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Research article

Identification of indole-3-acetic acid as an important hormone in post–pollination of *Dendrobium* orchids and interaction of other hormones

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Abstract

Importance of the work: A 'pollen hormone' in *Dendrobium* pollinia has been reported for over a century but the specific nature of such a compound or compounds has not been proven.

Objectives: This study investigated the 'pollen hormone' in pollinia of *Dendrobium* orchid cultivars that display contrasting post-pollination phenomena.

Materials & Methods: The aqueous extract of pollinia of two *Dendrobium* cultivars was analyzed using gas chromatography-mass spectrometry. Hormonal profiles of cytokinins (CKs), auxin (IAA), gibberellic acids (GAs), abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), and 1-aminocyclopropane-1-carboxylic acid (ACC) were analyzed using ultra-high-performance liquid chromatography-tandem mass spectrometry. The effects of the plant hormones on post-pollination of *Dendrobium* cv. Miss Teen was studied.

Results: An aqueous extract of pollinia showed a relatively high indole-3-acetic acid (IAA) concentration in Sakura (exhibits post pollination) was about 4.28 ng/µL, whereas no IAA concentration was detected in Miss Teen (fails to show post pollination). The IAA in the whole pollinia of *Dendrobium* cultivars showing post-pollination phenomena was an average of 227,070.25 ng/g pollen and higher concentrations than those cultivars that do not. Post-pollination effects were associated with elevated concentrations of IAA, ABA, SA, and gibberellins; however, no correlations were observed with JA and CKs. *Dendrobium* cv. Miss Teen, when treated with IAA singly or in combination with IAA plus ABA, SA, or GAs, induced the same post-pollination symptoms as found in a pollinated flower, while flowers treated with ACC did not induce a post-pollination response.

Main finding: The auxin presence in the pollinia of *Dendrobium* was associated with the occurrence of post-pollination events. These data strongly suggested that the main 'pollen hormone' in *Dendrobium* flowers was likely IAA and that it interacts with ABA, SA, and some GAs.

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Introduction

Generally, pollination in orchids induces rapid developmental changes including flower structure, change in color, early senescence and preparation of the ovary for the process of fertilization. For example, Hildebrand (1863) cited after Ketsa et al. (2006) reported that *Dendrobium nobile* flowers were fertilized several months after pollination, whereas visible early post-pollination effects occurred within 3 wk of pollination. The same findings were reported in commercial *Dendrobium* cultivars (O'Neill et al., 1993; Luangsuwalai et al., 2011). The progression of senescence of *Cymbidium pendulum* flower-related events was triggered by pollination (Attri and Nayyar, 2021).

Auxin (indole-3-acetic acid; IAA) was discovered in the late 1920s, and its structure was elucidated by the early 1940s, with IAA, or a closely related compound, being identified in orchid pollinia, based on experiments using paper chromatography and paper electrophoresis (Müller, 1953), methods that are now deemed insufficiently specific. However, the report of Yam et al. (2009) mentioned the argument that no one had proven that this 'pollen hormone' was actually IAA. Porat et al. (1998), using high-performance liquid chromatography, found no free IAA in an aqueous extract of *Phalaenopsis* orchid pollinia but reported the presence of a compound that showed auxin-like activity. Auxin-like compounds activate expression of several 1-aminocyclopropane-1-carboxylic acid synthase (ACC synthase: ACS) genes (O'Neill et al., 1993; O'Neill, 1997). In *Dendrobium*, the presence of an auxin-like compound, apparently in the pollinia, explained the initial growth of the column and ovary in response to pollination (van Doorn and Ketsa, 2021).

Using *Cymbidium* orchids, Arditti et al. (1971) observed that placement of the synthetic auxin 1-naphthylacetic acid (NAA) on the stigma produced all the post-pollination symptoms brought about by pollination, similar to *Dendrobium*, including swelling of the column and the ovary. In contrast, ethylene treatment induced several of the post-pollination effects, but did not induce straightening of the column or stigmatic closure or swelling of the column and the ovary (Arditti et al., 1973; Ketsa and Rugkong, 2000; Ketsa et al., 2006). After the discovery in the 1980s that ACC was the immediate precursor of ethylene, ACC was identified in the pollen of several species (Hoekstra and Weges, 1986), including the pollinia of *Dendrobium* (Ketsa and Luwangsuwalai, 1996) and *Phalaenopsis* (Porat et al., 1998) orchids. Exposure of *Dendrobium* flowers to exogenous ethylene or application of ACC to the stigma produced all the early post-pollination effects, except the growth of the column and the ovary, which required an auxin effect in addition to that of ethylene (Ketsa and Rugkong, 2000; Ketsa et al., 2006; Promyou et al., 2014).

Strauss and Arditti (1982) reported that auxin plays an important role in the induction and regulation of post-pollination phenomena in the orchid flowers of *Angraecum* and *Cattaleya*. Auxin might be at least one of the mobile factors transferring the pollination signal from the pollinia to the stigma and the ovary. It is also possible that other factors, possibly from pollinia, are important and may interact with auxin.

Whole pollinia of the *Dendrobium* cultivars Pompadour, Willie and Sakura that produced normal post-pollination effects, had ACC and IAA (Luangsuwalai et al., 2008); in addition, the pollinia diffusate of *Dendrobium* cv. Wanna had an auxin-like activity in an *Avena* bioassay (Promyou et al., 2014). In contrast, other cultivars, such as Karen and Miss Teen, showed no post-pollination effects. The ACC content in pollinia from the cultivars Karen and Kenny did not differ from those in the cultivar Pompadour (Luangsuwalai et al., 2008). In contrast, the ACC and IAA contents in *Dendrobium* cv. Karen (no post-pollination effect) were lower than those in cv. Sakura (normal post-pollination effect) (Luangsuwalai et al., 2013). The current study hypothesized that the pollinia-borne free auxin and ACC would be diffused into the stigma fluid and that the whole pollinia might also contain other plant hormones that play a role or interact with auxin. Therefore, this study aimed to identify the nature of the 'pollen hormone(s)' in *Dendrobium* primarily responsible for early post-pollination effects.

