DISCUSSION

Pollination with pollinia of *Dendrobium* ‘Pompadour’, *Dendrobium* ‘Sakura’, and *Dendrobium* ‘Willie’ stimulated a high level of ethylene production, and subsequently resulted an earlier perianth senescence of *Dendrobium* ‘Miss Teen’ similar to findings in *Dendrobium* ‘Pompadour’ (Ketsa and Rugkong, 2000), *Phalaenopsis* (Zhang and O’Neill, 1993), petunia (Singh *et al*., 1992) and tobacco (Hill *et al*., 1987). These three cultivars of orchid pollinia (‘Pompadour’, ‘Sakura’, and ‘Willie’) induced considerably ovary growth following pollination. In contrast, there was little or no effect of pollinia from *Dendrobium* ‘Karen’ and *Dendrobium* ‘Miss Teen’ on ovary growth.

In this study, ethylene production following compatible and incompatible pollination was clearly different. Ethylene production and ovary growth were accelerated upon compatible cross-pollination. Pollinia of ‘Karen’ and ‘Miss Teen’ showed incompatible cross-pollination while pollinia of ‘Pompadour’, ‘Sakura’ and ‘Willie’ showed compatible cross-pollination. Moreover, the various pollination developments were clearly observed after pollination with pollinia from ‘Pompadour’, ‘Sakura’ and ‘Willie’.

Since pollinia of *Dendrobium* are known to be rich in auxin (IAA) and ACC (Arditti, 1979; Stead, 1992). The concentrations of IAA and ACC in pollinia were analyzed, the results showed that ‘Miss Teen’ and ‘Karen’ pollinia contained low ACC and IAA levels, whereas the pollinia of ‘Pompadour’, ‘Sakura’ and ‘Willie’ held relatively high ACC and IAA levels. The IAA and ACC content in *Dendrobium* pollinia varied with the genetic background and ranged from 0.0311 to 0.5286 μg IAA per flower and 0.4105 to 1.8687 nmol ACC per flower. The variations in pollinia IAA and ACC content among different genetic backgrounds observed in this study may also explain the difference in the two groups of compatible and incompatible cross-pollination. Pollinia of ‘Pompadour’, ‘Sakura’ and ‘Willie’ exhibited compatible cross-pollination contained high amounts of IAA and ACC, while pollinia of

‘Karen’, and ‘Miss Teen’ exhibited incompatible cross-pollination contained low amounts of IAA and ACC.

Upon pollination pollen will germinate and pollen tube will penetrate down to ovules resulting in fertilization and post-pollination developmental processes including ovary growth (O’Neill, 1997). Pollinia of compatible cross-pollination exhibited a high percentage of pollinia germination and rapid pollinia tube growth whereas pollinia of incompatible cross-pollination did not. This suggested pollinia germination and pollinia tube growth depends on auxin and/or ethylene (McLeod, 1975). One possibility is lack of ovary growth following incompatible cross-pollination may be due to less pollinia germination and slow pollen tube growth.

The burst of ethylene production, ovary growth and premature senescence were triggered by compatible cross-pollination of pollinia of ‘Pompadour’, ‘Sakura’ and ‘Willie’ but these phenomena avoided in incompatible cross-pollination by pollinia of ‘Karen’ and ‘Miss Teen’. A major role of exerted compatible cross pollination on the ovary growth may be due to the presence of high levels of ACC and IAA in pollinia, which seems to have a direct effect on the ovary, and this effect was apparently related to pollen germination. It is yet unclear if premature senescence is also a direct effect of these two chemicals, which may act directly on the petals or via an effect on the ovary. A second part of the causal chain may be the difference in pollen germination and pollen tube growth. Both seem to require ethylene because application of AOA and/ or 1-MCP to the stigma before compatible cross-pollination reduced pollinia tube growth and ovary growth. It is also unclear to what extent they are required for the induction of premature senescence. In contrast in *Phalaenopsis*, the pollination-induced senescence syndrome cannot be related to pollen germination. Since pollen started to germinate only 7 days after pollination, long after the petals were completely wilted (Zhang and O’Neill, 1993).

