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A POSSIBLE ROLE OF A STYLAR PEROXIDASE GRADIENT IN THE REJECTION OF INCOMPATIBLE GROWING POLLEN TUBES

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SUMMARY

From stigma to basal end, styles of *Nicotiana alata* have a decreasing gradient of total peroxidase activity. As shown by starch gel electrophoresis this gradient is accompanied by differences in the contribution of particular peroxidase isoenzymes. Under the influence of pollination and subsequent pollen tube growth the activities of several peroxidase isoenzymes show changes which differ in the various stylar parts. The increase in activity of certain peroxidase isoenzymes is transmitted like a wave from the stigma to the basal end of the style. For some isoenzymes the distance covered by this activation wave depends on the pollen tube length, whereas for one of the peroxidases it only seems to depend on the time passed since pollination.

The increase of the peroxidase isoenzymes which was previously suggested to be necessary for continuation of pollen tube growth through the style appears to be a consequence of pollen tube growth.

The changes observed in the activity of one of the peroxidase isoenzymes (10) are extensively discussed. Preliminary evidence is provided which suggests that this isoenzyme is involved in the rejection of incompatible growing pollen tubes.

1. INTRODUCTION

The work of PANDEY (1967) revealed for the first time a link between peroxidase and incompatibility. The peroxidase isoenzyme composition of Nicotiana styles should be related to the S-genotype.

The influence of self- and cross-pollination on the total peroxidase activity and peroxidase isoenzyme composition of Nicotiana alata styles was studied previously (BREDEMEIJER 1974). Pollination and subsequent pollen tube growth cause an increase of the total peroxidase activity and an increase of several peroxidase isoenzymes. During the progamic phase two new peroxidase isoenzymes appear in cross-pollinated styles. Only one of these isoenzymes was found in self-pollinated styles but it was not possible to ascertain if this difference was a prerequisite or a consequence of the difference between compatible and incompatible pollen tube growth. In order to elucidate this problem peroxidase activity and peroxidase isoenzyme composition in the various parts of the style were examined by means of methods similar to those of ROGGEN (1967) who determined the activities of ketose-1-phosphate aldolase, aspartate aminotransferase and glutamate dehydrogenase in the various parts of cross-pollinated styles of *Petunia hybrida*.

2. MATERIAL AND METHODS

In these experiments with *Nicotiana alata* Link and Otto plants the general outlines of our former methods, used for studying changes in peroxidase activity and isoenzyme composition in whole styles (BREDEMEIJER 1973 and 1974), were applied.

For each analysis 44 unpollinated and as many self- (OWL × OWL), and cross-pollinated (OWL × OB-2) detached flowers were incubated. At chosen intervals after pollination styles were collected and cut into five sections of 1 cm starting from the stigma. The remaining basal part varying from 0.5 to 1.5 cm was used as section 6. Extracts were prepared by homogenizing 44 style segments for 2 min in an icecooled mortar with pure quartz sand and 0.45 ml 4% NaCl solution in distilled water per 0.1 g fresh weight. The supernatants of style extracts after 45 min centrifugation at 18,000 × g and at 2 °C were used for starch gel electrophoresis (BREDEMEIJER 1974) and peroxidase assay (SEEVERS et al. 1971). Determination of pollen tube length in the style by means of the U.V. microscope technique of MARTIN (1959) was carried out without fixation and maceration. The distance which the ten fastest-growing pollen tubes had covered through the style was measured.

3. RESULTS

3.1. Comparison of the total peroxidase activity in different segments of un-, self-, and cross-pollinated styles

The peroxidase activity in the various segments of unpollinated and pollinated styles 69 hours after pollination are presented in *table 1*. It is apparent from this

Table 1. Distribution of peroxidase activity (ΔA334/5min/g fresh weight) in un-, self-, and cross-pollinated styles of *Nicotiana alata* 69 hrs after pollination. Data are averages of 4–5 experiments. Minimum and maximum pollen tube lengths out of at least 20 styles are given.

