Turk J Bot
24 (2000) 337-346

Research Article

Structure of the Stigma and Style in *Capsicum eximium* and the Effects of Pollination

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Received: 21.09.1998
Accepted: 02.06.2000

Abstract : Since the stigma surface provides the first point of contact for pollen grains, the initial pollen-pistil interaction takes place between these two structures. It is, therefore, important to understand the stucture of the stigma, as well as the style, in order to reveal the nature of pollen-pistil interaction. The aim of this study was to gather more information about interactions between pollen and pistil in *Capsicum eximium* A.T.Hunz Solanaceae by studying immature, mature unpollinated and mature pollinated styles. It was found that most features of the stigma and style of *C. eximium* resembled those of other genera in the Solanaceae.

Key Words: *Capsicum eximium,* stigma, style, pollen-pistil interaction

Capsicum eximium'da Stigma ve Stilus'un Yapısı ve Tozlaşmanın Etkileri

Özet : Stigma yüzeyi polen tanelerinin bitki üzerinde ilk temas noktası ve polen-pistil etkileşiminin ilk başlangıç yeridir. Bu nedenle stigma ve stilus'un yapısının bilinmesi, polen-pistil etkileşiminin doğasının açıklanmasına yardımcı olacaktır. Bu çalışmada, çok genç, genç, olgun tozlaşmamış ve olgun tozlaşmış *Capsicum eximium* A.T.Hunz (*Solanaceae)* pistilleri üzerinde çalışılarak polen-pistil etkileşimi hakkında bilgi edinilmeye çalışılmıştır. Çalışmalar sonunda *C. eximium* stigma ve stilus'unun pek çok özelliğinin *Solanaceae* familyasında yer alan diğer cinslerle benzerlik gösterdiği bulunmuştur.

Anahtar Sözcükler: *Capsicum eximium*, stigma, stilus, polen-pistil etkileşimi

Introduction

Prefertilisation barriers on the stigma surface result in the arrest of pollen germination or pollen tube entry into the stigma. This is one of the frequent barriers particularly in pollination with distantly related species (1,2). The causative factors for the failure of pollen germination can be lack of effective adhesion, lack of full hydration and pollen germination factors on the stigma. Pollen adhesion largely depends on the nature and extent of the stigma surface, specifically for dry type stigma (3).

Failure of pollen germination may be the result of insufficient or uncontrolled hydration. Pollen hydration is the result of the transfer of water from the stigma to the pollen through an osmotic gradient. Even after effective adhesion and hydration, pollen grains require suitable conditions on the stigma for germination. For example, the pollen of many species requires calcium and boron for germination and it has been shown that the stigma provides these elements to pollen (4).

Park et al. (5), in a study on the lily, reported that flowering plants possess specialised extracellular matrices in the female organs of the flower that support pollen tube growth and sperm cell transfer along the transmitting tract of the gynoecium. Transport of the pollen tube cell and the sperm cells involves a cell adhesion and migration event in species such as the lily that possess a transmitting tract epidermis in the stigma, style, and ovary. They used a bioassay for adhesion to isolate from the lily stigma/stylar exudate the components that are responsible for in vivo pollen tube adhesion and found that at least two stylar components are necessary for adhesion.

Failure of the pollen tube to reach the ovary is perhaps the most common interspecific prefertilization barrier. It is now well established that growing pollen tubes utilise stylar nutrients. One of the possible reasons for the arrest of pollen tube growth is the inability of the pollen tubes to utilise stylar nutrients. This may be due to

a lack of suitable nutrients in the transmitting tissue or a lack of suitable enzymes in the pollen tubes (6).

As seen from all this information there is an interaction between the pollen and pistil. It is therefore important to understand the structure of the stigma and the style in order to reveal the nature of the pollen-pistil interaction.

Several researchers have investigated the structure of the stigma in different genera of the family *Solanaceae* as well as in other plant families. For example, Konar and Linskens (7) reported that the stigma of *Petunia* Lindl. (*Solanaceae*) is bilobed, with the middle part of each lobe raised upwards, and the surface of the stigma has a large number of papillae. They also reported that exudation begins at an early stage and takes place in two stages. In the first stage, epidermal cells release the exudate which accumulates between the cuticle and cellulose wall. Since the amount of exudate gradually increases, the cuticle starts to extend and eventually ruptures at various places. According to researchers, at the beginning the exudate comes out through these ruptured places and later on when the cuticle is entirely thrown off, all the stigma surface is covered with a thin layer of exudate. The second stage of exudation starts after anthesis in the petunia. It is also reported that *Petunia* has a solid style with transmitting tissue (7).

