Planta (Berl.) 71, 356—371 (1966)

# THE MORPHOLOGY AND ANATOMY OF THE STIGMA OF *PETUNIA HYBRIDA* *

R. N. KONAR and H. F. LINSKENS
Department of Botany, University, Nijmegen, The Netherlands

Received May 28, 1966

*Summary.* A mature stigma of *Petunia hybrida* ready for pollination shows 4—6 large shining drops of the exudate along with numerous smaller ones. A developing style and stigma have a columnar tissue that flares at the top. In the stigma there can be distinguished a secretory and a storage zone. In the former, schizogenous cavities are formed which are filled with the exudate. The mode of formation and secretion of the drop has been studied with light and electron microscope. The nature of reserves has been studied histochemically.

The exudation takes place in two stages. In the first stage, the epidermal and papillae cells release out the oily exudate upon rupture of the cuticle. The second phase of exudation begins with anthesis. The exudate from the schizogenous cavities is released between the epidermal cells. There are distinct loci on the stigma surface where more exudate is given out than at other places.

## Introduction

To understand the factors that control the reproductive processes in higher plants, it is essential to have a thorough knowledge of both the pollen and the gynoecium. While a considerable amount of information exists on the pollen (LINSKENS, 1964; STANLEY, 1964) and the ovary (MAHESHWARI, 1950) not much is known about the stigma. Some few studies do exist on mature stigma, stigma reaction etc. of plants but no detailed developmental studies have been made. The present investigation was undertaken with a view to study the morphology and functional anatomy of the stigma of *Petunia hybrida* and its role in the production and release of the exudate.

## Previous Work

As early as 1875, BEHRENS described the anatomy of the mature style and stigma of several plants including *Polygala venulosa*, *Pirola rotundifolia*, *Atropa belladona* and others. He also reported the presence of raphids in the stylar cavities. The stigmatic anatomy of *Digitalis*, *Gratiola* and *Torenia exappendiculata* were made by LUTZ (1911). The stylar tissue comprises two zones, viz. a central core surrounded by a parenchymatous zone. HANF (1935) distinguished three types of styles anatomically viz., open, solid and half-closed. VASIL and JOHRI (1964) made anatomical studies on the mature style and stigma of *Aegle marmelos*, *Fritillaria roylei*, *Lilium*

* Dedicated to the memory of the late Prof. P. MAHESHWARI FRS, our teacher (R. N. K) and friend (H. F. L) who read through the Ms in April at Paris, a month before his death.

*tigrinum*, *Catesbaea spinosa*, *Nicandra physaloides*, *Pavonia zeylanica* and *Zephyranthes ajax*. In *Aegle* and *Pavonia* the stigmatic secretion shows the maximum concentration of lipids at the time of pollination. Before pollination there was abundant starch in the stigma and style which is consumed at the time of pollination.

While crossing two species of *Orchis* (*O. masculata* × *O. morio*) STRASBURGER (1886) found that once the pollen grains had germinated, the stigma became disorganized and stigmatic papillae turned brown at the point of contact of the pollen tubes. He thought that the pollinia caused a certain change in the nature of the stigma cells.

In *Brassica* there is self-incompatibility at anthesis but within three to four days the stigma becomes self-compatible. This is because the effect of the secretion of substances responsible for self-incompatibility is weakened and their physiological functions are lost (KAKIZAKI, 1939; see MIKI-HIROSIGE, 1954). In *Secale cereale* the stigma cells differ in the stainability of the nucleus according to whether they are pollinated or not. The style of *Secale* is forked into two stigmas at its base and numerous stigmatic filaments are borne on them for their entire length. Under favourable conditions the pollen tube emerges from the germ pore within a minute or two. The pollinated stigmas can easily be stained with acetocarmine within a few minutes of pollination but unpollinated stigmas remain resistant to staining. As a consequence of pollination various other changes also take place in the stigma cells. They show better permeability, a change in the shape and size of the nucleus and a gradual withering and collapse. For all these physico-chemical changes which occur in stigma cells after pollination the term "*stigma reaction*" has been proposed. For this reaction it is not necessary for the pollen to be attached to the stigma by the germ pore but by any side of its surface (KATO, 1953; KATO and WATANABE, 1957).

In *Vicia faba* a large increase in seed setting has been reported by HOLDEN and BOND (1960) due to tripping (the release of the stigma and style from the enveloping keel petals). Careful examination has shown that in this process the stigmatic papillae are ruptured and the stigma surface rendered suitable for the germination of pollen.

FREYTAG (1959) studied the stigmatic papillae with polarizing optical method. He noticed that there are lipids in the walls of the papillae and most of them show the phenomenon of double refringence. After treating with lipid extracting solvents this phenomenon disappears. From this it is concluded by the author that lipids are radially oriented on the cellulose walls.

## Material and Methods

Two self sterile clones of *Petunia hybrida* $T_2U$ and W166K were used for this investigation. They were propagated throughout the year from stem cuttings under controlled conditions of light and temperature. The natural light in the glasshouse was supplemented with artifical light (Philips HPW 500, intensity 10,000 lux).

For developmental studies the stigmas were trimmed on parallel sides, fixed in FAA and later preserved in 70 per cent ethanol. They were dehydrated and cleared in the alcohol-xylol series and embedded in paraffin. Sections were cut in a rotary microtome at a thickness of 10—12 microns and stained with safranin-fast green. Much information was obtained by making free hand sections and staining them with Lugol's iodine and Sudan III (GURR, 1965). The free hand and the microtome sections were photographed with Leitz Orthomat using a light source of 560 mμ.

