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EFFECT OF BUD-POLLINATION AND DELAYED SELF-POLLINATION ON THE INDUCTION OF A POSSIBLE REJECTION PEROXIDASE IN STYLES OF NICOTIANA ALATA

G. M. M. BREDEMEIJER

Instituut voor Toepassing van Atoomenergie in de Landbouw (ITAL), Wageningen

SUMMARY

The pollination-induced wave of peroxidase isoenzyme 10 activity in mature *Nicotiana alata* styles does not occur in immature styles unless pollinated within one day before anthesis. The capacity of a style to increase peroxidase 10 activity arises at the same time as the shift from compatibility to incompatibility. In styles selfed at 4 days after anthesis the peroxidase 10 wave is transmitted with a higher speed as compared to styles selfed at anthesis.

The present results are discussed in relation to the hypothesis that peroxidase 10 is involved in the rejection of incompatible pollen tubes. It is not clear yet whether the presence of this isoenzyme is necessary to stop incompatible pollen tube growth. However, there exists a positive correlation between peroxidase 10 activity and the strength of the rejection reaction.

1. INTRODUCTION

In a previous study it has been suggested that peroxidase isoenzyme 10 in styles of *Nicotiana alata* might be involved in the rejection of incompatible growing pollen tubes (BREDEMEIJER & BLAAS 1975) because the tips of these tubes grow in a stylar part with a high peroxidase 10 activity, whereas compatible tube tips grow in a part with a very low activity of this isoenzyme. In order to confirm or reject this hypothesis, the influence of various techniques available for obtaining a temporary breakdown of the self-incompatibility reaction (DE NETTANCOURT 1972) on the activity of peroxidase 10 will be studied. The work reported here aimed at analysing the effect of bud self-pollination on the induction of peroxidase 10 and to find out if the shift from compatibility to incompatibility in *Nicotiana alata* buds is correlated to alterations in the pollination-induced increase in peroxidase 10 activity.

In 1964 LINSKENS suggested that the principle of self-compatibility following bud pollination is based on the fact that the incompatibility substances are absent or not fully effective in developing styles. It has recently been reported that the self-incompatibility proteins are present at very low concentrations (NASRALLAH 1974) in the immature stigma of *Brassica oleracea* where self-incompatibility is sporophytic. The shift from compatibility to incompatibility two days before anthesis is abrupt, a result which correlates with the sudden increase in the level of these proteins. Concerning the gametophytic incompatibility system no biochemical data are available to explain the absence of pollen tube rejection after bud self-pollination.

Since it is known that ageing processes in detached unpollinated flowers cause a slight increase in peroxidase 10 activity (BREDEMEIJER & BLAAS 1975) a second purpose of this study was to examine incompatible pollen tube growth rate and peroxidase 10 activity after delayed self-pollination. Assuming that such an increase is accompanied by a stronger inhibition of incompatible pollen tubes in aged flowers the hypothesis that peroxidase 10 is indeed involved in the rejection mechanism seems more likely.

2. MATERIAL AND METHODS

Plants of *Nicotiana alata* Link and Otto (OWL and OB-2 clone) were grown in a controlled environment room with a light intensity of 10,000 lux during a 16-hour photoperiod and a temperature of 23 and 18 °C during the light and dark period, respectively. Relative humidity was kept at 80%. OWL buds were collected at various developmental stages which were expressed as corolla-tube length. After anther removal and application of stigmatic secretion from 3 ripe OWL stigmas to the dry stigma of the immature style, the buds were self- or cross-pollinated and incubated in a climate room (light intensity 8,000 lux during 16 hours; temperature 15 °C; relative humidity 80%). In order to study seedset and changes in peroxidase 10 activity in attached flowers a number of pollinations were made on the plants in the climate room at 23 °C.

At chosen intervals after pollination, styles were collected and used for preparing extracts or determination of pollen tube lengths (BREDEMEIJER 1974, BREDEMEIJER & BLAAS 1975). The distance which the ten fastest-growing pollen tubes had covered through the style was measured. Extracts prepared from whole styles or from segments of one cm were used for electrophoretical separation of the peroxidase isoenzymes on starch gels (BREDEMEIJER 1973, 1974). After development, the sliced gels were photographed with a polaroid camera. The photographs were scanned by reflection with a Vitatron Manual TLD 100 densitometer.

