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PEROXIDASE ACTIVITY AND PEROXIDASE ISOENZYME COMPOSITION IN SELF-POLLINATED, CROSS-POLLINATED AND UNPOLLINATED STYLES OF NICOTIANA ALATA

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SUMMARY

By means of starch gel electrophoresis and peroxidase assays a detailed analysis was made of changes in peroxidase activities and variations in peroxidase isoenzyme composition in self-pollinated, cross-pollinated and unpollinated styles of the self-incompatible species *Nicotiana alata*.

Pollination and subsequent pollen tube growth cause an increase of the total peroxidase activity and of several peroxidase isoenzymes. This increase is more pronounced in cross- than in self-pollinated styles, especially after four days when many compatible growing pollen tubes have reached the ovules. During the progamic phase only one clear difference was observed between the isoenzyme patterns of self- and cross-pollinated styles. One of the new peroxidases only appeared after cross-pollination.

The significance of the observed differences is discussed in relation to the incompatibility reaction. The results are compared with data on changes in peroxidase activity after fungal infection in plant hosts.

1. INTRODUCTION

Although several reports suggest the involvement of enzymes in the expression of the monofactorial gametophytic system of self-incompatibility (for a review, see DE NETTANCOURT 1972 and KROES 1973) little is known about the nature of these enzymes. The studies dealing with changes in enzyme activities after self- and cross-pollination revealed a considerable increase in the activity of several glucan hydrolases in cross-pollinated styles, but none or only low increases after selfing (LINSKENS et al. 1969). Histochemical studies have shown a higher activity of cytochrome oxidase, amylase and acid phosphatase in incompatible pollen tubes (SCHLÖSSER 1961). With regard to esterases the analyses by DESBOROUGH & PELOQUIN (1968) failed to reveal any apparent association between these enzymes and the incompatibility reaction. None of these studies allowed any conclusion on a causal relationship between the presence or absence of these enzymes and the manifestation of the incompatibility reaction.

In an attempt to establish a correlation between specific S-alleles and isoenzyme patterns, PANDEY (1967) reported that in *Nicotiana* styles each S-allele has its own specific peroxidase isoenzymes. ROGGEN (1967) found a correlation between glutamate dehydrogenase isoenzymes and certain S-genotypes in

Petunia hybrida. This correlation may, however, be purely casual because only two different S-genotypes with strong variations in genetic backgrounds were compared with each other.

Since none of these analyses provided any unequivocal answer to the question whether the investigated enzymes really participate in the incompatibility reaction, a research programme has been initiated which aims at a more detailed investigation on the possible involvement of these enzymes. We started with peroxidase for several reasons. In the first place, as stated above, the peroxidase isoenzyme composition in tobacco styles should be related to the S-genotype. PANDEY (1967) proposed the formation of an activated peroxidase in self-pollinated styles. Only this peroxidase would be physiologically active in producing incompatibility. In the second place, peroxidase is reported to play a role in a different inhibition mechanism occurring when mycelial growth is prevented in a resistant host-plant (MACKO et al. 1968, SEEVERS & DALY 1970, HISLOP & STAHMANN 1971). Since several analogies have been found or suggested between pollen tube growth through stylar tissues and mycelial growth through plant tissues (for a review see HOGENBOOM 1973, LINSKENS 1968a), it seems reasonable to expect that peroxidase also plays a role in the inhibition of pollen tube growth. Both systems are characterized by gene to gene relationships between the recipient tissue and the intruding cells and depend upon this relationship for germination, penetration, growth, and exchanges of substances. There is little doubt, indeed, that pollen tubes penetrate the stigma and grow through the style in a manner (enzymatical dissolution of the intercellular substance in the transmitting tissue, see SCHOCH-BODMER & HUBER 1945 and 1947, LINSKENS 1968b, LINSKENS & KROH 1970) analogous to the breakdown of cutin, pectin and cellulose after fungal infection (LINSKENS & HEINEN 1962). A third argument for choosing peroxidase is the fact that it catalyses a number of reactions which may be important in the regulation of pollen tube growth through the style. One can think, in this connection, of a number of essential reactions, such as ethylene formation (MAPSON & WARDALE 1971), destruction of toxic hydrogen peroxide (COHEN & HOCHSTEIN 1963), destruction of auxins (RAY 1958) and hydroxylation of proline in the wall (RIDGE & OSBORNE 1970).