The style of *Lycopersicon* Mill. (*Solanaceae*) was investigated by Cresti et al. (8). They reported that the style of *Lycopersicon* has a thick epidermis with two vascular strands opposited each other. Transmitting tissue is located in the centre of the style but forks into two strands near the stigma in order to reach the two stigmatic lobes. They also reported that at the mature stage the transmitting tissues of *Petunia, Nicotiana L. (Solanaceae)* and *Lycopersicon* showed a very similar ultrastructure.

In another study. Matthews et al. (9) quantified the features of the transmitting tissue for a broad range of angiosperm species. They worked on ten different angiosperm species and concluded that the overall stylar structure and tissue areas were generally consistent within families but differed significantly among families.

Several researchers investigated the histochemistry of the style and stigma in the family *Solanaceae*. For example, it was reported that the stigmatic exudate of *Nicotiana* consists of lipids, phenols, proteins and polysaccharides (10). They reported that proteins were present in the layer covering the stigmatic papillae or dissolved in the exudate upon the stigma.

Cresti et al. (8) also reported that the transmitting tissue in the mature style of *Lycopersicon* contains intercellular spaces with materials reacting positively to tests for polysaccharides and proteins.

Herrero and Dickinson (11) working on *Petunia* reported that the final stage in stigmatic maturation is independent of pollination.

Vasil (12 and references therein) reported that while the surface of the stigma and stigmatic exudate primarily provide favourable conditions for the germination of the pollen grain, as in *Petunia,* they are not directly involved in the nutrition of the germinating grains.

The nutritive role of transmitting tissue for the growth of the pollen tubes through the style has been known for some time. For example, Herrero and Dickinson (11) reported that when the pollen tubes of *Petunia* reached the transmitting tissue, some degeneration took place in the transmitting tissue. When they checked the metabolism of the starch over the course of pollination, they found that a slight increase in stylar starch synthesis was stimulated by pollination.

Kroh and Helpser (13) working on *Petunia* reported that pollen tubes grew within the intercellular substance of the transmitting tissue and in *Petunia* this intercellular metarial appeared to be acidic carbohydrates. They assumed that in pollinated styles the pollen tubes broke down the intercellular material enzymatically and utilied it for growth.

In other crops outside the family *Solanaceae*, Cerovic et al. (14) investigated the localisation of insoluble and pectic polysaccharides in particular ovary base in sour cherry (*Prunus cerasus* L./*Rosaceae*). They reported that in the period of intensive pollen tube growth through the transition zone between pericarp and style, this zone stained intensively for pectic polysaccharides.

This study was conducted to understand the stucture of the stigma and style in *Capsicum eximium* and to reveal pollen-pistil interaction. It was thought that more knowledge about these issues can help plant breeders to develop different methods to overcome different breeding barriers at different sites and it can also be helpful for the scientists who are trying to reveal the evolutionary ties between different genera and species.

Materials and Methods

Plant material

Capsicum eximium A.T.Hunz (*Solanaceae*) accession Hawkes 3860 (J.G.Hawkes) was employed during this investigation. Pistils of C. *eximium* fixed for microscopic studies were as follows:

Age	Length	Pollination
Very young	1.0-1.5 mm	unpollinated
Immature	2.0-2.5 mm	unpollinated
Mature	3.5-4.5 mm	unpollinated
Mature	3.5-4.5 mm	pollinated

Mature buds at a stage just prior to anthesis were pollinated and pistils harvested after 24 hours. All the pollinated, mature unpollinated and young buds were collected and kept on ice to keep the tissue fresh as long as possible. Ovaries were excised using a razor blade and the lengths of the styles were measured and recorded to verify the age group of each bud.

Preparation for transmission electon microscopy (TEM)

Material was processed according to following schedule (15):

1. Fix for 4 hours at room temperature in fixative of 3% (v/v) Glutaraldehyde EM Grade 25% solution (GDA) and 4% (w/v) paraformaldehyde in 0.005 M phosphate buffer at pH 7.00.

