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Post-Pollination Biochemical Changes in the Floral Organs of *Rhynchostylis retusa* (L.) Bl. and *Aerides multiflora* Roxb. (Orchidaceae)

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If left unpollinated, the flowers of *Aerides multiflora* (Roxb.) and *Rhynchostylis retusa* (L.) Bl. can remain fresh for 17 and 24 d, respectively. However, they begin to wilt at 2 to 3 days after pollination (DAP) and 3 to 4 DAP, respectively, and become senescent at 5 DAP and 7 DAP, respectively. When measured at two developmental phases -- Stage 1, start of wilting and Stage 2, progression to senescence -- all the floral organs from pollinated flowers had higher contents of total soluble sugars, reducing sugars, and free amino acids than those from unpollinated flowers. A corresponding increase was noted in the activities of hydrolytic enzymes, i.e., α-amylase, β-amylase, and invertase, and proteolytic enzymes (proteases) in those organs. This indicated that signals related to pollination had up-regulated those activities, leading to a breakdown of complex molecules into simpler ones for mobilization. The amounts of sugars and enzyme activity were relatively greater in the pollinated flowers of *A. multiflora* compared with *R. retusa*, and levels were always higher in the floral lips and perianths. When inhibitors of auxin (0.25 mM TIBA) or ethylene (0.25 mM $AgNO_3$) were applied to the pollinated flowers, their senescence was partially prevented, thus signifying hormonal involvement in governing the pollination-induced biochemical alterations normally found in those organs.

Keywords: enzymes, floral organs, inhibitors (auxins and ethylene), orchids, post-pollination, sugars

Pollination regulates a complex of developmental responses that contribute to successful sexual reproduction in higher plants (O'Neill et al., 1993). The events affected by pollination collectively prepare the flower for fertilization and embryogenesis, while also bringing about the withering of floral organs that have completed their functions of pollen dispersal and reception. Orchid flowers are peculiar in having a prolonged life span; though they may stay fresh in an un-pollinated state for a few weeks to several months, they show rapid senescence upon pollination (O'Neill, 1997). Their development is strictly regulated by the pollination process, which then initiates alterations at various organizational levels, eventually leading to floral senescence (O'Neill et al., 1993). This rapid degeneration reduces their commercial value (Ketsa and Rugkong, 1999). Thus, it is imperative for researchers to trace out the events associated with floral senescence and to devise possible manipulations that prolong that life span.

The mechanisms underlying floral senescence in orchids are still enigmatic. Senescence, when it does occur, might be imperceptible for weeks and, even after becoming apparent, symptoms may develop slowly. In orchids, little is known about the response of various floral organs to pollination at the biochemical level, and about how these organs relate to each other in pollinated flowers. In other plant species, an efflux has been reported for some cellular constituents, e.g., the vacuolar pigments and electrolytes (Suttle and Kende, 1978; Celikel and van Doorn, 1995), that is related to the loss of turgor and the visible wilting that results from aging flowers.

Auxins and ethylene have been implicated as the primary molecules that control these pollination-induced responses (Burg and Dijkman, 1967; Ketsa et al., 2006). Pollen interaction with the stigma releases either auxin to stimulate ethylene synthesis or 1-amino-cyclopropane-1-carboxylic acid (ACC) (O'Neill et al., 1993). The latter, a precursor of ethylene, then traverses the floral organs, leading to the onset of their senescence by affecting metabolic events (O'Neill et al., 1993; Bui and O'Neill, 1998). Various developmental events that occur after pollination are reportedly influenced by auxin or ethylene (Zhang and O' Neill, 1993; van Doorn, 1997). However, information is lacking about the precise metabolic changes related to this hormonal involvement.

Therefore, the goal of our investigation of two commercially important orchid species, *Aerides multiflora* Roxb. and *Rhynchostylis retusa* (L.) Bl., was four-fold: 1) to elucidate some biochemical alterations pertaining to carbohydrate and nitrogen metabolism in different floral organs following pollination, 2) to determine whether these species, which vary in their floral life spans, also differ with respect to those biochemical parameters, 3) to detect any interrelationship among different organs, and 4) to probe the involvement of auxin and ethylene in affecting pollination-induced changes, hitherto not reported in these orchids.

MATERIALS AND METHODS

Materials

Orchid species [*Aerides multiflora Roxb.* and *Rhynchostylis retusa* (L.) Bl.; Fig. 1A, B] were collected from their natural habitats, Kangra and Palampur (H.P; India), and were maintained in the 'Orchid house', Department of Botany, Panjab University, Chandigarh, India. Their flowers were hand-pollinated at 9:00 a.m. and examined daily thereafter for visible morphological changes. Flowers were harvested at Stage 1 (first sign of wilting after pollination;

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Fig. 1, D) and at Stage 2 (as wilting progressed; Fig. 1E, F). These samples were then dissected into their perianth, lip, column, and ovary portions, and were analyzed for pigment contents, and for the activities of molecules and related enzymes associated with carbohydrate and nitrogen metabolism.

Measurement of Anthocyanins

Tissues were homogenized in 5 mL of methanol containing 1 N HCl (9:1 methanol:HCl) and maintained at 4°C for 4 h (Christe et al., 1994). Particulates were removed by centrifuging the homogenate at 10,000 g for 30 min. Absorbance of the clear supernatant was read at 530 nm and expressed on a per-gram basis (Kho et al., 1977).

Measurement of Carotenoids

Carotenoids were estimated per the method of Holm (1954). Tissues were homogenized in a clean mortar for 5 min in 5 mL of 80% acetone. The resulting liquid was suction-filtered in a Buchner funnel, and tissue-grinding was repeated twice in 5 mL of 80% acetone, with all three extracts being combined into one flask to ensure the full recovery of all carotenoids. Afterward, the slurry was filtered into the filtrate flask, all the containers being rinsed with 5 mL of 80% acetone to collect any remaining carotenoids. To conveniently calculate the amount of carotenoids present, the final filtrate volume was adjusted to 20 mL with 80% acetone.