Materials and Methods

Based upon current information, the present study was designed, where initially gas chromatography-mass spectrometry (GC-MS) was used to investigate the presence of IAA and ACC in aqueous extracts of *Dendrobium* pollinia, as the primary hormones that induce the early post-pollination effect. The pollinia of two cultivars were compared, namely, Sakura and Miss Teen. Secondly, the presence was investigated of IAA and other plant hormones in the whole pollinia (used for analysis) of two cultivars of *Dendrobium*. The *Dendrobium* cultivars Burana Jade, Sakura, Willie and Khao Chaimongkol were grouped as having normal post-pollination, whereas the cultivars Miss Teen, Miss Orchid and Karen were grouped as

having no post-pollination effect. The cultivars Sakura, Willie and Khao Chaimongkol shared a common genetic background while in the no post-pollination group, all cultivars shared a similar genetic background. The exogenous hormones, at concentrations found in the whole pollinia, were also studied, particularly for their effects (premature senescence).

Pollinia diffusate experiment

Cut *Dendrobium* flowers of the cultivars Sakura and Miss Teen were obtained from commercial growers in the suburban area of Bangkok, Thailand. The pollinia from recently opened flowers in the basal region of each inflorescence of each cultivar were carefully removed under a stereomicroscope. No damaged pollinia were used for producing the pollen diffusates.

Eighty pollinia of the *Dendrobium* cultivars Sakura and Miss Teen were extracted either in 2,000 µL of distilled water (0 MPa, pH 6–7) or in 0.25 M CAPSO ((3-cyclohexylamino)-2-hydroxy-1-propanesulphonic acid) (-0.5 MPa, pH 9.7), for 3 hr at 25°C. Each pollen diffusate was passed through a nylon membrane filter (13 mm diameter, 0.22 µm pore size) and immediately injected into a GC-MS system for analysis.

Free IAA in the pollen diffusate was analyzed immediately after filtration using gas chromatography (QP2010, Shimazu, Japan) equipped with a BP-20 capillary column (SGE Analytical Science; Australia) and a mass spectrometer. The operating conditions were as described by Dunlap and Guinn (1989). Free IAA levels were determined from peak areas generated by individual traces of ions at 51 m/z, 77 m/z and 103 m/z and the major fragment ion at 130 m/z. Free IAA was calculated by comparing samples with an IAA standard (Fluka; Switzerland) (R^2 = 0.99). The retention time for free IAA was 10.7 min and the concentration of IAA was expressed as nanograms per microliter.

It was initially inferred that the pollinia might contain IAA conjugated with sugars or amino acids. Thus, both IAA-myoinositol and IAA-aspartate were included as standards in the GC-MS analyses.

The ACC concentration in the same pollen diffusates was analyzed following the method of Lizada and Yang (1979). The ACC concentration was expressed as nanograms per microliter.

To assess pollen viability, pollinia from flowers of the *Dendrobium* cultivar Sakura were stained for 2 min using Evans Blue (0.5%; Sigma-Aldrich; India) and then washed with distilled water as described by Taylor and West (1980). Stained specimens (in triplicate) were observed under a light microscope. Dead pollen appeared blue.

Whole pollinia experiment

Hormonal profiles of cytokinins (zeatin, ZR, IPA, 2-iP), auxin (IAA), gibberellic acids (GAs: GA_1, GA_4, GA_9, GA_{19}, GA_{20}, GA_{24}), abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and ACC were analyzed using ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), as described by Müller and Munné-Bosch (2011).

Samples of 100 mg each of the whole pollinia from cut flowers of each of the *Dendrobium* cultivars Burana Jade, Sakura, Willie, Khao Chaimongkol, Miss Teen (also known as Kenny), Miss Orchid and Karen (also known as Sonia Bom#28) were extracted in a 2 mL microcentrifuge tube using 0.2 mL of ethanol:isopropanol:acetic acid (50:49:1, volume per volume) and ultrasonicated at 4°C. The extract was centrifuged at 10,000 rpm for 10 min at 4°C. Then, the supernatant was collected and the pellet re-extracted using the same solvent. Supernatants were pooled, passed through a polytetrafluoroethylene filter (Water; USA), and injected into the UHPLC-MS/MS system. Quantification of each compound was performed using the multiple reaction monitoring method and recovery rates were calculated for each sample using deuterated standards, as described by Müller and Munné-Bosch (2011).

Hand pollination and hormonal treatments on stigma of Dendrobium cv. Miss Teen

Flowers were cross pollinated by placing the pollinia from open flowers of *Dendrobium* cv. Sakura onto the stigmas of flowers of *Dendrobium* cv. Miss Teen. This was done without removal of the anther cap or the pollinia from the recipient flowers (Luangsuwalai et al., 2008) to avoid stimulation of any wounding responses and the associated production of ethylene.

