Acta Bot. Neerl. 21 (1): 1-16. 1972

CHANGES IN THE AUTOFLUORESCENCE OF THE POLLEN WALL DURING MICROSPOROGENESIS AND CHEMICAL TREATMENTS

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SUMMARY

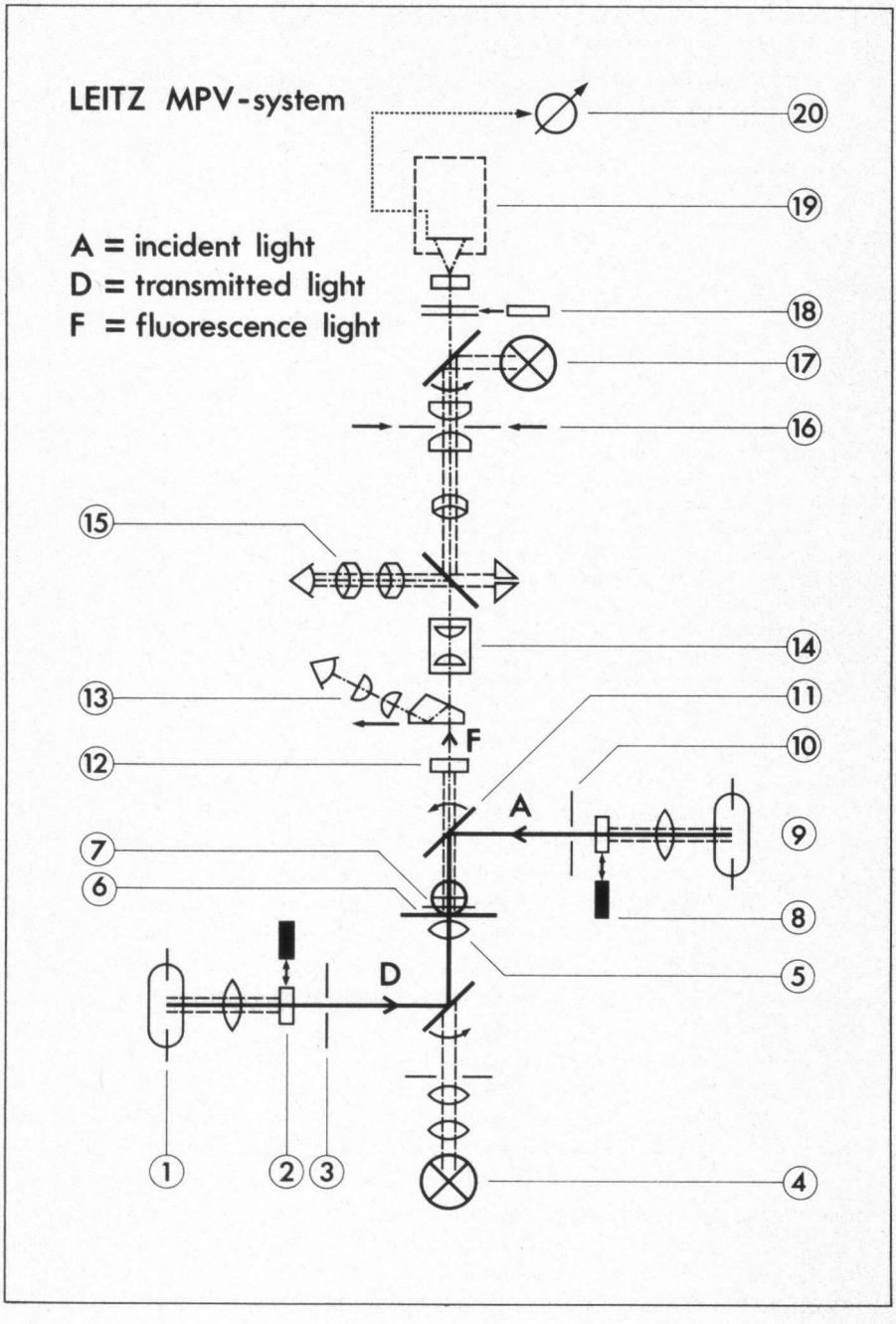
Measurements were made of the spectral maximum, the intensity, and fading of the autofluorescence spectrum during microsporogenesis and of pollen after some chemical treatments in *Gasteria*, *Pinus*, *Physostegia*, and some other plants.

Mainly the sporopollenin of the sexine and the footlayer of the pollen wall showed autofluorescence which appeared to be specific for each pollen species. The cytoplasm showed sometimes autofluorescence during the initiation of the pollen wall formation. The spectral maximum increased in wave-length and the percentage of fading decreased before the formation of the intine, but also when the intine of the pollen wall is removed.

The exine of the pollen wall of *Pinus* disappeared gradually after treatment with a mixture of acetic acid and hydrogen peroxyde. The pollen wall of *Gasteria* showed a higher resistance to the chemical treatments compared with *Pinus* pollen.

1. INTRODUCTION

In plants many cytoplasmic components, cell wall compounds and pollen grains show UV-fluorescence (GOODWIN 1953). BERGER (1934) and ASBECK (1955) described the colour of fluorescence of various pollen species. Their fluorescence colour and spectrum appeared to depend on type and geological age, due to various substances in the pollen wall (VAN GIJZEL 1967, 1971a). Changes in fluorescence spectrum during pollen development of *Pinus sylvestris* and *Gasteria verrucosa* have been observed (WILLEMSE 1971a). In the exine of the pollen of *Ambrosia trifida* a difference could be distinguished between the endexine and extexine by measuring the UV absorption spectra (SOUTHWORTH 1969). WATERKEYN & BIENFAIT (1971) reported that the footlayer and the nexine II show a less intensive secondary fluorescence than the sexine of the developing pollen wall of *Lilium* sp. and of *Ipomoea*. These differences may be due to the changes in the sporopollenin content or to the appearance of other products in the exine. The exine is chemically composed of a substance named sporopollenin, an oxidative polymer of carotenoids and/or carotenoid esters (BROOKS & SHAW 1968; SHAW 1971). The constitution of the microspore wall of *Lycopodium clavatum* and *Pinus sylvestris* was more extensively investigated by SHAW & YEADON (1966). In both plants the microspore walls consist of 10–15% cellulose, 10% xylan, 10–15% of a lignin-like fraction and a lipid fraction of 55–65%. In the pollen wall of *Pinus sylvestris* also callose is present (MARTENS *et al.* 1967).


