen grains float of the placenta before germination. The pistil material itself did not show any chemotropic activity, so the pollen grains lost by floating off took only by chance part in the growth over the placenta and probably in entering the micropyle. This fluid could also interfere with pollen tube penetration once they have reached the micropylar side of the ovules. In a series of experiments the fluid was removed with a pipet or by culturing on filterpaper. Placental pollination in dryer conditions after removing the fluid with a pipet did not influence the percentage of pollen tube penetration. Culturing on filterpaper did have a significant positive influence on the ovule penetration, but it also enhanced the material to turn brown at the cut surfaces after a longer period. A satisfactory start of the placental pollination is made by pushing the pistil material into the agar to achieve a full contact between the dissected septum and the culture medium, followed by a lift up, so that the fluid originating from both the pressed medium and the humid environment partly disappeared under the material. The influence of the medium on the pollen tubes is large, resulting in a bad germination or bursting pollen tubes at an unsuitable medium, e.g. MS-medium with a low sugar concentration.

- Receptivity and pretreatments of the ovary -

The experiments mentioned above were carried out at the ovary age of 4 days

after anthesis. Varying this age from 3 to 9 days after anthesis had no negative influence on the percentage of ovules penetrated by a pollen tube. Prepollination at two days after anthesis and using the ovules another 2 days later (i.e. before the pollen tubes had reached the ovary) led to a penetration percentage of 3%, the same as when the pistil was left unpollinated (average of 14 experiments carried out throughout the year). Prepollination for 3 - 4 days in case the pollen tubes had been blocked in the style or in the top of the ovary, did also not increase the percentage of penetrated ovules. Blocking the pollen tubes in the style is preferred above in the ovary because there is less infection in the subsequent vitro pollination experiment.

- Sperm cell formation and embryos -

Twenty four hours after placental pollination sperm cells are formed in one third of the pollen tubes (figs 11 and 12). This one third is low when compared with normal pollination on the stigma in which after the same time interval in the majority of the pollen tubes sperm cells are present. Incubating pollen in a liquid BK-medium with DAPI also reveals a large variation in sperm cell formation.

After placental pollination embryos are formed (fig. 13), but they are hard to detect because they remain small and starch is accumulating in the nucellus, the inner and outer integument. Placentas and ovules were cultured for up to subsequently 1.5 and 1 month without any development of larger, e.g. 1 mm embryos. Placental pollination with pollen of the Asiatic hybrid 'Whilito' and the ovary of 'Gelria' resulted in a penetration percentage of only 0.8 (BK-medium, a total of 238 ovules). The formation of interspecific embryos was out of the scope of these experiments.

Anthers placed on the medium improve the swelling of the ovules (fig. 14) although a part of the ovules did not swell (fig. 15). This swelling is independent upon the presence of pollen at the placenta, and so independent upon fertilization. After placental pollination the inner integument seldom shows a large swelling as observed in fig.16. The majority of the ovules swell due to the enlargement of mainly the outer integument (fig. 17). The ovary wall remains small compared with the in vivo situation. Another influence from the anthers placed at the medium is that the ovarian sectors turn less brown, a process always starting after a few days of culturing at the cut surfaces of the explants. Incubation of placentas for a longer period to obtain embryos in light caused more bending of the material when compared with incubation in the dark, both in the presence of anthers at the medium.

Pollinating the flowers and using the ovary in placental pollination experiments before it has been reached by the pollen tubes did not influence the swelling and also did not seem to enhance embryo formation. A total of 156 'Gelria' and 17 'Flevo' flowers were used in placental pollination experiments with a longer incubation to allow embryo formation.

- Applying pollen tubes to the placenta -

Pollen tubes isolated from the style at 24 or 48 hours after pollination or from a liquid BK-medium after 24 hours of incubation and applied to the placenta in vitro hardly resumed growing, with the result that usually no ovules were penetrated. The penetration percentage of the latter was 0.2% (BK-medium, 908 ovules). This is in contrast with an incubation in the same liquid medium for 4 hours in which after transfer to the placenta, the pollen tubes remained growing and penetration of the ovules took place in 2% of the ovules. This percentage decreased significantly when this pregermination was increased to 8 hours (0.4%). In the latter in 80% of the placentas no penetration of ovules was observed at all. Applying pollen grains to the placenta resulted in a penetration percentage of 5%, which is significantly better when compared with pollination using pregerminated pollen.

In table 1 the penetration percentages of several experiments with significant differences are summarized.

B. Grafted style pollination

To supply the placenta with actively growing pollen tubes instead of pollen grains, styles were grafted to the placenta. The passage of the pollen tubes from the style to the placenta was not always successful and only when pollen tubes did reach the placenta, which was checked with the aqueous cotton blue solution, the graft was included in the results of the experiment. Although the exclusion of grafts in which no pollen tubes reached the placenta, the variation in the percentage of penetrated ovules between different grafts was still large. Applying stigmatic exudate to the placenta before grafting

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Fig. 9. Penetration of an ovule by a pollen tube (PT) in the centre of the initially lobed micropyle, after placental pollination as observed by SEM. 3 DAPP, untreated pistil, BK-medium, magn. 703x.

Fig. 10. Penetration of an ovule after placental pollination as observed by light microscopy. Embryo sac (ES), inner integument (II), pollen tube (PT), magn. 96x.

Fig. 11. The generative and the vegetative nucleus (arrows) in a pollen tube after placental pollination at BK-medium, stained with DAPI, as observed by UV microscopy. Magn. 208x.

Fig. 12. One vegetative nucleus (arrow) and two sperm cell nuclei (arrowheads) as observed by UV microscopy after placental pollination with DAPI stained pollen. BK-medium, magn. 208x.

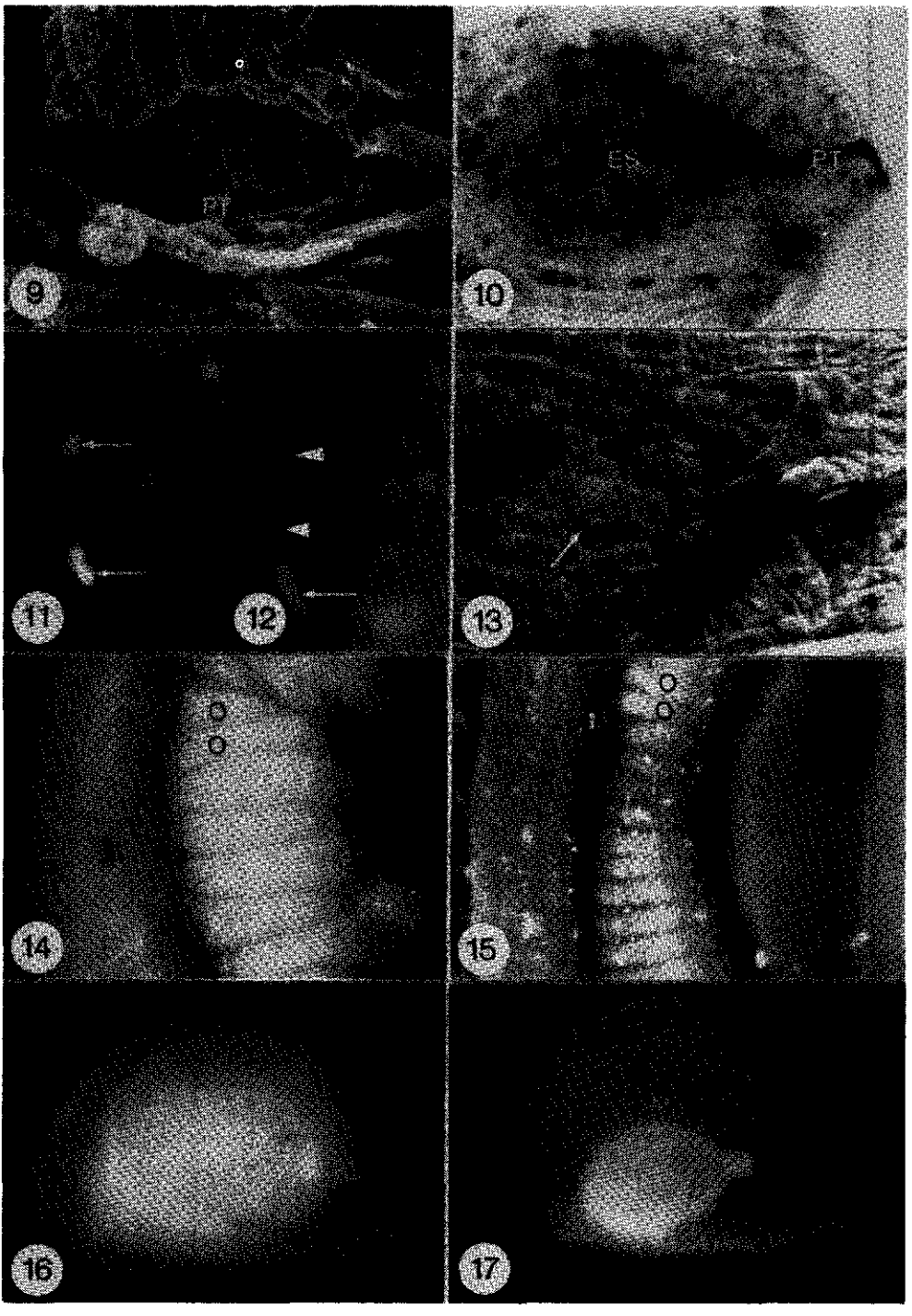
Fig. 13. Embryo (arrow) obtained after 21 days of placental pollination. Magn. 208x.

Fig. 14. The swelling of ovules (O) five weeks after placental pollination of *L. longiflorum* 'Flevo' at MS-medium supplemented with anthers. Magn. 27x.

Fig. 15. Ovules (O) after placental pollination with the same treatments as fig. 14. Swelling hardly took place. 35 DAPP, magn. 27x.

