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Effects of hormone and fertilizers on early flower induction of *Dendrobium anosmum* hybrid seedlings under *ex vitro* condition

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Dendrobium anosmum

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Early flowering

Hybrid.

Ex vivo

ABSTRACT

Early flowering of new orchids is important to save time for selecting valuable flowers and artificial induction of flowering is a critical consideration in the orchid production industry. In this study, a new *Dendrobium anosmum* hybrid was generated by cross-breeding between *D. anosmum* 'Chau Nhu' and *D. anosmum* 'Di Linh'. The ancestors and hybrid seedlings from *in-vitro* culture were trained in the net house and their growth and flowering were evaluated under *ex vivo* conditions with specific fertilizers and hormones. The results suggest that the hybrid plants grew better than their parents in terms of stem height, stem diameter, and leaf number. Growth hormones were applied to stimulate early flowering in matured hybrids and it was discovered that 'Keiki pro', a commercial hormone product, produced the best results, with a flowering rate of 66.67% after two applications. Hybrid flowers varied in width from 36.36% (3.0-6.0 cm) to 63.64 % (more than 6.0 cm) from ancestral width in medium-sized and large-sized flowers, respectively. Also, the hybrid flower colours was mostly a combination of pink/violet (75C) and purple/pink (68A), which is different from their parents. Importantly, the dorsal sepal, petal colours, and shape of hybrid flowers varied significantly among individual hybrids, between hybrids and their progenitors. Some mutations in the lips and columns of the novel hybrid flowers were also visualized. Hence, the *D. anosmum* hybrid seedlings

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successfully induced flowers after a year of culture under optimal hormones and fertilizers conditions. The results can serve as a critical reference for the early flowering of the orchid seedlings.

1 Introduction

Dendrobium is the third-largest genus in the Orchidaceae with an upper range of 1400 species, including a large number of hybrid (Da Silva et al. 2015; Da Silva and Ng 2017), and is also the most important global floricultural, ornamental, and medicinal plants (Leitch et al. 2009; Takamiya et al. 2011; Da Silva and Ng 2017). This genus is mainly distributed across Southeast Asia, including the Philippines, Indonesia, Vietnam, Papua New Guinea, Sri Lanka, Malaysia, and Thailand (Teoh 2005; Xiaohua et al. 2009). *Dendrobium* species have fascinated people since early times because of their sheer beauty with the myriad of shapes and vibrant colours (Da Silva et al. 2014). In 1986, the first species of the *Dendrobium* genus, introduced into Hawaii from the Philippines, was known as *D. anosmum* and became a favorite flower among gardeners (Kamemoto et al. 1999). In Vietnam, there are more than 100 indigenous *Dendrobium* species (Tran 1998), but their natural populations have contracted due to their low rate of regeneration and seed germination, global climate change, human population increase, and over-exploitation for illegal trade (Maharjan et al. 2020). Therefore, the conservation of *Dendrobium* has recently emerged as a global environmental strategy (Kishor et al. 2006).

Hybridization is a method of recombining the genotypes of parents to create hybrid plants with novel characteristics, which is an exciting dimension in the floriculture industry (Kishor et al. 2006). In particular, cross-breeding is an important hybridization method to generate new hybrid orchids, which serve as the primary material for selecting desired flowers with different shapes, free-blooming habits, superior quality, and a blend of coloursss. In addition, many *Dendrobium* hybrids produce flowers several times a year, and their flowers are in high demand for the cut-flower and pot plant markets (Da Silva et al. 2015). Some research groups have attempted to cross-pollinate between different orchid varieties to generate novel hybrids. Zheng et al. (2009) reported that the success rate of *D. nobile* cross-pollination was 58.62%, and two kinds of hybrid plants (No.5029 and No. 5041) were developed for mass production. Further, Galdiano et al. (2012) successfully applied cryopreservation protocols for *D.* Swarts hybrid 'Dong Yai' seed germination and seedling development. Hartati and Cahyono (2014) successfully cross-pollinated a female (♀) *D.liniale* and male (♂) *D. bigibbum*, ♀ *D.bigibbum* and ♂ *D.liniale*, ♀ *D. mirbelianum* and ♂ *D.liniale*, ♀ *D. liniale* and ♂ *D. mirbelianum*, and achieved 100% seed germination. Recently, at the Laboratory of Cells, Institute of Biotechnology, Hue University, Vietnam, hybrid seedlings, resulting from a cross between ♀ *D.anosmum* 'Chau Nhu' and ♂ *D.anosmum* 'Di Linh', was successfully transplanted to the orchid net house (unpublished works).