LEITZ MPV-system
A = incident light
D = transmitted light
F = fluorescence light
20
19
18
17
16
15
14
13
11
10
12
F
A
9
7
6
8
5
D
1
2
3
4

More details on the chemical composition of the lipid components of the microspore wall of *Lycopodium clavatum* and *Pinus pinaster* are given by DUNGWORTH *et al.* (1971).

After chemical treatments of fresh pollen, the autofluorescence may change in colour, which is caused mainly by changes in the composition of the pollen wall (VAN GIJZEL 1971a; WILLEMSE 1971a). Subject of this study are the changes in fluorescence during pollen development and under influence of chemical treatments of pollen combined with the morphological changes in the pollen wall.

2. MATERIAL AND METHODS

An investigation of the changes in the autofluorescence, in the following called fluorescence, was made during the pollen development of *Gasteria verrucosa* (Mill.) Haw. (Liliaceae), *Physostegia virginiana* (L.) Benth. (Lamiaceae) and a number of other plants (see Diagram I), of which some developmental stages were measured. All plants originated from the Botanical Garden of the University of Nijmegen. For the various chemical treatments mainly pollen of *Pinus pinaster* Ait., stored at –10 °C, and fresh *Gasteria* pollen were used.

The fluorescence microphotometry was carried out with the Leitz MPV-system mounted on an Orthoplan microscope, see figure.

The main excitation wave-length is 365 nm, which was obtained by means of a stabilized high pressure HBO 100 lamp and the UG 1 (4 mm) and BG 38 excitation filters. At transmitted illumination a dark field oil condensor was used with glycerin-phosphate buffer pH 7.2, 1:1 as oil immersion. Furthermore, an objective lens Pl 40/0,65 and a barrier filter K 430 were used. The diameter of

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Leitz MPV-system after VAN GIJZEL (1971b)
1: high pressure Hg-lamp HBO 100
2: excitation filters UG 1 (4 mm) and BG 38 (4 mm)
3: diaphragm
4: Tungsten lamp 6V 5A for normal light
5: microscope condensor
6: object stage
7: objective
8: excitation filters UG 1 (4 mm) and BG 38 (4 mm)
9: high pressure Hg-lamp HBO 100
10: diaphragm
11: dichroitic mirror of illuminator after PLOEM
12: barrier filter (K 430)
13: binocular tube
14: ocular in monocular tube
15: observation ocular for measuring diaphragm
16: variable measuring diaphragm
17: illumination of measuring diaphragm
18: filter with monochromatic stages
19: photocell with photomultiplier
20: recorder or potentiometer

the variable measuring diaphragm was fixed for all measurements at 100 scale units; for pollen grains smaller than this diaphragm a correction has been applied. All specimens were measured at about 23 °C in a 0.05 ml drop of distilled water (pH 5.5) under a cover glass. For a more detailed description of the methods of fluorescence microphotometry the reader may be referred to VAN GIJZEL (1967, 1971b).

The spectral maximum and the maximal intensity in arbitrary units were recorded after 4–8 seconds (average 6″) and 27–33 seconds (average 30″). The percentage of fading or increase in intensity during 30″ was calculated, equaling the value of the intensity after 6″ = 100%. In all cases the value of the intensity was calculated for 1.80 KV. The mean value and standard deviation of at least three measurements were determined. In all measurements the intensity of the background was less than 4%. The error in the measured wave-length amounts to ± 0.5 nm.

Some chemical treatments represent partly a modification of the methods used by SHAW & YEADON (1966). After homogenization all steps were boiled under reflux. After cleaning in ether, ethanol 96% and water, the pollen mass was divided into several samples. Each of them was treated either with 6% KOH, or with ethanolamine or with acetic anhydride and concentrated H_2SO_4 (9:1), or with acetic acid (98%) and H_2O_2 (30%) 1:1. After each treatment one or more washings with water were carried out. After the treatment with KOH and the washing with water the pollen samples were cleaned with ethanol 96% and ether. The medium in which the pollen was treated was also tested for fluorescence. The pollen grains were measured while mounted in this medium. The wings of pine pollen were measured separately.

For electron microscopy the pollen grains were fixed in buffered 1% OsO_4 for 30′ at pH 7.2. After washing with water the pollen was stained with uranyl acetate in 70% ethanol for 30′. After dehydration and embedding in Epon 812 sections were cut using a Porter Blumm ultramicrotome. The sections were examined by means of a Philips electron microscope EM 300 at 60 KV. For light microscopy pectic substances in the pollen were stained with ruthenium red (1:5000) in water. Callose was stained with a 0.005% solution of aniline blue in 50% ethanol.