Assay of α-amylase Activity

Enzyme activity was assayed (Shuster and Gifford, 1962) by adding 1 mL of starch substrate to 0.5 mL of the enzyme extract (extraction buffer: 0.1 M phosphate buffer, pH 7.0). A 0.2 mL aliquot was removed and combined with 3 mL of potassium iodide solution at Time Zero. After its absorbance was recorded at 620 nm, the reaction mixture was incubated for 30 min at 25°C before another 0.2 mL of aliquot was removed and added to 3 mL of potassium iodide solution. Absorbance of the violet-blue coloring was recorded at 620 nm. Blanks were run simultaneously, either without having substrate in the control or with the enzyme extract being replaced by 0.5 mL distilled water.

Assay of β-amylase Activity

Enzyme activity was assayed as described by Shuster and Gifford (1962). The reaction mixture, containing 0.2 mL of enzyme extract (0.067 M phosphate buffer, pH 6.0) plus 1.0 mL of freshly prepared starch solution, was incubated at 30°C for 1 h. This reaction was terminated by adding 1.0 mL DNSA. Afterward, the tubes were held in boiling water for 10 min, then cooled at room temperature (RT). Two milliliter of distilled water was added to each tube and absorbance was recorded at 560 nm. A control for each reaction mixture was run concurrently to check the level of endogenous sugars over time when the reaction was terminated by adding 1 mL DNSA reagent just before incubation. The reference curve was prepared by using a standard glucose solution.

Assay of Invertase Activity

Invertase activity was assayed (Hawker and Hatch, 1965; Nygaard, 1977) by combining the reaction mixture with 0.6 mL of 0.2 M acetate buffer (pH 4.8) and 0.3 mL of 0.4 M sucrose in 0.1 mL of extract. In the control tube, sucrose was added only when the enzyme preparation had been inactivated by boiling for 5 min. After incubation at 30°C for 30 min, 1 mL of DNSA reagent was added to this reaction mixture. The tubes were placed in a boiling-water bath for 10 min, then cooled to RT. Absorbance was recorded at 560 nm, and the reference curve was prepared with a standard glucose solution.

Assay of Protease Activity

Protease activities were estimated according to the methods of Basha and Beevers (1975) and Salmia et al. (1978). Tissues were homogenized in 5 mL of 0.1 M phosphate buffer (pH 6.0), then spun at low temperature in a high-speed centrifuge. The supernatant was used as our enzyme extract. A mixture containing 0.5 mL of casein solution (1% in 0.1 M phosphate buffer, pH 6.0) and 0.5 mL of enzyme extract was incubated at 45°C for 1 h before the reaction was terminated by the addition of 0.1 mL TCA (40%). Following centrifugation, the TCA soluble component was retained. Amino acid contents were measured after the reaction with Folin-phenol reagent, and absorbance of the reaction mixture was read at 620 nm.

Extraction of Sugars and Amino Acids

Oven-dried roots and shoots were homogenized in hot ethanol (80%), and centrifuged at 2000 rpm for 10 min. The supernatant was clearly decanted. Afterward, 3 mL of ethanol (80%) was added to the residue mixture, followed by re-centrifugation. The extraction was repeated twice to ensure the complete recovery of sugars and amino acids, and the residue was kept for starch estimations. The supernatant was cooled and evaporated to dryness on a china dish in a boiling-water bath, and the residue was eluted with 5 mL of 20% ethanol before being analyzed for sugars and amino acids.

Measurement of Total Sugars

To 4 mL of chilled anthrone reagent, 1 mL of ethanol extract was added. The tubes were shaken gently to mix the solution, then covered with glass marbles and immediately placed in a boiling-water bath for 10 min. After cooling in chilled water, absorbance of the blue green-colored solution was read spectrophotometrically at 625 nm against a blank containing 80% ethanol in place of the ethanol extract. The concentration of total sugars was calculated from a standard curve plotted with a known concentration of glucose (Yemm and Willis, 1954).

Measurement of Reducing Sugars

The amount of reducing sugars was estimated as described by Sumner (1935). To 1 mL DNSA reagent, 1 mL of ethanol extract, prepared as above, was added to a reaction mixture that had been boiled for 12 min. Next, 2 mL of

distilled water was added and absorbance was recorded at 560 nm against a blank containing 80% ethanol in place of the ethanol extract. The level of reducing sugars was calculated from a standard curve plotted with a known concentration of glucose.

Measurement of Free Amino Acids

Amino acid contents were estimated by the method of Lee and Takahashi (1966). To 1 mL of the ethanol extract, 3.8 mL ninhydrin reagent was added. The contents were shaken vigorously and heated in a boiling-water bath for 12 min. The tubes were cooled to RT under running tap water, and absorbance of the colored solution was read at 570 nm against a blank containing 80% ethanol. The concentration of free amino acids was calculated from a standard curve with a known concentration of glycine.

Treatment of Flowers with Inhibitors of Auxin and Ethylene

Intact pollinated flowers were sprayed with an inhibitor of either polar auxin transport (0.25 mM triiodobenzoic acid; TIBA) or ethylene action (0.25 mM silver nitrate) to restrict the endogenous effects of those hormones on flowers after pollination. The spray solutions were prepared by dissolving the appropriate compound in distilled water and adding 0.1% Tween 20 as a surfactant. Our observations were repeated thrice, and the collected data were statistically analyzed for standard errors, using ANOVA and SPSS software.

RESULTS

These two orchid taxa -- *Aerides multiflora* Roxb. and *Rhynchostylis retusa* (L.) Bl. -- differed in their floral life span, as observed with several of their growth parameters (Table 1). Whereas the unpollinated flowers of the former remained fresh for 17 d (Fig. 1A) but then attained senescence (Fig. 1C, E) at 7 days after pollination (DAP), those of the latter were fresh for 24 d (Fig. 1B) but senesced at 5 DAP (Fig. 1D, F). These observations are somewhat similar to those reported earlier for *Phalaenopsis* and *Cymbidium* species, where the flowers could live for up to 8 weeks if left unpollinated, but died within 7 d after the act of pollination. Likewise, *Paphiopedilum* blossoms lasted for 3 months in an unpollinated state but senesced within 3 weeks after pollination (Arditti, 1992).

