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PEROXIDASE LEAKAGE AND POLLEN TUBE GROWTH INHIBITION IN AGED NICOTIANA ALATA STYLES

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SUMMARY

An attempt was made to explain the influence of stylar age on the growth of compatible pollen tubes based on peroxidase data.

The growth of compatible pollen tubes in *Nicotiana alata* styles varied with the physiological age of the style. Especially in detached styles ageing caused changes by which the tube growth was retarded, or even totally inhibited. One of these changes might be the increase in the activity of several stylar peroxidase isoenzymes. Among them, there was the isoenzyme 10 which has previously been suggested to be involved in the inhibition of the growth of incompatible pollen tubes. The senescence-induced increase in the activity of this isoenzyme in unpollinated styles was 3 to 4 times higher when the styles were detached from the flower.

An *in situ* extraction of walls and intercellular material by centrifugation after vacuum infiltration of stylar sections revealed that after ageing during 7 days, the detached styles contained 9 times more extracellular peroxidase 10 than the styles which remained attached to the flower.

A possible relationship between peroxidase release from cytoplasm to walls and intercellular spaces, and inhibition of pollen tube growth was discussed.

1. INTRODUCTION

After pollination of *Nicotiana alata* styles a wave of peroxidase isoenzyme 10 arises just below the stigma and is then transmitted to the basal end of the style with a speed which is independent of the fact whether the style is self- or cross-pollinated. In self-pollinated styles pollen tube growth is retarded by the incompatibility reaction so that the front of the peroxidase 10 wave is able to pass the growing pollen tube tips. Consequently incompatible pollen tubes grow in a part of the style which contains a substantial amount of peroxidase 10. Compatible pollen tube tips, on the other hand, grow in a stylar part with a very low activity of peroxidase 10 because growth rate of these tubes is higher than the speed of the peroxidase 10 wave (BREDEMEIJER & BLAAS 1975). Based on these findings and on the fact that the peroxidase 10 wave is not induced upon self-pollination of immature styles (bud-pollination) which are not able to reject incompatible pollen tubes, it was suggested that peroxidase 10 might participate in the rejection of incompatible pollen tube growth (BREDEMEIJER & BLAAS 1975; BREDEMEIJER 1976).

In flowers pollinated at anthesis peroxidase 10 can not influence compatible

pollen tube growth because, as stated above, the tube tips grow in advance of the peroxidase 10 wave. However, with the increasing age of the flower, the speed by which the pollination-induced peroxidase 10 wave is transmitted also increases (BREDEMEIJER 1976), so that at a certain stylar age, the speed of this wave is high enough to pass the growing pollen tube tips. Finally, peroxidase 10 is induced even in the unpollinated style as a consequence of senescence (BREDEMEIJER & BLAAS 1975). Such aged styles, therefore, seem very much suitable to study the influence of peroxidase 10 on compatible pollen tube growth and to establish whether the general decrease in the growth of compatible pollen tubes during stylar senescence (ASCHER & PELOQUIN 1966; BREDEMEIJER & BLAAS 1975) is due to the action of peroxidase 10.

In all previous studies stylar extracts contained cytoplasmic, ionically wall bound and intercellular peroxidases. Since it is well known that in ageing tissues peroxidases are released from the cytoplasm to cell walls and intercellular spaces (BIRECKA & MILLER 1974) we extended our comparative studies to include both the cytoplasmic fraction and the wall and intercellular fraction.

2. MATERIAL AND METHODS

Flowers of *Nicotiana alata* Link and Otto were collected at anthesis. The flowers were incubated after the removal of anthers in a climate room (light intensity 8,000 lux during 16 hours; temperature 15 °C; relative humidity 80%) during 7 days (attached styles); in another series styles were detached from the flowers before incubation. Extraction and electrophoresis of stylar peroxidases was carried out according to the methods described previously (BREDEMEIJER 1973, 1974, 1976; BREDEMEIJER & BLAAS 1975).

