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# POSTPOLLINATION PHENOMENA IN ORCHID FLOWERS. XII. EFFECTS OF POLLINATION, EMASCULATION, AND AUXIN TREATMENT ON FLOWERS OF CATTLEYA PORCIA 'CANNIZARO' AND THE ROSTELLUM OF PHALAENOPSIS

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Pollination or treatment with naphthaleneacetic acid, 50 μg per flower, prevented the death of gynostemia on blossoms of *Cattleya* Porcia 'Cannizaro.' Auxin treatments, as well as emasculation and pollination, increased anthocyanin levels. After applications of indoleacetic acid (IAA)-2-$^{14}$C, stigmatic extracts contained IAA, IAA-aspartate, and other degradation products. Cycloheximide and Actinomycin D reduced the formation of IAA-aspartate. Some of the postpollination phenomena exhibited by *Cattleya* Porcia 'Cannizaro' are similar to those of other orchids. Light and electron microscopic studies showed that removal of the pollinia damages the outer cells of the rostellum of *Phalaenopsis* flowers. Cells in some regions of the ground tissue of the rostellum close to a vascular bundle contain relatively large numbers of mitochondria. These observations suggest that the rostellum is well adapted for ethylene production. The initial evolution of ethylene may result from wounding, which, in turn, probably causes increased production of the hormone.

## Introduction

Postpollination phenomena of orchid flowers are numerous as well as variable and may be species or genus specific, but relatively few have been characterized, and combinations of symptoms have been reported for only a small number of species (ARDITTI 1979). Because of the lack of information, generalizations regarding the postpollination phenomena in orchids and their nature and/or regulation must often be speculative. In this series most previous papers have dealt mainly with *Cymbidium,* but we have extended our observations to *Angraecum* (STRAUSS and ARDITTI 1973; STRAUSS and KOOPOWITZ 1973; STRAUSS 1976), *Phalaenopsis* (ARDITTI 1976*a,* 1976*b;* ARDITTI and FLICK 1976; STRAUSS 1976; STRAUSS and ARDITTI 1977), *Phaius* (GANDAWIJAJA and ARDITTI 1982), and *Cattleya.*

The rostellum, a structure typical of orchids, was apparently first described in *Ophrys* (RICHARD 1818; VERMEULEN 1955, 1959, 1970) and was later considered to be a modified stigma (BAUER and LINDLEY 1830–1838; BROWN 1833; HOOKER 1854, 1855; HENSLOW 1858; BENTHAM 1881; DARWIN 1904; HAGERUP 1952). Current suggestions are that the rostellum was a median stigma which has evolved into a specialized organ (GARAY 1960), which seems to initiate and regulate a number of postpollination phenomena (CURTIS 1943; DUNCAN and SCHUBERT 1943; STRAUSS and KOOPOWITZ 1973; ARDITTI and FLICK 1974; ARDITTI 1979) by producing ethylene (CHADWICK et al. 1980; CHADWICK and ARDITTI, unpublished). Very little is known about the structure and ultrastructure of the rostellum because it is extremely difficult to fix, embed, section, and stain.

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## Material and methods

All *Cattleya* flowers were cut from a very large single plant that produced more than 1,000 blooms. Subperianth portions of the blossoms were surface sterilized with saturated calcium hypochlorite and inserted through rubber caps into tubes of sterile medium (ITO 1961; ARDITTI and KNAUFT 1969). Culture tubes were allowed to stand for 24 h before insertion of flowers to dissipate any ethylene that may have been formed by autoclaving. For the duration of each experiment, flowers were maintained at 22 ± 2 C, 10-h photoperiods, and a light intensity of 283 μW/cm$^2$ produced by Sylvania Gro Lux lamps.

