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Effect of ethyl methanesulfonate on growth and morphology of *Doritis pulcherrima* Lindl.

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Abstract Results showed that 0.5 mg/l BA and 1 mg/l NAA promoted the greatest seedling growth, with increased leaf number, leaf length, plant height, and root development. In the third experiment, protocorm-like bodies were treated with EMS at 0, 0.5, and 1.0% for 60 or 90 minutes. Survival declined with increasing concentration and time, with LD_{50} values of 0.82% at 60 minutes and 0.54% at 90 minutes. Chlorophyll a, Chlorophyll b, and carotenoids were not significantly affected, but stomatal size changed. Stomatal width and length increased under 1.0% EMS, while density decreased from 220.17 to 149.47 stomata/mm². EMS treatment for 90 minutes showed an increased density relative to 60 min. RAPD analysis using 10 primers revealed high polymorphism indicating EMS induced genetic variation. Cluster analysis divided 22 samples into 9 groups at a similarity coefficient of 0.89, confirming genetic diversity despite no visible morphological changes.

Keywords: Plant tissue culture, Orchid, EMS, Mutation and RAPD marker

Introduction

In the Orchidaceae, *Doritis pulcherrima* Lindl. is a semi-epiphytic plant with short stems, thick leathery green leaves with a purple underside, and an upright inflorescence emerging from the base of the leaves. There are multiple color forms with varying shades, most commonly pink, white, and purple petals. (Arditti, 2008; Averyanov, 2009). The species is distributed in Assam, the Chinese Himalayas, and Myanmar, through Thailand, Malaysia, Laos, Cambodia, and Vietnam to Sumatra (Averyanov, 2009; Cristension, 2001). They are found in sandy loam-rich soil and on rock surfaces (Thipwong *et al*., 2022). A significant number of Thai wild orchid species are declining, with some species now classified as endangered (Kosir *et al*., 2004) as a result of their naturally slow growth, destruction of ecosystems, forest fires, and climate change. Moreover, orchid are threatened by illegally collection and transportation forest trees for the

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purpose of selling them (Utami and Hariyant, 2019). Plant tissue culture is a widely used method to achieve rapid mass propagation and the generation of disease free new plants (Park *et al*., 2003). This technique involves cultivating cells, tissue, organs, and other plant components through aseptic *in vitro* growth in a controlled environment. The following are important components of plant tissue culture: Firstly, the culture medium consists of both macro- and micronutrients. There are many medium, such as Murashige and Skoog medium (MS) (Murashige and Skoog, 1962), a widely used essential medium, and Vacin and Went medium (VW) (Vacin and Went, 1949), which is a suitable medium for orchid growing. Activated charcoal (AC) and coconut water (CW) were added into the medium to enhance seed germination. (Zeng *et al*., 2011) Secondly, The explants used in this cultivation method are important. The cultivation process has a particular impact on each plant's explant. Thirdly, cultivation conditions: a temperature of 25±1°C. Medium composition and enviromental conditions required for the optimal growth of orchids vary in different plant varieties. It is important to understand the requirements of the plant before starting the culturing process (Twaij *et al*., 2020). Paul *et al*. (2012) reported that MS medium gave a higher percentage of seed germination of *Dendrobium hookerianum* than B5 medium. MS medium is enriched with macronutrients and micronutrients, vitamins, and sugars (Murashige and Skoog, 1962). Utami and Hariyant (2019) also showed that VW medium was suitable for seedling formation and development from protocorm.

Furthermore, plant growth regulators (PGRs) control and modify morphological processes such as seed germination, leaf growth and development, shoot and root formation, and stem and root elongation (Trigiano and Gray, 2004). The most important group of plant growth regulators used in tissue culture are auxins and cytokinins. Auxins are transported from stems and roots to other parts of the plant, influencing growth and development, and adventitious root formation. Cytokinins produced in shoots assist in leaf production, lateral shoot growth, and overcoming apical dominance and delaying leaf aging (Rademacher, 2015). The response to auxin and cytokinin types and concentrations varies depending on the explant and orchid species. Tao *et al*. (2011) also reported that Half-Murashige and Skoog medium (½MS) supplemented with NAA 2.0 and 1.0 mg/l showed higher growth and multiplication of protocorm like-bodies (PLBs). The research of Thipwong *et al.* (2022) showed the highest leaf length and root number on VW medium supplemented with BA and NAA at 0.5 mg/l. Therefore, this study examined the influence of basal medium, types of breeding systems and plant growth regulators on the growth and development of Doritis. In addition to tissue culture and the use of plant growth regulators, chemical mutagens such as ethyl methanesulfonate (EMS) are also applied for orchid improvement. EMS

is recognized as an efficient mutagen that induces point mutations in DNA, resulting in genetic diversity. This variability can be utilized to create new cultivars with selected qualities or to produce unique physical and physiological properties. This effect can be seen in *in vitro* mutagenesis and characterization of mutants through morphological and genetic analysis in the orchid *Aeridas crispa* Lindl., where varying EMS concentrations and exposure times influenced reduced survival at higher concentrations while generating diverse morphological and physiological variations, including leaf variegation, pigment alterations, and growth abnormalities, making it a valuable tool to enhance genetic variability for breeding and conservation (Srivastava *et al.,* 2018). Therefore, the study aimed to improve *in vitro* propagation of *Doritis*. by evaluating the impacts of basal medium, plant growth regulators, and culture conditions on seed germination, protocorm development, plantlet growth and investigate the efficacy of Ethyl Methanesulfonate (EMS) as a chemical mutagen to induce genetic variability.