Little portions of the stigma were fixed in aqueous glutaric acid for $1^1/_2$ hours for electron microscopy. These were washed in phosphate buffer (pH 7.2) and pre-

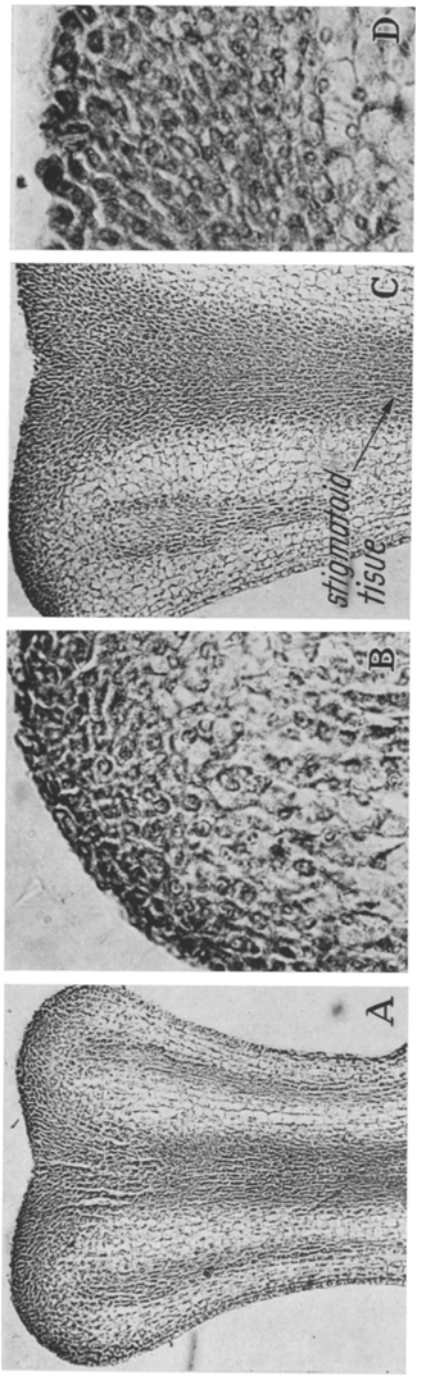


Fig. 1. A Microphotograph of longitudinal section of a developing stigma and style showing the central depression (length of style with stigma = 0.3 mm). ×76. B Portion from A magnified. ×319. C—D Same as A and B; later stage of development (length of style with stigma = 1 mm). C ×76, D ×319. E—F Stigma in longitudinal section at a still later stage of development (length of style with stigma = 6 mm). E ×76; F ×76, G Tip of the vascular bundle enlarged. ×319

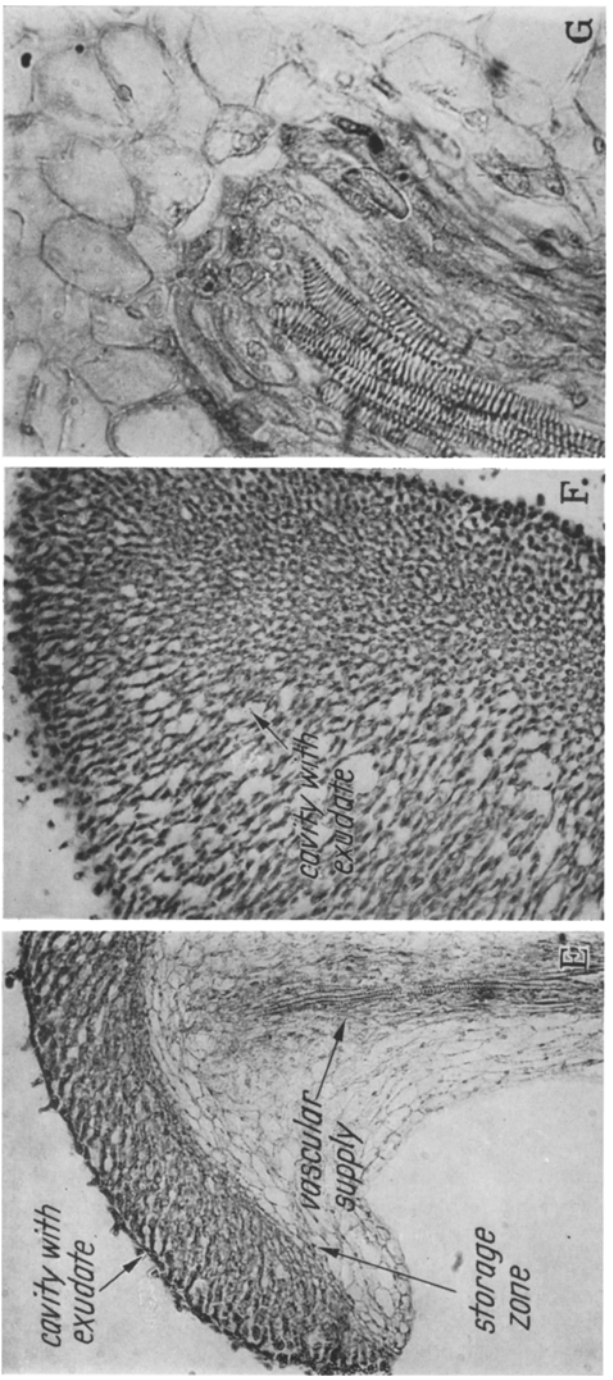
cavity with exudate
vascular supply
storage zone
E
cavity with exudate
F.
G