To extract intercellular and wall peroxidases, a modification of the methods adopted by RATHMELL & SEQUEIRA (1974) and MEUDT & STECHER (1972) was used. For each analysis 32 styles, excluding the topmost portion of 0.5 cm, were cut into 4 sections of 1,5 cm each and then put in a small glass beaker containing a 4% NaCl solution in distilled water placed in a desiccator. The desiccator was evacuated by means of a conventional water aspirator pump up to the time (5–10 min) the end of the stylar sections released no more air bubbles. The stylar sections were then removed from the solution, dried on a blotting paper and placed in a centrifuge tube provided with perforations at the bottom. This tube was placed in another centrifuge tube in such a way that it was held by the rim. After centrifugation in a swing out rotor at 1000 g for 10 min at 2 °C, the exudate was collected, diluted and used for separation of the peroxidase isoenzymes on starch gels and for glucose-6-phosphate dehydrogenase (G-6-PDH) assays; the remaining stylar sections were homogenized in a mortar with pure quartz sand and a 4% NaCl solution. The supernatant obtained after centrifugation (18,000 g; 45 min; 2 °C) was also used for electrophoretical separation of the isoperoxidases and for the G-6-PDH assay.

The G-6-PDH assay was carried out as described by Boehringer, Mann-

heim, W. Germany, (see also KORNBERG & HORECKER 1955) and used as a marker for contamination of the intercellular fluid by the cytoplasmic contents of style cells (RATHMELL & SEQUEIRA 1974).

The activity of peroxidase isoenzyme 10 was estimated from the triangular area corresponding to the peak on the recorder paper obtained by scanning photographs of starch gels by reflection with a Vitatron Manual TLD 100 densitometer.

3. RESULTS

3.1. Effect of stylar ageing on the growth of compatible pollen tubes

The growth of compatible pollen tubes was retarded in detached styles, especially when the style was aged during 7 days at 15 °C before pollination (*table 1*). In the aged styles, the pollen tube growth was completely stopped 3 days after pollination and the morphological features of these tubes observed under the fluorescence microscope were similar to those of incompatible pollen tubes in the styles of self-pollinated flowers at anthesis (strong callose deposition and swollen tube tips).

When the styles remain attached to the flower, ageing during 7 days has, however, no influence on the growth rate of compatible pollen tubes.

Table 1. Mean lengths of compatible pollen tubes (mm) after 2, 3 and 4 days growth in attached and detached styles (15–20) pollinated at anthesis or 7 days following anthesis.

Days after pollination	Mean length of pollen tubes (mm)			
	Attached styles (Flowers)		Detached styles	
	Fresh	Aged	Fresh	Aged
2	26.4	31.6	15.8	16.2
3	45.9	49.8	30.0	20.8
4	61.5	64.2	43.8	19.6

3.2. Senescence-induced increase in peroxidase isoenzyme 10 activity in the attached and the detached styles

The problem was thus to find out whether the difference between compatible pollen tube growth in the attached and detached styles was correlated with differences in peroxidase 10 activity. Comparison of peroxidase 10 activity in various stylar segments of attached and detached styles which were incubated during 7 days at 15 °C revealed a clear difference (*fig. 1*). In all segments peroxidase 10 activity was higher when the styles were detached from the flowers. Apparently, the senescence-induced peroxidase 10 wave was accelerated when the style was detached from the flower. To investigate possible

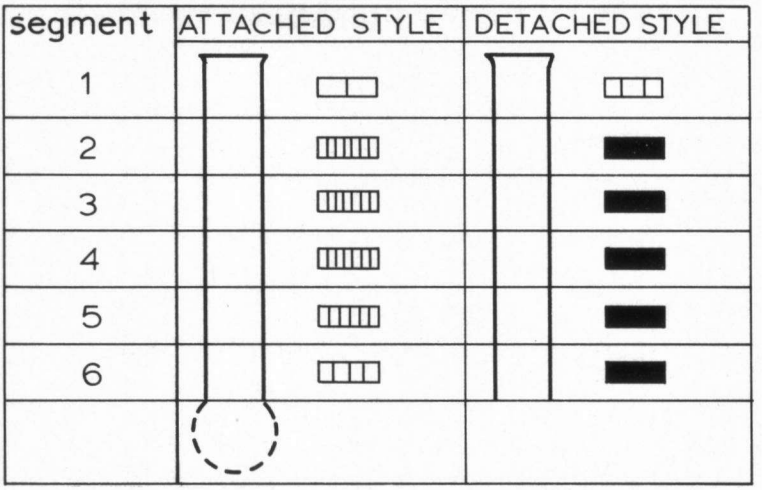

Fig. 1. Distribution of peroxidase 10 activity in unpollinated styles 7 days after anthesis.

