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Pollination-Induced Ethylene and Production of 1-Aminocyclopropane-1-Carboxylic Acid by Pollen of *Nicotiana tabacum* cv White Burley

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Abstract. Tobacco (*Nicotiana tabacum* cv White Burley) pollen contains high levels of 1-aminocyclopropane-1-carboxylic acid (ACC; ~2700 nmol/g). Such large amounts, however, do not appear until very late in the development of the anthers. Washing pollen in Kwacks medium (10% sucrose) removes nearly 40% of the pollen-held ACC. Pollination of isolated styles with washed pollen results in lower ethylene production than that of styles pollinated with unwashed pollen. No reduction in viability of washed pollen was observed in situ, although loss of viability occurred in vitro. Physical wounding of the stylar tissue induced a relatively small increase in ethylene production when compared to that induced by pollination. The results suggest that pollen-held ACC may participate in pollination-induced ethylene production in this species; however, further promotion of ethylene biosynthesis must occur in response to some other factor associated with pollination.

In many species pollination induces a rapid increase in ethylene production and in some it causes a hastening of corolla senescence: *Vanda* orchids (Burg and Dijkman 1967), *Dianthus caryophyllus* (Nichols 1971), and *Petunia hybrida* (Whitehead et al. 1984). In other species pollination may induce corolla abscission: *Digitalis purpurea* (Stead and Moore 1979). In the species that have been investigated the increase in ethylene production is initially detectable from the stylar tissues and only later do the ovary and petals produce increased amounts of ethylene (Stead and Moore 1979, Nichols et al. 1983). The increase in ethylene production from the ovary, however, occurs before fertilization. Therefore, if stylar activity induces ethylene evolution in the ovary and corolla, it is evident that some form of stimulus must be translocated

through the stylar tissue ahead of the growing pollen tubes. The nature of this stimulus is unclear; in *Lilium* changes in the electrical potential difference across the style have been detected (Spanjers 1981), while in *Dianthus* it has been suggested that the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) is translocated through the stylar tissues (Reid et al. 1984). In yet other species, however, the evidence is against ACC as the transmitted stimulus causing increased ethylene production following pollination (Gilissen and Hoekstra 1984). The recent observations that the pollen of a number of species contains high levels of the ethylene precursor ACC (Whitehead et al. 1983, Hoekstra 1984, Stead 1985) lead to the question of what role pollen-held ACC may have; it may contribute to the pollination-induced increase in ethylene production and the accelerated senescence of the corolla observed in some species, or it may be associated with some aspect of pollen germination and tube growth.

Cultivars of *Nicotiana tabacum* have been previously shown to contain exceptionally high levels of ACC in mature pollen (Stead 1985). This paper reports the production of ACC by the developing pollen of *N. tabacum*, the elution of this ACC from mature pollen grains, and compares ethylene production from physically wounded pistils to that from pistils pollinated with either washed or unwashed pollen.

Materials and Methods

Plants of *Nicotiana tabacum* cv White Burley were grown in a glasshouse under a photoperiod of 14 h minimum.

ACC in Pollen and Developing Anthers

Anthers (100 mg) or pollen (5 mg) from a range of floral stages were crushed in 6 ml 0.2 M TCA and left to stand for 1 h before being filtered (8 μm pore). The filtrate was then analyzed for ACC following a modified method of Lizada and Yang (1979), which depends upon the conversion of ACC to ethylene. $HgCl_2$ (10 mM, 100 μl) was added to 0.7 ml of each filtrate in 6.7 ml tubes followed by 100 μl 2 M NaOH and thoroughly mixed on a Vortex mixer. All reagents and reaction tubes were kept on ice for the duration of the analysis. The tubes were then sealed with Suba-seals and kept in ice until required, at which time 100 μl 1.7 M NaOCl in 2.5 M NaOH was injected into the tube. The reaction mixture was then agitated for 5 s prior to standing in ice for 5 min, after which it was agitated for a further 30 s. The concentration of ethylene in the tube was established by removing 1 ml of the headspace and assaying on a gas chromatograph (Hewlett-Packard 439) equipped with a Poropak N column and a flame-ionization detector. The area of the ethylene peak from the sample was compared with that of an ethylene standard. The efficiency of conversion of ACC to ethylene was determined by addition of a small volume of ACC standard to a duplicate tube of extract prior to the addition of $HgCl_2$. The amount of internal

standard was sufficient to produce an ethylene peak of about twice the height of that from the endogenous ACC.