2. Rinse in three changes of 0.005 M phosphate buffer over a period of an hour.

3. Post fix in a 1% aqueous (w/v) solution of osmium tetroxide (O_3O_4) for 3 hours at room temperature.

4. Rinse in three changes of distilled water.

5. Dehydrate through a graded ethanol series of 30%, 50%, 70%, 90%, 95%, 100% (v/v) and twice in absolute dry ethanol, leaving the material for 20 minute in each solution.

6. Infiltrate with a 1:1 and then 1:3 mixture of absolute ethanol:London Resin (L.R.) White for 20 minutes each time.

7. Infiltrate with three changes 100% L.R. White over a period of 24 hours for each change. Specimens are maintained on a rotator.

8. Embed in fresh 100% L.R. White in gelatine capsules, which are filled and capped.

9. Polymerise the resin by incubating the capsules for 24 hours in an oven at 60ºC.

10. Sections (50-100 nm) from the stigma through the style were cut from the L.R. White blocks using a diamond knife fitted to a Reichert-Jung Ultracut microtome.

Preparation method for scanning electron microscopy (SEM)

Samples were treated according to the schedule described for transmission electron microscopy with the following modifications:

1. Dehydrate through a graded acetone series of 30%,50%,70%,90%,95%,100% twice, for 20 minutes in each solution.

2.Dry in a critical point drier.

3. Attach specimens onto SEM stubs using double-sided adhesive tape.

4. Coat mounted specimens with 30 nm of gold.

Specimens were then examined and photographed in a Jeol T20 SEM operating at 20 KV.

Preparation for light microscopy

Material was cut into 1-2 mm lengths and processed according to the schedule as described for transmission electon microscopy. The only difference was the thickness of the sections, which were cut 2 µm thick and sections were put onto a slide on a water drop and then dried out on a hot plate. Prior to staining for carbohydrates or proteins, 1% periodic acid was applied to the unstained sections for 6-8 minutes.

Localisation of protein with Coomassie Brilliant Blue

Proteins were localised according to a method of Fischer (16). The tissue was immersed in a staining solution containing 0.2%(w/v) Coomassie Brilliant Blue R-250 dye dissolved in a methanolic solution, methanol:acetic acid: water (5:1:4) for ten minutes at 60ºC. The stain was filtered prior to use. Destaining was carried out in distilled water. The slides were immersed in the water for 5 minutes at room temperature, and

then dried and mounted in DPX. The presence of protein is indicated by the appearance of a blue colour.

Localisation of carbohydrate an starch by PAS treatment

Sections were treated according to a method of Feder and O'Brien (17). General background staining was avoided by blocking aldehyde groups with 2,4 Dinitrophenylhydrazine (DNPH). This solution was prepared by dissolving 0.5 g of DNPH in a 15% (v/v) aqueous solution of acetic acid, which was filtered prior to use. Aldehyde blocking was effected by immersion in a solution for 20 minutes at room temperature, followed by rinsing in running water for 30 minutes. After blocking, the slides were immersed in a solution of 1% (w/v) periodic acid for 20 minutes, and washed in running water for 10 minutes. Staining in Schiff's reagent was carried out for 30 minutes in the dark, after which the slides were transferred through three times 3 minute changes of 0.5% (w/v) sodium metabisulphite solution and then rinsed in running water for 15 minutes. Finally the slides were dried and mounted in a few drops of DPX with a coverslip. While the presence of carbohydrate in the tissue is indicated by the appearance of a purplish-red colour, the presence of starch is indicated by the presence of purplish-red granules.

Results

Stigma

Capsicum eximium possessed a "wet stigma" which was bilobed, and papillae. At the early stages (very young or young immature buds) the cells of the stigma were compactly arranged with no or very small intercellular spaces. But during the time from the immature to mature stage, the cells separated from each other more and more to form large intercellular spaces. These changes wereobserved to occur during stigma maturation and were independent of pollination.

Development of exudate appeared to take place in two stages, as in *Petunia* and *Lycopersicon*. In the very young stage (1.0-1.5) mm style length), the stigmatic papillae were relatively free from surface secretions, compared to immature and mature stigma (2.0-2.5 and 3.5-4.5) mm style lengths respectively) (see Figure 1).

SEM pictures indicate the differences in the amount of exudate present on the stigma of varying ages. The immature stigma had visibly less superficial exudate than the mature stigma. The integrity of the papillae could therefore be observed on an immature stigma, whereas when the stigma was mature, the papillae was obscured by the massive amount of exudate (see Figure 2).

