RESULTS

Experiment 1 Effects of pollination with compatible and incompatible pollinia on senescence symptoms and ethylene production

1.1 Timing of senescence after pollination with different pollinia

Dendrobium 'Miss Teen' flowers were either self-pollinated or pollinated with pollinia of four other cultivars and the appearance of petal senescence symptoms (epinasty, lip yellowing, venation and water soaking) were monitored during the postharvest life. Non pollinated flowers served as controls. Two types of behaviors were observed. Early senescence symptoms and early ovary growth appeared when *Dendrobium* 'Miss Teen' flowers were cross-pollinated with the 'Pompadour', 'Sakura', and 'Willie'cultivars (Table 1 and figure 3A). This type of behavior corresponds to compatible pollination. A second type of behavior with late senescence and ovary growth was observed after self-pollination of 'Miss Teen' flowers or after cross-pollination of 'Miss Teen' flowers with 'Karen' pollinia. It was considered as incompatible pollination. In this case, the timing of senescence was similar to non-pollinated flowers for some aspects of senescence such as lip yellowing, fading and browning and only slightly faster for some others such as epinasty, drop and venation (Table 1).

The flower senescence process comprised several symptoms that stepwise developed during the postharvest life (Figure 3). Flower epinasty (Figure 3B) was the earliest of the senescence symptoms. It occurred within 1-2 days in the case of compatible pollination of 'Miss Teen' flowers with pollinia of 'Pompadour', 'Sakura', and 'Willie'. Drooping (Figure 3C) was the next symptom to develop at 2-3 days after pollination (DAP). It was followed by color fading, yellowing of lip and water soaking.

When pollinia of 'Pompadour', 'Sakura', and 'Willie' were placed on the stigma of 'Miss Teen' a fast swelling of the column (tissue around the stigma) was

observed within 1 DAP. The pedicel color changed from white to green accompanied by enlargement of the proximal end of pedicel (Figure 3D). Such changes were not observed in non-pollinated flowers and in ‘Miss Teen’ flowers self-pollinated or pollinated with pollinia from ‘Karen’.

1.2 Effects of pollination on the distance between lip and peduncle

Pollination affected the distance between lip and peduncle within 12 hours after pollination (HAP). The distance between lip and peduncle of ‘Miss Teen’ flowers pollinated with compatible pollinia (‘Pompadour’, ‘Sakura’, and ‘Willie’), rapidly decreased, leading to a flower epinasty symptom (Figure 3B). The distance between lip and peduncle in the case of incompatible pollination, was only a slight decrease but with no upside down position of the flowers. The distance between lip and peduncle of unpollinated flower remained unchanged (Figure 4).

1.3 Ovary diameter

The ovary diameter of ‘Miss Teen’ flowers either unpollinated or self-pollinated or pollinated with pollinia of ‘Karen’ remained unchanged with time over the experimental period (Figure 5). In contrast, compatible pollination resulted in the steady increase of ovary diameter over the 7-day period (Figure 5).

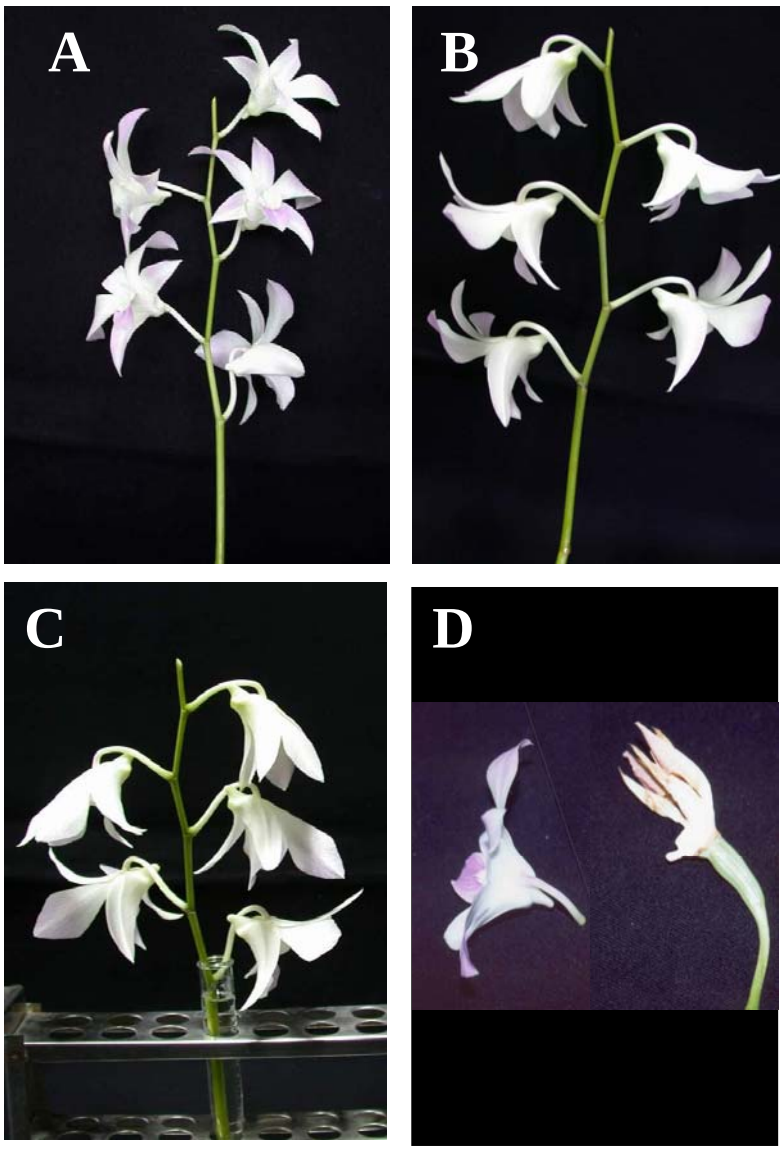


Figure 3 Unpollinated flowers (A), epinasty (B), droop (C), and ovary development (D) of *Dendrobium* 'Miss Teen'.

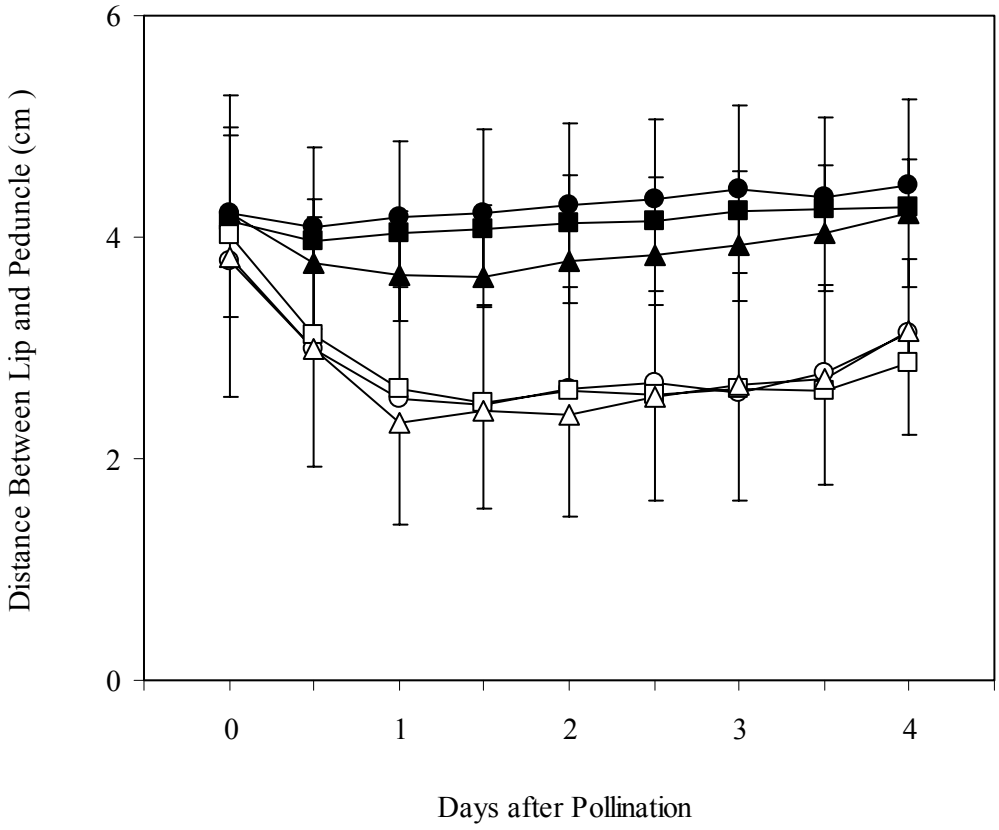


Figure 4 Changes in distance between lip and peduncle of unpollinated *Dendrobium* Miss Teen' flowers (●), pollinated with pollinia from 'Miss Teen' (■), 'Karen' (▲), 'Pompadour' (○), 'Sakura (□) and 'Willie' (△). Results are means of 25 flowers ± SD.

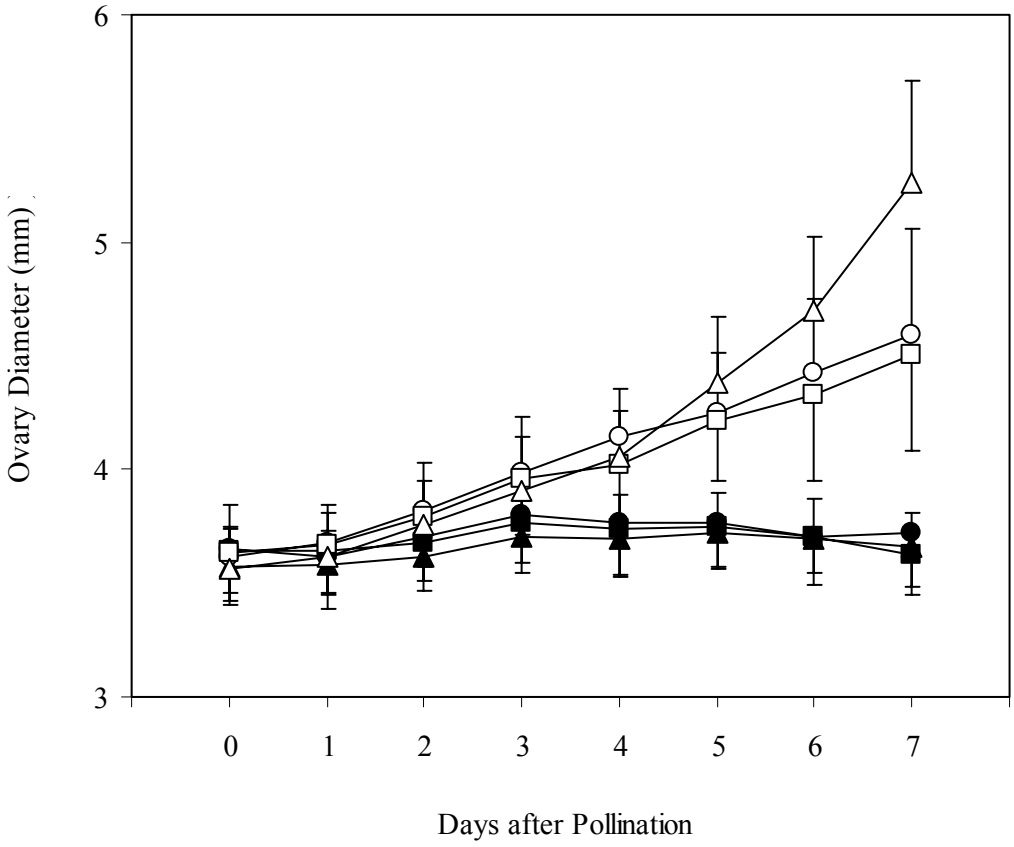


Figure 5 Ovary growth of unpollinated *Dendrobium* 'Miss Teen' flowers (●) and pollinated with pollinia from 'Miss Teen' (■), 'Karen' (▲), 'Pompadour' (□), 'Sakura (○) and 'Willie' (△). Results are means of 25 flowers ± SD.