differences between the localization of peroxidase 10 in attached and detached styles, an *in situ* extraction of walls and intercellular spaces by centrifugation after vacuum infiltration was carried out.

3.3. Peroxidase isoenzyme 10 in the fluid from intercellular spaces of aged styles

The intercellular fluid fraction obtained by vacuum infiltration of stylar sections with a 4% NaCl solution contained all the protoplast peroxidase isoenzymes except the slow moving No. 1, 3 and 4 (*fig. 2*). Most of the peroxidase activity in the intercellular fluid was due to isoenzymes 10 and 16 in the attached styles and 10 and 11 in the detached styles. It was also clear that the relative amount of a specific peroxidase isoenzyme present in the intercellular fluid differs very much from isoenzyme to isoenzyme, e.g. peroxidase isoenzyme 16 occurs at a relatively high concentration in the fluid fraction.

A comparison of the isoenzyme patterns of the cellular fraction and the intercellular fluid fraction appears difficult since the total quantity of the two fractions differs strongly (*fig. 2*). In the case of peroxidase 10 the distribution over the two fractions was calculated from the peaks obtained by reflection writing of gel-photographs taking into account the difference between the total quantity of both fractions. The data given in table 2 show that 3.9 and 9.1% of the total amount of the isoenzyme was present in the intercellular fluid respectively from attached and detached styles. In absolute amounts the fluid from detached styles had a 9 times higher concentration of peroxidase 10 when compared to attached styles. These values were already corrected for release due to diffusion from cut surfaces which amounted to 0.8% as estab-

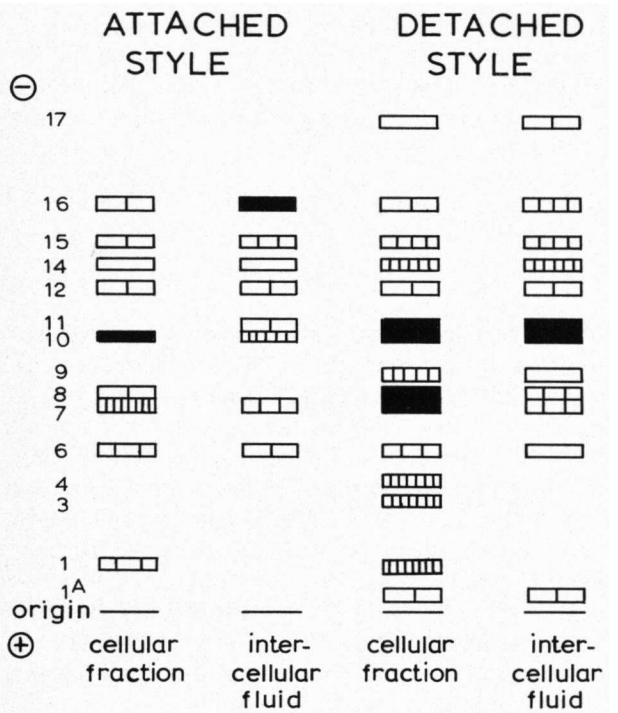

Fig. 2. Peroxidase isoenzyme patterns of the cellular and intercellular fluid fraction from aged styles. The intercellular fluid (0.070 ml and 0.088 ml from attached and detached styles respectively) was diluted 3 times before application to the gel; the cellular fraction (in both cases 2.10 ml) was not diluted. The degree of hatching approximates the staining intensity. No hatching means that the bands on the gels were just visible.