Fig. 16. An ovule of which both the outer and the inner integument with nucellus and embryo sac are swollen. 35 DAPP, magn. 40x.

Fig. 17. An ovule of which the outer integument had enlarged. 35 DAPP, magn. 40x.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did not have a significant positive influence (table 2), but there is a strong tendency. A barrier did not reduce the percentage of penetration (table 2) and also did not have a negative influence on the passage of the pollen tubes from the style to the ovary. The figures in table 2 were averaged over the different experiments with the same comparison of treatments.

The penetration of the pollen tubes was mainly restricted to the area of the placenta where the contact with the style was made. Some growth of the pollen tubes along the placenta and at the micropylar side of the ovules was detected, especially when the number of pollen tubes at the placenta was that large that counting was impossible. The pollen tubes never grew till the end of the grafted ovary part. Grafting a shorter placenta to the style did however not raise the percentage of penetration (table 2).

In the grafting experiments only a part of one placenta with ovules was attached to the style. The original position of that maximal one third of the placenta in respectively the top, the middle or the basis the ovary, is not significantly influencing the penetration percentage (table 2), nor does the age of the ovary when considered in between 3 to 8 days after anthesis at the start of the incubation (fig. 18). Ten days after anthesis the

Table 1. A survey of penetration percentages after intraspecific placental pollination experiments with placentas of *L. longiflorum* 'Gelria' and pollen of 'White American' and with significant differences between the treatments (Fisher exact probability test, $P \leq 0.05$) and the total number of ovules observed in those experiments.

Treatments	Penetration percentage	Total no. of ovules
duration of the experiment: 1 DAP	3	1743
2-4 DAP	8	
explant: with ovary wall	4	2184
without ovary wall	10	
culturing conditions: BK-medium	20	1868
mannitol	2	
culturing conditions: with filterpaper	13	2033
without filterpaper	9	
applied pollen: 8 hours preincubation	0.4	
4 hours preincubation	2	2352
pollen grains	5	
different experiments: placental pollination	5	
grafted style pollination	25	859
stigmatic pollination	94	

penetration percentage was still as high as 11%. In some experiments the age of the styles varied from 3 to 6 days after anthesis at the time the grafts were made, without influencing the penetration percentage. Because of the absence of a culture medium the experiments could only last for a short time but were still incubated for at least 5 days. With a longer incubation there was no rise in the percentage of penetration.

For grafting experiments not the whole style is strictly needed. Results equal to those observed after grafting a complete style were achieved with grafting just a basal style part as short as 2.5 cm, used two days after stigmatic pollination. Grafting a stigma and style with a length of 2.5 cm gave a strong reduction of the percentage of penetration but this reduction was not significant (table 2).

When a placenta of the same cultivar as the pollen tubes was grafted ('Flevo' style, 'Gelria' pollen tubes and placenta, 8 successful grafts, 11%), there was no decrease in the penetration percentage in comparison with a compatible combination, in which the style and ovary were of the same cultivar and the pollen tubes of another ('Flevo' style and ovary, 'Gelria' pollen tubes, 7 successful grafts, 11%). An interspecific combination of a 'Flevo' style, 'Gelria' pollen tubes and an ovary of the Asiatic hybrid 'Whilito' still had a penetration percentage of 1% (7 successful grafts).

- Activation of the ovary -

Apart from supplying the placenta with actively growing pollen tubes this series of experiments was also designed to determine the presence of a possible activation of the ovules causing a higher percentage of pollen tube entrance. Before grafting a part of the ovaries were activated by pollen tube growth in the style. The ovaries were used before the pollen tubes reached it. A barrier had to avoid signals from the stylar tissues to both the pretreated an the unpretreated ovaries during the graft. Nevertheless no influence of this pretreatment of the ovary was observed (table 2). The penetration percentages of the experiments mentioned in table 2 were generally lower when 'Flevo' was used as a placental donor, in comparison with 'Gelria'.

Grafting/placental pollination

In the combination of placental pollination with grafting an inducing style, no influence of the presence of pollen tubes in this style on the penetration of the ovules by the placental pollination derived pollen tubes was detected. The average penetration percentage was 4% in case of a style with and 5% in a style without pollen tubes (23 styles per treatment), which is not significantly different.

Comparison between different pollination techniques

In a direct comparison between placental pollination and grafting experiments, the first had a penetration percentage of 5%, the latter with an aluminium foil barrier 25%

(average of 24 grafts). Stigmatic pollination of a flower and counting sector 2 of the ovary (chapter 2) gave a penetration percentage of 94%.

Discussion

- Pollen tube pathway after placental pollination -

The placental pollination technique was used to get more insight in the communication between pollen tubes and the ovary/ovule. It was set up to explain the arrears in ovule penetration after cut-style pollination (chapter 4) in comparison with pollination at the stigma. The penetration percentages of cut-style and placental pollination are comparable, but the media through which the pollen tubes grow seems different. Using placental pollination the pollen tubes not only grow in the exudate produced by the pistil, as is the case after cut-style pollination, but also in the culture medium running over the placenta and in between the ovules and the pericarp. In this light it is striking that the pollen tubes still have their preference for the growing in between the ovules, both

Table 2. The percentage of ovules with a pollen tube in the micropyle after grafted style experiments with 'Gelria' pistils and 'White American' pollen, averaged per placenta at which pollen tube growth was observed using diluted cotton blue. The differences between the averaged penetration percentages were not significant (ANOVA, $P \geq 0.05$). σ_{n-1} standard deviation.

Treatment	%	σ_{n-1}	No. of experiments	Total no. of grafts
stigmatic exudate present	15	2	2	22
stigmatic exudate absent	8	10		
style with barrier	15	9	15	157
style without barrier	10	7		
short placenta	20	13	3	25
long placenta	28	5		
position in the ovary: top	17	13	2	23
middle	12	8		
basis	10	5		
'normal' style	7	5	5	27
short style (2.5 cm)	2	2		
pollinated placenta	12	7	7	83
unpollinated placenta	14	9		

towards the micropylar side and back towards the place of pollination, although to a lesser extend. The influence of the culture medium on the exudate production is unknown, but taking this pattern of pollen tube growth in mind, being a growth in the proximity of the exudate producing cells, it is likely that it is still in production. The secretory product will mix with the fluid present at the placenta and will diffuse into the culture medium. Therefore the growth of the pollen tubes towards the micropylar side of the ovules is a growth pattern towards a higher concentration of exudate. After passing the micropyle a growth in between the ovules might follow and then the pollen tube arrives again at the place of pollination. There the concentration of exudate is lower, due to the diffusion, which might induce the pollen tubes to turn around and grow again in between the ovules. This explains the growth pattern observed in fig. 2.

Despite this diffusion of exudate towards the medium, pollen tubes from pollen grains at the culture medium do not preferably enter the placenta. Not only the secretory product diffuses from the explant, but also substances from the cut surfaces, which might interfere with this process. Also the concentration of the medium mixing exudate can diminish quickly at an increasing distance from the explant and become too low for a reaction from the pollen tubes. The pollen tube growth is only guided by the proximity of exudate producing cells.

Next to the above mentioned difference in growth medium, another difference between placental and cut-style pollination (see chapter 4) is an appearance of pollen tube growth over the outer integument in the first method. Because of cutting the ovary in sectors with a piece of the ovary wall, the placenta and a row of ovules, the ovules might slightly bend away from the ovary wall, hereby creating some space between the outer integument and the pericarp. As observed with cryo SEM (chapter 2) in an undissected ovary those tissues are firmly pressed together with some exudate present in between them. With the creation of some space and the medium mixing with the exudate, a growth condition for some pollen tubes might be created. But this growth of the pollen tubes is not taking place close to the exudate producing cells, so the majority will still grow over the placenta and along the inner integument.

The growth over this inner integument also seems to be somewhat different from that observed after cut-style pollination. The contact between the inner integument and the pollen tubes is less firm after placental pollination. The pollen tubes seem to be more spherical in cross section. The decreasing contact might result from the bending back of the ovules, creating more space not only at the outer but also in front of the inner integument.

The mixture of culture medium with exudate produced by the placenta might interfere with the process of the pollen tube growth. The ultrastructure of pollen tubes grown in vitro differs from those grown in vivo (Rosen and Gawlik, 1966). Also the osmolarity of the growth substances might differ between both techniques, placental and cut-style pollination. This all might result in somewhat different pollen tubes. The consequences of this difference is not known, but placental pollination under dryer circumstances, i.e. culturing at filterpaper, obviously with less interfering medium at the

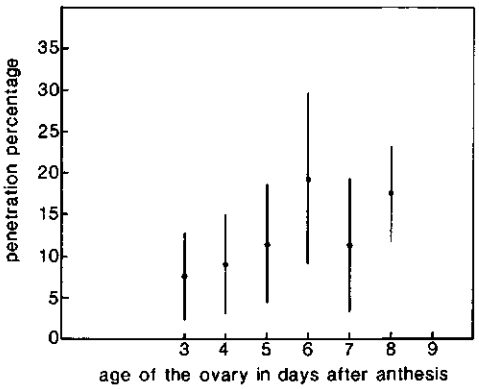


Fig. 18. The penetration percentage in grafted style experiments at different ages of the ovary of *L. longiflorum* 'Gelria'. The bars represent the standard deviation σ_{n-1}. The average of 132 grafts.

placenta, did increase the percentage of penetration. But in a dryer environment of the micropyle the chemotropic substances probably produced by especially the nucellus (chapter 3) are less diluted as well, which could also be the cause of this higher penetration percentage. Removing the ovary wall (a dissection originally carried out to allow ovule swelling) and possibly allowing an even higher diffusion of substances from the secretory cells and the nucellus and a larger influence of the culture medium, did however also have an unexpected positive influence on this penetration percentage. There is probably more space for pollen tube growth which than can reach a greater length, an important phenomenon as discussed in chapter 4. Also the accessibility of nutrients in the absence of the ovary wall might have increased, resulting in a better functioning of the ovules what might induce a higher production of the secrete by the nucellus. The absence of nutrients, as with culturing at an agar plate with just mannitol, could thus interfere with both the condition of the ovules and of the pollen tubes with the resulting in a very poor penetration. The decrease in the penetration percentage after placental pollination with pregerminated pollen, carried out to increase the pollen tube length, was more likely caused by the poor regrowth of the pollen tubes after the transfer to the placenta and the increase of fluid at the placenta, which is a remnant of the pregermination medium, hereby diluting secretory substances. The pollen tubes derived from pollen grains applied at the placenta are active for more than one day, because the penetration percentage still rises. Sperm cells are formed after placental pollination and penetration does not take place before the first sperm cells have been formed. Despite all these variations carried out in an attempt to improve the penetration percentage of the ovules after placental pollination, it stayed far behind that achieved after stigmatic pollination of an intact pistil.