However, hybrid orchid seedlings are often slow to flower in *ex vitro* conditions, usually taking more than one year after transplanting to the greenhouse (Kishor et al. 2006; Sim et al. 2007; Hartati et al. 2021). Kishor et al. (2006) highlighted those hybrid seedlings of *Vanda coerulea* ×*Ascocentrum auranticum* first flowered after two years under *ex vitro* conditions. Sim et al. (2007) showed that many commercial orchid hybrid species took from two to four years for their juvenile periods. Therefore, it takes a long time for researchers and gardeners to characterize novel hybrid species. To reduce juvenile periods, some research groups attempted to stimulate orchid flowering in the early plant growth regulators. Wang et al. (2009) showed the successful *in-vitro* flowering of *D.nobile* Lindl. seedlings on MS (Murashige and Skoog) medium supplemented with PBZ (paclobutrazol) and TDZ (thidiazuron) at different concentrations. The *in vitro* floral bud induction rate was 33.3% to 34.8% after 4 months of culture on ½ strength MS medium supplemented with 0.5 mgL^{-1}PBZ or on MS containing 0.1 mgL^{-1}TDZ. In addition, Guan and Shi (2009) demonstrated that floral buds of *D. denndanum* seedlings were induced at a rate of 10% by culturing on MS medium supplemented with 2.0 mgL^{-1} BA (6-benzylaminopurine). Cen et al. (2010) induced flowering in non-root plantlets of *D. officinate* by culturing on MS medium supplemented with 1.0 mgL^{-1} BA + 0.2 mgL^{-1}NAA (Naphthalene acetic acid), 3.0% sucrose and 0.1% activated carbon (AC). Zhao et al.(2012) reported 96% flower bud initiation in *D. strongylanthum* seedlings cultured on MS medium supplemented by 2 mgL^{-1}BA + 0.2 mgL^{-1}NAA and 3.0% sucrose after 70 days of culture. However, these studies focused on laboratory scale rather than a real application in orchid production. There is a huge market demand for the characterization of new orchid flowers (pot or cutting) in the *exvitro* condition instead of *in vitro* conditions. Unfortunately, studies of *ex vitro* growth and flowering are poorly reported. *D. tosaense* is the first *Dendrobium* orchid successfully acclimated and flowering from *in-vitro* seedlings after 18 months (Lu et al. 2014). Recently, *Anacamptis sancta*, a tuberous orchid was successful in symbiotic germination and flowering under *ex vitro* conditions after 30 months (Deniz et al. 2022). In Vietnam, this research field is currently under-investigatigation, especially in *D. anosmum* hybrid seedlings. Therefore, this study aimed to evaluate the effect of fertilizer and plant growth regulators on *D. anosmum* hybrid flowering induction and to characterize hybrid flower morphology.

Figure 1 *Dendrobium anosmum* seedlings a) 'Chau Nhu', b) 'Di Linh', and c) hybrid

2 Materials and methods

2.1 Plant materials and cultivation

Three cultivars of *D. anosmum* orchid seedlings were transplanted to *ex vitro* condition at approximately four months. Plants chosen for this study were *D. anosmum* 'Chau Nhu', *D. anosmum* 'Di Linh', and hybrid seedlings of ♀ *D. anosmum* 'Chau Nhu'× ♂*D. anosmum* 'Di Linh' and having 10 cm in height and 8 true leaves (Figure 1). These seedlings were provided by the Laboratory of Cells, Institute of Biotechnology, Hue University, Vietnam. Seedlings were grown under the orchid net house (Institute of Biotechnology, Hue University, Vietnam) without an air conditioning system. The net house was set up with an irrigation system to produce mist which reduces the temperature during hot weather, so the range of temperature in the house was usually 20^{o}C to 35^{o}C. Irrigation was applied to all cultivars following the instruction of the Vietnams National Standard of technical requirements for spray irrigation method (TCVN 9170-2012).

