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Selection of Suitable Media for *In vitro* Development of Seedling in an Ornamental Orchid: *Doritis pulcherrima* Lindl.

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Abstract

Doritis pulcherrima is an important ornamental orchid due to its aesthetic appeal and wide usage in hybrid breeding. This study evaluated several nutrient-rich growth media including MS, ½MS, Knudson's C (KC), KC with added peptone, Vacin and Went (VW), Lindemann Orchid (LO), Mitra Orchid (MT), and Gamborg's medium (GM) to determine the most suitable one for *in vitro* multiplication of protocorm-like bodies (PLBs) derived from asymbiotic seed suspension cultures. Development of the plantlets assessed over four and eight weeks based on the number of leaves, root, and plantlet length. Among the various media used KC showed the most vigorous growth with highest number of leaves (7.4 ± 1.356), roots (4.8 ± 1.469), and plantlet length (2.46 ± 0.454) after eight weeks of culture. In contrast, VW caused tissue browning, while KC with peptone slowed early growth, highlighting KC as the most effective media for *in vitro* growth and propagation of *D. pulcherrima.*

Introduction

Orchids represent one of the largest and most diverse families of flowering plants, with an estimated 30,000 to 35,000 species worldwide (Vendrame and Khoddamzadeh. 2016, Fay et al. 2025). Known for their stunning floral diversity, vibrant colours, and delightful fragrances, orchids hold a prominent place in global horticulture and floriculture markets (Christenson 2001). They are among the most captivating ornamental plants globally, commanding premium prices in both domestic and international markets due to their long-lasting and exquisite blooms. Comprising approximately 7% of all flowering plant species, orchids are one of the most valuable decorative crops currently available and hold a dominant position in the global cut flower industry (Bhowmik and Rahman 2017). *Doritis pulcherrima* Lindl., also known as *Phalaenopsis pulcherrima*, is a sympodial terrestrial orchid native to a broad region that spans from the Himalayas to Southeast

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Asia (Teuscher 1977). Valued for its striking flowers, *D. pulcherrima* has been extensively used in breeding programs to develop popular hybrids such as *Doritaenopsis* (Christenson 2001). This genus includes economically important species and varieties that are in high demand for ornamental horticulture.

In addition to their ornamental appeal, orchids are valued for their rich supply of bioactive compounds, which have been utilized in traditional medicine systems such as Ayurveda (Chugh et al. 2009). Their importance in both horticulture and therapeutics makes orchids increasingly significant as economic crops. However, natural orchid populations face serious threats. Over-harvesting, widespread habitat loss, and deforestation have led to steep population declines, placing many species on the International Union for Conservation of Nature (IUCN) Red List as threatened or endangered (Wraith and Pickering 2018). The conventional methods of orchid propagation via seed germination and proliferation are often slow, unreliable, and labour-intensive, making them inadequate for large-scale commercial production and conservation (Park et al. 2002). Due to their high genetic variability, orchids propagate vegetatively through methods such as rhizome division, bulb separation, or offshoot rooting, all of which are time-intensive and slow. Furthermore, achieving the target number of plants through these conventional approaches is often difficult. Consequently, it is crucial to implement techniques that enable large-scale multiplication and facilitate the successful establishment of orchids in their natural environments. Tissue culture has thus emerged as a highly effective alternative for the mass propagation of orchids (Mondal et al. 2013, Baltazar-Bernal et al. 2024). Plant tissue culture has revolutionized orchid propagation by enabling rapid multiplication of healthy, uniform plantlets under controlled conditions. Many orchids have been successfully micro-propagated using semi-solid culture media supplemented with appropriate combinations of plant growth regulators (PGRs), which support shoot and root development (Vendrame et al. 2007). Terrestrial orchids often require more complex germination media than epiphytic species (Yam and Arditti 2018). While MS medium is commonly used for *in vitro* propagation, some orchids do not respond well to it, highlighting the need for medium optimization. Specialized orchid media MT, VW, KC, have been developed to address this (Paramanik et al. 2024, Raju et al. 2025). Identifying or optimizing an appropriate germination medium is essential for protocorm proliferation and successful mass propagation in ex situ conservation efforts (Tinoammini et al. 2024).

Propagation of *D. pulcherrima in vitro* typically utilizes protocorm-like bodies (PLBs), which provide a rapid and effective means of clonal multiplication. To enhance growth and regeneration, it is crucial to optimize culture conditions, encompassing media composition, explant type, and Plant Growth Regulators (PGRs). Auxins like NAA promote root initiation and cell division, while Cytokinins such as Benzyladenine (BA) stimulate shoot proliferation (George et al. 2008, Teixeira Da Silva 2013). Orchids naturally reproduce through tiny, endosperm-free seeds that require specific fungal partners for germination, resulting in sexual propagation that is often slow and

unpredictable. Additionally, vegetative propagation, such as rhizome division and offshoot rooting, is also time-consuming and yields limited plant numbers (Chugh et al. 2009). While some orchid genera can easily generate PLBs from shoot tips, *D. pulcherrima*'s short stems make this approach challenging, as shoot tip culture risks damaging the mother plants (Roy et al. 2007). Young leaf explants offer an alternative source material for initiating PLBs without compromising the donor plants. Under optimal culture conditions, these explants can efficiently regenerate complete plantlets through organogenesis or callus formation (Arditti 1977). Therefore, the study was designed to systematically evaluate the efficacy of eight distinct basal nutrient media on the *in vitro* development of *D. pulcherrima* seedlings. By quantitatively assessing the key morphological parameters specifically leaf proliferation, velamen formation, and overall plantlet elongation at the interval of four and eight-week, this study determines the optimal nutritional formulation for the multiplication of protocorm-like bodies (PLBs) of *D. pulcherrima*. This optimized nutrient medium will provide a reliable and scalable framework for the micropropagation and *ex situ* conservation of this ecologically vulnerable ornamental orchid *D. pulcherrima*. Thus, establishing effective protocols using leaf primordia generated from seed suspension culture is therefore critical for mass clonal propagation and conservation of *D. pulcherrima*.