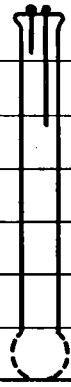
No	UNPOLLINATED	SELF-POLLINATED	CROSS-POLLINATED
1	18.46 ± 4.14	17.94 ± 2.00	20.30 ± 5.68
2	7.76 ± 0.58	12.70 ± 1.78	13.63 ± 3.13
3	7.05 ± 0.92	5.97 ± 1.10	8.41 ± 1.57
4	4.02 ± 1.00	4.71 ± 0.48	4.80 ± 0.60
5	4.56 ± 0.39	3.69 ± 1.18	4.07 ± 0.86
6	5.88 ± 0.76	4.59 ± 1.10	5.48 ± 0.44
			

table that both unpollinated and pollinated styles have a decreasing peroxidase gradient from segment 1 to segment 5, the trend being reversed at the level of segment 6. Subdivision of segment 1 in a stigma part and a stylar part revealed a 13 per cent higher peroxidase activity in the stigma as compared to the stylar part.

After 69 hours of compatible or incompatible pollen tube growth through the style the peroxidase activity in segment 2 has clearly increased and is much higher than in the corresponding segment of unpollinated styles; in the other segments, the changes in activity are much less pronounced but cross-pollinated styles display, as a rule, a somewhat higher peroxidase activity than the corresponding segments of self-pollinated styles.

Table 1 also shows that the peroxidase activities of the segments of the style where the pollen tubes have not yet grown are not higher than the corresponding activities in unpollinated styles. This means that at variance with what ROGGEN (1967) found for several other enzymes, there is no wave of peroxidase activity preceding the growth of the pollen tubes.

3.2. Comparison of the peroxidase isoenzyme composition of different segments from un-, self-, and cross-pollinated styles

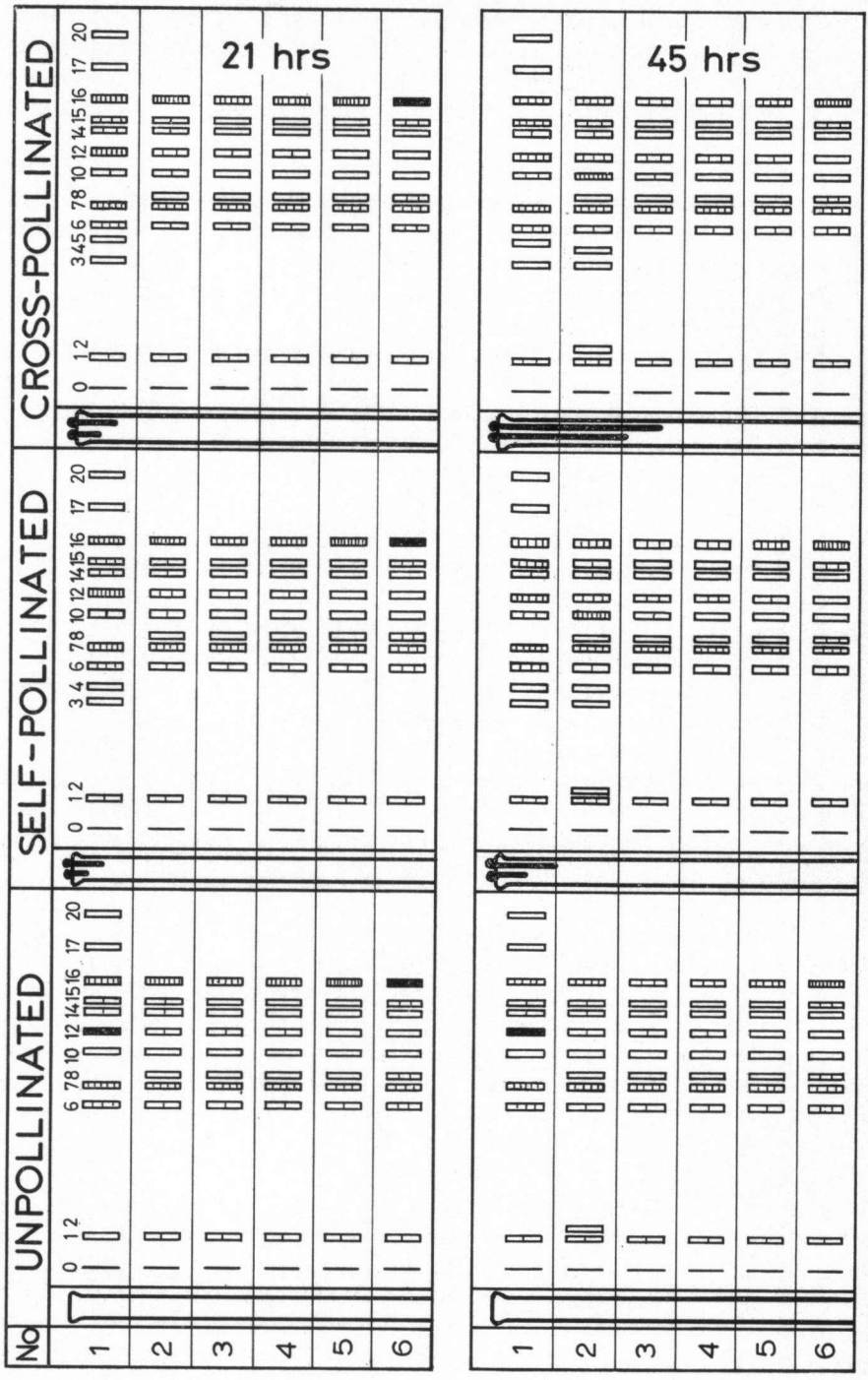
The distribution of peroxidase isoenzymes in unpollinated and pollinated styles at various times after pollination is presented in *fig. 1*. This figure illustrates that the activities of several peroxidase isoenzymes (e.g. 6, 12 and 16) vary along the length of the style. Moreover, some peroxidases (10, 12 and 16) change as a function of time during senescence (cf. patterns of unpollinated styles after various incubation periods) or pollen tube growth. The extent of these alterations depends on the location within the style. In the present paper only those changes will be discussed which might be related to differences between compatible and incompatible pollen tube growth.

