GROWTH HORMONES IN POLLEN, STYLES AND OVARIES OF PETUNIA HYBRIDA AND OF LILIUM SPECIES

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SUMMARY

The presence of auxins has been demonstrated in extracts from pollen and styles of *Petunia hybrida*. The presence of gibberellins was demonstrated in extracts of pollen, styles and ovaries of *Petunia hybrida* as well as of three *Lilium* species. Pollen contains relatively high amounts of auxins as well as bibberellins compared with styles or ovaries. No significant effect was found of either compatible or incompatible pollinations in *Petunia* on the auxin and the gibberellin content of the pollinated styles, since the small increases of auxins and gibberellins found could be attributed to the auxins and gibberellins present in the pollen used for pollination.

The gibberellin content of pollen of *Lilium henryi* dropped sharply during the first hour of germination, indicating the importance of gibberellins for germination.

1. INTRODUCTION

Pollen of many species has been found to contain auxin, e.g. *Hibiscus* (LAIBACH 1932), *Curcurbita* (LAIBACH & MASCHMANN 1933), *Sequoia* (THIMANN 1934), maize and sunflower (LAIBACH & MEYER 1935), *Cyclamen*, *Antirrhinum*, *Nicotiana*, and *Datura* (MUIR 1947). A particularly high content is found in the pollinia of orchids (LAIBACH 1932, LAIBACH & MASCHMANN 1933). No extractable auxin could be demonstrated in the pollen of *Acacia*, *Anoda*, *Abutilon*, *Lilium*, *Hippeastrum*, and *Strelitzia* (LAIBACH & MASCHMANN 1933). In some instances a large part of the activity did not appear until after alkaline hydrolysis (*e.g.* in *Antirrhinum* and *Nicotiana*), so that its physiological significance is open to question.

LAIBACH & MASCHMANN (1933) demonstrated that the growing pollen tubes of orchids, prepared free from the stigma, also contain considerable amounts of auxin which they feel are not derived from the pollen, but are produced in the tubes themselves. However, application of auxin (IAA) was shown to inhibit pollen germination and pollen tube growth in almost all plant species tested (ADDICOTT, 1943; POHL, 1951). MUIR (1942) found that styles and ovaries of unpollinated flowers of *Nicotiana* contained very little growth hormone compared with those of pollinated flowers. Furthermore, he found that the quantity of growth hormone extracted from pollen was in all cases insufficient to account for the quantities present in styles or ovaries.

Thus probably *de novo* synthesis of auxins, induced by pollination, occurs in the styles and ovaries. However, another possibility is the release of bound

auxins present in the styles and ovaries through the production of enzymes induced by the growing pollen tube (LUND 1956a, b). Together, the data in the literature suggest that auxins are involved in the fertilization process although their exact role is as yet still unknown.

Still less is known about the involvement of another group of hormones during fertilization *e.g.* the gibberellins. KATO (1955) obtained stimulation of pollen germination as well as of pollen tube growth by adding gibberellic acid to the medium. A more extensive investigation by CHANDLER (1957) revealed that of 27 plant species tested only 10 species, including *Petunia* and *Lilium*, showed a stimulatory effect of gibberellic acid on germination and pollen tube growth.

To our knowledge gibberellin-like substances have so far been extracted from pollen, but not from styles or ovaries. MICHALSKI (1967) demonstrated unquestionably the presence of gibberellins in extracts from pollen of pine.

This paper describes the extraction of auxins and gibberellins from pollen, styles and ovaries of *Petunia hybrida* Hort., *Lilium henryi* Bak., *Lilium umbellatum* Hort., and *Lilium* cv. "Corsage". A few pollination experiments have been carried out also in order to get some additional information on the involvement of these growth hormones in the fertilization process.

2. MATERIALS AND METHODS

2.1. Plant material

Pollen, styles and in some cases also ovaries from flowers of *Petunia hybrida* Hort., clone W 166 K, *Lilium henryi* Bak., *Lilium umbellatum* Hort., and *Lilium* cv. "Corsage" were used for analysis. *Petunia* plants were grown in the greenhouse at approximately 25 °C and continuously illuminated with 400 Watt HPL (Philips) lamps.

The *Lilium* species used were grown either in the greenhouse or in the field depending on the time of the year. Unless stated otherwise, *Petunia* as well as *Lilium* flowers were collected one day before anthesis and their pollen, styles or ovaries removed which were immediately frozen at –20 °C for future analysis.