Although the pollen tubes look different from those after cut-style pollination, while growing over the inner integument, still a reaction of the pollen tubes near the micropyle can regularly be observed. The pollen tubes seem to grow along and some times happen to bend towards a micropyle. This reaction can result in a penetration. In *Papaver nudicaule* a directional growth of some tubes produced by grains in direct contact with the ovules towards the micropyle was observed as well after placental and ovule pollination

(Olson and Cass, 1981). The reason why pollen tubes sometimes react to the micropyle without penetration remains unsolved. Probably a stimulus to penetrate as proposed by Van Roggen et al. (1988) is received but disappears thereafter or is too weak to actually induce penetration. A possible activation of the ovary and probably the ovules by prepollination did not change this behaviour. This activation might also disappear when the ovary is cut prior to in vitro culture. A hormone treatment of IAA and GA, which triggered prefertilization phenomena in cotton (Jensen et al., 1983), had no effect on the penetration in lily. The fusion of the polar nuclei and the degeneration of a synergid, as were triggered by the hormone treatment in cotton, were in *L. longiflorum* not occurring prior to the arrival of a pollen tube in the micropyle (chapter 3).

- Embryos after placental pollination -

Embryo formation, as a proof that fertilization has taken place, was found after placental pollination. The initial growth of the embryos is slightly retarded compared with pollination at the stigma of an intact pistil (see chapter 2) and larger embryos of e.g. 1 mm were not found at all. Therefore an enhancement of embryo development after placental pollination is missing. This enhancement is taking place in a flower within 5 days after compatible pollination at the stigma (Van Tuyl et al., 1991) after which the embryos do develop vigorously on a suitable medium in vitro.

After placental pollination the ovary wall was not enlarging as much as the ovules, which was also observed in *L. formosanum* by Hayashi et al. (1986) who found embryo formation after ovary slice culture carried out at 20 days after self-pollination. So, the enlargement of the ovary wall does not seem to have a large influence on embryo development. The production of haploid plants via gynogenesis might be a step closer to the enhancement of the embryo formation in vitro. According to Prakash and Giles (1986) the production of doubled haploids originating from the embryo sac in oriental lilies is possible. Haploids were also produced in *Gerbera jamesonii* (Meynet and Sibi, 1984) and other species (review Yang and Zhou, 1982).

Van Tuyl et al. (1991) interpreted ovule swelling as a fertilization reaction. A swelling is a sign for embryo development because, plantlets do not germinate from a hardly swollen ovule. In our experiments, however, a swelling of the ovules without pollinating the placenta could be made artificially by applying anthers to the medium. The swelling did unfortunately not coincide with a vigorous development of the embryos.

- Grafted style pollination -

In the grafting experiments pollen tubes derived from the stigma and style were applied at the placenta. A barrier at the basal end of the style, avoiding diffusion of substances from the stylar tissues towards the placenta, did not reduce the penetration percentage. This also indicates that there is no substance from the stylar tissue enhancing penetration of the ovules in grafting experiments. This is in line with the discussion in

chapter 2 and 4 in which the pollen tubes seem to grow separated from the stigma and stylar tissues, and that the aging of the pollen tubes seem to be a more important factor for penetration. The pollen tubes emerging from the style have grown for two days, which should be old enough to achieve a high percentage of penetration. The penetration percentage was higher when compared with placental pollination, but is still low when compared with stigmatic pollination. Although only the grafts in which pollen tubes grew from the style to the placenta were included, there was no differentiation between the number of pollen tubes passing. In case that only one or a few pollen tubes passed, which might have been the result of an insufficient contact between the stylar canal and the placenta, the penetration percentage will be low, resulting in a lower average penetration percentage. But still the penetration percentage was never as high as reached on average in a flower. The reason for that might be the dissection of the placenta, being deprived from external nutrients as a culture medium and consequently being unable to support pollen tube growth into the ovules. The preferential penetration of ovules with a position close to the grafted style could hereby also be explained. The pollen tubes growing from the style are well fed but do not grow far from the grafting point because the placenta has no nutrition to offer. The reason why a shorter placenta did not increase the average penetration percentage might be the consequence of a too short placenta to support the ovules well. And therefore these ovules are now not a healthy partner in pollen tube penetration. The result is a disruption of the interaction between the pollen tube and the ovule.

Another reason for a poor penetration, in comparison with stigmatic pollination of an intact pistil, might also be that a penetration inducing substance from the style is only transported through e.g. the vascular system which is not continuous anymore in the grafts. But grafting a placenta originating from a prepollinated pistil did not increase the penetration percentage. It might be that this activation was lost during the experiment.

Grafting a shorter style with shorter pollen tubes to the placenta decreased the percentage penetration, parallel to the cut-style method carried out at different style lengths as discussed in chapter 4.

Placental pollination with an 'inducing' style did not increase the penetration percentage. This 'inducing' style, whether or not pollinated, did also not seem to have any influence on the pattern of ovule penetration in relation to the application place of the style at the placenta. Therefore the local ingrowth of the pollen tubes after applying stigma derived pollen tubes with the style to the placenta might not be the result from a local stimulation, as concluded before. And also the placental derived pollen tubes might still be too young to reply to any activation of the ovules.

- Receptivity -

In both the placental and grafted style pollination varying the age of the ovary between 3 and 9 or 10 days after anthesis did not influence the results. A long receptivity was also found in flowers (chapter 2). The penetration percentage of the ovules was

highest after normal stigmatic pollination of an intact flower. Grafting decreased the penetration, but this was still better than after placental pollination. In conclusion the length which is possibly correlated with the physiological state of the pollen tube seems to be the most important factor.

- General conclusion -

After pollination at the stigma of an intact pistil the pollen tubes grow through the stigmatic lobes into and through the style, followed by an entrance in the ovary. Their pathway till the entrance of the micropyle is lined with secretory cells. They produce the medium and the nutrition which make pollen tube growth possible. After cut-style pollination just above the ovary and placental pollination, the pollen tubes only meet the last part of the pollen tube traject. Still the pollen tubes follow the path of secretory cells. Those cells again produce a growth medium and nutrition. In case of placental pollination carried out at a growth medium, additional substances not supplemented by the medium but produced by the secretory cells induce the pollen tube growth to take place in their proximity. The result is again a pollen tube traject comparable to that found after cut-style pollination. The growth medium and the dissection of the ovary creates however a somewhat different pattern, but the result is about the same: a poor penetration percentage.

Ovule activation did not induce a higher penetration percentage. Also after placental pollination in a flower (see chapter 4) where the ovules were still in contact with the style, no increase in that percentage was observed. We could not prove any activation inducing a higher penetration.

The penetration of the ovule after stigmatic pollination is probably helped by a substance secreted by the nucellus (see chapter 3). The pollen tube is mature enough to respond to this substance. Leaving the style longer with cut-style pollination and grafting a style to the placenta, both implying a longer pollen tube, did raise the penetration percentage. The pollen tube length is thus an important factor in the poor penetration after cut-style (just above the ovary) and placental pollination. The experiments with the grafted styles also indicated the importance of a well supported ovule and placenta, giving the opportunity to secrete the substances needed for a good interaction between ovule and pollen tube.

A reproductive calendar of *Lilium longiflorum* Thunb. 'Gelria'.

6

The combination of reproductive data in one diagram was not often presented. Venturelli (1981, as discussed by Johri and Ambegaokar, 1984) produced a reproductive calendar including microsporogenesis, megasporogenesis, fertilization and the development of the fruit of *Struthanthus vulgaris* which covered a period from January till November. It was built by drawings. Pacini and Sarfatti (1978) covered almost the same development by describing the events in *Lycopersicum peruvianum*. In this species this line of processes only took just over one month. Recently the calendar of *Rhododendron macgregoriae* was published (Williams et al., 1991) to serve plant breeding and protandry was concluded.

In this chapter a part of the data of chapters 2 and 3 are included in a reproductive calendar to enlarge the insight in the reproductive system of *Lilium longiflorum*. The megasporogenesis is omitted because the ovules are in different developmental stages at one age of the flower as was discussed in chapter 2. Instead, the increase in maturation of embryo sacs around anthesis is indicated. Also the development of the embryo showed a large variation, but this item is included in the diagram because it was not given much attention in chapter 2. The microsporogenesis was not studied in detail. This development in relation to flower bud length of the *Lilium* hybrid 'Enchantment' was described by Willemse and Reznickova (1980). No calendar of this process was however given. The amount of stigmatic and stylar exudate was determined after inspection with a binocular, cryo scanning electron microscopy and by centrifugation of the different flower parts.

