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POSTPOLLINATION PHENOMENA IN ORCHID FLOWERS. IX. INDUCTION AND INHIBITION OF ETHYLENE EVOLUTION, ANTHOCYANIN SYNTHESIS, AND PERIANTH SENESCENCE[1]

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Auxin (naphthaleneacetic acid [NAA]) application to *Cymbidium* stigmas (25 μg/flower) induced high rates of ethylene evolution by the flowers within 18 h of the treatment. Actinomycin D reduced the rate of ethylene evolution if applied with the auxin or 2 h after it. Cycloheximide reduced ethylene production regardless of time of application. Puromycin had an inhibitory effect if applied 1 or 2 h after the auxin. Ethionine, if applied with the auxin or 1 or 3 h after it, induced more rapid ethylene production and higher evolution rates than NAA alone. Applications of ethionine 2 h after the auxin reduced the levels of ethylene evolution but not the rates or induction time. Anthocyanin levels decreased when each inhibitor was applied together with the auxin. Actinomycin D, cycloheximide, or puromycin, given 1 h after auxin application, did not reduce anthocyanin levels, whereas ethionine did. These results indicate that (1) initial synthesis of anthocyanins may depend primarily on preexisting RNA and proteins, (2) subsequent production requires de novo RNA and protein synthesis, and (3) ethionine may be a specific inhibitor of anthocyanin production. Of the additional postpollination phenomena exhibited by *Cymbidium* flowers, some are sensitive to RNA and protein synthesis inhibitors, and others are not.

Introduction

Orchid flowers, if unpollinated, remain alive for long periods of time. Some flowers, which stay fresh for up to 3–4 mo in the absence of pollination, start to show signs of senescence or exhibit typical postpollination phenomena shortly (in some orchids, within 15 min) after being pollinated (Decker 1941; Poddubnaya-Arnoldi and Selezneva 1957; van der Pijl and Dodson 1966; Arditti 1979).

Depending on species or genus, pollination can cause a variety of developmental, physiological, and biochemical events, including wilting, senescence, greening, fading, or anthocyanin formation in some or all segments of the perianth (Arditti 1976, 1979). Auxins can mimic certain pollen effects and initiate a number of postpollination phenomena in many orchids, including *Cymbidium* (Arditti 1979). One of these is ethylene evolution; this hormone has been implicated in control of several postpollination processes (Burg and Dijkman 1967; Arditti, Hogan, and Chadwick 1973; Arditti 1979). However, the time course of ethylene production and factors which regulate it and its effects remain unresolved.

Material and methods

Plants and maintenance procedures.—Flowers of *Cymbidium* 'Samarkand' (Dos Pueblos Orchid Co.) were selected, surface-sterilized, and maintained as described by Arditti et al. (1973).

Treatments.—Naphthaleneacetic acid (NAA) (25 μg/flower), ethionine (10 μg/flower), and puromycin (1 μmol/flower) were applied in liquified lanolin pastes. Actinomycin D (10 μg/flower) and cycloheximide (10 μg/flower) were administered in 0.2% aqueous agar. All substances were applied to stigmas in 5–10 μl drops. Previous work indicated that these substances were effective when administered in this fashion (Arditti and Knauft 1969). Treated flowers were placed singly in 1-liter jars covered by lids fitted with ampule caps (Arditti et al. 1973) and maintained under 16-h photoperiods and 22 C. Untreated, pollinated, and emasculated flowers, as well as blossoms treated with agar, lanolin, and agar plus lanolin, both in and out of jars, served as controls. All treatments were replicated three times.

Ethylene determinations.—Evolution of ethylene was determined by gas chromatography (Steen and Chadwick 1973) at 2, 5, 9, 18, 20, 24, 40, 42, 66, and 70 h after treatments. A 5-ml sample of gas was removed from the flask for chromatography and replaced with room air at each time interval. The rate is expressed as nanomoles ethylene per flower per hour.