To estimate the effects of the plant hormones on post-pollination, whole inflorescences of *Dendrobium* cv. Miss Teen bearing only open flowers were either left unpollinated (negative control) or were pollinated (positive control) and treated with 30 ng/µL IAA, 0.01 ng/µL ABA, 0.2 ng/µL GA_3, 0.025 ng/µL SA and 0.01 ng/µL ACC applied in a 20 µL amount to the stigma. These growth regulators were either applied singly or in various combinations of IAA plus ABA or GA or SA, ABA plus GA or SA, ABA plus GA and SA, and also the combination of all four hormones together. All treatments were based on the results of the analysis of concentrations of plant hormones found in the whole pollinia of *Dendrobium,* as represented in Table 2. All chemicals were dissolved in 18.2 MΩ water. Flowers were assessed daily for 15 d and symptoms of epinasty, drooping and venation were recorded.

Statistical analysis

The data were analyzed using analysis of variance and mean comparisons were performed using least significant differences. The results were considered significant when $p < 0.05$. Principal component analysis (PCA), facilitated by the PAST version 3.0 software (Oyvind Hammer, Norway), was used to identify patterns of the *Dendrobium* cultivars and the hormonal profiles in pollinia, and to eliminate redundancy in univariate analyses, when multicollinear data were involved.

Results

Compounds in aqueous pollinia extracts

The diffusate of pollinia that had been placed in distilled water for 3 hr at 25°C and then analyzed using GC-MS showed two maxima, one being IAA (compared with the retention time of the IAA standard; Fig. 1A), and the other an unknown compound with 65% similarity to 2-(hydroxymethyl)-5-(octylsulphanylmethyl)-oxolane-3,4-diol. The IAA concentration was considerably higher in the extracts of Sakura than in those of Miss Teen (Table 1). When the pollinia were dipped in distilled water for less than 1 s, the same two compounds were present in the extract, albeit at low concentrations (Fig. 1B).

Staining of the pollinia with Evans Blue revealed that a portion of the pollen grains were dead after soaking in aqueous solution for 3 hr. Similarly, after placing the pollinia in distilled water for less than 1 s, it was also found that some of the pollen grains were dead (data not shown). Furthermore, the pollinia soaked in water extract for 3 hr showed some pollen membrane rupture. These results indicated that some pollen lacked viability under natural conditions.

Compounds in CAPSO pollinia extracts

CAPSO buffer (pH 9.7) can release IAA conjugated with sugars or protein after mild hydrolysis at approximately pH 9 or above; it was used at a concentration that produced an osmotic potential equivalent to that of the stigma fluid. The pollinia diffusate produced after extraction for 3 hr at 25°C showed no peaks in the GC-MS spectral range (data not shown). Evans Blue staining of pollinia after these treatments showed some dead pollen, similar to that observed following aqueous extraction; however, there was no pollen rupture. These data confirmed that the auxin in the pollinia of *Dendrobium* was free IAA.

As indicated above, it was initially inferred that the pollinia might contain IAA conjugated with sugars or amino acids. Thus, IAA-myoinositol and IAA-aspartate were used as standards in the GC-MS assessments (data not shown); however, these compounds were not detected.

1-Aminocyclopropane-1-carboxylic acid concentrations in extracts

The ACC concentrations in the 3 hr distilled water and 3 hr CAPSO pH 9.7 buffer extracts are shown in Table 1. The ACC concentrations in either of these 3 hr extracts of Sakura and Miss Teen pollinia were not significantly different (Table 1).

Table 1 Concentrations of indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylic acid (ACC) extracted by soaking pollinia from *Dendrobium* cultivars Sakura and Miss Teen for 3 hr in distilled water or in CAPSO buffer

Cultivar	Concentration in pollen diffusate (ng/μL)		Total content in pollen diffusate (ng)	
	Distilled water	CAPSO	Distilled water	CAPSO
		IAA		
Post-pollination effects				
Sakura	4.28±2.7	nd	8557.1±5314.9	nd
No post-pollination effects				
Miss Teen	nd	nd	nd	nd
		ACC		
Post-pollination effects				
Sakura	0.013±0.01[a]	0.253±0.15[a]	25.85±13.9[a]	505.89±319.8[a]
No post-pollination effects				
Miss Teen	0.004±0.002[a]	0.110±0.07[a]	7.84±3.6[a]	221.063±156.8[a]

CAPSO = N-cyclohexyl-2-hydroxyl-3-aminopropanesulfonic acid; nd = not detected;
Values (mean ± SD, 4 replications) within a column superscripted with different lowercase letters are significantly ($p < 0.05$) different.

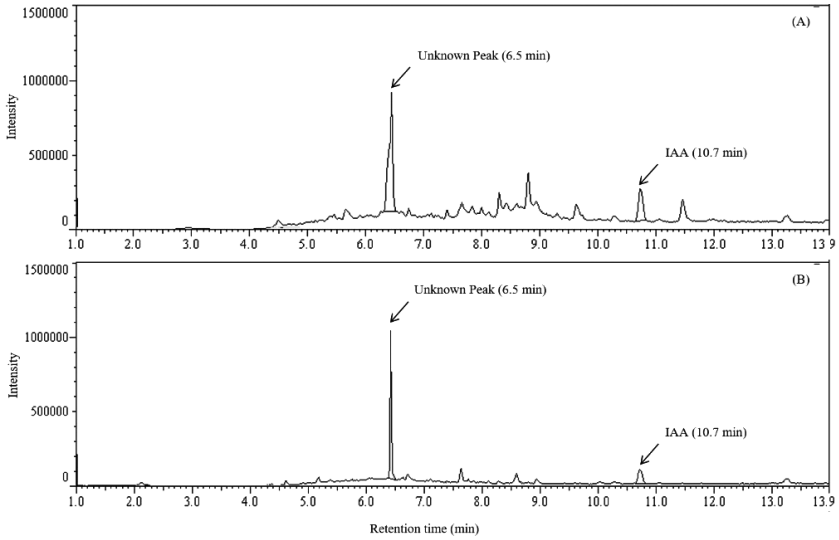

Fig. 1 Gas chromatography-mass spectrometry analysis of extract showing two maxima, one being indole-3-acetic acid (IAA) at 10.7 min and an unknown compound at 6.5 min: (A) pollinia of *Dendrobium* flowers placed in distilled water for 3 hr at 25°C; (B) pollinia of *Dendrobium* flowers dipped in distilled water for less than 1 s, where the same two compounds were present in the extract, albeit at low concentrations

However, the ACC concentration in 3 hr CAPSO pH 9.7 buffer was higher than that in the aqueous extract (Table 1). No ACC was detected in the initial extracts (dipped in extraction for less than 1 s) using either aqueous or CAPSO buffer extraction.