2.2 Methods

2.2.1 Application of fertilizer in optimizing hybrid seedling growth and development

The fertilizer experiments were based on Diem et al. (2021) study of *D. anosmum* 'Di Linh' seedlings cultivation. This experiment was designed to compare the growth indicators of hybrid seedlings with their parent. All the treatments were sprayed once a week with foliar fertilizer which was prepared by dissolving 1 gram of N (nitrogen): P (phosphorus): K (Potassium) 30:10:10 (Grow More; Gardena, CA, USA) and 2 grams of Vitamin B1 (Thailand T-REX) in 1 litter of water. A slow-release fertilizer Hyponex MagamP (NPKMg 6: 40: 6: 15) (Hyponex Japan, Co. Ltd, Osaka, Japan) was applied 5 grams on the surface of every pot, which contained the seedlings after acclimation a month (Diem et al. 2021). Experimental treatments were arranged randomly in a Randomized Complete Block Design (RCBD), each treatment had 3 replicates, and each replicate had 50 plants. Plant growth parameters, including the number of new leaves, shoot height, stem diameter, leaf length, and width were measured at intervals of 10 days for 120 days. Shoot height (in cm) was measured using a measuring tape from the base to the tip of the apical leaf. The leaf length and width (cm) were measured at the longest and widest points with a measuring tape, respectively. The number of new leaves was counted during plant growth, and the stem diameter was measured at the widest point (cm).

2.2.2 Effect of plant growth regulator application on *D. anosmum* hybrid seedling flower induction

This experiment was carried out to identify the effect of plant growth regulators on the diverse flowering of hybrid seedlings. After 4 months of growth in *ex vitro* conditions, the flower induction with PGR was carried out as outlined in Table 1.

Firstly, the apical leaf was slightly injured to exposure meristem and the designed dose of PGR (plant growth hormone) was spread on it as mentioned in table 1. Repeat treatments were carried out on the second and third days. Treated hybrid plants were moved to the shaded area without water for 24 hours to avoid removing the PGR. The treatment had three replicates, and five plants were used for each. After three weeks, data was collected and counting the rate of floral bud, shoot bulb, top shoot disease, inflorescence number, and flower number.

Table 1 The Plant Growth Regulator treatment on the precocious flower of *D. anosmum* hybrid seedlings

Plant Growth Regulator (PGR)	Concentrations	Volume using	Time repeat	References
Keiki pro orchid growth hormone	Proprietary	20 µl	1, 2, or 3 times*	Commercial product from Kelley's Korner Orchid Supplies
TDZ and PBZ	0.1 mgL^{-1} TDZ + 0.5 mgL^{-1} PBZ	20 µl	1, 2 or 3 times*	Wang et al. 2009
BA and NAA	2 mgL^{-1} BA + 0.2 mgL^{-1} NAA	20 µl	1, 2, or 3 times*	Zhao et al. 2012
Control	Without PGR			

* Note: Each time repeat is one day later

Table 2 Growth indicators of three D. anosmum seedlings

Times	Beginning					After four months				
Species	Stem height (cm)	Stem diameter (cm)	Leaf number	Leaf length (cm)	Leaf width (cm)	Stem height (cm)	zStem diameter (cm)	Leaf number	Leaf length (cm)	Leaf width (cm)
Chaunhu	10.11^{az}	0.40^{a}	8.00^{a}	4.40^{c}	1.51^{b}	18.57^{b}	0.57^{c}	11.85^{b}	5.99^{c}	2.00^{b}
Dilinh	10.15^{a}	0.40^{a}	8.00^{a}	5.44^{a}	1.52^{b}	18.59^{b}	0.50^{b}	12.05^{b}	7.47^{a}	2.55^{a}
Hybrid	10.03^{a}	0.40^{a}	8.00^{a}	5.02^{b}	1.57^{a}	19.56^{a}	0.60^{a}	12.45^{a}	6.54^{b}	2.53^{a}
LSD<0.05	ns	ns	ns	<0.05	<0.05	<0.05	<0.001	<0.05	<0.001	<0.05

[z]Means labeled with the same letter in a column were not significantly different at the P <0.05 (Tukey's HSD)

2.2.3 Estimation of morphological characters

The morphological characteristic of hybrid plant flowers was based on De (2020) and colours was assessed following the Royal Horticultural Society (RHS) colours chart. When the blooming of floral buds reached around 80%, the characteristics of flowers were assessed to identify flower variation of hybrid plants including a) flower width (cm) in front view (tip distance of two lateral petals), predominant flower colours, flower fragrance, flower longevity on plants (days); b) dorsal sepal outside and inside colours, outside and inside ornamentation, shape, apex, and curvature; c) lateral sepal outside and inside colours, outside and inside ornamentation, shape, apex, and curvature; d) lip outside and inside colours, outside and inside ornamentation, shape, apex, and curvature; e) column colours and shape (Vu et al., 2022).

2.3 Statistical analysis

Statistical analysis was performed by Microsoft Excel 2016, and by one-way analysis of variance (ANOVA) followed by Turkey's test, using the SPSS statistic 20.0 software (SPSS Inc., Chicago, IL, USA). The difference in the table represents significant differences as p< 0.05.