Fig. 1 E—G

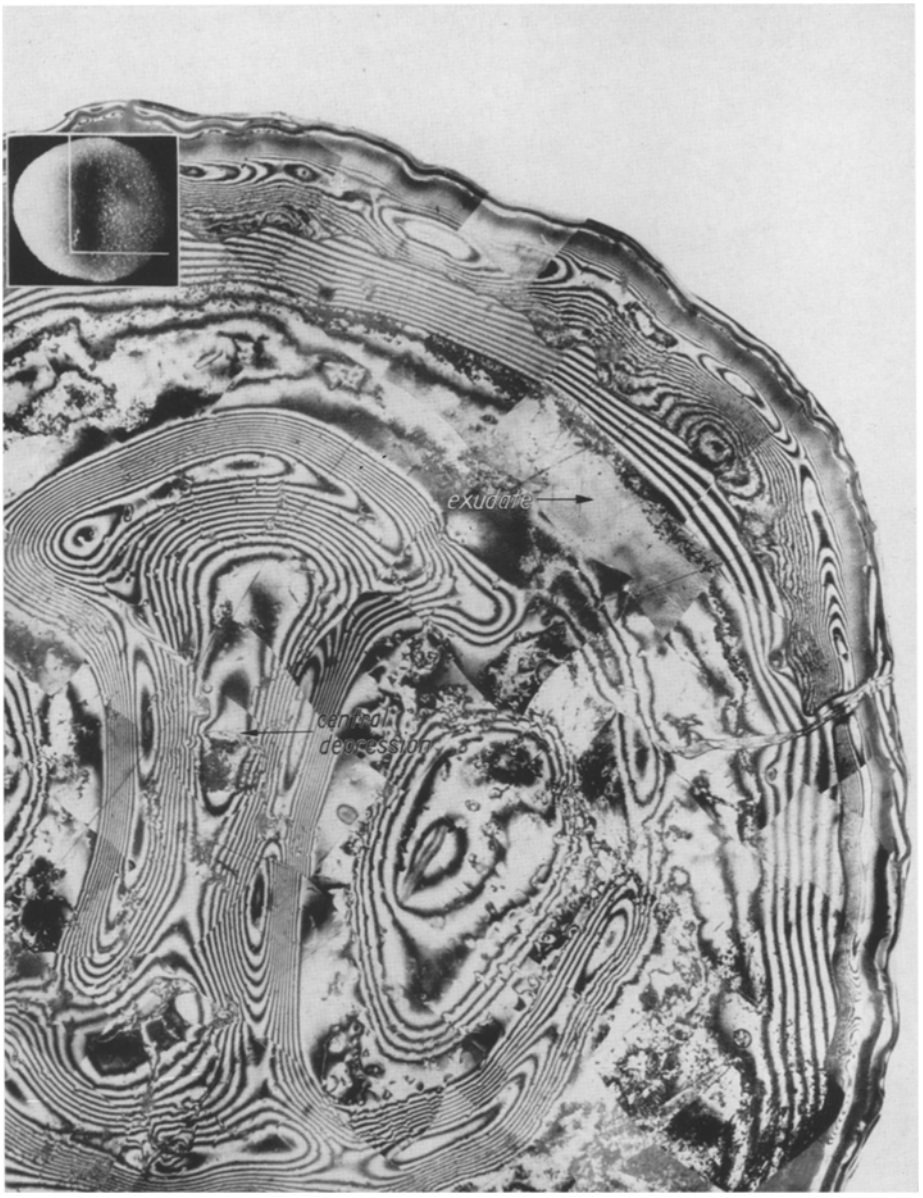


Fig. 2. Interference picture of the upper surface of the stigma using a surface interference microscope (A. Kohaut; reconstructed from serial photographs). Plastic replica in air; line distance = 0.54 μ. The inset shows the top view of the stigma along with the demarcation of the portion photographed

served overnight in the buffer. Later they were transferred to potassium permanganate solution for half an hour followed by an hour in 2 per cent osmium tetraoxide solution. Subsequently these were immersed in a solution of uranyl acetate (1 per

cent) for 2 hours and dehydrated in progressively increasing concentrations of ethanol (10 minutes in each). Four changes were given in absolute alcohol (20 minutes each time). These were then placed in alcohol-methacrylate solution of increasing concentrations (3:1, 1:1, 1:3) for an hour each and finally transferred to pure methacrylate.

For embedding, individual gelatine capsules were filled with methacrylate and a piece of stigma was placed in each. These were then covered with gelatine caps

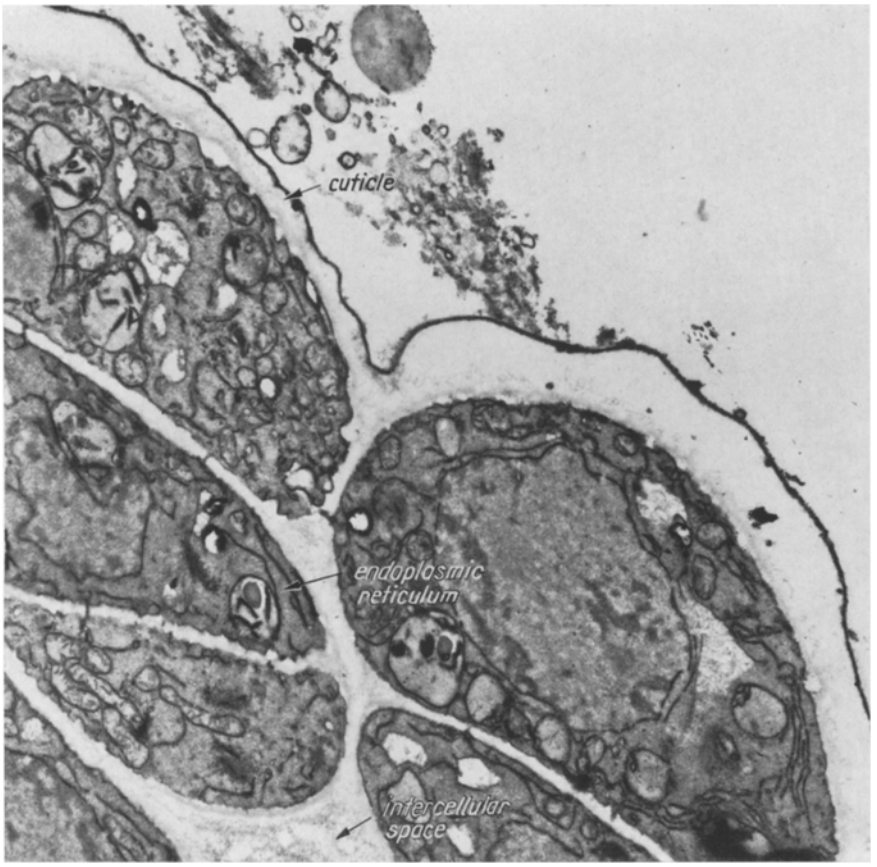


Fig. 3. Electron micrograph of a stigma at a very young stage (stigma diam. = 0.3 mm) showing a continuous cuticle above the epidermal cells. × 7,600

and polymerised at 60° C for 24 hours. Next the capsules were transferred to water where the gelatine dissolved leaving the material embedded in methacrylate.

Sections were cut with glass knives in an LKB Ultrotome and studied with a Philips EM 100C.