Materials and methods

Plant materials

Pods of Doritis were obtained from Chumphon province of Thailand. Pods were collected 100 days after pollination (DAP) (Figure 1).

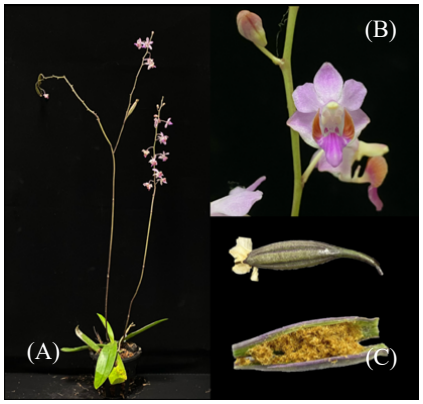

Figure 1. Doritis inflorescence in greenhouse: (A) Doritis plant, (B) flowering, (C) Pod

Surface sterilization

Pods were surface sterilized by immersion in 95% ethanol for 3-5 s and then flaming 3 times. The pod was slit longitudinally with a sterile surgical blade

to isolate the seeds. Seeds were suspended in 10 ml sterilized distilled water. This seed suspension contained 0.5 ml per culture flask. Seed suspension was dispersed uniformly on the medium in each culture flask.

Influence of medium and types of breeding on seed germination and development

A 3x2 factorial in a completely randomized design (factorial in CRD) was used for this experiment; 5 replicates were used for each treatment. Factor 1: a basal medium for 3 treatments, such as Murashige and Skooog medium (MS), half-strength MS medium (½MS), and Vacin and Went medium (VW) supplemented with 150 ml coconut water (CW), 20 g/l sucrose, and 2 g/l activated charcoal (AC). Factor 2: Types of breeding of Doritis, which includes 2 treatments such as self-pollination and cross-pollination. Seeds were counted using a hemocytometer and analyze cell viability under an Olympuscx31 microscope. Each treatment had 280 seeds; all cultures were maintained at 25±1°C under a 16-h photoperiod. Seed germination and development were estimated for each treatment by 7 parameters: percentage of seed germination, growth index, the number, length and width of leaves, number of roots, and plant height.

Seed morphology and data collection

The percentage of seed germination was calculated by dividing the amount of seeds in each development stage by the total amount of seeds x 100:

$$= \frac{\text{the amount of seeds in stage1 + stage2 +…+stage 4}}{\text{total amount of seeds}} \times 100$$

The seed germination and development process of Doritis was categorized into five groups (Table 1), modified from Stewart and Settler (2002).

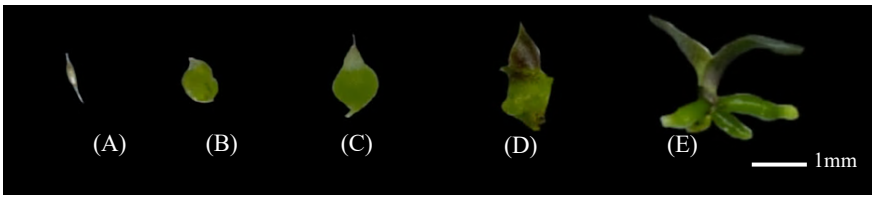

Figure 2. Seed germination and development stage of Doritis: (A) 0 = no germination, (B) stage 1 germination = swollen embryo, (C) stage 2 protocorm-like bodies (PLBs), (D) stage 3 = seedling with one leaf, (E) stage 4 seedling = seedling with two leaves and at least one root. (scale bar = 1 mm)

Effect of plant growth regulators and ethyl methanesulfonate on growth and morphology

Explant materials were obtained from 10 week old seedlings of Doritis (Stage 3 PLBs; Figure 2D). The PLBs were divided into two experiments, which were used to examine the effects of plant growth regulators (PGRs) and ethyl methanesulfonate (EMS), respectively. For the first experiment, the effect of plant growth regulators (PGRs) on seedling development was examined. Seedlings were cultured on VW medium supplemented with 150 ml CW, 20 g/l sucrose, and 2 g/l AC, containing different concentrations of BA and NAA (0, 0.5, 1, and 2 mg/l). Cultures were maintained at 25 ± 1°C under a 16-h photoperiod. A completely randomized design (CRD) with 16 treatments and 5 replicates per treatment was applied. Seedling development was evaluated based on five parameters: number, length, and width of leaves, number of roots, and plant height. For the second experiment, the effect of ethyl methanesulfonate (EMS) on growth and morphology was investigated. Stage 3 PLBs were treated with EMS at 0, 0.5, and 1.0% (v/v) for 60 and 90 min, rinsed 3 times with sterile distilled water, and cultured on VW medium. A factorial completely randomized design (Factorial in CRD) with 3 replicates per treatment and 15 PLBs per replicate. Data collection was to determine the lethal dose (LD_{50}). Survival of PLBs was recorded weekly for 12 weeks. Additional analyses included mutation detection using Random Amplified Polymorphic DNA (RAPD) markers, evaluation of genetic variation based on morphological characteristics, stomatal size and density under light microscopy, and quantification of chlorophyll a, chlorophyll b, and carotenoid contents spectrophotometrically.