In order to confirm the relationship between pollinia germination and pollinia tube growth and ethylene production, flowers were treated with AOA and 1-MCP before pollination with compatible pollinia. The results showed a considerable

reduction pollinia tube growth compared with the control (Figure 20). The experiment indicated that the endogenous ethylene is required for pollinia germination and pollinia tube growth, thus ovary growth. In tomato, ethylene promoted pollen germination and pollen tube growth (McLeod, 1975). Ethylene was reported to increase germination and fruit growth of pear (Search and Stanley, 1970) and peach (Buchanan and Biggs, 1969). De Martinis *et al*. (2002) have recently demonstrated that ethylene evolution upon pollination is correlated with the response to pollen tube growth in the tobacco flowers.

Using *Dendrobium* 'Sakura' pollinia for compatible pollination, and *Dendrobium* 'Karen' pollinia for incompatible pollination, it was found that the compatibly pollinated flower produced a burst of ethylene. The marked increase in ethylene may have resulted in a rapid rise of ACO activity. The compatible pollination not only increased in ethylene production but also increased in sensitivity to ethylene (Porat *et al.*, 1994).

The incompatibly pollinated flowers produced low levels of ethylene. The patterns of ethylene production of compatibly pollinated, incompatibly pollinated, and non-pollinated flowers were similar to findings in petunia (Singh *et al*., 1992). Incompatible pollination was unable to stimulate early senescence as fast as compatible pollination (Table 1). For compatible pollination, it is possible that the stigma perceived the primary pollination signal from pollinia and then transmitted and amplified this primary pollen signal serving to coordinate interorgan postpollination responses (O'Niell, 1997). Whereas incompatible pollination slightly affected ethylene production then decreased and stayed very low as in unpollinated flowers. It seemed that the signal from incompatible pollination was not enough to elicit early petal senescence such as lip yellowing and fading.

As a result, compatibly pollinated flowers revealed higher ACC content, ACS activity, and ACO activity than incompatibly pollinated and unpollinated flowers (Ketsa *et al*., 2001). The increased ACO activity following pollination was similarly found in *Phalaenopsis* and *Petunia* (Nadeau *et al*., 1993; Tang *et al*., 1994 ; and Tang

and Woodson, 1996). Furthermore, column plus pedicel of compatibly pollinated flowers produced higher ethylene levels and ACO activity than petal, sepal plus lip. These results indicated that most of ethylene generated in the gynoecium triggers the onset of early senescence in the petals as found in petunia and carnation (Woltering *et al*., 1997; Shibuya *et al*., 2000). Ketsa and Rugkong (2000) reported that pollination induced an increase in ACC content and ACS activity of *Dendrobium* flowers. In corn, ACC content increased both in the silk and ovaries within 20 min after pollination (Mol *et al*., 2004). Furthermore, ACO activity increased significantly in stigma of *Phalaenopsis* following pollination due to *de novo* synthesis of *ACO* mRNA (Nadeau *et al*., 1993). The increment of ACC content, and activities of ACS and ACO indicating that ethylene production of pollinated flowers is an autocatalysis similar to climacteric fruits (O'Neill *et al*., 1993) and a burst of ethylene production plays important role in the transduction of the pollination signal and the coordination of post-pollination development in many flowers (Mudalige and Kuehnle, 2004).

In carnation flowers, ACS activity in senescing styles was approximately 6 folds greater than in petals (Woodson *et al*., 1992). In *Phalaenopsis* stigma, ACS activity was examined after pollination. The level of enzyme activity in the stigma increased within 1.5 h after pollination. ACS activity in *Phalaenopsis* stigma at 1.5 h after pollination was higher than that observed in the ovary and labellum at 12 h after pollination, indicating that ACS activity is induced first in the stigma of pollinated flowers (Bui and O'Neill 1998). However, ACS activities of *Dendrobium* 'Miss Teen' column plus pedicel were not significantly different from that of the sepal plus petal plus lip. This might be due to a long interval measurement (every 24 h).