The surface relief of the stigma was obtained by interference microscopy (Linskens, 1966; Linskens and Kroes, 1966). A drop of acetone was put on the stigmatic surface and a little piece of thin (0.07 mm) cellulose-acetate film was gently rolled down into it. The film was bowed slightly and started at one edge so that no air bubble was trapped. After a minute the film was uniformly pres-

sed on the surface with the back of a scalpel. Fifteen minutes later the film was removed and placed in the "Zehender chamber" (see Linskens, 1966) and viewed under a surface interference microscope using thallium lamp for illumination.

We are thankful to Dr. (Miss) M. Kroh for the electron micrographs.

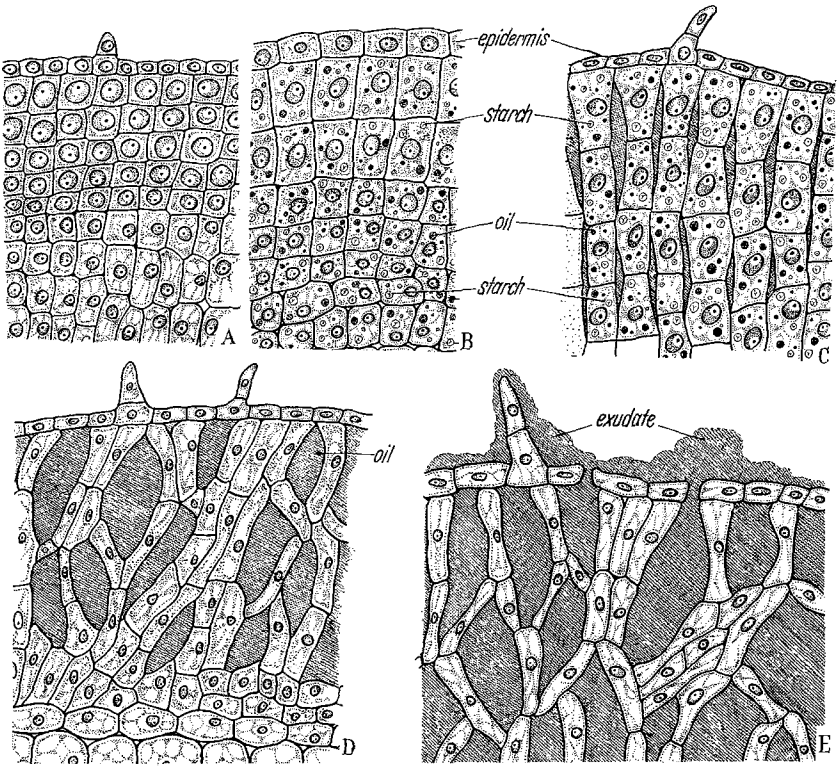


Fig. 4. A Very young stigmatic cells showing prominent nuclei. × 369. B Portion of the stigma cells showing starch and oil. × 369. C Stigmatic cells showing accumulation of exudate in the intercellular spaces. × 369. D Schizogenous cavities in the secretory zone of the stigma. × 298. E Section from a fresh material showing the separated epidermal cells and the release of the exudate. × 298

## Observations

A vertical section of the slightly zygomorphic, pentamerous flower shows five epipetalous stamens which terminate at various heights below the level of the stigma. The stamens are of three types. Two have long filaments (17 mm) and dehisce first followed by two others with slightly smaller filaments (14 mm). The fifth and smallest stamen has a filament length of 10 mm and is last to dehisce. The style is slightly bent in the bud condition but becomes straight at anthesis. The total length of style and stigma varies from 25—30 mm at maturity. The stigma is a flat, bilobed structure with the middle part of each lobe slightly

raised upwards (Fig. 1 A). There is a central depression which is slit-like and gradually narrows down into the style.

A mature stigma, ready for pollination, shows 4—6 large, shining drops of the exudate. These correspond to the places where the production is maximum. Numerous smaller drops of various sizes are also seen. The surface of the stigma has a large number of papillate hairs distri-

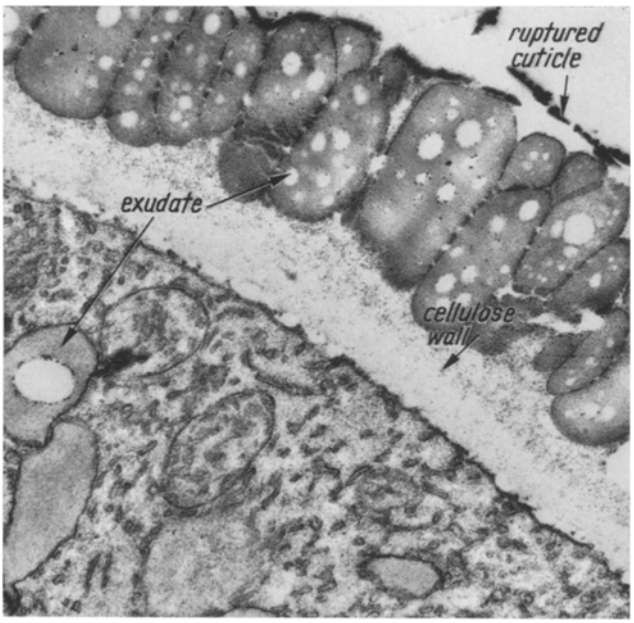


Fig. 5. Electron micrograph of a portion of the epidermal cell to show the accumulation of the exudate between the cuticle and the cellulose wall. Also note the ruptured cuticle. ×21,330

buted all over. An interference picture of the surface is shown in Fig. 2. The distance between the lines is 0.54 μ. It is clearly shown that the surface is not smooth but has raised areas and depressions.

The style is solid and the stigmatoid tissue is single stranded and multi-layered. After reaching the stigma, the pollen tube moves through this tissue by intrusive growth.

