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PEROXIDASE ACTIVITIES AND PEROXIDASE ISOENZYME PATTERNS DURING GROWTH AND SENESCENCE OF THE UNPOLLINATED STYLE AND COROLLA OF TOBACCO PLANTS

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SUMMARY

During flower development in tobacco (*Nicotiana alata* Link & Otto) changes in peroxidase activity in the unpollinated style and in the corolla were determined both qualitatively and quantitatively. In both the peroxidase activity increases during maturation, especially towards the end of the growth period. Immediately after anthesis the peroxidase activity in the style decreases during two days, followed by a strong increase. The changes in peroxidase activity in the style are different from those in the corolla.

The alterations of isoenzyme patterns during flower development are primarily quantitative rather than qualitative. Only one new isoenzyme appears during the growth in style and corolla.

The possible relationship between the variations in peroxidase activity in the style and the physiological barrier preventing self-fertilization is discussed.

1. INTRODUCTION

The possible correlation between the peroxidase isoenzyme pattern of the style and its S-genotype (specificity for self-incompatibility) was studied by PANDEY (1967), DESBOROUGH & PELOQUIN (1968), and NASRALLAH et al. (1970). Only PANDEY (1967) found a positive correlation: each S-allele in the self-incompatible species *Nicotiana alata* had its own specific peroxidase isoenzymes. He suggested that the polypeptide molecule specified by the structural cistron of the S-gene complex is peroxidase.

In immature styles the incompatibility substances are absent or not fully effective. The physiological barrier preventing self-fertilization is built up during the growth of the style (LINSKENS 1964). This principle has been used in bud-pollinations of self-incompatible species (DE NETTANCOURT 1972). In *Nicotiana alata*, for example, seed set was increased more than two hundred-fold by self-pollination (with pollen harvested from a mature flower) in the bud stage (PANDEY 1963).

Assuming that a number of peroxidase isoenzymes is really specified by the S-gene, it seems likely that the peroxidase isoenzyme composition depends on the developmental stage of the style. Using immature styles it may be possible to study the building up of the biochemical barrier controlling self-incompatibility. Therefore, we studied developmental changes in peroxidase activity and isoenzyme polymorphism in the style. Because of its influence on style elongation the corolla was used for an identical study.

Another purpose of our investigation was to explore the potential application of starch gel electrophoresis for studying the relationship S-genotype – peroxidase isoenzymes in styles, leaves, and pollen. Some preliminary results dealing with peroxidases from leaves with various S-alleles have been published (BREDEMEIJER 1972).

2. MATERIAL AND METHODS

2.1. Plant material

The self-incompatible clone (S_3S_3) of *Nicotiana alata* Link & Otto was propagated by adventitious bud formation (DE NETTANCOURT et al. 1971). Leaves from a plant obtained by self-pollinating a S_2S_3 plant were used. The plants grew in a regular greenhouse (day temperature 25°–30°C; night temperature 22°–25°C). Flowers were collected at various developmental stages. The length of the style was used for determining the stage of development until anthesis; thereafter intervals of age measured in hours were used.

2.2. Extraction procedure

Different extraction procedures were tried out (VAN LOON & VAN KAMMEN 1968; DESBOROUGH & PELOQUIN 1968; DOEKES 1968; BREWBAKER et al. 1968) but the best result was obtained by using 3 to 5% NaCl solutions. This method was selected because these extracts revealed a higher peroxidase activity and a higher number of isoenzymic bands than the extracts prepared with the other procedures.

The extracts were prepared by homogenizing equal portions (0.4 g fresh weight) of styles or corollas for 3 minutes in an icecooled mortar with pure quartz sand and 1.2 ml 4% NaCl solution in distilled water. The supernatant obtained after centrifugation for 45 minutes at 18,000 g at 2°C was immediately used for electrophoresis and the peroxidase assay.