1.4 Ethylene production

In the case of incompatible pollination (‘Miss Teen’ flowers pollinated with ‘Miss Teen’ or ‘Karen’ pollinia), there was no burst of in ethylene production (Figure 6). Ethylene levels slightly increased to a maximum 12 h following pollination and slowly decreased thereafter. In contrast, in the case of compatible pollination (‘Miss Teen’ flowers pollinated with ‘Sakura’, ‘Willie’, or ‘Pompadour’ pollinia), there was a burst of ethylene production that reached a maximum 12 HAP and declined slowly thereafter (Figure 6).

Experiment 2 Pollen tube growth, IAA and ACC content in pollinia

2.1 Pollen tube growth after compatible and incompatible pollination

After self pollination of ‘Miss Teen’ flowers, the pollen looked unclear if germinated or not and there was no increase in length (Figure 7). Upon pllination of ‘Miss Teen’ with ‘Karen’ pollinia, slight germination of pollen was observed with small increse in length from day 4 to day 7. This was in contrast with compatible pollination (‘Miss Teen’ flowers pollinated with ‘Sakura’, ‘Willie’, or ‘Pompadour’ pollinia), where the pollen tube exhibited rapid growth from day 2 to day 7 (Figure 7).

2.2 IAA and ACC content in pollinia

The levels of IAA and ACC in the pollinia depended on the cultivars. Incompatible pollinia contained low amounts of IAA and ACC, while compatible pollinia contained relatively high amounts of both IAA and ACC as shown in Figures 8 and 9. The IAA concentration from five cultivars pollinia were 43.32-352.39 μg per g FW. ACC content in pollinia were 419.63-1,245.80 nmol per g FW.

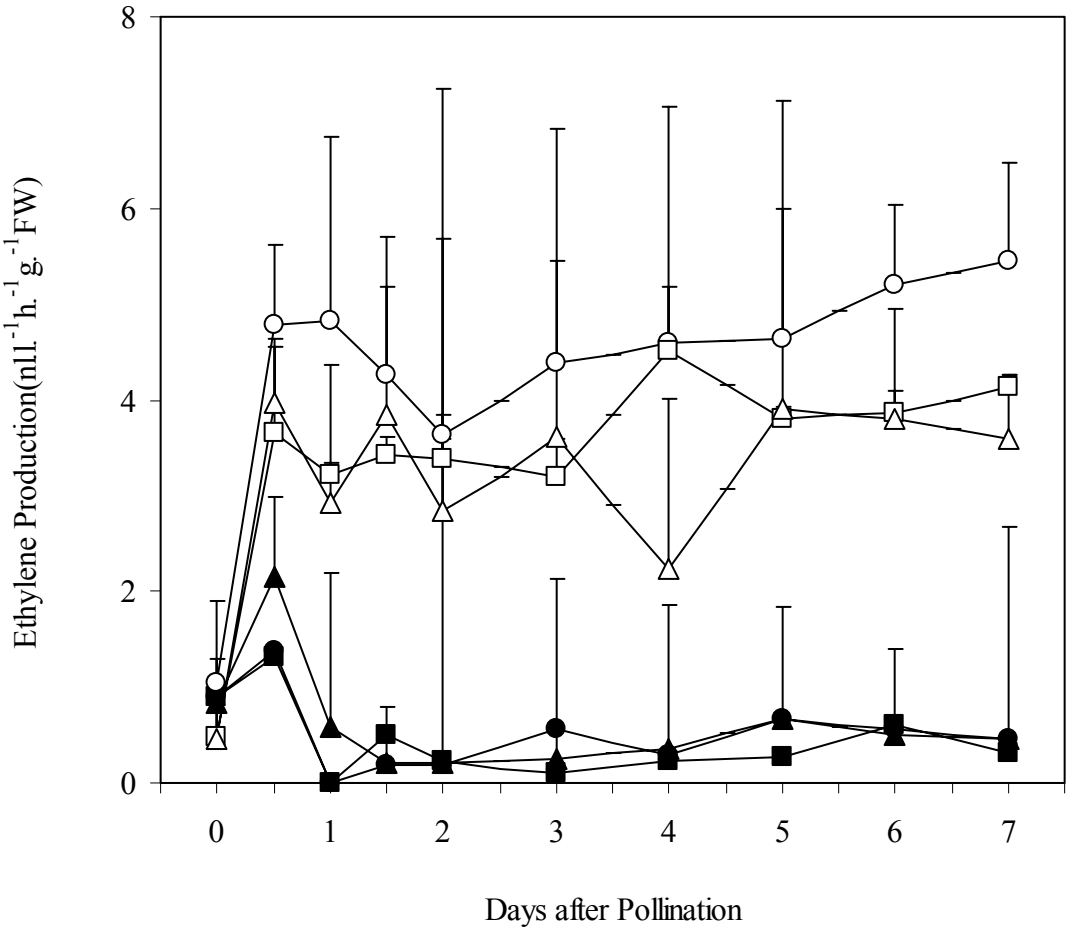


Figure 6 Ethylene production of unpollinated *Dendrobium* 'Miss Teen' flowers (●), pollinated with pollinia from 'Miss Teen' (■), 'Karen' (▲), 'Pompadour' (□), 'Sakura (○) and 'Willie' (△). Results are means of 10 bottles ± SD.

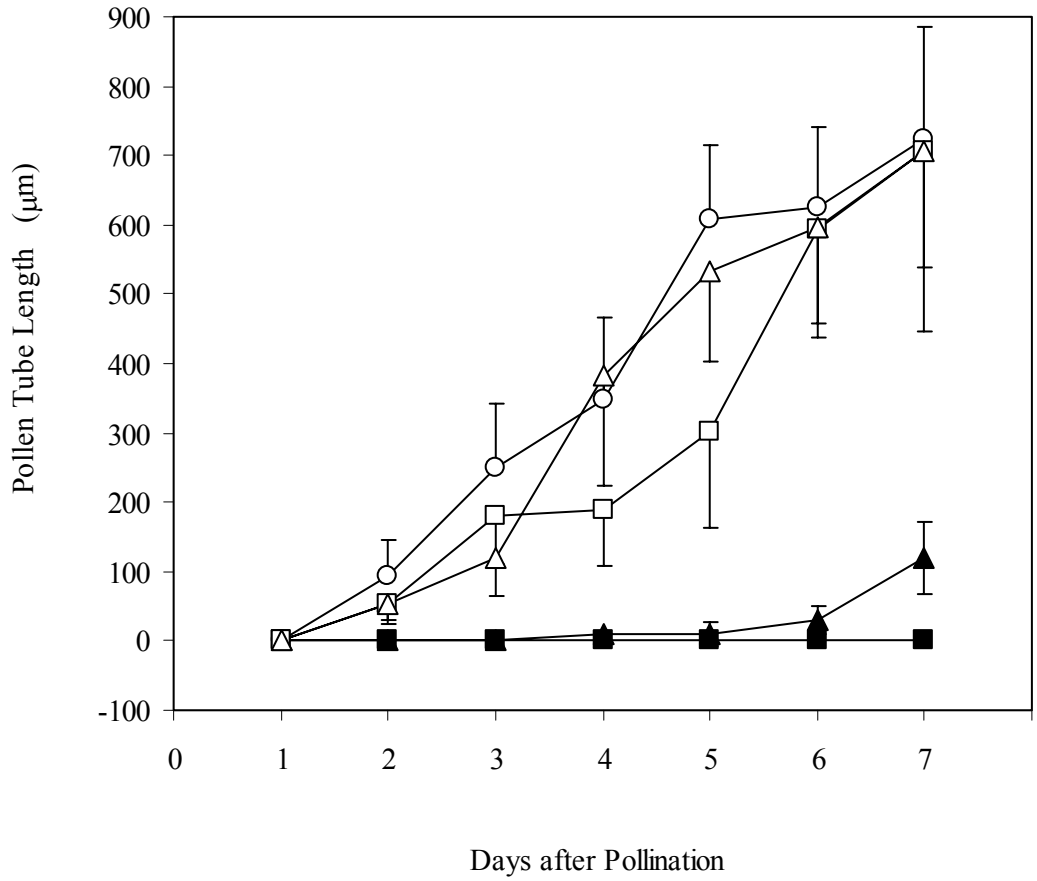


Figure 7 Pollen tube growth of pollinia from 'Miss Teen' (■), 'Karen' (▲), 'Pompadour' (□), 'Sakura' (○) and 'Willie' (△) placed on the stigma surface of 'Miss Teen' flowers. Results are means of 30 pollen tubes ± SD.

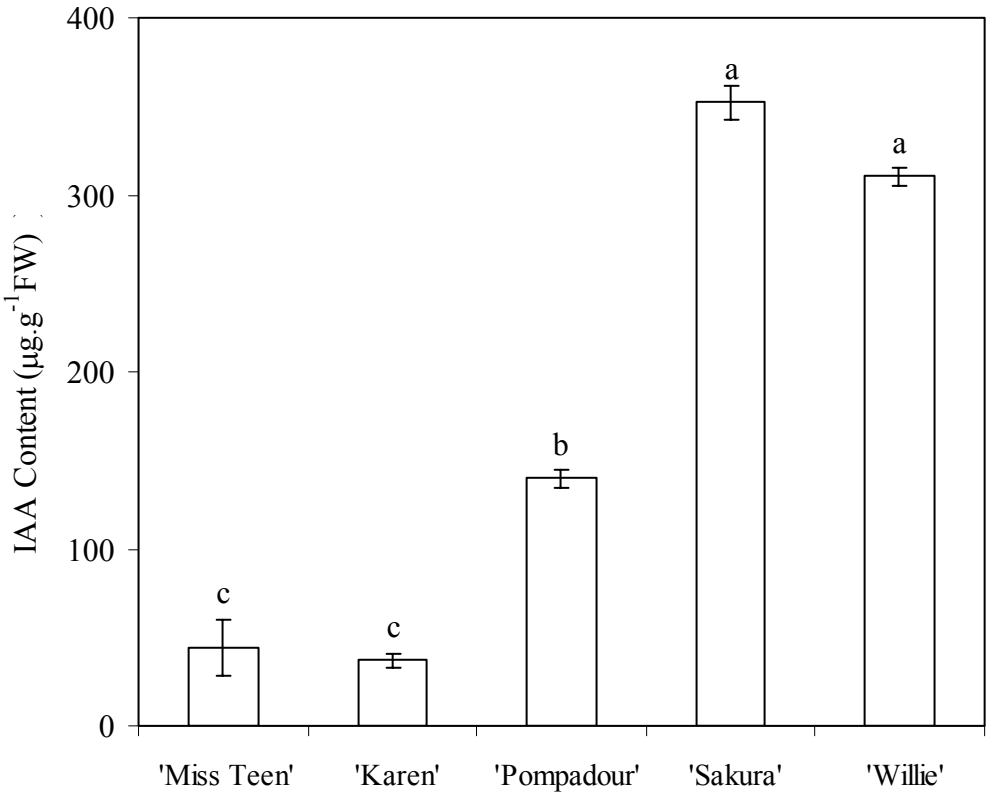


Figure 8 IAA concentration of pollinia from *Dendrobium* 'Miss Teen', 'Karen', 'Pompadour', 'Sakura', and 'Willie'. Results are means of 3 replications ± SD. The different letters are significantly different at $p \leq 0.05$ by DMRT.