Histologically, a developing style and stigma show a columnar tissue which flares up at the top with a slight depression in the center (Fig. 1 A). The stigma at this stage has a single layered epidermis followed by sub-epidermal cells. There is a thin cuticle over the epidermis which is clearly seen under the electron microscope (Fig. 3). The sub-epidermal cells are densely cytoplasmic and meristematic (Fig. 1 B, 4 A). The center of the developing style or the stigmatoid tissue is composed of small cells with prominent nuclei and cytoplasm which is in continuity with the

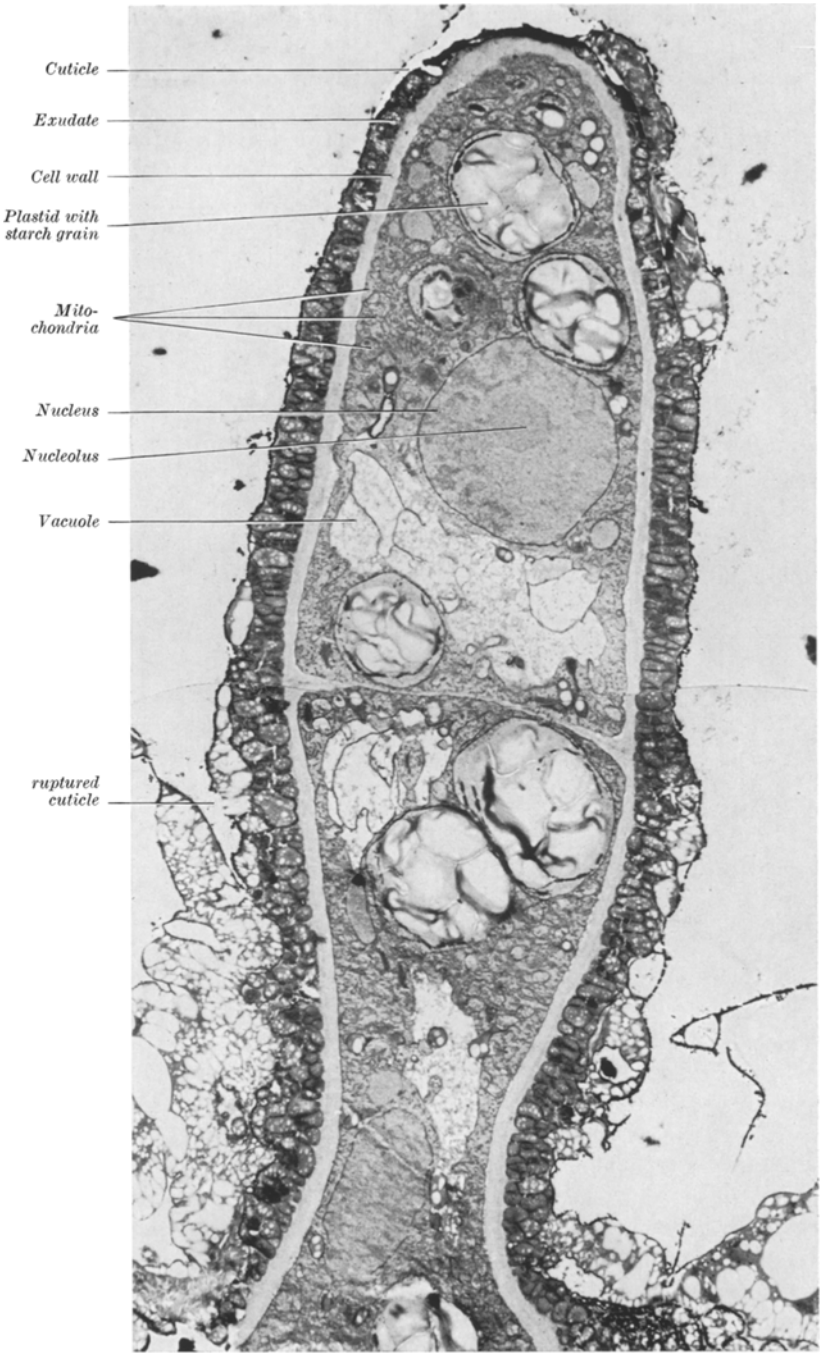


Fig. 6. Stigmatic papilla as seen under the electron microscope. The accumulated exudate is seen between the cellulose wall and the cuticle × 7000

sub-epidermal cells of the stigma (Fig. 1 A, C). Two provascular strands traverse the ground tissue (Fig. 1 A) and terminate at the base of the stigma. The cells of the subepidermal region of the stigma near the central depression are more or less vertically oriented while those at the periphery are arranged tangentially (Fig. 1 D). These cells also show slight vacuolation. At this stage many of the epidermal cells undergo

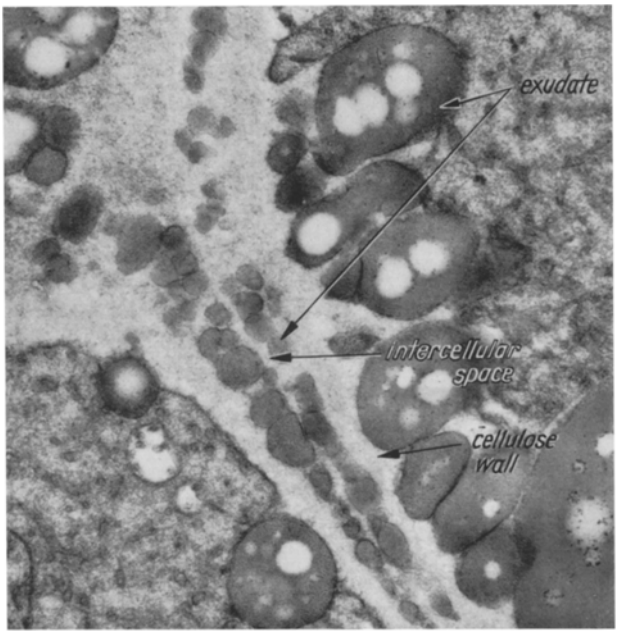


Fig. 7. Electron micrograph showing the migration of oily drops into the intercellular space. ×27,810

transverse divisions giving rise to two cells which finally develop into bi-celled stigmatic papillae (Figs. 4 C, D; 6).

Histochemical studies done on the developing and differentiating cells of the stigma revealed that quite early in ontogeny, the cells in this region become filled with starch and oil globules. Even the epidermis and the papillae are not free from such inclusions (Fig. 4 B).