Determinations of chlorophyll a, chlorophyll b and carotenoids

The chlorophyll content (mg/g FW) of Doritis seedlings treated with EMS was determined following a modified method of Lichtenthaler (1987). Fresh leaf samples (0.2 g) were extracted with 5 mL of 80% acetone. The leaves were homogenized with a mortar and pestle, filtered through filter paper, and centrifuged at 10,000 rpm for 10 minutes. The final volume was adjusted to 5–7 mL with 80% acetone, and the clear supernatant was used to measure absorbance at 470 nm (carotenoids), 663 nm (chlorophyll a) and 647 nm (chlorophyll b) using a spectrophotometer.

The chlorophyll content was calculated as follows:

chlorophyll a (µg/ml) = 12.25(A663) - 2.79(A647),

chlorophyll b (µg/ml) = 21.50(A647) - 5.10(A663), and

carotenoids (µg/ml) = [1000(A470) - 1.82(chl a) – 85.02(chl b)] /198

Measurement of stomatal size and stomata density

The stomatal size and density of Doritis. seedlings were examined using fully expanded leaves. The upper epidermis was carefully peeled off with a razor blade to obtain the lower epidermal layer. Sections of the epidermal tissue were placed on glass slides, a drop of distilled water was added, and the samples were covered with a coverslip. Stomatal morphology and the number of chloroplasts were then observed under a light microscope (Olympus CX23, Olympus Corporation, Tokyo, Japan).

Genetic variation of Doritis

Genomic DNA was extracted from fresh leaves using the CTAB. RAPD analysis was performed with 14 primers (Table 1) in 25 µl PCR reactions containing genomic DNA, primer, and Taq Mix Red. Amplification consisted of an initial denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 1 minute, primer-specific annealing at 30-34°C for 1 minute, extension at 72°C for 2 minutes and a final extension at 72°C for 10 minutes. DNA quality and quantity were assessed on 2% agarose gels. A total of 22 samples were analyzed, and only clear, unambiguous bands were scored (‘1’ for presence, ‘0’ for absence). Genetic distances were estimated using Jaccard’s coefficient, and a dendrogram was generated with the TREE procedure in NTSYS 2.10p using the UPGMA method.

Table 1. Types and nucleotide sequences of the 14 primers used to evaluate the genetic variation in EMS treated Doritis

Primer	Primer sequence
OPA01	5'-CAG GCC CTT C-3'
OPA02	5'-TGC CGA GCT G-3'
OPA03	5'-AGT CAG CCA C-3'
OPA05	5'-AGG GGT CTT G-3'
OPA08	5'-GTG ACG TAG G-3'
OPA09	5'-GTG ATC GCA G-3'
OPA10	5'-CAA TCG CCG T-3'
OPA11	5'-CAG CAC CCA C-3'
OPA12	5'-GGG TAA CGC C-3'
OPA18	5'-GGG TAA CGC C-3'
OPA19	5'-CAA ACG TCG G-3'
OPA20	5'-GTT GCG ATC C-3'
OPC19	5'-GTT GCC AGC C-3'
OPD07	5'-AGT CAG CCA C-3'

Data analysis

Data were subjected to analysis of variance (ANOVA) using the SAS version 9.0 program (SAS Institute, Inc., Cary, NC). The differences between the means were tested according to Duncan's multiple test (DMRT) with a significance level of $p \leq 0.05$.

Results

Influence of medium and types of breeding on seed germination and development

Seeds of Doritis started germination in week 2 and proceeded to protocorm-like bodies (PLBs) by week 6, eventually generating leaves and roots by week 12.The development and germination of each breeding type showed significant. At 4-8 weeks, ½MS medium revealed higher germination rates compared to other medium (Figure 3A-B). At 10–12 weeks, VW medium had the most effective PLBS growth and development (Figure 3C). The percentage of seed germination cross-pollination pods was superior to that of self-pollination pods, measuring 65.73% and 70.94%, respectively. Seed were cultured on VW medium showed the highest germination rate at 84.24% and also supported better PLB and seedling development than ½MS and MS, followed by ½MS medium at 63.21% and MS medium at 57.56%, respectively (Figure 3).

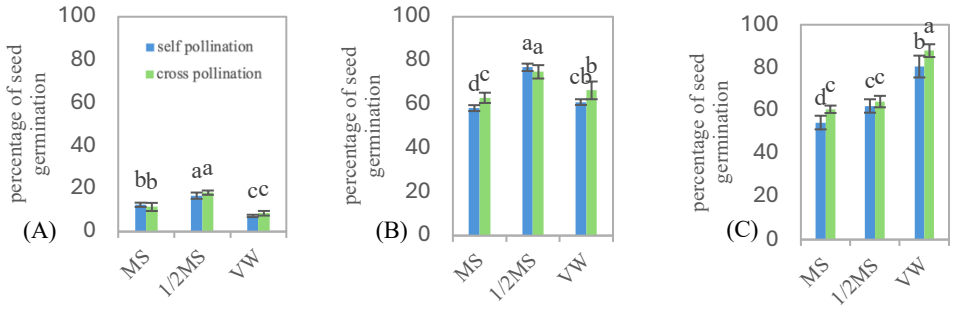

Figure 3. Percentage of seed germination of Doritis for 12 weeks: (A) week4, (B) week8, (C) week12. Values are mean ± S.D. Different letters indicate significant differences ($P < 0.05$, Duncan's test)

VW medium promoted the highest number of leaves after 12 weeks. According to morphology, cross-pollination pods cultivated on VW medium had a higher number of leaves, along with width, length, and root size, when

compared to those grown on MS medium and ½MS medium, whereas cross-pollination pods showed better growth relative to self-pollination pods. Therefore, the VW medium was the most effective for overall seedling development (Table 2).