Pollen germination occurred within 24 h for *A. multiflora* and 48 h for *R. retusa*. Their floral lips began to fade at 2 DAP in the former and at 4 to 5 DAP in the latter, while the columns and ovaries from both species did not differ in the degree of increase in their dimensions after pollination. Wilting began at 2 to 3 DAP in *A. multiflora* and at 3 to 4 DAP in *R. retusa*. The first detectable symptom was a darkening of the lips, a result of elevated anthocyanin contents (Table 2). This change in color continued until the perianth wilted and shrank to its minimum size. Such a phenomenon occurred almost simultaneously in both the taxa, the rate of darkening in color accelerating more rapidly in the lip than in the perianth at both developmental stages. These observations agree with those of Arditti et al. (1973) and Woltering (1990a, b), who reported altered pigmentation, especially enhanced anthocyanin levels, in the perianths of pollinated *Cymbidium* species. Accompanying these color variations was a rise in carotenoid contents during Stage 1 in both species, followed by a decline at Stage 2 (Table 3). This change in pigment presumably serves as a signal to pollinators that a flower has been visited or pollinated. Although the specific underlying biochemical events that affect this are not well understood, they have been associated with carotenoid or anthocyanin biosynthesis (Mohan Ram and Mathur, 1984), anthocyanin degradation (Procter and Creasy, 1969), and a pH change in the floral tissues (Asen et al., 1977).

At both development stages, all organs sampled in the pollinated flowers had higher levels of total soluble sugars (Table 4), reducing sugars (Table 5), and amino acids (Table 6) compared with the unpollinated flowers. The greatest amounts were measured in the lips, followed by the perianths. We noted, with few exceptions, a corresponding

Table 1. Comparison of floral changes in *Rhynchostylis retusa* (L.) Bl. and *Aerides multiflora* Roxb. over time. DAP = days after pollination.

Step	Event	Time	
		R. retusa	*A. multiflora*
1	Senescence in unpollinated flowers	24 d	17 d
2	Senescence in pollinated flowers	7 DAP	5 DAP
3	Change in lip	Within 24 h	Within 24 h
4	Initiation of increase in column size	1 DAP	1 DAP
5	Initiation of increase in diameter of ovary	1 DAP	1 DAP
6	Pollen germination	24-48 h	Within 24 h
7	Initiation of perianth wilting (Stage 1)	3-4 DAP	2-3 DAP
8	Perianth wilting progresses (Stage 2), just before shrinkage	5-6 DAP	3-4 DAP
9	Maximum diameter of ovary	30 mm at 46-50 DAP	30 mm at 70-74 DAP
10	Maximum ovary length	30 mm	23 mm
11	Structure of ovule at anthesis	Absent	Absent
12	Placental ridges become meristematically active	After pollination	After pollination
13	Number of cells in axial filament	5-7 cells	5-7 cells

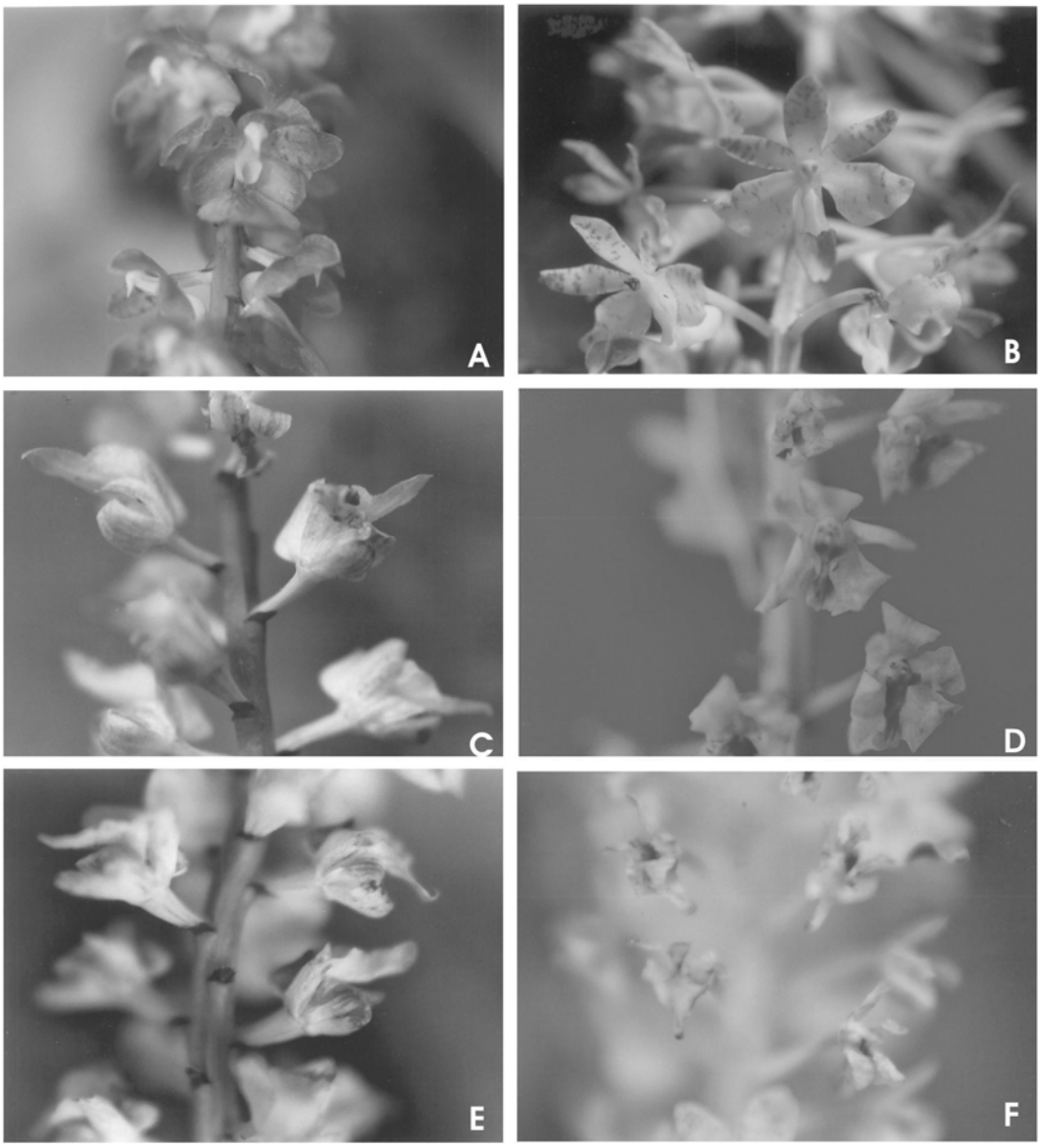

Figure 1. Morphology and post-pollination changes in orchid flowers. **A and B,** Inflorescences showing unpollinated flowers from *Aerides multiflora* and *Rhynchostylis. retusa*, respectively. **C and D,** Initiation of perianth senescence during Stage 1, at 2-3 days after pollination (DAP) for *A. multiflora* or at 3-4 DAP for *R. retusa*. **E and F,** Progression of senescence (Stage 2) in flowers at 3-4 DAP for *A. multiflora* or 5-6 DAP for *R. retusa*.