To determine the ACC content of pollen from developing anthers, the pollen was removed from undehisced anthers, extracted, and analyzed as above.

ACC Content: Extraction Medium

Fresh pollen collected from flowers at stages 5 and 6 was extracted (for 1 h) in 6 ml of either 0.2 M TCA, Kwacks medium containing 10% sucrose (Brewbaker and Kwack 1963), or in distilled water with or without sonication for 2 min (MSE Ultrasonic disintegrator Mk2 with an exponential probe). The samples were then filtered (8 μm pore) and analyzed for ACC, each extract was assayed at least twice, and each extraction procedure replicated seven times.

Washed Pollen: ACC Content

Fresh pollen was weighed onto millipore filter papers (8 μm pore) and washed with Kwacks medium. The pollen was allowed to air-dry before pollinating isolated styles or extracting and analyzing for ACC. The washing medium was also analyzed for ACC.

Effect of Pollen Load on Ethylene Production

Styles were excised from flowers when the corolla was just opening (stage 5) and in which the anthers had not dehisced, and placed with their bases in 100 μl distilled water for 1 h before pollination to reduce the influence of wound-induced ethylene formation. Pollen load was varied by first masking the stigma surface and then covering the exposed area with fresh pollen. The masks used allowed 100, 50, 25, or less than 10% of the stigma surface to be covered with pollen, at least 9 replicate styles were used for each level of pollen load. Ethylene production was measured every hour for 5 h by enclosing the styles in 4.4 ml vials with a Suba-seal. After 15 min a 1 ml sample of the headspace was assayed for ethylene.

Washed Pollen and Wounding: Effect on Ethylene Production

Styles were prepared as above. In some experiments they were then either pollinated with unwashed and washed pollen, or unpollinated; in others they were pollinated, brushed with inert glass beads, or pierced along their length with a fine needle. Ethylene production was measured every hour by sampling the headspace of the tubes after 15 min enclosure. Each experiment was repeated 5 times and there were at least 3 replicates of each treatment in each experiment.

Washed Pollen Viability

Pollen germination on the stigma was observed using incident ultraviolet illumination on a Leitz Dialux 20 microscope after staining with water-soluble aniline blue (Martin 1959). Pollen tube growth in vitro was measured by germinating washed and unwashed pollen (1 mg/ml) in Kwacks medium, supplemented with a range of sucrose concentrations (5–20%), for 4 h at 18°C on a wheel rotating to maintain adequate aeration. Germination of 200 pollen grains and their mean pollen tube lengths were measured.

Results

Floral Development in N. tabacum

The corolla of the flowers of tobacco protrude beyond the calyx from an early stage (Fig. 1, stage 1). The flowers take about 3 days to develop from stage 1 to 3 and approximately 5 days from stage 3 to stage 6; the anthers dehisce just after corolla opening (i.e., between stages 5 and 6).

ACC Content of Developing Anthers

Analysis of ACC content of anthers from a range of floral stages (Fig. 1) showed that ACC is accumulated in the anthers late in their development, the greatest accumulation of ACC occurring just prior to, or coinciding with, anther dehiscence. Single anther fresh weight measurements (Fig. 1) declined soon after the corolla began to emerge through the sepals and continued to decrease up until anther dehiscence. Removal of the pollen from the anthers of flowers at stages 3 and 5 revealed significant amounts of ACC associated with the pollen (156 ± 23.7 and 1687 ± 166.6 nmol/g fresh weight pollen); corresponding values for the complete anther were 0.014 ± 0.011 and 124 ± 55.0 nmol/g fresh weight.