Longitudinal section of a mature stigma shows single layered epidermis bearing a large number of two-cell hairs (Fig. 1 E). Next comes the secreting zone which is spongy and shows a large number of schizogenous cavities filled with exudate (Fig. 1 E, F). The cavities are bounded by elongated parenchymatous cells. The inner side of the secretory zone is limited by a few layers (1—3) of parenchymatous cells which are quite distinct from the rest of the style and stigma. They constitute the storage region. The cells between the tips of the vascular supply and the

storage region are highly vacuolated and thin walled (Fig. 1 E). The vascular strand has a larger proportion of xylem as compared to phloem (Fig. 1 G).

The cells of the entire stigmatic region are filled with chloroplasts. In the mature bud all the cells of the stigma, the stigmatoid tissue and the

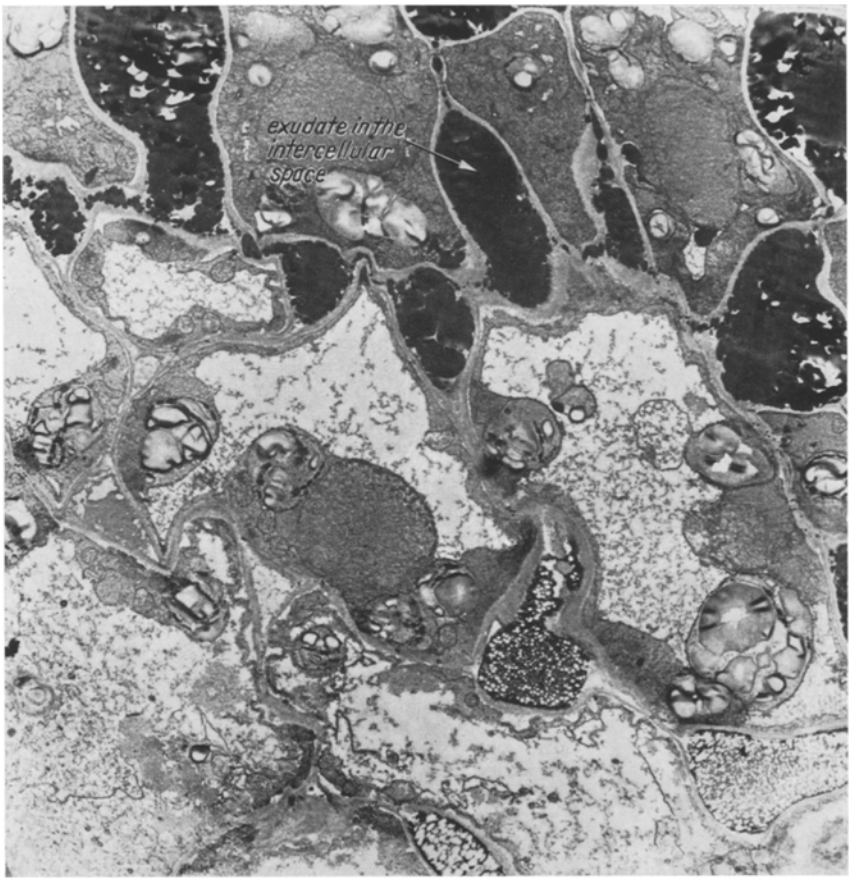


Fig. 8. Electron micrograph of a part of the secretory zone of a stigma (2 mm diam.) showing the electron dense exudate in the intercellular spaces. ×4,000

ground tissue show considerable quantities of starch in their cells. With anthesis the starch content markedly falls in the stigma and the stigmatoid region.

The oily bodies which are of various sizes migrate to the periphery of the cells and pass out through the cellulose wall. In the case of epidermal and the papillae cells the oil globules accumulate between the cuticle and the cellulose wall (Figs. 5, 6). The oil globules are electron dense. In the cells of the secreting zone the exudate gradually accumulates