increase in the activities of α-amylase (Table 7) and β-amylase (Table 8), two enzymes involved in the hydrolysis of starch, as well as invertase (for sucrose; Table 9) and proteases (proteins; Table 10). This was true for both species during at least Stage 1, indicating that pollination-related signals up-regulated those activities and that the degradation of complex molecules to simple ones may have occurred during senescence. Ovaries had relatively lower levels of sugars and enzymes compared with other organs, although those former tissues showed greater contents of simple molecules in the second stage, thereby demonstrating their mobilization into the ovary from the lip and perianth.

The amount of sugars and the activity of enzymes were relatively greater in the pollinated flowers of *A. multiflora* compared with *R. retusa*, reflecting the faster rate of senescence in the former. In previous studies, sugar contents were found to increase post-pollination in the gynostemia and perianth segments from *Cattleya* but not from *Cymbidium* (Hsiang, 1951). Moreover, the concentration of sugar in *Cymbidium sinense* declined over time while the opposite trend was reported for *Dendrobium nobile* (Wen et al., 1990).

Here, the lip and perianth showed higher enzyme activities and sugar amounts in Stage 2, suggesting that, upon pollination, a signal may emanate from the pollen itself or be induced by metabolic changes in the column that are transferred to other organs, making this a time-dependent process. O'Neill et al. (1993) have determined that both auxin and 1-amino-cyclopropane-1-carboxylic acid (a precursor of ethylene) are released by the pollen and stimulate the synthesis of ethylene, which then affects the entire flower and induces senescence-related events. The elevation of sugar

Table 2. Anthocyanin contents (ΔOD wt/g fw) at Stage 1 and Stage 2 in orchid floral organs after treatments. Values represent mean ± S.E.

Stage	Treatment	Floral organ							
		Aerides multiflora[1]				*Rhynchostylis retusa*[2]			
		Column	Ovary	Lip	Perianth	Column	Ovary	Lip	Perianth
1	UP[a]	0.031±0.002	0.016±.002	0.026±.003	0.03±0.01	0.02±0.008	0.014±0.004	0.04±0.009	0.02±0.006
	P[b]	0.025±0.003	0.080±0.008	0.15±0.06	0.23±0.03	0.01±0.008	0.02±0.007	0.04±0.006	0.03±0.008
	TIBA[c]	0.052±0.008	0.074±0.008	0.09±0.009	0.10±0.02	0.01±0.008	0.02±0.008	0.06±0.007	0.07±0.005
	$AgNO_3$[d]	0.026±0.008	0.056±0.007	0.06±0.008	0.08±0.02	0.02±0.007	0.04 ±0.008	0.11±0.02	0.07±0.006
2	UP[a]	0.015±0.003	0.081±0.005	0.03±0.006	0.03±0.008	0.01±0.008	0.02±0.009	0.03±0.007	0.015±0.006
	P[b]	0.025±0.008	0.06±0.006	0.14±0.02	0.14±0.01	0.02±0.006	0.014±0.003	0.05±0.004	0.03±0.007
	TIBA[c]	0.035±0.007	0.03±0.008	0.05±0.009	0.09±0.008	0.05±0.007	0.02±0.008	0.08±0.007	0.03±0.009
	$AgNO_3$[d]	0.026±0.004	0.048±0.010	0.04±0.008	0.10± 0.02	0.02±0.008	0.01±0.007	0.04±0.005	0.02±0.004

C.D. (P=0.05) Critical difference (C.D.) indicates weather the difference between two treatments or organs is statistically significant or not. [1]first stage (S-1)-for treatments: 0.003, for organs: 0.005 and second stage (S-2)-for treatments: 0.004, for organs: 0.003. [2]first stage (S-1)-for treatments: 0.002, for organs:0.003 and second stage (S-2)- for treatments: 0.003, for organs:0.003. [a]Unpollinated; [b]Pollinated; [c]Triiodobenzoic acid; [d]Silver nitrate

Table 3. Carotenoid contents (μg g^{-1} fw) at Stage 1 and Stage 2 in orchid floral organs after treatments. Values represent mean ± S.E.

Stage	Treatment	Floral organs							
		Aerides multiflora[1]				*Rhynchostylis retusa*[2]			
		Column	Ovary	Lip	Perianth	Column	Ovary	Lip	Perianth
1	UP[a]	0.033±0.004	0.021±0.003	0.05±0.008	0.16±0.008	0.10±0.02	0.02±0.008	0.024±0.009	0.07±0.008
	P[b]	0.015±0.003	0.03±0.008	0.08±0.009	0.19±0.02	0.25±0.02	0.05±0.009	0.10±0.01	0.04±0.009
	TIBA[c]	0.042±0.005	0.08±0.007	0.12±0.02	0.24±0.03	0.46±0.03	0.06±0.008	0.06±0.008	0.06±0.008
	$AgNO_3$[d]	0.048±0.004	0.10±0.006	0.20±0.02	0.28±0.02	0.164±0.009	0.05±0.008	0.04±0.008	0.10±0.01
2	UP[a]	0.045±0.003	0.02±0.006	0.09±0.009	0.15±0.02	0.125±0.008	0.03±0.008	0.11±0.02	0.10±0.02
	P[b]	0.029±0.003	0.04±0.008	0.05±0.006	0.14±0.02	0.15±0.02	0.05±0.009	0.12±0.02	0.20±0.02
	TIBA[c]	0.058±0.005	0.05±0.009	0.08±0.009	0.30±0.03	0.08±0.008	0.07±0.006	0.16±0.02	0.175±0.011
	$AgNO_3$[d]	0.045±0.003	0.08±0.007	0.08±0.009	0.30±0.04	0.30±0.03	0.05±0.009	0.13±0.01	0.104±0.008

C.D. (P=0.05): Critical difference (C.D.) indicates weather the difference between two treatments or organs is statistically significant or not [1]first stage (S-1)-for treatments: 0.018, for organs: 0.016 and second stage (S-2)-for treatments: 0.019, for organs: 0.017. [2]first stage (S-1)-for treatments: 0.014, for organs: 0.019 and second stage (S-2)-for treatments: 0.016, for organs: 0.026. [a]Unpollinated; [b]Pollinated; [c]Triiodobenzoic acid; [d]Silver nitrate

Table 4. Amounts of total sugars (μg g^{-1} dw) at Stage 1 and Stage 2 in orchid floral organs after treatments. Values represent mean ± S.E.

