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POST-POLLINATION PHENOMENA IN ORCHID FLOWERS

II. INDUCTION OF SYMPTOMS BY ABSCISIC ACID AND ITS INTERACTIONS WITH AUXIN, GIBBERELIC ACID AND KINETIN*

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SUMMARY

Applications of ABA to *Cymbidium* flowers induce some, but not all, post-pollination symptoms. Anthocyanin levels in sepals, petals, columns and labella are raised; flowers wilt; dorsal sepals become hooded; calli develop colouration while losing turgidity; columns do not swell, lose very little curvature; and stigmas do not close. Combinations of ABA and NAA induce all post-pollination phenomena, but lower anthocyanin content than treatments with ABA only. ABA plus GA₃ have effects which are similar to those of ABA alone, except that anthocyanin levels are reduced. The same is essentially true of ABA-kinetin mixtures but intensities of the effects are different and with some concentration ratios, stigmatic closure also occurs. The effects of ABA and its interactions with GA₃, kinetin or NAA are explained in terms of the roles these hormones may play in synthesis of nucleic acids and enzymes.

INTRODUCTION

Orchid flowers, including those of *Cymbidium*, undergo remarkable changes following pollination. The perianth wilts or some of its segments may become green and leaf-like. Stigmas close while columns swell and lose their curvature. Both columns and labella produce anthocyanins whereas the usually yellow calli (in *Cymbidium*) turn red or orange and lose their turgidity. Within the ovary, ovule development is initiated, continues at a rate commensurate with pollen tube growth and climaxes in fertilization 26–45 days later (Wirth and Withner, 1959). Applications of auxins to the stigma initiate most of these changes, although ovule development is aborted (for a short review see Arditti and Knauff, 1969). This is of particular interest since either pollination or auxin initiate events of such diversity that they would seem to be normally unrelated. Wilting of the perianth is an aspect of senescence; closing of the stigma and swelling of the column represent growth due to cell enlargement; greening and persistence of the column are modifications in function; changes in segments of the perianth involve organogenesis; ovule development is morphogenesis; synthesis of anthocyanins results from newly expressed biochemical capabilities; and the reduced turgidity of the calli implies changes in water relations.

* See Arditti and Knauff (1969) for part I.

It is not clear at present whether pollination or auxin treatment initiate the production of additional hormones other than ethylene (Burg and Dijkman, 1967) and, if so, what their roles might be. Even if these were known it would be difficult, on the basis of current knowledge, to ascribe specific roles to any of the known plant hormones (Crane, 1964, 1969). In fact, '... the roles of the endogenous hormones [in fruit set and development] have become even more nebulous particularly in view of the implications that abscisic acid and ethylene are intimately involved' (Crane, 1969). By implication this suggestion may be generally pertinent to many post-pollination phenomena.

Orchid flowers, and particularly those of *Cymbidium*, are singularly suited to studies of the effects that various plant hormones may have in post-pollination phenomena. They are large, easy to maintain *in vitro*, exhibit several easily observable and well-defined reactions to pollination or hormone treatments and are readily available during the flowering season (Arditti and Knaft, 1969). Thus, on the assumption that the known plant hormones may play different roles in anthocyanin production and the induction of other post-pollination phenomena in orchid flowers, and that our investigation of their interactions will contribute to the understanding of fruit-set in general, we have studied the effects of abscisic acid (ABA), an auxin (NAA), one cytokinin (kinetin) and gibberellin (GA_3) singly and in combinations with one of the other three. Furthermore, ABA has been applied to *Cymbidium* plants in attempts to delay flowering (Brewer, Gradowski and Meyer, 1969). It did not produce the desired results, but inhibited growth and accelerated leaf abscission. Its effects, if any, on flower colour or senescence have not been reported and are therefore of interest.

A previous paper (Arditti and Knaft, 1969) reported our findings with auxin, actinomycin D, ethionine and puromycin. The present one deals mainly with ABA. An article in preparation will report in detail on the effects of kinetin and GA_3 as well as their interactions.