Petunia flowers were pollinated one day before anthesis after their anthers had been removed. For the pollination of 1000 styles approximately 0.4 g pollen was used. Pollinated as well as unpollinated flowers were kept in an incubator at 25 °C for 24 hrs before extraction.

Compatible cross pollinations of *Petunia* were carried out by bringing pollen of clone T_2U on styles from clone W 166 K. Incompatible self pollinations were carried out with clone W 166 K.

2.2. Extraction procedures

Auxins were extracted three times with ether at 4 °C for 1, 22 and 1 hr successively according to LARSEN (1955). After filtration and subsequent evaporation the residue was taken up in ethanol. Further purification was obtained by

partitioning the ether-extract with sodium bicarbonate. In this way the basic substances remain in the ether (basic fraction).

The aqueous phase is then adjusted to pH 3.0 with HCl and subsequently partitioned with ether (acid fraction). The remaining residue after extraction was in some cases hydrolysed with 0.1 N NaOH, the pH readjusted to pH 6 and again extracted with ether in order to demonstrate the presence of so-called bound auxins.

Gibberellins from pollen were extracted with methanol in a glass-homogenizer. Little alcoa-powder was added in order to crush the pollen more efficiently. Styles and ovaries were homogenized with cold methanol in a Sorvall omnimixer at maximum speed for two minutes. The mixing chamber was cooled with ice. In both cases the extracts were filtered and washed with methanol on a Buchner funnel under reduced pressure. The residue was once more extracted with methanol at room temperature for 30 minutes followed by extraction 3 times 24 hrs. The combined filtrates were evaporated to dryness in a Büchi flash evaporator. The residue after evaporation was taken up in 100 ml 0.1 M phosphate buffer pH 8.4 .The lipid material was removed by partitioning the buffer phase with petroleum ether (60–80 °C) until the petroleum ether remained clear. The petroleum ether was discarded. Subsequently the buffer phase was shaken with ethyl acetate in the same way and the ethyl acetate was also discarded. The buffer was then adjusted to pH 2.5 and again extracted 5 times with 100 ml ethyl acetate. The combined ethyl acetate fractions were evaporated to dryness and stored at –20°C for future chromatography. In some cases the buffer phase was hydrolysed with 0.04 N HCl at 60 °C for one hour in order to demonstrate the presence of so-called bound gibberellins. After hydrolysis the pH was readjusted to pH 2.5 and extracted 5 times with 100 ml ethyl acetate, evaporated to dryness and also stored for future chromatography.

2.3. Chromatography

The auxin extracts were analysed by ascending paper chromatography on 3 cm wide strips of Whatman paper no. 3 MM. The solvent system consisted of isopropanol/ammonia 25%/water (8:1:1, v/v). For further identification sometimes the following solvent systems were used; butanol/ethanol/water (4:1:1, v/v) and carbon tetrachloride/acetic acid (50:1, v/v).

Synthetic indole acetic acid (IAA) was always used as a reference and localized with the formaldehyde-HCl-water (1:2:2, v/v) reagent of PROCHAZKA *et al.* (1959). Gibberellin extracts were analysed by thin layer chromatography with silica gel G. The solvent system consisted of chloroform/ethyl acetate/acetic acid (60:40:5, v/v).

After development the chromatograms were divided into 10 equal fractions which were individually eluted, 3 times with 5 ml ethyl acetate and subsequently twice with 5 ml methanol. The combined eluate was evaporated to dryness and kept for bioassay.

2.4. Bioassays

To measure the auxin-like activity the *Avena*-mesocotyl assay according to NITSCH & NITSCH (1956; see also PILET 1961, pages 38–40) was used.

The fractions containing the gibberellin-like substances were taken up in 1 ml distilled water containing 0.05% Tween 20, 0.4 ml of which was used for the dwarf corn (d_5) assay. For a detailed description of the procedures see BARENDSE *c.s.* (1968), Of the remaining solution 0.5 ml has been used occasionally for the barley endosperm assay according to JONES & VARNER (1967) to verify the results obtained with the dwarf corn assay.