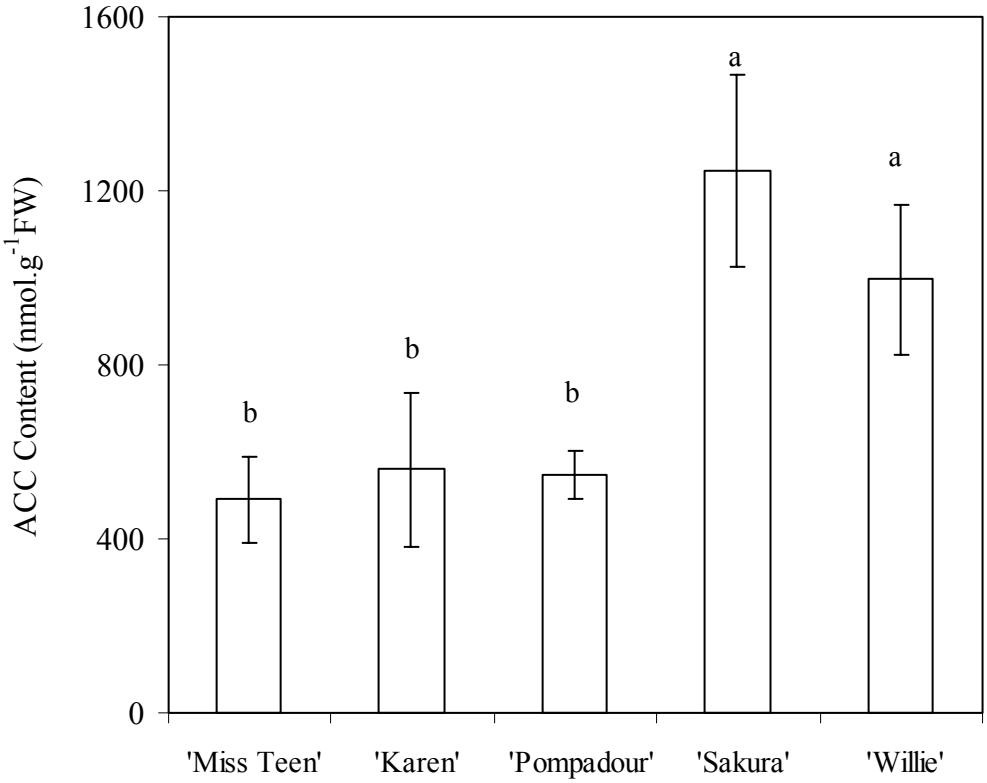


Figure 9 ACC content of pollinia from *Dendrobium* 'Miss Teen', 'Karen', 'Pompadour', 'Sakura', and 'Willie'. Results are means of 3 replications ± SD. The different letters are significantly different at $p \leq 0.05$ by DMRT.

Experiment 3 Comparison of ACC content, ACC synthase activity, and ACC oxidase activity in *Dendrobium* 'Miss Teen' flowers following compatible and incompatible pollination

3.1 Ethylene production

It is well known that pollination generally stimulates ethylene production. Flowers with compatible pollination (x 'Sakura') produced higher levels of ethylene than flowers with incompatible pollination (x 'Karen'). The ethylene production of compatibly pollinated flowers increased and reached the first peak within 12 HAP. The ethylene level stayed high and reached the second peak at day 4 whereas unpollinated flowers produced very low ethylene levels (Figure 10). The high levels of ethylene induced by pollination were produced by the column plus pedicel while the petal, sepal plus lip produced very low levels of ethylene (Figure 11).

3.2 ACC content

The column plus pedicel of flowers undergoing compatible pollination contained high level of ACC. ACC levels markedly increased and reached a maximum at 3 DAP, then declined sharply. ACC concentration of column plus pedicel of unpollinated and incompatibly pollinated flowers followed the same trend with an increase at 1 DAP, and a decrease at 2 and 3 DAP. ACC content of the unpollinated and incompatibly pollinated petal, sepal plus lip exhibited a similar pattern. In contrast, ACC contents of compatibly pollinated petal, sepal plus lip were lower than in the column plus pedicel (Figure12).

3.3 ACC synthase (ACS) activity

ACS activities of compatibly pollinated petal, sepal, plus lip rapidly increased 2 DAP, then declined sharply. ACS activities of unpollinated, compatibly pollinated and incompatibly pollinated petal, sepal plus lip were higher than that of column plus pedicel. However, there was no significant difference in ACS activity of column plus pedicel of compatibly, incompatibly pollinated and non-pollinated flowers (Figure 13).

3.4 ACC oxidase (ACO) activity

Compatible pollination resulted an increment in ACO activity than was higher than that of incompatible pollination. ACO activity of compatibly pollinated flowers rapidly increased within 12 HAP and stayed at high activity until 4 DAP in both column plus pedicel and petal plus sepal and lip. Nonpollinated flowers showed very low ACO activity, while column plus pedicel contained higher ACO activity than petal plus sepal and lip (Figure 14).

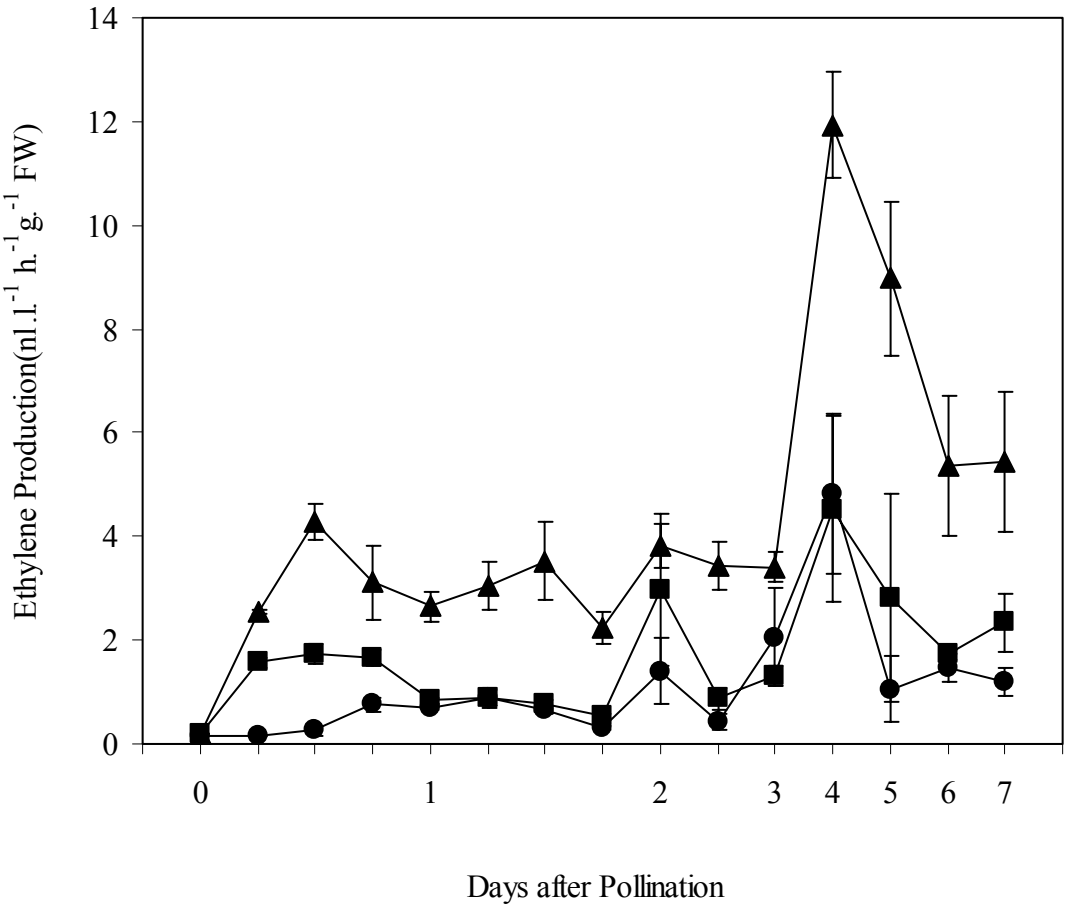


Figure 10 Ethylene production of unpollinated *Dendrobium* 'Miss Teen' flowers (●), compatibly pollinated flower (x 'Sakura') (▲), and incompatibly pollinated flowers (x 'Karen) (■). Results are means of 10 bottles ± SD.

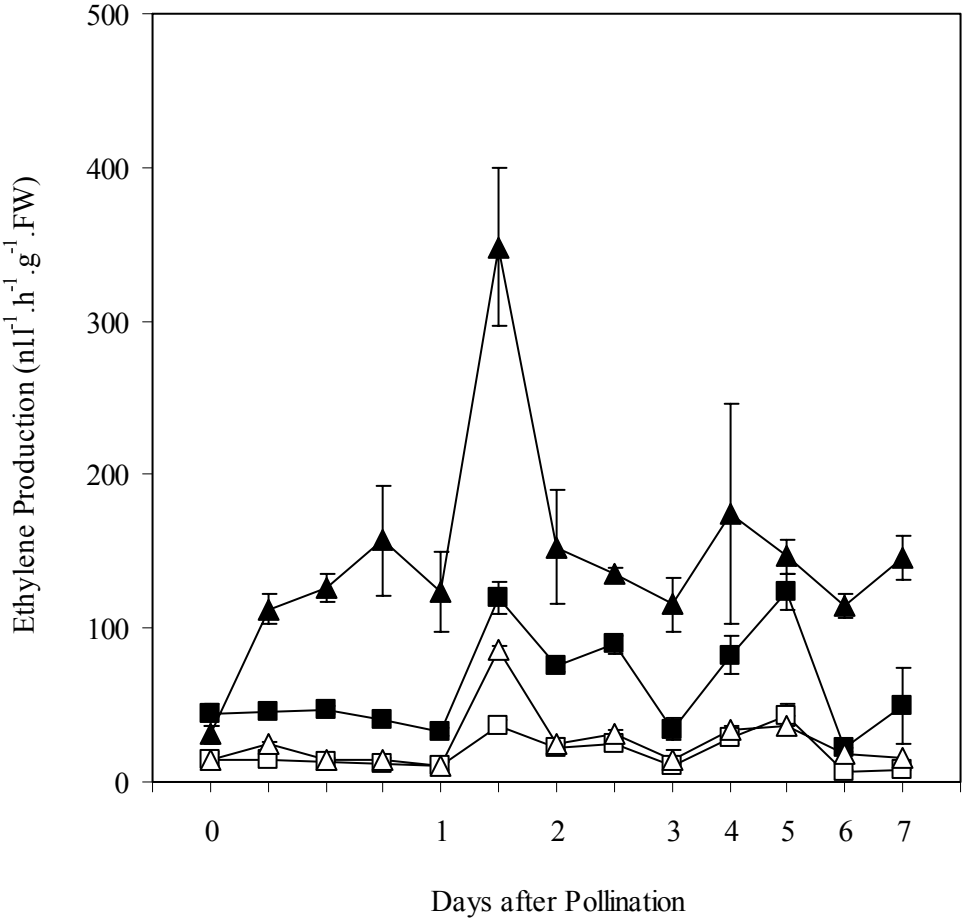


Figure 11 Ethylene production of unpollinated column plus pedicel (■), unpollinated petal, sepal plus lip (□), compatibly pollinated column plus pedicel (▲), and compatibly pollinated petal, sepal plus lip (Δ) of *Dendrobium* 'Miss Teen'. Results are means of 5 bottles ± SD.