In order to confirm or reject the hypothesis of an involvement of peroxidases in incompatibility reactions, the present study essentially aimed at the analysis of differences in peroxidase activity and peroxidase isoenzyme composition after self- and cross-pollinations.

2. MATERIAL AND METHODS

2.1. Plant material

The self-incompatible clones OWL (self-incompatibility alleles S_2S_3) and OB-2 (S_6S_7) of *Nicotiana alata* Link and Otto were grown in a climate room with 16 hours illumination (intensity about 10,000 lux) and 8 hours darkness. Temperature was maintained at 23 °C during light and 18 °C at darkness. Relative humidity was kept at 80 %.

OWL buds were collected just before anthesis. After anther removal 18 flowers were cross-pollinated with fresh OB-2 pollen. The same number of flowers was self-pollinated and left unpollinated. The flowers were placed in distilled water and stored in a climate room (16 hours illumination of approximately 8,000 lux; 8 hours darkness; temperature 15°C; relative humidity 80%) for the chosen intervals of time. Then the styles were collected and used for extraction or determination of pollen tube length by means of the U.V. microscope technique of MARTIN (1959).

2.2. Pollen germination and tube growth in vitro

Pollen germination occurred in an aerated gas washing tube placed in a water bath at 25°C. The culture vessel contained a medium consisting of 15% sucrose and 30 ppm boric acid as recommended by DEAN (1964) for tobacco pollen. A concentration of 10 mg pollen in 1 ml of the germination medium was used.

2.3. Extraction procedure, electrophoresis and peroxidase assay

Style extracts were prepared as described earlier (BREDEMEIJER 1973a). Pollen extracts were prepared by homogenizing 50 mg pollen at 0°C in a Potter Elvehjem homogenizer with 0.8 ml 4% NaCl. The supernatants of style and pollen extracts after 45 minutes centrifugation at 18,000 g and 2°C were used for electrophoresis and peroxidase assay (BREDEMEIJER 1973a).

Starch gel electrophoresis and staining method were modified for a better resolution and stainability of the bands (DOEKES 1968; BREDEMEIJER 1973a). Horizontal starch gel electrophoresis occurred in a Shandon electrophoresis apparatus (model U77) using aluminium lactate-lactic acid buffer, pH 3.40, with an ionic strength of 0.06 in the gel and electrode boxes. Gels (17%; final pH 4.7) containing 3M urea were prepared by pouring the gelatinised and degassed starch into trays (18.5 × 10 × 0.5 cm). After three hours of cooling and drying under constant temperature (22°C) and relative humidity (about 75%) the gels were covered with PVC film and kept at 0–4°C for 2 days. Samples were applied to the gel in slots (8 × 0.8 × 5 mm) cut with the aid of a holder with pieces of a razor blade. The electrophoresis apparatus was placed in a refrigerator at -1 ± 1°C and a voltage of 300 V at 16 mA was applied to the gel for 5½ hours. After electrophoresis the topmost layer of the gel was removed with the Shandon slicer.

The gel was stained for peroxidases by incubation during 15 minutes in 0.1% benzidine solved in 100 ml of 0.2 M acetate buffer pH 5.0 and 2 ml 3% H_2O_2. Then it was rinsed in tap water and photographed with a polaroid camera.

As small differences in pH, molarity of the buffer, and percentage of the gel may cause qualitative and quantitative variations in the isoenzyme patterns, all procedures and especially those concerning the preparation of the gels, were carried out under exactly the same conditions.

3. RESULTS

3.1. Variations in the total peroxidase activity of self- and cross-pollinated styles

The alterations in peroxidase activity per pollinated style were calculated by substracting the value for activity of unpollinated styles from the comparable pollinated styles and are shown in *fig. 1.*

Pollination has no immediate effect on the total peroxidase activity of the style (see t = 0 hr). The addition of the peroxidase content of the pollen used for pollination could not be detected. This can be explained by the fact that the peroxidase activity of the 0.5 mg pollen used to pollinate one style is very small as compared to the activity of one unpollinated style ($\triangle$A470/min/ 0.5 mg pollen is about 0.001 as found by calculation from the activity of 50 mg pollen; $\triangle$A470/min/style = 0.056).