3. RESULTS

3.1 Pollen tube growth in detached buds of varying developmental stages

According to PANDEY (1963), the critical stage for successful bud pollination in *Nicotiana alata* corresponds to a bud length of about half the length of a mature flower, younger or older buds failing to respond. To determine more precisely the time at which the incompatibility reaction becomes effective, the influence of the developmental stage, expressed as corolla-tube length, on pollen tube growth was investigated.

In buls of length 3.0–5.5 cm incompatible pollen tubes almost attain the same length as compatible tubes (*fig. 1*). In these buds, pollen tubes increase in length with increasing bud length. Maximum growth rate of compatible pollen tubes is reached in buds of length 5.5–6.5 cm, whereas maximum growth

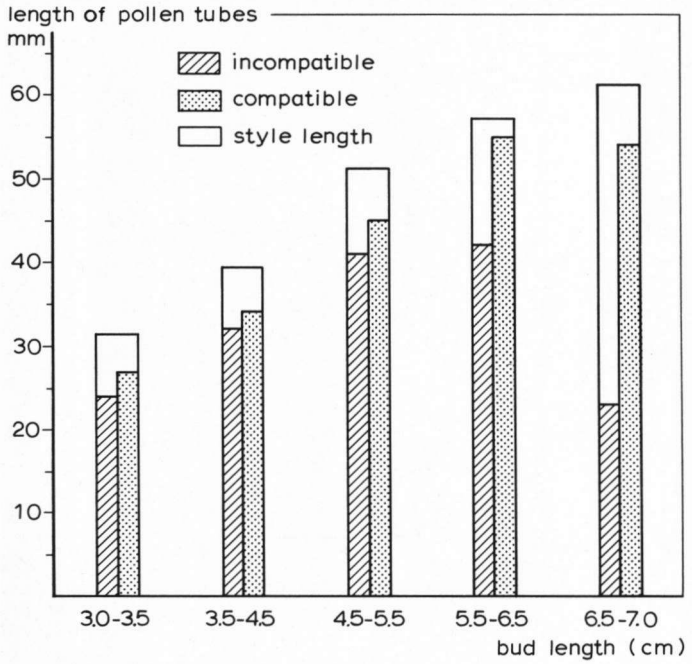

Fig. 1. Mean length of compatible and incompatible pollen tubes after 3 days of growth at 15 °C in detached *Nicotiana alata* buds of different lengths. Values represented were obtained from no fewer than 20 styles. Bud lengths of 6.5–7.0 cm are reached at anthesis.

of incompatible tubes is already reached in buds of length 4.5–5.5 cm. Apparently, the shift from compatibility to incompatibility starts in buds of length 5.5–6.5 cm. In buds of length 6.5–7.0 cm, that is to say at anthesis, incompatible pollen tubes reach about one-half the length of compatible tubes.

Buds of 3.5–6.5 cm left on the plant set seed upon selfing. The number of seeds is, however, strongly reduced when the bud length exceeds 6.2 cm. Selfed buds of length 6.0–6.2 cm produce an average of 300 seeds per fruit, whereas buds of 6.3–6.5 cm produce always less than 150 seeds per fruit. In other words the incompatibility character increases clearly when the bud length exceeds 6.2 cm, that is to say within one day before anthesis.

3.2 Comparison of the peroxidase isoenzyme patterns of styles selfed at various developmental stages

The problem was thus to find out if the shift from compatibility to incompatibility during the last day before anthesis is accompanied by a change in the pollination-induced increase in peroxidase 10 activity. The first step for answering this question involved a comparison of peroxidase isoenzyme patterns of immature and mature styles at 3 days after selfing. The densitograms presented in *fig. 2* show a higher peroxidase 10 activity in mature styles as

