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Full Length Research Paper

Hybridization of several *Aerides* species and *in vitro* germination of its hybrid

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The wild species of *Aerides* namely *Aerides odorata*, *A. odorata* var.'Yellow', *Aerides flabellata* and *Aerides quinquevulnera* var. *calayana* are fragrant and distributed in Malaysia. Crosses among them attempted to produce primary hybrids which were used to investigate the effects of kinetin and BAP concentrations on seed germination percentage (%) of *A. odorata* × *A. quinquevulnera* var. *calayana* and *A. quinquevulnera* var. *calayana* × *A. odorata*. The total six crosses were: *A. odorata* var.'Yellow' × *A. quinquevulnera* var. *calayana*, *A. odorata* × *A. quinquevulnera* var. *calayana* and *A. odorata* × *A. flabellata* and their reciprocals. The highest percentage of successful crosses were between *A. odorata* var.'Yellow × *A. quinquevulnera* var. *calayana* (80%) and lowest were *A. flabellata* × *A. odorata* (25%), *A. odorata* × *A. quinquevulnera* var. *calayana* and its reciprocal were 60 and 62%, respectively. Crosses of *A. quinquevulnera* var. *calayana* × *A.odorata* var.'Yellow' and *A. odorata* and *A.flabellata* showed 0% success. Pods of *A. odorata* var.'Yellow' × *A. quinquevulnera* var. *calayana* matured at 76 days, *A. flabellata* × *A. odorata* at 116 days, *A. odorata* × *A. quinquevulnera* var. *calayana* at 179 days and *A. quinquevulnera* var. *calayana* × *A. odorata* at 184 days. Pods of *A. flabellata* × *A. odorata* were the largest and the smallest were *A. odorata* var.'Yellow' × *A. quinquevulnera* var. *calayana*. There was a significant difference in seed germination where the highest germination percentage were with 1.5 mg/L kinetin and the lowest were in 2.0 mg/L BAP for *A. odorata* × *A. quinquevulnera* var. *calayana*. Germination of *A. quinquevulnera* var. *calayana* × *A. odorata* was highest in 1.5 mg/L BAP and lowest in 2.0 mg/L kinetin. Seeds of *A. odorata* × *A. quinquevulnera* var. *calayana* cultured in 1.5 mg/L kinetin took the shortest time for yellowing and greening, 54 and 76 days respectively. 1.0 mg/L kinetin produced first root, as early as 91 days and 1.0 mg/L BAP was the earliest to produce leaf primordial at day 96. The longest time taken for all these stages were in control treatment. The earliest seeds of *A. quinquevulnera* var. *calayana* × *A. odorata* to yellow were at 1.5 mg/L kinetin within 13 days, greening at 1.0 mg/L BAP for 24 days, first root and leaf primordial at 1.5 mg/L BAP at 33 and 54 days respectively. The longest time taken for this species to yellow and green were at control treatment at days 38 and 50, first root in 1.5 mg/L BAP at day 33 and to produce leaf primordia were in 0.5 mg/L kinetin at day 74.

Key words: Aerides, reciprocal, crosses, hybrids.

INTRODUCTION

Orchids have their own devotees because of the uniqueness of the family, Orchidaceae which is reflected in its huge amount of diversity coupled with peculiar pollination contrivances and wide natural hybridization. The orchid genus *Aerides* Lour. consists of approximately 21 Southeast Asian species ranging from India to Papua New Guinea (Kocyan et al., 2008). The flowers exhibit an excessively wide range of variation in shape, form, size and colouration surpassing flowers of all the other Angiosperms. As a result, they are highly valued in flori-trade as species or hybrids for pot plant or cut flower and

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holds enormous promise (Kishor et al., 2006). Endemic orchids of the world are facing the grim possibility of extinction under intense biotic pressures like forest fires, indiscriminate wild collection and illegal trade by people. Continuing loss of native orchids has lead to an increased emphasis on orchid conservation. The loss of diversity in orchid population is worrying and conservation method including hybridization and *in-vitro* propagation are essential. Hybridization of orchids introduces a new dimension in the floriculture industry with constant production of better breeds (Vij, 1998). Orchid requires pollinators for pod production and levels of genetic variation in a natural population highly depend on cross fertilization. However, with the change in climate and destruction of forests, the ecology of the pollinators is threatened.