3 Results and Discussion

3.1 Effect of fertilizer on optimizing hybrid seedling growth and development

A combination of foliar fertilizer NPK 30:10:10 and vitamin B1 with a slow-dissolving fertilizer NPKMg (6:40:6:15) showed a positive effect on the growth characteristics of three *D. anosmum* seedlings after four months of experiment (Table 2).

The shoot height of each seedling cultivar dramatically increased from 10 cm to approximately 19 cm The hybrid height was estimated at 19.56 cm which was higher than that of 'Chau Nhu' (18.57 cm) and 'Di Linh' (18.59 cm). Further, the stem diameter in hybrid seedlings was 0.60 cm, which was higher than their parents. In addition, there were more leaves in hybrid seedlings (12.45 leaves) compared to their parent (11.85 leaves in the mother and 12.05 leaves in the father). Interestingly, the leaf length and leaf width of hybrid plants were intermediate, between those of their progenitors (Table 2).

Supplying adequate types and kinds of fertilizer was critical for the vegetative growth stage of all cultivars. The use of fertilizer in this study was consistent with the findings of previous studies (De 2020; Diem et al. 2021; Khuraijam et al. 2017). These authors highlighted that an ample supply of NPK 30:10:10 fertilizer during the vegetative stage of *Dendrobium* species was required not only to facilitate robust shoot and leaf production but also for subsequent orchid flowering. In addition, a slow-release fertilizer NPKMg (6:40:6:15) was also investigated and had been identified as the best for orchid root growth (Kawakami et al. 2008; Sendo et al. 2010). Vitamin B1 helps in increasing plant root system development, chlorophyll production, light photosynthesis, and extreme hot and cold water resistance (Fitzpatrick and Chapman 2020; Herastuti and Siwi Hardiastuti 2020). Both hybrid and parent seedlings of *D. anosmum* in the present study showed improved growth compared to the study on *D.* 'Sunya Sunshine' by Zhang et al. (2019). One year after transplanting, *D.*"Sunya Sunshine" was approximately just 16 cm tall with six leaves (Zhang et al. 2019), whereas, *D. anosmum* reached approximately 19 cm and had 12 leaves.

Therefore, the use of fertilizers in the recent study optimized the growth of hybrid seedlings. Moreover, the growth parameters of hybrid seedlings, including plant height, stem diameter, and leaf numbers, were significantly greater than those in their progenitor. These outcomes determined the success of the hybridization experiment

3.2 Effect of plant growth regulator on *D. anosmum* hybrid seedling flower induction

The application of PGR significantly influenced the floral bud initiation of the *D. anosmum* hybrid (♀ *D. anosmum* "Chau Nhu" × ♂*D. anosmum* "Di Linh") (Figure 2). Over 40% of hybrid plants were induced floral buds by Keiki pro with the highest induction rate obtained 66.67%, after two treatments. Moreover, the hybrid plants treated with the mixture of TDZ and PBZ showed 53.33% of induced plants had floral buds after three treatments, which was remarkably higher than a single treatment (33.33%) or two treatments (40.00%) (Figure 2). The combination of BA and NAA was less effective on hybrid plant flower induction than Keiki pro, or the mixture of TDZ and PBZ, with the rate of hybrid plant floral bud induction was 20.00% (treatment one time), 33.33% (treatment two times), and 13.33% (treatment three times) (Figure 2).

PGR played a key role in the specific flowering time, especially after the dormancy of hybrid plants. The results revealed a significant finding of this study that precocious flowering can be initiated in hybrid plants one year after transplanting, notwithstanding different effect levels among the treatments. Remarkably, the PGR commercial product was more effective than the two other PGR treatments. This may be due to the particular combination of phytohormones, which may have more PRG factors that activate dormant orchid node growth (Cen et al. 2010). Furthermore, the mixture of PBZ and TDZ induced floral buds of the hybrid plants better than that of BA and NAA, implying that PBZ and TDZ could enhance meristematic activity and influence inflorescence initiation (Huang et al. 2021). In addition, the percentage of hybrids treated with PBZ and TDZ combination achieved significantly higher floral bud initiation and it was higher than those in *in-vitro* culture. For instance, around 34% of *D. nobile* Lindl seedlings induced floral buds when seedlings were treated with 0.5mgL^{-1} PBZ or 0.1 mgL^{-1} TDZ; while it was only 29% in Friederick's *D.orchid* when treated seedlings with 0.05 mg L^{-1} PBZ under *in vitro* condition, respectively (Te-Chato et al. 2009; Wang et al. 2009). The explanation was that PGR had different effects on different kinds of *Dendrobium.*