2.3. Starch gel electrophoresis

Horizontal starch gel electrophoresis was performed according to the technique of DOEKES (1968) using aluminium lactate – lactic acid buffer, pH 3.1, with an ionic strength of 0.06. A voltage of 300 V at 16 mA was applied to a gel (18.5 × 10 × 0.5 cm) during 5 hrs at 4°C. After slicing off the topmost layer, the gel was stained for the peroxidases by incubating for 15 min in a solution containing 0.1% benzidine in 70 ml of 96% ethanol, 28 ml of 0.2 M acetate buffer at pH 5.0 and 2 ml 3% H_2O_2. The gels were fixed in 2% acetic acid. Since several bands faded rapidly registration of the results was carried out within half an hour.

2.4. Peroxidase assay and protein determination

Peroxidase activity was measured by following spectrophotometrically (Unicam Sp. 700) the change in absorbance at 470 nm and 25°C which is due to guaiacol oxidation in the presence of hydrogen peroxide and enzyme. The reaction

mixtures contained 0.1 ml of 0.5% guaiacol, 0.1 ml of extract, 2.80 ml of 0.05 M acetate buffer at pH 5.0, and 0.1 ml of H_2O_2 solution (0.1 ml perhydrol from Merck in 50 ml distilled water). The peroxidase activity was expressed as ΔA 470/5 min/mg protein.

After precipitation with 10% trichloroacetic aced proteins were determined according to the method of LOWRY et al. (1951). Crystalline bovine albumine, Cohn Fraction V (Calbiochem., Lucerne, Switzerland) was used as a standard.

3. RESULTS

3.1. Variations in the total peroxidase activities during growth and senescence of the style and the corolla of unpollinated flowers

This experiment was carried out three times. In spite of the fact that the extraction and assay procedures in the three independent experiments were the same, the level of peroxidase activity varied somewhat from experiment to experiment. However, the trend was always the same. Differences in illumination (SIEGEL & GALSTON 1967) and variations from plant to plant (LAVEE & GALSTON 1968) are probably involved in these discrepancies.

The results are represented in *fig. 1*. During elongation of the style from 17 mm to 50 mm, only a slight increase of the specific peroxidase activity occurs. On the contrary, towards the end of the growth period the peroxidase activity increases strongly. The optimum is reached during opening of the flower bud (anthesis) when the style ceases growing (maximum length 59.2 $\pm$ 0.9 mm). From that moment the peroxidase activity shows a decrease during about two days, followed by a steep increase. During the last period the corolla becomes limp and starts browning.

If the same experiment is carried out with the corolla (*fig. 1*) the alterations of the peroxidase activity give a somewhat different pattern. Prior to anthesis the peroxidase activity of the corolla oscillates, whereas the activity in the style shows a steady increase. In the corolla the following optimum in peroxidase activity is reached just before anthesis (9 hours in daylight); in the style it is reached during anthesis. Moreover, it appears that the peroxidase activity of the corolla is much higher than that of the style.

Fig. 2 shows that the protein content, expressed as mg protein/mg dry weight, changes during growth and senescence. This means that the changes in specific peroxidase activity are due both to changes in the effective amount of peroxidase and to alterations in the protein content. Nevertheless, should the amount of dry weight be used as reference for peroxidase activity, the shape of the curves would not show striking deviations, except that the upward slope during senescence would be less pronounced for both style and corolla.

3.2. Comparison of the peroxidase isoenzyme patterns of styles and corollas at different developmental stages

The electrophoretic patterns for styles and corollas at various developmental stages are presented in diagrams (*figs. 3* and *4*). The distribution of peroxidase