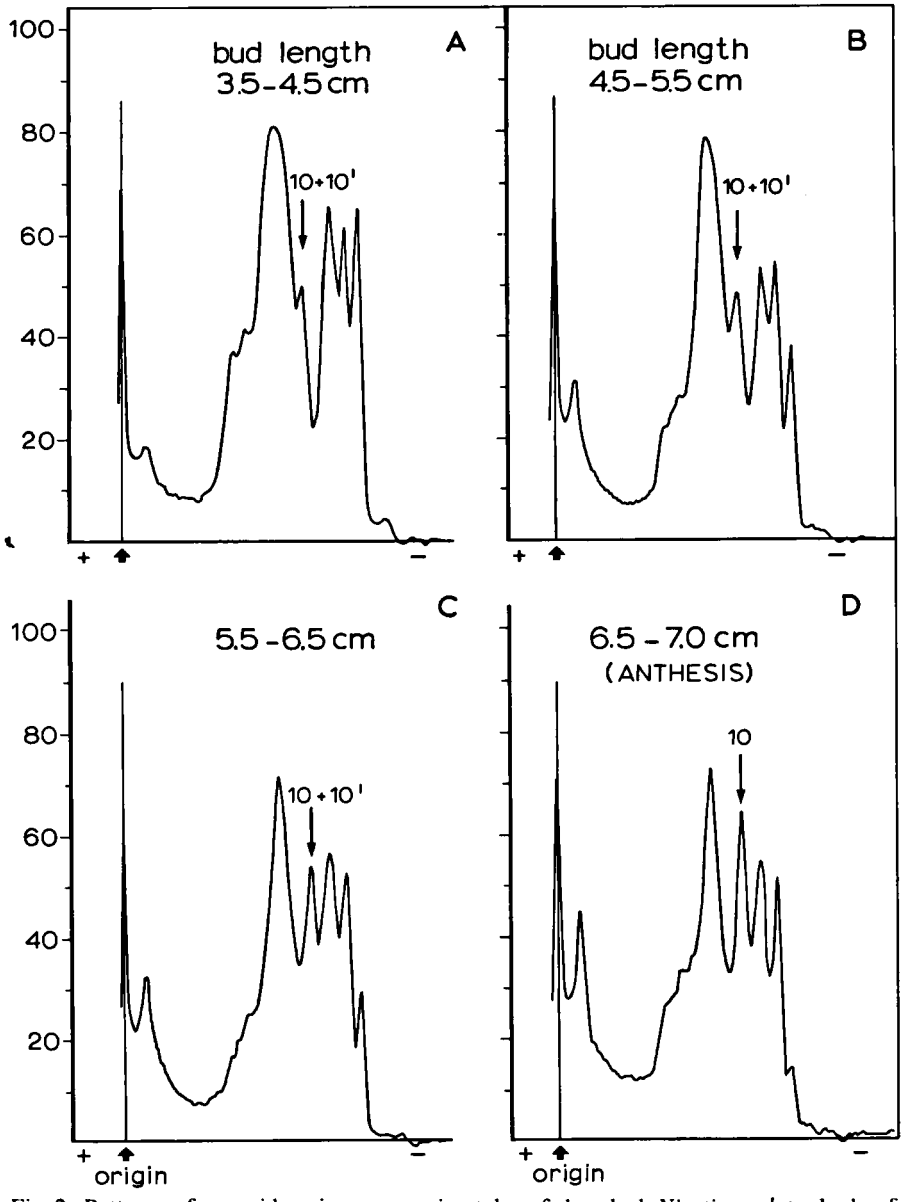

Fig. 2. Patterns of peroxidase isoenzymes in styles of detached *Nicotiana alata* buds of various lengths at 3 days after self-pollination (15° C) obtained by scanning the photographs of starch gels by reflection with a densitometer.

compared to that of immature styles. In fact, the difference between peroxidase 10 activity in young styles *(fig. 2a)* and mature styles *(fig. 2d)* is even greater than it seems from the densitograms because the immature styles also contain a peroxidase 10' which can hardly be separated from peroxidase 10 by the method used. The peroxidase 10 peak in the densitograms thus represents the sum of the two peroxidases in the buds and only peroxidase 10 in mature styles because interpretation of the gels revealed that peroxidase 10' decreases during stylar development and is not detectable in mature styles *(fig. 3)*. Whatever the actual increase in peroxidase 10 would turn out to be if one could clearly dissociate peroxidase 10 from peroxidase 10', it is clear that the pollination-induced increase in peroxidase 10 activity is strongest in mature styles. The main shift in this increase takes place during the last day before anthesis (compare *fig. 2c* and *d*).

3.3 Distribution of peroxidase isoenzyme 10 in styles selfed at varying developmental stage

In self-pollinated buds of length 3.0–5.5 cm peroxidases 10 and 10' only occur in stylar segment 1 *(fig. 3)*. In buds selfed at anthesis, on the contrary, peroxidase 10 is present in all segments with a considerable activity in segment 2. Buds of 5.5–6.5 cm represent an intermediate situation where peroxidase 10 is weakly detectable in segments 2 and 3.

