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BRIEF COMMUNICATION

Post-pollination changes in the floral organs of two *Cymbidium* species

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Abstract

It was observed that the unpollinated flowers of *Cymbidium pendulum* (Roxb.) Sw. and *C. aloifolium* (L.) Sw. stayed fresh for 20 and 18 d, respectively, but attained senescence in 8 and 7 d, respectively, after pollination. The higher content of total soluble sugars, reducing sugars and free amino acids was observed in all the floral organs of pollinated flowers than in unpollinated ones. Pollination also up-regulated the activity of hydrolytic (α-amylase, β-amylase, invertase) and proteolytic enzymes (proteases) in floral organs. Amongst floral organs, the lip and perianth possessed highest contents of metabolites. Application of auxin inhibitor (0.25 μM triiodobenzoic acid) and ethylene inhibitor (0.25 μM $AgNO_3$) to the pollinated flowers partially prevented the process of senescence.

Additional keywords: amylases, auxins, ethylene, invertase, proteases, senescence, sugars.

The development of orchid flowers is strictly regulated by pollination that initiates alterations at various organization levels eventually leading to floral senescence. The developmental events affected by pollination collectively prepare the flower for fertilization and embryogenesis while bringing about the withering of the floral organs that have completed their function in pollen dispersal and reception. Orchid flowers are peculiar in having a prolonged life span and their development is almost strictly regulated by pollination and associated signals (O'Neill *et al.* 1993). Their flowers may stay fresh in un-pollinated state ranging from few weeks to months but show rapid senescence upon pollination (O'Neill 1997). Pollination induces rapid senescence of orchid flowers thereby reducing their commercial value (Ketsa and Rugkong 1999).

The mechanisms underlying floral senescence in orchids are still enigmatic. Senescence, when it does occur, may be imperceptible for weeks, even after becoming apparent, symptoms develop slowly. In orchids, little is known as how various floral organs respond to pollination at biochemical level and how these organs relate to each other in pollinated flowers. An efflux of some cellular constituents like vacuolar pigments and electrolytes occur in floral organs (Celikel and Van Doorn 1995) that are related to loss of pressure potential and visible wilting as a result of flower senescence.

Auxins and ethylene have been implicated in controlling the pollination-induced response in flowers (Ketsa *et al.* 2006, Attri *et al*. 2007). It has been reported that pollen interaction with stigma either releases auxin that stimulates ethylene synthesis or 1-amino-cyclopropane-1-carboxylic acid (ACC) a precursor of ethylene (O'Neill *et al.* 1993), which traverses across the flower organs leading to onset of their senescence by affecting metabolic events (Bui and O'Neill 1998). Various developmental events occurring after pollination are also reported to be influenced by auxin or ethylene (Van Doorn 1997, Attri *et al.* 2007). Information about the precise metabolic alterations in relation to involvement of these hormones is lacking.

Keeping in view the above, the present investigations were conducted in *Cymbidium pendulum* (Roxb.)Sw. and *Cymbidium aloifolium* (L.) Sw., the two commercially important orchid species to fill the gap related to above study.

Orchid species (*Cymbidium pendulum* (Roxb.) Sw. and *Cymbidium aloifolium* (L.) Sw. used for these studies were collected from their natural habitat (Sikkim and Manipur, north-east India) and maintained in the Orchid House, Department of Botany, Panjab University,

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Abbreviations: DAP - days after pollination; TIBA - triiodobenzoic acid.
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Chandigarh, India. The flowers were hand pollinated in the morning (09:00) and observed daily for visual changes. The flowers were harvested at stage 1 (first sign of wilting) and at stage 2 (when the wilting was progressed) after pollination. The harvested flowers were dissected into perianth, lip, column and ovary and analyzed for pigments, sugars, proteins and related enzymes. The amount of anthocyanins was estimated according to the methods of Christe *et al.* (1994) and Kho *et al.* (1977). The contents of carotenoids were estimated according to Holm (1954). The activities of α-amylase and β-amylase were assayed as described by Shuster and Gifford (1962) with some modifications using starch as a substrate. The activity of invertase was assayed according to methods of Hawker and Hatch (1965) and Nygaard (1977) and activities of proteases were estimated using the methods of Basha and Beevers (1975) and Salmia *et al.* (1978). For extraction of sugars and amino acids, the oven-dried plant material was homogenized in hot ethanol (80 %) and centrifuged at 10 000 *g* for 10 min. The supernatant was clearly decanted off, 3 cm^3 of ethanol (80 %) was added to the residue mixture and re-centrifuged. The extraction was repeated twice to ensure the complete recovery of sugars and amino acids. The supernatant was cooled and evaporated to dryness in a china dish on a boiling water bath. The residue was eluted with 5 cm^3 of 20 % ethanol and subjected to analysis for sugars and amino acids. For the measurement of total sugars, the method given by Yemm and Willis (1954) was used. The reducing sugars were estimated as per method of Sumner (1935) using dinitrosalicylic acid (DNSA) reagent and the estimation of amino acids was done by the method of Lee and Takahashi (1966) using ninhydrin reagent.