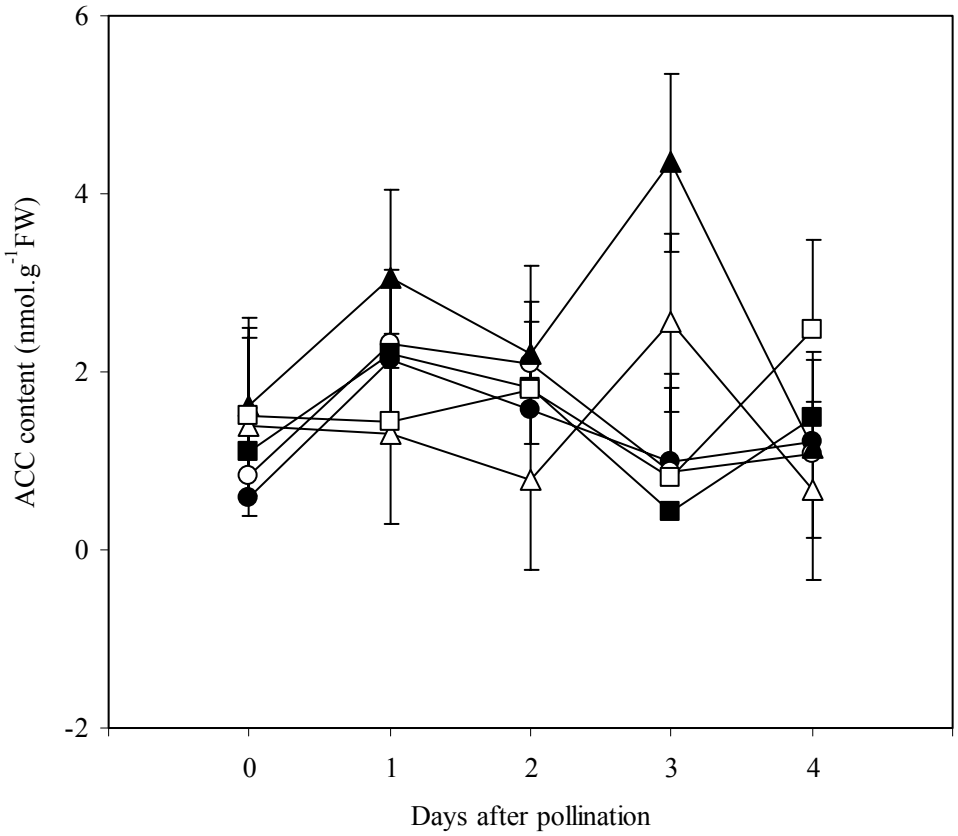


Figure 12 ACC content in unpollinated column plus pedicel (●), unpollinated petal, sepals plus lip (○), compatibly pollinated column plus pedicel (▲), compatibly pollinated petal, sepal plus lip (△), incompatible pollinated column plus pedicel (■), and incompatibly pollinated petal, sepal plus lip (□) of *Dendrobium* 'Miss Teen'. Results are means of 3 replications ± SD.

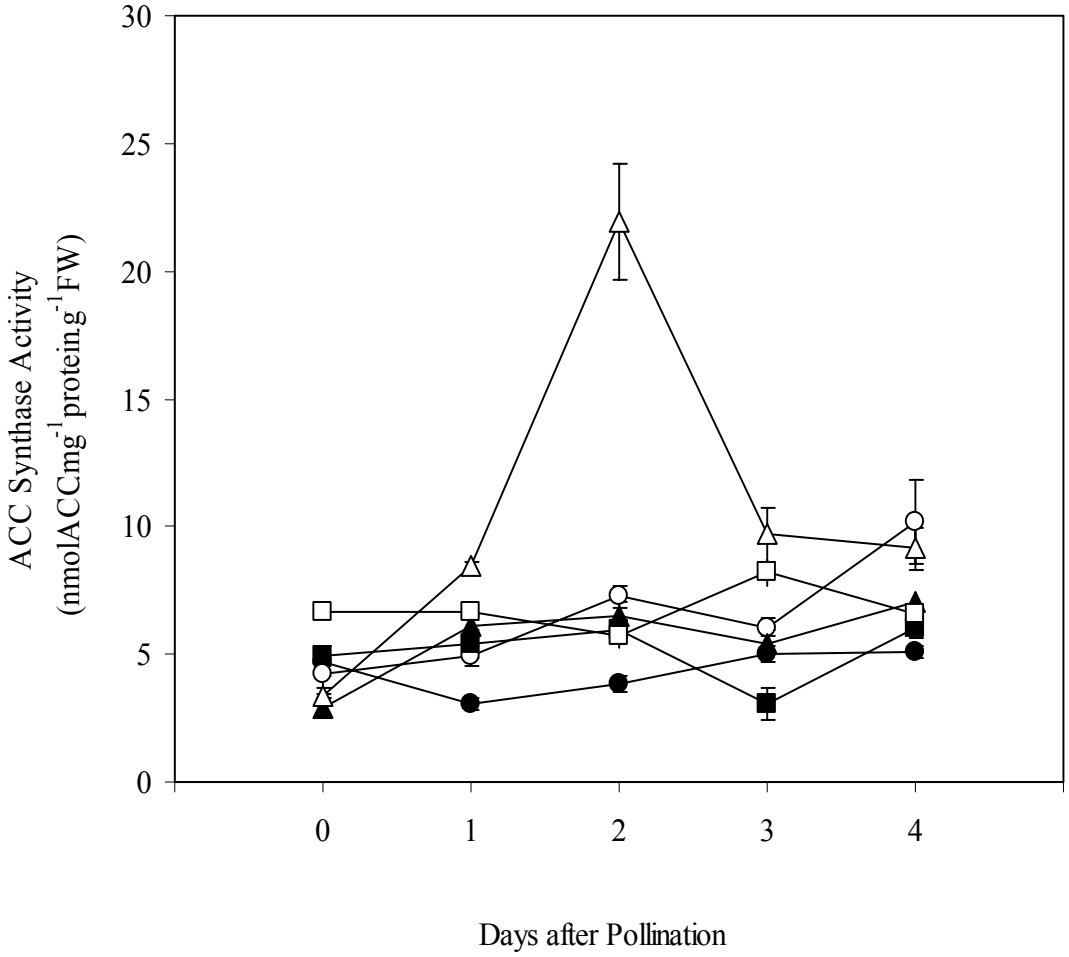


Figure 13 ACC synthase activity in unpollinated column plus pedicel (●), unpollinated petal, sepal plus lip (○), compatibly pollinated column plus pedicel (▲), compatibly pollinated petal, sepal plus lip (△), incompatibly pollinated column plus pedicel (■), and incompatibly pollinated petal, sepal plus lip (□) of *Dendrobium* 'Miss Teen'. Results are means of 3 replications ± SD.

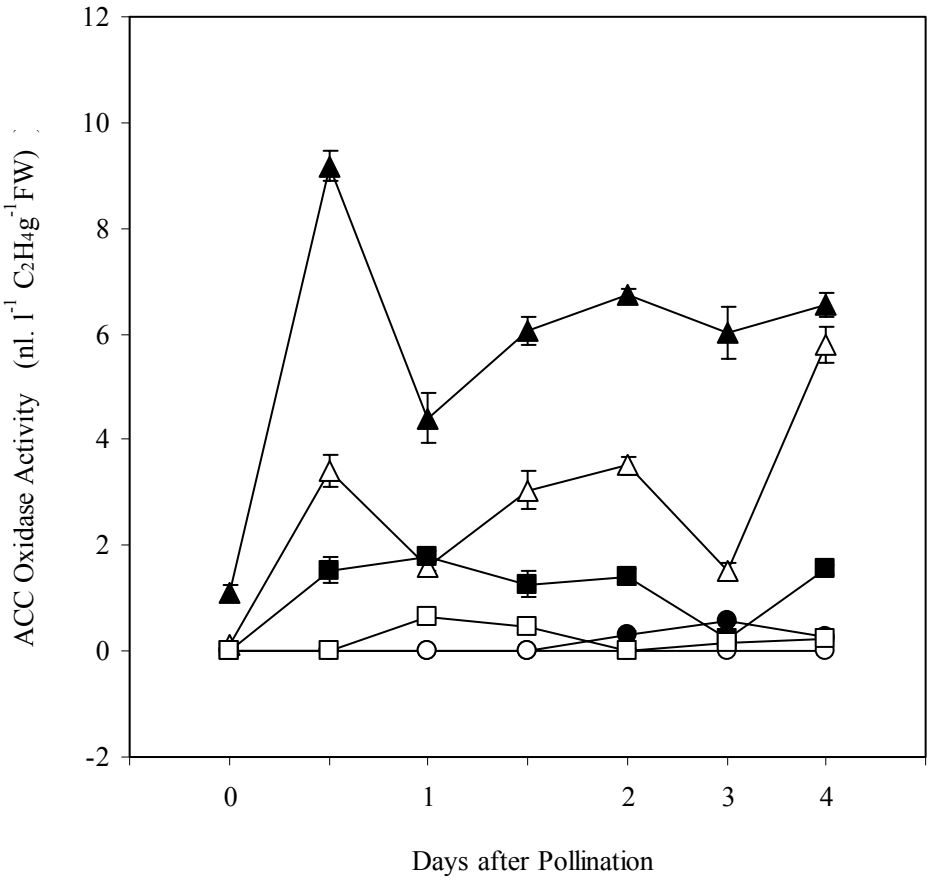


Figure 14 ACC oxidase activity in unpollinated column plus pedicel (●), unpollinated petal, sepal plus lip (○), compatibly pollinated column plus pedicel (▲), compatibly pollinated petal, sepal plus lip (△), incompatibly pollinated column plus pedicel (■), and incompatibly pollinated petal, sepal plus lip (□) of *Dendrobium* 'Miss Teen'. Results are means of 3 replications ± SD.

Experiment 4 Role of signals and effectors on pollination

The biochemical and physiological changes occurring after pollination are considered as mediated by signals such as hormones (Woodson and Lawton, 1998) and other effectors such as octanoic acid (Halevy *et al*., 1995). The possible role of these signals or effectors was studied by soaking the pollinia in water to depletion some signals and then applying them on the stigma of 'Miss Teen' flowers.

4.1 Time to exhibit symptom of senescence

The early senescence symptoms of the flowers those pollinated with soaked pollinia were similar. Different soaking time of *Dendrobium* 'Sakura' pollinia in water before pollination did not alter early senescence induced by pollination. Longer soaking time did not delay effect of pollination (Table 2).

4.2 Distance between lip and peduncle

Regardless of soaking pollinia, the distance between lip and peduncle decreased rapidly. There was no significant difference in distance between lip and pedicle of the pollinated flower with *Dendrobium* 'Sakura' pollinia with and without soaking (Figure 15).

4.3 Ovary diameter

Soaked pollinia still induced the ovary growth of *Dendrobium* 'Kenny'. However, soaking pollinia for longer time seemed to reduce the increment of ovary diameter of pollinated *Dendrobium* 'Kenny' (Figure 16).

4.4 Effects of soaking pollinia in water on IAA content

Soaking ‘Sakura’ pollinia in the water caused a significant reduction of IAA concentration in pollinia from around 350 μg g^{-1}FW to around 250 μg g^{-1}FW independently of the duration of soaking for the first 5 minutes. After 10 minutes, there was additional significant loss of IAA to less than 200 μg (Figure17).