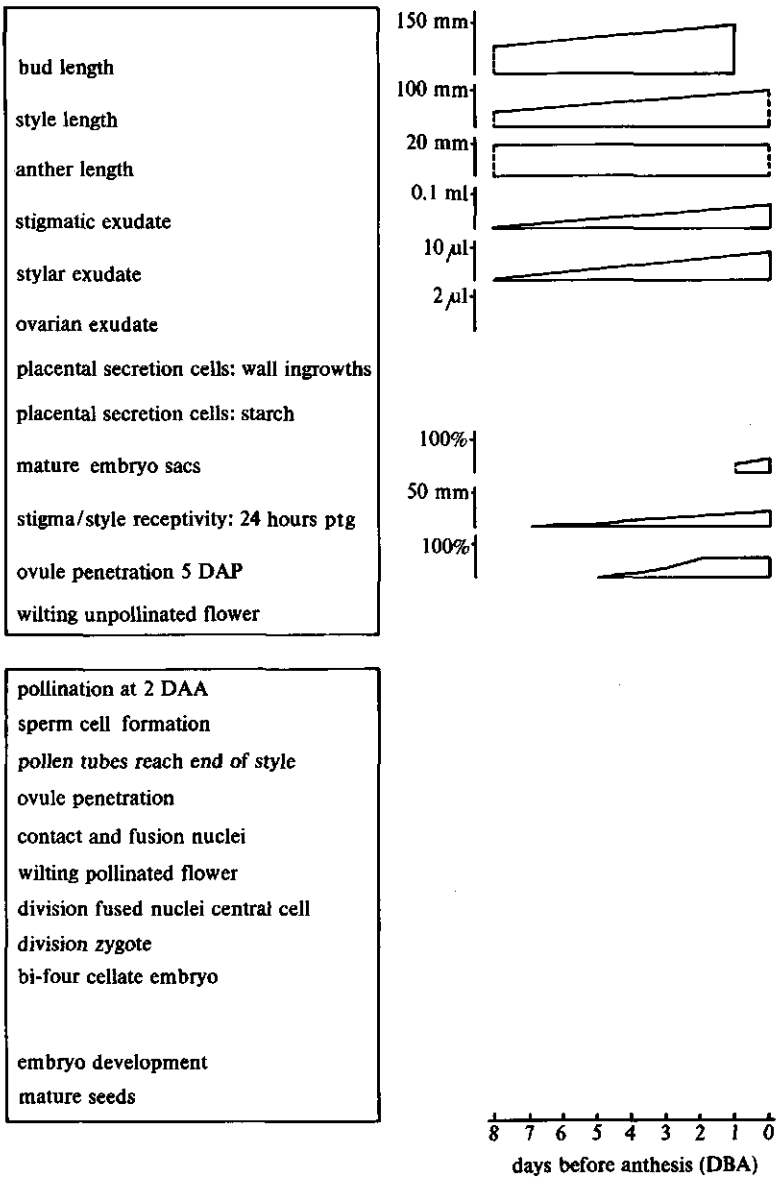
Interesting points of the reproductive calendar are (apart from the ones discussed in chapters 2 and 3):

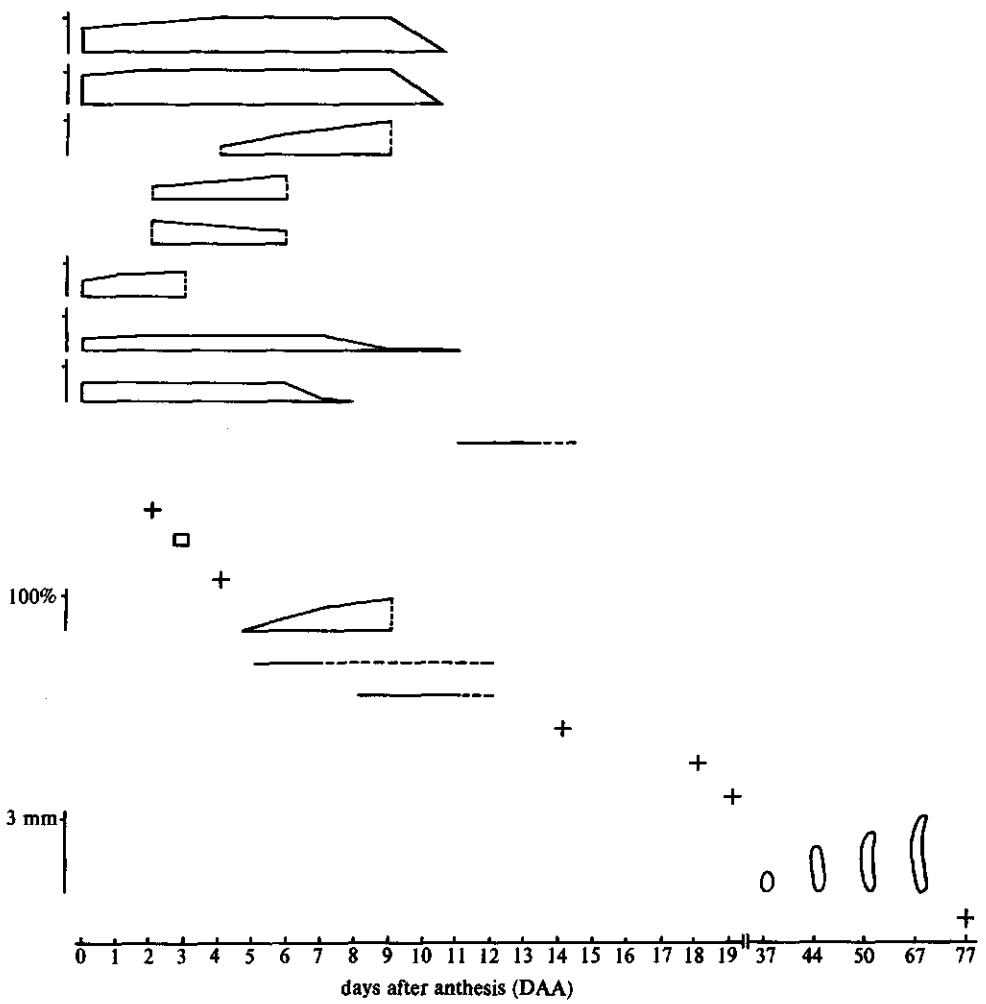
* The amount of exudate, both stigmatic and stylar, still increases after anthesis. The increase of ovarian exudate is coincident with an increase of wall ingrowths and a decrease of starch in the placental cells.

* The receptivity as measured by the pollen tube growth (ptg) in the style starts earlier in flower development than penetration of ovules after 5 days of pollen tube growth is observed. At 6 or 7 days before anthesis (DBA) a slow pollen tube growth is possible. Penetration is only observed in ovules with a mature embryo sac (see chapter 2). The result of a pollination at 6 DBA is measured at 1 DBA when a part of the embryo sacs is mature, but no penetration was observed. The pollen tube growth is probably too slow to reach the ovules in time. This retarded pollen tube growth is the result of the quantity

A reproductive calendar of *Lilium longiflorum* Thunb. 'Gelria'.

The vertical dotted lines present in some blocks indicate that data outside the given interval were not determined.




100%
3 mm
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 37 44 50 67 77
days after anthesis (DAA)

and/or the quality of the exudate as discussed in chapter 2.

* The period of ovule penetration ends earlier than the presence of stigmatic and stylar exudate. The length of the pollen tubes reached in 24 hours also declines earlier than the decrease in the amount of exudates. Substances needed from the exudate are probably unstable, inactive or absent after some period of time. It is striking that pollen tube growth in the style is still possible in older flowers: good pollen tube growth at 7 DAA and retarded pollen tube growth at 8 and 9 DAA, whereas penetration of ovules is already diminishing or absent when pollinated at those days. The decline in quality, but not yet in quantity, of the exudate might already be effective. The pollen tubes do not reach the ovules within 5 days after pollination and the ovules are probably no longer receptive.

* Long before wilting of an unpollinated flower as determined by a change of the colour of the perianth from white into brown, the flowers have lost their receptivity.

* Conspicuous again is the relatively long period of about 13 days from pollen tube penetration in the embryo sac till the completion of the fusion of the nuclei and prior to the division of the zygote. But once the zygote has divided the following divisions for embryo development take place in a much higher frequency. At 35 DAP, 19 days after the division of the zygote, an embryo of less than 1 mm is already formed and at 59 days after the division of the zygote the seeds are mature. This points towards a period of rest followed by a more active period of development.

General discussion

7

In this thesis interactions in the reproductive process between the pollen tubes and the pistil of *Lilium longiflorum* were studied. First an intact system was studied, followed by a manipulated system in vivo and in vitro. The reason for carrying out this research was the poor ovule penetration after interspecific cut-style pollination. This was soon also observed after cut-style pollination within the species. The interaction between the pollen tube and the pistil is disturbed in such a way that ovule penetration hardly took place. The work was focused on a possible ovule activation, which should led to the guidance of the pollen tube into the micropyle. In other words, pollination at the stigma and/or pollen tube growth in the style should induce processes leading to ovule penetration. First the field of interaction between pollen tube growth and the pistil after stigmatic pollination will be discussed.

Pollen tube growth at the stigma and in the style

Exudate at the stigma of *L. longiflorum* is partly secreted before anthesis. Germination of pollen grains is then possible. After anthesis hugh amounts of exudate are observed. The pollen grains do not strictly need that much exudate to hydrate and for initial pollen tube growth at the stigma, because in earlier stages of the flower, when less exudate is present, pollen tube growth is already possible. In an open flower drops of exudate may even hang down the stigma allowing pollen tube growth from the stigma to the outside of the style at places were stigmatic exudate is present (Janson, unpublished). This takes place because of the partly undirected pollen tube growth at the stigma. The pollen tubes thus grow dependent upon the presence of stigmatic exudate rather than on the contact with or the shape of the stigmatic papillae. The large quantity of exudate probably also intervenes with the growth towards the slits in the stigma to penetrate the style. The larger the quantity of exudate the longer it will on average take to find these slits by chance. So the large quantity of exudate is not just present to benefit the pollen tubes. It might also serve as a source of nutrition for pollinators although *L. philadelphicum* is mainly visited by butterflies which collect nectar (Edwards and Jordan, 1992).