In flowers selfed at 4 days after anthesis the front of the peroxidase 10 wave has passed a longer distance than in flowers pollinated at anthesis. Apparently, the speed of this wave depends on the developmental stage of the flower.

Concerning pollen tube growth, *fig. 3* clearly demonstrates that in immature styles pollen tube tips grow in a stylar part without peroxidase 10, whereas in mature styles the tube tips grow in a stylar part with a high peroxidase 10

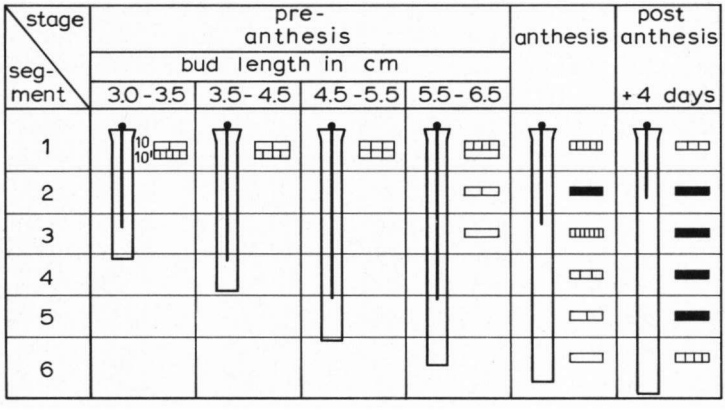

Fig. 3. Peroxidase 10 activity in various stylar segments at 3 days after self-pollinating detached buds or flowers (15 °C). Average pollen tube length and style length from at least 20 styles is given.

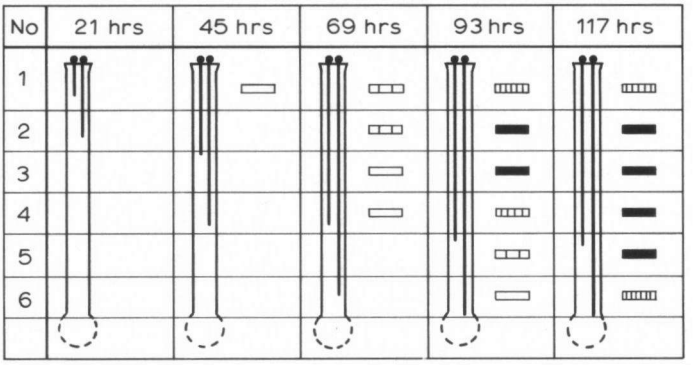

Fig. 4. Peroxidase 10 activity in stylar segments at various times after self-pollinating attached buds of 6.3–6.5 cm at 23 C. The two pollen tubes in each style in this figure represent the shortest and the longest distance covered by 10 pollen tubes observed among at least 17 styles.

activity. Comparison of peroxidase 10 activity and pollen tube growth in styles selfed at anthesis and 4 days after anthesis also reveals a positive correlation between tube growth inhibition and peroxidase 10 activity.

3.4 Pollen tube growth and peroxidase 10 activity during the shift from compatibility to incompatibility

A time course study of incompatible pollen tube growth in attached buds of length 6.3–6.5 cm revealed two growth phases: during the first 3 days after pollination incompatible pollen tube growth rate approximates an average of 70 per cent of compatible growth rate; afterwards, in a number of styles, incompatible tubes are strongly inhibited and completely cease growth, whereas in other styles the initial inhibition of the tubes remains more or less the same.

Concerning peroxidase 10 in these buds, *fig. 4* illustrates a wave of activity arising in segment 1 approximately 2 days after pollination and transmitted afterwards to the basal end of the style. During the first 3 days after pollination, pollen tube tips grow in front of the peroxidase 10 wave; afterwards, in a number of styles, pollen tube tips grow in a stylar part with a high peroxidase 10 activity, whereas in other styles the tips still grow in front of the peroxidase 10 wave.

The initiation of the phase of strong inhibition takes place when the front of the peroxidase 10 wave has passed the tube tips. This only happens when the initial growth rate is relatively low (short tubes in *fig. 4*). In styles containing pollen tubes with a relatively high growth rate (long tubes in *fig. 4*) pollen tube tips reach the ovary before being overtaken by the peroxidase 10 wave.