Table 2. Influence of medium and types of breeding on seed germination and development of *Doritis* for 12 weeks

Culture media (mg/l)	Type of breeding	No. of leaves (no./explant) (Mean±SE)	Size of Leaves (cm)		Plant height (cm) (Mean±SE)	No. of roots (no./explant) (Mean±SE)
			length (Mean±SE)	width (Mean±SE)		
MS	Self	2.89±0.10^{c}	0.56±0.03^{b}	0.33±0.02^{c}	0.39±0.05^{c}	2.61±0.19^{b}
	Cross	2.94±0.42^{c}	0.80±0.06^{a}	0.41±0.06^{bc}	0.34±0.04^{c}	3.06±0.77^{b}
½ MS	Self	3.22±0.10^{c}	0.57±0.03^{b}	0.34±0.01^{c}	0.37±0.05^{c}	2.89±0.10^{b}
	Cross	3.22±0.10^{c}	0.78±0.04^{a}	0.43±0.04^{bc}	0.32±0.02^{c}	3.00±0.29^{b}
VW	Self	3.67±0.33^{b}	0.73±0.09^{a}	0.44±0.04^{b}	0.52±0.03^{b}	3.06±0.10^{b}
	Cross	4.56±0.10^{a}	0.82±0.12^{a}	0.69±0.08^{a}	0.72±0.06^{a}	5.00±0.44^{a}
F-test A		**	**	**	**	**
F-test B		**	**	**	ns	**
F-test A*B		**	**	**	**	**
C.V.(%)		6.79	9.92	10.90	9.83	11.99

** Significant different at P≤0.01/ ns = non significant difference; Means within column followed by the same later are not significant different at the 99% (P≤0.01) as determined by Duncan's multiple range test.

Effect of plant growth regulators on seedling development

After 8 weeks of culture, seedlings grown on VW medium with 0.5 mg/l NAA showed the greatest height. VW medium supplemented with 0.5 mg/l BA and 1 mg/l NAA promoted the highest number and length of leaves, plant height, and number of roots. Although VW with 0.5 mg/l BA produced the most shoots, the difference was not significant (Figure 4B). However, BA concentration increased to 1 and 2 mg/l the rate of number of root induction was reduced. Particularly in VW medium without NAA supplementation (Table 3)

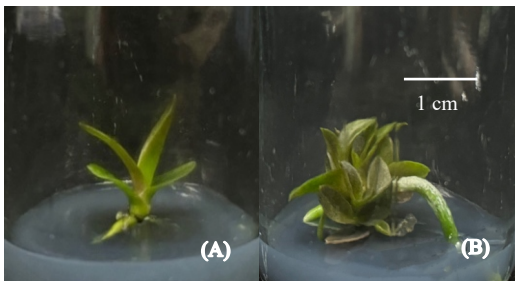

Figure 4. Seedling of Doritis were cultured on VW medium after 16 weeks (A) control (B) with 0.5 mg/l BA and NAA (Treatment 6)

Table 3. Effect of BA and NAA on growth and development on seedling of Doritis after 8 weeks