The pollinated flowers (as described above) were sprayed with tri-iodobenzoic acid (TIBA), the inhibitors of auxin polar transport, and silver nitrate (ethylene inhigitor) to restrict effects of these hormones in flowers after pollination. The solution of these inhibitors was prepared fresh by dissolving TIBA (0.25μm) and silver nitrate (0.25 μm) in distilled water and sprayed on the flowers, along with 0.1 % *Tween 20* (surfactant). The observations were repeated thrice, the data was analyzed statistically, *ANOVA* test was applied and critical difference (C.D.) was worked out between treatments and between organs using *Agristat* software (Tamil Nadu, India).

The flowers of *Phalaenopsis* and *Cymbidium* species were found to live up to 8 weeks, if unpollinated, but died within 7 d after pollination. Likewise, *Paphiopedilum* blossoms lasted for 3 months in unpollinated state but senesced within 3 weeks after pollination (Arditti 1992). Similarly, the flowers of *Aerides multiflora* and *Rhynchostylis retusa* stays fresh for 17 and 24 d, respectively, but perished within 5 and 7 d after pollination (Attri *et al*. 2007). In the present study also, the unpollinated flowers of *Cymbidium pendulum* stayed fresh for 20 d and attained senescence in 6 - 7 d after pollination (DAP), while those of *Cymbidium aloifolium* remained fresh for 18 d without pollination and senesced in 8 DAP. In both the taxa, the first detectable symptom after pollination was alteration in colour of the lip that became darker, due to elevation of anthocyanin content (Table 1). This increase antocyanins continued till the perianth wilted and shrunk. The lip showed more marked increase in anthocyanins than perianth. Elevated anthocyanin contents in the perianth of pollinated *Cymbidium* species

Table 1. Amount of anthocyanins, carotenoids and sugars in *C. pendulum* (CP) and *C. aloifolium* (CA) in unpollinated, pollinated, pollinated TIBA treated and pollinated silver nitrate treated flowers at first and second stage of development.

Plants	Stages	Treat-ments	Anthocyanin [μg g^{-1}(f.m.)]				Carotenoids [μg g^{-1}(f.m.)]				Total sugars [μg g^{-1}(d.m.)]			
			column	ovary	lip	perianth	column	ovary	lip	perianth	column	ovary	lip	perianth
CP	S 1	UP	1.50	0.45	0.48	1.20	20	13	65	62	264	222	334	274
		P	1.80	0.60	0.71	1.50	56	22	71	61	386	287	464	508
		TIBA	1.50	0.60	0.77	1.50	45	17	56	59	424	310	426	496
		$AGNO_3$	1.50	0.60	0.60	1.20	31	14	42	37	285	210	168	216
	S 2	UP	0.15	0.15	0.47	0.90	21	11	59	55	309	284	386	284
		P	0.15	0.15	0.71	1.20	45	19	66	60	492	512	552	672
		TIBA	0.15	0.15	0.62	1.05	42	18	61	52	400	490	520	610
		$AGNO_3$	0.15	0.15	0.53	0.90	36	16	38	28	310	225	202	404
CA	S 1	UP	0.90	0.45	0.72	1.35	9	8	6	7	45	41	76	80
		P	1.20	0.45	1.53	1.50	12	10	9	10	69	53	92	110
		TIBA	1.05	0.45	1.47	1.50	6	6	5	9	65	50	86	98
		$AGNO_3$	0.90	0.45	1.02	0.75	5	4	6	8	57	47	83	76
	S 2	UP	0.75	0.45	0.16	1.50	8	7	6	7	59	49	85	97
		P	1.20	0.45	1.73	1.80	8	6	6	9	74	59	102	123
		TIBA	1.20	0.45	1.53	1.65	5	5	6	7	70	56	97	32
		$AGNO_3$					5	4	5	7	58	52	87	23

Table 2. Amount of reducing sugars, amino acids and protease activity in *C. pendulum* (CP) and *C. aloifolium* (CA) in unpollinated, pollinated, pollinated TIBA treated and pollinated silver nitrate treated flowers at first and second stage of development.