Hormone concentrations in the whole pollinia and hormonal treatments

PCA based on a correlation matrix showed that the first principal component (PC1) with an eigenvalue greater than 1.0 constituted about 67.5% of the total variation (data not shown). This was mainly determined by positive values for IAA, ABA and SA and a negative value for ACC (Fig. 2A). The highest loadings in PC1 indicated the importance of this component, with these hormones representing the largest portion of those associated with the post-pollination effect. Therefore, PCA analysis revealed that IAA concentrations were strongly associated with the post-pollination effects (Table 2 and Fig. 2A). Strong associations were also found with the concentrations of ABA, SA and a number of GAs (Table 2, Table 3 and Fig. 2B). However, the ACC concentrations were not well correlated with the post-pollination effects, with values being considerably higher where no post pollination effects were observed (Table 2 and Fig. 2A). Similarly, poor correlations were observed with concentrations of JA and a range of cytokinins (Table 2 and Table 4).

Table 2 Concentrations of indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylic acid (ACC), abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) in ground pollinia of several *Dendrobium* cultivars, with or without normal post-pollination events

Cultivar	Concentration (ng/g pollen)				
	IAA	ACC	ABA	JA	SA
Post-pollination effects					
Burana Jade	67132.5±13120^{c}	79.3±27.0^{bc}	199.0±45.2^{a}	20.7±9.8^{a}	490.8±41.3^{ab}
Sakura	591638.0±71236^{a}	22.6±6.0^{c}	105.6±19.0^{b}	10.4±5.6^{a}	504.6±19.7^{a}
Willie	96771.0±46418^{c}	49.9±16.4^{bc}	48.2±14.2^{c}	18.9±4.6^{a}	373.5±89.7^{b}
Khao Chaimongkol	152739.5±47255^{b}	48.7±13.9^{bc}	46.5±17.0^{c}	11.4±5.8^{a}	388.6±98.9^{ab}
No post-pollination effects					
Miss Teen	1244.3±513^{d}	110.6±39.6^{b}	24.7±7.8^{d}	7.7±3.8^{a}	147.8±11.8^{c}
Miss Orchid	2304.4±419^{d}	203.4±99.5^{a}	13.2±3.4^{d}	18.7±11.0^{a}	220.5±60.7^{c}
Karen	14629.7±3182^{d}	87.5±24.0^{bc}	25.7±9.9^{d}	12.0±3.9^{a}	116.7±28.4^{c}

Values (mean ± SD, 5 replications) within a column superscripted with different lowercase lettersare significantly ($p < 0.05$) different.

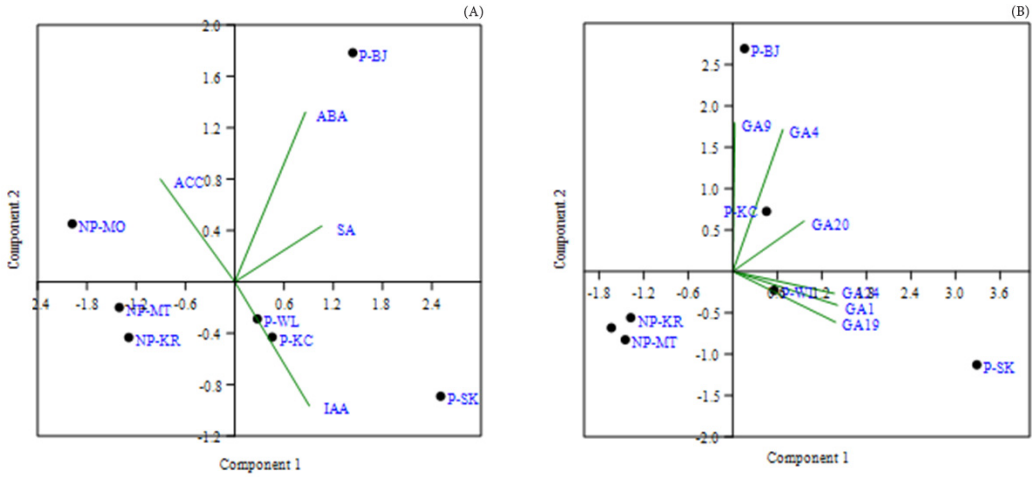

Fig. 2 Principal component analysis of hormonal profiles in whole pollinia of *Dendrobium* (indicated by green lines) and position of *Dendrobium* cultivars (denoted by abbreviations): (A) for indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC), abscisic acid (ABA) and salicylic acid (SA); (B) for gibberellins (GAs), where P = post-pollination effects and NP = no post-pollination effects and *Dendrobium* cultivars are Burana Jade (P-BJ**),** Sakura (P-SK), Willie (P-WL), Khao Chaimongkol (P-KC), Miss Teen (NP-MT), Miss Orchid (NP-MO) and Karen (NP-KR)