Stage	Treatment	Floral organ							
		Aerides multiflora[1]				*Rhynchostylis retusa*[2]			
		Column	Ovary	Lip	Perianth	Column	Ovary	Lip	Perianth
1	UP[a]	28 ± 1.4	34.3 ± 1.4	39 ± 1.4	42.1 ± 2.3	5.63 ± 1.2	5.8 ± 1.5	10.5 ± 1.4	7.2 ± 1.5
	P[b]	53 ± 2.3	47.3 ± 1.5	61 ± 2.5	64.1 ± 2.4	11.5 ± 2.2	11.7 ± 1.6	19.4± 1.3	12.4 ± 1.4
	TIBA[c]	35.7 ± 2.2	45.3 ± 1.3	56 ± 2.1	57.7 ± 2.3	6.4 ± 2.3	11.3 ± 1.3	14.6± 1.5	9.9 ± 1.3
	$AgNO_3$[d]	33 ± 1.5	36.3 ± 1.5	50 ± 1.3	50.6 ± 2.3	3.6 ± 1.4	9.5 ± 1.5	10.3± 1.4	6.9 ± 1.6
2	UP[a]	33.7 ± 1.4	42.3 ± 1.5	48.5 ± 1.4	52.2 ± 2.4	9.2 ± 1.5	13 ± 1.3	5.7 ± 1.6	5.4 ± 1.4
	P[b]	28 ± 2.2	52.3 ± 1.5	69 ± 2.3	70 ± 2.3	6.1 ± 1.6	15.5 ± 1.5	7.5 ± 1.1	10.5 ± 1.4
	TIBA[c]	42 ± 2.4	51.3 ± 1.3	54 ± 2.4	69.8 ± 2.5	4.2 ± 1.4	15.6 ± 1.4	12.5 ± 1.5	10.3 ± 1.3
	$AgNO_3$[d]	37.3 ± 1.5	50 ± 1.2	51.3 ± 1.2	64.8 ± 1.1	6.9 ± 1.4	11.6 ± 1.1	11.7 ± 1.4	11.2 ± 1.2

C.D. (P=0.05) Critical difference (C.D.) indicates weather the difference between two treatments or organs is statistically significant or not [1]first stage (S-1) - for treatments: 2.4, for organs: 2.8 and second stage (S-2) - for treatments: 1.5, for organs: 2.1.[2]first stage (S-1)- for treatments: 2.4, for organs: 1.4 and second stage (S-2)- for treatments: 2.1, for organs: 1.7. [a]Unpollinated; [b]Pollinated; [c]Triiodobenzoic acid; [d]Silver nitrate

Table 5. Amounts of reducing sugars (μg g^{-1} dw) at Stage 1 and Stage 2 in orchid floral organs after treatments. Values represent mean ± S.E.

Stage	Treatment	Floral organ							
		Aerides multiflora[1]				*Rhynchostylis retusa*[2]			
		Column	Ovary	Lip	Perianth	Column	Ovary	Lip	Perianth
1	UP[a]	18 ± 2.1	8 ± 1.1	20 ± 1.2	16.2 ± 1.2	0.11 ± 0.02	1.77 ± 0.12	0.72 ± 0.04	0.77 ± 0.03
	P[b]	12.3 ± 2.3	20. 7 ± 2.3	32.5 ± 2.3	26.4 ± 1.4	0.58 ± 0.05	1.83 ± 0.13	1.4 ± 0.03	1.06 ± 0.04
	TIBA[c]	16.3 ± 1.4	22.3 ± 1.2	17.2 ± 1.4	27.7 ± 1.5	0.40 ± 0.02	1.30 ± 0.12	0.33 ± 0.04	0.24 ± 0.03
	$AgNO_3$[d]	12 ± 1.4	15.3 ± 1.4	15 ± 1.2	20.4 ± 1.4	0.50 ± 0.03	1.5 ± 0.15	0.43 ± 0.02	0.55 ± 0.03
2	UP[a]	13.7 ± 1.1	11.3 ± 1.3	27 ± 1.4	21.5 ± 1.3	1.9 ± 0.2	1.42 ± 0.13	1.41 ± 0.05	0.33 ± 0.02
	P[b]	7 ± 1.3	18 ± 1.4	42.6 ± 1.5	25.5 ± 1.5	1.04 ± 0.07	2.4 ± o.23	1.53 ± 0.04	1.86 ± 0.05
	TIBA[c]	9 ± 1.1	21.7 ± 1.2	31.7 ± 1.3	18.4 ± 1.3	1.35 ± 0.05	0.30 ± 0.03	0.34 ± 0.03	0.34 ± 0.02
	$AgNO_3$[d]	10.7 ± 1.2	15 ± 1.4	36 ± 1.4	25.7 ± 1.4	1.3 ± 0.2	0.76 ± 0.04	0.14 ± 0.02	0.21 ± 0.04

C.D. (P=0.05) Critical difference (C.D.) indicates weather the difference between two treatments or organs is statistically significant or not [1]first stage (S-1) - for treatments: 1.6, for organs: 1.5 and second stage (S-2) - for treatments: 2.3, for organs: 15. [2]first stage (S-1)- for treatments: 0.12, for organs: 0.18 and second stage (S-2)- for treatments: 0.16, for organs: 0.21. [a]Unpollinated; [b]Pollinated; [c]Triiodobenzoic acid; [d]Silver nitrate

Table 6. Amounts of amino acids (μg g^{-1} dw) at Stage 1 and Stage 2 in orchid floral organs after treatments. Values represent mean ± S.E.