3.2.1. Increase of peroxidase 8 activity by cross-pollination

This isoenzyme which had been, in the past, only observed in the patterns of cross-pollinated styles (BREDEMEIJER 1974), also appears to occur at a very low concentration in un- and self-pollinated styles. The increase in activity of peroxidase 8 after cross-pollination is restricted to the stylar segments (except no. 1) which have already been passed by the pollen tube tips (see *fig. 1;* $t = 69$ and 93 hrs); in the peroxidase patterns of un- and self-pollinated styles no changes of band 8 are visible.

3.2.2. Wave of peroxidase 10 activity induced by pollination

After pollination, a wave of peroxidase 10 activity arises in segment 1 and is then transmitted to the basal end of the style. It is clear, from a comparison of the patterns of self- and cross-pollinated styles that the changes in the activity of peroxidase 10 through the various segments are independent of the type of pollination performed. The highest peroxidase 10 activity is reached in the seg-


No
UNPOLLINATED
SELF-POLLINATED
CROSS-POLLINATED
0 1 2 6 7 8 10 12 14 15 16 17 20
0 1 2 3 4 6 7 8 10 12 14 15 16 17 20
0 1 2 3 4 5 6 7 8 10 12 14 15 16 17 20
1
2
3
4
5
6
21 hrs
45 hrs