3. RESULTS

3.1. Extraction of auxin from pollen of *Petunia*

A quantity of 0.2 g pollen was extracted and purified following the procedures described under section 2, except that the basic and neutral fractions were not separated. The results are presented in *fig. 1.*

Two peaks of activity were observed with maxima at Rf 0.45–0.50 and Rf 0.85–0.90 respectively. The activity found at Rf 0.4–0.6 cochromatographed with indole acetic acid (IAA). When other solvent systems were used also two peaks of activity were found. The inhibition found at Rf 0.7–0.8 was observed in most experiments. The inhibition at Rf 0.15–0.30 was observed only occasionally. The total growth promoting activity was calculated from two identical experiments and found to be 0.15 μg equivalent IAA / g mature pollen average.

3.2. Extraction of auxins from styles of *Petunia*

An extract was made of 1000 styles weighing 7.6 g. In this experiment the basic and acidic fraction were separated and both chromatographed and subsequently tested with the bioassay. The results are presented in *fig. 2.*

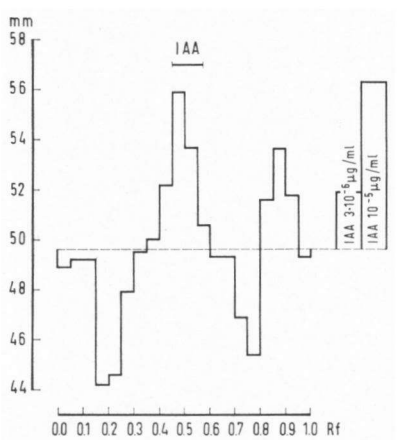

Fig. 1. Chromatogram of an auxin extract from pollen of *Petunia*, developed in isopropanol/ammonia 25%/water (8:1:1, v/v) and assayed with the *Avena*-mesocotyl assay.

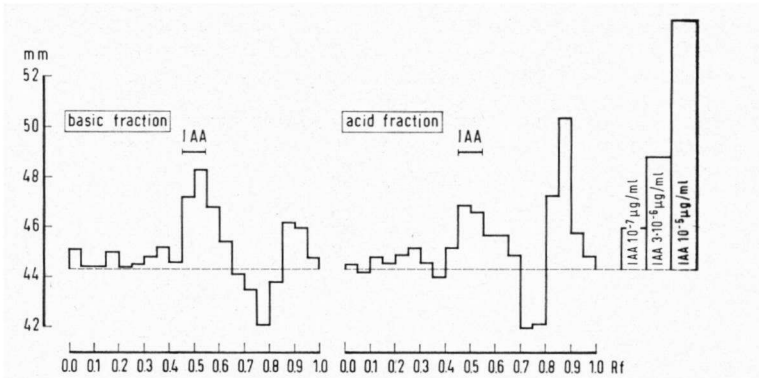

Fig. 2. Chromatograms of the basic and acid fractions of an auxin extract from styles of *Petunia*, developed in isopropanol/ammonia 25%/water (8:1:1, v/v) and assayed with the *Avena*-mesocotyl assay.

There appears to be only a quantitative difference between the basic and the acidic fraction. Two peaks of growth promoting activity were found with Rf values similar to those in pollen extracts. The inhibition observed at Rf 0.7–0.8 corresponds with the inhibition found in pollen. No inhibition was found at Rf 0.15–0.30. The total activity was calculated from three identical experiments: 0.06 µg equivalent IAA / g fresh weight.

3.3. Extraction of auxin from pollinated and unpollinated styles of *Petunia*

Compatible cross pollinations and incompatible self pollinations were carried out. After pollination both pollinated and unpollinated styles were kept in an incubator for 24 hrs at 25° before extraction. The basic and acid fractions were not separated.

It was found that extracts of styles from experiments carried out in the summer contained much less growth promoting activity than those from experiments done in the fall. Extracts from material collected in the fall showed also less inhibition than similar extracts from summer material. For this reason only the results of experiments done in the fall are presented.

The total activities per experiment were calculated from the chromatograms with the aid of the IAA standards used. The result are compiled in *table 1*.

For the pollination of one thousand styles approximately 0.4 g pollen is used, which contained approx. 0.06 µg equivalent IAA.