The ' Sakura' pollinia were soaked in distilled water at various times, before placing on the stigma, resulted in decreased IAA content in pollinia and reduced effect of pollination on ovary growth, but did not delay premature senescence (Table 4). Auxin diffusion or loss of auxin signals from soaked pollinia may reduce stimulatory effect on ethylene production resulting in less ovary growth. In tobacco, pollen washing in Kwacks medium (10% sucrose) causes the loss of *in vitro* viability

and ACC in pollen (Hill *et al*., 1987). The result indicated that auxin in pollinia plays an important role in the ovary growth of pollinated flowers. It is probably auxin in pollinia causes *de novo* ethylene synthesis since auxin induces *ACS* (Yang and Hoffman, 1984). It has been found that auxin inducible *ACS* gene were expressed in *Phalaenopsis* flowers (Bui and O'Neill, 1998). In addition, application of NOA and/or NAA, the synthetic auxin, to the stigma surface of unpollinated orchid flowers mimicked several effects of pollination such as an increase in ethylene production, petals senescence and the commencement of ovary growth. This indicated that auxin in pollinia may promote ethylene synthesis in *Dendrobium* flowers following pollination.

Application of AOA (an inhibitor of ACC synthase) to the stigma before pollination with 'Sakura' pollinia prevented epinasty, drooping, and fading (Table 6). Since AOA reduced ethylene production (Ketsa and Rungkong, 2000). AOA and/or 1-MCP also inhibited effect of NAA or pollination-induced ovary growth, suggested that ethylene is required for initiation of ovary growth of orchid flowers (Reid *et al*., 1984; Zhang and O'Neill, 1993). A comparative study on the effect of auxin and ethylene showed that AOA, an inhibitor of ACS, strongly inhibited effect of pollination. Similarly, exposure of 1-MCP prior compatible pollination prevented post-pollination syndromes. This result indicated that auxin action via ethylene. Zhang and O'Neill (1993) also found evidence for a role of ethylene in the pollen germination and pollen tube growth of *Phalaenopsis* orchids by using aminoethoxy-vinylglycine (AVG) to inhibit ethylene synthesis. Besides, pretreatment of TIBA (an anti-auxin transport), and PCIB (an inhibitor of auxin action) caused a reduction effect of pollination-induced ovary growth. This indicated that both auxin and ethylene play a key role to regulate ovary development (Ketsa *et al*., 2006)

ACC is an intermediate of ethylene biosynthetic pathway, hastened ethylene production and premature senescence of *Dendrobium* 'Pompadour' flowers (Ketsa and Luangsuwalai, 1996; Ketsa and Rugkong, 2000). In contrast, application ACC on stigma surface of unpollinated *Dendrobium* 'Miss Teen' orchid flowers did not substitute effect of pollination induced early senescence of petal (Table 5). The result

indicated that ACC does not invoke the full post-pollination syndrome and the pollination signal probably consists of a more complex set of stimulation (Weterings *et al*., 2002). Exogenous application of ACC to *Phalaenopsis* flowers unable to mimic pollination by stimulating a rapid increase in ACC synthase activity in the stigma and ovary and inducing *Phal-ACS2* and *Phal-ACS3* mRNA accumulation in the stigma and ovary (Bui and O'Neill, 1998). However, ACC elicited ethylene production induced premature senescence in *Dendrobium* 'Pompadour' flowers (Ketsa and Rungkong, 2000).

The short-chain fatty acids (C_7 to C_{10}) especially octanoic acids (C_8 : OA) are candidates of the ethylene sensitivity inducible factors that increased soon after pollination. Application of OA to the stigma of unpollinated *Phalaenopsis*, carnation, and petunia flowers greatly increased the sensitivity of the the flowers to ethylene. Both pollination and application of OA increased the fluidity of gynoecium and perianth membrane. This indicated that the effect of OA mediated via membrane function (Halevy, 1995[1, 2]; Porat *et al*., 1995). However application of 160-500 ng on the stigma surface of unpollinated *Dendrobium* 'Miss Teen' did not increase the sensitivity to ethylene as found in *Phalaenopsis*, carnation, and petunia flowers. This suggested that OA may not be a pollination signal induced sensitivity to ethylene as reported in *Phalaenopsis*, petunia, and carnation (Halevy, 1995[1,2]; Whitehead and Halevy, 1989). Application of 1 ppm ethylene to unpollinated *Dendrobium* 'Miss Teen' flowers was unable to hasten premature senescences, except a slight epinasty. This suggested that *Dendrobium* 'Miss Teen' flowers may be insensitive to ethylene.