Table 2. Total activity of peroxidase isoenzyme 10 and of glucose-6-phosphate dehydrogenase in the intercellular fluid from 32 aged styles and in the residual cell material. Peroxidase 10 activity is expressed as peak area (mm^2) after reflection writing of gel-photographs. G-6-PDH activity is expressed as change in absorbance at 340 nm during 10 min at 25 °C. Data are the average of 3–5 experiments.

	Peroxidase 10		G-6-PDH
	Attached styles	Detached styles	Attached styles
Cellular fraction			
amount present/32 styles	81080	284790	0.951
Intercellular fluid			
amount present/32 styles	3290	28650	0.020
percentage of total amount	3.9	9.1	2.1

lished by determining the amount of the cytoplasmic marker enzyme glucose-6-phosphate dehydrogenase present in the intercellular fluid from fresh styles collected at anthesis. The G-6-PDH activity in the fluid from aged styles could be determined only when the styles remained attached to the flower. It appeared that 2.1 % of the total amount of G-6-PDH was present in this fluid. The total activity in detached styles was too low for reliable determination.

4. DISCUSSION

Ageing of unpollinated styles is accompanied by induction of peroxidase isoenzyme 10. A certain proportion of this isoenzyme is released from the cytoplasm to the walls and the intercellular spaces. This release is partially due to natural leakage because the intercellular fluid contained plasma marker enzyme G-6-PDH. The fact that the percentage intercellular peroxidase 10 is higher than the percentage intercellular G-6-PDH indicates, however, that at least a certain proportion of peroxidase 10 is released from the cytoplasm by another process than leakage. This idea is also supported by the fact that the release of peroxidase isoenzymes is not a general, but a selective process; the various isoenzymes are released at different degrees which is in agreement with the findings of RITZERT & TURIN (1970) and BIRECKA & MILLER (1974). In 1974 RATHMELL & SEQUEIRA suggested that a mechanism exists for the selective export of peroxidases from cell to intercellular space.

In spite of the fact that peroxidase 10 activity is induced in both attached and detached styles, compatible pollen tube growth is retarded only in the latter. This findings seems, at first sight, to deny the hypothesis that peroxidase 10 is involved in the inhibition of pollen tube growth. It is, however, possible that other factors like availability of substrate and hydrogen donors or localization of the enzyme play a role. Concerning the last possibility, the present investigations clearly demonstrate differences between the localization of peroxidase 10 in attached and detached styles. During ageing of attached styles there was only a small release of peroxidase 10 from the cytoplasm to the walls and intercellular spaces, whereas it was very high in the detached styles. At the time the aged styles were pollinated, the intercellular fluid from detached styles contained a 9-fold amount of peroxidase 10 as compared to the fluid from attached styles. Assuming the idea, that peroxidase 10 is involved in the inhibition of pollen tube growth to be correct the present results demonstrate that only the wall and/or intercellular fraction of this isoenzyme is responsible for the inhibition.

Preliminary studies have indicated that the senescence-induced release of stylar peroxidase isoenzymes in unpollinated flowers was increased considerably by pollination so that incompatible pollen tubes had to grow in a stylar part containing wall and intercellular peroxidases. The fact that the morphological features of pollen tubes after cross-compatible pollination of aged styles and after self-pollination of flowers at anthesis are similar may

indicate that incompatibility leads to inhibition of pollen tube growth by a process which takes place also during ageing of unpollinated styles.

In both cases pollen tubes have to grow in a stylar part characterized by a considerably increased permeability of the cell membranes as indicated by the increased peroxidase release. It is, therefore, suggested here that rejection of incompatible pollen tubes occurs by way of an increased permeability of stylar cell membranes for various substances including peroxidase 10. It is very reasonable to assume that the peroxidases are not the only compounds present in the intercellular fluid from styles because the fluid obtained from tobacco leaves contains, except peroxidases, also amino acids, phenolic compounds, sugars and electrolytes (RATHMELL & SEQUEIRA 1974). The question whether peroxidase 10 and/or other compounds released from the cytoplasm to walls and intercellular spaces are involved in the inhibition of pollen tube growth needs further investigation.

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