ACC Content of Mature Pollen

The ACC content of mature *N. tabacum* pollen differed depending on the extraction technique used (Table 1) and on the length of the extraction time. Extraction in 0.2 M TCA released the greatest amount of ACC (2675 ± 457.2 nmol ACC/g pollen), with lesser amounts being released by washing in water and Kwacks media. Extraction times less than 1 h were also used and showed that the diffusion of ACC from the pollen was exceptionally rapid, with up to 175 nmol ACC/g detected in the Kwacks media after rinsing the pollen for as little as 30 s. The authenticity of the extracted ACC was checked by co-chromatography using ascending paper chromatography with butanol/acetic acid/water (4:1:5). The paper strips were eluted with water and the ACC content assayed as before, in each case the R_f of the extracted ACC corresponded with that of the authentic ACC.

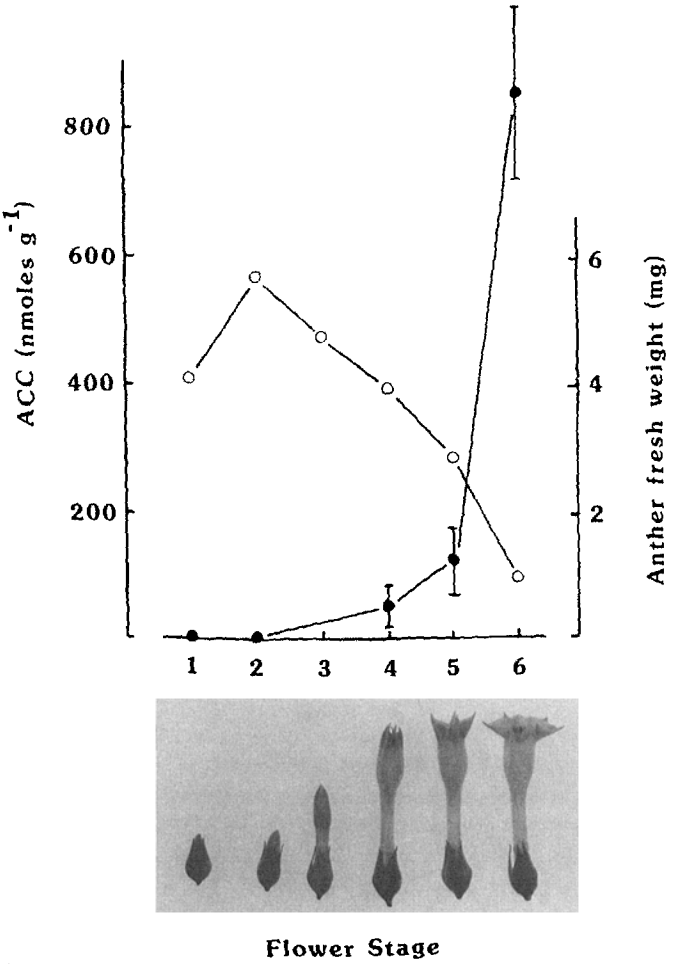

Fig. 1. ACC content (●) and fresh weight (○) of developing tobacco anthers (vertical bars represent standard errors), and the various stages of floral development in *Nicotiana*.

Effect of Pollen Load on Ethylene Production

Increasing the area of the stigma covered in pollen increased the amount of ethylene produced (Fig. 2). When the stigma was completely covered with pollen the rate of production continued to rise throughout the experiment; with lesser amounts of pollen the maximum rate of ethylene production was reached progressively earlier. When 25% or less of the stigma surface was covered with pollen the rate of ethylene production was not significantly different from the unpollinated controls until 3 h after pollination; with greater amounts of pollen, however, the rate of production was noticeably larger after only 2 h. The standard errors (not shown) associated with these data were large due to

Table 1. ACC extracted from *N. tabacum* cv White Burley pollen after 1 h using different media.

Extraction medium	ACC content in extraction media[a] ($\bar{x}$)	(SE)
Distilled water	1986	(171.8)
Kwacks	1662	(469.5)
0.2 M TCA	2675	(457.2)
Sonication (distilled water)	1597	(165.5)

[a] ACC content = nmol/g.