Hence, hybridization by man is required. The great popularity of hybrid orchids is due to a variety of reasons like superior quality, ease of cultivation, free-blooming habit, incredible array of shapes, blend of colours and longer shelf-life. However, development of new hybrid orchid is a tedious work that calls for great patience. Breeding orchids for the synthesis of hybrids with superior characters was extensively performed during the last century (Kishor et al., 2006). Propagation through conventional method such as vegetative propagation is a very slow process. Besides that propagation of orchids by seeds in the natural require mycorrhizal association for germination. Therefore, germinating seeds in nutrient containing medium can be a fast tool. This method of cultivation was revolutionized after the discovery of Knudson (1922), that orchid seeds can be germinated on a simple sugar containing medium without fungal association. *Aerides odorota* (AO) (Figure 1a), *A. odorota* var 'Yellow' (AOY) (Figure 1b), *Aerides quinquevulnera* var. *calayana* (AQ) (Figure 1c) and *Aerides flabellata* (AF) (Figure 1d) are wild and have not been domesticated. They are well known for their fragrance.

The flowers of *A. odorota* are small, milky and purplish at the edges of sepals and petals compared to *A. odorota* var. 'Yellow' which are slightly smaller and has yellow spur. *A. quinquevulnera* var. *calayana* has deep purple colour with well developed petals with spur pointed downwards while *A. flabellata* has spotted bicolour showy flowers with the yellow upper part and lower light purple (Figure 1). Synthesis of better hybrid orchids will certainly reduce the threatening pressure on their wild parents and be helpful for conservation. Besides that many modifications of medium have also been done by many researchers by adding plant growth regulators to enhance and fasten the germination of orchid seeds, yet there were no reports on *in-vitro* germination of *Aerides* species. In this study, hybridity confirmation of several *Aerides* species was made and different concentration levels of benzylaminopurine (BAP) and kinetin was inves-tigated in order to determine the highest percentage of seed germination and the duration of time taken for seed development until it produced leaf primordial.

MATERIALS AND METHODS

Hybridization studies were carried out at Orchid Shade House, Horticultural Unit, MARDI, Serdang. Plant materials, *A.odorata* and *A. odorota* var.'Yellow' were obtained from MARDI, Serdang while *A. quinquevulnera* var. *calayana* and *A. flabellata* were bought from United Malaysian Orchid Farm, Rawang, Selangor. Hybridization was performed in October 2009 when *A. odorata, A. odorota* var.'Yellow', *A. quinquevulnera* var. *calayana* and *A. flabellata* were flowering synchronously. Hybridizations were done in the morning between 8 and 10 am. After 5 to 6 days of flower opening, the pollinia from the species were separately removed, using fine sterilized forceps, and deposited on the stigma of the respective female parents. Pollinia from the female parents were also removed to be used for the reciprocal crosses and to prevent self-pollination. The hand pollinated flowers were labelled individually with tags giving the date and the time of pollination. The total crosses were six, *A. odorata* var.'Yellow' × *A. quinquevulnera* var. *calayana*, *A. odorata* x *A. quinquevulnera* var. *calayana* and *A. odorata* × *A. flabellata* and their reciprocals.

After hybridization, the pods were allowed to develop by maintaining the plants in the orchid shade house until it reaches its maturity. F1 seeds of *A. odorata* × *A. quinquevulnera* var. *calayana* and *A. quinquevulnera* var. *calayana* × *A.odorata* were chosen for *in-vitro* germination study. The pods were collected from the field after maturation. Pods were first washed with soap solution and then surface sterilized with 70% alcohol for 5 min. The pods were rinsed 4 times with sterile distilled water before being transferred to the laminar air flow. The seeds were cultured in Murashique and Skoog (MS) medium supplemented with four concentration levels (0.5, 1.0, 1.5, and 2.0 mg/L) of BAP and kinetin. The cultures were arranged in completely randomized design (CRD) with five replications. Cultures were placed at 25±1℃ and 70 to 80% of relative humidity, with a light intensity of 180 $\mu molm^{-2}S^{-2}$ provided by cool white fluorescent lamps for a light period of 16 h per day.