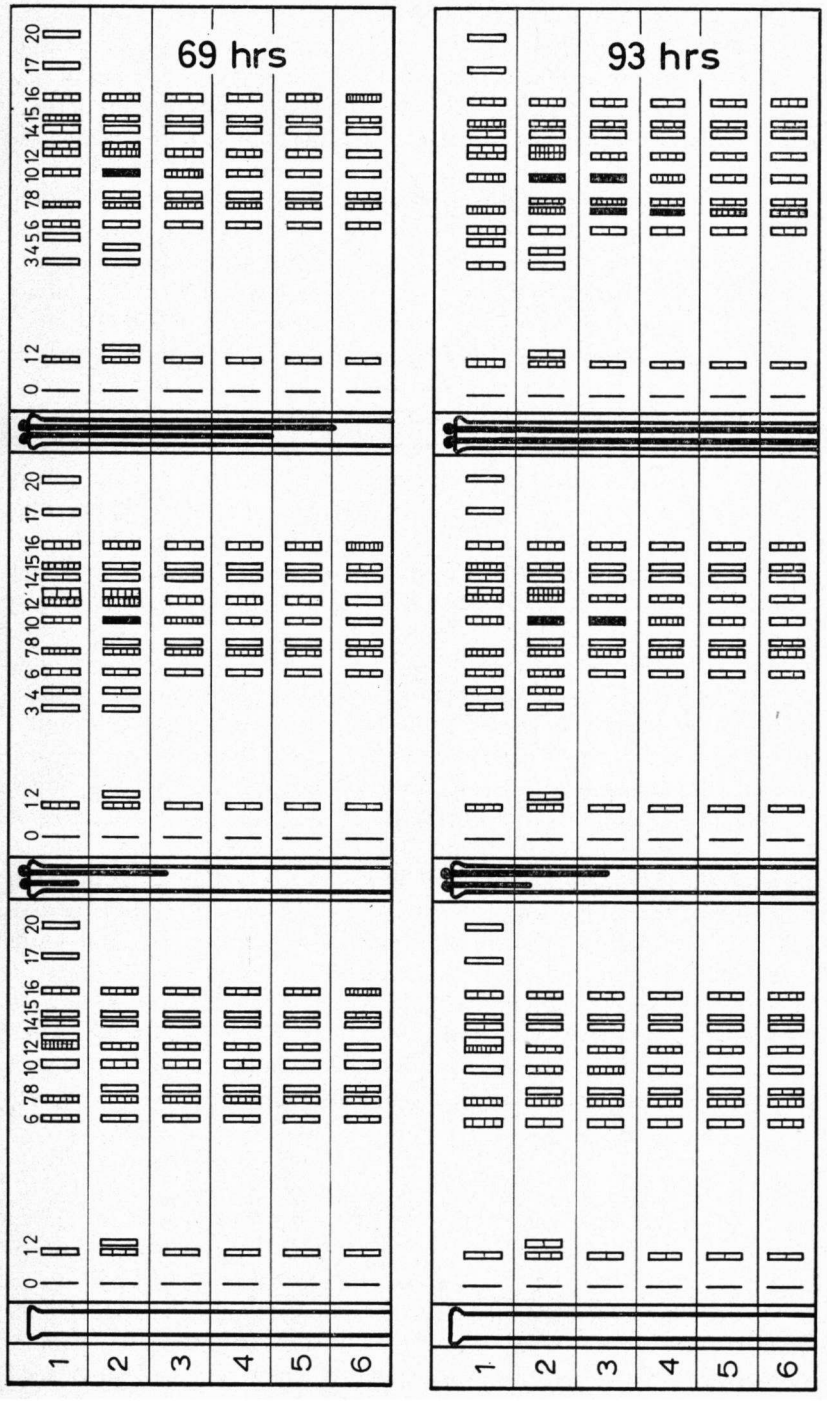


Fig. 1. Distribution of cationic peroxidase isoenzymes in un-, self-, and cross-pollinated styles of Nicotiana alata at various times after pollination. The degree of hatching approximates the staining intensity. No hatching means that the bands on the gels were just visible. 0 = origin.

ments 2 and 3 at respectively 69 hrs and 93 hrs after pollination. In spite of the fact that the activation wave has passed segment 1 for a longer period of time the increase for peroxidase 10 is, in this segment, less pronounced than in segments 2 and 3 (see *fig. 3*). No attempts have been made to find out if the activity of peroxidase 10 in the segments 4, 5 and 6 reaches the high level observed in the segments 2 and 3.

The peroxidase isoenzyme patterns of those stylar parts in which most of the incompatible (Is) or most of the compatible (IIc) pollen tube tips are located 69 hours after pollination are presented in *fig. 2*. From this photograph it can clearly be seen that peroxidase 10, although it has the same levels of activity in the corresponding parts, of self- and cross-pollinated styles, displays a much lower activity in part II than in part I. This means that the tips of incompatible pollen tubes grow in a part of the style (Is) with a high peroxidase 10 activity, whereas the tips of compatible pollen tubes are present in a stylar part (IIc) with a low peroxidase 10 activity. During the first day after pollination both compatible and incompatible pollen tubes grow in segment 1, which has a very low peroxidase 10 activity (*fig. 1;* t = 21 hrs). Thereafter, tips of compatible pollen tubes grow in front of the activation wave of peroxidase 10, whereas tips of incompatible pollen tubes grow behind this front in a zone with a high peroxidase 10 activity (*fig. 1;* t = 45, 69 and 93 hrs).