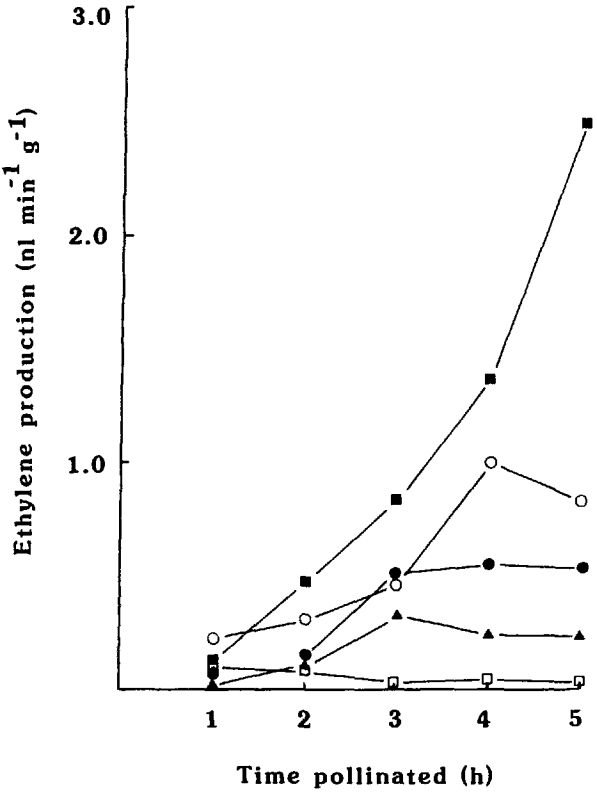

Fig. 2. Rates of ethylene production from unpollinated styles (□) and styles covered with pollen over less than 10% (▲), 25% (●), 50% (○), or 100% (■) of the stigma surface.

the difficulty in reproducing similar percentage cover of the stigma with pollen. In subsequent experiments, pollen was applied very much more thinly over all of the stigma surface so that the amount applied was approximately equal to a dense covering over 25–50% of the stigma surface.

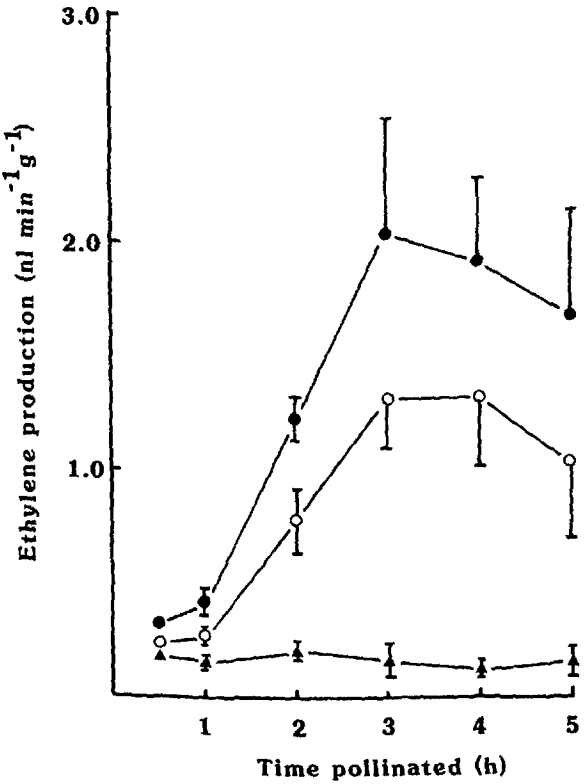

Fig. 3. Rates of ethylene production from unpollinated styles (▲) and styles pollinated with washed (○) or unwashed (●) pollen. Vertical bars represent standard errors.

Comparison of Washed and Unwashed Pollen on Pollination-Induced Ethylene Production by Isolated Styles

Pollination of excised styles with unwashed pollen resulted in a rapid increase in ethylene production (Fig. 3), significant at 1 h and rising to a peak at 3 h, after which the rate of production began to decline. Ethylene production of unpollinated styles remained at or less than 0.2 nl $min^{-1} \cdot g^{-1}$ of stylar tissue throughout. When pollen had been rinsed in Kwacks medium prior to pollination, stylar ethylene production was lower than from styles pollinated with unwashed pollen but followed a similar pattern with maximum production 3 h after pollination. The rate of production of ethylene from pollen germinating in vitro was found to be below the level of detection irrespective of washing.