Extraction and measurement of anthocyanins.—Anthocyanins were extracted from preweighed labella (lips, median petals) and gynostemia (columns) by maceration with a glass rod and steeping in 1% HCl in methanol (Arditti and Knauft 1969). Following centrifugation to remove debris, the extracts were adjusted to a constant volume (25 ml) and their absorbance at 525 nm was determined with a Beckman DBG spectrophotometer. Anthocyanin concentration is expressed as A_{525}/g fresh weight (FW) tissue (Furuya and Thimann 1964). Since swelling may dilute anthocyanin content, data from swollen gynostemia were corrected using average

[1] Dedicated to the memory of Robert I. Norton, formerly of Dos Pueblos Orchid Co., a longtime friend and a strong supporter of orchid research.

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weights of untreated ones from freshly cut flowers as a correction factor.

FLORAL SEGMENTS.—Wilting, aging, loss of curvature, and stigmatic closure are described in subjective terms. Swelling was measured as increase in width along the lower edge of the stigma.

Results

ETHYLENE EVOLUTION.—Application of NAA and pollination resulted in marked increases of ethylene production (fig. 1). Ethylene evolution was not affected by agar or lanolin alone or in combination (fig. 2).

Ethylene evolution was reduced by actinomycin D to levels lower than those of untreated flowers (fig. 3). When the inhibitor was applied concurrently with NAA or 2 h after it, ethylene production was reduced noticeably. If applied 1 or 3 h after the auxin, actinomycin D reduced only slightly the NAA-induced ethylene evolution (fig. 3).

The rate of ethylene evolution by cycloheximide-treated flowers was slightly higher than that of unpollinated ones (fig. 4). Cycloheximide applied together with NAA or 1, 2, or 3 h after it considerably reduced the auxin-induced ethylene production.

Application of ethionine resulted in a rapid stimulation of ethylene evolution (fig. 5). In the presence of NAA, ethionine applications at 0, 1, or 3 h brought about an immediate increase which was initially much higher than that resulting from auxin treatments (fig. 5). During the first 18 h, rates of ethylene evolution by ethionine-treated flowers were always higher than those by blossoms supplied with auxin alone.

Evolution of ethylene was not appreciably affected by puromycin alone during the initial 40 h, but subsequently it was lower than in the controls (fig. 6). Application of puromycin at 0 h slightly enhanced the rate of ethylene production; if applied later it was inhibitory.

ANTHOCYANIN LEVELS.—Pollination, emasculation, and NAA application all induced anthocyanin synthesis (fig. 1) in both labella and gynostemia (columns). Puromycin raised anthocyanin levels in labella substantially; actinomycin D and cycloheximide had a lesser effect (fig. 2). Lanolin, agar, and agar plus lanolin brought about limited increases (fig. 1). Ethionine did not raise anthocyanin levels (fig. 2). The NAA-treated flowers maintained in jars contained more anthocyanins than those placed on a bench top (NAA vs. NAA out in figs. 1, 2).

Auxin-initiated anthocyanin production in both labella and gynostemia was reduced when actinomycin D was applied together with NAA. This held true for both corrected and uncorrected values (Act D application curves vs. NAA bars in the insert of fig. 3). Anthocyanin levels, with the exception of corrected columns at 3 h, were raised to above those of the control when actinomycin D was applied 2 or 3 h after NAA treatments (fig. 3). Actinomycin D alone raised anthocyanin levels in comparison with those in unpollinated flowers (figs. 2, 3).

The NAA-enhanced anthocyanin production in labella was reduced when cycloheximide was applied at time 0, 1 h later, or 3 h after the auxin (fig. 4). Anthocyanin levels in both gynostemia and labella increased when cycloheximide was applied 2 h after the auxin. Pigment concentrations in gynostemia and labella were raised by treatments with cycloheximide only (fig. 2, insert of fig. 4).

No increases in anthocyanin content resulted from ethionine treatments. The NAA-induced anthocyanin synthesis in both gynostemia and labella was inhibited by this analog of methionine, even when applied 3 h after the auxin (figs. 2, 5). Applications of NAA together with ethionine (time zero) reduced anthocyanin levels to below those brought about by either substance alone (figs. 2, 5).