Plants	Stages	Treat-ments	Reducing sugars [μg g^{-1}(d.m.)]				Amino acids [μg g^{-1}(d.m.)]				Protease activity [μg(amino acids) g^{-1}(f.m.)]			
			column	ovary	lip	perianth	column	ovary	lip	perianth	column	ovary	lip	perianth
CP	S 1	UP	19	44	57	60	54	56	34	75	7	7	10	7
		P	33	68	112	102	68	145	96	121	10	7	14	10
		TIBA	31	61	102	111	76	106	86	103	10	7	13	9
		$AGNO_3$	22	51	88	62	32	26	42	68	7	4	10	6
	S 2	UP	24	49	61	45	64	62	41	81	8	6	7	8
		P	41	73	125	112	87	182	137	129	6	8	11	10
		TIBA	36	63	105	107	92	102	112	108	7	7	12	8
		$AGNO_3$	22	53	92	71	72	42	52	72	7	5	9	5
CA	S 1	UP	18	17	26	30	26	33	27	40	14	24	17	13
		P	31	24	41	52	73	20	55	69	18	29	33	18
		TIBA	26	23	37	43	68	18	46	61	12	26	29	14
		$AGNO_3$	23	19	29	40	58	15	41	56	12	18	21	12
	S 2	UP	23	21	33	41	30	27	43	49	11	15	19	15
		P	33	36	48	58	87	32	34	74	12	21	22	12
		TIBA	29	22	39	47	74	21	30	71	9	22	21	11
		$AGNO_3$	25	21	32	42	63	19	27	62	6	17	15	10

Table 3. Enzyme activities in *C. pendulum* (CP) and *C. aloifolium* (CA) in unpollinated, pollinated, pollinated TIBA treated and pollinated silver nitrate treated flowers at first and second stage of development.

Plants	Stages	Treat-ments	α-Amylase [μg(starch) g^{-1}(f.m.)]				β-Amylase [μg(sugar) g^{-1}(f.m.)]				Invertase [μg(sugar) g^{-1}(f.m.)]			
			column	ovary	lip	perianth	column	ovary	lip	perianth	column	ovary	lip	perianth
CP	S 1	UP	9.2	3.5	13.7	13.0	5	11	15	13	11	18	17	17
		P	13.9	6.0	18.1	20.0	6	14	21	20	20	28	26	24
	S 1	TIBA	10.2	5.1	16.4	17.0	5	12	10	17	17	24	23	18
		$AGNO_3$	10.4	4.3	15.6	15.0	3	6	8	15	8	12	19	14
	S 2	UP	8.4	3.6	11.4	15.0	5	10	14	15	9	13	16	14
		P	19.2	8.2	23.2	22.0	8	16	25	22	17	36	24	28
		TIBA	10.1	9.8	20.4	19.0	4	11	13	19	14	28	21	20
		$AGNO_3$	13.1	6.2	14.5	17.0	3	7	14	17	6	17	18	14
CA	S 1	UP	4.7	4.2	7.8	10.6	12	11	15	16	16	13	18	25
		P	8.5	5.8	11.4	14.4	18	21	38	27	27	19	27	37
		TIBA	8.1	5.2	10.8	13.4	14	18	32	24	20	16	29	39
		$AGNO_3$	6.2	4.7	9.2	11.6	11	14	22	13	19	14	23	29
	S 2	UP	5.2	5.1	8.2	11.2	18	14	18	23	17	13	19	28
		P	7.6	6.1	10.6	18.4	21	24	26	31	25	17	26	40
		TIBA	7.2	5.4	10.2	16.2	27	21	30	29	21	16	24	38
		$AGNO_3$	6.4	5.2	9.6	13.4	14	18	20	16	19	15	23	32

were previously reported by Arditti *et al.* (1973), Woltering (1990a,b) and Attri *et al*. (2007). The contents of carotenoids showed increase in both the species at first stage but reduction at the second stage (Table 1). These changes in pigments are suggested to act presumably as a signal to the pollinators indicating that the flower has been visited or pollinated. The change in colour has been associated with carotenoid or anthocyanin biosynthesis (Mohan Ram and Mathur 1984), anthocyanin degradation (Procter and Creasy 1969) or tissue pH change (Asen *et al.* 1977).