Table 3 Concentrations of some gibberellins (GAs) in ground pollinia of several *Dendrobium* cultivars, with or without normal post-pollination symptoms

Cultivar	Concentration (ng/g pollen)					
	GA1	GA4	GA9	GA19	GA20	GA24
Post-pollination effects						
Burana Jade	67.1±68.7^{b}	1305.4±142.3^{a}	215.9±54.8^{a}	68.0±33.4^{bc}	25.3±11.1^{bcd}	817.4±233.7^{b}
Sakura	260.8±207.9^{a}	563.4±70.4^{c}	15.6±5.3^{b}	928.8±320.3^{a}	31.3±14.4^{ab}	1929.8±400.6^{a}
Willie	59.8±27.9^{b}	169.0±44.5^{d}	49.3±19.3^{b}	88.6±44.3^{bc}	39.6±18.5^{ab}	1675.6±297.1^{a}
Khao Chaimongkol	25.1±18.0^{b}	936.2±209.9^{b}	17.6±8.1^{b}	230.0±86.3^{b}	50.2±27.3^{a}	722.9±131.8^{b}
No post-pollination effects						
Miss Teen	15.2±14.1^{b}	48.7±8.9^{d}	19.9±4.6^{b}	18.5±3.6^{c}	8.2±1.3^{d}	612.5±59.2^{bc}
Miss Orchid	15.5±28.2^{b}	85.0±8.8^{d}	19.0±6.3^{b}	34.0±16.2^{bc}	12.0±3.8^{cd}	205.6±71.9^{d}
Karen	18.7±4.7^{b}	73.6±16.8^{d}	35.0±10.0^{b}	64.7±15.6^{bc}	14.8±1.5^{cd}	373.7±97.2^{cd}

Values (mean ± SD, 5 replications) within a column superscripted with different lowercase letters are significantly ($p < 0.05$) different.

Table 4 Concentrations of cytokinins in ground pollinia of several *Dendrobium* cultivars, with or without normal post-pollination symptoms

Cultivar	Concentration (ng/g DW)			
	Zeatin	ZR	IPA	2-iP
Post-pollination effects				
Burana Jade	4.2±1.9^{a}	933.9±58.7^{a}	155.8±17.4^{a}	39.3±12.3^{a}
Sakura	0.5±0.9^{c}	141.0±24.7^{c}	15.5±3.9^{de}	15.5±9.4^{cd}
Willie	1.0±0.7^{bc}	310.4±113.7^{b}	66.4±27.3^{b}	18.0±7.7^{cd}
Khao Chaimongkol	2.1±1.4^{b}	160.4±23.1^{c}	39.7±6.4^{c}	24.3±7.2^{bc}
No post-pollination effects				
Miss Teen	0.47±0.1^{c}	263.4±41.8^{b}	33.1±7.8^{cd}	9.7±5.7^{d}
Miss Orchid	0.5±0.1^{c}	104.5±17.4^{c}	11.8±3.7^{e}	33.4±11.6^{ab}
Karen	1.1±0.6^{bc}	266.4±55.0^{b}	19.1±4.4^{de}	15.4±6.8^{cd}

ZR = zeatin riboside, IPA = isopentenyl adenosine, 2-iP = 2-isopentenyl adenine

Values (mean ± SD, 5 replications) within a column superscripted with different lowercase letters are significantly ($p < 0.05$) different.

Treatment of unpollinated flowers with the combined IAA+ABA+GA+SA solution resulted in epinasty, drooping and venation like that in pollinated flowers or in those that had been treated with IAA. Unpollinated flowers treated with IAA plus ABA, GA or SA showed epinasty faster than those flowers treated with ABA plus GA or SA. In contrast, treatment of unpollinated flowers with ACC did not show any visible post-pollination symptoms of senescence (epinasty, drooping, or venation), as presented in Table 5.

Discussion

The following key points are discussed: 1) the presence of IAA and ACC in aqueous extracts of *Dendrobium* pollinia; 2) the presence of IAA and a number of other plant hormones in whole pollinia of two cultivars of *Dendrobium*; and 3) the effects of exogenous hormones on flower senescence.

Previous research on post-pollination in *Dendrobium* revealed that ACC concentrations and auxin activity in pollinia were closely related to early post-pollination effects (Promyou et al., 2014), as was also found in *Phalaenopsis* (Porat et al., 1998). In the past, the techniques used for identifying plant hormones, particularly IAA, were not sufficiently specific (Fitting, 1910; Müller, 1953; Porat et al., 1998). In the present study, GC-MS was used for the identification of free IAA in pollen diffusates and UHPLC-MS/MS was used for determination of the hormone profile in the whole pollinia.

The results indicated first that the auxin-like chemical in the pollen diffusates (aqueous extracts) of the *Dendrobium* cultivar Sakura was IAA, which was not found in the *Dendrobium* cultivar Miss Teen. Second, IAA was found in the whole pollinia of all the *Dendrobium* cultivars tested.

However, the IAA contents in the whole pollinia of *Dendrobium* cultivars in which normal post-pollination effects were induced, were higher than for the *Dendrobium* cultivars in which post-pollination effects were not induced. These data confirmed that free IAA is the 'pollen hormone' in *Dendrobium* orchid pollinia (as first inferred by Fitting [1910] who used the term 'pollen hormone' in his study of orchid pollinia phenomena; see also Yam et al., 2009). Therefore, it was hypothesized that the auxin-like compound in the pollinia of *Dendrobium* might be an IAA conjugate and/or free IAA. IAA conjugated with sugars or protein can be released after mild hydrolysis at approximately pH 9 or above (Baldi et al., 1989; Kowalczyk and Sandberg, 2001). To prove this hypothesis, using CAPSO buffer (pH 9.7) the extraction of the stigma fluid and the pollinia diffusate for 3 hr showed no peaks in the GC-MS spectral range (data not shown), compared with IAA-myoinositol and IAA-aspartate as standards, but there was no presence of these compounds. These data confirmed that the auxin in the pollinia of *Dendrobium* was free IAA.