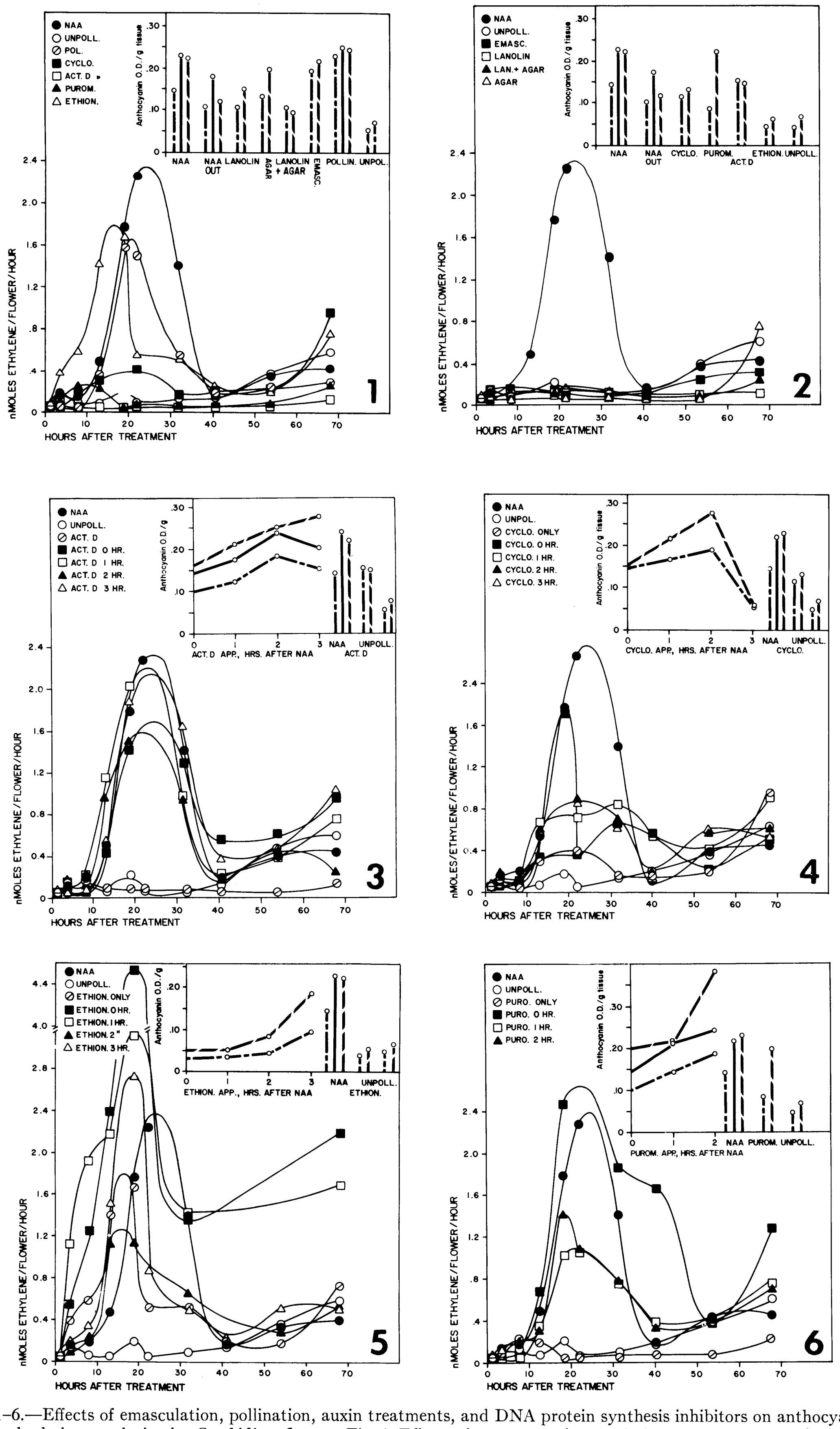
Anthocyanin content was raised by puromycin in both gynostemia and labella (fig. 6). The anthocyanin-enhancing effect of NAA was reduced when puromycin was applied at time zero, but the inhibitor had no effect if applied 1 h after the auxin (fig. 6). Anthocyanin concentration was enhanced slightly in gynostemia and considerably in labella when puromycin was applied 2 h after NAA (figs. 2, 6).