The contents of total soluble sugars, reducing sugars and amino acids (Table 1) were elevated in both the species in pollinated flowers in comparison to the unpollinated ones at both the stages, the highest level possessed by lip at both the stages followed by perianth. A corresponding increase in the activity of enzymes involved in hydrolysis of proteins (protease; Table 2), starch (α-amylase and β-amylase; Table 3) as well as sucrose (invertase; Table 3) was evident with few

exceptions. These observations indicated that pollination related signals up-regulated the activities of these enzymes for degradation of complex molecules into simple ones. In both the species, the ovary had relatively lesser contents of sugars and lower enzyme activities as compared to other organs. However, contents of sugars and amino acids in ovary were considerably higher at stage 2 than at stage 1, suggesting translocation of these compounds from the lip and perianth to the ovary. The remobilization of nutrients including sugars from the senescing organs like perianth and lip might contribute effectively towards the success of overall reproductive process (O'Neill and Nadeau 1997). Nevertheless, the lip and perianth also possessed higher enzyme activities and sugar contents at second stage compared to first stage.

The amount of sugars and enzyme activities were higher in pollinated flowers of *C. pendulum* than in those of *C. aloifolium* with few exceptions, which explains faster senescence in the former species. In the earlier studies, the sugar content increased in the gynostemia and the perianth segments after pollination in *Cattleya, Rhynchostylis* and *Aerides* but not in *Cymbidium* (Hsiang 1951, Attri *et al*. 2007). The sugar content in *Cymbidium sinense* was observed to decline with age while in *Dendrobium nobile*, it was vice versa (Wen *et al.* 1990).

It has been reported earlier that auxin as well as 1-amino-cyclopropane-1-carboxylic acid (ACC - a precursor of ethylene) released by the pollen and both stimulate senescence related events (O'Neill *et al.* 1993). The metabolic changes are activated initially in the column and subsequently in other organs. These metabolic alterations appear to be a part of general response of the flowers during senescence, which breakdown of the complex compounds into the simpler ones (Van Doorn and Woltering 2004). These simple molecules may be mobilized to the mother plant and utilized subsequently in various metabolic pathways (Erdelská and Ovecká 2004, Procházková and Wilhelmová 2007). The increase in sugarcontents may have variety roles in senescing organs (Olley *et al.* 1996, Attri *et al*. 2007). On the other hand, petal senescence has been speculated to be linked to sugars starvation since exogenous application of sugars has been found to prolong the longevity (Van Doorn 2004). Especially, sucrose appears to have a predominant role since it decreases markedly from the onset of senescence in pollinated flowers compared to the unpollinated ones. It may be possible that sugars act as components of multiple signals (Rolland *et al.* 2002) of pollination that originate in the style at the site of pollination and are translocated to the ovary and petals where they trigger ovary development and corolla senescence. Another possibility of sugars' involvement might be as component of secondary signals in senescence (Rolland *et al.* 2002). Thus, in the present context, sugars might be acting as secondary signals induced by primary signals like auxin or ethylene (O'Neill and Nadeau 1997).

The flowers treated with TIBA and $AgNO_3$ showed relatively lesser amount of reducing and soluble sugars along with reduced activities of corresponding enzymes than untreated pollinated flowers. In general, it was observed that application of these inhibitors to pollinated flowers partially suppressed the pollination-induced changes implying the interaction of these hormones with sugar and nitrogen metabolism. $AgNO_3$ had more pronounced effect than TIBA indicating greater involvement of ethylene in mediating pollination caused effects as compared to auxins. Our observations in this regard also supported the previous results, where inhibitors of ethylene and auxins caused substantial delay in flower senescence in other plant species (Hilioti *et al.* 2000, Ketsa *et al.* 2006, Attri *et al.* 2007). The effects of these inhibitors also varied at first and second stage of pollination in both the plant species which might be ascribed to the differences in endogenous auxin and ethylene content. The floral senescence was delayed by 5 and 4 d in *C. pendulum* and *C. aloifolium* with TIBA treatment, respectively, while silver nitrate delayed the senescence by 8 and 7 d in *C. pendulum* and *C. aloifolium*, respectively. Sensitivity to ethylene or any other molecule inducing senescence might be associated with variation in senescence time (Woltering *et al.* 1997).

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