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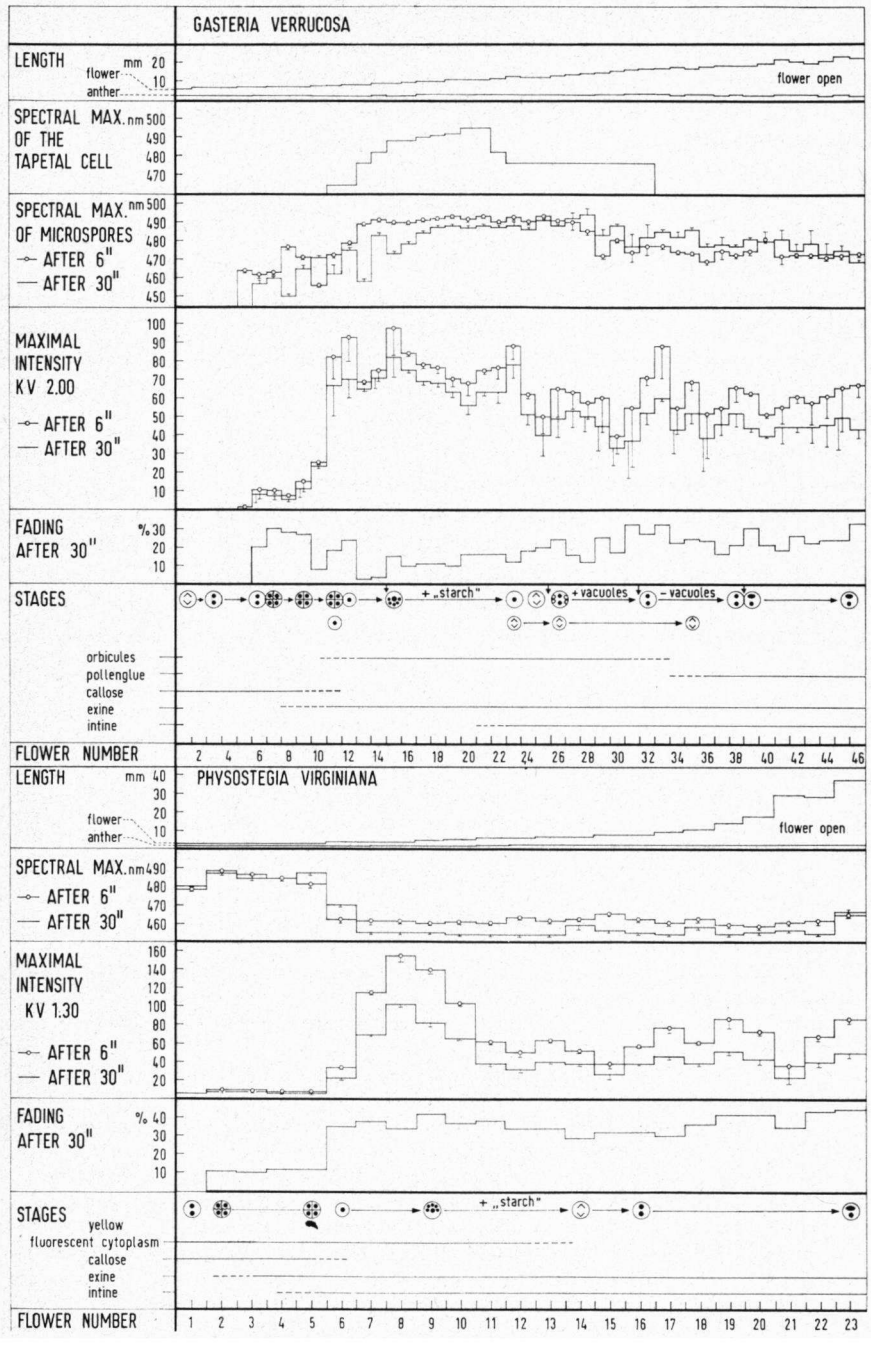
Diagram 1. Changes in fluorescence during microsporogenesis of *Gasteria* and *Physostegia*. The mean values with standard deviations are given of the spectral maximum after 6″ and 30″, of the intensity after 6″ and 30″ and the percentage of fading during 30″ for the different stages of microsporogenesis. The standard deviation is drawn as a line only on one side of the mean value point. The fluorescence of the tapetal cell of *Gasteria* has also been measured. Because of the irregularity of the cell only the mean value of the spectral maximum is noted. The appearance of the exine, intine, pollen glue, orbicules and also the fluorescence of the cytoplasm and the disappearance of the callose wall are shown by a line in relation to the stages of development. The increase in length of the flower and the anther are also presented. The length of the inflorescence of *Gasteria* is 14.9 cm, of *Physostegia* 14.5 cm; of the latter the flowers of one row only were measured.


GASTERIA VERRUCOSA
LENGTH
mm 20
flower
anther
10
flower open
SPECTRAL MAX. OF THE TAPETAL CELL
nm 500
490
480
470
SPECTRAL MAX. OF MICROSPORES
AFTER 6"
AFTER 30"
nm 500
490
480
470
460
450
MAXIMAL INTENSITY KV 2.00
AFTER 6"
AFTER 30"
100
90
80
70
60
50
40
30
20
10
FADING AFTER 30"
% 30
20
10
STAGES
+ „starch"
+ vacuoles
- vacuoles
orbicules
pollenglue
callose
exine
intine
FLOWER NUMBER
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46
PHYSOSTEGIA VIRGINIANA
LENGTH
mm 40
30
20
flower
anther
10
flower open
SPECTRAL MAX.
AFTER 6"
AFTER 30"
nm 490
480
470
460
MAXIMAL INTENSITY KV 1.30
AFTER 6"
AFTER 30"
160
140
120
100
80
60
40
20
FADING AFTER 30"
% 40
30
20
10
STAGES
yellow fluorescent cytoplasm
+ „starch"
callose
exine
intine
FLOWER NUMBER
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

The infrared spectrum of the solution of acetic acid with H_2O_2, in which the pollen was treated during different periods, was measured at a frequency of 4000–800 cm^{-1} with a Perkin-Elmer 257 recording spectrophotometer for potassium bromide discs. At the start of the measurements some KBr was added to the solution.

3. RESULTS

3.1. Changes in fluorescence during microsporogenesis

During microsporogenesis the fluorescence changes in the pollen, which has a broad fluorescence spectrum with one maximum. The racemose inflorescence of *Gasteria* and *Physostegia* permits the easy collecting of many different stages of development in a normal sequence. The results of the changes in fluorescence and in morphology are compiled in *diagram 1*.

A comparison between *Gasteria* and *Physostegia* reveals many remarkable differences in fluorescence during microsporogenesis.

The spectral maximum in *Gasteria* lies between 455–470 nm in cells in the tetrad stage, increases in wave-length after the break-out of the microspore to 480–490 nm, and decreases in wave-length around 470 nm when the intine formation starts. A shift in the spectral maximum occurs up to the beginning of the intine formation (flower 26) in the direction of 450 nm, thereafter in the