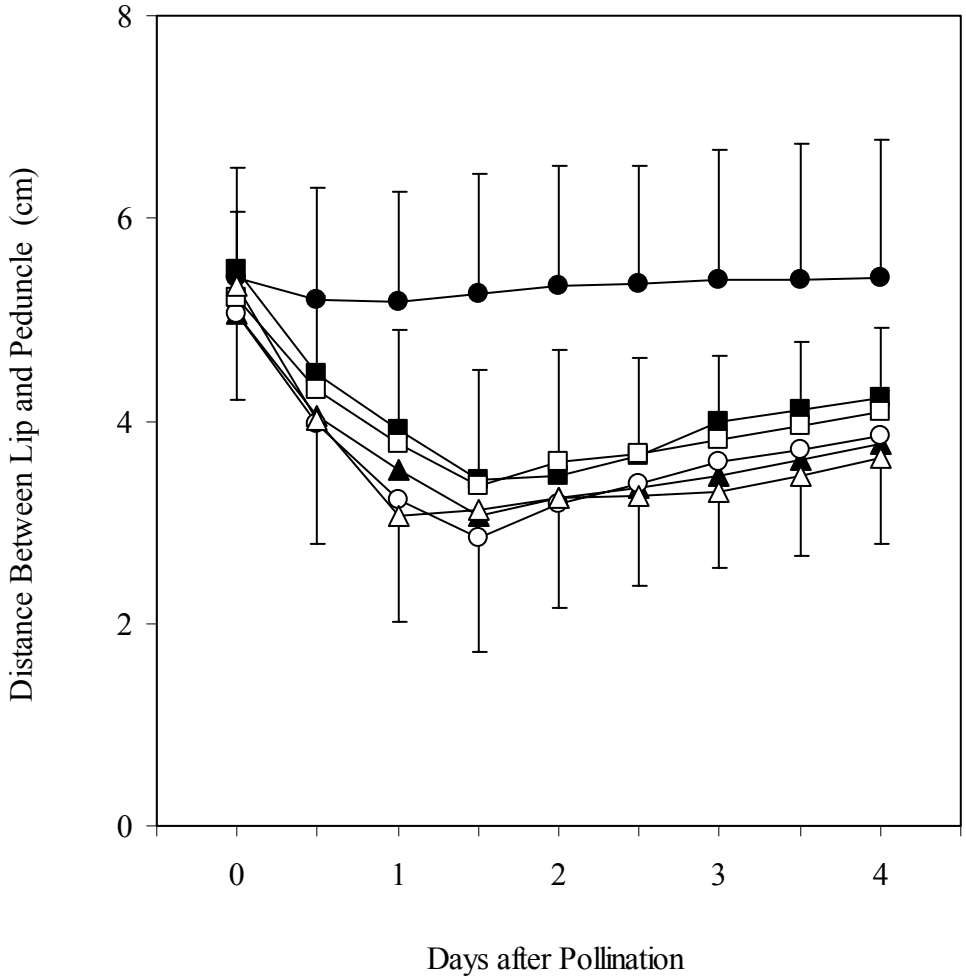


Figure 15 Changes in distance between lip and peduncle of unpollinated *Dendrobium* 'Miss Teen' flowers (●), pollinated with pollinia from *Dendrobium* 'Sakura' (■) and soaked in water for 1 (▲), 3 (○), 5 (□) and 10 (△) min. Results are means of 25 flowers ± SD.

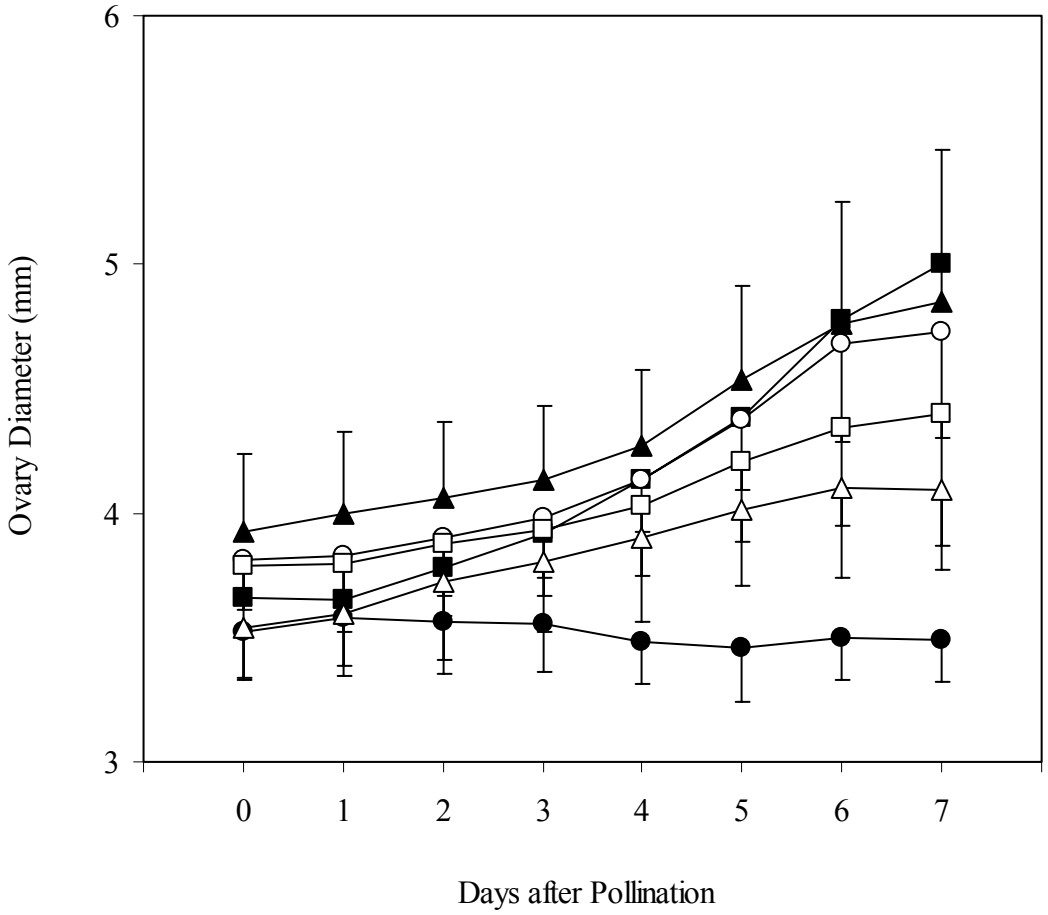


Figure 16 Ovary growth of unpollinated *Dendrobium* 'Miss Teen' flowers (●), pollinated with pollinia from *Dendrobium* 'Sakura' (■), and soaked in water for 1 (▲), 3 (○), 5 (□) and, 10 (△) min. Results are means of 25 flowers ± SD.

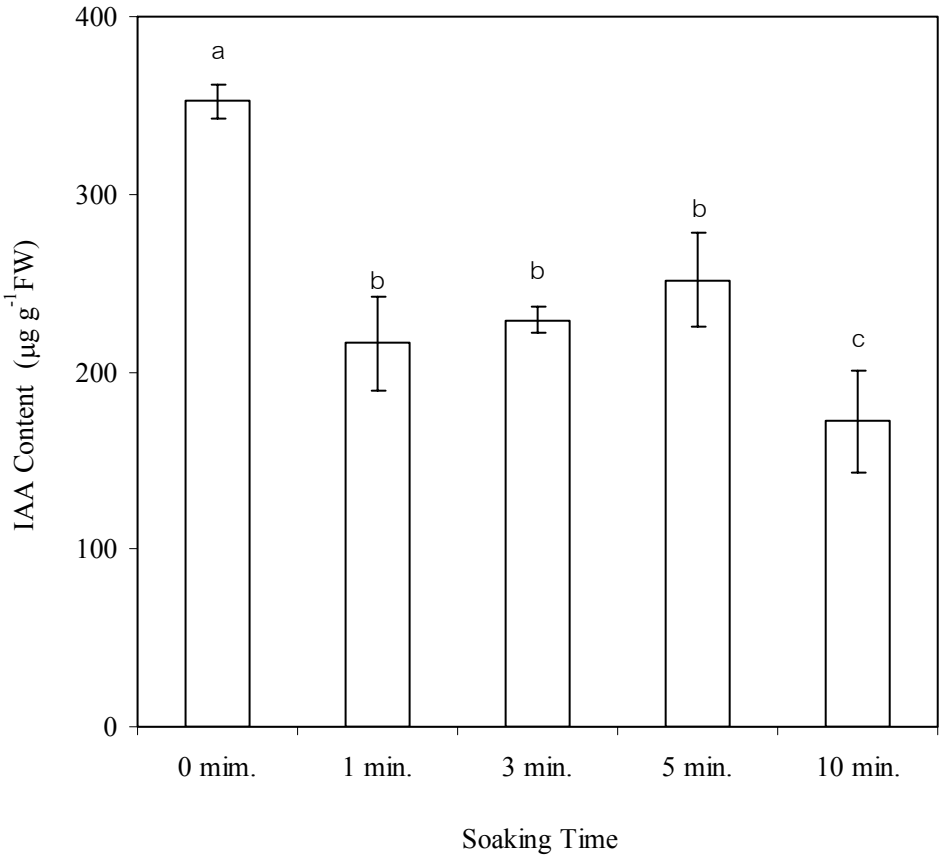


Figure 17 IAA concentration in pollinia of *Dendrobium* 'Sakura' without soaking, and soaked in water for 1, 3, 5, and 10 min. Results are means of 3 replications. The different letters are significantly different at $p \leq 0.05$ by DMRT.

Experiment 5 Effect of inhibitors on postpollination changes

5.1 Effect of auxin inhibitors

Application of 5 μg PCIB (an anti-auxin action) or 5 μg TIBA (an anti-auxin transportation), both inhibitors slightly delayed effect of compatible pollination which induced ovary enlargement (Table 3). Besides, it was found that TIBA inhibited ovary growth of compatatibly pollinated flowers more effectively than PCIB (Figure 18).

5.2 Effect of ethylene inhibitor (AOA and 1-MCP) on pollinated flowers

Application of 0.3 nmol AOA, an ACS inhibitor, to *Dendrobium* 'Miss Teen' stigma prior pollination with *Dendrobium* 'Sakura' pollinia delayed effect of pollination induced premature senescence. The early senescence symptoms did not occur 7 DAP (Table 3). AOA completely inhibited ovary growth of compatibly pollinated flowers (Figure 19). Both AOA and 1-MCP prevented effect of NAA and pollination induced ovary growth (Figures 20 and 21)**.** In addition, They also reduced the length of pollen tube (Figure 22).

5.3 Effect of synthetic auxin

Application of 5 μg NOA, a synthetic auxin, to the stigma also significantly induced ovary growth (Figure 23) similar to pollination with compatible pollinia (Table 3). It was more effective when applied at higher concentrations (Figure 24).

5.4 Effect of octanoic acid

Application of 500 ng of octanoic acid to the stigma of *Dendrobium* 'Miss Teen' before pollination with *Dendrobium* 'Karen' pollinia or application of ACC and/or ethylene did not increase the sensitivity to ethylene of *Dendrobium* 'Miss Teen' flowers. All treated flowers exhibited indifferent symptoms from flowers without octanoic acid application (Table 4).

Table 3 Time to exhibit symptom of senescence after chemical application.

Treatment	Time (days after chemical application)			
	Epinasty[1]	Droop[2]	Venation[3]	Fading[4]
50% EtOH+Nonpollination (control)	> 8a	> 8a	> 8a	> 8a
50% EtOH+Pollination	1e	2.2c	3.8b	5.5b
5 μg NOA +Nonpollination	1.3d	2.4c	2.4d	4.2c
5 μg NOA+Pollination	1.1e	2.2c	2.3d	4.1c
5 μg TIBA +Nonpollination	> 7.7b	> 8a	> 7.8a	> 8a
5 μg TIBA+Pollination	1.6c	2.7b	3.2c	4.2c
5 μg PCIB +Nonpollination	> 7.8b	> 8a	> 7.7a	> 8a
5 μg PCIB+Pollination	1.8c	2.8b	3.2c	4.16c
0.3 nmol AOA+Nonpollination	> 8a	> 8a	> 8a	> 8a
0.3 nmol AOA+Pollination	> 8a	> 8a	> 8a	> 8a
F-test	**	**	**	**

[1,2,3,4] Means within columns not sharing the same letter are significantly different at $P = 0.05$ (DMRT) and ** = significant at $P = 0.01$