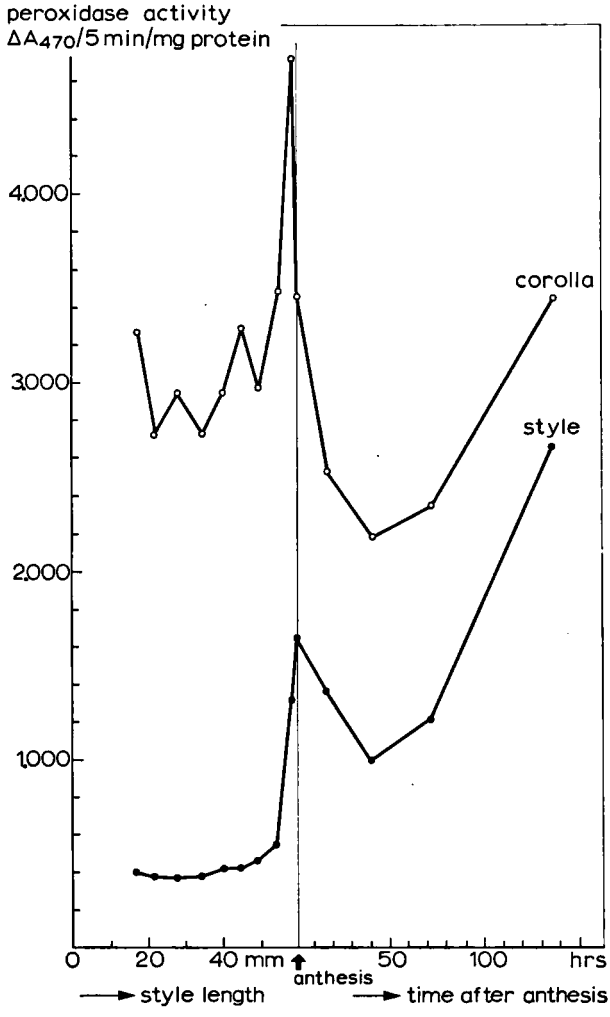

Fig. 1. Variations in the specific peroxidase activity of the corolla and the style of *Nicotiana alata* during development and senescence of the flowers.

isoenzymes is tissue-specific. The style has up to 13 isoenzymes and the corolla up to 14 isoenzymes. Most isoenzymes of the style and the corolla are common ones, i.e. peroxidases with the same electrophoretic mobility; a few are specific ones. It is difficult to compare both patterns quantitatively since the protein content of the extracts used was not the same. Until anthesis style extracts contain approximately twice as much protein as corolla extracts (*fig. 2*). In spite of this fact, several peroxidase bands of the corolla have a higher staining intensity than the corresponding bands in the isoenzyme pattern of the style. So there are quantitative differences indeed.

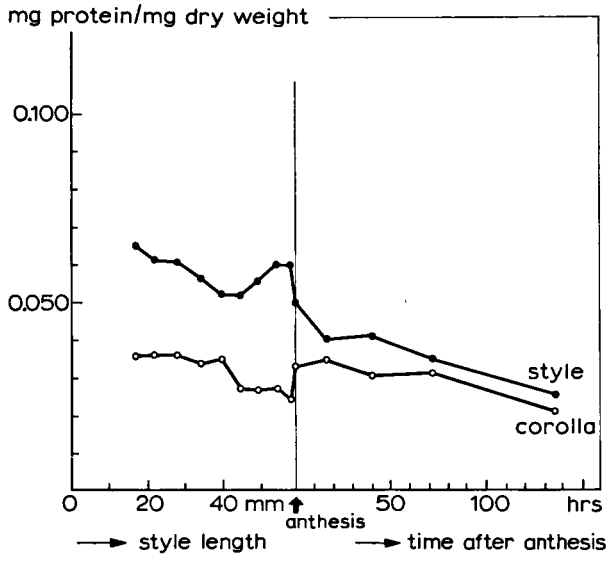

Fig. 2. Changes in protein content of the corolla and the style of *Nicotiana alata* during development and senescence of the flowers.

From *figs. 3* and *4* it can also be seen that a number of peroxidase isoenzymes exhibit quantitative differences at different developmental stages. The developmental differences observed in the corolla are not identical with those observed in the style. The increase of the staining intensity of band 2 in the pattern of the style is stronger than in the pattern of the corolla. During growth the increase in intensity of band 4 of the style is attended with a decrease of the corresponding band of the corolla. Moreover, the activity of peroxidase isoenzyme 11 in the corolla reaches an optimum before that of the same peroxidase in the style. The decrease of peroxidase isoenzyme 13 in the style and the increase of the numbers 6, 7 and 8 in the corolla could not be compared with possible alterations in the corresponding bands of the other flower part since the latter bands were too weak.