Results from studies of comparable resin-embedded material in the light microscope confirmed the differences noted in the SEM. While within the matrix of the exudate present on the stigma of the pollinated pistil, discrete dark granules were observed (see Figure 3), there were no granules within the matrix of the exudate present on the unpollinated stigma (data not shown). These discrete dark granules were not seen on the TEM pictures. It was assumed that these dark granules might have been calcium oxalate, as suggested by Herrero and Dickinson (11). Due to an interaction between calcium oxalate and electron beam or an interaction between calcium oxalate and any other chemical used for tissue preparation for TEM, calcim oxalate was removed from the tissue and left shiny areas on the surface (see Figure 4). Further research is needed to clarify this assumption.

In a mature unpollinated stigma, a few starch grains were present in the outer layer of stigmatic cells, but there were very few in the inner cells. The walls of the stigma cells appeared dark pink, indicating a strong PAS-positive reaction. In addition to cell wall components, the extracellular material between the cells also appeared dark pink, indicating PAS-positive material. In immature stigmas, all stigma cell walls and extracellular material between the cells gave a weak PAS-positive reaction.

When the stigma sections were stained for proteins, a blue coloration was observed in the cytoplasm. In addition, dark blue proteinaceous bodies were noted in the cytoplasm or in the vacuole. No protein-positive material was noted in the cell wall and very little if any protein-positive staining was observed in the extracellular matrix of the stigmatic cells. Similar observations were also obtained for immature pistils, but staining was weaker. However, a protein-positive staining of extracellular material was observed in a region close to the transmitting tissue (around the neck).

Transmitting tissue

Capsicum had a solid style like other genera in *Solanaceae*. There were two strands of transmitting tissue. The transmitting tissue appeared to be large enough to accommodate many pollen tubes. The cells in

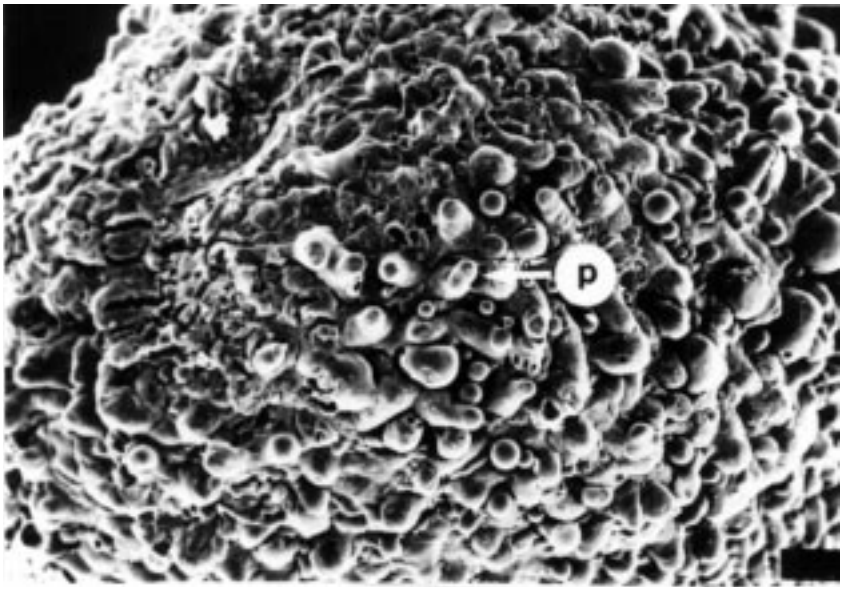

Figure 1. SEM pictures showing development of stigma and amount of exudate present on the very young stigma of C.*eximium* (1.0-1.5 mm) (274x) (aerial view). Arrow and letter (p) indicate the papillae.