Thus in the case of cross pollination the increase in auxin merely represents the sum of auxin present in unpollinated styles and the pollen used for pollination i.e. $0.04 + 0.06 = 0.10$ µg equivalent IAA which is not significantly different from the 0.09 µg equivalent IAA found in the cross pollinated styles. Apparently there is no effect of pollination on the auxin production of styles

Table 1. Extraction of auxin from pollinated and unpollinated styles of *Petunia*. Per experiment 1000 styles i.e. 7.6 g were extracted.
The activities are expressed as μg equivalent IAA/1000 styles.

experiment	unpollinated	cross pollinated	self pollinated
1	0.03	0.07	0.05
2	0.04	0.08	– —
3	0.05	0.11	0.08
4	0.05	0.11	0.06
average	0.04	0.09	0.06

in the case of the compatible cross pollination. The auxin activity found in self pollinated styles is lower (0.06 μg equivalent IAA) than the sum of the amounts of auxin found in unpollinated styles and pollen. This is hard to explain unless one assumes that auxin destruction takes place in either pollen or styles in the case of an incompatible self pollination.

3.4. Extracts of auxins tested on the dwarf corn and lettuce hypocotyl bioassay

Since the *Avena*-mesocotyl assay, which was used in most experiments, is not specific for auxins but is also affected by gibberellins, a number of style extracts were tested on the dwarf corn and lettuce hypocotyl assay (FRANKLAND & WAREING 1960) which are specific for gibberellins. None of the extracts have revealed any activity in either assay. Thus it is reasonably safe to assume that the activities found in the above described experiments are indeed due to auxins and not to gibberellins.

3.5. Extraction of bound auxins in *Petunia*

After the usual extraction the residue was hydrolysed with 0.1 N NaOH and again extracted with ether in order to establish whether so-called bound auxins

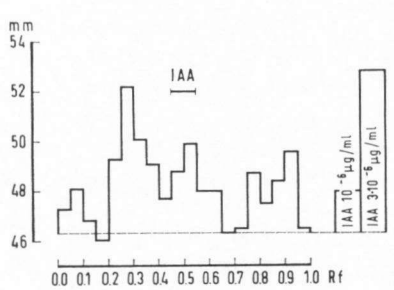

Fig. 3. Chromatogram of the "bound" auxin fraction of *Petunia* after hydrolysis, developed in isopropanol/ammonia 25%/water (8:1:1, v/v) and assayed with the *Avena*-mesocotyl assay.

Table 2. Extraction of gibberellins from pollen of *Petunia*. The total growth promoting activity per experiment is expressed as μg equivalent GA_3/g fresh weight.

experiment	stage	quantity extracted	μg equivalent GA_3/g fresh weight
1	immature pollen	15.3 g	0.007
2	immature pollen	5.0 g	0.004
1	mature pollen	15.3 g	0.057
2	mature pollen	5.1 g	0.056

are present in extracts. The extracts were chromatographed and assayed in the usual manner. The results of such an experiment are presented in *fig. 3.*

One notices that the two peaks of activity found in the basic or acidic fractions are again present (compare with the *figs. 1* and *2*). However, the greatest activity is found at Rf 0.2–0.4 which most likely represents an intermediate between the bound forms of auxins and the free auxin. The total activity found in this experiment was 0.2 μg/1000 styles i.e. 0.03 μg equivalent IAA / g fresh weight, which is a relatively high activity compared with the activities found in the basic or acidic fractions.

One cannot, however, interpret these results easily, since it is still unknown whether the so-called bound auxins have any physiological significance at all. For this reason the above described experiments were confined to measuring the free auxin content only.

3.6. Extraction of gibberellins from pollen of *Petunia*

Immature pollen *i.e.* pollen 24 hrs before opening of the anthers as well as mature pollen were extracted together with their theca, since it is difficult to separate immature pollen from their surrounding tissue in *Petunia*. The extracts were purified and subsequently chromatographed, followed by the dwarf corn bioassay. The chromatograms revealed only one peak of activity at Rf 0.2–0.4. The total growth promoting activity per experiment was calculated from the chromatograms with the aid of the gibberellic acid (GA_3) standards used, and expressed as μg equivalent GA_3/g fresh weight. The results of two experiments are compiled in *table 2.*

It is obvious that mature pollen of *Petunia* contains considerably more gibberellins than immature pollen. The possibility that immature pollen contains so-called bound gibberellins which are released during maturation has also been tested. The aqueous fractions which may contain the so-called bound gibberellins (BARENDSE *c.s.* 1968) were hydrolysed and subsequently again extracted and purified in the usual manner. However, no activity was detected in such extracts from either immature or mature pollen.

Thus the increase of gibberellins during maturation is most likely due to *de novo* synthesis.