MATERIALS AND METHODS

Flowers of *Cymbidium* 'Samarkand' (Dos Pueblos Orchid Company, Goleta, California) were selected, treated, maintained and observed as described previously (Arditti and Knaft, 1969) except that the hormones were applied in aqueous solutions or 0.3% agar with Eppendorf micropipettes (Brinkmann Instruments, Westbury, N.Y., 11590) rather than in lanolin pastes. GA_3 (Merck and Co.) was dissolved in a minimal amount of ethanol and adjusted to volume with distilled water; 0.05 M NaOH was used to dissolve kinetin; the NAA solvent was 0.125% NH_4OH and ABA (R. J. Reynolds Co.) was applied in distilled water. All solvents, liquid, or in 0.3% agar, were used as controls in addition to self-pollinated, cross-pollinated, untreated and emasculated flowers (Table 1, Fig. 1). Swelling of the column (Table 1) was measured as increase in width 5 mm below the anther cap (Arditti and Knaft, 1969). Stigmatic closure (Table 1), hooding (an inward bending of the sides and tip to form a cup or hood shape), curvature of the column, condition of the calli and wilting are described in subjective terms.

Anthocyanins were extracted from pre-weighed columns, sepals and petals, and labella by steeping (Thimann and Edmondson, 1949) at room temperature in the dark for 48 hours in 1% HCl in methanol (Arditti and Knaft, 1969). After drying, extract residues were redissolved quantitatively in 0.01% HCl in methanol and the optical density of the solution determined at 525 nm (Arditti and Knaft, 1969). Anthocyanin content is expressed as OD^{525}/g fresh weight tissue (Furuya and Thimann, 1964; Arditti and Knaft, 1969).

RESULTS

All previously reported (Arditti and Knauff, 1969) post-pollination phenomena were induced by pollination or auxin treatments and to some extent by emasculation (Table 1, Fig. 1).

Gibberellic acid applications of 100 μg and 10 μg caused slight stigmatic closure. Limited swelling of the columns was brought on by 100–0.1 μg GA_3 /flower. Slight wilting, some loss of turgidity and development of purple by the calli, but not loss of column curvature were also initiated by GA_3 . Anthocyanin content was generally raised following GA_3 applications (unpublished results).

Fig. 1. Anthocyanin content in sepals and petals (closed columns), labela (open columns) and columns (stippled columns) of *Cymbidium* 'Samarkand' 1 week after application of (a) abscisic acid, (b) ABA plus auxin, (c) ABA plus gibberellic acid, (d) ABA plus kinetin singly and (e) controls. Applications in μg per flower. ABA above, and NAA, GA and kinetin below, braces.

Kinetin application did not, on the whole, initiate post-pollination symptoms (unpublished results).

Absciscic acid

Concentrations of 1×10^{-2} μg /flower raised anthocyanin content to levels exceeding those of all controls (Fig. 1) and brought on excessive hooding of the dorsal sepal.

Applications of 5×10^{-3} μg and 1×10^{-3} $\mu\text{g}/\text{flower}$ reduced pigment concentration relative to cross- or self-pollinated flowers (Fig. 1). ABA also caused colour formation in the sepals and petals (Fig. 1); limited loss of curvature by the column; wilting of the perianth; development of orange-red colouration and loss of turgidity by the calli; but not swelling of the column or stigmatic closure (Table 1).

Table 1. *Effects of abscisic acid (ABA), gibberellin (GA_3), kinetin and naphthaleneacetic acid (NAA) singly and in combination on stigmatic closure and swelling of columns in Cymbidium 'Samarkand' flowers*

Treatment	Width of column (mm)*	Condition of stigma
None	10	Open
Water agar	10	Open
Emasculated	10.6	Open
Cross-pollinated	16.0	Closed
Self-pollinated	15.6	Closed
ABA($\mu\text{g}/\text{fl}$)	10	Open
0.5	10	Open
0.1	10	Open
0.05	10	Open
0.001	10	Open
0.005	10	Open
0.001	11	Open
ABA($\mu\text{g}/\text{fl}$) NAA($\mu\text{g}/\text{fl}$)		
0 2.5	13	Closed
0.05 2.5	14	Closed
0 25	17	Closed
0.1 25	16	Closed
0.05 25	16	Closed
0.01 25	16	Closed
0 250	14	Closed
0.05 250	17	Closed
ABA($\mu\text{g}/\text{fl}$) GA_3 ($\mu\text{g}/\text{fl}$)		
0 1	11	Open
0.05 1	9.6	Open
0 10	11	Slightly closed
0.1 10	10	Open
0.05 10	10.3	Open
0.01 10	11	Open
0 100	12	Slightly closed
0.05 100	11	Open
ABA($\mu\text{g}/\text{fl}$) kinetin($\mu\text{g}/\text{fl}$)		
0 1	10	Open
0.05 1	10	Open
0 10	10	Open
0.1 10	10	Closed
0.05 10	10	Closed
0.01 10	10	Closed
0 100	10.5	Open
0.05 100	10	Open