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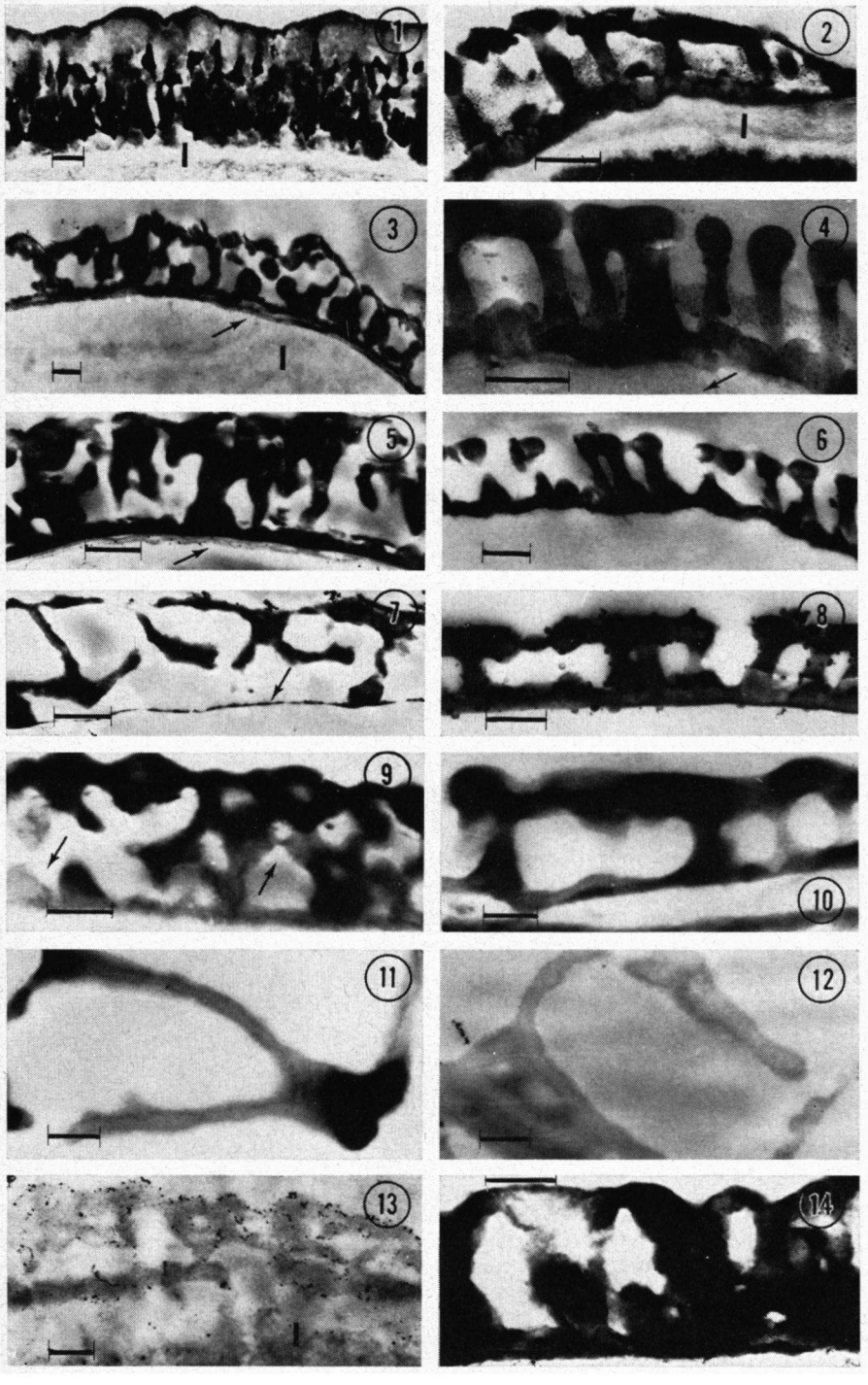
Fig. 1. Pollen wall of *Physostegia*. Exine with electron dense material in less electron dense material. I = intine, × 10,000.
Fig. 2. Pollen wall of *Gasteria*. Note the material between the bacules. I = intine, × 20,000.
Fig. 3. Pollen wall of *Pinus* after treatment with ether, ethanol and water. Nexine II layered (arrow). I = intine, × 8,500.
Fig. 4. *Gasteria:* pollen wall after treatment with ether, ethanol and water. Note the affected intine (arrow), × 25,400.
Fig. 5. *Pinus:* pollen wall after treatment with KOH and washing with water. The nexine II is less electron dense (arrow), × 16,200.
Fig. 6. *Gasteria*: pollen wall after treatment with KOH and washing with water. The material between the bacula and the intine is absent, × 14,800.
Fig. 7. *Pinus*: pollen wall after treatment with ethanolamine. Note the footlayer (arrow) and disappearing bacula and tectum, × 18,500.
Fig. 8. *Gasteria*: pollen wall after treatment with KOH and washing with water, ethanol and ether, × 18,000.
Fig. 9. Pollen wall of fresh *Pinus* pollen after treatment in acetic acid – H_2O_2 during 5′. The electron dense material starts to disappear (arrow), × 19,700.
Fig. 10. *Gasteria*: pollen wall after treatment in acetic acid – H_2O_2 during 30′, × 17,000.
Fig. 11. Pollen wall of fresh *Pinus* pollen after treatment in acetic acid – H_2O_2 during 5′. Note the disappearing electron dense material in the sexine, × 14,500.
Fig. 12. Pollen wall of fresh *Pinus* pollen after treatment in acetic acid – H_2O_2 during 10′. The sexine lacks electron dense material, × 14,000.
Fig. 13. Pollen wall of *Pinus* pollen treated in acetic acid – H_2O_2. The pollen wall, without electron dense material, is setting free from the intine (I), × 13,600.
Fig. 14. Pollen wall of *Pinus* treated in acetic anhydride – H_2SO_4, × 20,500.
The line on the figure represents a length of 0.5 μm.


1
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14

direction of 500 nm. When the pollen is exposed for a longer time to daylight, the shift is in the direction of 450 nm (flower 46 and 23 of *Physostegia*). The mean value of the spectral maximum of the tapetal cell agrees with the mean value found in the pollen. When the intine formation starts, the spectral maximum of the tapetal cell decreases in wave-length to around 475 nm. Particularly the orbicules show fluorescence.

The intensity of fluorescence in the tetrad stage is low, but increases quickly after the break-out of the microspore. The mean value of the intensity decreases when the formation of the intine starts (flower 24), while the standard deviation shows more fluctuations. The cell stages are less synchronized. During the increase of the intensity after the break-out of the microspores, the fading percentage is low but increases slowly up to around 25%.

The intine of *Gasteria*, which lies mainly in the area of the colpus, gives a positive reaction for the presence of callose and pectine.

The cytoplasm of *Physostegia* microspore cells has a yellow fluorescent colour, which after the break-out of the microspores diminishes and finally disappears. As shown in *fig. 1*, the pollen wall contains less osmiophilic material and has an irregular shape.

During the tetrad stage the spectral maximum is about 480 nm, after break-out of the microspores it decreases in wave-length to 460 nm. The shift in the spectral maximum is mainly in the direction of 450 nm. After the tetrad stage the intensity increases. When the cytoplasm shows no more fluorescence the intensity decreases. The fading is slow during the tetrad stage. In all measurements the standard deviation is low. The intensity is much higher in *Physostegia* than in *Gasteria*.

The pollen of *Physostegia* is tricolpate; the colpi areas show less fluorescence and no reaction to the aniline blue staining for callose. The areas around the colpi have an intensive fluorescence and contain callose. The whole pollen wall reacts positively to the ruthenium red stain for pectine.

Changes in fluorescence during microsporogenesis have been investigated in some other plants as well. The whole tetrads, young microspores just delivered from the tetrads, and the ripe pollen were measured. The results are given in *diagram 2*.