Besides floral bud induction, PGR also affected the shoot bud stimulation. The rate of shoot bud induction in plants was approximately 20% in all experiments, except for those involving three treatments, which induced 13.33% of plants to form shoot buds (Figure 2). There was no shoot induction in the untreated hybrid plants. TDZ, BA, and IAA are the three main PGRs used in shoot stimulation for the orchid *in vitro* propagation (Naing et al. 2011; Sjahril et al. 2016; Regmi et al. 2017; Luan et al. 2019), so these could also initiate shoot bud formation in orchid plants under *ex vitro* conditions. Moreover, disease at the top of the

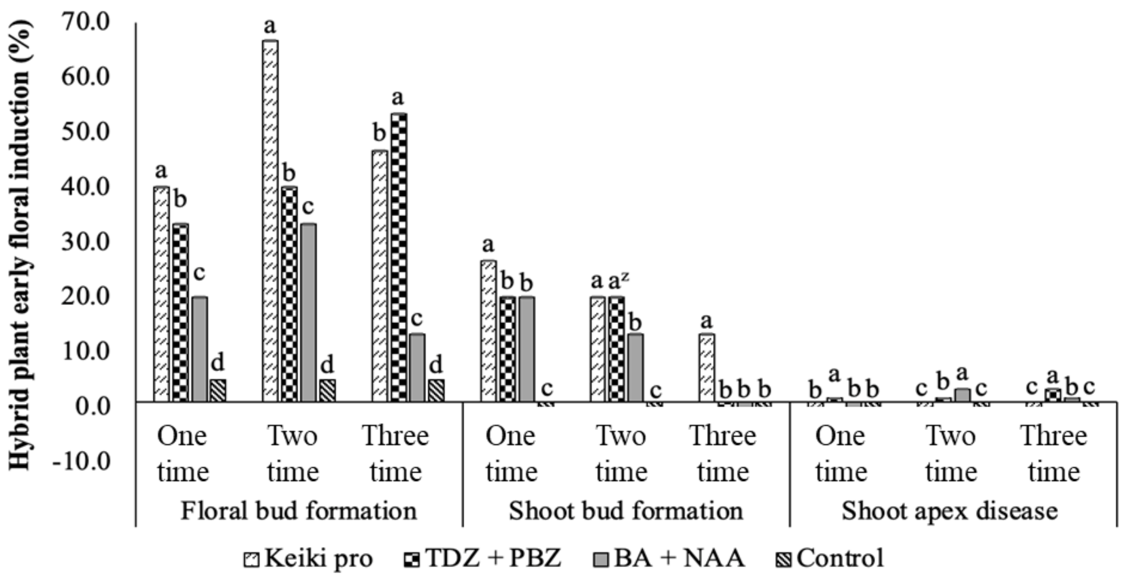

Figure 2 The percentage of hybrid plants induced to form floral buds and shoot buds and the percentage with a disease of the shoot apex when treated with three PGR preparations; [z]Means labeled with the same letter were not significantly different at the $P < 0.05$ level (Tukey's HSD)

Table 3 The Influence of PGR on hybrid plant inflorescence and flower number

Type	Inflorescence number			Flower number		
PGR	One time	Two time	Three times	One time	Two time	^{z}Three times
Keiki pro	1.0^{az}	1.6^{a}	1.0^{b}	2.0^{a}	3.6^{a}	1.6^{b}
TDZ + PBZ	1.0^{a}	1.2^{b}	1.4^{a}	1.6^{b}	2.0^{b}	2.6^{a}
BA + NAA	1.0^{a}	1.0^{b}	1.2^{b}	1.6^{b}	1.6^{c}	1.6^{b}
Control	1.0^{a}	1.0^{b}	1.0^{b}	1.5^{b}	1.5^{c}	1.5^{b}
LSD<0.05	Ns	<0.05	<0.05	<0.05	<0.05	<0.05

^{z}Means labeled with the same letter within a column were not significantly different at the $P < 0.05$ level (Tukey's HSD)

Figure 3 Flower characteristics of a) *D.anosmum*'Chau Nhu', b) *D.anosmum*'Di Linh',c) hybrid flowers, d) hybrid flower treated twice with Keiki pro, e) hybrid flower of medium size and f) large hybrid flower

shoot occurred when hybrid plants were treated with PGR. However, this phenomenon did not cause concern as it was less than 4% (Figure 2).