Table 5 Time to epinasty (first visible post-pollination symptoms), drooping and venation of unpollinated flowers of *Dendrobium* cv. Miss Teen, pollinated flowers with pollinia from *Dendrobium* cv. Sakura, and unpollinated flowers treated with indole-3-acetic acid (IAA), abscisic acid (ABA), salicylic acid (SA), gibberellic acid (GA) or combinations of these hormones and ACC (1-aminocyclopropane-1-carboxylic acid, as an immediate precursor of ethylene)

Treatment	Time (days after pollination)		
	Epinasty	Drooping	Venation
Unpollinated (Miss Teen)	>10.0^{a}	>10.0^{a}	> 10.0^{a}
Pollinated (Miss Teen × Sakura)	1.0^{d}	4.0^{c}	4.4^{bc}
IAA (600 ng/floret)	1.0^{d}	3.8^{c}	3.8^{bc}
ABA (0.2 ng/floret)	3.0^{b}	>10.0^{a}	>10.0^{a}
GA (4 ng/floret)	3.0^{b}	4.6^{b}	5.0^{bc}
SA (0.5 ng/floret)	3.0^{b}	4.6^{b}	5.0^{bc}
IAA+ABA	1.4^{cd}	3.6^{c}	4.5^{bc}
IAA+GA	1.9^{bcd}	4.0^{c}	5.2^{bc}
IAA+SA	1.7^{cd}	3.2^{c}	5.8^{b}
ABA+GA	2.6^{bc}	3.2^{c}	5.7^{bc}
ABA+SA	2.2^{bcd}	3.4^{c}	5.4^{bc}
ABA+GA+SA	3.0^{b}	4.7^{b}	4.9^{bc}
IAA+ABA+GA+SA	1.0^{d}	3.3^{c}	3.7^{c}
ACC (0.2 ng/floret)	>10.0^{a}	>10.0^{a}	> 10.0^{a}

Values (mean ± SD, 5 replications) within a column superscripted with different lowercase letters are significantly ($p < 0.05$) different.

The unknown compound detected using GC-MS showed 65% similarity to an alkylated pentose, as determined from the Wiley 7 library of GC-MS. The present study hypothesized that the unknown compound did not have auxin activity, as its structure was dissimilar to compounds having such activity. The literature reports on similar structures, such as batzellasides, which are C-alkylated iminosugars with an alkylated 'tail' of 9–11 C atoms (Segraves and Crews, 2005), but further research is required to identify the specific nature of this compound found in aqueous extracts of *Dendrobium* pollinia.

The data showed that the concentration of IAA in Sakura pollinia diffusates was lower than that in the whole pollinia of this cultivar. This suggested that IAA diffuses from the pollinia and induces the early post-pollination effects in *Dendrobium*; similar findings have been reported (Promyou et al., 2014). The IAA content in whole pollinia of Sakura was 592 µg/g pollinia, which was in agreement with Müller (1953), who indicated that orchid pollinia are a rich source of IAA, with as much as 100 µg/g pollinia being detected in the cultivars that were assessed in that study. Furthermore, IAA might be the first signal in the induction of early post-pollination by accelerating enhanced ethylene production. The absence of post-pollination effects in the Kenny and Karen cultivars may have been due to the low ethylene production induced by the pollinia of those cultivars (Luangsuwalai et al., 2008). This implied that the low IAA concentrations determined in pollinia of the *Dendrobium* cultivars Miss Teen, Miss Orchid and Karen in the present study may have been insufficient to induce ethylene production in these cultivars. However, the higher IAA concentrations in *Dendrobium* orchid cultivars that produced the post-pollination effect, such as Sakura and Khao Chaimongkol, appeared to be sufficient to induce ethylene production. Therefore, it was apparent that IAA in the pollinia of *Dendrobium* orchids can be confirmed as being one of the hormones associated with pollination in this species.

In general, the *Dendrobium* cultivars that produced normal post-pollination symptoms had higher concentrations of ABA, SA and a range of GAs (GA_1, GA_4, GA_9, GA_{19}, GA_{20}, GA_{24}), than those cultivars that did not show these effects. However, GA_1 and GA_4 are the bioactive forms in plants and GA_{19} and GA_{24} are the precursors of GA_{20} and GA_9, respectively (Yamaguchi, 2008). The introduction of a 3β-hydroxyl group converts inactive precursors (GA_{20} and GA_9) into bioactive GAs (Yamaguchi, 2008). The combinations of exogenous IAA, ABA, GA and SA applied to *Dendrobium* cv. Miss Teen, which does not normally produce post-pollination symptoms, did produce symptoms similar to those in flowers pollinated by pollinia from *Dendrobium* cv. Sakura. This implied that in addition to the auxin IAA, other hormones may be involved in the pollination process (Yam et al., 2009). Indeed, post-pollination symptoms in *Cymbidium* orchids were induced by exogenous ABA, GA_3 and NAA applied either alone or in combination, but not by kinetin, a hormone that retards senescence. The application of GA_3 on *Cymbidium* flowers was sufficient to mimic the effects induced by NAA, suggesting that gibberellins may act by increasing auxin levels (Arditti et al., 1971). Therefore, it is likely that ABA and GA may be involved in the induction of post-pollination effects in *Dendrobium* flowers.