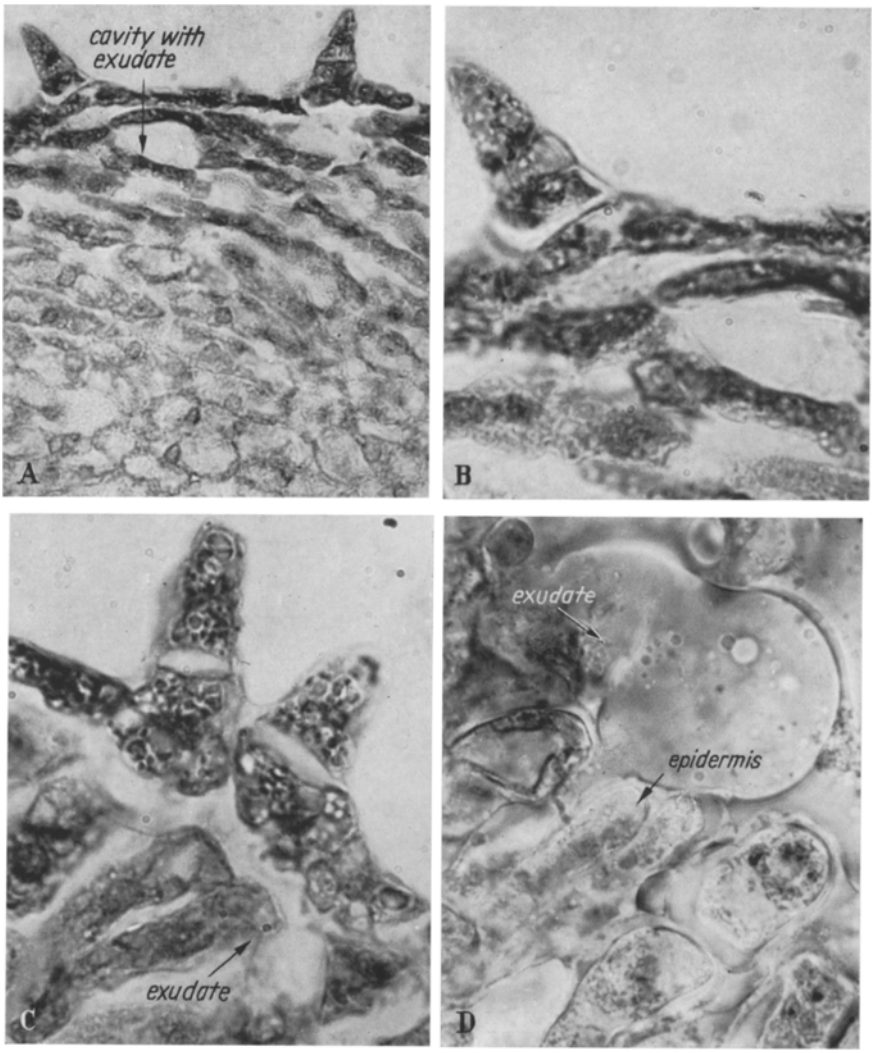


Fig. 9. A Photomicrograph of the secretory zone of the stigma showing a few schizogenous cavities. ×319. B A part from A enlarged. ×319. C Stage of the stigma just before the release of the exudate. ×319. D Same as above showing the release of the exudate between the epidermal cells. ×319

in the intercellular region (Figs. 4C—E, 7). As the accumulation of the exudate continues the cavities become larger and larger (Figs. 4D; 8; 9A, B). The exudation also continues to accumulate between the cuticle and the cells of the epidermis.

Soon the cuticle is ruptured at many places (Figs. 5, 6) and the oily exudate spreads over the surface of the stigma. This happens fairly early in the stage of development of the buds. The exudate from the schizo-

genous cavities of the secretory zone of the stigma is given out soon after anthesis. At this stage the epidermal cells become loose and the exudate is released between the cells (Figs. 4E, 9C, D). The exudation soon appears on the surface and within a few hours most of it is released.

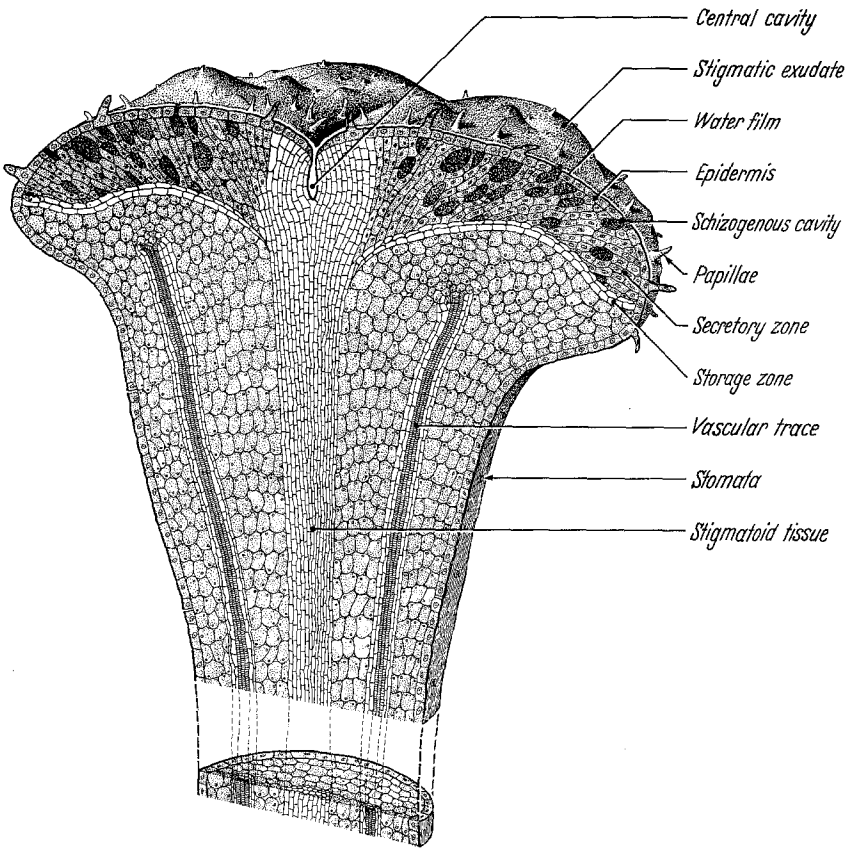


Fig. 10. Semi-diagrammatic reconstruction of a stigma of *Petunia hybrida* just before pollination. × 35

## Discussion

The stigma of *Petunia* is a bilobed structure with a central depression. The undulated surface is studded with a large number of papillate hairs. At maturity it shows large amount of oily exudate. The presence of water has been demonstrated with the help of cobalt chloride paper (KONAR and LINSKENS, 1965, 1966). It is at its maximum in the central depression.

A study of its developmental anatomy shows that the stigma can be separated into two zones: an upper zone with the epidermis constitut-

ing the secretory zone, and a lower 1—3 layers of laterally extended cells forming the storage zone. The exudation begins to accumulate at an early stage in the intercellular spaces (schizogenous cavities) and is released soon after anthesis.

The cells of the secretory zone including the papillae and the epidermis are very rich in chloroplasts and on staining with LUGOL's iodine reveal the presence of starch in them. The cells of the storage region also show considerable accumulation of starch.

The exudation takes place in two stages. In the first stage the epidermal and papillate cells release the oily exudate that accumulate between the cuticle and the cellulose wall. This happens fairly early in ontogeny. With gradually increasing amount of the exudate the cuticle more and more extends and finally ruptures at various places. Initially the exudate comes out through these discontinuities but later the cuticle is thrown off in the form of flakes and the entire stigmatic surface is covered with a thin layer of oily exudate.

The second stage starts soon after anthesis and is much more vigorous. The exudate which initially accumulates in the schizogenous cavities located in the secretory zone is released rather quickly between the epidermal cells. The latter are loose at maturity. It is known that the cells of the stigmatic epidermis become loose in several plants at the time of pollination (ESAU, 1960). When the exudation begins it carries the remnants of the cuticle with it so that nothing of it is left at maturity. In their studies on *Lilium candidum*, *Funkia ovata* and others ROSENTHALER and KOLLE (1921) report that in the course of development the cuticle partly disappears.