During the first day after self- and cross-pollination the peroxidase activity remains more or less constant. The period between one and four days after pollination is characterized by a slow but steady increase in peroxidase activity. This increase is most pronounced in the cross-pollinated styles. After about 88 hrs at the relatively low temperature of 15 °C many compatible growing pollen tubes are observed at the end of the style and peroxidase activity then increases strongly. In the same period, incompatible tube growth stops nearly half-way in the style and the peroxidase activity of the style only slightly increases.

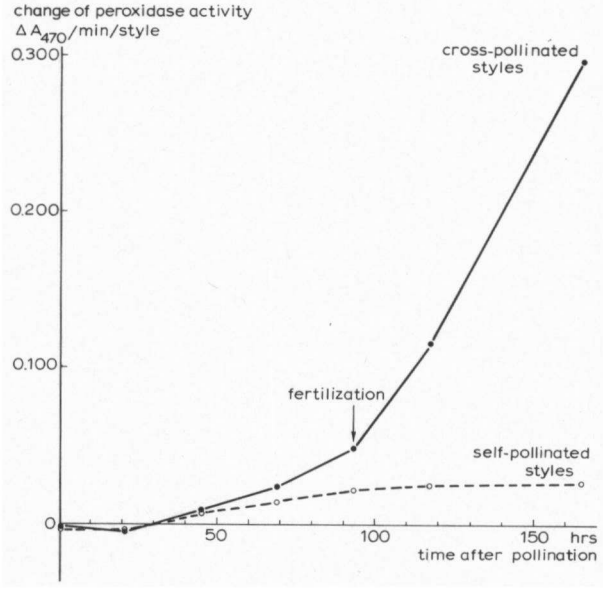

Fig. 1. Changes in peroxidase activity in self- and cross-pollinated tobacco styles. The values in this figure are averages of the results of 2–5 experiments.

3.2. Comparison of the peroxidase isoenzyme patterns of un-, self-, and cross-pollinated styles

The electrophoretic patterns for un-, self-, and cross-pollinated styles at various times after pollination are presented in a diagram *(fig. 2)*. *Fig. 3* shows a photograph of the peroxidase patterns of styles 7 days after pollination. The first effect of pollination on the peroxidase isoenzyme pattern of the style is the appearance of the faint bands 3, 4 and 5 which correspond to pollen peroxidases (for a comparison between peroxidase patterns of the style at t = 0 hr and of the pollen, see *fig. 2*).

During pollen tube growth through the style the activity of several peroxidase isoenzymes gradually increases, whereas only one (16) decreases. Peroxidase 1, 2 and 10 also increase in senescing unpollinated styles, but this effect is less pronounced than in pollinated styles. Compatible growing pollen tubes need almost four days to pass through the whole style. During this period (progamic phase) two new peroxidases (8 and 13) appear in cross-pollinated styles, whereas only one new peroxidase (13) was observed after selfing.

After 93 hrs differences between self- and cross-pollinated styles become very clear (see also *fig. 3*). Many peroxidase isoenzymes in cross-pollinated styles increase strongly, whereas the corresponding isoenzymes in the self-pollinated styles increase slightly. Furthermore, a number of new peroxidases (9, 11, 19 and 21) becomes only visible in the pattern of the cross-pollinated styles.

As indicated in *fig. 2*, OB-2 pollen peroxidases could not be detected in unpollinated OWL styles. Pollen germination and tube growth in vitro (5 hrs) cause only a decrease of peroxidase 22. No peroxidases occurring in OWL styles