No other studies have reported on the involvement of SA in post-pollination effects in flowers. However, one study showed that SA retards orchid flower senescence and reduces ethylene production (De et al., 2014). Zhang et al. (2013) reported that SA was found in the pollen of *Cedrus atlantica* and *Acacia dealbata*. Furthermore, SA induces metabolic activity which enhances pollen sterility in the TGMS line of rice (Praba and Thangaraj, 2005). Therefore, it is likely that SA may also contribute to the post-pollination effects in *Dendrobium* flowers.

ACC is the direct precursor of ethylene that induced post-pollination symptoms by application of ACC on the stigma of *Dendrobium* flowers (Wisuttiamonkul and Ketsa, 2013). The absence of a high rate of ethylene production seems important in explaining the lack of effects after pollination with cv. Kenny (Miss Teen) (Luangsuwalai et al., 2008). The ACC concentration was low in pollinia of *Dendrobium* cultivars that produced normal post-pollination effects and was similar to that in other cultivars that did not show these effects, as was reported for two groups of *Dendrobium* cultivars (Luangsuwalai et al., 2008). Treatment of the stigma of unpollinated flowers of *Dendrobium* cv. Miss Teen with ACC did not produce post-pollination effects. Other research found that the application of ACC to the stigma induced premature senescence (Wisuttiamonkul and Ketsa, 2013) because the ACC concentration was about 20 µmol/floret (2 µg/floret) (Wisuttiamonkul and Ketsa, 2013) which was higher than that identified in the present research using 0.2 ng/floret. The present research indicated that ACC was at very low concentrations and was insufficient to induce the post-pollination effects. Hence, it appeared that ACC in the pollinia of *Dendrobium* orchids was not a factor that induced early post-pollination symptoms.

IAA alone and in combination with ABA, SA, and several different GAs are pollen hormones in *Dendrobium* flowers that induce early post-pollination responses. Their specific modes of action warrant further investigation.

Conclusion

GC-MS and UHPLC-MS/MS were used for identification of the free-IAA and plant hormones in *Dendrobium* orchids from both normal post-pollination and no post-pollination effects groups. IAA, an auxin-like chemical, was identified in the pollen diffusates of the *Dendrobium* cultivar Sakura that first acted as a signal in the induction of early post-pollination development changes by accelerating enhanced ethylene production, but was absent in the Miss Teen cultivar that failed to show post-pollination changes. *Dendrobium* cultivars that produced normal post-pollination symptoms had high concentrations of IAA, ABA, SA and a range of GAs. The exogenous IAA alone or combined with ABA, GA or SA applied to *Dendrobium* cv. Miss Teen induced post-pollination symptoms as found in a pollinated flower, indicating the interaction of IAA with ABA, SA and a number of GAs. On the other hand, the ACC application with flowers did not induce a post-pollination response.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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References

Arditti, J., Hogan, N.M., Chadwick, A.V. 1973. Post-pollination phenomena in orchid flowers. IV. Effects of ethylene. Am J. Bot. 60: 883−888. doi.org/10.2307/2441068

Arditti, J., Jeffrey, D.C., Flick, B.H. 1971. Post-pollination phenomena in orchid flowers. III. Effects and interactions of auxin, kinetin or gibberellin. New Phytol. 70: 1125−1141. doi.org/10.1111/j.1469-8137.1971.tb04595.x

Attri, L.K., Nayyar, H. 2021. Pollination related temporal sequences of reproductive development and tracing the path of pollen tubes in *Cymbidium pendulum* (Roxb.) Sw., an ornamental orchid. Flora 279: 151813. doi.org/10.1016/j.flora.2021.151813

Baldi, B.G., Maher, B.R., Cohen, J.D. 1989. Hydrolysis of indole-3-acetic acid esters exposed to mild alkaline conditions. Plant Physiol. 91: 9−12. doi.org/10.1104/pp.91.1.9

De, L.C., Vij, S.P., Medhi, R.P. 2014. Post-harvest physiology and technology in orchids. J. Hortic. 1: 102. doi.org/10.4172/2376-0354.1000102

Dunlap, J.R., Guinn, G. 1989. A simple purification of indole-3-acetic acid and abscisic acid for GC-SIM-MS analysis by microfiltration of aqueous samples through nylon. Plant Physiol. 90: 197−201. doi.org/10.1104/pp.90.1.197

Fitting, H. 1910. Developmental studies on orchid flowers. Z. Bot. 2: 225267. [in German]

Hildebrand, F. 1863. The fruiting of orchids, proof of the double effect of the pollen. cited by Ketsa, S., Wisutiamonkul, A., van Doorn, W.G. 2006. Auxin is required for pollination-induced ovary growth in *Dendrobium* orchids. Funct. Plant Biol. 33: 887–892. doi.org/10.1071/FP06034 [in German]

Hoekstra, F.A., Weges, R. 1986. Lack of control by early pistillate ethylene of the accelerated wilting of *Petunia hybrida* flowers. Plant Physiol. 80: 403−408. doi.org/10.1104/pp.80.2.403

Ketsa, S., Luangsuwalai, K. 1996. The relationship between 1-aminocyclopropane-1-carboxylic acid content in pollinia, ethylene production and senescence of pollinated *Dendrobium* orchid flowers. Postharv. Biol. Technol. 8: 57−64. doi.org/10.1016/0925-5214(95)00053-4

Ketsa, S., Rugkong, A. 2000. The role of ethylene in enhancing the initial ovary growth of *Dendrobium* 'Pompadour' flowers following pollination. J. Hort. Sci. Biotechnol. 75: 451−454. doi.org/10.1080/14620316.2000.11511267

Ketsa, S., Wisutiamonkul, A., van Doorn, W.G. 2006. Auxin is required for pollination-induced ovary growth in *Dendrobium* orchids. Funct. Plant Biol. 33: 887−892. doi.org/10.1071/FP06034

Kowalczyk, M., Sandberg, G. 2001. Quantitative analysis of Indole-3-Acetic acid metabolites in Arabidopsis. Plant Physiol. 127: 1845–1853.