Miki-Hirosige et al. (1987) determined that the exudates of stigma and style differ. Because the pollen tubes grow in both the stigmatic and the stylar (see below) exudate,

a slower pollen tube growth at the stigma than in the style must be a consequence of differences in exudate. Close to the slits in the stigma the stigmatic and stylar exudates mix and induce a bending of the pollen tubes towards the slits when they grow within a certain range. This directing of pollen tubes can only take place when a gradient stays present. Evaporation results in an exudate with a higher osmolarity. Supplements from the style with a lower osmolarity can create this gradient. The pathway is thus determined by the exudate and changes in its composition.

Also in the style, especially in the top, a large amount of exudate is present. The style provides exudate at least partly independent upon the presence of pollen tubes. The exudate is the medium through which the pollen tubes grow and from which substances are taken up (Kroh et al., 1970b; Labarca and Loewus, 1972; 1973). In figure 22 of chapter 2, in which a transverse cryo section through a style two days after pollination is presented, space in the exudate is present between the large number of pollen tubes, between the secretory cells and the pollen tubes and between the pollen tubes and the stylar canal. This style was estimated to contain around two thousand pollen tubes just underneath the stigma, which is about five times as much pollen tubes as the number of ovules in the ovary. These pollen tubes still have to cross the style and some of them will not reach the end. But regarding the quantity of exudate, the style seems to be able to support even more pollen tubes. Why is there such a large quantity of exudate in the style as well?

When the pistil is not mature there is less exudate and probably together with its poorer quality, e.g. osmolarity, the pollen tube growth is retarded. The length reached after 24 hours of pollen tube growth is smaller. The pollen tube growth in the style is regulated by the pistil.

Bud-pollination does not influence the development of the embryo sacs. A part of the embryo sacs will be mature at the moment the retarded pollen tubes finally arrive in the ovary. Penetration is then only observed in ovules with a mature embryo sac. The pistil thus regulates the pollen tube growth by the quantity and quality of exudate it produces, hereby determining that the pollen tubes do not reach the ovules before maturity. A slow pollen tube growth is possible from seven days before anthesis.

When the flower opens, the quantity of exudate is large enough, although still increasing, and the quality of the exudate is suited even better for pollen tube growth, because they now have a higher speed compared with before anthesis. More embryo sacs become mature. The maturity of the ovules does not induce a faster pollen tube growth in the style because removal of the ovary did not influence the speed of pollen tube growth. The quantity and quality of exudate thus seems responsible for controlling the speed.

At four days after anthesis and two days of pollen tube growth the exudate in the style is not evenly spread over the secretory surface due to the lobed style. Half way down the style the pollen tubes are more flattened at places where less exudate is present compared with places with more exudate. If the production and the quality of the exudate is evenly spread over the secretory surface this flattening can be caused by the surface

tension or the inability of the pollen tube to grow in the gas phase in the centre of the style. Differences in quality, if present, will diminish due to diffusion. But exudate is still in production and more will be present for later arriving pollen tubes. Still pollen tube growth is possible in smaller amounts of exudate than observed at two days after anthesis in the top of the style.

As concluded before, stylar exudate dilutes stigmatic exudate, hereby creating a gradient which directs the pollen tubes towards the slits. Herefore large amounts of exudate might be needed. This exudate might serve as a buffer for the germination medium at the stigma which is exposed to evaporation. The exudate produced by the stigmatic papillae will also lower the osmolarity of the stigmatic exudate, when the concentration of the secreted substances remains or declines.

The large quantity of exudate after anthesis is probably also needed to absorb the influence of just a few pollen tubes in a way that the development of the ovary is not triggered and that other pollen tubes can grow through the style at a later moment. In this way the quantity of the pollen tubes determine the interaction. To support the pollen tube growth substances have to be offered to the pollen tubes. A large amount of exudate can supply more pollen tubes. In *L. longiflorum* there are no cell walls nor cuticles to dissolve in the style. The substances needed either have to be secreted on arrival of the pollen tubes or have to be present before that. The influence of the pollen tubes on the pistil is not very large since pollination was not correlated with marked changes in the ultrastructure of the secretory cells of the style (Rosen and Thomas, 1970).

The flowers of *L. longiflorum* have a long receptivity. Penetration of the ovules within five days of pollen tube growth is possible during a period of 12 days of pollination: from four days before till seven days after anthesis. Shortly after this period exudate is still present and pollen tube growth is possible, but the pollen tubes do not penetrate the ovules. Either the quality of the exudate has decreased with the result that the pollen tube growth slows down, as was observed in our experiments, and the ovules are not reached in time or the ovules are no longer receptive. If the quality of the exudate declines during aging, the exudate has to be kept in shape. The increase in the amount of exudate observed after anthesis might thus also be a prolongation of the quality of the growth medium.

According to the views above, the pathway of the pollen tubes seems paved or is still in construction but will be build by the pistil. The role of the pollen tube in this construction was not clear. It is probably not a passive role since changes occur in the style prior to the arrival of pollen tubes and those changes sometimes depend on the kind of pollen tubes arriving: compatible or incompatible (Amaki and Yamamoto, 1988; Amaki et al., 1989). Pollen tubes are able to adapt their medium through which they are going to grow. Incompatible pollen tubes grow faster in cross-pre-pollinated pistils (Amaki and Yamamoto, 1988). This might also explain why pollen tube growth is enhanced when a certain number of pollen tubes is present in the style (Janson , unpublished). How this adaption takes place remains unsolved. Also a reaction to the pollen tubes when they have passed takes place e.g. necrotic processes in the cells adjacent to the secretory cells in

the style (Crang, 1969). That something is happening to the pistil is also supported by the observation of a variation of the voltage potential in this structure after pollination (Spanjers, 1978 and 1981). So the assumption that the pollen tubes only deal with the exudate is just partly true.

Pollen tube growth in the ovary

Arriving at the end of the style, the pollen tube bundle spreads out while entering one of the three ovarian cavities. The pollen tubes thus react autonomously to the pathway of exudate. The shape of the secretory cells lining the pollen tube pathway gradually changes from elongated to spherical. This does not have to influence the pollen tube growth since this is taking place in the exudate rather than in contact with those cells. The production of ovarian exudate coincides with an increase in complexity of the wall ingrowths and a decrease of starch in the placental cells. The composition of the exudate and thus the growth medium of the pollen tubes is probably changing on entering the ovary. In the style the pollen tube growth is again taking place at a higher speed compared with that in the ovary. The slowing down of the pollen tube growth might have a relation with the growth pattern in the ovary and ovule penetration.

After stigmatic pollination the first few ovules are neglected, resulting in bending of the pollen tubes and penetration of ovules in an area in the flower where the embryo sacs are mature first (Van Went et al., 1985; chapter 2). The penetration percentage in sector two (sector one is at the top and four is at the basis of the ovary) is also higher than the average penetration of the whole row of ovules. The pattern of pollen tube growth thus matches the development of ovules.

There are different theories explaining the pollen tube growth pattern in the ovary where at the top of the pollen tube bundle the pollen tubes bend one by one to grow in between the ovules (see discussion in chapter 2). The most likely one is built on a theory of Mascarenhas (1975) which implies that a straight pollen tube growth occurs when no external stimuli, e.g. chemotropic and pistil structure, are changing the longitudinal growth axis. There might be a stimulus to bend and once this has been carried out the stimulus disappears, probably consumed, because later arriving pollen tubes do at first not bend to grow in between the same two ovules. The stimulus does recover in a later instant. The stimulus must be present in the exudate, because this is the growth medium of the pollen tubes.

There is also a possibility that pollen tubes take some stylar exudate with them into the ovary and once this is used the pollen tube growth slows down and the tubes start to curve. Another theory finds its basis in the autonomous curving of the pollen tubes into the ovary. Depending upon the place in the pollen tube bundle relatively to the occurrence of the split in the ovarian cavities, the pollen tubes are sooner or later led to the central placenta in between the two rows of ovules. This autonomous reaction is also observed in the ovary where the pollen tubes grow one by one in between the ovules towards the micropylar side. Tips arriving from the ovary entrance grow curved through the exudate

into the ovary finally resulting in a growth in between the ovules. Later arriving pollen tubes grow further down because their curved growth is first corrected by already present pollen tubes. The latter theory can not exclusively explain the observed growth pattern in the ovary in which the first few ovule are neglected by the earliest pollen tubes. Later arriving pollen tubes do curve in between those ovules.

Whatever theory is correct, this curving in between the ovules is not the bottle neck in the poor ovule penetration after cut-style pollination, because the pollen tube behaviour here looks very much like the one observed after stigmatic pollination. The following curve which is made to grow along the inner integuments is also observed after both pollination techniques. This curve is again, like the one entering the ovary, a growth in the proximity of the secretory cells.

After the growth along the micropylar side of the ovules some pollen tubes are observed to return to the pollen tube bundle in between the two rows of ovules, which is again taking place after both pollination techniques. And even a second turn towards the micropylar side of the ovules was observed. No ultrastructural differences were observed between the placental cells bordering the central bundle and those at the micropylar side of the ovules. The exudate produced at the micropylar side is probably of the same quality and if not the possible difference will be diminished by diffusion helped by the short distance. Again it is likely that the pollen tubes grow at places where exudate is present, with a preference to grow close to the producing cells, without a clear guidance other than the secretory pathway.