Effect of Wounding on Stylar Ethylene Production

Wounding tobacco styles caused an increase in ethylene production within 1 h and the effect persisted for at least 5 h; however, the increase was much

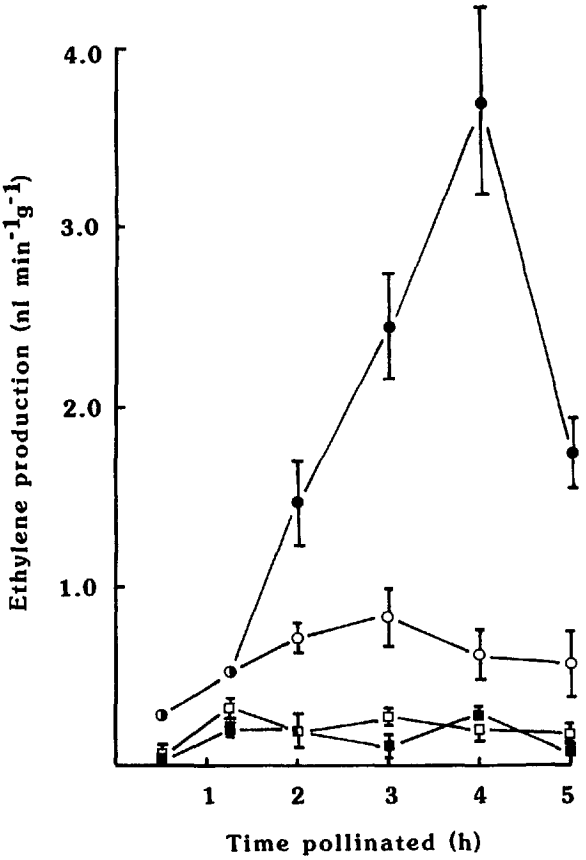

Fig. 4. Rates of ethylene production from unpollinated styles (□), styles brushed with inert glass beads (■), wounded by piercing the length of the style with a needle (○) or pollinated with normal viable pollen (●). Vertical bars represent standard errors.

smaller than that induced by pollination (Fig. 4). At 2 h after treatment the wounded styles produced approximately three times more ethylene than the untreated styles whereas the pollinated styles produced ethylene at nearly six times the rate of the controls; 2 h later the pollinated styles produced nearly 15 times more ethylene than the unpollinated styles, while ethylene production from the wounded styles remained constant. There was no significant difference between the production of ethylene from styles treated with washed glass beads and that from untouched, unpollinated styles (Fig. 4).

Pollen Viability

In 5 separate experiments the germination and rate of growth of the pollen tubes in situ, as revealed by fluorescence microscopy, during the first few hours after pollination, were similar for both washed and unwashed pollen.

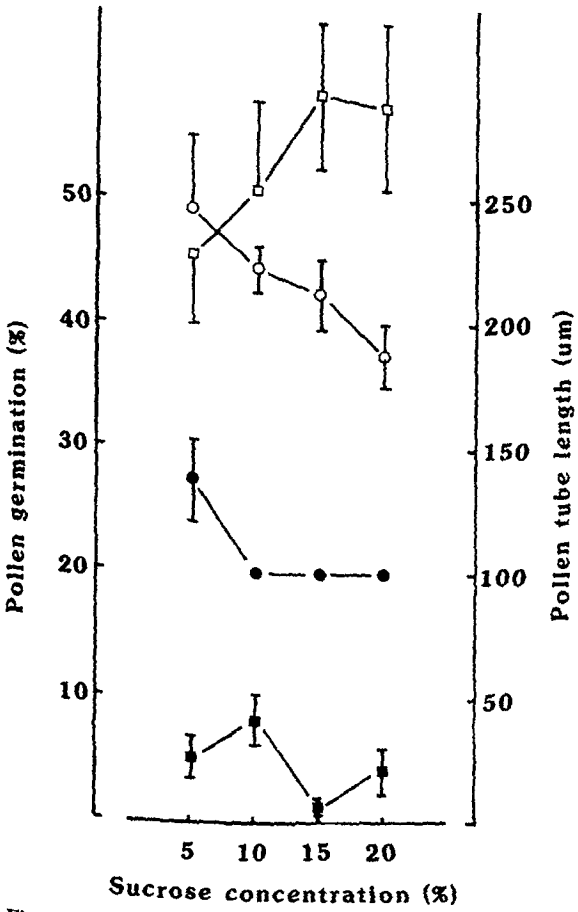

Fig. 5. Percentage germinated (■,□) and pollen tube lengths (●,○) of washed (closed symbols) and unwashed (open symbols) pollen in vitro. Vertical bars represent standard errors.