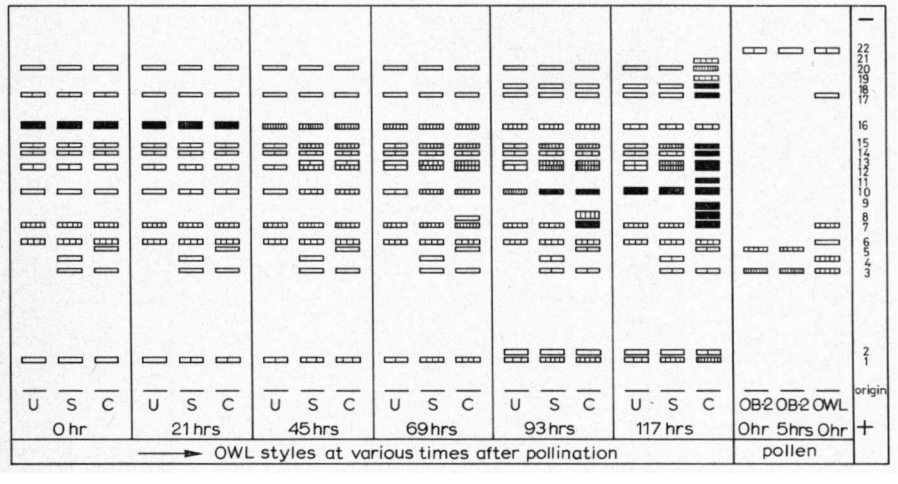

Fig. 2. Diagram of the electrophoretic patterns of peroxidases from un- (u), self- (s), and cross-pollinated (c) OWL styles at various times after pollination and from germinated OB-2 (5 hrs) and ungerminated OB-2 and OWL pollen (0 hrs). The degree of hatching approximates the staining intensity. No hatching means that the bands on the gels were just visible.

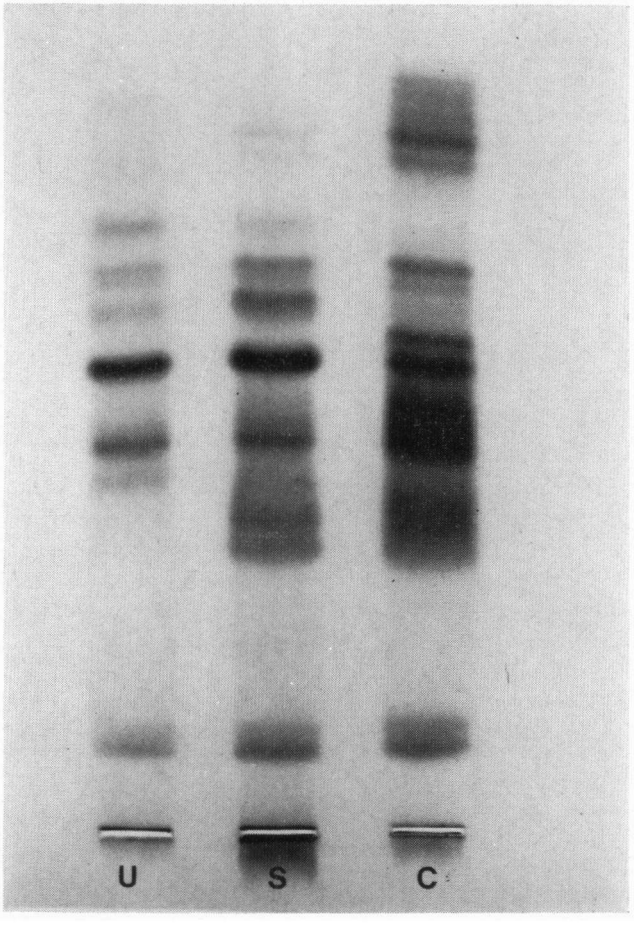

Fig. 3. Photograph of the peroxidase isoenzyme patterns of un- (u), self- (s), and cross-pollinated (c) OWL styles 7 days after pollination. The extract of the cross-pollinated styles was diluted three times.

were found in germinated OB-2 pollen. It is, therefore, likely that the increase of style peroxidases is not only caused by addition of the pollen peroxidase content but also by the interaction between growing pollen tubes and style tissue.

Although pollen peroxidases 3, 4 and 5 appeared to increase during the progamic phase, the staining intensities of these bands were too low to study a possible relation between changes in pollen peroxidases and incompatibility reaction.

4. DISCUSSION

4.1. Changes in peroxidase activity and peroxidase isoenzyme composition in cross-pollinated styles

The data presented here demonstrate that the progamic phase during which the compatible pollen tubes grow through the style to the ovary is characterized by

an increase in total peroxidase activity. This is in agreement with the work of ROGGEN (1967) and LINSKENS et al. (1969) who reported an increase in activity of several enzymes after cross-pollination. The peroxidase isoenzyme patterns show that several peroxidases increase in activity and that two new isoenzymes are formed.