PGR had a critical influence on the inflorescence and flower numbers of hybrid plants (Table 3). In general, hybrid plants had approximately one inflorescence each when treated once with PGR. However, the inflorescence number significantly increased in the plants treated twice with Keiki pro 100000 ppm (1.6 inflorescences/plant) or three times with the mixture of TDZ and PBZ (1.4 inflorescences/plant). Consequently, the highest flower number was 3.6 flowers/plant in plants treated twice with Keiki pro (Table 3).

The number was approximately 2.6 flowers/plant in hybrid plants treated three times with TDZ and PBZ and around 1.6 flowers/plants in all other treatments. These results were in contrast to Mishra et al. (2018), who studied the effect of PGR (GA3, BA, Kinetin, and IAA) on early flower induction in *Phalaenopsis* hybrids and reported that the application of different PGR did not affect the number of spikes/inflorescences per plant. Conversely, Zhang et al. (2019) determined that applying TDZ 30mgL^{-1} as a spray to *D*. "Sunya Sunshine" for early flower stimulation increased the number of inflorescences per plant. These divergent findings could be a result of using different *Dendrobium* in each study.

3.3 Hybrid flower characteristics

Four weeks after floral bud induction, most of the buds were flowering. Hybrid flower characteristics showed dramatic variation compared to those in their parents (Table 4).

Table 4 Comparative analysis of parental and hybrid flower characteristics

Type	Characteristic	Cultivars: *D. anosmum* 'Chau Nhu'	Cultivars: *D. anosmum* 'Di Linh'	Cultivars: Hybrid	ᶻPercentage (%)	Type of assessment
Flower	Flower width (cm)	Large (> 7.0 cm)	Large (>7.0 cm)	Medium (3.0-7.0 cm)	36.36	MS
		-	-	Large (>7.0 cm)	63.64	
	Flower predominant colours	Light violet (69C), purple/pink (68A)	Early white (N155B), purple/pink (68A)	Pink violet (75C), Purple pink (68A)	100.00	
	Flower fragrance	Heady	Sweet	Slightly + sweet	58.33	
				Heady	41.67	
	Flower longevity on plants (days)	20	20	20	100.00	
Dorsal sepal	Outside dorsal sepal colours	Light violet (69C),	Early white (N155B), purple/pink (73A)	Early white (N155B), Pink violet (75C)	100.00	VS
	Inside dorsal sepal colours	Light violet (69C),	Early white (N155B), purple/pink (73A)	Early white (N155B), Pink violet (75C)	91.67	
		-	-	Yellow-green (149D), Pink/violet (75C)	8.33	
	Outside dorsal sepal ornamentation	Absent	Straight stripe	Straight stripe	100.00	
	Inside dorsal sepal ornamentation	Absent	Straight stripe	Straight stripe	100.00	
	Dorsal sepal shape	Lanceolate	Lanceolate	Lanceolate	83.33	
		-	-	Oblong	16.67	
	Dorsal sepal apex	Acute	Obtuse	Acute	100.00	
	Dorsal sepal curvature	Deflexed	Deflexed	Deflexed	100.00	
Lateral sepal	Outside lateral sepal colours	Light violet (69C),	Early white (N155B), purple/pink (73A)	Early white (N155B), Pink violet (75C)	100.00	VS
	Inside lateral sepal colours	Light violet (69C),	Early white (N155B), purple/pink (73A)	Early white (N155B), Pink violet (75C)	100.00	
	Outside lateral sepal ornamentation	Absent	Absent	Straight stripe	100	
	Inside lateral sepal ornamentation	Absent	Straight stripe	Absent	83.33	
				Stripe/ Netted	16.67	
	Lateral sepal shape	Lanceolate	Lanceolate	Lanceolate	100.00	
	Lateral sepal apex	Acute	Acute	Acute	100.00	
	Lateral sepal curvature	Deflexed	Deflexed	Deflexed	100.00	