3.2.3. Changes in pollen peroxidases

In an earlier report, it had been pointed out that the peroxidases 3, 4 and 5 originate from the pollen (BREDEMEIJER 1974) and that peroxidase 4 and 5 appeared to be specific for respectively OWL and OB-2 pollen. The present results, however, demonstrate that peroxidase 4 is not specific for OWL pollen because cross-pollinated (OWL × OB-2) styles also contain this isoenzyme (*fig. 1*).

The peroxidases 3, 4 and 5 only occur in segment 1 within 21 hours after pollination and in segments 1 and 2 afterwards. The activities of these peroxidases are very low during the first three days after pollination; afterwards peroxidase 4 clearly increases in self-pollinated styles whereas its activity remains at a low level in cross-pollinated styles (*fig. 3*). The fact that peroxidase 4 only increases after three days of incompatible pollen tube growth indicates that this increase which might be due to an increase of the pollen as well of the style enzyme is rather a consequence than a cause of incompatible pollen tube growth.

4. DISCUSSION

4.1. Increase in peroxidase 8 activity induced by compatible pollen tube growth

In a previous study (BREDEMEIJER 1974) an attempt was made to find a correlation between the incompatibility reaction and peroxidase. The results obtained in that study did not reveal, however, a positive contribution of one or more peroxidase isoenzymes to the incompatibility reaction. Since peroxidase 8