Every type of pollen showed its very specific pollen wall fluorescence. During the tetrad stage the fluorescence in the pollen wall is caused by the presence of the callosic cell wall and the exine. During the young microspore stage the thickening of the exine and probably the formation of the intine give the

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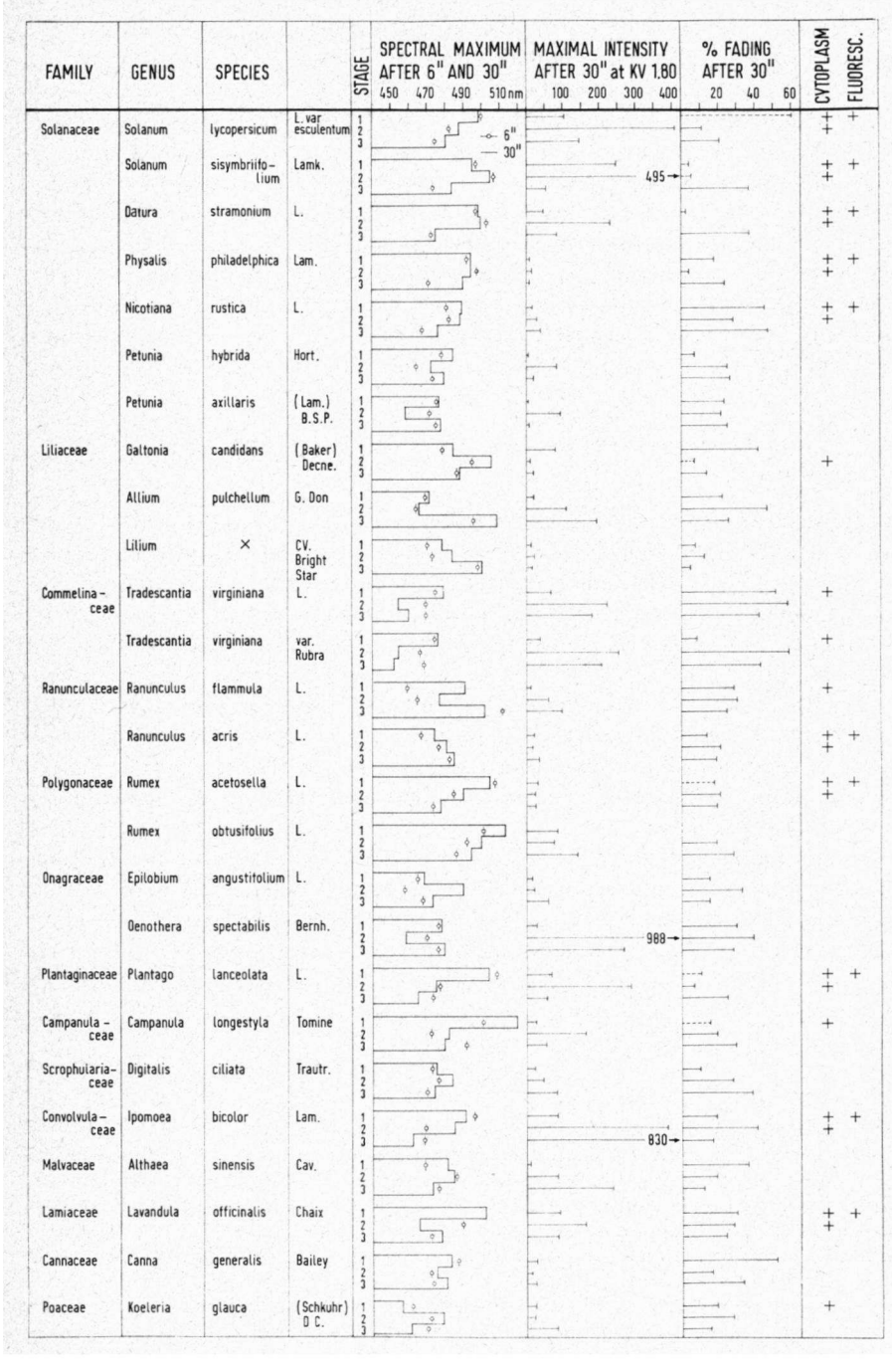
Diagram 2. Changes in fluorescence during microsporogenesis of some plants.
The mean value is given of the spectral maximum after 6″ and 30″, of the intensity and of the percentage of fading or the percentage of increase in intensity (dotted line) after 30″ during the tetrad stage (1), during the young microspore stage (2) and of the ripe pollen (3). The standard deviations of the mean values were very low. The intensity has been corrected for 1.80 KV. The fluorescence of the cytoplasm at the different stages is also presented.


FAMILY
GENUS
SPECIES
STAGE
SPECTRAL MAXIMUM AFTER 6" AND 30"
450 470 490 510 nm
6"
30"
MAXIMAL INTENSITY AFTER 30" at KV 1.80
100 200 300 400
495
988
830
% FADING AFTER 30"
20 40 60
CYTOPLASM
FLUORESC.
Solanaceae Solanum lycopersicum L. var. esculentum
Solanum sisymbriifolium Lamk.
Datura stramonium L.
Physalis philadelphica Lam.
Nicotiana rustica L.
Petunia hybrida Hort.
Petunia axillaris (Lam.) B.S.P.
Liliaceae Galtonia candidans (Baker) Decne.
Allium pulchellum G. Don
Lilium × CV. Bright Star
Commelinaceae Tradescantia virginiana L.
Tradescantia virginiana var. Rubra
Ranunculaceae Ranunculus flammula L.
Ranunculus acris L.
Polygonaceae Rumex acetosella L.
Rumex obtusifolius L.
Onagraceae Epilobium angustifolium L.
Oenothera spectabilis Bernh.
Plantaginaceae Plantago lanceolata L.
Campanulaceae Campanula longestyla Tomine
Scrophulariaceae Digitalis ciliata Trautr.
Convolvulaceae Ipomoea bicolor Lam.
Malvaceae Althaea sinensis Cav.
Lamiaceae Lavandula officinalis Chaix
Cannaceae Canna generalis Bailey
Poaceae Koeleria glauca (Schkuhr) D C.

fluorescence. In the ripe pollen the probably pigmented pollen wall with or without pollen glue causes the fluorescence.

In general very few similarities are found between the investigated plants, even within the same family. Two species of *Tradescantia*, *Rumex*, and *Petunia* each show some similarity in the spectral maximum, intensity and fading. No relations were observed between fluorescence and the morphology or pigmentation of the pollen. No distinct correlations were found between the different values of the spectral maximum, intensity and fading. A distinction can be made between developing pollen with and without a fluorescent cytoplasm during the tetrad and the young microspore stage. In general an increase in intensity takes place after the tetrad stage. The shift in the spectral maximum is in most of the investigated pollen in the direction of 550 nm.