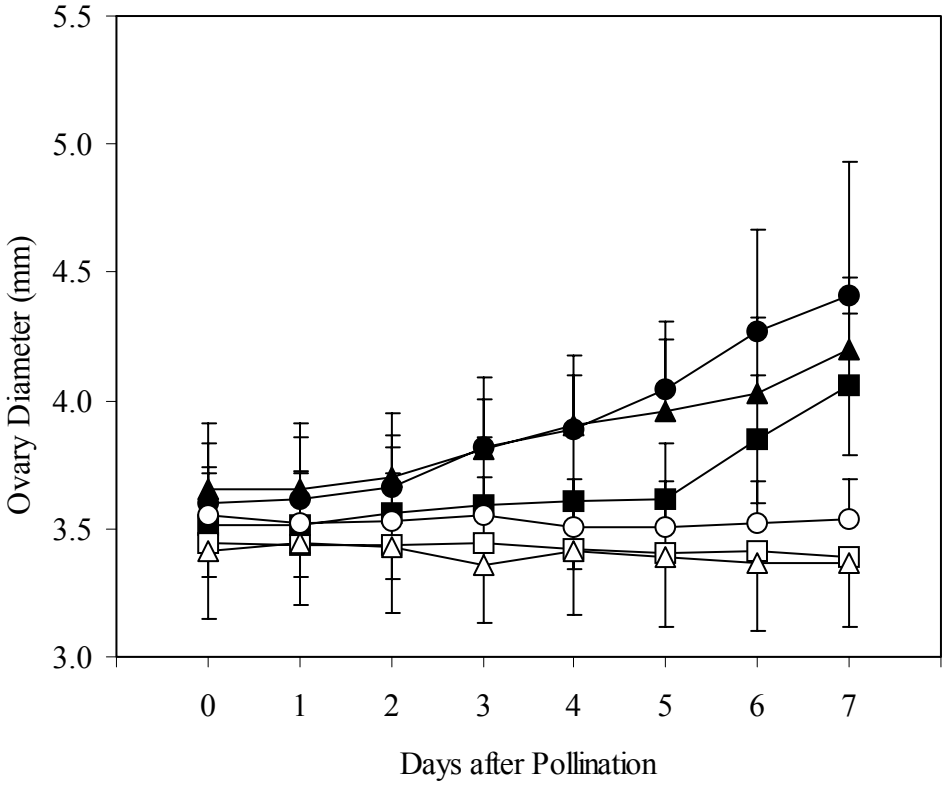


Figure 18 Ovary growth of pollinated and unpollinated *Dendrobium* 'Miss Teen' flowers (●,○), application of TIBA 5 μg per flower with and without pollination with pollinia from *Dendrobium* 'Sakura' (■,□), and application of PCIB 5 μg per flower with and without pollination (▲,△) Results are means of 25 flowers ± SD.

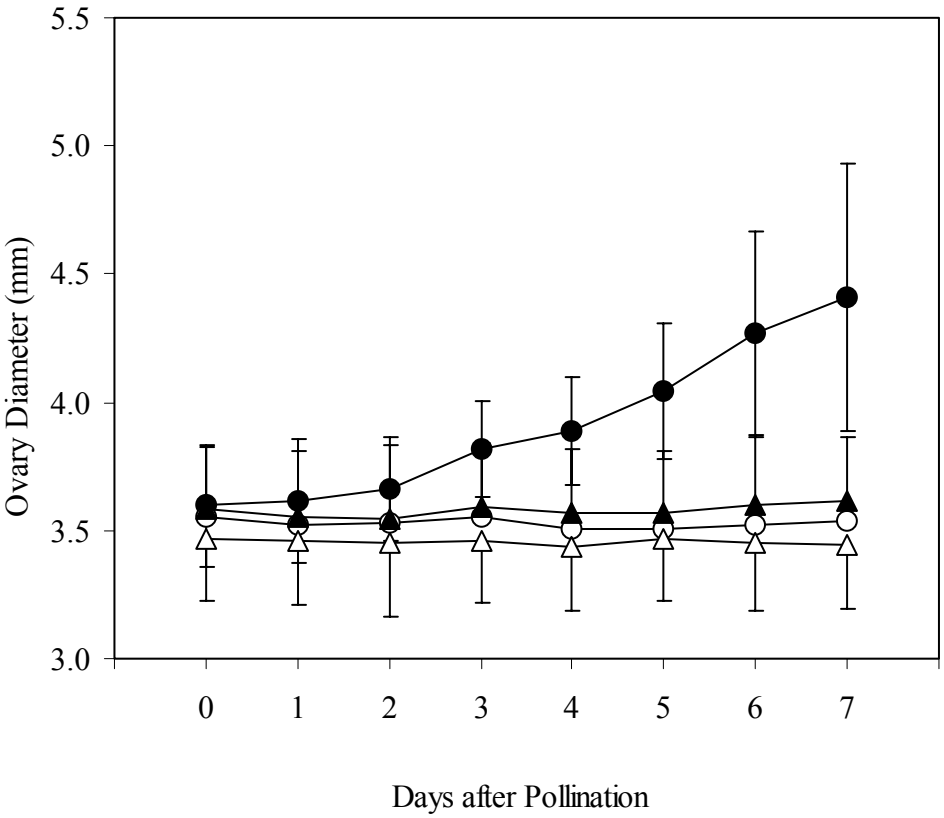


Figure 19 Ovary growth of pollinated and unpollinated *Dendrobium* 'Miss Teen' flowers (●,○), application of AOA 0.3 nmol per flower with and without (△,▲) pollination with pollinia from *Dendrobium* 'Sakura'. Results are means of 25 flowers ± SD.

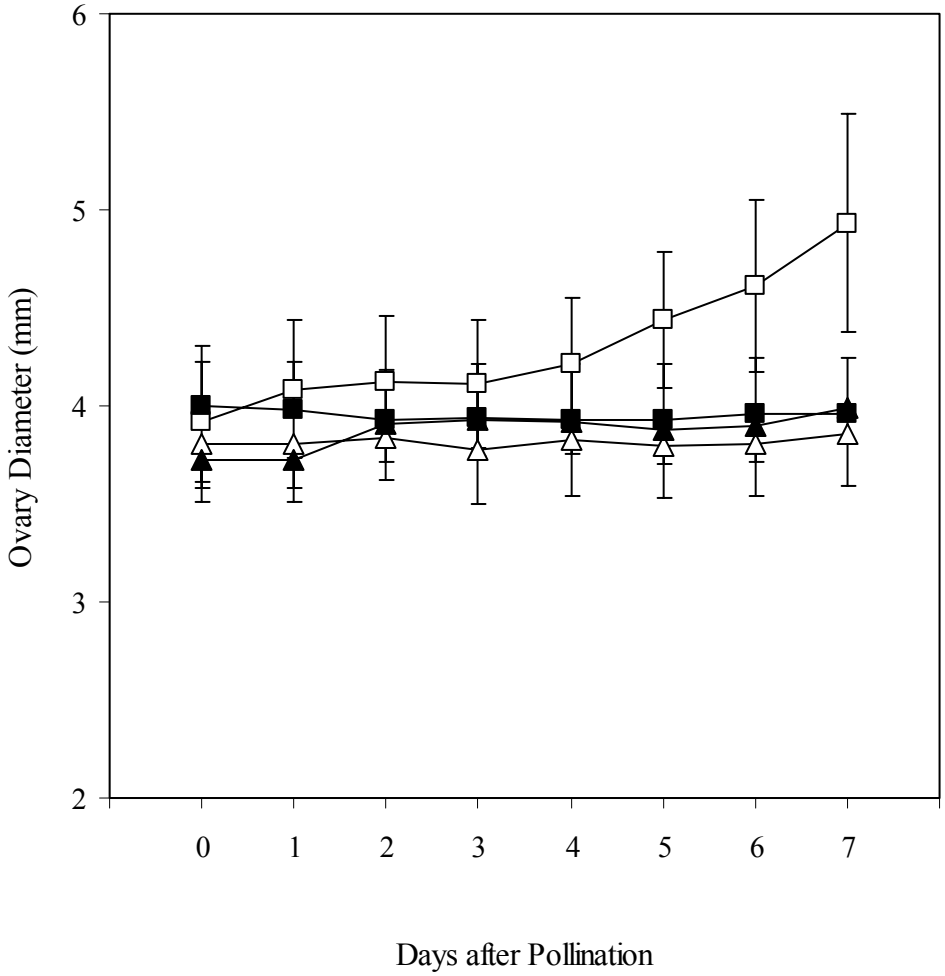


Figure 20 Ovary growth of unpollinated *Dendrobium* 'Miss Teen' flowers (■), pollinated with pollinia from *Dendrobium* 'Sakura' (□), fumigated with 500 ppb 1-MCP for 4 h prior to pollination with pollinia from *Dendrobium* 'Sakura' (▲), and application of 0.15 μmol AOA prior to pollination with pollinia from *Dendrobium* 'Sakura' (△). Results are means of 25 flowers ± SD.

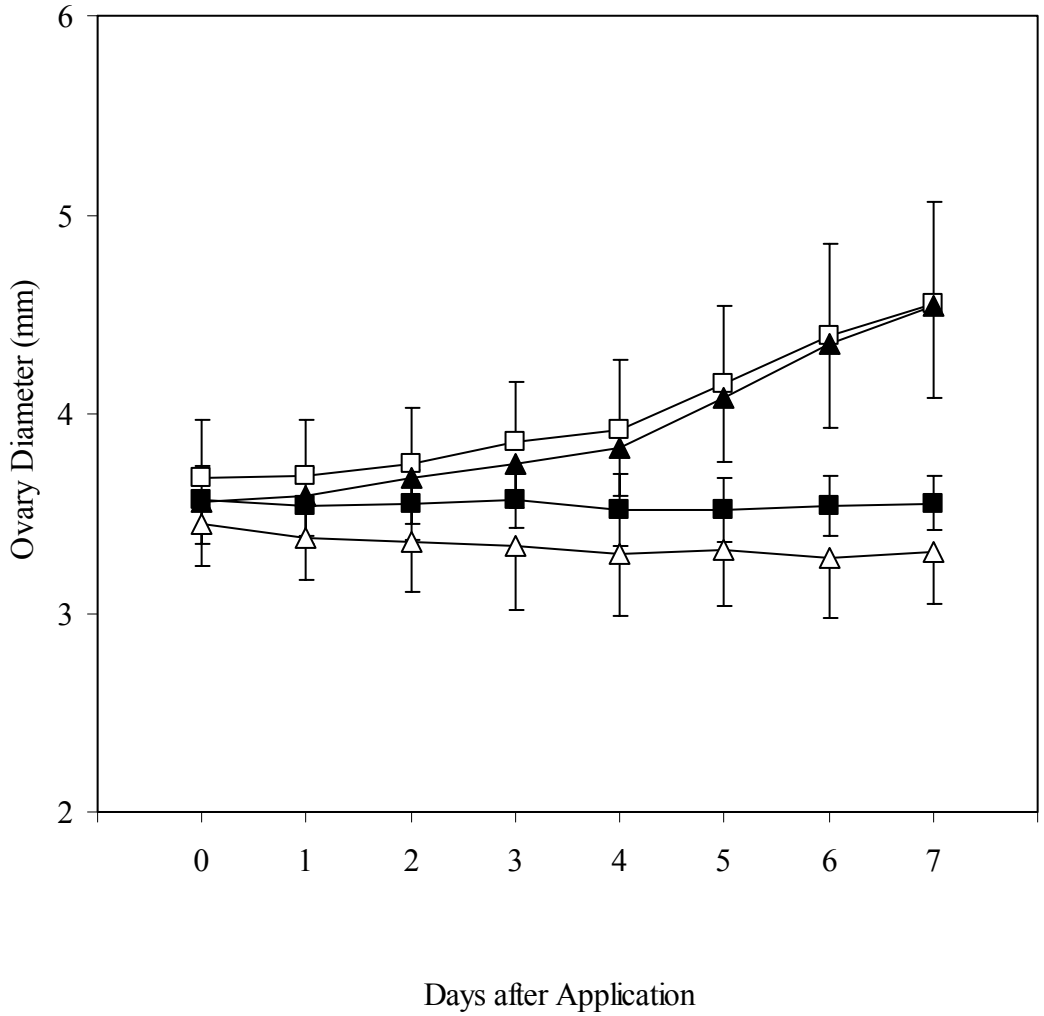


Figure 21 Ovary growth of unpollinated *Dendrobium* 'Miss Teen' flowers (■), application of NAA 0.5 μmol per flower with (□), and without (▲) pollination with pollinia from *Dendrobium* 'Sakura', and application of AOA 0.3 nmol per flower prior to application of NAA 0.5 μmol per flower (△). Results are means of 25 flowers ± SD.