3.7. Extraction of gibberellins from pollinated and unpollinated styles of *Petunia*

Only incompatible self pollinations were carried out. Chromatography of the extracts from pollinated as well as from unpollinated styles revealed again only one peak of activity at Rf 0.2–0.4. In three experiments approximately 2000 unpollinated styles weighing 16.3 gram fresh weight were extracted. The activities measured in these experiments were 0.012 μg, 0.024 μg, and 0.051 μg equivalent GA_3/16.3 g fresh weight i.e. 0.007 μg, 0.015 μg, and 0.031 μg equivalent GA_3/g fresh weight respectively. In one experiment, carried out concurrently with the third of the above experiments, 2000 pollinated styles were extracted and the activity determined, which amounted to 0.089 μg equivalent GA_3/2000 styles. For the pollination of 2000 styles approximately 0.8 g pollen was used, containing 0.045 μg equivalent GA_3. The higher amount of gibberellin found in pollinated styles can be attributed to the gibberellin present in pollen, since the sum of gibberellins present in the pollen used for pollination and in the unpollinated styles does not differ significantly from the amount of gibberellin found in the pollinated styles i.e. 0.051 μg + 0.045 μg = 0.096 μg equivalent GA_3 and 0.089 g equivalent GA_3 respectively.

3.8. Extraction of gibberellins from ovaries of *Petunia*

Ovaries were collected from pollinated as well as unpollinated flowers and subsequently extracted.

Chromatography again revealed only one peak of activity at Rf 0.2–0.4. The total activity of two experiments, in which twice 16.3 g ovaries from unpollinated flowers and twice 16.1 g ovaries from pollinated flowers were extracted, was calculated and expressed as μg equivalent GA_3/g fresh weight. The results were as follows: 0.003 μg and 0.006 μg equivalent GA_3/g fresh weight, and 0.003 μg and 0.003 μg equivalent GA_3/g fresh weight for ovaria from unpollinated and pollinated flowers respectively. Compared with unpollinated styles, the ovaries appear to contain a fairly high amount of gibberellings. However, there appears to be no effect of pollination on the gibberellin content of ovaries.

3.9. Extraction of gibberellins from pollen of *Lilium*

Lilium has been included in this investigation, since it possesses some advantages in comparison with *Petunia*. For instance, immature pollen of *Lilium* can be separated easily from the loculus and thus can be extracted without the theca; secondly less flowers are necessary to obtain sufficient material for extraction. Preliminary experiments had already shown that gibberellins are present in pollen of *Lilium*. In the following experiment immature, mature, germinating pollen of *Lilium henryi* as well as the germinating medium were extracted. Chromatography of the extract revealed two peaks of activity with maxima at Rf 0.2–0.3 and Rf 0.5–0.7 after the dwarf corn as well as after the barley endosperm assay. A representative chromatogram is shown in *fig. 4*.

The activities were calculated and are presented in *table 3*. Each quantity

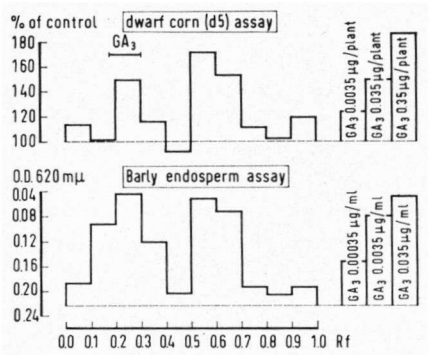

Fig. 4. Chromatograms of a gibberellin extract from pollen of *Lilium henryi*, developed in chloroform/ethyl acetate/acetic acid (60:40:5, v/v) and assayed both with the dwarf corn (d_5) and the barley endosperm assay.

of activity represents the average of two experiments. The so-called immature pollen was collected from two bud sizes.

Pollen collected from the young buds contains somewhat less gibberellins than pollen from the mature buds i.e. one day before anthesis.

Mature pollen collected from flowers which have just opened appears to contain only half the amount of gibberellins found in pollen from mature buds. Thus there seems to be a great loss of gibberellins during the last 24 hrs of maturation. When the pollen is germinated a great loss of gibberellins occurs during the first hr of germination, but the gibberellin content decreases only slightly thereafter. Furthermore, some excretion of gibberellins into the medium is observed. It is striking that the sum of the amount of gibberellin present in the germinating pollen and the amount excreted in the medium remains approximately constant. These results suggest that gibberellins are used particularly during germination, but hardly for the further growth of the pollen tube.