Flowers were emasculated with a small probe or a pair of tweezers by removing the pollinia, which were deposited into stigmas. Naphthaleneacetic acid (NAA, 50, 100, and 150 μg/flower), indoleacetic acid (IAA, 50 μg and 0.25 μCi $^{14}$C/flower), cycloheximide (CHI, 25 μg/flower), and Actinomycin D (Act D, 10 μg/flower) in 0.2% agar were applied in 5-μl drops. All treatments were replicated three

times. Untreated flowers and blossoms given 0.2% distilled water agar served as controls.

Flowers were inspected 2, 4, and 8 days after treatments. The state of gynostemia (one per flower), labella (i.e., a modified petal, one per flower), and the perianth (three sepals and two petals) is described subjectively. Anthocyanins were extracted with 1% HCl in methanol. The extracts were cleared by centrifugation and brought to equal volume, and their absorption (A) was determined at 525 nm. Results were expressed as $A_{525}$/g FW (ARDITTI and KNAUFT 1969).

Flowers treated with IAA-2-$^{14}$C alone or in combination with CHI or Act D were harvested 24 h after treatment, and their parts were extracted with 80% methyl alcohol for three 24-h periods at 4 C. Extracts were combined and reduced to dryness in a 50 C oven; the residues were resuspended in 1 ml methanol to which scintillation fluid (BRAY 1960) was added. Quenching was determined with an internal standard. Transport of $^{14}$C from the flowers into the maintenance medium was determined by combining the solution from several tubes after removal of the blossoms, lyophilizing, and assaying the residue for radioactivity. Production of $^{14}CO_2$ was measured by placing several $^{14}$C-IAA treated flowers in a desiccator, trapping $CO_2$ in 6 N NaOH, and assaying it for radioactivity by liquid scintillation.

Samples were spotted on Whatman no. 1 paper and developed (ascending) in isopropanol: ammonia:water (8:1:2) or isopropanol:water (8:2, vol/vol). Radioactivity distribution was measured with a Vanguard Model 880 Chromatograph Scanner from which relative amounts of each metabolite were determined. The presence or absence of metabolites was also determined from autoradiographs (Kodak No-Screen X-ray film).

Gynostemia from flowers of commercial *Phalaenopsis* hybrids, obtained from the University of California, Irvine, orchid collection, were excised immediately after the flowers were brought into the laboratory. For light microscopy, the gynostemia were fixed in 10% acrolein (FEDER and O'BRIEN 1968) and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, at 0–4 C for 18–20 h. Following dehydration in a graded acetone series, the tissues were embedded in Tissuemat, cut into 10–15-μm serial sections, and stained with Heidenhein's iron hematoxylin-orange G for mitochondria and other cellular organelles (JENSEN 1962; PURVIS et al. 1966). Safranin and fast green were used as a general stain for study of organ structure (JENSEN 1962). A mordant was used prior to staining with safranin (JOHANSEN 1940).

For electron microscopy, rostella (figs. 6, 7) of *Phalaenopsis* were fixed as above, with and without 10% acrolein, and postfixed 18–20 h in 2% osmium tetroxide buffered with 0.1 M phosphate buffer, pH 7.3. Dehydration was by a graded acetone series. The tissues were held overnight in 70% acetone containing 2% uranyl acetate or were passed from 2-methoxy-ethanol to propylene oxide. All samples were embedded in Epon-Araldite. Thin sections were cut on a Reichert Om U2 ultramicrotome and stained with uranyl acetate and lead citrate (REYNOLDS 1963). Observations were made with a Zeiss EM9A-2 electron microscope.

## Results

Gynostemia of *Cattleya* turned black after 8 days in untreated, emasculated, and distilled water agar–treated flowers; they were green 8 days after pollination or treatment with 50 μg NAA/flower (table 1). Those treated with 100 and 150 μg NAA remained white (table 1). Treatments did not cause stigmatic closure, but they affected wilting and folding of the labella and perianth as well as the intensity of fragrance (table 1).