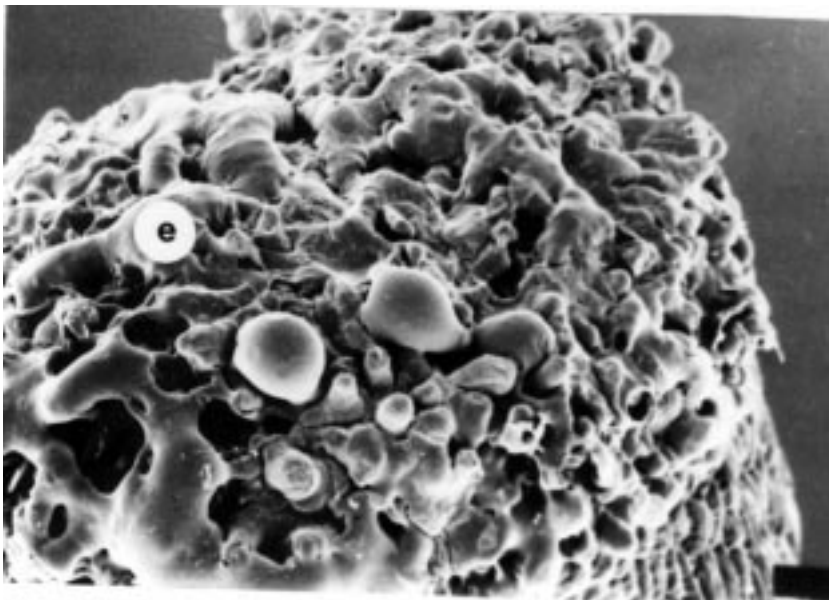

Figure 2. SEM pictures showing development of stigma and amount of exudate present on the mature stigma of C.*eximium* (3.5-4.5 mm) (274x) (aerial view). Letter (e) indicates the exudate.

the transmitting tissue of the mature style were elongate and more loosely packed than in the immature style. The walls of the cells of the transmitting tissue in the style were also found to be PAS reactive and large intercellular spaces were filled with exudate and stained heavily with PAS.

The cortical cells around the transmitting tissue were thin-walled and they contained numerous PAS-positive granules (possibly starch.) However, transmitting tissue cells contained few positive granules.

In an immature pistil, the walls of the cells of the transmitting tissue in the style and the intercellular spaces gave weak positive reactions, compared to mature pistils.

Cortical cells around the transmitting tissue already contained numerous PAS positive granules (possibly starch). It appeared that there was not a large amount of protein-rich bodies in the transmitting tissue.

Intercellular spaces in the transmitting tissue of immature pistils stained weakly for proteins.

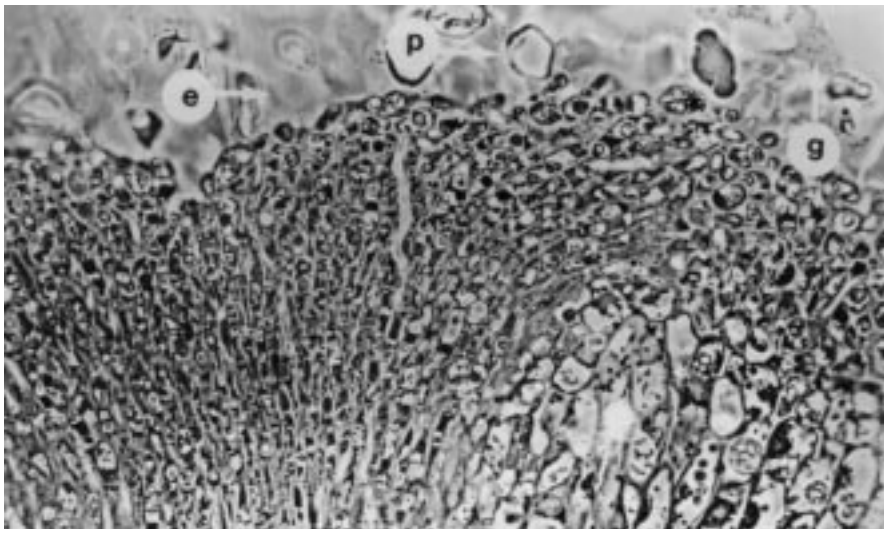

Figure 3. Light microscope picture showing amount of exudate on the stigma after pollination. (276x) (Longitudinal Section). Letter (e) indicates exudate, letter (p) indicates pollen grain and letter (g) indicates granules