The *ACO* gene from *Dendrobium* 'Miss Teen' flower is strong homology with the *ACO* gene of *Dendrobium cruenatum* (91% at amino acid level, GenBank accession number AF038840). Northern blot analysis revealed that the accumulation of *ACO* transcript in columns plus pedicels and sepals plus petals plus lip of *Dendrobium* 'Miss Teen' following compatible pollination at various times had correlation with ACC oxidase activity. It was found that high *ACO* transcript accumulation result high ACC oxidase activity and ethylene production. The accumulation *ACO* mRNA increased in compatibly pollinated samples as found in

carnation and tobacco (Jones and Woodson, 1997; Weterings *et al.*, 2002). In sepal plus petal plus lip, the transcript of *ACO* gene was much less abundance. This result was similar to the *ACO* transcripts accumulated in tobacco transmitting tissue (Weterings *et al.*, 2002), cut rose petals (Ma *et al*., 2006), tomato leaves (Moeder *et al*., 2002), tomato petal and pistil (Llop-Tous *et al*., 2000).

According to RT-PCR analysis, the *ACO* gene was differentially expressed in column plus pedicel samples at 36 HAP. This result confirmed that the pollination promoted the *ACO* expression. Besides, *ACO* higher expressed in compatible pollinated column plus pedicel than that incompatibly pollinated column plus pedicel. In sepal plus petal plus lip, the *ACO* mRNA accumulation was very low but did not correlate with the ethylene production, after compatible pollination .

CONCLUSIONS

The results of post-pollination development of *Dendrobium* flowers with pollinia from different cultivars of *Dendrobium* can be summarized as following:

1. Pollinia of *Dendrobium* 'Sakura', *Dendrobium* 'Willie' and *Dendrobium* 'Pompadour' gave compatible pollination, whereas pollinia of *Dendrobium* 'Karen' and *Dendrobium* 'Miss Teen' gave incompatible pollination to *Dendrobium* flowers.

2. Compatible pollination induced more ethylene production, larger ovary growth and earlier petal senescence than incompatible pollination and column plus pedicel produced higher level of ethylene than that of petal, sepal plus lip.

3. ACC oxidase activities of compatibly pollinated flowers (column plus pedicel and sepal plus petal plus lip) were higher than incompatibly pollinated flowers. ACC oxidase activities were low in sepal plus petal plus lip.

4. ACC synthase activites of compatibly pollinated flowers were not different from that of incompatibly pollinated and non-pollinated flowers.

5. Compatible pollinia exhibited a higher percentage of germination and more rapid pollen tube growth than incompatible pollinia and compatible pollinia contained higher ACC and auxin content than that of incompatible pollinia.

6. Application synthetic auxins NAA and/or NOA, to nonpollinated flowers stimulated early senescence of petal and ovary growth similar to pollination. In contrast, application of auxin transport inhibitors TIBA and PCIB, delayed the ovary growth of compatibly pollinated flowers.Application of ethylene inhibitors AOA and 1-MCP prior to pollination reduced pollen tube growth and ovary growth of pollinated flowers.Application of octanoic acid to incompatibly pollinated flowers did not increase in ethylene sensitivity of incompatibly pollinated flowers.

9. Pollination had effect on the accumulation of *Den-ACO* transcript in both incompatible and compatible pollination. However, the *Den-ACO* transcript was found at higher level in compatible pollination than in incompatible pollination.

10. The accumulation of *Den-ACO* transcript in column plus pedicel was higher than that in sepal plus petal and lip.