During development of the style and the corolla only one new peroxidase isoenzyme becomes visible in the zymograms. This band (No. 11 in *figs. 3* and *4*) is not detectable until the length of the style reaches 50 mm. Its activity appears to become progressively stronger, reaching an optimum level just before anthesis in the corolla and during anthesis in the style. Two days after anthesis only a weakly detectable band 11 can be observed.

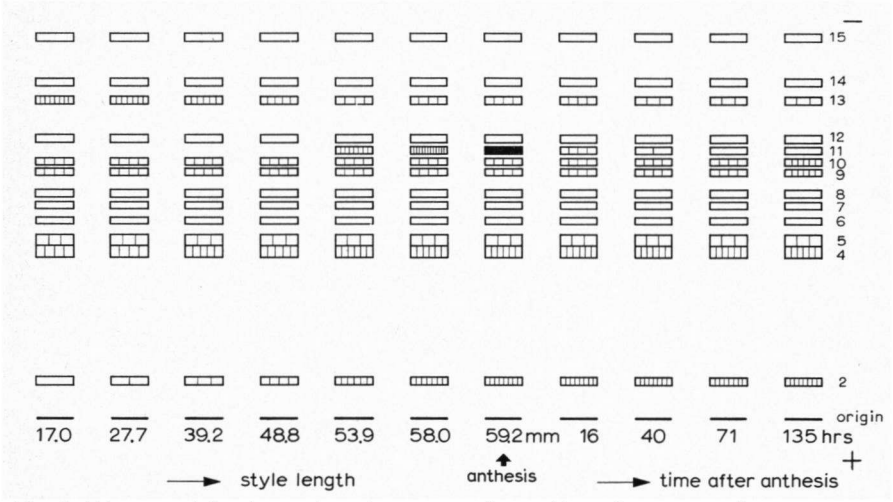

Fig. 3. Diagram of the electrophoretic patterns of peroxidases from styles of *Nicotiana alata* at different developmental stages. The degree of hatching approximates the staining intensity. No hatching means that the bands on the gels were just visible.

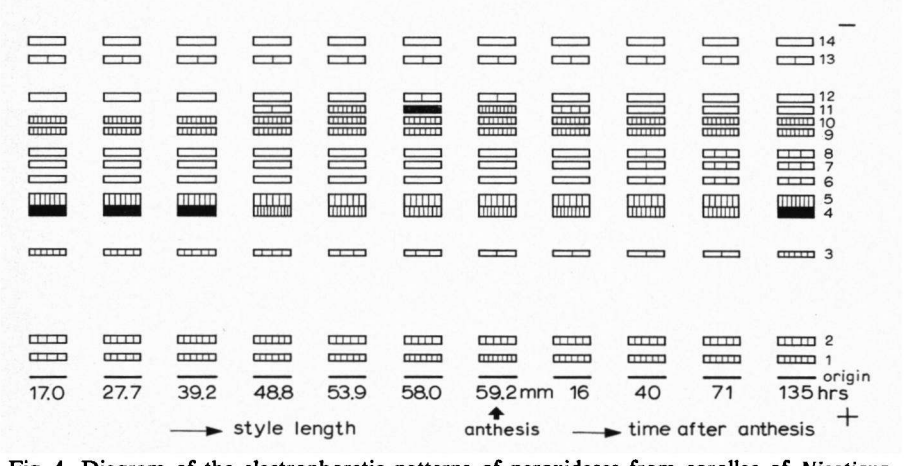

Fig. 4. Diagram of the electrophoretic patterns of peroxidases from corollas of *Nicotiana alata* at different developmental stages. The degree of hatching and the numbering of the isoenzymes correspond to those in *fig. 3.*

4. DISCUSSION

From the literature it is known that development and senescence of plant tissues involves many alterations in protein and enzyme composition (FLETCHER & OSBORNE 1965; SHANNON 1968; SCANDALIOS 1969; SPENCER & TITUS 1972). Similar changes were found in several parts (pollen, tapetal cells, style, and corolla) of the flower (LINSKENS 1966; DESBOROUGH & PELOQUIN 1968; TRIPPI & TRAN THANH VAN 1971).