RESULTS

Observations on the crossability between *A. odorata*, *A. quinquevulnera* var. *calayana* and *A. flabellata* showed the same development pattern but different successful percentages. The highest percentage of successful cross were between *A. odorata* var.'Yellow' × *A. quinquevulnera* var. *calayana*, which was 80% and the lowest percentage was *A. flabellata* × *A. odorata*, 25%. The successful crosses between *A. odorata* × *A. quinquevulnera* var. *calayana* and its reciprocal were 60 and 62% respectively. Crosses of *A. quinquevulnera* var. *calayana* × *A. odorata* var.'Yellow' and *A. odorata* and *A. flabellata* were not compatible for intergeneric hybridization, as crossability between them showed 0% success (Table 1). This unsuccessful cross was due to the bigger pollen size of male parents when inserted into narrow stigma surface of female parent. Flowers of *A. flabellata* are bigger than *A. odorata*; therefore, pollen of *A.flabellata* is larger and may damage the stigma surface of *A. odorata*. The same phenomena happen in the crosses between *A. quinquevulnera* var. *calayana* × *A. odorata* var.'Yellow'. The successful crosses developed into pods. One pollination event leads to the production of hundreds and thousands of seeds (Benzing, 1987). Observations have shown that the duration of time taken

Figure 1. Flowers of (a) *Aerides odorata* var.'Yellow' (b) *Aerides odorata* (c) *A. flabellata* (d) *A. quinquevulnera* var. *calayana*.

Table 1. Successful and unsuccessful crosses between *A. odorata*, *A. odorata* var.'Yellow', *A. quinquevulnera* var. *calayana*, and *A. flabellata*.

Crosses		Reciprocals	
♀	♂	♀	♂
AO X AF		AF X AO	
AO X AQ		AQ X AO	
AOY X AQ		AQ X AOY	

AO: *Aerides odorata*, AOY: *Aerides odorata var "*Yellow*"*, AF: *Aerides flabellata*, AQ: *Aerides quinquevulnera*, : successful cross, : Unsuccessful cross.

Table 2. Results of crosses and reciprocal crosses performed for the hybridization study using *A.odorata*, *A.odorata* var.'Yellow', *A.quinquevulnera* var. *calayana* and *A.flabellata*

S/N	Parentage		Time (day) for matured pod	Number of flower pollinated	Number of pod formed	Successful cross (%)
	Female	Male				
1	*A. odorata* var.'Yellow'	*A. quinquevulnera* var. *calayana*	76	10	8	80
2	*A. quinquevulnera* var. *calayana*	*A. odorata* var.'Yellow'	0	10	0	0
3	*A. odorata*	*A. quinquevulnera* var. *calayana*	179	15	9	60
4	*A. quinquevulnera* var. *calayana*	*A. odorata*	184	13	8	62
5	*A. odorata*	*A. flabellata*	0	5	0	0
6	*A. flabellata*	*A. odorata*	116	8	2	25

Table 3. Morphometric characteristics of seed pods of successful crosses.

Crosses	Colour	Length (cm)	Width (cm)	Weight (g)
AOY X AQ	166B	3.96±0.062	0.85±0.018	0.13±0.019
AF X AO	166B	5.15±0.150	1.45±0.350	1.4±0.096
AO X AQ	152A	5.05±0.084	1.07±0.033	1.27±0.076
AQ X AO	153C	4.75±0.084	0.93±0.042	0.82±0.032

± Standard error; N199B = grey brown group; 14A = yellow orange group; 152A = yellow green group; 153B = yellow green group.

to develop into the mature pods vary between the crosses (Table 2). *A. odorata* var.'Yellow' × *A. quinquevulnera* var. *calayana* produced mature pod as early as 76 days, followed by *A. flabellata* × *A. odorata* at 116 days, *A. odorata* × *A. quinquevulnera* var. *calayana* at 179 days and the longest time taken was 184 days by *A. quinquevulnera* var. *calayana* × *A. odorata*. The colour, length, width, length/width (L/W) ratio of seed pods differ between the crosses (Table 3).