3.2. Chemical treatments and changes in the morphology of ripe pollen

Some steps in pollen wall formation could be simulated by chemical treatment as done for ripe pollen of *Pinus pinaster* and *Gasteria verrucosa*. Also the change in the pollen wall could be investigated. Both fluorescence and the morphology of the pollen wall were studied during the following treatments. The results of the changes in fluorescence during the different chemical treatments are given in *diagram 3*.

The fluorescence of the pollen wall of *Pinus* and *Gasteria* after some chemical treatments changes. Between the wings, consisting of sexine only, and the pollen body of *Pinus* few differences occur. It seems that the fluorescence in *Pinus* is caused mainly by the sexine. Pollen of neither *Gasteria* nor *Pinus* show fluorescence of the cytoplasm.

After washing with ether, ethanol and water, the spectral maximum decreases in wave-length, the intensity as well as the fading percentage increase. This occurs to a greater extent in *Gasteria* than in *Pinus*. The morphology of the pollen wall after these treatments shows fewer changes. In *Gasteria* the intine shows a fibrillar structure compared with the normal wall (*fig. 2, 4*). In the nexine II of *Pinus* some layers are clearly visible (*fig. 3*). Both pollen walls react positively to the ruthenium red stain.

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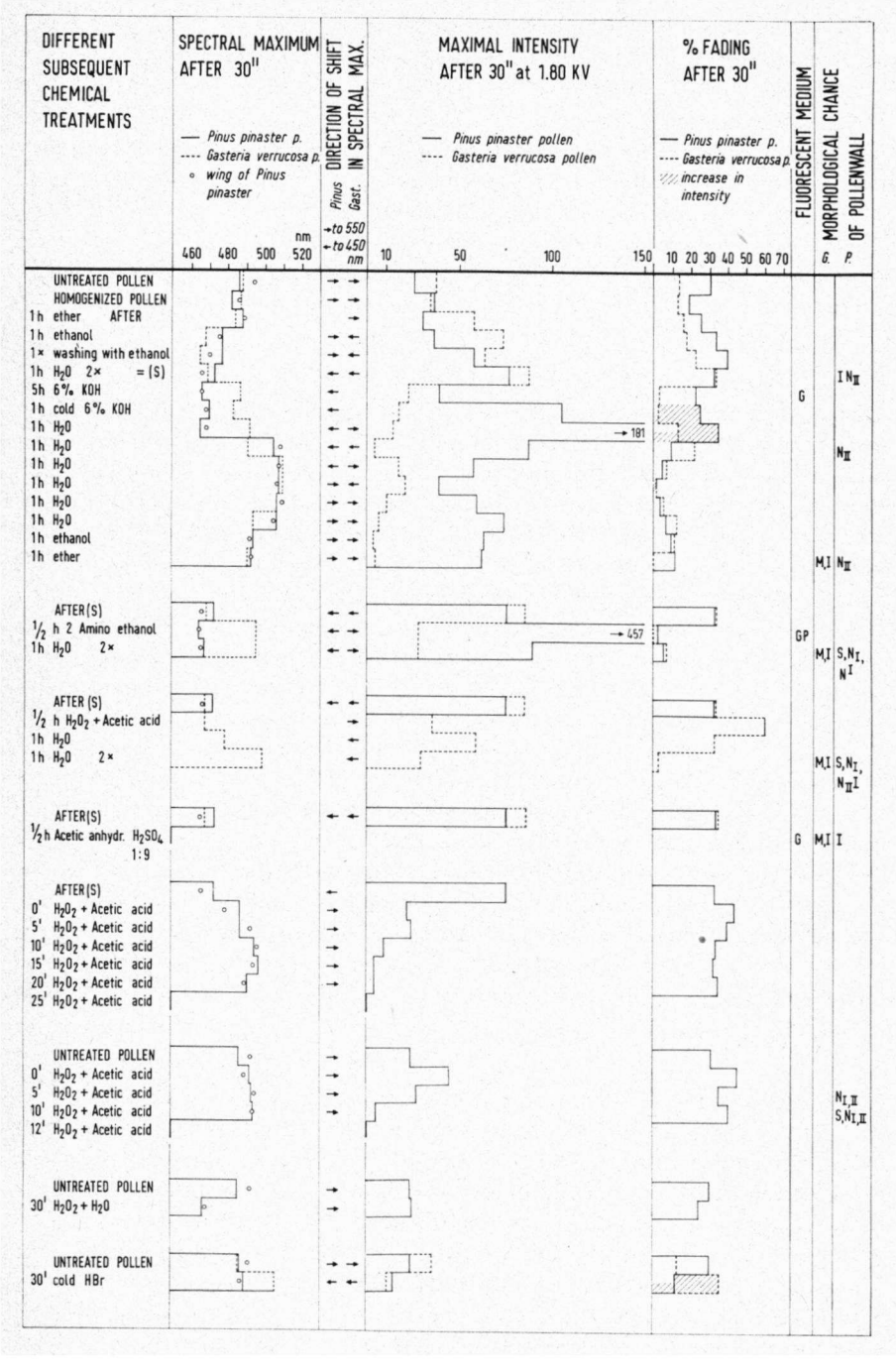
Diagram 3. Changes in fluorescence during chemical treatments of the pollen wall.

Of each step of the different subsequent chemical treatments of *Gasteria* and *Pinus* pollen the value of the spectral maximum after 30″, the direction of the shift in the spectral maximum, the maximal intensity in arbitrary units after 30″ at 1.80 KV and the percentage of fading or increase in intensity (shaded columns) of the fluorescence spectrum are given. The morphological changes and the fluorescence of the medium in which the pollen were treated are noted. G = *Gasteria*, P = *Pinus*, S = sexine, N I and N II = nexine I and II, I = intine and M = material between the bacula of *Gasteria*. Some treatments were started with untreated pollen, others with treated or cleaned pollen = (S). All treatments of the pollen took place in boiling media, except at the HBr treatment. The degradation of pollen wall material of *Pinus* in acetic acid – H_2O_2 at different time intervals is given for fresh and treated pollen.