Concerning peroxidase, it was observed that the total activity and the isoenzyme polymorphism increase with increasing maturity of the tissue (OCKERSE et al. 1966; HART & BHATIA 1967; LAVEE & GALSTON 1968; HAMILL & BREWBAKER 1969; CHAPPET 1970; KAMINSKI 1971). The results of our experiments suggest the same: during maturation the peroxidase activities of the style and the corolla increase and the peroxidase isoenzymes show qualitative and quantitative alterations. Only one new peroxidase isoenzyme is induced in the style and in the corolla of *Nicotiana alata*, whereas five new peroxidases appear in the corolla of *Phalaenopsis amabilis* (TRIPPI & TRAN THANH VAN 1971). This difference is probably due to variations in experimental procedures.

During style elongation a slight increase of peroxidase activity occurs. Towards the end of the growth period this activity, however, rises sharply. These findings corroborate the existence of an inverse correlation between peroxidase activity and growth potential (intensity and duration of growth) reported by LAVEE & GALSTON (1968) and CHAPPET (1970). The increase in total peroxidase activity is caused by the increase of certain peroxidase isoenzymes (2, 4 and 11 in the style; 1 and 11 in the corolla). As it is known that several peroxidase isoenzymes are active in auxin destruction (SHANNON 1968; FRENKEL 1972) and that variations in peroxidase activity in growing coleoptiles run parallel with variations in auxin oxidase activity (CHAPPET 1970), it seems likely that certain of the peroxidase isoenzymes which increase during growth of the style and the corolla are involved in the suppression of auxin activity and, consequently, in growth regulation.

The developmental alterations in the peroxidases occurring in the style and in the corolla are not identical in both tissues (see section 3.2.). Some of these differences, for example the increase of a peroxidase isoenzyme in one part of the flower combined with a decrease of the same isoenzyme in another part, may be explained by assuming a transport of the isoenzyme itself or rather a transport of substances regulating synthesis or activity of peroxidase isoenzymes. Auxin, for example, may induce some and repress other peroxidase isoenzymes (SHANNON 1968). Several substances are known to be transported from the corolla to other parts of the flower (TRIPPI & TRAN THANH VAN 1971). Moreover, the strong inhibition of style elongation caused by damaging or taking away parts of the corolla (LINSKENS 1964) also suggests a transport of certain substances.

Two lines of evidence appear to indicate a specific relation between peroxidase isoenzymes and the biochemical mechanism preventing self-fertilization. Firstly,

each S-allele has its own specific peroxidase isoenzymes (PANDEY 1967). Secondly, the present study shows that the increase of peroxidase isoenzyme 11 during the growth of the style and its decrease after anthesis (*fig. 3*) run more or less parallel with the effect of floral development and floral ageing on incompatible pollen tube growth (PANDEY 1963; ASCHER & PELOQUIN 1966). Some other facts, however, seem to deny the relationship as stated above. The alterations in the esterase isoenzyme pattern which is not related to the S-genotype also run parallel with the effect of ageing on incompatible pollen tube growth. Moreover, the new peroxidase isoenzyme also appears in the corolla, which tissue does not participate in the incompatibility reaction. Therefore, the alterations in peroxidase isoenzyme 11 might only reflect metabolic changes related to ageing.

None of these arguments, however, exclude the possibility of a non-specific influence on the incompatibility reaction by peroxidases which play a role in several important processes (MCCUNE 1961; MACNICOL 1966). This means that changes in the other peroxidase isoenzymes which do not show parallelism with the effect of ageing on self-incompatible pollen tube growth may also cause alterations in the incompatibility barrier. It is obvious that a more detailed study will be necessary for defining the exact role of peroxidases in the manifestation of self-incompatibility.

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