Plant Growth Regulators		No. of leaves (no./explant) (Mean±SE)	Leaves length (cm) (Mean±SE)	Plant height (cm) (Mean±SE)	No. of roots (no./explant) (Mean±SE)	No. of shoots (no./explant) (Mean±SE)
BA (mg/l)	NAA (mg/l)					
0	0	3.83±0.17 ^{bcd}	1.28±0.06 ^{bcd}	0.61±0.06 ^{a}	5.83±0.44 ^{abc}	1.00±0.00
0	0.5	5.33±0.67 ^{a}	1.13±0.07 ^{cd}	0.63±0.04 ^{a}	6.50±1.04 ^{ab}	1.33±0.17
0	1	4.67±0.33 ^{abc}	1.20±0.06 ^{bcd}	0.51±0.02 ^{abc}	5.66±0.33 ^{abcd}	1.83±0.44
0	2	4.66±0.17 ^{abc}	1.36±0.09 ^{abc}	0.56±0.02 ^{abc}	5.33±0.60 ^{abcd}	1.33±0.33
0.5	0	4.50±0.50 ^{abcd}	1.20±0.13 ^{bcd}	0.58±0.04 ^{ab}	5.66±1.42 ^{abcd}	2.33±0.60
0.5	0.5	4.67±0.44 ^{abc}	1.25±0.03 ^{bcd}	0.63±0.11 ^{a}	4.33±0.17 ^{bcd}	2.50±0.76
0.5	1	4.16±0.17 ^{abcd}	1.56±0.17 ^{a}	0.58±0.04 ^{ab}	7.16±0.93 ^{a}	1.67±0.33
0.5	2	4.17±0.17 ^{abcd}	1.42±0.02 ^{ab}	0.51±0.02 ^{ab}	4.50±0.29 ^{bcd}	1.16±0.17
1	0	3.33±0.17 ^{d}	1.26±0.07 ^{bcd}	0.41±0.02 ^{c}	3.33±0.88 ^{d}	1.16±0.17
1	0.5	5.00±0.29 ^{ab}	1.23±0.10 ^{bcd}	0.53±0.03 ^{abc}	4.16±0.17 ^{bcd}	1.50±0.29
1	1	4.83±0.33 ^{abc}	1.25±0.03 ^{bcd}	0.56±0.02 ^{abc}	4.33±0.33 ^{bcd}	1.00±0.00
1	2	4.83±0.44 ^{abc}	1.41±0.10 ^{ab}	0.56±0.07 ^{abc}	5.83±0.60 ^{abc}	1.50±0.29
2	0	4.83±0.17 ^{abc}	1.01±0.03 ^{d}	0.45±0.03 ^{bc}	3.33±0.44 ^{d}	1.33±0.17
2	0.5	4.00±0.29 ^{bcd}	1.30±0.09 ^{abc}	0.56±0.07 ^{abc}	4.66±1.01 ^{bcd}	1.50±0.50
2	1	3.67±0.17 ^{cd}	1.31±0.04 ^{abc}	0.50±0.03 ^{abc}	4.16±0.33 ^{bcd}	1.00±0.00
2	2	4.16±0.67 ^{abcd}	1.18±0.07 ^{bcd}	0.43±0.03^{bc}	3.83±0.60 ^{cd}	1.00±0.00
F-test		*	*	*	*	ns
C.V. (%)		14.24	11.20	15.04	24.47	41.10

* Significant different at P≤ 0.05/ ns = non significant difference; Means within column followed by the same later are not significant different at the 95% (P<0.05) as determined by Duncan's multiple range test

Effect of ethyl methanesulfonate on growth and morphology

In this experiment, PLBs of Doritis were treated with EMS at 0, 0.5, and 1.0% for 60 and 90 min. PLB survival declined with increasing EMS concentration and exposure time, with LD_{50} values 0.82% at 60 minutes and 0.54% at 90 minutes (Figure 5), indicating that extended exposure enhanced EMS toxicity. Chlorophyll a, chlorophyll b, and carotenoid contents in 12 month old leaves that had been transferred ex vitro for 2 weeks for two weeks showed no significant differences among concentrations or times. This suggesting that pigment biosynthesis was relatively resistant to EMS stress. In contrast, stomatal size was markedly affected. At 1.0% EMS (Figure 6 C, F), stomata width and length increased compared with the control (Figure 6 A, D).

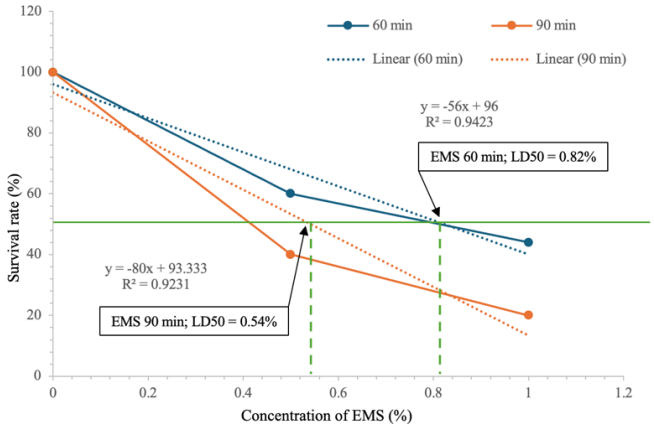

Figure 5. Survival of PLBs of Doritis after treating with various concentration of EMS and culture 12 weeks, LD_{50} values were determined using linear regression by substituting y = 50 into the regression equations

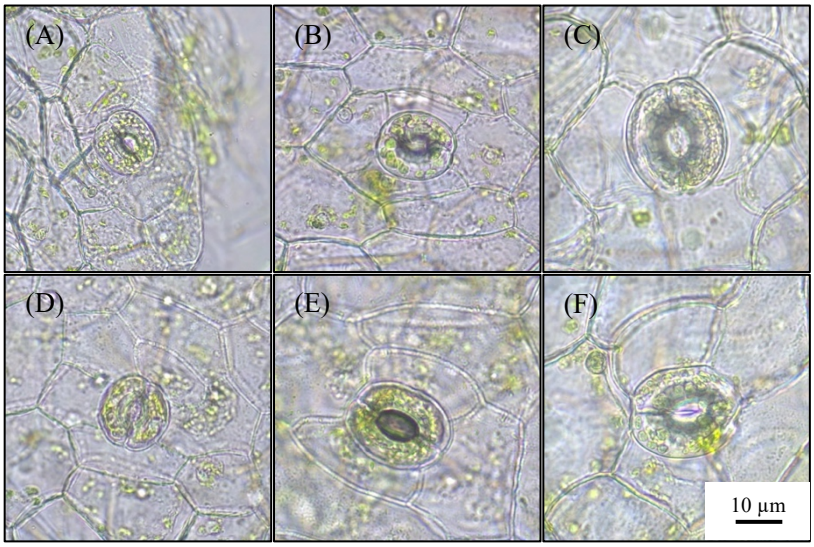

Figure 6. Size of stomata from plantlets obtained from PLBs with EMS: (A) 0% EMS for 60 minutes, (B) 0.5% EMS for 60 minutes, (C) 1% EMS for 60 minutes, (D) 0% EMS for 90 minutes, (E) 0.5% EMS for 90 minutes and (F) 1% EMS for 90 minutes after culturing for 12 month

While stomata density decreased from 220.17 to 149.47 stomata/mm² (Table 4). Immersion time also influenced stomatal density, with higher values at 90 minutes than at 60 minutes. These results suggest EMS concentration and

time revealed a consistent trend of reduced stomatal density under higher EMS levels.