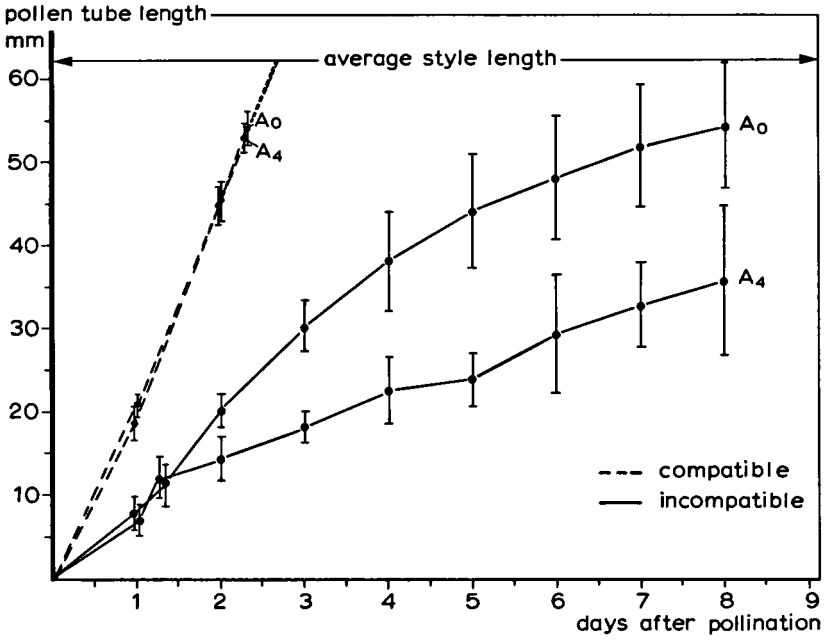

Fig. 5. Growth curves of compatible and incompatible pollen tubes in attached flowers pollinated at anthesis (Ao) or 4 days following anthesis (A4) at 23 °C. Each value is an average of pollen tube lengths observed in at least 20 styles.

3.5 Influence of the age of the mature flower on pollen tube growth and pollination-induced increase in peroxidase 10 activity

A time course study of pollen tube growth in styles selfed at 4 days after anthesis revealed a growth curve which exhibits two phases *(fig. 5)*: a first phase during which pollen tube growth rate is about 40 per cent of compatible growth rate and a second phase during which incompatible pollen tube growth rate is reduced in remarkably larger proportions. In flowers selfed at anthesis the initial inhibition of pollen tube growth is as strong as that observed in flowers selfed at 4 days after anthesis; the subsequent increase in pollen tube inhibition, however, is more gradual than abrupt.

In *fig. 3* it has already been shown that differences in pollen tube length at 3 days after selfing at anthesis or 4 days following anthesis are accompanied by differences in peroxidase 10 activity. The question thus arises as to whether or not peroxidase 10 activity is already different from the moment that the two growth curves shown in *fig. 5* begin to diverge? One should expect such a situation if peroxidase 10 is indeed involved in the initiation of the second growth phase. Comparison of peroxidase 10 activity in the various segments of styles selfed at anthesis or 4 days following anthesis revealed indeed a clear differ-

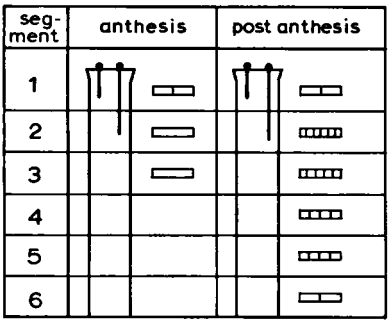

Fig. 6. Distribution of peroxidase 10 activity in styles at 31 hrs after selfing attached flowers at anthesis or 4 days after anthesis (23° C). Minimum and maximum pollen tube lengths out of at least 20 styles are given.

ence at 31 hours after pollination *(fig. 6)*. In segment 2 peroxidase 10 activity is much higher when selfing was carried out 4 days after anthesis than when selfing was performed on the day of flower opening. This segment corresponds exactly to the stylar part in which differences in pollen tube growth rate are initiated *(fig. 5)*. In the segments 1 peroxidase 10 activity is low in both cases and, in both cases, the rates of pollen tube growth remain identical.

4. DISCUSSION

The pollination-induced increase in peroxidase 10 activity observed in mature styles (BREDEMEIJER & BLAAS 1975) does not take place in immature styles unless the pollinations are carried out within one day before anthesis. Apparently, the metabolic changes which enable the style to increase peroxidase 10 activity upon pollination takes place during the last day before anthesis.