* Measured 5 mm below the anther cap (Arditti and Knauff, 1969). Average of three replicas.

Combinations of abscisic acid and naphthaleneacetic acid

Applications of 250, 25 and 2.5 μg NAA/flower alone or with all ABA concentrations caused most of the previously reported effects (Table 1, Fig. 1; Arditti and Knauff, 1969), although wilting of the perianth was somewhat retarded by 25 μg NAA plus 1×10^{-1} μg or 5×10^{-2} μg ABA. In all instances combinations of ABA and NAA reduced anthocyanin content relative to applications of ABA only (Fig. 1). Combinations of 25 μg

NAA with either 5×10^{-2} or 1×10^{-2} μg ABA induced more anthocyanins in labella, but less in columns, than when the auxin was applied alone (Fig. 1). NAA, 2.5 μg plus 5×10^{-2} μg ABA had a similar effect (Fig. 1). In all other cases ABA-NAA combinations resulted in lower anthocyanin content than in treatments with NAA only (Fig. 1).

Combinations of abscisic acid and gibberellic acid

Swelling or loss of curvature by columns and stigmatic closure were not induced by any of the ABA- GA_3 combinations (Table 1). Wilting of the perianth was evident and progressed with increased ABA concentrations in the presence of 10 μg GA_3 /flower. ABA at a concentration of 5×10^{-2} μg induced wilting when combined with 1 μg GA_3 but not in combination with 10–100 μg GA_3 . Anthocyanin content was mostly lower than in flowers treated only with ABA (Fig. 1). All combinations of ABA with 10 μg GA_3 /flower reduced anthocyanin content to below that of gibberellin-only treated flowers. The combination of 1 μg GA_3 with 5×10^{-2} μg ABA increased anthocyanin content relative to GA_3 -only at the the same concentration.

Combinations of abscisic acid and kinetin

Swelling of the column was not induced by either kinetin alone or any of the ABA-kinetin combinations (Table 1). Paradoxically, stigmas closed when flowers were treated with any of the ABA concentrations in the presence of 10 μg kinetin (Table 1). Yet, stigmatic closure was not caused when 5×10^{-2} μg ABA were applied per flower together with either 100 or 1 μg kinetin (Table 1). Calli developed colour and lost their turgidity, but columns retained their curvature with all ABA-kinetin treatments. Labella and columns of flowers treated with 1×10^{-1} , 1×10^{-2} or 1×10^{-2} μg ABA plus 10 μg kinetin contained less anthocyanins than those treated with ABA only. Combinations of 5×10^{-2} μg ABA with either 100 or 1 μg kinetin increased anthocyanin content relative to the same concentrations of the cytokinin when applied alone. However, with 5×10^{-2} μg of ABA plus 10 μg kinetin, anthocyanin content in labella was lower, and that of columns higher than in flowers treated with an equal amount of kinetin only (Fig. 1).

DISCUSSION

Absciscic acid

Some indication of a possible interaction between ABA and anthocyanin formation may be obtained from coloured autumn leaves. These leaves depend in part on anthocyanins for their colouration (Brian, Petty and Richmond, 1959). Strong growth-inhibiting substances accumulate in such leaves and ABA has been shown to be the principal inhibitor involved (Cornforth *et al.*, 1965; Milborrow, 1967; Addicott and Lyon, 1969). It is, therefore, not inconceivable that ABA may be capable of stimulating anthocyanin synthesis. Also, if ABA is a senescence-inducing hormone and if anthocyanin formation by orchid labella is an aspect of senescence then the observed effects of ABA are those that might be expected.