Emasculation and pollination had no effect on anthocyanin levels in labella 2 and 4 days after treatment (fig. 1). NAA applications showed no effect after 2 days but caused slight increases 4 days after treatment (fig. 1). All treatments produced increases in anthocyanin concentration after 8 days. The increase following emasculation was relatively low, but pigment levels following pollination increased considerably (fig. 1). NAA applications resulted in notable increases (fig. 1). Changes in anthocyanin levels of sepals and petals (including labellum) were relatively small following all treatments except 100 μg NAA/flower (fig. 2).

After IAA-2-$^{14}$C applications, chromatograms of stigma extracts of *Cattleya* had at least three radioactive zones (table 2): IAA-aspartate (low $R_f$), free IAA (medium range $R_f$), and one or more degradation products of IAA (high $R_f$). Application of CHI with IAA sharply reduced the amount of IAA-aspartate formed while increasing the levels of free IAA and high $R_f$ compounds (table 2). Treatment with Act D resulted in a reduction of high $R_f$ product and conjugate formation and an increase in free IAA (table 2). Following CHI and Act D treatments, the levels of free IAA were 71.9% and 74.4% higher, respectively, than in the controls (table 2). Extracts of labella contained none of the low $R_f$ metabolites (fig. 3).

The rostellum of *Phalaenopsis* (figs. 4–6) is composed of thin-walled, elongated (prosenchymatous), vacuolate cells. Subtending gynostemium cells are parenchymatous. A vascular strand extends through the organ (fig. 7), and surface cells occasionally contain enlarged raphides. Staining with Heidenhein's iron hematoxylin revealed a large number of small dark organelles in the cells of the rostellum that were not seen in the subtending gynostemium tissue (fig. 8). Electron microscopy of the rostellum showed numerous mitochondria with

readily apparent cristae (fig. 8). The number of mitochondria did not change noticeably following pollination.

The viscid disk is attached to the rostellum by a layer of thick-walled cells (fig. 9). These and the viscid disk are changed (fig. 9) or removed when the pollinia are detached. Within 24 h after pollination or emasculation, cells in a layer on the outside of the organ (where the viscid disk was attached) shrivel and eventually die (fig. 9).

## Discussion

Orchid blossoms live a long time, but eventually all parts of unpollinated orchid flowers senesce, and their segments disintegrate. Senescence of segments as well as cessation of scent production clearly render pollinated flowers unattractive to pollination vectors and serve to conserve "pollinator power" (ARDITTI 1976*a*, 1976*b*, 1979). Following pollination, ovaries and often gynostemia turn green and survive, e.g., *Cymbidium* blossoms (ARDITTI 1979). This is also true of the untreated, emasculated, and water agar–treated flowers of *Cattleya* Porcia 'Cannizaro.' At appropriate concentrations, NAA mimicked pollination in some orchids (ARDITTI 1979), including *Cattleya* Porcia 'Cannizaro,' while higher NAA concentrations (100 and 150 μg/flower) prevented blackening but did not cause greening.

Anthocyanin production by pollinated *Cymbidium* flowers is ethylene mediated (ARDITTI et al. 1973; ARDITTI and FISCH 1977; ARDITTI 1979; CHADWICK et al. 1980). Ethylene also induces anthocyanin destruction in pollinated *Vanda* blossoms (BURG and DIJKMAN 1967; DIJKMAN and BURG 1970; ARDITTI 1979). Ethylene production is initiated by emasculation, pollen (a rich source of IAA), auxin, and presumably the gas itself (BURG and DIJKMAN 1967; DIJKMAN and BURG 1970; ARDITTI 1979; CHADWICK et al. 1980). Consequently, treatments that initiate ethylene evolution by or-