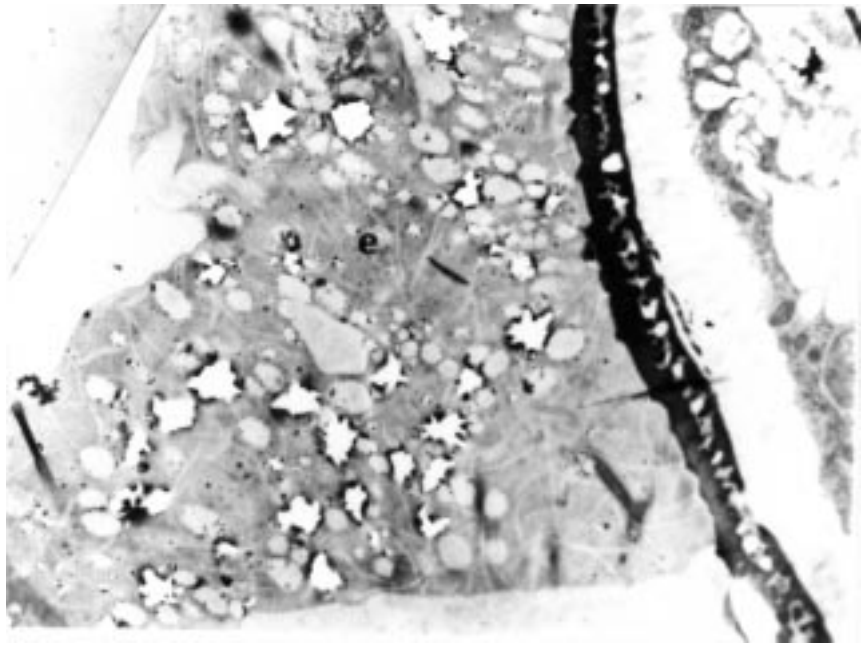

Figure 4. TEM picture showing amount of exudate on the stigma of pollinated pistil. (33684) (Longitudinal Section). Letter (e) indicates the exudate. Exudate of pollinated stigma displays marked heterogeneity.

Changes in the stigma and style after pollination

The SEM picture indicated possible differences between mature unpollinated and pollinated stigma in terms of amount of exudate. Notably, on a pollinated stigma, pollen grains were inundated by a considerable amount of exudate (see Figure 5), whereas on an unpollinated stigma there seemed less exudate than on a pollinated stigma (data not shown).

The TEM pictures indicated that the exudate of an unpollinated stigma appeared to be homogenous all around the sample, while the exudate of a pollinated stigma displayed marked heterogeneity as if in some regions on the stigma, some material had been taken away from the exudate (see Figues 4 and 6).

In the sections of the style from the neck of the stigma down to the top of the ovary, some differences were observed in terms of the relative amount of the PAS-reactive granules in the cortical cells around the transmitting tissue. It appeared that PAS-reactive granules in the pollinated styles were fewer than unpollinated styles (see Figues 7 and 8).

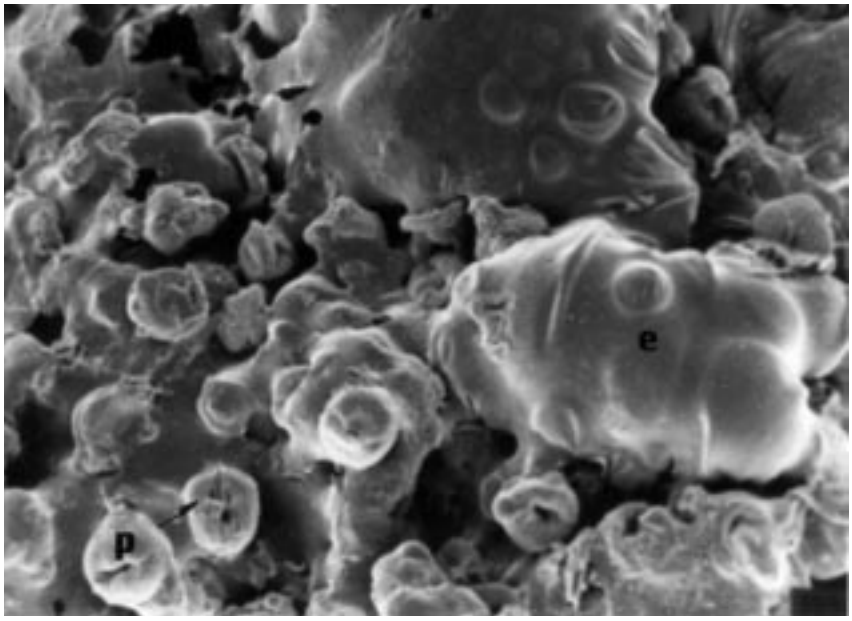

Figure 5. SEm pictures showing the amount of exudate on the stigma after pollination (274x) (aerial view). Arrow and letter (p) indicate the pollen grain and letter (e) indicates the exudate.