Stage	Treatment	Floral organ							
		Aerides multiflora[1]				*Rhynchostylis retusa*[2]			
		Column	Ovary	Lip	Perianth	Column	Ovary	Lip	Perianth
1	UP[a]	17.7 ± 2.3	15 ± 1.2	26.6 ± 1.4	40.8 ± 1.2	1.1 ± 0.2	0.50 ± 0.04	0.64 ± 0.04	0.56 ± 0.07
	P[b]	29 ± 2.1	25 ± 1.4	36 ± 1.5	56.8 ± 1.5	1.23 ± 0.10	0.42 ± 0.05	0.77 ± 0.05	1.57 ± 0.08
	TIBA[c]	23.3 ± 1.3	21.7 ± 1.4	32.5 ± 1.3	48.8 ± 1.3	1.16 ± 0.11	1.44 ± 0.04	0.63 ± 0.04	0.72 ± 0.09
	$AgNO_3$[d]	22.3 ± 1.5	22 ± 1.3	28 ± 1.4	47.1 ± 1.1	1.46 ± 0.10	1.0 ± 0.05	1.51 ± 0.11	0.40 ± 0.07
2	UP[a]	22.7 ± 1.4	21.7 ± 1.1	31.6 ± 1.3	37 ± 2.1	2.05 ± 0.12	2.14 ± 0.06	0.57 ± 0.08	1.7 ± 0.11
	P[b]	34 ± 1.3	31 ± 1.4	34.7 ± 1.5	45.7 ± 1.4	0.40 ± 0.07	2.7 ± 0.04	1.1 ± 0.08	1.54 ± 0.10
	TIBA[c]	33.3 ± 1.3	27.3 ± 1.3	28.9 ± 1.3	40 ± 1.6	1.65 ± 0.08	1.33 ± 0.03	1.50 ± 0.11	1.40 ± 0.09
	$AgNO_3$[d]	31 ± 1.3	25.7 ± 1.2	25.3 ± 1.6	39.8 ± 1.4	1.4 ± 0.07	1.06 ± 0.04	1.4 ± 0.09	1.56 ± 0.11

C.D. (P=0.05) Critical difference (C.D.) indicates weather the difference between two treatments or organs is statistically significant or not [1]first stage (S-1) - for treatments: 1.3, for organs: 1.8 and second stage (S-2) - for treatments: 1.6, for organs: 1.8. [2]first stage (S-1) - for treatments: 0.13, for organs: 0.18 and second stage (S-2)- for treatments: 0.16, for organs: 0.20. [a]Unpollinated; [b]Pollinated; [c]Triiodobenzoic acid; [d]Silver nitrate

Table 7. Activity of α-amylase (μg starch hydrolyzed g^{-1} fw) at Stage 1 and Stage 2 in orchid floral organs after treatments. Values represent mean ± S.E.

Stage	Treatment	Floral organ							
		Aerides multiflora[1]				*Rhynchostylis retusa*[2]			
		Column	Ovary	Lip	Perianth	Column	Ovary	Lip	Perianth
1	UP[a]	22 ± 1.8	13.3 ± 1.8	31.2 ± 1.9	37.3 ± 1.8	3.4 ± 0.9	5.9 ± 1.3	2.2 ± 0.8	21.7 ± 2.3
	P[b]	32 ± 1.7	28 ± 1.6	43.0 ± 2.1	47.3 ± 2.4	11.3 ± 1.1	11.7 ± 1.2	11.6 ± 1.1	29.7 ± 2.4
	TIBA[c]	28.7 ± 2.1	21.3 ± 1.7	38.3 ± 1.7	38 ± 2.5	4.1 ± 1.1	11.4 ± 1.3	4.03 ± 1.2	29 ± 2.4
	$AgNO_3$[d]	28 ± 1.8	16 ± 1.5	32.6 ± 2.3	34.5 ± 1.8	10.9 ± 1.5	10 ± 1.4	3.2 ± 0.9	22.2 ± 2.1
2	UP[a]	25.7 ± 1.6	18.3 ± 1.8	38.2 ± 2.1	41.8 ± 2.5	1.7 ± 0.7	8.0 ± 1.2	1.8 ± 0.9	29 ± 2.5
	P[b]	31.3 ± 1.8	12 ± 1.6	33 ± 1.7	37.7 ± 2.3	3.4 ± 0.6	12.8 ± 1.8	2.9 ± 0.8	35.6 ± 3.1
	TIBA[c]	28.7 ± 1.9	15.7 ± 1.5	25.9 ± 1.5	29.4 ± 2.4	3.5 ± 0.5	10.1 ± 1.8	2.4 ± 0.7	28.6 ± 2.3
	$AgNO_3$[d]	29.3 ± 1.8	10.7 ± 1.8	22.9 ± 1.7	32 ± 2.1	2.9 ± 0.7	7.4 ± 1.4	1.2 ± 0.4	21.6 ± 2.4

C.D. (P=0.05) Critical difference (C.D.) indicates weather the difference between two treatments or organs is statistically significant or not [1]first stage (S-1) - for treatments: 1.8, for organs: 2.2 and second stage (S-2) - for treatments: 1.6, for organs: 1.8. [2]first stage (S-1)- for treatments: 1.1, for organs: 1.6 and second stage (S-2)- for treatments: 1.4, for organs: 1.4. [a]Unpollinated; [b]Pollinated; [c]Triiodobenzoic acid; [d]Silver nitrate

Table 8. Activity of β-amylase (μg reducing sugar formed g^{-1} fw) at Stage 1 and Stage 2 in orchid floral organs after treatments. Values represent mean ± S.E.