TABLE 1

EFFECTS OF EMASCULATION, POLLINATION, AND NAA APPLICATION ON FLOWERS OF CATTLEYA PORCIA 'CANNIZARO'

| | GYNOSTEMIUM[a] | LABELLUM | | PERIANTH | | |
|---|---|---|---|---|---|---|
| TREATMENT AND DAYS | Color[b] | Wilting[c] | Folding[d] | Wilting | Folding[d] | FRAGRANCE[e] |
| Untreated: | | | | | | |
| 0 | wh | NW | NF | NW | NF | VFr |
| 2 | wh | NW | NF | NW | NF | Fr |
| 4 | wh | SW | F | SW | SF | NFr |
| 8 | bl | VW | F | VW | NF | NFr |
| Water agar: | | | | | | |
| 0 | wh | NW | NF | NW | NF | VFr |
| 2 | wh | NW | NF | NW | NF | NFr |
| 4 | wh | SW | SF | SW | SF | NFr |
| 8 | bl | W | F | VW | NF | NFr |
| Emasculated: | | | | | | |
| 2 | wh | NW | NF | NW | NF | NFr |
| 4 | wh | W | F | W | SF | NFr |
| 8 | bl | VW | F | VW | NF | NFr-SFr |
| Pollinated: | | | | | | |
| 2 | wh | NW | SF | SW | SF | NFr |
| 4 | wh | W | F | VW | VF | NFr |
| 8 | gn | VW | VF | VW | VF | NFr-SFr |
| NAA, 50 μg/flower: | | | | | | |
| 2 | wh | NW | NF | NW | NF | NFr |
| 4 | wh | W | SF | W | SF | NFr |
| 8 | gn | VW | F | VW | F | NFr |
| NAA, 100 μg/flower: | | | | | | |
| 2 | wh | NW | SF | NW | NF | NFr |
| 4 | wh | NW | VF | W | F | NFr |
| 8 | wh | VW | VF | VW | F | NFr |
| NAA, 150 μg/flower: | | | | | | |
| 2 | wh | SW | SF | SW | SF | NFr |
| 4 | wh | W | VF | W | F | NFr |
| 8 | wh | VW | VF | VW | NF | NFr |

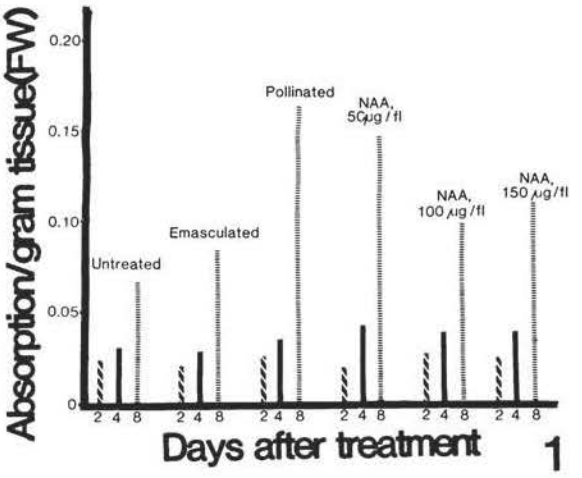
[a] Stigma was open in all flowers.
[b] wh = white; bl = black; gn = green.
[c] NW = not wilted; SW = slightly wilted; W = wilted; VW = very wilted.
[d] NF = not folded; SF = slightly folded; F = folded; VF = very folded.
[e] NFr = not fragrant; SFr = slightly fragrant; Fr = fragrant; VFr = very fragrant.

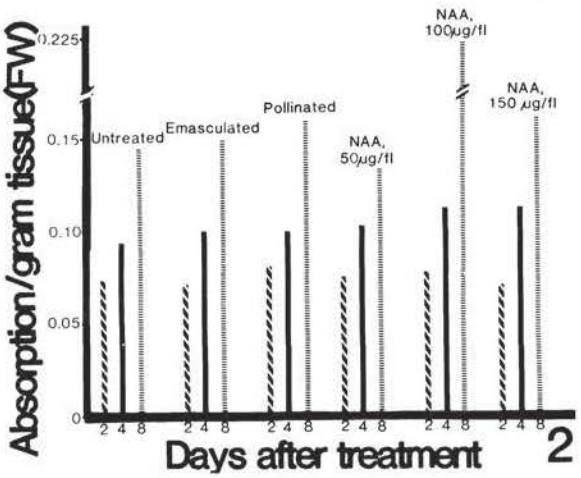
chid flowers may also cause anthocyanin production or destruction. In *Cattleya* Porcia 'Cannizaro,' all treatments resulted in anthocyanin production in the labellum, but only 100 μg NAA/flower had a similar effect on sepals and petals. Such differences in (a "split") response have not been reported in orchids before and suggest different thresholds of sensitivity.