Table 4. Effects of EMS concentration and time on chlorophyll content and stomatal characteristics of 12 month old Doritis leaves

		Chlorophyll contents (µg/cm²) (±SD)[1/]			Guard cell size		Density (stomata/mm²)
		Chlorophyll a (µg/cm²)	Chlorophyll b (µg/cm²)	Caroteniods (µg/cm²)	Width of stomata (µm)	Length of stomata (µm)	
EMS (%)	0	2.48±0.23	1.96±0.22	3.04±0.06	27.90±1.89	31.16±2.57	220.17±42.15a
	0.5	2.50±0.19	1.79±0.22	3.08±0.33	26.74±2.64	33.32±3.26	181.77±20.46b
	1.0	2.43±0.21	1.64±0.14	2.95±0.35	34.81±4.54	34.45±2.50	149.47±11.52c
F-test A		ns	ns	ns	ns	ns	**
Time	60	2.42±0.23	1.83±0.30	2.99±0.28	30.98±5.93	32.42±2.40	165.96±21.51b
(minutes)	90	2.52±0.17	1.77±0.14	3.06±0.27	28.66±2.96	33.53±3.59	201.64±46.42a
F-test B		ns	ns	ns	ns	ns	**
EMS (%)	Time (minutes)						
0	60	2.34±0.24	2.08±0.28	3.09±0.02	27.02±1.45	31.22±2.50	186.67±16.20b
	90	2.61±0.16	1.84±0.03	2.98±0.05	28.78±1.93	31.10±2.78	253.67±28.50a
0.5	60	2.57±0.11	1.78±0.32	3.11±0.49	27.37±2.89	33.05±2.13	166.87±13.58bc
	90	2.42±0.25	1.80±0.13	3.05±0.19	26.12±2.34	33.58±4.22	196.67±14.01b
1.0	60	2.34±0.30	1.62±0.11	2.87±0.17	38.55±2.67	32.99±2.31	144.33±7.57c
	90	2.53±0.03	1.66±0.19	3.04±0.52	31.07±2.32	35.91±1.78	154.60±13.97c
F-test A*B		ns	ns	ns	ns	ns	**
CV%		8.38	11.49	10.32	16.35	9.56	9.18

[1/]Mean ± SD values followed by different letters are significantly different according to Duncan's New Multiple Range Test (DMRT). ns = not significant; ** = significant at P ≤ 0.01."

Table 5. Effects of EMS concentration and time on the morphology of Doritis at 12 months

		Leaf			Stem Height	Number of roots
		Width	Length	Number		
EMS (%)	0	1.45±0.10a	4.62±0.28a	4.83±0.75a	4.08±0.27a	3.00±0.63
	0.5	1.05±0.10b	3.38±0.47b	4.00±0.63b	3.00±0.39b	2.67±0.52
	1.0	0.93±0.15b	2.80±0.26c	3.67±0.52b	2.50±0.18c	2.83±0.75
F-test		**	**	**	**	ns
time	60	1.14±0.30	3.70±0.88	4.11±0.60	3.24±0.71	2.56±0.53
(minutes)	90	1.14±0.22	3.50±0.85	4.22±0.97	3.14±0.80	3.11±0.60
F-test		ns	ns	ns	ns	ns
EMS (%)	Time (minutes)					
0	60	1.50±0.10a	4.67±0.15a	4.33±0.58ab	4.00±0.20a	2.67±0.58
	90	1.40±0.10a	4.57±0.40a	5.33±0.58a	4.17±0.35a	3.33±0.58
0.5	60	1.10±0.10b	3.77±0.25b	4.33±0.58ab	3.33±0.15b	2.67±0.58
	90	1.00±0.10bc	3.00±0.20c	3.67±0.58b	2.67±0.15c	2.67±0.58
1.0	60	0.83±0.06c	2.67±0.15c	3.67±0.58b	2.40±0.10c	2.33±0.58
	90	1.03±0.15b	2.93±0.31c	3.67±0.58b	2.60±0.20c	3.33±0.58
F-test		**	**	**	**	ns
CV%		9.21	7.23	13.86	6.52	20.37

[1/]Mean ± SD values followed by different letters are significantly different according to Duncan's New Multiple Range Test (DMRT). ns = not significant; ** = significant at P ≤ 0.01"

After 12 months, morphological were significantly reduced by EMS concentration, while exposure time had no independent effect. The control showed the greatest growth in leaf width 1.45 cm, leaf length 4.62 cm and stem height 4.08 cm. Whereas seedlings at 1% EMS exhibited the greatest reduction in leaf width 0.93 cm, leaf length 2.80 cm and stem height 2.50 cm (Table 5). Interaction analysis indicated that controls consistently outperfomed EMS treatments, with 1.0% EMS strongly inhibiting seedling growth. suppresses seedling growth. Modifying stomatal development results in a reduced number of larger stomata.