Stage	Treatment	Floral organ							
		Aerides multiflora[1]				*Rhynchostylis retusa*[2]			
		Column	Ovary	Lip	Perianth	Column	Ovary	Lip	Perianth
1	UP[a]	12 ± 0.7	15. 7 ± 1.3	35 ± 2.3	39.2 ± 1.4	6.97 ± 1.2	9 ± 1.1	11.5 ± 1.3	16.2 ± 1.3
	P[b]	11.2 ± 0.8	11.4 ± 1.2	10.5 ± 1.4	45 ± 1.3	8.1 ± 1.1	14 ± 1.3	14.5 ± 1.4	27 ± 2.1
	TIBA[c]	7.9 ± 1.2	11.3 ± 1.2	33.8 ± 1.7	40.8 ± 1.3	11.2 ± 1.2	10.3 ± 1.1	15.2 ± 1.1	23.3 ± 2.0
	$AgNO_3$[d]	6.2 ± 1.1	11.2 ± 1.1	5.0 ± 1.1	35.6 ± 1.5	11.7 ± 1.1	9 ± 1.2	16.3 ± 1.2	17.8 ± 1.4
2	UP[a]	14.9 ± 2.1	15 ± 1.3	17.4 ± 1.4	28 ± 1.3	10.7 ± 1.1	12.7 ± 1.2	17.2 ± 1.2	19.7 ± 1.3
	P[b]	34.3 ± 1.2	67 ± 1.4	54.4 ± 2.3	49.2 ± 2.1	13 ± 1.4	17 ± 1.3	25.3 ± 2.3	32.3 ± 2.2
	TIBA[c]	28.1 ± 1.3	24 ± 1.2	33.6 ± 2.4	39.5 ± 1.4	9.5 ± 1.3	14 ± 1.1	18 ± 2.1	25.8 ± 2.1
	$AgNO_3$[d]	17.5 ± 1.3	8.3 ± 1.3	5.9 ± 1.2	38.5 ± 1.1	9.7 ± 1.2	11.3 ± 1.4	15.2 ± 1.4	17.7 ± 2.2

C.D. (P=0.05)Critical difference (C.D.) indicates weather the difference between two treatments or organs is statistically significant or not [1]first stage (S-1) - for treatments: 2.1, for organs: 1.7 and second stage (S-2) - for treatments: 1.7, for organs: 1.5. [2]first stage (S-1)- for treatments: 1.8, for organs: 2.1and second stage (S-2)- for treatments: 1.6, for organs: 1.3. [a]Unpollinated; [b]Pollinated; [c]Triiodobenzoic acid; [d]Silver nitrate

Table 9. Invertase activity (μg reducing sugar formed g^{-1} fw) at Stage 1 and Stage 2 in orchid floral organs after treatments. Values represent mean ± S.E.

Stage	Treatment	Floral organ							
		Aerides multiflora[1]				*Rhynchostylis retusa*[2]			
		Column	Ovary	Lip	Perianth	Column	Ovary	Lip	Perianth
1	UP[a]	53.3 ± 2.2	44.7 ± 2.2	56 ± 3.2	63.5 ± 2.4	11.3 ± 1.4	11 ± 1.1	18.7 ± 1.5	20 ± 1.3
	P[b]	97.7 ± 2.1	97.7 ± 2.4	97 ± 4.5	104 ± 3.	16 ± 1.6	7 ± 1.3	12.8 ± 1.6	24.8 ± 1.4
	TIBA[c]	86 ± 2.1	77.3 ± 2.1	77.3 ± 5.2	218 ± 4.3	20.3 ± 1.6	9.7 ± 1.2	17.5 ± 1.5	18.3 ± 1.3
	$AgNO_3$[d]	54.7 ± 2.4	32.3 ± 2.4	54.6 ± 3.5	77 ± 5.4	16.3 ± 1.6	11.7 ± 1.4	8.7 ± 1.3	15.6 ± 1.6
2	UP[a]	44 ± 1.6	52 ± 2.4	62 ± 4.6	85.5 ± 2.4	8 ± 1.4	12 ± 1.3	12.5 ± 1.7	21.3 ± 1.3
	P[b]	78.7 ± 2.3	200 ± 7.8	155 ± 5.2	109 ± 3.5	21 ± 1.4	31 ± 2.1	39.2 ± 1.8	38.2 ± 1.9
	TIBA[c]	69.3 ± 1.8	184 ± 6.9	78 ± 3.6	54.9 ± 4.7	14 ± 1.3	28 ± 2.3	33.6 ± 1.3	23.3 ± 1.3
	$AgNO_3$[d]	64 ± 1.3	194 ± 8.9	68.9 ± 3.3	45.7 ± 3.6	12.7 ± 1.1	25 ± 1.5	25.6 ± 1.6	20.1 ± 1.7

C.D. (P=0.05) Critical difference (C.D.) indicates weather the difference between two treatments or organs is statistically significant or not [1]first stage (S-1) - for treatments: 2.2, for organs: 2.7 and second stage (S-2) - for treatments: 2.6, for organs: 2.4. [2]first stage (S-1)- for treatments: 1.2, for organs: 1.8 and second stage (S-2)- for treatments: 2.2, for organs: 2.8. [a]Unpollinated; [b]Pollinated; [c]Triiodobenzoic acid; [d]Silver nitrate

Table 10. Protease activity (μg amino acids formed g^{-1} fw) at Stage 1 and Stage 2 in orchid floral organs after treatments. Values represent mean ± S.E.

Stage	Treatment	Floral organ							
		Aerides multiflora[1]				*Rhynchostylis retusa*[2]			
		Column	Ovary	Lip	Perianth	Column	Ovary	Lip	Perianth
1	UP[a]	6.3 ± 1.1	7 ± 1.2	5.1 ± 1.4	5.8 ± 1.2	6.3 ± 1.3	7 ± 1.2	5.0 ± 1.1	5.8 ± 1.3
	P[b]	13.2 ± 1.3	12.3 ± 1.3	23 ± 2.1	12.2 ± 1.5	13.2 ± 2.1	12.3 ± 1.3	23.2 ± 2.2	12.2 ± 1.2
	TIBA[c]	7.2 ± 1.1	5. 7 ± 1.2	7.5 ± 2.2	10.2 ± 1.6	7.1 ± 1.2	5.6 ± 1.4	7.5 ± 1.3	10.2 ± 1.2
	$AgNO_3$[d]	8 ± 1.2	8 ± 1.5	10.6 ± 2.4	10.4 ± 1.2	8 ± 1.3	8.3 ± 1.3	10.6 ± 1.2	10.4 ± 1.3
2	UP[a]	8.8 ± 1.1	11 ± 1.4	5.7 ± 1.4	4.5 ± 1.3	8.7 ± 1,8	11 ± 1.3	5.7 ± 1.1	4.5 ± 1.2
	P[b]	14.7 ± 1.6	14.3 ± 1.3	19.2 ± 1.5	14.1 ± 2.2	14.7 ± 1.3	14.3 ± 1.4	19.2 ± 2.3	14.1 ± 1.4
	TIBA[c]	10.3 ± 1.3	13 ± 1.6	18.3 ± 1.1	13.4 ± 1.5	10.3 ± 1.2	13 ± 1.4	18.2 ± 1.4	13.4 ± 1.5
	$AgNO_3$[d]	5.3 ± 1.1	11 ± 1.2	5.5 ± 1.3	11 ± 1.1	5.3 ± 1.4	11 ± 1.3	5.5 ± 1.2	10.6 ± 1.2