Some of the auxin applied to *Vanda* flowers is transported to perianth segments (BURG and DIJKMAN 1967). The presence of radioactive auxin in the labellum is an indication that similar transport mechanisms operate in *Cattleya* Porcia 'Cannizaro.' Slightly more than a third of the auxin applied to stigmas is conjugated with aspartate; nearly 30% are converted into other metabolites; and 20% are recovered as free IAA. The IAA-aspartate is not mobile (STRAUSS 1976; STRAUSS and ARDITTI 1982), which explains why only labeled IAA and other metabolites are present in labella. Application of CHI together with $^{14}$C-IAA reduces conjugation considerably. Since auxin is transported from stigmas to labella even in the presence of CHI, it is obvious that the lack of conjugation is not the result of inhibition of uptake. Rather, CHI appears to either inhibit the conjugation itself or prevent enzyme synthesis required for conjugation. The enzymes that produce the metabolites are either already present in sufficient concentration or remain unaffected by the inhibitor.

The slight inhibition of conjugation and production of other metabolites by Act D suggest (1) a possible slow turnover of messengers, (2) the possibility that Act D itself is somewhat inhibitory to IAA conjugation or metabolism, and/or (3) limited requirements for de novo synthesis of RNA, or (4) that, as in *Taraxacum,* Act D may be inactivated by the tissue, which could prevent complete inhibition of auxin-induced phenomena (RUTHERFORD and DEACON 1973).

The mode of action of Act D and CHI in affecting postpollination phenomena of orchid flowers is not clear. One possibility is that these compounds act by inhibiting RNA and protein synthesis. Another is an effect on cell ultrastructure, as in barley aleurone cells, where Act D was shown to partially




FIGS. 1, 2.—Anthocyanins and auxin in flowers of *Cattleya* Porcia 'Cannizaro.' Fig. 1, Concentrations of anthocyanins in labella following pollination, emasculation, or NAA application. Fig. 2, Anthocyanin levels in sepals and petals (excluding labella) following pollination, emasculation, or NAA applications.

TABLE 2

DISTRIBUTION OF RADIOACTIVITY IN EXTRACTS OF STIGMAS OF CATTLEYA PORCIA 'CANNIZARO' FOLLOWING APPLICATION OF IAA-2-$^{14}$C, ACT D, OR CHI

| RADIOACTIVITY AND TREATMENT | RADIOACTIVITY RECOVERED FROM CHROMATOGRAMS[a] Total | % of IAA treatment |
|---|---|---|
| $R_f$ .0–.15:[b] | | |
| IAA only | 36.2 ± .21 | 100 |
| IAA + Act D | 26.6 ± 1.21 | 73.5 |
| IAA + CHI | 11.1 ± 2.05 | 30.7 |
| $R_f$ .31–.40:[c] | | |
| IAA only | 19.9 ± .28 | 100 |
| IAA + Act D | 34.7 ± 0 | 174.4 |
| IAA + CHI | 34.2 ± .5 | 171.9 |
| $R_f$ .7–1.0:[d] | | |
| IAA only | 29.3 ± .57 | 100 |
| IAA + Act D | 23.8 ± 4.67 | 81.2 |
| IAA + CHI | 43.0 ± 2.12 | 146.8 |
| Recovery: | | |
| IAA only | 85.9 ± .06 | 100 |
| IAA + Act D | 85.1 ± 3.39 | 99.1 |
| IAA + CHI | 88.2 ± .42 | 102.7 |

[a] Average of replicates ± SD.