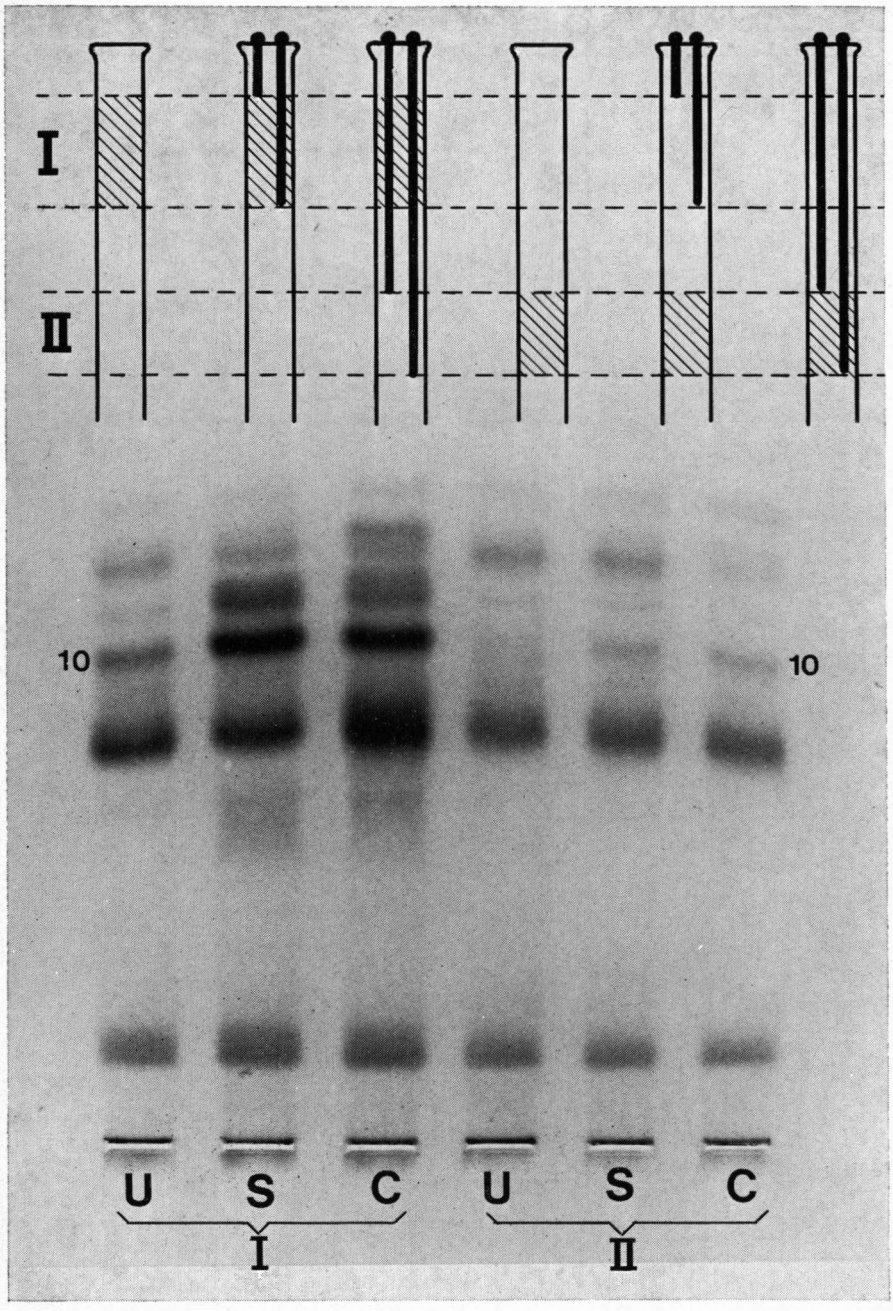


Fig. 2. Photograph of the peroxidase isoenzyme patterns of the stylar parts in which most of the incompatible (Is) or most of the compatible (IIc) pollen tube tips grow at 69 hrs after pollination. For comparison the patterns of the corresponding parts in unpollinated (Iu and IIu) and self- (IIs), or cross-pollinated styles (Ic) are given. Each of the 6 isoenzyme patterns (below) belongs to the hatched part of the corresponding style (above).