The fact that the shift from compatibility to incompatibility corresponds and is parallel to the shift in the capacity to induce peroxidase 10 activity upon pollination supports the hypothesis of an involvement of this isoenzyme in the rejection of incompatible growing pollen tubes. The finding that in buds of length 6.3–6.5 cm, pollen tubes are already retarded before a peroxidase 10 wave arises *(fig. 4)* seems at first sight to deny the validity of such a hypothesis but the objection can easily be ruled out by assuming, as shown by the two distinct phases in the growth curve of incompatible tubes in aged flowers *(fig. 5)*, that two different rejection mechanisms are involved. The first, resulting in a reduced growth rate of the pollen tubes starts shortly after pollination; the second resulting in a strong inhibition of tube growth and growth cessation starts at a later stage. Similar growth curves have also been found in *Petunia hybrida* (LINSKENS & TUPY 1966, LINSKENS & KROH 1967).

According to this interpretation, during the first growth phase which occurs in a stylar part with a low peroxidase 10 activity, the initial retardation of incompatible pollen tube growth would result from the action of another

compound than peroxidase 10. In an earlier report it was suggested that the initial retardation would correspond to the recognition reaction (BREDEMEIJER & BLAAS 1975). This suggestion was based upon the fact that the peroxidase 10 wave passed the incompatible pollen tube tips in such a short time after pollination that the period of initial inhibition was short. The present results, however, demonstrate that this suggestion is not necessarily correct because in buds of length 6.3–6.5 cm it is now clear that the first growth phase of incompatible pollen tubes can last several days. It is unlikely that the inhibition of pollen tube growth during such a long period is caused by the recognition reaction which is known to take place during the very early contact period between the pollen grains and the pistil (LINSKENS 1974, VAN DER DONK 1974). It is much more likely that the low molecular weight proteins found by VAN DER DONK (1974) are responsible for the inhibition during the first growth phase of the incompatible pollen tubes.

During the second growth phase, that of strong reduction of growth rate, pollen tube tips always grow in a stylar part with a considerable peroxidase 10 activity. The fact that the two pollen tube growth curves in fig. 5 diverge at a moment that the pollen tubes have just entered stylar parts which differ in peroxidase 10 activity *(fig. 6)* supports the hypothesis that this isoenzyme is involved in the rejection of incompatible growing pollen tubes.

The fact that the shift in inhibition of pollen tube growth is gradual in flowers selfed at anthesis and is abrupt in flowers selfed at 4 days after anthesis *(fig. 5)* can be explained by the observed differences in peroxidase 10 activity. In the first case the peroxidase 10 activity wave has to reach and pass the growing pollen tube tips so that the enzyme activity around these tips increases gradually; in the second case pollen tube tips are abruptly confrontated with a considerable peroxidase 10 activity.

It is not likely that the decline in incompatible pollen tube growth rate in aged flowers is due to a sudden strengthening of the initial rejection reaction at 31 hours after pollination *(fig. 5)* because this reaction tends to weaken in still older flowers. In flowers selfed at 7 days after anthesis, pollen tubes grow 8.7 ± 1.5 mm during the first day, whereas they grow 6.9 ± 1.7 mm in flowers selfed at 4 days after anthesis. The possibility that the decline in incompatible pollen tube growth at 4 days after anthesis is due to stylar degeneration seems also excluded since compatible tubes grow quite similarly at anthesis and after pollinations carried out on flowers having opened 4 days earlier *(fig. 5)*.

One does not yet know if the presence of peroxidase 10 is necessary to stop incompatible pollen tube growth or if the initial rejection reaction alone, is able, through the action of certain low molecular weight proteins (VAN DER DONK 1974), to establish complete inhibition. At any rate, the present results do indicate that the presence of peroxidase 10 in the stylar part where the incompatible pollen tube tips are growing causes a stronger inhibition of tube growth. In aged flowers in which the peroxidase 10 wave is transmitted with a higher speed than in flowers pollinated at anthesis, the front of this wave passes the tube tips at an earlier stage. This means that the influence of peroxidase 10 on

pollen tube growth is also initiated earlier and that in aged flowers incompatible pollen tubes grow only a fraction of the length that they achieve in flowers pollinated on the day of anthesis.

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