DIFFERENT SUBSEQUENT CHEMICAL TREATMENTS
SPECTRAL MAXIMUM AFTER 30"
Pinus pinaster p.
Gasteria verrucosa p.
wing of Pinus pinaster
nm
460 480 500 520
DIRECTION OF SHIFT IN SPECTRAL MAX.
Pinus
Gast.
→ to 550 nm
← to 450 nm
MAXIMAL INTENSITY AFTER 30" at 1.80 KV
Pinus pinaster pollen
Gasteria verrucosa pollen
10 50 100 150
181
457
% FADING AFTER 30"
Pinus pinaster p.
Gasteria verrucosa p.
increase in intensity
10 20 30 40 50 60 70
FLUORESCENT MEDIUM
MORPHOLOGICAL CHANCE OF POLLENWALL
G. P.
UNTREATED POLLEN
HOMOGENIZED POLLEN
1 h ether AFTER
1 h ethanol
1 × washing with ethanol
1 h H2O 2 × = (S)
5 h 6 % KOH
1 h cold 6 % KOH
1 h H2O
1 h H2O
1 h H2O
1 h H2O
1 h H2O
1 h H2O
1 h ethanol
1 h ether
G
I NII
NII
M,I NII
AFTER (S)
½ h 2 Amino ethanol
1 h H2O 2 ×
GP
M,I S,NI, NI
AFTER (S)
½ h H2O2 + Acetic acid
1 h H2O
1 h H2O 2 ×
M,I S,NI, NII
AFTER (S)
½ h Acetic anhydr. H2SO4 1:9
G M,I I
AFTER (S)
0' H2O2 + Acetic acid
5' H2O2 + Acetic acid
10' H2O2 + Acetic acid
15' H2O2 + Acetic acid
20' H2O2 + Acetic acid
25' H2O2 + Acetic acid
UNTREATED POLLEN
0' H2O2 + Acetic acid
5' H2O2 + Acetic acid
10' H2O2 + Acetic acid
12' H2O2 + Acetic acid
NI,II
S,NI,II
UNTREATED POLLEN
30' H2O2 + H2O
UNTREATED POLLEN
30' cold HBr

3.2.1. The influence of pH on the fluorescence of pollen

At first the influence of pH on the fluorescence of *Pinus* and *Gasteria* pollen at 23 °C was checked. A gradual change of the pH from 1.2 up to pH 11.2 (obtained from acetic acid and/or KOH) had no effect on the fluorescence spectrum. At pH 13.5 (= 6% KOH) the value of the intensity doubled in *Pinus*, the percentage of fading of −20% changed into an increase of intensity of +7%, the spectral maximum shifted from 490 to 480 nm. At pH 13.5 *Gasteria* pollen show no increase in intensity, the percentage of fading of −20% changed in an increase in intensity of +7%, the spectral maximum changed from 480 to 530 nm. Only pH 13.5 affects the fluorescence of both pollen species.

3.2.2. Treatment with KOH and with ethanolamine

During treatment with 6% KOH, water, ethanol and ether, in both kinds of pollen the value of the spectral maximum shifted to 505 nm, whereas the percentage of fading decreases. In *Pinus* the intensity increases for a short while and there is no fading. In *Gasteria* a strong decrease in intensity occurs. The treatments with ethanol and ether result in few changes in the pollen wall of both species. The morphology of the pollen wall changes in both. In *Gasteria* the intine is no more observed while the exine remains unaltered after the complete treatment. The material between the bacula has disappeared (*fig. 6, 8*). After washing with water in *Pinus* the nexine II is affected and the intine has partly disappeared (*fig. 5*). The sexine and nexine I show the normal morphology after the treatment with ether, the nexine II has disappeared.

After the treatments the pollen wall of both species shows no reaction to the staining on pectine and callose. The probably total disappearance of the intine in both species may cause the increase in wave-length of the spectral maximum and the decrease of the percentage of fading.

The effect of the treatment with ethanolamine on the fluorescence of the pollen wall is comparable with that of KOH. The spectral maximum and intensity increase, whereas the fading decreases. Morphological observations show that the intine resolves for a large part. In *Gasteria* the exine does not change. In *Pinus* the nexine II has disappeared and the nexine I and sexine are strongly affected (*fig. 7*).

3.2.3. Treatment with H_2O_2-acetic acid

In *Gasteria* the effect of H_2O_2-acetic acid during 30″ results in an increase of the fading percentage. After washing with water the spectral maximum changes in the direction of 500 nm, the fading percentage decreases. The intine disappears, however, the exine has locally a low contrast (*fig. 10*). It appears that the microspores of *Gasteria* and *Physostegia* during the tetrad stage totally resolve after 30″. Ripe pollen of *Physostegia* has disappeared within 30″.

In *Pinus* the acetic acid-H_2O_2 mixture dissolves the pollen wall exine, only the cellulose of the intine remains intact. Fresh pollen loses the pollen wall and the fluorescence after 10″; the treated pollen (= S) after 20″. In both cases the

spectral maximum increases in wave-length, whereas the intensity decreases immediately. The percentage of fading remains constant.

After 5′ the changes in the morphology of the fresh pollen become visible. The contrast containing material of the pollen wall exine starts to disappear and converts into electron transparent material. This takes place first in the nexine, thereafter in the sexine (*fig. 9, 11*). After 10′ in the electron microscope the pollen wall exine lacks all contrast, but tectum, bacula, and footlayer remain recognizable (*fig. 12*). This pollen wall shows no fluorescence. After 15 minutes the footlayer loses the contact with the cellulose of the intine and the exine dissolves slowly. The exine is electron transparent and has a fine fibrillar structure (*fig. 13*). *Pinus* pollen treated with 15% H_2O_2 only show a decrease in the wave-length of the spectral maximum.

During the treatment with acetic acid-H_2O_2 the infrared spectrum of the solution with *Gasteria* and *Pinus* pollen shows only one absorption band of 1640 cm^{-1} (6100 nm), which changes in intensity when the medium is measured after 0′, 10′, 15′ and 30′. The solution in which *Gasteria* pollen were treated shows the band of 1640 cm^{-1}, which increases in intensity up to 15 minutes and which thereafter decreases. In the solution in which *Pinus* pollen was treated, the same absorption band increases continuously in intensity. This band may represent the aromatic C = C or C = N bonds.

3.2.4. Treatment with acetic anhydride – H_2SO_4 and with HBr

The fluorescence of the pollen wall in both species disappears completely after treatment with acetic anhydride – H_2SO_4. The intine disappears, but the exine remains morphologically intact (*fig. 14*).

With HBr *Gasteria* pollen reacts with an increase in wave-length of the spectral maximum and intensity. The reaction of *Pinus* is less intensive.

During all treatments the cytoplasm of the pollen gradually disappears. The standard deviation of the mean values was very low during all measurements.