Type	Characteristic	Cultivars *D. anosmum* 'Chau Nhu'	*D. anosmum* 'Di Linh'	Hybrid	zPercentage (%)	Type of assessment
Petal	Outside petal colours	Light violet (69C),	Early white (N155B), purple/pink (73A)	White (N999D)	16.67	VS
		-	-	Early white (N155B), Pink/violet (75C)	83.33	
	Inside petal colours	Light violet (69C)	Early white (N155B), purple/pink (73A)	White (N999D)	16.67	
		-	-	Early white (N155B), pink/violet (75C)	16.67	
		-	-	Early white (N155B), pink/violet (75D)	66.66	
	Outside petal ornamentation	Absent	Absent	Absent	100.00	
	Inside petalornamentation	Absent	Straight stripe	Absent	83.33	
		-	-	Stripe/ Netted	16.67	
	Petal shape	Oblong	Oblong	Oblong	16.67	
		-	-	Ovate	66.66	
		-	-	Elliptic	16.67	
	Petal apex	Obtuse	Acute	Acute	40.00	
				Obtuse	60.00	
	Petal curvature	Deflexed	Deflexed	Deflexed	100.00	
Lip	Lip outside colours	Base: purple/pink (68A); Apex: white (155A)	Base: purple/pink (68A); Apex: pink violet (75C)	Base: Purple/pink (68A); Apex: white (155A)	100.00	VS
	Lip inside colours	Base: purple/pink (68A); Apex: white (155A)	Base: purple/pink (68A); Apex: pink/violet (75C)	Base: Purple/pink (68A); Apex: white (155A)	100.00	
	Lip shape	Ovate	Heart	Heart	28.94	
		-	-	Obovate	66.66	
		-	-	Mutation	4.40	
	Lip apex	Acute	Acute	Obtuse	50.00	
		-	-	Acute	33.33	
		-	-	Retuse	16.67	
	Lip curvature	Straight	Straight	Straight	100.00	
	Lip margin	Fimbriate	Fimbriate	Fimbriate	100.00	
Column	Column colours	Purple/pink (68A)	Purple/pink (68A)	Purple/pink (68A)	100.00	VS
	Column shape	Round	Round	Round	95.60	
		-	-	Mutation	4.40	

zPercentage of hybrid flower varieties; MS: measurement of individual plant number or parts of plants; VS: visual assessment by observations of individual plants or parts of plants

In general, hybrid flowers have one dorsal sepal, two lateral sepals, two petals, one lip, and a column, similar to their ancestors, however, some other traits were significantly varied (Figure 3a, 3b, 3c, and 3d). Taking large (> 6 cm) parental flower width as 100%, hybrid flower width was 36.36% in a medium-sized (3.0-6.0 cm) and 63.64% in large-sized flowers (Table 4, Figure 3e, 3f). The segregation of this trait may be due to early flower induction, and the vegetative growth of some hybrid plants was not as pronounced as that of others. The cross-breeding of the mother flower, which was light violet (69C) and purple/pink (68A), and the father flower, which was early white (N155B) and purple/pink (68A) generated hybrids with the dominant colours was a combination of pink/violet (75C) and purple/pink (68A) (Figure 3a, 3b, and 3c). The fragrance of novel flowers was a combination of their originator fragrance with slightly sweet and heady aromas (Table 4), whereas flower longevity was the same as their parent's (20 days).

The dorsal sepal colours of hybrid flowers was significantly different from those of their progenitors. The dorsal sepal coloursss of 91.67% of plants were early white (N155B) and pink/violet (75C) while those of 8.33 % were yellow-green (149D) and pink/violet (75C) coloursss compared to those of their mother (light violet -69C) and father (early white - N155B) and purple/pink (73A) (Figure 3a, 3b, and 4a). Outside and inside dorsal sepal ornamentation of the hybrid flowers involved a straight stripe that was similar to their father (Table 4). The dorsal sepal apex of the hybrids flowers was 83.33% similar to their parents, which showed a lanceolate shape, and 6.67% was oblong shaped, which differed from their ancestors (Figure 3a, 3b, 3e and 3f).

The lateral sepal colours of hybrid flowers was the same as their dorsal sepal colours and differed only slightly from those of their ancestors. Excitingly, the most variable change was in the petal shape of hybrid flowers. The petal shape was not only oblong (16.67%) as seen in their parents but also ovate (66.66%) and ellipse (16.67%) (Figure 4b). Outside and inside lip colour were inherited from their mother plants, but lip shape and apex traits were more varied than in the parents (Table 4). Hybrid lips were heart-shaped (28.94%), obovate-shaped (66.66%) or mutated (4.40%) (Figure 4c, 4d, and 4e), however, the mother and father flower lip shapes were ovate and heart-shaped, respectively (Figure 3a and 3b). Hybrid flower lip apex was obtuse (50%), acute (33.33%), or retuse (16.67%) as compared to only acute in their ancestors (Table 4). Mutation of hybrid flowers also occurred in the column shape of 4.40% of plants (Figures 4f and 4g).