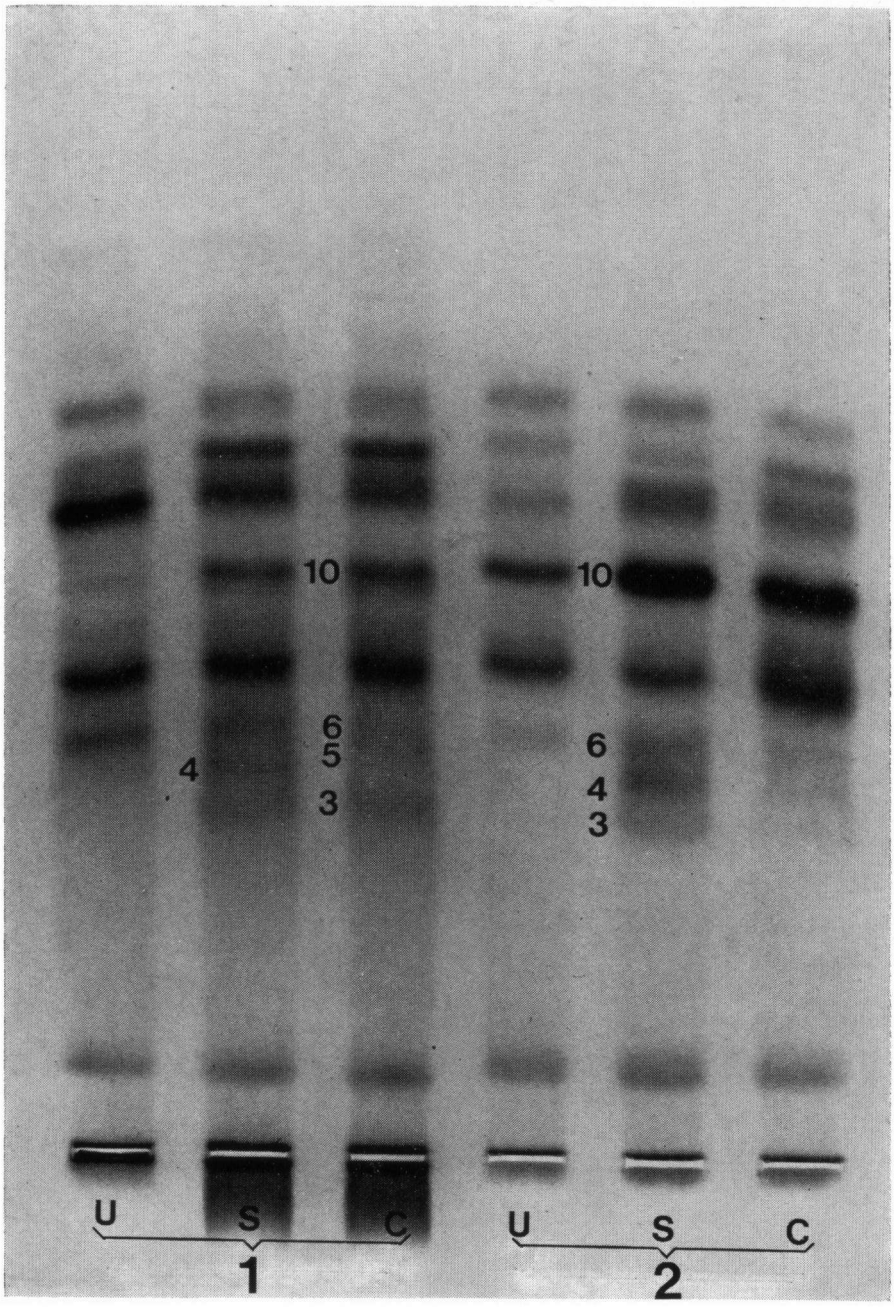


Fig. 3. Photograph of the peroxidase isoenzyme patterns of the two top segments no. 1 and 2 of un- (U), self- (S), and cross-pollinated (C) styles 93 hrs after pollination.

was only observed in cross-pollinated styles it was assumed that prevention of formation or inhibition of this isoenzyme might be a part of the incompatibility reaction. The present results, however, indicate that this assumption is unlikely. Firstly, peroxidase 8 also appears to occur in self-pollinated styles and secondly the increase of this isoenzyme in cross-pollinated styles takes only place in stylar parts which have already been passed by the pollen tube tips. This means that the increase of peroxidase 8 is rather a consequence than a prerequisite of pollen tube growth.

4.2. Possible role of peroxidase 10 in the inhibition of incompatible growing pollen tubes

A much more important change in the zymograms of both self- and cross-pollinated styles is the increase of peroxidase 10. As shown previously peroxidase 10 activity is hardly detectable in fresh unpollinated styles and increases steadily after pollination (BREDEMEIJER 1974). The increase of peroxidase 10 arises in segment 1 and is then transmitted to the base of the style with a speed which is independent of the type of cross performed. Since the distance covered by the activation front is independent of pollen tube length it is clear that the increase in activity of peroxidase 10 is a consequence of the pollination or the penetration of the pollen tubes in the stigma rather than a consequence of pollen tube growth. The fact that incompatible pollen tube tips grow in a stylar part with a high peroxidase 10 activity, while compatible pollen tube tips grow in a part with a low activity of this isoenzyme might indicate that peroxidase 10 is directly or indirectly involved in the inhibition of incompatible pollen tubes.

Assuming this idea to be correct one would like to know what mechanism leads the compatible tube tips to grow in front of the peroxidase 10 wave and causes the incompatible tube tips to remain behind this front. The initial retardation of incompatible pollen tubes is probably not due to peroxidase 10 itself, since the activity of this isoenzyme is equally low, at the onset of germination, after self- and cross-pollination. It therefore appears that there should be another inhibiting mechanism which retards the incompatible pollen tube growth at an early stage in such a way that the activation front of peroxidase 10 can pass the incompatible pollen tube tips. The hypothesis is consistent with the assumption by LINSKENS (1974) that the incompatibility reaction does indeed consists of two different steps, a "recognition reaction" and a "rejection reaction". It is suggested here that the initial inhibition of incompatible pollen tubes might be due to the "recognition reaction" in a manner possibly similar to the one described by HESLOP-HARRISON et al. (1973) who consider that pollen intine-held "recognition substances" are discharged from the apertural intine within a few minutes after pollen moistening; the rejection would then be caused by peroxidase 10 *(fig. 4)*. Compatible pollen tubes are not inhibited by the high peroxidase 10 activity because pollen tube growth only occurs at the apex of the pollen tube (e.g. LARSON 1965, CRANG & HEIN 1971) which is surrounded by stylar tissue with a low peroxidase 10 activity. Moreover, as the growing pollen tube tip is isolated from the rest of the tube by callose plugs,