●○

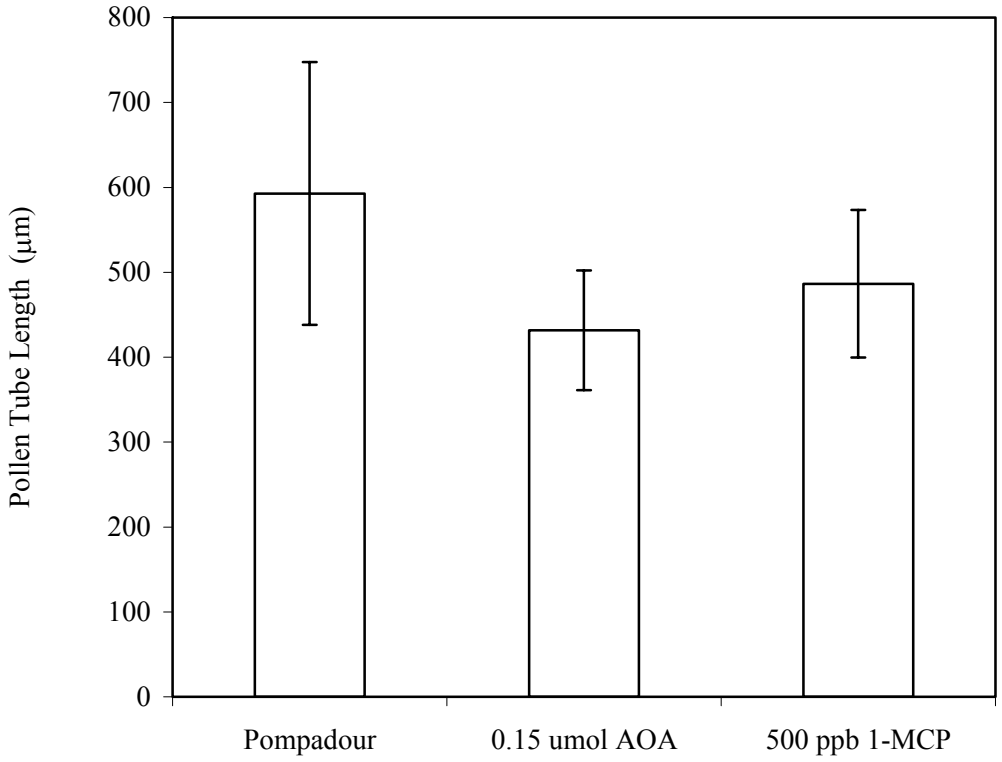


Figure 22 Effect of AOA and 1-MCP on the pollen tube length of *Dendrobium* 'Pompadour' placed on stigma of *Dendrobium* 'Miss Teen' flowers ± SD.

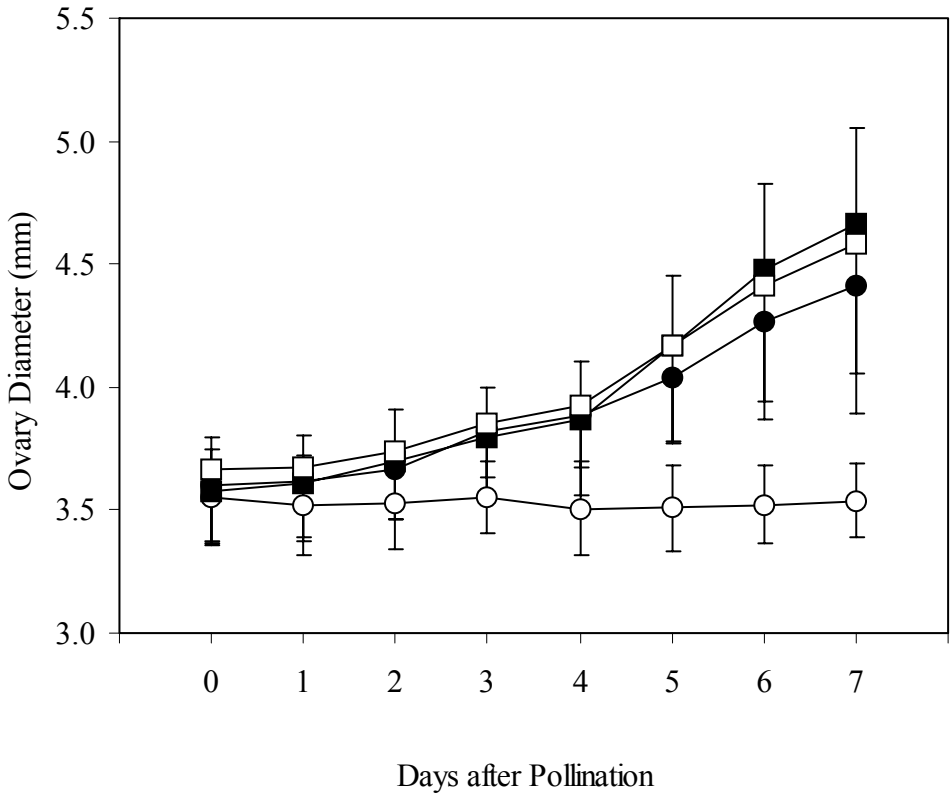


Figure 23 Ovary growth of pollinated and unpollinated *Dendrobium* 'Miss Teen' flowers (●,○), application of NOA 5 μg per flower with and without (■,□) pollination with pollinia from *Dendrobium* 'Sakura'. Results are means of 25 flowers ± SD.

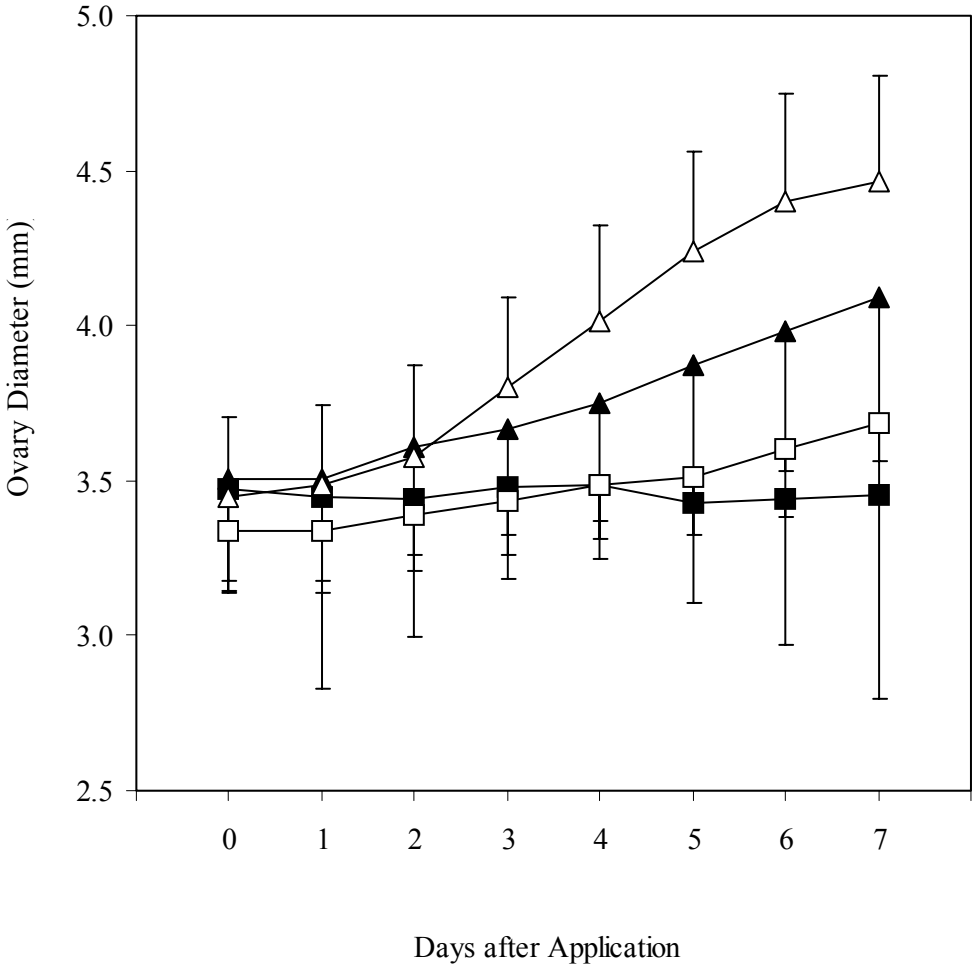


Figure 24 Ovary growth of unpollinated *Dendrobium* 'Miss Teen' flowers (■), pollinated with pollinia from *Dendrobium* 'Sakura' (□), application of NOA 5 (▲), and 10 μg (△) per flower. Results are means of 25 flowers ± SD.

Table 4 Time to exhibit early senescence symptoms of *Dendrobium* 'Miss Teen' after application of octanoic acid (OA).

Treatment	Time (days after octanoic acid application)			
	Epinasty[1]	Drooping[2]	Venation[3]	Fading[4]
1. Nonpollination	>9.8a	>10a	>11.9a	>12a
2. Pollination (x Sakura)	2.2c	3.5d	4.1d	4.3b
3. 500 ng OA	>9.8a	>10.8a	>11.9a	>12a
4. x Karen	>10.1a	>10.3a	>11.6a	>12a
5. 500 ng OA + x Karen	>9.9a	>10.7a	>11.8a	>12a
6. 0.8 nmol ACC	>9.4a	>10.4a	>11.9a	>12a
7. 500 ng OA + 0.8 nmol ACC	>9.8a	>10.4a	>11.3a	>12a
8. 1 nl/l ethylene	3.4b	>8.8b	>9.7b	>12a
9. 500 ng OA + 1 nl/l Ethylene	3.6b	>8.8b	>9.4b	>12a
10. 500 ng OA + 1 nl/l ethylene + 0.8 nmol ACC	3.4b	>7.6c	>8.6c	>12a
F-test	**	**	**	**

[1,2,3,4] Means within columns not sharing the same letter are significantly different at $P = 0.05$ (DMRT) and ** = significant at $P = 0.01$

Experiment 6 Gene expression in *Dendrobium* 'Miss Teen' following compatible and incompatible pollination

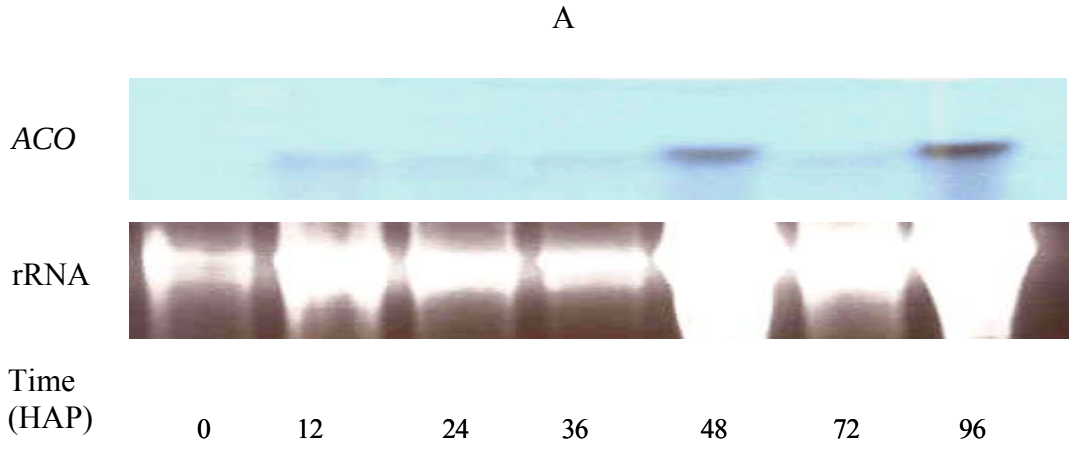
The ACC oxidase (*ACO*) gene was amplified from total RNA extracted from a pollinated *Dendrobium* 'Miss Teen' column plus pedicel by RT-PCR. The PCR product (product size approximately 950 bps) was inserted into the expression pGEM-T Easy vectors (Promega, USA.). The sequences were determined by DNA Technology Laboratory, BIOTEC service. DNA sequence was similar to *ACO* of *Dendrobium crumenatum* flowers (91% identical at amino acid levels in GenBank accession number AF038840. The cell contained inserted *ACO* gene was used as a temlpate to synthesize an *ACO* probe for northern blot analysis.