The colour of mature pods of *A. odorata* var.'Yellow' × *A. quinquevulnera* var. *calayana* at harvested stage were grey brown group (N199B) as referred to Royal Horticulture Society (RHS) colour chart. Meanwhile, pods of *A. flabellata* × *A. odorata* were in yellow orange group (14A). Pods *A. odorata* × *A. quinquevulnera* var. *calayana* and its reciprocal crosses were in the group of yellow green (152A) and (153B) respectively. Pods of *A. flabellata* × *A. odorata* were the largest compared to the other pods, as it had the mean of longest length, 5.15 cm and widest width 1.45 cm with 1.4 g of weight. Meanwhile, pods of *A. odorata* × *A. quinquevulnera* var. *calayana* were 5.05 cm in length, 1.07 cm in width and 1.27 g of weight. The following size of pods was *A. quinquevulnera* var. *calayana* × *A. odorata* with length of 4.75 cm, width, 0.93 cm and 0.82 g weight. The smallest and lightest pods were *A. odorata* var.'Yellow' × *A. quinquevulnera* var. *calayana* with 3.96 cm length, 0.85 cm width and 0.13 g weight. The size of pods determines the total seeds that have been produced from the fertilization and it is useful for *in-vitro* culture. Observation revealed that seeds of *A. quinquevulnera* var. *calayana* × *A. odorata* and *A. flabellata* × *A. odorata* were loose and does not stick to each other while seeds of *A. odorata* x *A. quinquevulnera* var. *calayana* and *A. odorata* var.'Yellow' × *A. quinquevulnera* var. *calayana* were spongy as other vandaceous orchid seeds. The successfully harvested pods of the present cross revealed 90% full seeds as examined before inoculation, indicating success of the cross (Figure 2).

A. odorata × *A. quinquevulnera* var. *calayana* and *A. quinquevulnera* var. *calayana* × *A. odorata* are the most compatible cross therefore this two hybrids were chosen for *in-vitro* germination studies. Seed germination percentage (%), was recorded at the fourth month after *in-vitro* culture and the duration of time taken by seeds to become green, following yellowing, first root and leaf primordial formation was determined. Supplemented concentration of 1.5 mg/L kinetin in MS media had proved the best for seed germination of *A. odorata* × *A. quinquevulnera* var. *calayana* with the highest percentage (78%) (Table 4) and the least germination percentage for this hybrid was at 2.0 mg/L BAP concentration level (Figure 3). For hybrid *A. quinquevulnera* var. *calayana* × *A.odorata*, seeds cultured in MS media supplemented with 1.5 mg/L BAP has also shown highest germination percentage of 78% (Figure 4). Seed germination of this hybrid was least (18%) at 2.0 mg/L kinetin. It clearly showed that BAP and also kinetin concentration of 2.0 mg/L inhibits the seed germination and also delay the development process of both *A. odorata* × *A. quinquevulnera* var. *calayana* and *A. quinquevulnera* var. *calayana* × *A. odorata* hybrids. Seeds of *A. odorata* × *A. quinquevulnera* cultured in MS media supplemented with 1.5 mg/L kinetin took the shortest time for yellowing and greening, 54 and 76 days respectively, concentration of 1.0 mg/L kinetin produced first root as early as 91 days,

Figure 2. (a) Globular shaped pollen of *A. odorata* (20x); (b) Successful cross of *A. quinquevulnera* var. *calayana* (♀) x *A. odorata* (♂); (c) Pod development one month after pollination; (d) Pod development four months after pollination; (e) Matured capsules containing hybridized seeds (6 months after crossing).

Table 4. Effects of kinetin and Benzylaminopurine in MS media on the development stages of *A. odorata* x *A. quinquevulnera* var. *calayana* and *A. quinquevulnera* var. *calayana* x *A. odorata*.

Trt	***A. odorata* x *A. quinquevulnera***				***A. quinquevulnera* x *A. odorata***			
	Mean (day taken by seed for development)							
	Yellowing	**Greening**	**1st root**	**Leaf primordia**	**Yellowing**	**Greening**	**1st root**	**Leaf primordia**
0	120^{a}	156^{a}	180^{a}	170^{a}	28^{a}	50^{a}	60^{b}	67^{b}
B0.5	80^{cd}	100^{cd}	140^{b}	120^{cd}	20^{bc}	44^{ab}	55^{b}	60^{cd}
B1.0	62^{ef}	82^{de}	109^{cd}	96^{e}	17^{cd}	24^{d}	40^{c}	55^{d}
B1.5	74^{de}	90^{de}	120^{c}	110^{de}	14^{d}	26^{cd}	33^{d}	54^{d}
B2.0	96^{b}	145^{ab}	178^{a}	161^{ab}	24^{ab}	46^{ab}	59^{b}	64^{bc}
K0.5	94^{bc}	123^{bc}	162^{a}	140^{bc}	21^{bc}	41^{b}	60^{b}	74^{a}
K1.0	64^{ef}	80^{de}	91^{d}	110^{de}	14^{d}	28^{cd}	45^{c}	64^{bc}
K1.5	54^{f}	76^{e}	92^{d}	106^{de}	13^{d}	33^{c}	40^{c}	56^{d}
K2.0	100^{b}	151^{a}	172^{a}	160^{ab}	24^{ab}	47^{ab}	68^{a}	70^{ab}