C.D. (P=0.05) Critical difference (C.D.) indicates weather the difference between two treatments or organs is statistically significant or not [1]first stage (S-1) - for treatments: 1.4, for organs: 2.1 and second stage (S-2) first stage (S-1)- for treatments: 2.1, for organs: 2.6 and second stage (S-2)- for treatments: 1.8, for organs: 1.6. [a]Unpollinated; [b]Pollinated; [c]Triiodobenzoic acid; [d]Silver nitrate

concentrations in a sequential pattern over those two stages also demonstrated that metabolic events are initially activated in the column, then in the other organs. Earlier research had shown that the sugar content declines over time in *Cymbidium sinense* (Wen et al., 1990) but rises with age in *Dendrobium nobile*. Levels of sugar also increase following pollination in the gynostemia and perianth segments of *Cattleya* sp. but not *Cymbidium* (Hsiang, 1951).

These metabolic alterations appear to be part of a general floral response during the organized degenerative process of senescence, involving several hydrolytic reactions that break down more complex molecules (van Doorn and Woltering, 2004). These simple molecules may then be mobilized to the mother plant and subsequently utilized after they are diverted to other metabolic pathways (Erdelska and Oveèka, 2004). The rise in sugar concentrations may aid in a variety of roles in senescing organs (Olley et al., 1996; Rolland et al., 2002), perhaps serving as a critical source of energy during programmed cell death (van Doorn and Woltering, 2004). Petal senescence also may be linked to sugar starvation because the exogenous application of those molecules seems to prolong the floral life span (van Doorn, 2004). Sucrose in particular appears to play a predominant role, as suggested by its marked decrease from the onset of senescence in pollinated flowers. These sugars may possibly act as components of multiple signals (Rolland et al., 2002) that originate in the style at the site of pollination and are then translocated to the ovary and petals where they trigger ovary development and corolla senescence. They may also be components of secondary signals during senescence (Rolland et al., 2002), either as the primary signalling molecules themselves or as something induced by the primary signals, e.g., auxin or ethylene (as seen here), during pollen-pistil interactions. Sugar enhancement that originates in the style is presumably followed by translocation to the ovary and petals where it may prompt further development and corolla senescence. The remobilization of nutrients, including sugars, from aging organs such as the perianth and lip might contribute effectively towards the success of the overall reproductive process (O'Neill and Nadeau, 1997).

Effects of Auxin and Ethylene Inhibitors on Post-Pollination Changes

The involvement of auxin and ethylene can be suitably investigated with direct measurements as well as through the application of known hormone inhibitors. Here, we treated pollinated flowers with either anti-auxin triiodobenzoic acid, which blocks auxin transport, or silver nitrate, a potent inhibitor of ethylene action.

With few exceptions, all of the organs from the flowers of both species sprayed with 0.25 mM TIBA or 0.25 mM $AgNO_3$ had relatively lower amounts of reducing and soluble sugars as well as diminished enzymatic activities. We might conclude that application of those inhibitors partially suppressed the pollination-induced changes. This implies that the hormones tested here interact with carbohydrate and nitrogen metabolism, possibly by down-regulating their enzymes. Silver nitrate had a more pronounced effect than TIBA, indicating the greater involvement of ethylene in mediating pollination-related outcomes. Using this same hormonal approach, Hilioti et al. (2000) clearly demonstrated the participation of auxin and ethylene in regulating flower senescence in other plant species (van Doorn, 1997). Our observations also corroborated those from previous investigations (Zhang and O'Neill, 1993; Jones and Woodson, 1997; Ketsa et al., 2000) in which the inhibitors of ethylene and auxins caused a substantial delay in floral senescence.

Although auxin is unlikely to be a secondary pollination signal that regulates perianth senescence (Strauss and Arditti, 1982), ethylene has been strongly implicated as a potential transmissible secondary signal to floral organs distant from the stigma, thereby promoting a number of post-pollination events, including perianth senescence. This greater involvement of ethylene in governing that process might suggest that its inhibitor is more effective than the auxin inhibitor tested here. In fact, our observations are in accord with previous findings (Zhang and O' Neill, 1993; van Doorn, 1997). Furthermore, the varying influence of these inhibitors between the first and second stages of pollination might be ascribed to differences in the endogenous levels of auxins and ethylene. Here, floral senescence was delayed 3 and 4 d in *A. multiflora* and *R. retusa*, respectively, after TIBA treatment, while silver nitrate deferred this senescence by 5 and 7 d, respectively. Future studies with more inhibitors, and at a broad range of concentrations, would test their capacity for extending the floral life span.

DISCUSSION

Our findings indicate that pollination-induced flower senescence in orchids involves several hydrolytic processes responsible for causing the degradation of floral organs, especially the perianth and lip. This is evident by the elevated levels of certain enzymes, i.e., α-amylase, β-amylase, invertase, and proteases, which lead to the breakdown of complex molecules, such as carbohydrates and proteins, into simpler products (total sugars, reducing sugars, and amino acids). Changes in the contents of anthocyanin and carotenoid pigments also appear to be a part of this degenerative process. The resultant simple molecules are possibly re-mobilized into the mother plant for their utilization. A comparative evaluation of various parameters showed similarities among the basic events affecting senescence in the pollinated and unpollinated flowers of both orchid species; pollination seemed to accelerate those events to induce early senescence. However, the two taxa differed in their floral longevity in both the unpollinated and the pollinated stages, as well as in the expression of molecules in some organs. Future studies of orchids and other species should focus on monitoring the mRNA transcripts of certain molecules in order to probe the specific markers that govern those variations in life span. Nevertheless, we have here been able to corroborate the involvement of auxins and ethylene in affecting flower senescence because their inhibitors partially prevented related events. In this context, the influence of ethylene appeared to be greater than auxin. Further experiments might include time-course analyses of those

hormones, as well as biosynthetic enzymes, in different organs if we are to understand some of the finer regulatory mechanisms that underlie pollination-related flower senescence in orchids.

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