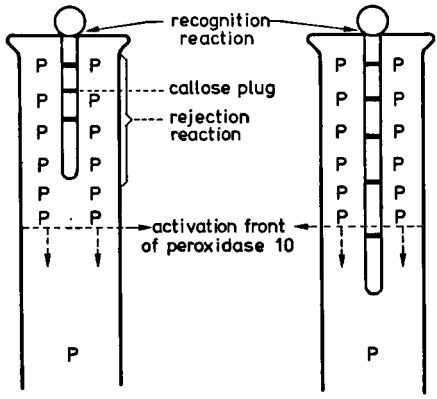


Fig. 4. Hypothesis of the role of peroxidase isoenzyme 10 in the incompatibility reaction shown schematically.

no growth inhibiting substances formed by the action of peroxidase 10 in the tube part behind the growing tip may be transported to the tip.

The idea of involvement of peroxidase 10 in the "rejection reaction" is of course rather speculative and the fact that compatible pollen tube tips grow in front of the activation wave of peroxidase 10, whereas incompatible tube tips grow behind this front might be a coincidence. In that case the increase in peroxidase 10 activity is probably only related to senescence which is accelerated by pollination (TRIPPI & TRAN THANH VAN (1971). Yet a few preliminary results appear to support, at the moment, the hypothesis of pollen tube rejection by peroxidase 10. In the first place it has been demonstrated that peroxidase is able to inhibit and stop pollen tube growth in vitro (BREDEMEIJER 1975). Furthermore, peroxidase 10 has a very low activity in self-pollinated immature styles in which the pollen tube growth is not inhibited (bud-pollination). Finally, it was found that compatible pollen tube growth is retarded in isolated styles from which peroxidase 10 activity has been increased before pollination by senescence during 7 days (BREDEMEIJER 1974). This increase already starts after two days as can be seen by comparing *fig. 1; 45, 69 and 93 hrs.* The fact that compatible pollen tube growth is also inhibited by the action of peroxidase 10 would mean that the "rejection reaction" by this isoenzyme is independent from the S-gene, in contrast with the "recognition reaction" in which S-gene specific proteins (LINSKENS 1960, MAKINEN & LEWIS 1962, LEWIS et al. 1967, KNOX & HESLOP-HARRISON 1971) are involved.

PANDEY (1967) suggested that peroxidase isoenzymes are involved in the expression of the S-alleles. He proposed that, in the case of an incompatible pollination, two identical peroxidase dimers (composed of S-dependent peroxidases), one from pollen and one from style, combine to form a peroxidase tetramer which is in some way responsible for the inhibition of pollen tube growth. As such peroxidase tetramer, according to this author, should be

formed only in self-pollinated styles, it is unlikely that this peroxidase is identical with the peroxidase 10 occurring in both self- and cross-pollinated styles. This, however, does not mean that the S-allele specific peroxidases found by PANDEY (1967) are not concerned with the incompatibility reaction. It might be possible that these peroxidases are involved in the "recognition reaction" which depends on S-allele specific substances.

4.3. Comparison of the changes in peroxidase activity during style-pollen interactions and host-parasite interactions

In an earlier report changes in peroxidase activity in self-pollinated styles were compared with data on changes in peroxidase activity in a resistant host after fungal infection (BREDEMEIJER 1974). It was suggested that the action of peroxidase in the inhibition of mycelial growth should be totally different from its possible role in the inhibition of incompatible pollen tubes. This conclusion was based upon the fact that the peroxidase activity which increases strongly during mycelial growth in a resistant host, showed only a small increase after self-pollination. Our present results do show that the interpretation is not necessarily correct because it is now clear, in the case of peroxidase 10, that the growth of incompatible pollen tube tips is associated to a high activity of this isoenzyme. Hence one may well imagine that the increase in activity of specific peroxidase isoenzymes characterizes both the self-incompatibility and the host-parasite reaction. The analogy may be of great importance in further studies on the mode of action of peroxidase 10 because many studies have been made on the role of peroxidases in the inhibition of mycelial growth in a resistant host.

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