and 1.0 mg/L BAP was the earliest to produce leaf primordial at day 96. The longest time taken for yellowing, greening, first root and also leaf primordial were in the control treatment when there were no kinetin and BAP added. The earliest seeds of *A. quinquevulnera* var. *calayana* × *A. odorata* to yellow were in MS media

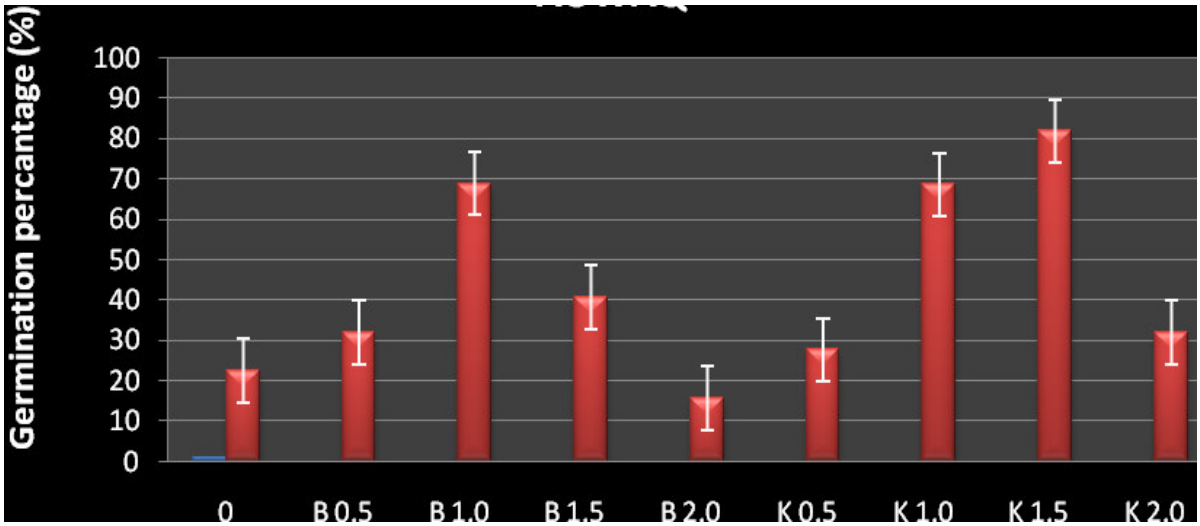

Figure 3. Effects of kinetin and benzylaminopurine in MS media on the *in-vitro* germination of *A. odorata* x *A. quinquevulnera* var. *calayana*