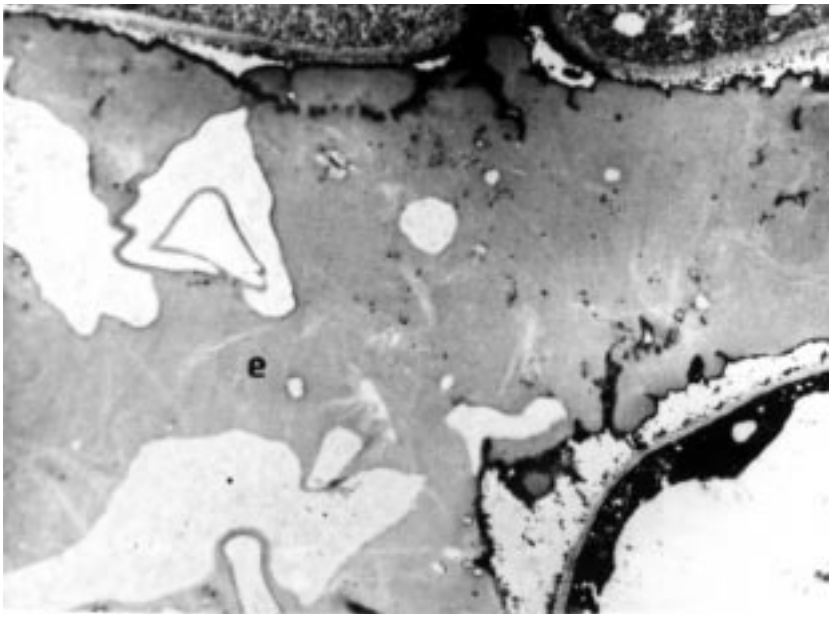

Figure 6. TEM picture showing amount of exudate on the stigma of unpollinated pistil. (33684) (Longitudinal Section). Letter (e) indicates the exudate. Exudate of unpollinated stigma appears homogenous all around the sample.

No differences were observed in terms of protein distribution between pollinated and unpollinated pistils.

Discussion

Capsicum had a "wet", bilobed stigma like other genera in the family *Solanaceae* (1,7). In the very young and immature stigmas, the developing stigma had relatively less exudate on its surface than mature stigmas and therefore individual papillae can be observed on an immature stigma, whereas when the stigma was mature, papillae were obscured by the massive amount of exudate. This result may indicate that secretion of exudate took place in two stages as reported for *Petunia.*

In immature stigmas, stigma cells were compact with small intercellular spaces. During maturation, exudate accumulated between cells, separating them from each other and leaving large intercellular spaces between them

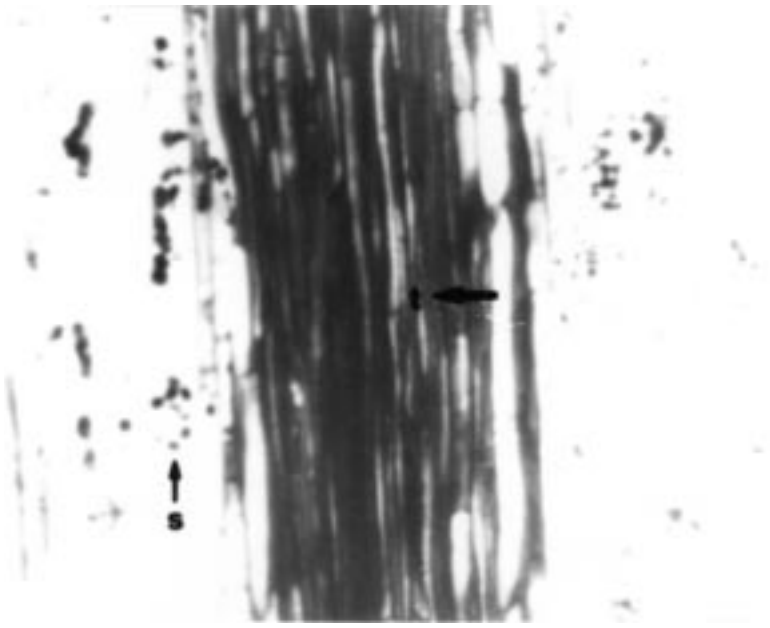

Figure 7. Localisation of carbohydrates in the transmitting tissue and in outer cortex walls of pollinated pistil (276x) (Longitudinal Section). Letter (t) indicates the transmitting tissue and letter (s) indicates the starch granules.