The marked increase in peroxidase activity starting four days after cross-pollination is certainly not a prerequisite of pollen tube growth as many tubes already have reached the ovary. This increase is the result not only of the interaction between pollen tubes and style but also of the fact that pollen tubes have reached the ovary. Certain style peroxidases (2, 17, 19, 20 and 21) which started increasing four days after cross-pollination of detached flowers did not increase when isolated styles were cross-pollinated. Apparently the increase of these isoenzymes is caused by an activation of the growth centre in the ovary which may regulate the enzyme pattern (TRIPPI & TRAN THANH VAN 1971).

Pollination and subsequent pollen tube growth accelerate the increase of some peroxidase isoenzymes which also takes place in senescing unpollinated styles. This is in agreement with the finding that pollination accelerates the increase in activity of corolla peroxidases caused by senescence (TRIPPI & TRAN THANH VAN 1971). Several style peroxidase isoenzymes, however, which increased in activity in pollinated styles did not do so in unpollinated styles, even after 22 days of senescence, when the flowers already became quite brown. This suggests that the increase of style peroxidases after pollination is not only related to accelerated senescence but probably also to another process caused by an interaction between pollen tubes and styles.

4.2. Differences in peroxidase activity and peroxidase isoenzyme patterns between self- and cross-pollinated styles in the progamic phase

The increase in total peroxidase activity of the style is lower after self- than after cross-pollination. During the progamic phase (0–88 hrs) only one clear difference was observed between the peroxidase isoenzyme patterns of self- and cross-pollinated styles. Two new peroxidases (8 and 13) were formed in cross-pollinated styles, whereas only one of them (13) was formed after selfing. It is not known yet whether this difference is a prerequisite or a consequence of differences in pollen tube growth. If the activity of the new peroxidase (8) is a prerequisite for continuation of pollen tube growth, prevention of formation or inhibition of this isoenzyme in the self-pollinated style might be a part of the chain of biochemical events leading to inhibition of pollen tube growth. The present results suggest that peroxidase is probably not involved in the incompatibility reaction in the manner proposed by PANDEY (1967) since no new peroxidase isoenzyme specific for self-pollinated styles could be observed (see previous discussion by BREDEMEIJER 1973b). Therefore, further investigations will be carried out in order to establish how the changes in peroxidase activity are induced and to what extent they influence pollen tube growth.

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

When the results obtained are compared with fungal infection it is clear that with our system peroxidase probably does not play a role in the inhibition of incompatible pollen tube growth in a way as proposed for inhibition of mycelial growth in a resistant host. The marked increase in peroxidase activity after fungal infection in a resistant host is suggested to be responsible for inhibition of mycelial growth through participation in biosynthesis of phenolic compounds or by direct inhibition of fungal growth (for a review, see SEEVERS et al. 1971). Although fungal infection in a plant host (MACKO et al. 1968, HISLOP & STAHMANN 1971, JOHNSON & CUNNINGHAM 1972) and pollination of a style both induce an increase in total peroxidase activity and the formation of new isoenzymes, there are some essential differences. Inhibition of mycelial growth by a resistant host is accompanied by a considerable increase in peroxidase activity as compared to the increase in a susceptible host, whereas inhibition of incompatible pollen tube growth is characterized by a lower increase in peroxidase activity as compared to cross-pollinated styles. This should mean that, thinking in terms of disease resistance, cross-pollinated styles should contain a higher amount of phenolic oxidation products than self-pollinated styles. In that case, neither phenolic oxidation products nor peroxidase itself apparently inhibit pollen tube growth. It is, however, also possible that the higher increase in peroxidase activity after cross-pollination is a consequence of, not a determinant in continuation of pollen tube growth analogous to the idea of LUDDEN & DALY (1970) and SEEVERS et al. (1971) that the association of peroxidase with resistance is a consequence of resistance. The proposition that variations in peroxidase activity can be considered as an additional common point between pollen tube-style incompatibility relationships and host-parasite associations can probably be ruled out.

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