Ovule penetration and fertilization

The next question is whether there is any excretion from the micropyle attracting the pollen tubes and whether this substance is present once the ovule is mature or is induced by pollination at the stigma and/or pollen tube growth in the style. The synergids in the egg apparatus are generally metabolic very active cells as in *Ornithogalum caudatum* (Tilton, 1981), *Agave parryi* (Tilton and Mogensen, 1979) and *Capsella bursa-pastoris* (Schulz and Jensen, 1968). These cells or their degeneration are in some species held responsible for attracting the pollen tube. In *Pennisetum glaucum* high levels of calcium were present in the synergids, in which a filiform apparatus is present, and less in the cytoplasm of the egg cell, the central cell and the antipodal cells (Chaubal and Reger, 1992). Jensen et al. (1983) postulated a theory in cotton in which the degeneration of the synergid, which takes place before the arrival of the pollen tube, results in a release of calcium. This is attracting the pollen tube and also induces the opening of the pollen tube in the degenerated synergid (Jensen et al., 1983). The chemical composition of the cells of the egg apparatus of *L. longiflorum* was not determined in this research. The pollen tubes of lily were in vitro not attracted to calcium (Rosen, 1964).

In *L. longiflorum* the synergids were metabolic not very active and did not contain a filiform apparatus nor a degeneration was observed prior to pollen tube penetration in the micropyle. The whole embryo sac did not show a high degree of metabolic activity.

A chemotropic substance seems thus not produced or secreted by the synergids. The probably metabolic more active nucellus and to a lesser extent the strongly vacuolate inner integument might be responsible for the production of a chemotropic substance which is not likely to be calcium. A problem hereby is also the diffusion of secreted substances from the micropyle. The substance is either constantly produced or of a high viscosity resulting in a slow diffusion. The secrete is likely to be produced as a developmental stage independent upon pollen tube growth, because ultrastructural data did not show a difference in activity in those tissues between pollinated and unpollinated ovules.

The micropyle is penetrated by just one pollen tube. The cuticle bordering the nucellus is partly ruptured and is lifted up by the pollen tube. The nucellus is the first site in the life of the now at least three days old pollen tube, in case of stigmatic pollination, where it has to dissolve cell walls. Inability of the pollen tube to dissolve cell walls of the pistil, which was not tested before in its live cycle, will lead to an absence of embryo sac penetration.

No consistent differences were observed between the three cells of the egg apparatus, neither in structure nor in position. The pollen tube of *L. longiflorum* grows towards a cell which is unlikely to degenerate prior to pollen tube penetration, hereby creating a gradient. If it did degenerate prior to pollen tube penetration and created this gradient, a strand of the degenerated plasma has to flow in between the persistent cells of the egg apparatus and the nucellus. Although in the ultrastructural studies degenerated plasma is observed between the central cell on one hand and the cells of the egg apparatus on the other and between the cells of the egg apparatus themselves, it was absent between those cells and the nucellus. This is the pathway of the pollen tube and it thus seems unlikely that the degeneration of a synergid creates a gradient in the embryo sac of either calcium or anything else and that the pollen tubes just picks one of the three cells of the egg apparatus to penetrate. But, as expected, in this research the pollen tube was never observed to directly penetrate the central cell. The pollen tube is probably able to distinguish the central cell from the cells of the egg apparatus. It is however more likely that the pollen tube growth in the embryo sac is more guided than described and if so by its growth pattern. In the embryo sac the pollen tube grows along the nucellus towards the micropylar side of the embryo sac. The curving of the pollen tube into one of the cells and its opening has to be triggered in time.

Cell walls in the egg apparatus partly disappear during or after pollen tube penetration. Not only between the degenerated synergid and its neighbours, but also between persistent cells. This is thus not a distinguishing process. Because of the disappearance of the plasmamembrane of the degenerated synergid, the sperm cells can directly border the plasma membranes of the egg cell and of the central cell. The enucleate cytoplasmic bodies, remnants of the sperm cells, are observed to border both these membranes and each other. A regulation of sperm nuclei discharge by the position of the sperm cells in the degenerated cytoplasm is thus not possible. A difference between the membranes of the cells of the egg apparatus on one hand and the one of the central cell on the other is more likely. Information about the kind of cell has to be exchanged

between the sperm cells before the discharge with the result that one sperm nucleus appears in the central cell and one in the egg cell. Here as well it is possible that the future egg cell is picked by chance from one of the two persistent cells of the egg apparatus. After transfer of the sperm nuclei the enucleate cytoplasmic bodies are still present allowing a longer period of information exchange. They differ in ribosome number and one of the bodies will eventually fuse with one of the cells of the embryo sac.

In the central cell the sperm nucleus is probably transported by ER which was present in this cell as CSER and LER prior to fertilization. It first contacts the haploid nucleus after which the triploid polar nucleus follows. In the egg cell the nuclei first line up. The way of nuclear transport is unknown. In both transports little energy seems to be involved, because the embryo sac is metabolic still not very active.

The role of the embryo sac during penetration is probably a passive one. The three cells of the egg apparatus are probably each capable of functioning as either egg cell or synergid. The polar nuclei are still not fused. No ultrastructural changes were observed in the embryo sac and the nucellus prior to the arrival of the pollen tube. The pistil has a long period of receptivity. The protein pattern of the ovules does not change after pollen tube penetration, at least not until seven days after pollination. Not until after penetration and fertilization the embryo sac seems to gain in metabolic activity and starch is formed in the plastids. The period between fusion of nuclei after pollen tube penetration in the embryo sac till the division of the zygote is long, 13 days. This is followed by a relatively rapid embryo development. The ovule is now activated.

On which level is the interaction between pollen tubes and pistil realized?

As mentioned above it seems that the pistil is developing independent upon pollen tube growth. The interaction between the flower and the pollen tube does hardly show signs of a communication between the two partners. Bud-pollination does not influence the development of the embryo sacs. Stigmatic, stylar and ovarian exudate and also the secrete from the nucellus are produced as part of a developmental stage. The embryo sac development seems to have stopped in an early stage: no filiform apparatus is formed and the cells of the egg apparatus are metabolically not very active. During penetration the condition of the embryo sac is passive as well. The pollen tube growth in the style is independent on the presence of an ovary. Damaging or removing the perianth does not influence the number of seeds with an embryo. The system of the pistil with its exudate is built or in construction and functions when used after compatible stigmatic pollination. But from literature as discussed above the conclusion must still be that the interaction between the pistil and the pollen tube is more complicated. No events as a reaction to pollen tube growth were observed in our experiments.

Cut-style and placental pollination

After cut-style pollination the majority of the pollen tubes do not penetrate the

micropyles, although some of them even grow over and react to the micropyles without penetration. As mentioned above the pathway from just above the ovary towards the inner integument is very much like the one observed after pollination at the stigma. This is, as discussed in chapter 5, just partly the case after placental pollination. But here as well the penetration percentages are similar to those reached after cut-style pollination just above the ovary. After placental pollination the pollen tubes do find the inner integument, but this hardly results in penetration. A return of the pollen tube from the micropylar side to the central bundle between the two rows of ovules as observed after cut-style pollination was also taking place after placental pollination in which a return to the place of pollen application was observed.

After cut-style pollination the result of a disturbance of the interaction between the pollen tube and the pistil is obvious just in front of the micropyle. Grafting a stigma to the cut-style prior to pollination does not improve the penetration. Pretreatment of the ovary by allowing pollen tube growth in the style did not induce pollen tube penetration. But if the stylar length at the ovary is kept longer, in other words if less of the style is removed, prior to cut-style pollination more ovules are penetrated. The disturbance in the interaction is thus more at the account of the pollen tubes. The increase in ovule penetration after cut-style pollination is gradual with increasing style, and also pollen tube, length. The longer the interaction with the style, the higher the chances of ovule penetration. In analogy, grafting a style to the placenta, as was carried out in chapter 5, did also give better results concerning ovule penetration compared with placental pollination in which pollen grains were applied at the placenta. Something happens with the pollen tube during the growth through the style. Sperm cell formation does also take place in case of placental and cut-style pollination. The question is if the absence of this formation can influence the penetration behaviour since only the vegetative cell organises pollen tube growth (see review Steer and Steer, 1989). Sperm cell formation in *L. longiflorum* did not influence the constant speed of pollen tube growth in the style, which might point towards independent mechanisms.

No difference in pollen tube behaviour between pollination at the stigma and cut-style pollination shows until the arrival at the micropyle. Whether or not pollen tube penetration is going to take place is determined during the passage in the style. The growth in the stylar exudate thus intervenes with the reaction of the pollen tube in the ovary. After a slow pollen tube growth at the stigma a faster growth is observed in the style, followed by a decrease in speed in the ovary. The exudate in the micropyle is likely to be produced independent upon pollen tube growth in the style. It is probably of a low viscosity, which might again induce an even slower pollen tube growth. After both cut-style and placental pollination the pollen tubes are able to react to the presence of the micropylar exudate, but this reaction results more as an exception in penetration. In the absence of the essential stylar phase, the pollen tubes are probably unable to grow through this micropylar exudate or a pathway in the more fluid ovarian exudate is preferred. This inability to penetrate is not the result of a late acting incompatibility, because pollen tube growth is not actively stopped and the reaction is not built on a

genetic basis like self-incompatibility. The reaction is thus more a neglecting of or an inability to penetrate the mature ovules.

The embryos obtained after placental pollination do not develop vigorously. The support by the ovary is probably to small. Placing anthers at the medium enhanced ovule swelling, as was also found in Van Tuyl et al. (1991), and so partly overcame the lack of ovary support. It did not have a recovering effect on embryo formation. After pollination at the stigma the embryo sac gained in metabolic activity not until after fertilization. This slow activation might be a problem in placental pollination.