Figure 8. Localisation of carbohydrates in the transmitting tissue and in outer cortex walls of unpollinated pistil (276x) (Longitudinal Section). Letter (t) indicates the transmitting tissue and letter (s) indicates the starch granules.

and the cells became loosely arranged. Similar structural changes were also described for *Petunia* and *Nicotiana.*

An increase in the amount of exudate and structural changes in arrangements of the stigma cells happened regardless of pollination. Similar result were also reported by Herrero and Dickinson (11) for *Petunia*. They reported that the final stages in stigmatic maturation were clearly independent of pollination.

There was a considerable amount of the exudate on the pollinated stigma surface. It is known that compatible pollination may increase the amount of the exudate secreted onto the stigma following pollination. No promotive effect has been reported in the family *Solanaceae*, but Sedgley and Scholefield (18) reported that pollination increases the secretion of exudate in the family *Compositae*.

Exudate on the stigma surface did not stain for carbohydrates or proteins, although extracellular exudate in the intercellular spaces did stain for carbohydrates and proteins. In wet types of stigma, carbohydrates and

proteins are normally detected in the exudate. SEM pictures and pictures of unstained sections studied under the light microscope showed that exudate was present on the stigma surface. Therefore, some steps during the staining might have prevented the exudate on the stigma surface getting stained. The only difference between specimens processed for SEM or light microscopy of unstained sections and specimens for staining was the application of 1% periodic acid. All the sections taken for staining were immersed in that solution for 6-8 minutes. It was thought that this process either took the exudate from the stigma surface away or somehow prevented it staining.

It was found that exudate of the pollinated stigma was not homogenous and it seemed as if in some regions on the stigma, some of the material was taken away from the exudate. Shivanna (19,20) reported that phenolic compounds may play a role in pollen nutrition as well as promotoin or inhibition of pollen germination on the stigma. Since exudate of the pollinated stigma appeared heterogeneous in composition and had something taken away from it, it is possible to suggest that pollen grains might have taken some phenolic compounds or other substances such as proteins and carbohydrates away and used them as some sort of energy resource, although this assumption is highly speculative.

Another striking point arose, when the stigma and style sections were stained for carbohydrates. In the unpollinated pistil, the outer cortical cells of the style carry more starch grains than the pollinated pistils. This difference is more obvious in the sections from the neck of the stigma down to the ovary. Pollen grains use their own energy resources to initiate germination and early growth, and then switch to heterotrophic growth, which relies on external sources of nutrients, e.g. possibly from the style. Therefore, one can assume that somehow pollen grains send some sort of signal which causes starch in the style to be mobilised and used as an energy source.

It is a well known fact that in interspecific incompatibility in *Capsicum*, pollen tube growth was inhibited just below the stigma around the neck (21). One, thus, may think that incompatible pollen grains do not send any signal to mobilise the starch and cannot use it. Knox (22) reported that mentor pollen can be used to overcome incompatibility assuming that mentor pollen may send a message to prevent blockage or activate the starch mobilisation. In future work, mentor pollen may be useful in *Capsicum* as well to overcome interspecific incompatibility and should be tried by plant breeders.

The presence of extracellular protein on the surface of stigmas as well as in the intercellular spaces of the transmitting tissue has been reported in *Petunia* (11). In this study, some extracellular protein was found around the neck of the stigma, close to the transmitting tissue, and in the region inside the transmitting tissue itself. Localisation of the extracellular proteins around the neck of the stigma is important, because this region is the site of inhibition for unilateral incompatibility (21), as stated above, Shivanna (19) reported that "considerable significance is attached to the presence of extracellular proteins on the stigma in the path of pollen tube growth, because of the possibility of its involvement in pollen recognition and incompatibility responses". Therefore not seeing any extracellular protein on the stigma in this present study, and seeing it around neck of the stigma, may tempt one to say that extracellular proteins around the neck of the stigma may play a initial role in unilateral incompatibility, although it is necessary to get more information about what these proteins are.

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