The solid style of *Petunia* has a single multilayered stigmatoid tissue. This tissue has been variously named as the conducting tissue (HANF, 1935), transmitting tissue (HUNT, 1937; VASIL and JOHRI, 1964) and inductive tissue (IWANAMI, 1959). In agreement with ESAU (1960) we have called it "stigmatoid tissue" as it is continuous with the stigmatic tissue. Structurally it also resembles the cells of the stigma and later the pollen tube grows through this tissue.

Fig. 10 is a semi-diagrammatic representation of the stigma and part of style of *Petunia hybrida* at the time of pollination.

## Zusammenfassung

Bei der Durchsicht der einschlägigen Literatur ergab sich, daß die Kenntnisse über die Entwicklungsgeschichte und den Sekretionsmechanismus des Narben-Schleimes äußerst lückenhaft sind, obgleich dieser im Verlauf des Bestäubungs-Vorganges bei vielen Pflanzen eine bedeutende Rolle spielt. Die vorliegende Untersuchung versuchte daher eine detaillierte Einsicht in die Morphologie und Anatomie der Narbe

von *Petunia hybrida* zu geben, da diese Art als Objekt für zahlreiche physiologische Untersuchungen auf dem Gebiet der Befruchtungsinkompatibilität dient.

Die Blüten von *Petunia* sind zwittrig, zygomorph und fünfzählig. Sie besitzen fünf epipetale Antheren mit drei verschiedenen Filament-Längen; diese öffnen sich zu verschiedenen Zeitpunkten. Die reife Narbe zeigt zum Zeitpunkt der Bestäubung 4—6 große sowie zahlreiche kleinere, transparente Flüssigkeits-Tropfen.

Der sich entwickelnde Griffel mit der Narbe besteht aus einem säulenartigen Gewebe, das sich an der Spitze verbreitert. Bei der Narbe kann eine Sekretions- und eine Reservematerial-Zone unterschieden werden. In der Sekretionszone werden schizogen Hohlräume gebildet, die sich mit dem Exsudat füllen. Die Bildung und Sekretion der Narbenflüssigkeit wurde licht- und elektronenmikroskopisch untersucht. Mittels histochemischer Methoden wurden die Substanzen der Reservematerial-Zone näher charakterisiert.

Die Freisetzung der Narben-Flüssigkeit findet in zwei Schritten statt: Während der ersten Phase wird das ölige Exsudat von den epidermalen Zellen und den Papillen der Narbe nach Zerreißen der Narben-Cuticula freigesetzt. In der zweiten Phase jedoch tritt das Exsudat aus den schizogenen Hohlräumen durch die Zwischenräume in der Narben-Epidermis aus. Auf der Narben-Oberfläche gibt es Bezirke, welche offensichtlich in reichlicherem Maße Flüssigkeit produzieren als andere Bezirke.

## Literature

Behrens, W. J.: Untersuchungen über den anatomischen Bau des Griffels und der Narbe einiger Pflanzenarten. Inaug.-Diss. Göttingen 1875.

Esau, K.: Anatomy of seed plants. New York: Wiley & Sons, Inc. 1960.

Freytag, K.: Doppelbrechende und leicht lösliche Lipoide in den Zellwänden der Narbenpapillen verschiedener Blütenpflanzen. Z. Bot. **47**, 113—120 (1959).

Gurr, E.: The rational use of dyes in biology. London: L. Hill 1965

Hanf, M.: Vergleichende und entwicklungsgeschichtliche Untersuchungen über Morphologie und Anatomie der Griffel und Griffeläste. Beiheft Bot. Cbl. A **54**, 99—141 (1935).

Holden, J. H. W., and D. A. Bond: Studies on the breeding system of the field bean *Vicia faba* (L). Heridity **15**, 175—192 (1960).

Hunt, K. W.: A study of style and stigma with reference to the nature of carpel. Amer. J. Bot. **24**, 288—295 (1937).

Iwanami, Y.: Physiological researches of pollen V. On the inductive tissue and growth of the pollen tube in the style. Bot. Mag. (Tokyo) **66**, 189—196 (1959).

Kato, K.: A phenomena in the early stage of pollination in *Secale cereale*; the stigma reaction. Mem. Coll. Sci., Univ. Kyoto B **20**, 203—206 (1953).

—, and K. Watanabe: The stigma reaction II. The presence of the stigma reaction in intraspecific and intergeneric pollination in the *Gramineae*. Bot. Mag. (Tokyo) **70**, 96—101 (1957).

KONAR, R.N., and H.F. LINSKENS: Some observations on the stigmatic exudate in *Petunia hybrida*. Naturwissenschaften **52**, 625 (1965).
— — The physiology and biochemistry of the stigmatic exudate of *Petunia hybrida* Planta, (Berl). **71** 372—387 (1966).
LINSKENS, H.F.: Biochemistry of incompatibility. Genetics Today **3**, 629—636 (1964).
— Das Relief der Blattoberfläche. Planta (Berl.) **68**, 1—14 (1966).
—, and H. KROES, Interference microscopy of the pattern of leaf surfaces. Nature (Lond.) **210**, 968 (1966).
LUTZ, C.: Untersuchungen über reizbare Narben. Z. Bot. **3**, 342—347 (1911).
MAHESHWARI, P.: An introduction to the embryology of angiosperms. New York: McGraw-Hill Book Co. 1950.
MIKI-HIROSIGE, H.: A study of tropism of pollen tubes to the pistils VI. Mem. Coll. Sci., Univ. Kyoto B **29**, 75—80 (1962).
ROSENTHALER, L., u. F. KOLLE: Über die äußerste Schicht der Pflanzen. Ber. dtsch. pharm. Ges. **31**, 446—453 (1921).
STANLEY, R.G.: Physiology of pollen and pistil. Sci. Progr. **52**, 122—132 (1964).
STRASBURGER, E.: Über fremdartige Bestäubung. J. wiss. Bot. **17**, 50—98 (1886).
VASIL, I.K., and M.M. JOHRI: The style, stigma and pollen tube I. Phytomorphology **14**, 352—369 (1964).

Dr. R.N. KONAR
Reader, Department of Botany,
University of Delhi
Delhi — 7, India

Prof. Dr. H.F. LINSKENS
Botanisch Laboratorium, Universiteit
Driehuizerweg 200, Nijmegen, Nederland