Since the experiments reported here only relate to the initial response after pollination, no attempt was made to compare seed set between those pollinated with washed and unwashed pollen. The germination and tube growth of washed pollen in vitro, however, were greatly reduced compared with unwashed pollen (Fig. 5). Attempts to improve the viability of the washed pollen by varying the concentration of sucrose had little or no effect on the percentage germination for either washed or unwashed pollen (Fig. 5); however, pollen tube lengths were somewhat longer in the lower sucrose concentrations.

Discussion

During early floral development the anthers contain less than 1 nmol ACC/g fresh weight (Fig. 1), but at the stage when the corolla begins to open there is a

sharp rise in the ACC present in the anther tissue. Analysis of pollen removed from immature and mature anthers showed that higher concentrations of ACC are associated with the pollen rather than the anther tissue, suggesting that most, if not all, of the ACC found in the anther is associated with the pollen grains. Furthermore, although there is a substantial decrease in the fresh weight of the anther during the later stages of development it is insufficient to account for the increasing concentration of ACC in the anther tissue. Thus ACC accumulates within the anther, particularly during the later stages of pollen development and over 50% of the final ACC content of the anthers accumulates in the few hours between corolla opening and anther dehiscence (stages 5–6). This is in contrast to *D. caryophyllus*, in which it has been suggested that ACC is produced in the anther over a prolonged period (Whitehead et al. 1983). That such high levels of a free amino acid should occur in pollen is not unique; in some species certain amino acids occur at a concentration 10 times that of ACC in tobacco (Bieberdorf et al. 1961).

The site of synthesis of ACC within the anther is still unclear; the ACC may be produced either within the pollen grains or by the tapetal cells from which it is transported to the pollen grains. The site of accumulation of ACC within the pollen grain is also unclear; since a large proportion of the total ACC held is released by a brief washing, it seems likely that it is held in the exine cavities of the pollen grain. If this is so, the site of synthesis would most likely be the diploid tapetal cells, and deposition onto the pollen grain would occur when the tapetal cells break up and invest the pollen grains, a process which in some species may also occur just prior to anther dehiscence (Dickinson and Lewis 1973). However, some intine products have been reported to be released from pollen grains within the first few minutes of hydration (Knox and Heslop-Harrison 1970) and if this is the source of the pollen-held ACC the site of synthesis would most likely be the cytoplasm of the haploid pollen grain.

Although a large proportion of the total pollen-held ACC is removed from the pollen by washing with Kwacks medium, it has consistently been possible to detect only about 20% of this ACC in the Kwacks medium; the fate of the remainder, approximately 700 nmol/g of pollen is unclear but it is not due to any interference between the Kwacks media and the assay procedure, since added ACC can be accurately assayed in this media (Stead unpublished data). The elution of ACC from the pollen is very rapid, with the ACC content significantly reduced by only a few seconds washing. Further washing removes more ACC up to a maximum after about 20–30 min; at this stage approximately 60% of the ACC has been eluted. Additional washing, however removes only a small proportion of remaining ACC, suggesting that some of the ACC at least is held more tightly, or that there may be several sites of storage of ACC in the tobacco pollen. The rapid elution of materials from pollen grains has been previously reported for a number of species (Makinen and Brewbaker 1967, Search and Stanley 1971, Roggen 1975).

Ethylene production from isolated styles pollinated with washed pollen was significantly lower than that from those pollinated with unwashed pollen, whereas ethylene production from unpollinated styles was considerably less than that from any of the pollinated styles. The large standard errors associated with these data are due, in part at least, to the addition of differing