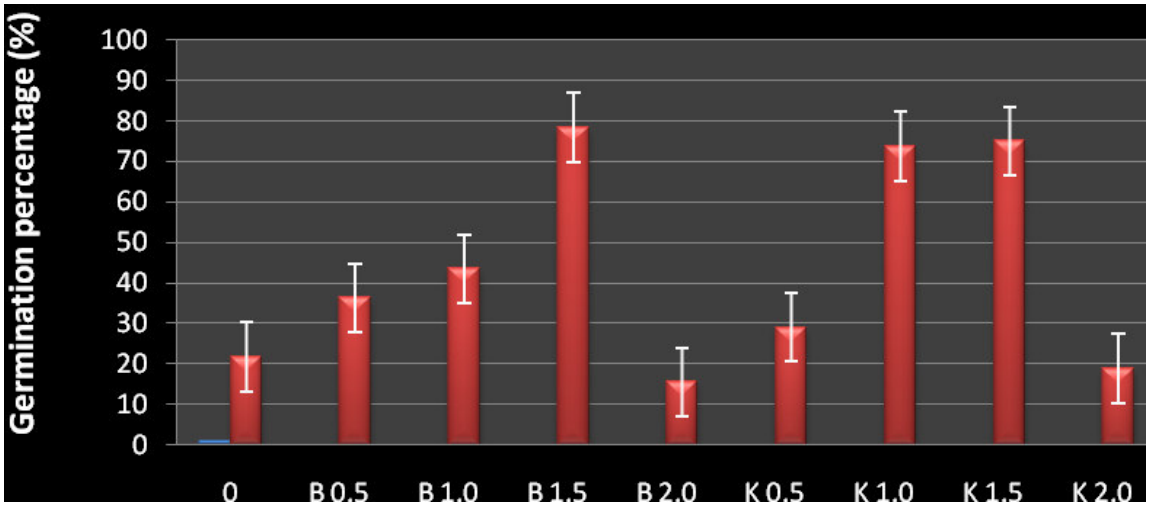

Figure 4. Effects of kinetin and benzylaminopurine in MS media on the *in-vitro* germination of *A. quinquevulnera* var. *calayana* x *A. odorata.*

supplemented with 1.5 mg/L kinetin within 13 days, greening at 1.0 mg/L BAP 24 days, first root and leaf primordial at 1.5 mg/L BAP at 33 and 54 days respectively. The longest time taken for this hybrid to turn into yellow and green were at control treatment at day 38 and 50, first root in 1.5 mg/L BAP at day 33 and to produce leaf primordia were in 0.5 mg/L kinetin at day 74.

DISCUSSION

Fertilization occurs when a pollen grain is deposited into the stigma of a female parent. The flower starts to wilt a day after pollination. The ovary swells and develops into seed pods. One pollination event leads to the production of hundreds and thousands of seeds (Benzing, 1987). A successful pollination depends on pollen size and also flower age. *Aerides* flowers remain open for several days, while inflorescences continually flower for the following weeks. Pollinating young, fully open flowers is recommended since pollen is most receptive 1 to 8 days after flowers are open (Proctor, 1998; Shiau et al., 2002; Lo et al., 2004). Likewise, using young flowers less than 1 week from opening ensures that the stigmatic surface is receptive to pollen. After 2 weeks, flowers close and pollen becomes brown and unreceptive. It is important to attain flowering in different individuals at the same time for hybridization through artificial pollination. Taking

opportunity of the off-season flowering of *A. odorata*, its hybridization with *A. quinquevulnera* var. *calayana* and *A. flabellata* was achieved.

According to Hartman et al. (1997), specific endogenous growth promoting and inhibiting compounds are involved directly in the control of seed development, dormancy, and germination. Evidence for hormone or plant growth regulators (PGR) involvement comes from correlations of PGRs concentration with specific developmental stages, effects of applied PGRs, and the relationship of PGRs to metabolic activities. The activation of the metabolic machinery of the embryo leading to the emergence of a new seedling plant is known as germination. It is therefore essential to investigate the PGR requirement to induce *in-vitro* germination and morphogenesis of rare orchids and its hybrids. Cytokinins such as 6-benzylaminopurine (BAP) and kinetin have also been included in germination media, but with varying results ranging from inhibition, no effect or stimulation. Protocorms and germinating seeds have different sensitivities to cytokinins with seeds being more sensitive. The mechanism for differential sensitivity of seeds to cytokinins is not clear. Either the germinating seed has a lower cytokinin requirement or it can synthesize enough cytokinins by itself. Cytokinin activity has been detected in seeds of *Epidendrum* orchids (Taylor et al., 1982; Mercier and Kerbauy, 1991). The optimum concentration level of both BAP and kinetin was 1.5 mg/L. This is similar to the result found by Jaime et al. (2005) that the highest concentration of kinetin (1.5 mg/L) developed a maximum percentage seed germination in combination both with or without IAA and GA3, indicating an overall positive effect on *Comparettia falcata* seed germination.

In conclusion, a successful pollination depends on pollen size and also flower age. Fruit setting provides a common measure of reproductive success in orchid breeding (Proctor, 1998). The lower percentage of fruit setting in the successful crosses was possibly due to intergeneric incompatibilities, experimental mishandling, and selective abortion or reduced plant vigor (Shiau et al., 2002). Besides, BAP and kinetin stimulates germination and growth in *A. odorata* x *A. quinquevulnera* var. *calayana* and *A. quinquevulnera* var. *calayana* x *A. odorata* hybrids at concentration level of 1.5 mg/L and higher concentration inhibits these processes.

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