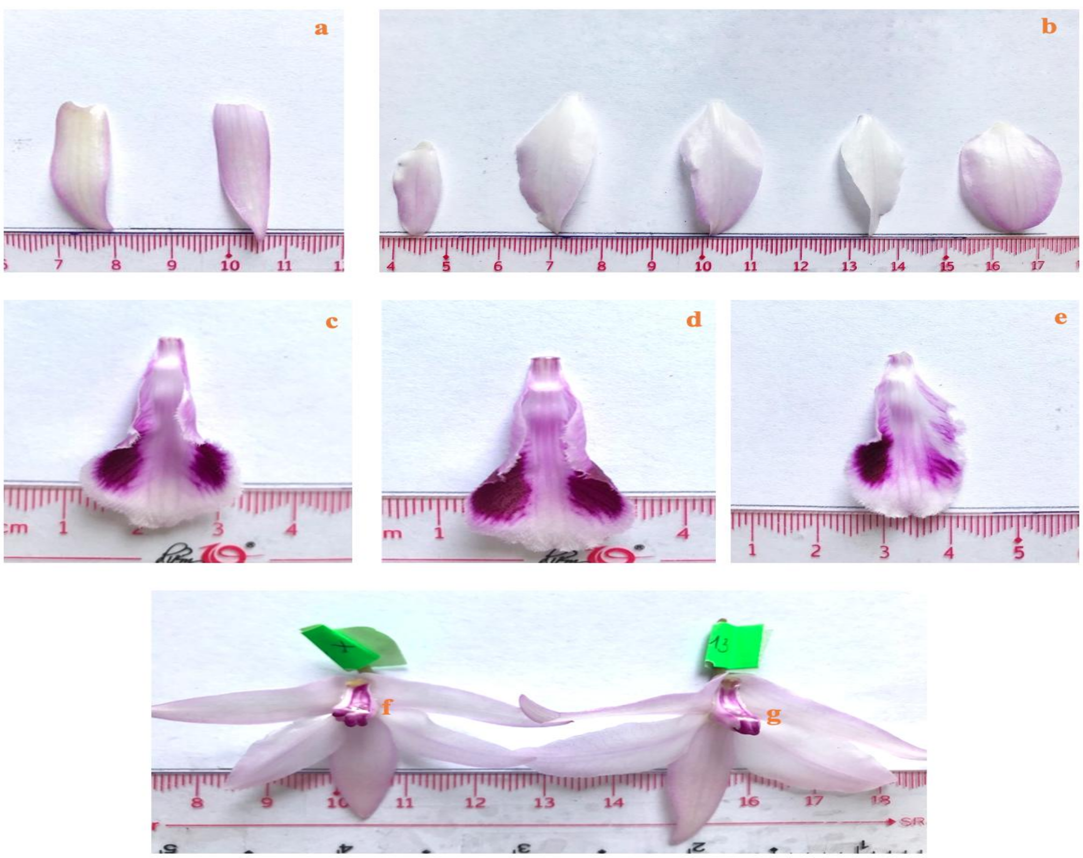

Figure 4 Hybrid flower characteristic. a) dorsal sepal colours and shape assessment, b) petal colours and shape assessment, c) lip heart shape, d) lip obovate shape, e) lip mutation, f) column mutation and g) normal column

The current study reports for the first time that the characteristics of hybrid varieties of ♀ *D. anosmum* 'Chau Nhu' X ♂*D. anosmum* 'Di Linh', despite previous attempts to investigate the commercially important, flowering hybrid orchid family (Mishra et al. 2018; Zhang et al. 2019). The results of this study revealed variations in the traits of *D. anosmum* hybrid flowers, especially the segregation of dorsal sepal and petal traits. In addition, mutations occurred in lip and column traits of hybrid flowers. These followed Mendel's theory of inheritance that cross-pollination uncovers variations inherited by hybrid plants from their ancestor (Bateson and Mendel 2013; Ellis et al. 2011). Therefore, the outcomes of this study would be a valuable resource for further study on the genetic variation of *D. anosmum* hybrid flowers.

Conclusions

The Fertilizer was optimized to maximize the vegetative growth of *D. ansomum* hybrid plants. The application of PGR at a specific time point induces the highest level of precocious flowering in hybrid plants around one year after transplanting to *ex-vitro* conditions. The study identifies various traits of hybrid flowers, adding to *D. anosmum* orchid historical research. Further research is needed to study the genetic variation of these hybrid flowers and the genetic events behind the phenotypic changes.

Conflicts of interest and financial disclosures

The authors declare no competing interests.

Author contribution

T.O.N, T.D.N. and T.K.C.N. designed the experiment. T.D.N., T.O.N., T.T.H and H.T.N carried out the experiments and data analyses. T.D.N. and T.O.N. prepared all of the figures, and all authors contributed to data interpretation. T.D.N. wrote the first draft of the manuscript, T.T.H and T.K.C.N edited the draft. All authors reviewed the manuscript.

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