WILTING OF SEPALS AND PETALS.—Emasculation, pollination, and NAA treatments caused some wilting of sepals and petals (table 1). Lanolin and agar, applied singly or in combination, did not. The NAA-induced wilting was inhibited by actinomycin D, cycloheximide, ethionine, or puromycin regardless of application time (table 1).

GYNOSTEMIA AND STIGMAS.—Pollination and NAA treatments induced swelling and straightening of gynostemia as well as stigmatic closure (table 1). The effects of NAA were inhibited by ethionine and cycloheximide but not by actinomycin D or puromycin (table 1).

Discussion

ETHYLENE.—Production of ethylene can be blocked by RNA synthesis inhibitors (ABELES 1973). In *Cymbidium*, evolution of the gas during the first 18 h was reduced only slightly by actinomycin D (fig. 3). One reason for this may be that de novo RNA synthesis is involved minimally or not at all in the initial response. A second possibility is that actinomycin D may not be reaching the site(s) of ethylene evolution in sufficient amounts. Auxin applied to orchid stigmas is translocated to parts of the gynostemium (BURG and DIJKMAN 1967) and the rostellum, the major site of ethylene production in orchid flowers (ARDITTI 1979), where it induces evolution of the gas. Uptake and translocation of actinomycin D may be limited (TAO and KHAN 1976) and slower than auxin movement (ABELES and HOLM 1967).



FIGS. 1–6.—Effects of emasculation, pollination, auxin treatments, and DNA protein synthesis inhibitors on anthocyanin production and ethylene evolution by *Cymbidium* flowers. Fig. 1, Effects of treatments (emasculation, NAA, pollination) and carriers (agar, lanolin). Fig. 2, Results of inhibitor (actinomycin D, cycloheximide, ethionine, puromycin) applications. Fig. 3, Effects of NAA and actinomycin D. Fig. 4, Cycloheximide and NAA effects. Fig. 5, Effects of NAA and ethionine. Fig. 6, NAA and puromycin effects. Abbreviations: Act. D = actinomycin D; cyclo. = cycloheximide; emasc. = emasculation; ethion. = ethionine; NAA = naphthaleneacetic acid; Pollin. = pollinated; Unpoll. = unpollinated. Time: 0 h, simultaneous application of inhibitor and NAA; 1 h, inhibitor applied 1 h after NAA; 2 h, inhibitor applied 2 h after NAA; 3 h, inhibitor applied 3 h after NAA. Larger figures, ethylene evolution. Insert: anthocyanin content, pigment levels in labella (line broken with slants) and columns uncorrected for swelling (lines broken with dashes) and corrected for swelling (solid lines).

High rates of ethylene evolution require protein synthesis (STEEN and CHADWICK 1973), and some of our findings indicate the same. Production of the gas is reduced by administration of cycloheximide (fig. 4), ethionine 2 h after the auxin (fig. 5), and puromycin 1 or 2 h following NAA (fig. 6). Exceptions are the lack of inhibition by puromycin and when applied at time zero (fig. 6) and ethionine treatments at 0, 1, and 3 h (fig. 5).

Increases in ethylene evolution which follow application of ethionine (fig. 5) suggest that this methionine analog serves as a precursor for the extant system (STEEN and CHADWICK 1973) in *Cymbidium* as it does in *Ipomea tricolor* flowers (KONZE, SCHILLING, and KENDE 1978), but not in apple plugs (YANG 1974). In another model system, ethylene was formed from the ethyl moiety of ethionine (SHIMOKAWA and KASAI 1967).

The failure of ethionine applied at 2 h (fig. 5) to cause a marked rise in ethylene evolution may suggest that (1) during this time neither the inducible nor the extant system is fully operative and so cannot utilize it as a substrate, and/or (2) ethionine acts as an inhibitor of protein synthesis required for activation of the inducible system.