Another possibility is suggested by the report that anthocyanin production in *Spirodela* can be inhibited by ribonuclease (Radner and Thimann, 1963) and may depend on an unstable RNA which must be continuously resynthesized (Thimann and Radner, 1962). Possibly this lack of stability is due to a highly active ribonuclease whose production along with that of other ribonucleases (Chrispeels and Varner, 1967) may be inhibited by ABA.

Swelling of the column and closing of the stigma, which are not induced by ABA (Table 1), may be regarded as being aspects of growth and possibly redifferentiation (physiological, biochemical and/or morphological). Thus, there is no reason to expect that they would be initiated by a senescence hormone (Addicott and Lyon, 1969) like ABA.

Abscisic acid-naphthaleneacetic acid interactions

Combinations of ABA and NAA inhibit the increase in anthocyanin content which each can induce separately. This agrees in principle with reports that auxin and ABA are antagonistic. In combination with IAA, ABA has counteracted some auxin effects (Addicott and Lyon, 1969), and ABA inhibition of cell division has been partially reversed by IAA (Thomas, Wareing and Robinson, 1965). Decreasing ABA levels in the presence of a constant amount of NAA can elevate anthocyanin content (Fig. 1). Reduced amounts of NAA (from 250 to 2.5 $\mu\text{g}/\text{flower}$) in the presence of an unchanging level of ABA decrease anthocyanin content of labella slightly but first increase, and then lower it in columns.

Swelling of the column and closing of stigmas in orchid flowers can be caused by NAA and are due to increased water uptake, not cell division (Hubert and Maton, 1939; Hsiang, 1951; Oertli and Kohl, 1960). They cannot be inhibited by actinomycin D, puromycin and ethionine, which is a probable indication that newly synthesized RNA or proteins are not required (Arditti and Knauff, 1969). Although the mode of ABA action is not entirely clear at present, the hormone has been implicated in alteration of nucleotide composition or rapidly labelled RNA (Khan and Anojulu, 1970), translation (Walton, Soofi and Sondheimer, 1970), the events following mRNA formation (Gayler and Glasziou, 1969), RNA formation and prevention of its incorporation into enzyme synthesizing units or enzyme synthesis (Chrispeels and Varner, 1967). This, and the suggestion that NAA tends to stabilize invertase-mRNA in sugar cane (Gayler and Glasziou, 1969), raise interesting possibilities regarding the role of ABA and lend further support to our previous conclusions regarding the mode of action of auxin in orchid flowers. Still, it is necessary to keep in mind that the time lapse between exposure to the hormone and measurement of responses may be long enough to allow for a great deal of other biochemical activity (Addicott and Lyon, 1969). It is, therefore, important to approach such interpretations with some restraint (Addicott and Lyon, 1969; Chrispeels and Varner, 1967) and the realization that they contain an element of speculation.

Abscisic acid-gibberellic acid interactions

It is difficult to draw parallels between our results and earlier reports due to lack of consistency in the literature. Gibberellins have been reported to reduce or inhibit anthocyanin content in some instances (Brian, Petty and Richmond, 1959; Arnold and Albert, 1964; Bachelard, 1965; Russell and Galston, 1969) but stimulate it in others (Brian *et al.*, 1959; Furuya and Thimann, 1964).

Decreasing GA_3 concentrations in the presence of a constant amount of ABA result in increased anthocyanin content (Fig. 1). This indicates that GA_3 may act as an ABA antagonist in anthocyanin synthesis. Reductions in the amount of ABA in the presence of 10 μg GA_3 first reduce then raise and subsequently depress anthocyanin content in both columns and labella (Fig. 1). Previous reports indicate that ABA does generally counteract GA induced responses (Addicott and Lyon, 1969; Chrispeels and Varner, 1967) and that gibberellins can reverse ABA inhibited germination (Aspinall, Paleg and

Addicott, 1967; Khan, 1968) and root growth (Sondheimer and Galson, 1966; Khan, 1969) as well as certain types of senescence (Ruddat and Pharis, 1966). Thus, our findings are in agreement with these reports although the mechanism of action remains obscure. A possible speculation is that ABA acts by inhibiting production of a ribonuclease which destroys the unstable RNA required for anthocyanin production (Thimann and Radner, 1962; Radner and Thimann, 1963). Gibberellins may, on the other hand, promote production of this ribonuclease.