Genetic variation of Doritis

The genetic variance analysis of 22 samples of Doritis .The DNA extracted from all samples was tested for genetic variance using 14 RAPD primers revealed clear genetic variation (Table 1). Among the 10 primers that produced reliable bands included OPA01, OPA02, OPA03, OPA08, OPA10, OPA10, OPA11, OPA12, OPA18, OPA19 and OPC19. The percentage of polymorphism indicated the effectiveness of each primer in detecting variability (Table 6). For sample, OPA10 at 100% (Figure 7A) and OPA19 at 90% (Figure 7B) were highly informative, while OPA11 at 25% revealed limited variability.

Table 6. Polymorphism information of 10 primers responded during RAPD analysis of EMS and their combinations induced mutants of Doritis

primer	Primer sequence	Amplified Bands	Number of polymorphic bands	% Polymorphism
OPA01	5'-CAG GCC CTT C-3'	6	3	50
OPA02	5'-TGC CGA GCT G-3'	5	3	60
OPA03	5'-AGT CAG CCA C-3'	11	6	54.54
OPA08	5'-GTG ACG TAG G-3'	5	4	80
OPA10	5'-CAA TCG CCG T-3'	5	5	100
OPA11	5'-CAG CAC CCA C-3'	8	2	25
OPA12	5'-GGG TAA CGC C-3'	7	6	85.71
OPA18	5'-GGG TAA CGC C-3'	8	5	62.5
OPA19	5'-CAA ACG TCG G-3'	10	9	90
OPC19	5'-GTT GCC AGC C-3'	4	2	50

A dendrogram of Doritis treated with different concentrations and durations of EMS was generated using NTSYSpc 2.10p. At a similarity coefficient threshold of 0.89, the 22 samples were divided into 9 clusters. which indicating genetic diversity induced by different EMS concentrations, despite the absence of visible morphological variation (Figure 8).

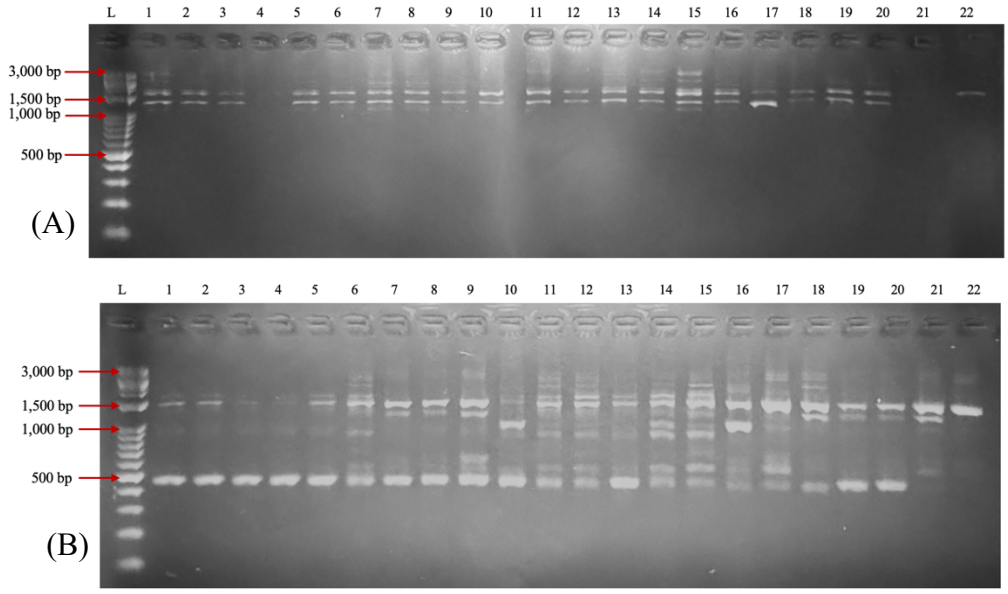

Figure 7. DNA bands were amplified using primer: (A)OPA-10, (B)OPA-19; Lane L = 100 bp DNA Ladder, Lanes 1–5 = EMS 0% for 60 min, Lanes 6–7 = EMS 0.5% for 60 min, Lanes 8–9 = EMS 1% for 60 min, Lanes 10–14 = EMS 0% for 90 min, Lanes 15–19 = EMS 0.5% for 90 min, Lanes 20–22 = EMS 1% for 90 min