Total RNA were isolated from column plus pedicel and sepal plus petal plus lip of *Dendrobium* 'Miss Teen' flowers following pollination at various times and analyzed by northern blot analysis. The *ACO* transcrips increased in response to compatibly pollinated column plus pedicel (Fig. 25A), and accumulation of *ACO* transcript in various times after pollination correlated with ACC oxidase activity (Fig. 25 A and B). The increase in *ACO* transcript accumulation was found in the compatibly pollinated column plus pedicel at 12, 48, and 96 HAP.

RT-PCR analysis showed a differentially accumulated *ACO* gene in compatibly pollinated columm plus pedicel of *Dendrobium* 'Miss Teen'. The highest accumulation of *ACO* transcript was detected in compatibly pollinated columm plus pedicel at 36 HAP (Fig. 26).

The accumulation pattern of *ACO* transcripts in compatibly pollinated column plus pedicel was higher than that of compatibly pollinated sepal plus petal plus lip (Fig. 27).

Pollination incresed both ACC oxidase activity and *ACO* transcript in column plus pedicel of *Dendrobium* ‘Miss Teen’. The accumulation of *ACO* transcript in 36 HAP column plus pedicel of nonpollination, incompatible pollination and compatible pollination were analyzed by RT-PCR. The *ACO* transcript accumulation showed high correlation with ACC oxidase activity (Fig. 28A and B). The compatibly pollinated column plus pedicel showed higher accumalation of *ACO* and ACC oxidase activity than that of incompatibly pollinated column plus pedicel. The accumulation of *ACO* transcript and ACC oxidase activity in unpollinated column plus pedicel was very low, whereas it was very high in compatibly pollinated column plus pedicel (Fig. 28A).



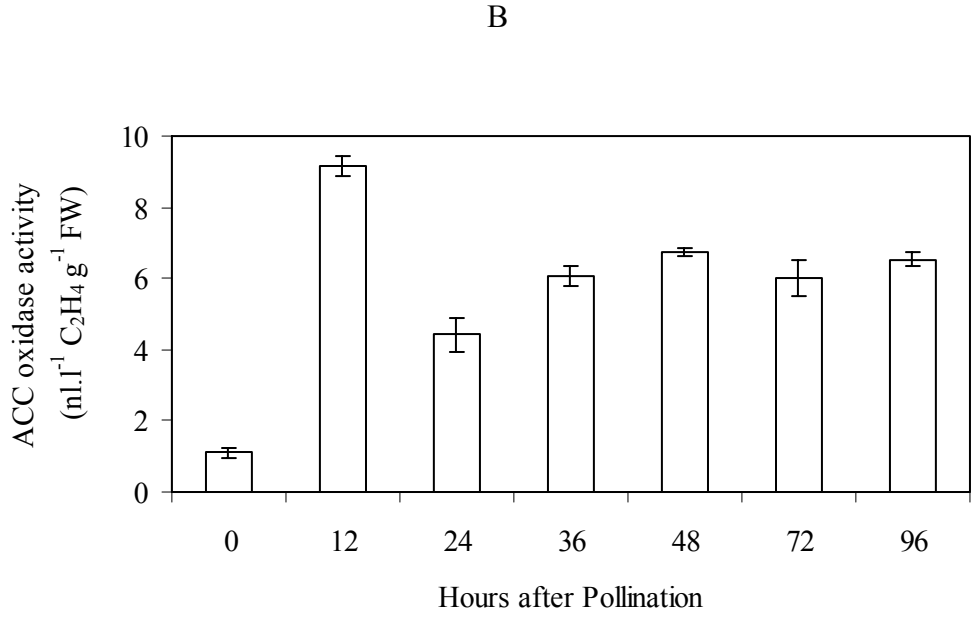


Figure 25 The accumulation of *ACO* transcript analyzed by northern blot analysis (A) and ACC oxidase activity (B) of compatibly pollinated column plus pedicel of *Dendrobium* 'Miss Teen' flowers following pollination at various times. Data are means of 3 biological replications ± SD.

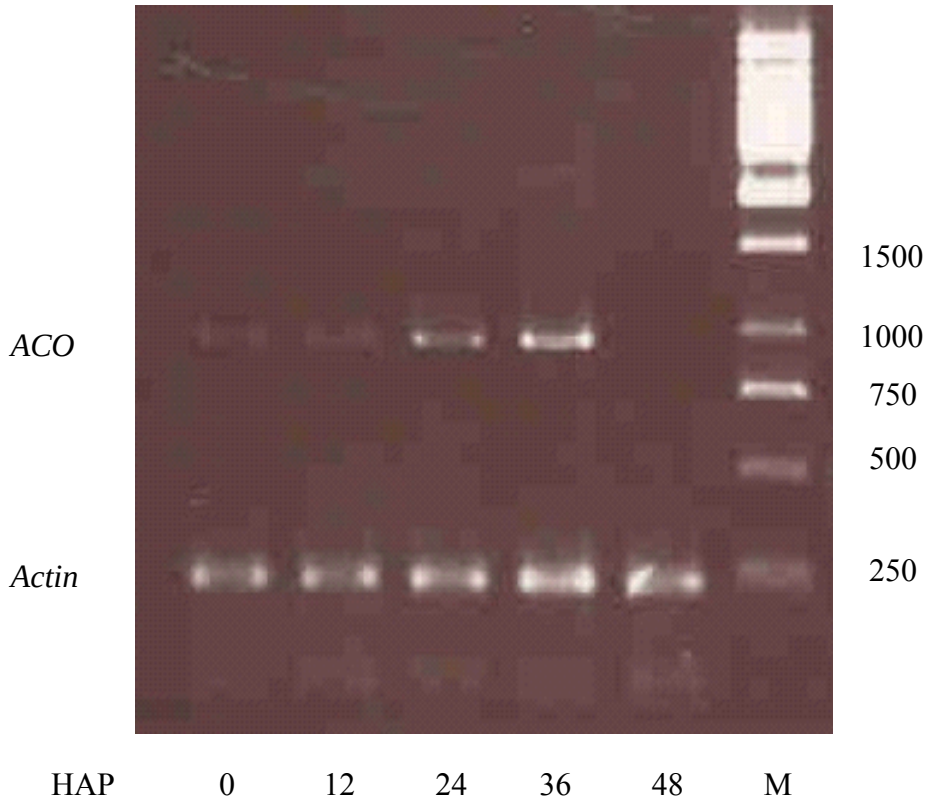


Figure 26 Time courses of the accumulation of *ACO* transcript in compatibly pollinated column plus pedicel of *Dendrobium* 'Miss Teen' flowers analyzed by RT-PCR; *Actin* was used as an internal control; (M = 1 Kb DNA marker).

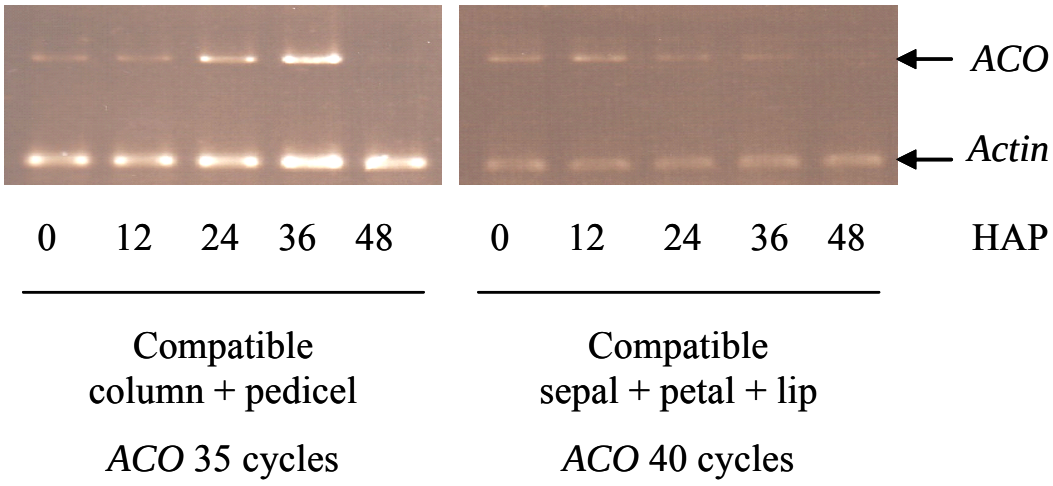


Figure 27 Comparison of *ACO* transcript accumulation in compatibly pollinated column plus pedicel and sepal plus petal plus lip of *Dendrobium* 'Miss Teen' flowers following pollination at 0, 12, 24, 36, and 48 h; *Actin* was used as an internal control.

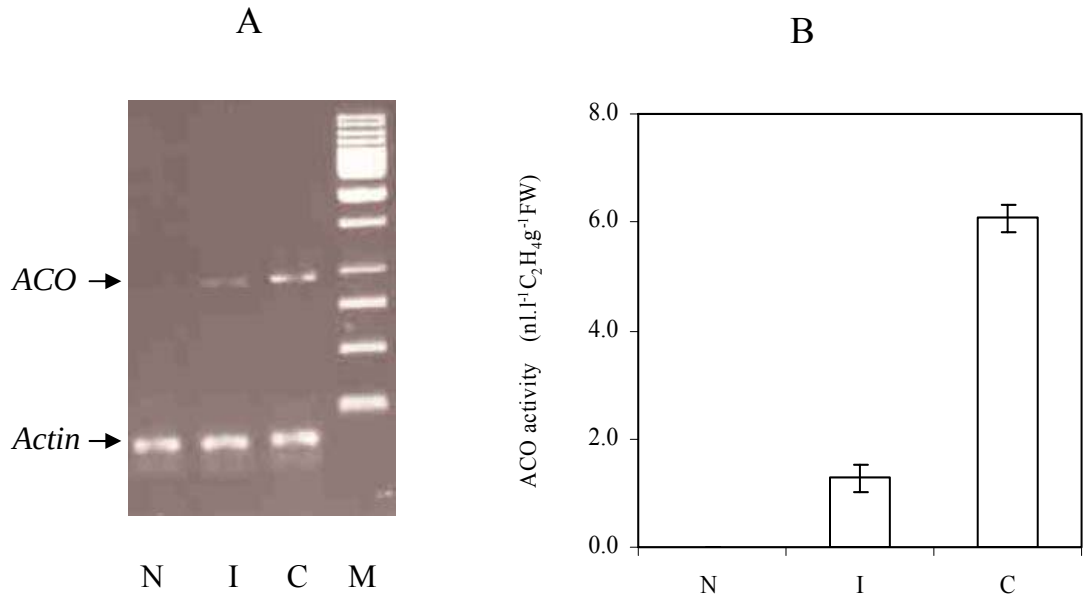


Figure 28 Accumulation of *ACO* transcript in column plus pedicel at 36 HAP analyzed by RT-PCR analysis (A) and ACC oxidase activity in column plus pedicel (B) of *Dendrobium* 'Miss Teen' flowers; *Actin* was used as an internal control. N = Nonpollination, I = incompatible pollination C = compatible pollination and M = 1 Kb DNA marker. Data are means of 3 biological replications ± SD.