Cycloheximide increases movement of auxin into perianth segments of *Angraecum* and reduces its levels in gynostemia (STRAUSS 1976). In pea roots, cycloheximide blocks the protein synthesis-dependent phase of ethylene production (STEEN and CHADWICK 1973). If it has the same effects in *Cymbidium*, cycloheximide would inhibit the early burst of ethylene synthesis by (1) reducing auxin levels and/or (2) limiting the inducible system (STEEN and CHADWICK 1973) through the blocking of de novo protein synthesis. Some of the effects of puromycin can also be explained in terms of its effects on the protein-synthesis-dependent pathway of ethylene evolution.

ANTHOCYANINS.—Our findings that auxin and ethylene increase anthocyanin levels in *Cymbidium* flowers confirm previous reports (ARDITTI and FISCH 1977). One possibility is that NAA (either directly or via enhanced ethylene evolution) stimulates production of an unstable RNA required for anthocyanin synthesis or at least stabilizes it (RADNER and THIMANN 1963; MOHR 1969). Increased RNA synthesis in *Phalaenopsis* following pollination (ARDITTI and FLICK 1976, p. 37) lends support to this view. Under such circumstances it is reasonable to assume that a certain amount of de novo protein

TABLE 1

POSTPOLLINATION PHENOMENA IN CYMBIDIUM 'SAMARKAND' FLOWERS AFTER POLLINATION, EMASCULATION, AND AUXIN APPLICATIONS AS WELL AS NAA ACCOMPANIED OR FOLLOWED BY ACTINOMYCIN D, CYCLOHEXIMIDE, ETHIONINE, OR PUROMYCIN

Treatment[a]	Column swelling (mm)	Condition of			
		Stigma	Column[b]	Calli[c]	Sepals and Petals
In treatment jars:					
Unpollinated	10.7	Open	Cur	Or	Not wilted
Pollinated	14.3	Closed	Str	Or-rd	Sl wilted
Emasculated	11	Open	Cur	Rd	Sl wilted
NAA	16.3	Closed	Str	Or-rd	Sl wilted
Lanolin	11	Open	Cur	Yl	Not wilted
Agar	11	Open	Cur	Yl-or	Not wilted
Agar-lanolin	11	Open	Cur	Yl-or	Not wilted
Actinomycin D	11.3	Open	Cur	Yl	Not wilted
NAA and act D, 0 h	13.3	Closed	Str	Yl-or	Not wilted
NAA and act D, 1 h	14	Closed	Str	Or	Not wilted
NAA and act D, 2 h	15	Closed	Str	Or-rd	Not wilted
NAA and act D, 3 h	15.3	Closed	Str	Rd	Not wilted
Cycloheximide	11	Open	Cur	Yl-or	Not wilted
NAA and chi, 0 h	11.5	Open	Cur	Yl-or	Not wilted
NAA and chi, 1 h	12.5	Open	Sl cur	Or-rd	Not wilted
NAA and chi, 2 h	11	Open	Sl cur	Rd	Not wilted
NAA and chi, 3 h	11	Open	Sl cur	Or	Not wilted
Ethionine	11.5	Open	Cur	Yl	Not wilted
NAA and eth, 0 h	11.6	Open	Cur	Yl	Not wilted
NAA and eth, 1 h	12	Open	Cur	Yl	Not wilted
NAA and eth, 2 h	12	Open	Cur	Yl-or	Not wilted
NAA and eth, 3 h	14.5	Sl closed	Sl cur	Yl-or	Not wilted
Puromycin	11	Open	Cur	Yl-or	Not wilted
NAA and pur, 1 h	15.3	Closed	Str	Or-rd	Not wilted
NAA and pur, 1 h	16	Closed	Sl cur	Or-rd	Not wilted
NAA and pur, 2 h	15	Closed	Str	Rd	Not wilted
Outside treatment jars:					
Unpollinated	11	Open	Cur	Yl	Not wilted
NAA	15.6	Closed	Str	Or-rd	Sl wilted

[a] Act D, actinomycin D; chi, cycloheximide; eth, ethionine; NAA, naphthaleneacetic acid; pur, puromycin.
[b] Cur, curved; sl cur, slightly curved; str, straight.
[c] Or, orange; rd, red; yl, yellow.

synthesis may also occur, and our results suggest that this is indeed so.