[b] Presumed to be IAA-aspartate (ANDRAE and VAN YSSELSTEIN 1960; ROBINSON et al. 1968; IVERSEN and AASHEIM 1970; HALLIDAY and WANGERMANN 1972*a*, 1972*b*).

[c] Free IAA (identified by cochromatography with IAA standard).

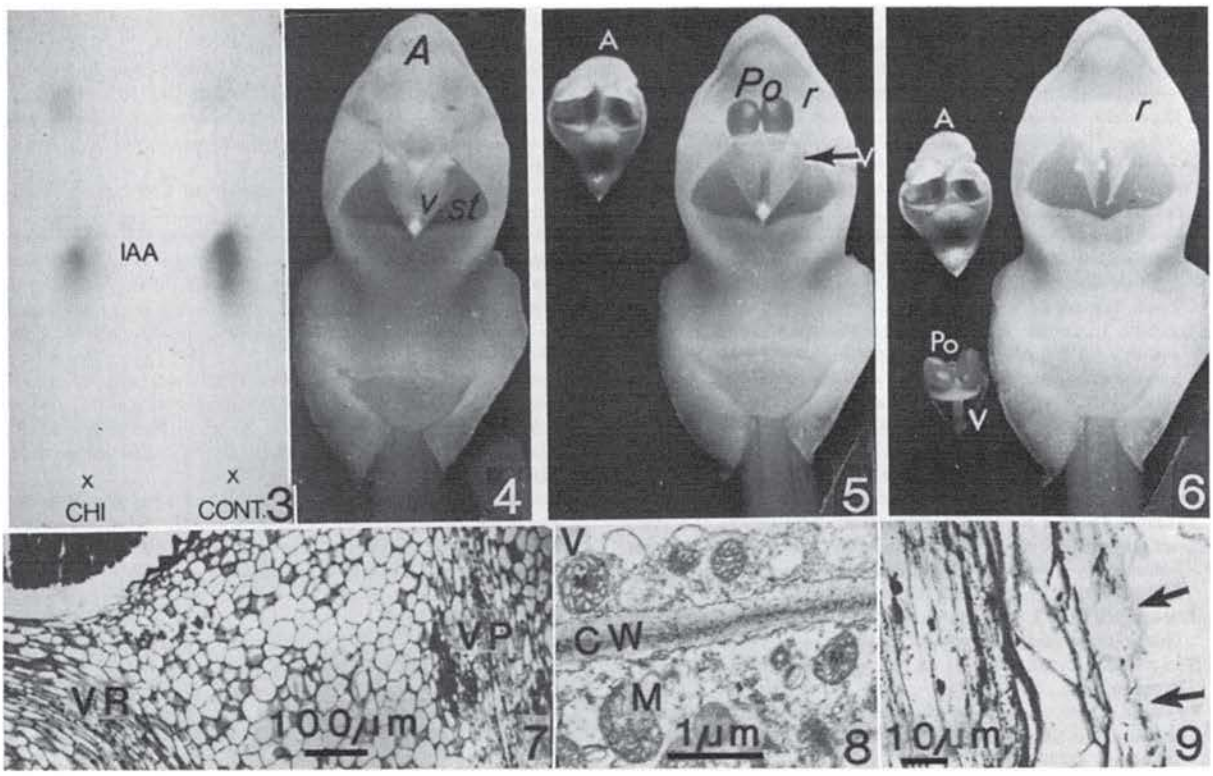
[d] Other metabolites (IVERSEN and AASHEIM 1970).

inhibit α-amylase synthesis by interfering with the development of rough endoplasmic reticulum (VIGIL and RUDDAT 1973). A third possibility is suggested by reports that CHI partially inhibits oxidative phosphorylation in isolated mitochondria (WILSON and MOORE 1973; VIGIL and RUDDAT 1973; ROSS 1974). Thus, the marked effect of CHI may be, in part, the result of inhibition of ATP formation. The presence of numerous mitochondria in the rostellum, an organ that plays an important role in the induction of postpollination phenomena (ARDITTI and FLICK 1974; STRAUSS 1976; ARDITTI 1979), suggests that oxidative phosphorylation may be necessary for these processes.