Table 3. Extraction of gibberellins from pollen of *Lilium henryi*. The total activities were calculated from the chromatograms and expressed as µg equivalent GA_3/g fresh weight. To indicate the stage from which immature pollen was collected the length of the buds is given in parentheses. All data represent the average of two experiments. The quantity of pollen extracted varied from 0.9 g to 1.2 g.

material	activity in pollen	activity in medium	germination (%)
immature pollen (buds, 30–40 mm)	1.79		
immature pollen (buds, 45–55 mm)	2.19		
mature pollen (anthesis, buds >65 mm)	1.08		
mature pollen:			
germinated for 1 hr	0.22	0.02	48
germinated for 2.5 hrs	0.24	0.03	65
germinated for 3.5 hrs	0.17	0.07	70
germinated for 6 hrs	0.05	0.07	–

3.10 Extraction of gibberellins from styles of *Lilium*

Extracts were made from styles of *Lilium henryi*, *Lilium umbellatum*, and *Lilium* cv. "Corsage". The extracts were purified and chromatographed in the way described in section 2. After assaying on dwarf corn the total activities were calculated. The chromatograms showed the same two peaks of activity described for pollen (see *fig. 4*) of the three *Lilium* species.

The results were as follows:

Lilium henryi; 17.1 g extracted; 0.005 μg equivalent GA_3/g fresh weight

Lilium umbellatum; 18.4 g extracted; 0.002 μg equivalent GA_3/g fresh weight

Lilium cv. "Corsage"; 12.3 g extracted; 0.002 μg equivalent GA_3/g fresh weight.

3.11. Extraction of gibberellins from ovaries of *Lilium*

Extracts were made from ovaria of *Lilium henryi*, *Lilium umbellatum* and *Lilium* cv. "Corsage". The extracts were purified, chromatographed and subsequently tested on the dwarf corn bioassay as described in section 2. The chromatograms showed again two peaks of activity in all three *Lilium* species (see *fig. 4*).

The total activities were:

Lilium henryi; 20.8 g extracted; 0.002 μg equivalent GA_3/g fresh weight.

Lilium umbellatum; 21.1 g extracted; no activity detected.

Lilium cv. "Corsage"; 11.1 g extracted; 0.005 μg equivalent GA_3/g fresh weight.

4. DISCUSSION

Our investigation mainly demonstrates the presence of auxins in pollen and styles of *Petunia hybrida* and of gibberellins in pollen, styles and ovaries of *Petunia hybrida* as well as of three *Lilium* species. No effect was found of either compatible or incompatible pollination in *Petunia* on the activity of auxins and/or gibberellins. The consumption theory ot incompatibility (STRAUB, 1947, 1948) can therefore not be simply attributed to the consumption of growth hormones of the auxin and gibberellin type.

On the other hand gibberellins were shown to be of importance for the initial stages of germination of pollen of *Lilium henryi* which is in accordance with the findings in the literature (CHANDLER 1957). Our work confirms the evidence that at least two growth hormones may occur in pollen, styles and ovaries. However, to our knowledge the possible presence of other hormones like for instance cytokinins has not yet been investigated.

We know for instance that auxins, gibberellins as well as cytokinins promote pollen tube growth in *Lilium longiflorum* (BOSE 1959), but also that cytokinins as well as indole acetic acid diminish the promoting effect of gibberellin when applied together. On the other hand, applied auxin (IAA) was found to inhibit pollen germination and pollen tube growth in almost all plant species tested by ADDICOTT (1943) and POHL (1951).

Generally, in connection with experiments like the ones described here, one should never lose sight of the fact that the different groups of growth hormones are continually interacting with one another. So, viewed in this light, it is unwarranted to propose a specific role in fertilization for one of these hormones in particular as has previously been done for auxin. The more so, because the content in auxin and gibberellin in styles and ovaries after pollination is never greater than the sum of the contents of these substances in pollen and styles and ovaries of the non-pollinated flowers.

It is known that both auxins and gibberellins (VARGA 1963) may induce or promote development of seedless (parthenocarpic) fruits. So it seems conceivable that the growth substances found to be present in the pollen are of importance for pollen germination and/or pollen tube growth, whereas those present in the ovaries will promote fruit growth at a later stage.

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