Lizada, M.C.C., Yang, S.F. 1979. A simple and sensitive assay for 1-aminocyclopropane-1-carboxylic acid. Anal. Biochem. 100: 140−145. doi.org/10.1016/0003-2697(79)90123-4

Luangsuwalai, K., Ketsa, S., Wisutiamonkul, A., van Doorn, W.G. 2008. Lack of visible post-pollination effects in pollen grains of two *Dendrobium* cultivars: Relationship with pollinia ACC, pollen germination, and pollen tube growth. Funct. Plant Biol. 35: 152158. doi.org/10.1071/FP07245

Luangsuwalai, K., Ketsa, S., van Doorn, W.G. 2011. Ethylene-regulated hastening of perianth senescence after pollination in *Dendrobium* flowers is not due to an increase in perianth ethylene production. Postharv. Biol. Technol. 62: 338–341. doi.org/10.1016/j.postharvbio.2011.07.011

Luangsuwalai, K., Paull, R.E., Ketsa, S. 2013. Compatible and incompatible pollination and the senescence and ovary growth of *Dendrobium* flowers. Eur. J. Environ. Sci. 3: 35–42. doi.org/10.14712/23361964.2015.21

Müller, M., Munné-Bosch, S. 2011. Rapid and sensitive hormonal profiling of complex plant samples by liquid chromatography coupled to electrospray ionization tandem mass spectrometry. Plant Methods 7: 37. doi.org/10.1186/1746-4811-7-37

Müller, R. 1953. The quantitative determination of indole acetic acid by using paper chromatography and paper electrophoresis. Beitr. Biol. Pfl. 30: 1–32. [in German]

O'Neill, S.D. 1997. Pollination regulation of flower development. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 547–574. doi.org/10.1146/annurev.arplant.48.1.547

O'Neill, S.D., Nadeau, J.A., Zhang, X.S., Bui, A.Q., Halevy, A.H. 1993. Interorgan regulation of ethylene biosynthetic genes by pollination. Plant Cell 5: 419–432. doi.org/10.1105/tpc.5.4.419

Praba, M.L., Thangaraj, M. 2005. Effect of growth regulators and chemicals on pollen sterility in TGMS lines of rice. Plant Growth Regul. 46: 117–124. doi.org/10.1007/s10725-005-7362-5

Porat, R., Nadeau, J.A., Kirby, J.A, Sutter, E.G., O' Neill, S.D. 1998. Characterization of the primary pollen signal in the post-pollination syndrome of *Phalaenopsis* flowers. Plant Growth Regul. 24: 109–117. doi.org/10.1023/A:1005964711229

Promyou, S., Ketsa, S., van Doorn, W.G. 2014. Pollinia-borne chemicals that induce early postpollination effects in *Dendrobium* flowers move rapidly into agar blocks and include ACC and compounds with auxin activity. J. Plant Physiol. 171: 1782–1786. doi.org/10.1016/j.jplph.2014.08.008

Strauss, M.S., Arditti, J. 1982. Postpollination phenomena in orchid flower X. Transport and fate of auxin. Bot. Gaz. 143: 286–293. doi.org/10.1086/337302

Segraves, N.L., Crews, P. 2005. A Madagascar sponge *Batzella* sp. as a source of alkylated iminosugars. J. Nat. Prod. 68: 118–121. doi.org/10.1021/np049763g

Taylor, J.A., West, D.W. 1980. The use of Evan's Blue stain to test the survival of plant cells after exposure to high salt and high osmotic pressure. J. Exp. Bot., 31: 571–576. doi.org/10.1093/jxb/31.2.571

van Doorn, W.G., Ketsa, S. 2021. Pollination-induced changes in the morphology and physiology of *Dendrobium* orchid flowers prior to fertilization: The roles of ethylene and auxin. In: Warrington, I. (Ed.). Horticultural Reviews, Vol 48. John Wiley and Sons Inc. Hoboken, NJ, USA, pp. 1–36.

Wisuttiamonkul, A., Ketsa, S. 2013. Role of stigma fluid in ovary growth and senescence of pollinated *Dendrobium* flowers. Eur. J. Environ. Sci. 3: 43–47. doi.org/10.14712/23361964.2015.22

Yam, T.W, Chow, Y.N., Avadhani, P.N., Hew, C.S., Arditti, J., Kurzweil, H. 2009. Pollination effects on orchid flowers and the first suggestion by Professor Hans Fitting (1877–1970) that plants produce hormones. In: Kull, T., Arditti, J., Wong, S.M. (Eds.). Orchid Biology: Reviews and Perspectives, Vol 10. Springer. Dordrecht, the Netherlands, pp. 37–140.

Yamaguchi, S. 2008. Gibberellin metabolism and its regulation. Annu. Rev. Plant Biol. 59: 225–251. doi.org/10.1146/annurev.arplant.59.032607.092804

Zhang, K., Halitschke, R., Yin, C., Liu, C.J., Gan, S.S. 2013. Salicylic acid 3-hydroxylase regulates *Arabidopsis* leaf longevity by mediating salicylic acid catabolism. Proc. Natl. Acad. Sci. USA. 110: 14807–14812. doi.org/10.1073/pnas.1302702110