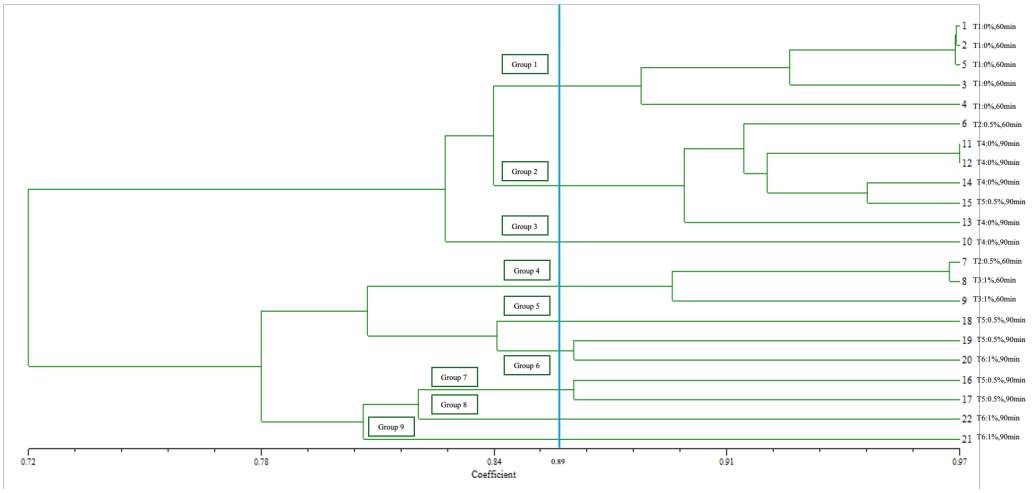

Figure 8. The phylogenetic tree of Doritis treated with various concentrations of EMS was constructed using the NTSYSpc 2.10p program based on RAPD markers

Discussion

The application of tissue culture for Dorit*is* successfully induced seed germination, PLB formation, and seedling development. The type of breeding influenced to seed germination. Cross-pollination showed higher percentage of seed germination than self-pollination, consistent with Ritti *et al.* (2022), who reported higher seed viability from cross-pollination at 52.07% than self-pollination at 32.2%. Similarly, Ackerman *et al.* (2023) noted that 76% of orchid species are cross-pollinating and 88% self-pollinating, with 54% relying on rewards and 46% on deceit. Nutrient composition of the medium strongly influenced germination. The results showed ½MS supported higher initial germination than MS, while VW medium promoted superior PLB and seedling development due to coconut water and organic additives (Stewart and Kane, 2006; Gnasekaran *et al.,* 2010). Zeng (2009) reported that higher seed germination on ½MS than MS medium, as the higher nitrogen and salt content of MS can inhibit germination (Stewart and Kane, 2006). Initially, the germination process is effective, but an excess of nutrients beyond the plant's requirements can cause stress, resulting in stunted or decreased growth. Conversely, VW medium supplemented with coconut water provides amino acids, sugars, vitamins, and cytokinins that stimulate cell division and regeneration (Gnasekaran *et al.,* 2010). The effects of BA and NAA demonstrated that types and concentrations of plant growth regulators roles in seedling development. Auxins stimulate cell division and root formation, while cytokinins promote shoot growth but can inhibit root formation at high levels (Trigiano and Gray, 2000). Similarly, Mose *et al.* (2020) reported the highest number of PLBs at 36.45 ± 0.26 in NP medium supplemented with 3 mg/l TDZ and 1 mg/l NAA. EMS treatments exerted concentration and time dependent effects on PLBs, reducing survival with prolonged exposure, in agreement with findings in Dendrobium (Martha *et al.,* 2023) and Curcuma hybrids (Yoosumran *et al.,* 2025). Chlorophyll a, b, and carotenoid contents in Doritis were not significantly affected by EMS, consistent with findings in *Setaria* EMS mutants (Tang *et al.,* 2022), indicating pigment biosynthesis is preserved under mutagenic stress. In contrast, stomatal were highly responsive. EMS increased stomata size but reduced density at higher EMS concentrations, indicating guard cell development and potential effects on gas exchange and photosynthetic performance. This pattern differs from *Aerides odoratum*, where EMS increased stomatal density (Jyothsna *et al.,* 2024), highlighting species-specific responses. Reduced stomatal density in Doritis may serve as an adaptive strategy to limit water loss, whereas increased density in *A. odoratum* enhanced osmolyte accumulation and stress tolerance. Overall, these findings highlight that stomatal should be

evaluated together with chlorophyll content and osmolyte traits (e.g., soluble sugars, amino acids) as integrated biomarkers of stress tolerance in EMS induced mutants. At the molecular level, RAPD analysis revealed high polymorphism, with OPA10 (100%) and OPA19 (90%) being the most informative, clustering 22 samples into 9 groups. This confirmed EMS induced genetic variation despite the absence of visible morphological changes, consistent with reports in *Aerides crispa* (Srivastava *et al.,* 2018). It was determined that only some of the mutant morphological characteristics had been verified as true mutations by the RAPD technique. Although RAPD analysis confirmed the genetic variability induced by EMS. Although RAPD is effective for detecting mutagenic polymorphism, multi-generational evaluation is recommended to distinguish stable heritable mutations from transient somaclonal or epigenetic variation.

In summary, ½MS medium was suitable for seed germination, whereas VW medium was optimal for PLB and seedling development. VW supplemented with 0.5 mg/l BA and 1 mg/l NAA is further recommended for growth. EMS extered concentration and time dependent effects on PLBs, reducing survival and altering stomatal , while chlorophyll and carotenoid contents remained stable. RAPD confirmed induced genetic diversity. These findings highlight tissue culture as an effective method to mass propagation to solve the problem of naturally sluggish development and help with conservation efforts and EMS as a useful mutagenic tool for orchid breeding.

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Conflicts of interest

The authors declare no conflict of interest.

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