The evidence presented here demonstrates that most postpollination phenomena of *Cattleya* Porcia 'Cannizaro' are generally similar to those in other orchids. This provides further support to the hypothesis that many of these phenomena are common or even universal among the Orchidaceae.

For reasons which are still not clear, it was extremely difficult to fix gynostemium and rostellum tissues, especially for electron microscopy. These difficulties are not unique to the gynostemium and have posed problems in studies of orchid seeds (HARRISON 1973, 1977).

Initiation of postpollination phenomena following disturbance of the rostellum has been reported for *Angraecum* (STRAUSS and KOOPOWITZ 1973), *Cymbidium* (DUNCAN and SCHUBERT 1947; ARDITTI and FLICK 1974), *Phalaenopsis* (CURTIS 1943; DUNCAN and SCHUBERT 1943), *Vanda* (BURG and DIJKMAN 1967; DIJKMAN and BURG 1970), and other orchids (ARDITTI 1979). In *Cymbidium* the rostellum may affect anthocyanin synthesis but not stigmatic closure (ARDITTI and FLICK 1974). In both *Vanda* (BURG and DIJKMAN 1967; DIJKMAN and BURG 1970) and *Cymbidium* (CHADWICK et al. 1980; CHADWICK and ARDITTI, unpublished) the rostellum may also be one of the earliest sites of ethylene synthesis following pollination, emasculation, and NAA treatments. Removal of the viscid disk in *Phalaenopsis* and other orchids (GELLERT 1923) injures the rostellum cells that were in




FIGS. 3–9.—Extracts and parts of orchid flowers. Fig. 3, Autoradiogram of labellar extracts. Flowers were treated with IAA (CONT.) and IAA plus cycloheximide (CHI). The origin is marked by *X*. Fig. 4, Intact gynostemium of *Phalaenopsis*, ×3.5. Fig. 5, Gynostemium of *Phalaenopsis* without anther cap, ×3.5. Fig. 6, *Phalaenopsis* gynostemium without pollinia, ×3.5. Fig. 7, Off-median longitudinal section of *Phalaenopsis* gynostemium (stained with safranin and fast green) showing the vascular strands which lead to the rostellum (*VR*) and pollinia (*VP*). Fig. 8, Rostellum cell with numerous mitochondria. Fig. 9, Outer edge of rostellum where the viscid disk was attached 24 h earlier. Some cells (arrows) are degenerating (Heidenhein's hematoxylin and orange G). *A*, anther cap (one per flower); *CW*, cell wall; *M*, mitochondria; *Po*, pollinia; *r*, rostellum; *st*, stigma; *V*, viscid disk; *Va*, vacuole; *VP*, vascular bundle leading to pollinia; *VR*, vascular bundle leading to rostellum.

contact with it. The injury probably induces wound ethylene production, which stimulates further autocatalytic evolution of the hormone, inducing senescence of some floral segments in turn.

Ethylene production by the rostellum, a modified stigma, is in line with reports on styles and stigmas of *Petunia* (GILLISSEN 1976, 1977), *Vaccinium* (HALL and FORSYTH 1967; FORSYTH and HALL 1969), and other flowers (HALEVY and MAYAK 1979, 1981; MAYAK and HALEVY 1980). The sole difference is that, in *Petunia* and *Vaccinium,* the process is initiated only by deposition of pollen on the stigma, whereas in orchids both the removal of pollinia from a flower and pollination induce ethylene evolution.

Rostellum cells, located close to the vascular strand and containing large numbers of mitochondria, were identifiable as either xylem or phloem parenchyma. The mitochondria in these cells may relate to their role in the metabolic control of phloem and/or xylem function. However, these cells may also supply the energy needed for ethylene production.

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