amounts of pollen to the stigma since, in the first few hours after pollination, ethylene production is proportional to the amount of pollen added in *Nicotiana* as it is in other genera (Stead 1985). Secondly, since fresh pollen was washed immediately prior to each experiment there was considerable variation in the amount of pollen-held ACC removed for each experiment; such variability is evident from the large standard errors associated with values in Table 1. The reduced ethylene production by the styles pollinated with washed pollen may be due to the loss of eluted ACC. The amount of ACC lost from the pollen used to pollinate a single style is equivalent to between 1.0 and 1.5 nl ethylene; this is sufficient to account for the differences in ethylene production between those styles pollinated with washed and unwashed pollen during these experiments. Calculation of the amount of stylar ethylene expected from the ACC applied in pollen shows that it is only sufficient to account for ethylene production in the first 5 h after pollination. Therefore it would seem that some of the pollination-induced ethylene production must be produced by de novo synthesis of ACC within the gynoecial tissues, and certainly that produced 5 or 6 h after pollination must be derived from ACC synthesized within either the gynoecial tissues or the growing pollen tubes. The use of aminoethoxyvinylglycine (AVG) to investigate the de novo synthesis of ACC within the style after pollination was inconclusive since application of AVG directly to the stigma surface reduced the subsequent germination of pollen (unpublished data).

In *Petunia* it has been suggested that, since physical damage to the style causes corolla wilting in a manner similar to pollination (Gilissen 1977), the damage caused by the growing pollen tubes may be sufficient to induce wound ethylene production and hence corolla wilting. If this is so, any reduction in pollen viability will drastically reduce stylar-produced ethylene and hence the effectiveness of pollination in causing corolla senescence. In this study the viability of washed *Nicotiana* pollen was reduced in vitro, but in vivo the viability of washed pollen was apparently unaffected, and the apparent rate of pollen tube growth in the style was similar for both washed and unwashed pollen. Thus the amount of damage to the stylar cells caused by growing pollen tubes would be very similar for both washed and unwashed pollen and it is therefore not possible to distinguish between the role of wound-induced ethylene formation and that derived from pollen-held ACC in this experiment. Artificial wounding of the stylar tissues, by either crushing or piercing with a needle, does not induce as large an increase in ethylene production as does pollination. It therefore seems unlikely that pollination induces increased ethylene production solely because of the physical wounding of the stylar tissue in this species. In *P. hybrida,* however, the effect of pollination on ethylene production and corolla wilting can be mimicked by wounding the style (Gilissen 1977, Whitehead et al. 1984) and the effect of wounding also induces ACC synthesis in a manner similar to that following pollination (Nichols and Frost 1985).

The loss of viability of washed pollen in vitro may be attributed to the loss of ACC, although it has long been known that many other compounds are eluted from pollen grains by only very brief washing (Makinen and Brewbaker 1967, Stanley and Search 1971). The fact that viability in vivo is unaffected suggests that something is removed by washing the pollen which can be replaced by the

stigma or style; one possibility might be sugars or lipids, since the stigma exudes a fluid rich in carbohydrate (Portnoi and Horovitz 1977) and lipid (Dumas 1974). However, different concentrations of sucrose in the media did not overcome the inability of washed pollen to germinate in vitro. Although seed set was not recorded in this study, in *Lilium* it has been shown that washing pollen does not reduce seed set (Fett et al. 1976), while in *Petunia* washed pollen also showed good viability in vivo but only poor germination in vitro (Gilissen and Brantjes 1978).

In conclusion, some, but not all, of the ethylene produced during the first few hours after pollination is derived from pollen-held ACC since removing some of the ACC by washing significantly reduced the rate of ethylene production without affecting pollen viability. However although *Nicotiana* pollen contains the highest levels of ACC so far reported (Stead 1985) the total amount is only sufficient to account for the ethylene produced in the first 5 h after pollination; for continued ethylene production ACC must be synthesised de novo within the gynoecial tissue.

Recent work published by Hoekstra and Weges (1986) suggests that in *Petunia* pollination-induced ethylene production during the first few hours after pollination is derived not from pollen-held ACC at all, but from ACC synthesized de novo in the style, since treatment of the stigma with AVG blocked early pistillate ethylene production. In the current study such an approach was not practical because treatment of the stigma with AVG, or any other solution, caused the stigmatic papillae to collapse and reduce pollen germination. The use of washed pollen to pollinate styles in this study did significantly reduce pollination-induced ethylene production, suggesting that pollen-held factors are responsible for the increased production of ethylene from the style following pollination. This suggests that pollen-held ACC may play an important role in pollination-induced ethylene production in *Nicotiana*, at least in the first few hours after pollination.

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