After cut-style pollination ovule penetration is low, even when intraspecific compatible pollen is used. The question remains to which extend interspecific pollen tube growth following cut-style pollination can be compared with that in an intraspecific combination. The images of pollen tube growth from scanning electron microscopy are similar after comparing the results in this thesis (intraspecific) with Van Roggen et al. (1988, interspecific). In an intraspecific combination the penetration percentage increases with an increasing length of the style left at the ovary prior to cut-style pollination. Interspecific pollen tubes stop upon reaching the stylar canal or somewhere in the style (Ascher and Peloquin, 1968). Reciprocal differences in the pollen tube length reached in the style occur (Ascher and Drewlow, 1971). If the penetration percentage after cut-style pollination was also poor due to the development of the pollen tube, an increase in length of the style as was carried out in an intraspecific combination, might improve the results. But care should be taken because the pollen tubes might stop in the style. There is no localized place of arrest; the pollen tube growth stops after a certain period of interaction with the style (Ascher, 1977). And then it still has to be determined whether pollen tubes which have been growing in an interspecific style are capable of entering the ovule or not. There might also be a barrier for interspecific pollen tubes to enter the ovules, independent of a growth through a style.

In this thesis the importance of the exudates of the pistil in determining and regulating pollen tube growth is stressed. The interaction between the pollen tube and the stylar exudate, which is largely absent after cut-style pollination, is important in ovule penetration. The basis for this interaction, the production of exudate, is largely prefabricated, without the influence of the pollen tube. Future research should therefore focus on processes taking place in the pollen tube during its interaction with the stylar exudate.

Summary

In this thesis the interaction between pollen tube growth and the pistil with the subsequent fertilization was studied both in intact flowers and after different flower manipulations and in vitro pollination. Light and electron microscopy and electrophoresis were used.

To achieve interspecific crosses in lily the cut-style pollination, in which the style is cut off just above the ovary and pollen grains are applied at the cut surface, is used. Just a few ovules are penetrated by a pollen tube. In an intraspecific compatible combination the penetration percentage of the ovules after cut-style pollination is however low as well. Complications occur in the interaction between the pollen tube and the ovule. It might be that the ovules lack an enhancement which takes place during pollination or germination at the stigma or during pollen tube growth in the style, which is absent after cut-style pollination.

In chapter 1 a review is given of compatible, incompatible and interspecific pollen tube growth in *Lilium*, the guidance of the pollen tube and other aspects of the interactions between the pollen tubes and the pistil. Following this chapter interactions are first studied in an intact system of *Lilium longiflorum*.

In chapter 2 the exudate production in the pistil, embryo sac development and pollen tube growth in *L. longiflorum* is studied and related to flower bud length and flowering stage. The exudate production on the stigma and in the style starts before the bud opens, as determined by cryo scanning electron microscopy. Just underneath the stigma the exudate first accumulates at the top of each secretory cell, followed by a merging of those accumulations as exudate production proceeds.

After germination the pollen tubes grow across the stigma and enter the style in between the three stigma lobes. This growth over the stigma seems at least at first not directed. In the style the pollen tubes grow straight downward at a constant speed and are covered by exudate. As the pollen tube bundle reaches the ovary, the secretory pathway and thus also the pollen tube bundle is divided into three ovarian cavities. Hereby they spread out, but their growth is restricted to the area with secretory cells. The secretory cells covering the placenta are similar to those present in the stylar canal, although their surface shape is more spherical rather than elongated as in the style. The transfer wall of the placental cell is originating from fusing Golgi vesicles but the endoplasmic reticulum (ER) seems to have an important role as well.

In between the two rows of ovules in one ovarian cavity a pollen tube bundle is formed in the exudate produced by the placental cells. After neglecting the first few ovules the pollen tubes bend from this bundle in between the ovules and grow towards the micropylar side. There they bend again to stay close to the secretory cells. At anthesis a part of the embryo sacs are in their seven-nucleate and six-cellate (the cell walls in the embryo sac were hard to detect after clearing) stage, i.e. mature. Penetration of the pollen tubes into the micropyle has only been observed in these ovules.

About 8 days before anthesis exudate is observed in a flower bud. Pollen tube

growth in the style is possible from seven days before anthesis. The pollen tube growth is then however strongly retarded compared with the pollen tube growth in a flower at anthesis. It seems that some pollen tubes are not covered by an exudate layer. Newly appearing pollen tube tips have a tendency to grow close to the secretory cells, resulting in a growth between these cells and preceding pollen tubes. If there is still little exudate produced it results in a lifting up of the pollen tubes, out of the exudate.

The development of the embryo sacs in sector two (sector one is at the top and four is at the basis of the ovary) is ahead of the embryo sacs in the other sectors of the ovary. Pollination or pollen tube growth did not influence the development of the embryo sacs. When the pollen tubes formed after pollination of a flower bud finally reach the ovary, a part of the ovules have matured. From four days before till seven days after anthesis, pollination results in penetration of the ovules. The protein pattern of the ovules observed after electrophoresis did not show a consequent change in the period from anthesis till 9 days after anthesis or 7 days after pollination.

In chapter 3 the ultrastructure of the embryo sac, the nucellus and parts of the micropyle of *L. longiflorum* is studied both before and after pollination. Before pollen tube penetration the three cells of the egg apparatus cannot be distinguished, neither in structure nor in their position. No filiform apparatus was detected, no degeneration of a synergid occurs without pollen tube penetration. The polar nuclei in the central cell do not fuse until fertilization. The metabolic activity of the cells of the egg apparatus and the central cell seems low. The nucleus of the most chalazal of the two antipodals has an irregular shape and in some embryo sacs a third antipodal cell, small in size and without a nucleus, is present. Pollen tube growth does not induce changes in the embryo sac.

When the pollen tube arrives at the nucellus the cuticle surrounding this nucellus is lifted up. Enzymatic digestion of the cell wall of the at this place one cell layer thick nucellus has to take place to create a pathway for the pollen tube to enter the embryo sac. After entering the embryo sac, the pollen tube grows along the inside of the nucellus and finally penetrates one of the three cells of the egg apparatus, now distinguished as the degenerated synergid. Shortly after fertilization two enucleate cytoplasmic bodies of a different ribosome density were observed in the degenerated plasma of the synergid and the pollen tube. These structures border both the central cell, the egg cell and each other and are most likely the two empty sperm cells. The sperm nucleus in the central cell is probably transported by ER and first makes contact with the haploid polar nucleus which is, as the triploid polar nucleus, connected with ER as well. In the egg cell another process is more likely, because here strands of ER were not observed. Here the nuclei line up before fusion. The cells of the embryo sac become more metabolic active after pollen tube penetration. In this chapter an attempt is made to relate ultrastructure to function and processes.

In chapter 4 the pollen tube growth in the ovary after cut-style pollination was observed with scanning electron microscopy. Different flower manipulations were carried out in an attempt to elucidate the interaction between the pollen tube growth and the pistil. Until the arrival of the pollen tube at the inner integument, the pollen tube growth

did not show any difference between cut-style and stigmatic pollination, as studied in chapter 2. Using cut-style pollination the pollen tubes either grew past the inner integument and ignored it, or grew along but not into the micropyle or penetrated the micropyle.

Grafting a stigma just above the ovary did not influence the penetration percentage, nor did a possible activation of the ovary induced by pollination or pollen tube growth in the style or even in the ovary itself, preceding or during cut-style, interstylar or placental pollination in a pistil. The percentage of penetration after cut-style pollination increased however when the stylar part present at the ovary was left longer.

The presence of the ovary did not influence the pollen tube growth in the style as determined after isolation of styles from the ovary and comparing the pollen tube length.

When pollen grains and stigmatic exudate were applied through a slit half-way down the style of an intact pistil the pollen tube growth was not influenced by a simultaneous pollination at the stigma.

In chapter 5 placental pollination was carried out predominantly with *L. longiflorum* to study the interaction between the pollen tube and the placenta with ovules. The percentage of penetrated ovules is low when compared with compatible pollination at the stigma. After placental pollination the pollen tube growth between the ovules seems directed and the pollen tubes do find the inner integument. A reaction to the inner integument or the micropyle is observed, but hardly results in ovule penetration. Embryos were found, but did not develop vigorously. The similarities and differences with cut-style pollination, in which the percentage of ovule penetration is also low, are discussed. Grafting a style with pollen tubes to the placenta increased the penetration percentage obtained after placental pollination five times.

In chapter 6 the data from chapter 2 and 3 are combined in a reproductive calendar.

In chapter 7 the reproduction is considered as a regulated interaction process. Previous experimental results are considered as aspects of the system and are discussed in this context.

Samenvatting

In dit proefschrift is de interactie tussen pollenbuisgroei en de stamper met de daarop volgende bevruchting bestudeerd, zowel in intacte bloemen als na verschillende bloemmanipulaties en in vitro bestuiving. Licht- en electronenmicroscopie en electroforese werden daarbij gebruikt.

Bij het maken van interspecifieke kruisingen in lelie wordt de zgn. cut-style bestuiving toegepast, een methode waarbij de stijl net boven het vruchtbeginsel wordt afgesneden, waarna de pollenkorrels op het snijvlak worden aangebracht. In slechts weinig zaadknoppen groeien pollenbuizen naar binnen. Na cut-style bestuiving in een intraspecifieke, compatibele combinatie, is het percentage zaadknoppen met een pollenbuis in de micropyle ook laag. In de interactie tussen pollenbuis en zaadknop treden blijkbaar complicaties op. Het is mogelijk dat bij cut-style bestuiving een activering van de zaadknoppen afwezig is, welke normaliter plaatsvindt tijdens bestuiving of kieming op de stempel of tijdens pollenbuisgroei in de stijl.

Hoofdstuk 1 geeft een literatuur overzicht van de compatibele, incompatibele en interspecifieke pollenbuisgroei in *Lilium*, de geleiding van de pollenbuis en andere aspecten van de interacties tussen pollenbuizen en stamper. Na dit hoofdstuk worden eerst de interacties in een intact systeem in *Lilium longiflorum* bestudeerd.