Absciscic acid-kinetin interactions

A most interesting aspect of this interaction is stigmatic closure which is not accompanied by swelling of the column in the presence of $10\text{ }\mu\text{g}$ kinetin regardless of ABA concentration (Table 1). This highly localized and specific effect does not occur when $100\text{ }\mu\text{g}$ or $1\text{ }\mu\text{g}$ of kinetin are combined with $5 \times 10^{-2}\text{ }\mu\text{g}$ ABA (Table 1). A slight wilting of the perianth and senescence-type changes in the calli were also evident. This is surprising since cytokinins generally inhibit ageing and overcome ABA-induced effects (Aspinall *et al.*, 1967; Khan, 1967; van Overbeek, Loefer and Mason, 1967; Khan and Downing, 1968) although abscisic acid inhibition of GA_3 -induced α -amylase synthesis cannot be reversed by kinetin (Khan, 1969).

Anthocyanin synthesis in *Impatiens balsamina* is promoted by kinetin, but this promotion is erased by ethionine (Klein and Hagen, 1961). In orchid flowers kinetin does not promote anthocyanin synthesis, although ethionine inhibits it (Arditti and Knauff, 1969) as it does in *Spirodela* (Thimann and Radner, 1955) and apple skin discs (Faust, 1965). At a constant $5 \times 10^{-2}\text{ }\mu\text{g}$ ABA/flower, decreased kinetin concentrations first reduce and then enhance anthocyanin content (Fig. 1). Applications of $10\text{ }\mu\text{g}$ kinetin with decreasing amounts of ABA lowered anthocyanin content of labella and caused a slight increase followed by a decrease in columns (Fig. 1). These results are generally in agreement with previously reported interactions between cytokinins and ABA (Addicott and Lyon, 1969; Aspinall, Paleg and Addicott, 1967; Khan, 1967, 1968; Khan and Downing, 1968; van Overbeek *et al.*, 1967).

General comments

Of the hormones studied to date, exogenous auxin and possibly ethylene (Burg and Dijkman, 1967) can initiate all or most post-pollination phenomena. Other hormones cause only selected symptoms. This suggests the possibility that several control sites or mechanisms may be involved. It may be that of the hormones we have applied only auxin normally reaches *Cymbidium* flowers during pollination due to its presence in orchid pollen. The others, if present later, could be synthesized by flowers following pollination or auxin application. This has been demonstrated for ethylene (Burg and Dijkman, 1967) and is very likely true for the rest since they have all been implicated in fruit set and development (van Overbeek, 1962; Crane, 1964, 1969). As a rule when one (or more) hormone(s) are supplied exogenously to a plant organ *in vitro* it is not easily possible to determine from the effects alone whether other substances become involved and what their nature or concentration ratios might be since hormonal treatments may initiate production of several factors or at least changes in their levels. An excellent example of this, at least in *Vanda* flowers, is auxin-initiated ethylene evolution (Burg and Dijkman, 1967).

Anthocyanin production in pollinated or hormone-treated orchid flowers is probably initiated by several mechanisms. Auxin may act principally by stimulating synthesis of

new, specific RNAs, or by stabilizing existing ones (Gayler and Glasziou, 1969). ABA could function mainly by inhibiting production of a specific ribonuclease which destroys the RNA required for anthocyanin synthesis. Gibberellins probably affect an early step (Furuya and Thimann, 1964) or perhaps participate in a roundabout way through increased production of hydrolytic enzymes which raise the concentration of monosaccharides in labella and columns (Gessner, 1948; Oertli and Kohl, 1960) thereby enhancing anthocyanin synthesis (Thimann, Edmondson and Radner, 1951; Harborne, 1967). Cytokinins appear to have no direct effect on anthocyanin levels in *Cymbidium* flowers although they may somewhat increase or decrease synthesis in other systems. Our observations so far have been concentrated on the external or the visible effects of pollination and hormone treatment. The primary site of action or interactions for plant hormones is undoubtedly on the protein synthesis or nucleic acid levels (Key, 1969). The sensitivity of orchid flowers to hormone treatments and the variety of effects produced make them a very suitable system for future work in this area.

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