3.3. Spectral maxima and fading percentage of some other plant cell walls or cell wall substances

The fluorescence spectrum of the measured plant cell walls or cell wall substances has only one maximum. The spectral maximum and percentage of fading after 30″ were measured in other plant cell walls and substances which may have a relation to the pollen wall. The values are given in *table 1*.

The suggestion is that the spectral maxima below the value of 480 nm are the result of polysaccharide components in the cell wall.

In preliminary experiments the pollen of *Gasteria* and *Pinus* were put in a number of solutions each of which contained one enzyme with a concentration of 1 mg/ml. The fluorescence of the pollen does not change after 30′ and after 20 h, when the pollen is placed in a solution with: lipase (pH 8.0, 23 °C), papaine (pH 6.6, 23 °C), trypsine (pH 7.0, 23 °C), pronase (pH 7.0 and 9.3, 23 °C), pepsine (pH 6.0, 30 °C), α amylase (pH 6.0, 23 °C), chitinase (pH 5.0, 30 °C), pectin esterase (pH 7.0, 23 °C), pectinase (pH 4.0, 23 °C + 0.25% NaEDTA), cellulase (pH 6.6, 23 °C). With the method used no effect could be registered.

Table 1. Spectral maximum and fading percentage of some plant cell walls. Callose and pectin show fluorescence, but with a very low intensity.

Material	Spectral maximum after 30″	Fading percentage after 30″
Cellulose (cell wall of *Gasteria*)	476 nm	−18
Lignine (cell wall of *Gasteria*)	477 nm	−8
Cutin (cell wall of *Gasteria*)	481 nm	−1
Callose (cell wall of *Gasteria*)	not measurable (± 475)	
Pectin (extracted from apples)	not measurable (± 475)	
β Carotene $C_{40}H_{56}$ (synthetic)	no fluorescence	
Oxidative polymer of β Carotene (data obtained from Dr. P. VAN GIJZEL)	510 nm	+57 (after 30′)

4. DISCUSSION AND CONCLUSION

The fluorescence of the pollen wall seems quite characteristic for the individual pollen species. Only within a plant family similarities may occur. The result of the treatment with HBr compared with the result in *Lycopodium* (VAN GIJZEL 1971a) shows also the specificity of microspore wall composition. Only developing pollen can be distinguished through the absence or presence of fluorescent cytoplasm. This cytoplasmic fluorescence may start during the interphase II; the relation between the formation of the pollen wall and the fluorescent cytoplasm is not yet clear.

In general, polysaccharides in the cell wall have a spectral maximum around 475 nm and a low fading percentage. The carotenoid esters may have a higher value of the spectral maximum, probably around 510 nm. During the development of the pollen the production of sporopollenin is connected with a spectral maximum of about 490 nm and a low fading. When the intine appears the spectral maximum changes to about 475 nm and the fading increases. The opposite occurs when the intine is removed by the KOH or ethanolamine treatment; then the spectral maximum increases in wave-length and the fading decreases. The orbicules of the tapetal cell show the same phenomenon. The influence of the strong alkalinity during the treatment with KOH is also repressed, probably by the resolving of intine substances. The spectral maximum does not change much compared with pollen put in a solution at pH 13.5. Elements of the intine strongly influence the fluorescence. The intensity is high after the removal or before the arrival of the intine. There may be a relation between the intensity and the content of sporopollenin, but this relation is influenced by the presence of the intine.

In ethanolamine the whole pollen wall starts to dissolve (ROWLEY & FLYNN 1966). In acetic acid-H_2O_2 first the electron density disappears and the structure remains, but shows no autofluorescence. Either the electron dense material dissolves from the nexine and sexine or the electron dense material changes into electron transparent material. The latter explanation is preferred because of the fact that the structure of the nexine I and sexine in *Pinus* does not change immediately. When the pollen wall exine starts splitting from the intine, the

material is altered and shows more fine fibrils. The change in the infrared absorption band suggests a gradual process of a loss of C = C of an aromatic ring, probably of a β-carotene (BROOKS & SHAW 1968). This means that the pollen wall sexine and nexine I may contain polymer carotenoids, which give the electron density and cause the fluorescence. The precursor of these polymer carotenoids can be electron transparent. During pollen wall formation in *Pinus sylvestris* (WILLEMSE 1971b) electron dense material, probably sporopollenin, impregnates the fibrillar material derived from the content of Golgi vesicles. This fibrillar material precipitates against the callosic wall and probably consists of polysaccharides. During the ontogeny of the pollen wall two components could be distinguished in the sexine and nexine I of *Pinus*. By the treatment with the H_2O_2-acetic acid mixture these two components are also demonstrated, probably consisting, respectively, of carotenoids and a polysaccharide which is less resistant than cellulose. The nexine II consists of a different material, because it dissolves quickly in KOH and ethanolamine. The difference between nexine II and sexine with nexine I is in agreement with the results of SOUTHWORTH (1969) and WATERKEYN & BIENFAIT (1971).

Treated pollen seems to be more resistant against the acetic acid-H_2O_2 treatment than fresh pollen. The presence of acetic acid is thereby necessary to affect the pollen wall within 30'. The pollen wall of *Gasteria* is more resistant than the *Pinus* pollen wall. The mainly callosic-pectin-like intine of *Gasteria* disappears quickly, including the material between the bacula, which may indicate that less cellulose is present. The exine of *Gasteria* is more resistant than the exine of *Pinus*. It should be noted that the fluorescence may be altered without morphological changes in the pollen wall. This can be seen from the acetic anhydride-H_2SO_4 treated pollen walls, which show an intact structure but no fluorescence, due to molecular changes only. Therefore, during preparation of pollen walls, either for electron microscopy or palynology, it can not be excluded that changes in the pollen wall occur. Even ethanol seems to have an effect on the fluorescence of the pollen wall.

Because of the complexity of the chemical composition of the pollen wall, which among others may contain sporopollenin, polysaccharides, pigments and many other substances, no explanation can be given of the causes of fluorescence on the molecular level. The unanswered questions on the change of the shift in the spectral maximum illustrate this problem. Only some relations could be indicated between the developing or affected pollen wall and the fluorescence.

ACKNOWLEDGEMENTS

The author is much indebted to Prof. Dr. H. F. Linskens for his stimulating interest, to Dr. M. M. A. Sassen and Dr. P. van Gijzel for the critical reading of the manuscript, and to Dr. G. W. M. Barendse for the correction. The author is grateful to Miss E. B. F. Pey for her skilful assistance, to Miss E. A. J. Derksen for typing the manuscript, and to Mr. J. Gerritsen for drawing the diagrams. The author is much obliged to Mr. W. Flokstra for supplying the plant material.

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