In hoofdstuk 2 worden de productie van exudaat in de stamper, de embryozak ontwikkeling en de pollenbuisgroei in *L. longiflorum* bestudeerd en gerelateerd aan de lengte van de bloemknop en het stadium van de bloem. Met cryo scanning electronen microscopie werd vastgesteld dat de productie van exudaat op de stempel en in de stijl begint voordat de bloemknop opent. Net onder de stempel hoopt het exudaat eerst op de top van elke secretie cel op, gevolgd door een samenvloeiing van die opeenhopingen tot een continue laag van exudaat.

Na kieming groeien de pollenbuizen over de stempel en komen de stijl binnen door tussen de stempellobben door te groeien. In de beginfase is de groei over de stempel ongericht. In de stijl groeien de pollenbuizen in het exudaat met een constante snelheid recht naar beneden. Wanneer de pollenbuisbundel het vruchtbeginsel bereikt verdeelt de secretieve weg en dus ook de pollenbuisbundel zich over de drie vruchthokken. Hierbij spreiden de pollenbuizen zich uit, maar hun groei blijft beperkt tot het gebied met secretiecellen.

De secretiecellen die de placenta bekleden lijken op die van het stijlkanaal, hoewel hun vorm meer afgerond is dan de langwerpige cellen in de stijl. De wandverdikkingen van de secretiecellen van de placenta zijn afkomstig van fuserende Golgi blaasjes, maar het endoplasmatisch reticulum (ER) lijkt ook een belangrijke rol te spelen.

Tussen de twee rijen zaadknoppen in een vruchthok wordt geleidelijk aan een pollenbuisbundel gevormd in het exudaat, geproduceerd door de placentale cellen. Na het negeren van de eerste zaadknoppen buigen de pollenbuizen van deze bundel af en groeien naar de micropylaire zijde van de zaadknoppen. Daar buigen ze wederom en blijven zo in de buurt van de aldaar aanwezige secretiecellen. Tijdens anthese is een deel van de

embryozakken in hun zevenkernige en zes-cellige (na opheldering zijn de celwanden in de embryozak moeilijk waar te nemen) stadium, d.w.z. rijp. Ingroei van pollenbuizen in de micropyle is alleen waargenomen in deze zaadknoppen.

In een bloemknop is exudaat waargenomen vanaf ongeveer acht dagen voor anthese. Pollenbuisgroei is dan echter sterk geremd in vergelijking met de pollenbuisgroei in een bloem tijdens anthese. Het lijkt alsof sommige pollenbuizen niet bedekt zijn door een laag exudaat. Nieuw verschijnende pollenbuistoppen zijn geneigd om dicht bij de secretiecellen te groeien, wat resulteert in een groei tussen die cellen en de voorgaande pollenbuizen. Als er nog steeds weinig exudaat geproduceerd wordt, resulteert dit in het optillen van pollenbuizen, uit het exudaat.

De ontwikkeling van de embryozakken in sectie twee (sectie één is in de top en vier is aan de basis van het vruchtbeginsel) is verder dan die in de overige secties van het vruchtbeginsel. De ontwikkeling van de embryozakken werd niet beïnvloed door bestuiving of pollenbuisgroei. Wanneer de pollenbuizen gevormd na bestuiving van een bloemknop eindelijk het vruchtbeginsel bereiken, is een aantal van de zaadknoppen rijp. Bestuiving resulteert in penetratie van de zaadknoppen als deze is uitgevoerd in de periode van vier dagen voor tot zeven dagen na anthese. Het eiwitpatroon van de zaadknoppen verkregen via electroforese, blijft onveranderd in de periode van anthese tot 9 dagen na anthese of zeven dagen na bestuiving.

In hoofdstuk 3 is de ultrastructuur van de embryozak, de nucellus en delen van de micropyle van *L. longiflorum* zowel voor als na bestuiving bestudeerd. Vóór pollenbuispenetratie kunnen de drie cellen van het eiapparaat niet van elkaar onderscheiden worden, noch in structuur, noch in hun positie. Een filiform apparaat werd niet waargenomen en degeneratie van een synergide trad niet op zonder pollenbuis penetratie. De poolkernen in de centrale cel fuseren niet voor de bevruchting. De metabolische activiteit van de cellen van het eiapparaat en van de centrale cel lijkt laag. De kern van de meest chalazale van de twee antipoden heeft een onregelmatige vorm en in sommige embryozakken is een kleine derde antipode waargenomen, waarin een kern ontbreekt. Pollenbuisgroei induceert geen veranderingen in de embryozak.

Wanneer de pollenbuis bij de nucellus arriveert, wordt de daar aanwezige cuticula opgetild. Enzymatische oplossing van de celwand van de hier één cellaag dikke nucellus moet plaatsvinden om een doorgang voor de pollenbuis te creëren. Na het binnengroeien van de embryozak groeit de pollenbuis langs de binnenkant van de nucellus uiteindelijk één van de drie cellen van het eiapparaat in, die nu de gedegenereerde synergide blijkt te zijn. Kort na bevruchting werden in het gedegenereerde cytoplasma van de synergide en de pollenbuis twee cytoplasmatische lichamen, verschillend in ribosoom dichtheid en beide zonder kern, waargenomen, vermoedelijk de achtergelaten lege spermacellen. Deze structuren grenzen ieder zowel aan de centrale cel, de eicel als aan elkaar. In de centrale cel wordt de kern van de spermacel mogelijk door het ER getransporteerd en maakt eerst contact met de haploïde poolkern welke, net als de triploïde poolkern, verbonden is met ER. In de eicel is een ander proces meer waarschijnlijk, omdat hier geen ER strengen waargenomen werden. Hier gaan de kernen voor de fusie eerst naast elkaar liggen. De

cellen van de embryozak worden na pollenbuis penetratie metabolisch meer actief. In dit hoofdstuk is een poging gedaan om de ultrastructuur aan functie en processen te relateren.

In hoofdstuk 4 werd na cut-style bestuiving de pollenbuisgroei in het vruchtbeginsel bestudeerd met scanning electronen microscopie en werden verschillende bloemmanipulaties uitgevoerd om meer inzicht in de interactie tussen pollenbuisgroei en stamper te verkrijgen. De pollenbuisgroei na cut-style bestuiving vertoonde geen verschil met die na bestuiving op de stempel, zoals beschreven in hoofdstuk 2, tot de aankomst van de pollenbuis bij het binnenste integument. De pollenbuizen groeiden óf langs het binnenste integument en negeerden het, óf over maar niet in de micropyle óf penetreerden de micropyle.

Het penetratiepercentage werd niet beïnvloed door de enting van een stempel net boven het vruchtbeginsel. Een mogelijke activering van het vruchtbeginsel geïnduceerd door bestuiving of pollenbuisgroei in de stijl of zelfs in het vruchtbeginsel had eveneens geen invloed. Het penetratiepercentage na cut-style bestuiving nam echter wel toe naarmate het deel van de stijl dat nog op het vruchtbeginsel aanwezig was langer was.

Via geïsoleerde stijlen en vergelijking van pollenbuisgroei werd vastgesteld dat de aanwezigheid van het vruchtbeginsel geen invloed op pollenbuislengte in de stijl heeft.

De pollenbuisgroei van pollenkorrels die samen met stempel exudaat via een spleet halverwege de stijl aangebracht werden in een verder intacte stamper, werd niet beïnvloed door een simultane bestuiving van de stempel.

In hoofdstuk 5 werd placentale bestuiving uitgevoerd hoofdzakelijk binnen de soort *L. longiflorum* met als doel de interactie tussen de pollenbuis en de placenta met zaadknoppen te bestuderen. Het penetratie percentage is laag vergeleken met dat na compatibele bestuiving op de stempel. Na placentale bestuiving lijken de pollenbuizen tussen de zaadknoppen geleid te worden en het binnenste integument te vinden. Een reactie op dit binnenste integument of de micropyle is waargenomen, maar resulteert zelden in zaadknop penetratie. Embryo's werden aangetroffen, maar ontwikkelden zich slecht. Overeenkomsten en verschillen met cut-style bestuiving, waarbij het percentage penetratie van de zaadknop eveneens laag is, worden besproken. Enting van een stijl met pollenbuizen op de placenta resulteerde in een vervijfvoudiging van het penetratie-percentage ten opzichte van placentale bestuiving.

In hoofdstuk 6 zijn de resultaten van de hoofdstukken 2 en 3 gecombineerd in een voortplantingskalender.

In hoofdstuk 7 is de voortplanting beschouwd als een gereguleerd interactief proces. Voorgaande experimentele resultaten worden besproken als aspecten van dit systeem.

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Curriculum vitae

Juliette Janson werd op 28 augustus 1964 geboren in een bovenwoning te Eindhoven. In 1982 behaalde zij het VWO diploma op de Koninklijke Scholengemeenschap Apeldoorn. Hierop volgde de studie Plantenveredeling waarbij zij deel uitmaakte van de eerste lichting twee-fasen studenten aan de Landbouwhogeschool Wageningen, waarvan de naam al snel veranderd werd in Landbouwuniversiteit Wageningen. Als onderdeel van deze studie ging zij vier maanden naar het Scottish Crop Research Institute in Invergowrie, Schotland. Afstudeervakken werden uitgevoerd in het vlak van veredeling/plantencytologie- en morfologie/plantenziektekunde en weefselkweek. Het in dit proefschrift beschreven onderzoek werd uitgevoerd op de Vakgroep Plantencytologie en -morfologie van de Landbouwuniversiteit Wageningen, in samenwerking met het IVT, dat tegenwoordig deel uitmaakt van het CPRO-DLO. Zij begon in 1987 als assistent in opleiding aan dit onderzoek. Tijdens deze periode heeft zij bij Prof. Dickinson aan de universiteit in Reading, Engeland, drie maanden onderzoek gedaan met *Petunia*. In maart 1992 trad zij in dienst van de Zaadunie te Enkhuizen.