Anthocyanin content is lowered when each inhibitor is applied at time zero (figs. 3–6). One hour after the application of NAA, anthocyanin synthesis is inhibited only by ethionine (figs. 3–6). This agrees well with the time required for disturbance effects to spread from the column (GESSNER 1948) and the results of surgical experiments (ARDITTI and FLICK 1974).

Pigment levels are actually increased by actinomycin D, cycloheximide, and puromycin when applied 2 h after the auxin (figs. 3, 4, 6), whereas NAA-induced anthocyanin synthesis is blocked by cycloheximide applications 3 h after the auxin (fig. 4). These results suggest that (1) initial production of anthocyanins may require de novo synthesis of RNA and protein but is primarily dependent on preexisting substances; (2) subsequent biosynthesis of anthocyanins in *Cymbidium* flowers depends almost entirely on newly produced RNA and/or proteins as it does in *Sorghum vulgare* (CRAKER 1971); such two-stage hormonal effects are not uncommon and have been reported in barley seeds (BEN TAL and VARNER 1974) and pea root-tips (STEEN and CHADWICK 1973); or (3) due to faster translocation from the stigma, auxin reaches the site of action and acts prior to the arrival of inhibitors.

Ethionine inhibits protein synthesis and appears to be a specific inhibitor of anthocyanin production (THIMANN and RADNER 1955; SCHRANK 1956; FAUST 1965; ARDITTI and KNAUFT 1969). This explains its effects on anthocyanin production in *Cymbidium* flowers (fig. 5).

ETHYLENE AND ANTHOCYANIN PRODUCTION.—Production or destruction of anthocyanins in orchids can be initiated by either auxin or ethylene (BURG and DIJKMAN 1967; ARDITTI et al. 1973), and since NAA applications bring about ethylene evolution, it may be that auxin effects are ethylene mediated. Our results indicate that (1) auxin can initiate ethylene evolution and anthocyanin synthesis simultaneously, and (2) anthocyanin production can be initiated by either auxin or ethylene. The latter initiated anthocyanin synthesis in orchids (ARDITTI et al. 1973), cranberries (CRAKER 1971), and sorghum (CRAKER, STANDLEY, and STARBUCK 1971).

Ethylene- and auxin-regulated phenomena can be blocked by inhibitors of DNA-dependent RNA synthesis such as actinomycin D (ARDITTI and KNAUFT 1969; ABELES 1973). There are, however, also instances where actinomycin D does not have much of an effect, even on processes which are inhibited by cycloheximide, suggesting that (1) actinomycin D is not reaching the tissues where RNA synthesis is occurring (JACOBSEN 1977), or (2) some ethylene-regulated phenomena do require RNA production (and subsequently probably also protein synthesis) whereas others do not (even if these, too, involve the manufacture of new proteins). The latter may be the reason why swelling and straightening of columns as well as stigmatic closure, although blocked by cycloheximide, are not strongly affected by actinomycin D.

WILTING.—Senescence and wilting of the perianth can be induced by either NAA or ethylene, are initiated at approximately the same time, and are difficult to separate visually (ARDITTI et al. 1973). Wilting is the result of water and dry weight losses by *Cymbidium* orchids and other flowers (MAROUSKY 1973; ROGERS 1973; ARDITTI and HARRISON 1979). Senescence, which in *Cymbidium* flowers accompanies the wilting, is undoubtedly no different from aging in other plant systems and may, therefore, require the production of new RNAs or proteins. This is indeed so in *Nicotiana* (TUPY and RANGASWAMY 1973) and *Phalaenopsis* (ARDITTI and FLICK 1976). Hence, it is not surprising that inhibitors of protein and RNA synthesis can also inhibit wilting and aging (table 1).

The initial responses of orchid flowers to pollination, auxin applications, and ethylene treatments are relatively rapid. Some postpollination phenomena are sensitive to inhibitors of protein and RNA synthesis, whereas others are not. This sensitivity changes with time of application of the inhibitor, suggesting that postpollination phenomena are brought about by two sets of proteins and RNA: one preexisting, the other newly produced.

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