Materials and Methods

This study was conducted in 2024 at the Cytology and Plant Tissue Culture Laboratory of the Department of Botany, Visva-Bharati, Santiniketan, and West Bengal, India. Mature capsules of *D. pulcherrima* (Fig. 1) were obtained via manual pollination of mother plants maintained in the departmental medicinal plant garden. To maximize seedling yield and efficiency, seed suspension culture technique, previously established in our laboratory, was employed to generate the initial plantlets and PLBs. The PLBs obtained from the seed suspension culture were further used as the explant for the experiments. To identify the optimal nutritional requirements for *in vitro* growth and development of *D. pulcherrima*, eight distinct basal media (Table 1) were evaluated: (i) MS, (ii) ½MS, (iii) KC,

Fig. 1. Diagrammatic flowchart of the experimental methodology.

(iv) KC supplemented with 0.1% peptone, (v) VW, (vi) LO, (vii) MT, and (viii) GB (Park et al. 2018, Nongdam et al. 2023). All media were solidified with 0.8% (w/v) agar, and the pH was adjusted to 5.8 prior to sterilization. For the sucrose-free media (½MS, VW, and LO), 3% (w/v) sucrose was added as the carbon source.

Table 1. Media (with batch number, modifications and concentration) used for *in vitro* growth and development of *D. pulcherrima*.

Name of the media and its abbreviation	Batch No.	Readymade Modifications	Media concentration
(MS Media)	HIMEDIA, PT099-10X1L	With $CaCl_2$, vitamins and sucrose; without agar	34.4 g/l readymade powdery MS media was used to make the media.
Half Strength (½MS)	HIMEDIA PT091-5L	With vitamins; without sucrose and agar	2.3 g/l of readymade powder MS media was mixed with sucrose at 3% concentration.
Knudson C (KC)	HIMEDIA PT006-25L	with sucrose; without vitamins and agar	21.59 g/l of readymade powdery KC media was used to make the media.
Modified Knudson's C medium with peptone (0.1%) (KCP)	HIMMEDIA PT006-25L + Peptone - HIMEDIA RM001-500g	With sucrose; without peptone powder, vitamins and agar	21.59 g/l of readymade powdery KC media was mixed with Peptone powder. Peptone concentration was 1 g/l.
Vacin and Went (VW)	HIMEDIA PT041-5L	Without vitamins, sucrose and agar	1.6 g/l of readymade powdery VW media was used to make the media.
Lindeman Orchid (LM)	HIMEDIA, PT039-5L	Without vitamins, sucrose and agar	2.60 g/l of readymade powdery LM media was used to make the media.
Mitra Orchid (MT)	HIMEDIA PT106-5L	With vitamins and sucrose; without agar	22.70 g/l of readymade powdery MT media was used to make the media.
Gamborg's media (GB)	HIMEDIA PT127-5L	With sucrose; without agar, kinetin, IAA	23.2 g/l of readymade powdery GB media was used to make the media.

The experiments were performed using culture tubes containing 25 ml of the respective media. Each treatment consisted of five replicates. The media were sterilized by autoclaving at 121°C (15 psi) for 15 min. Under aseptic conditions, two PLBs derived from the seed suspension culture were inoculated into each tube. Cultures were maintained at 25 ± 2°C under a 16/8 hrs (light/dark) photoperiod provided by cool white fluorescent lamps (2500-3000 lux). Observations were recorded after four weeks and eight weeks of culture.

Results and Discussion

An experiment evaluating the efficacy of eight basal media (KC, VW, MS, ½MS, KCP, LM, MT, and GB) on the vegetative growth and root morphogenesis of *D. pulcherrima* seedlings over four and eight weeks revealed that the KC medium consistently demonstrated the most vigorous growth (Figs 2 and 3). Specifically, seedlings cultured in KC medium exhibited an average of 3.8 ± 0.748 leaves, 2 ± 0.894 velamen, and a plantlet

length of 2.42 ± 0.722 cm at four weeks. These metrics progressed to 7.4 ± 1.356 leaves, 4.8 ± 1.469 velamen, and a length of 2.46 ± 0.454 cm by the eight weeks mark. During the initial four-week period, KC, ½MS, and KCP optimally promoted vegetative elongation; MS, KC, and GB yielded the highest leaf counts; and KC, ½MS, and MS facilitated the greatest velamen formation. Other media, including LM, MT, and GB, generally provided moderate developmental supports. The growth of the explants during four weeks of culture has been exhibited in Fig. 4. Moreover, the growth analysis of the explants following four weeks of culture using different media has been presented in Table 2. By eight weeks, KC maintained its clear superiority and ½MS continued to show a high success rate. In contrast, full-strength MS plateaued, failing to enhance growth beyond its four-week levels, while LM, MT, and GB exhibited stagnant performance. Notably, the VW medium proved to be a complete failure across observation periods, resulting in pronounced growth inhibition, an absence of root structure, and extensive tissue necrosis. Fig. 5 demonstrators the growth of the explants for a period of eight weeks of culture.

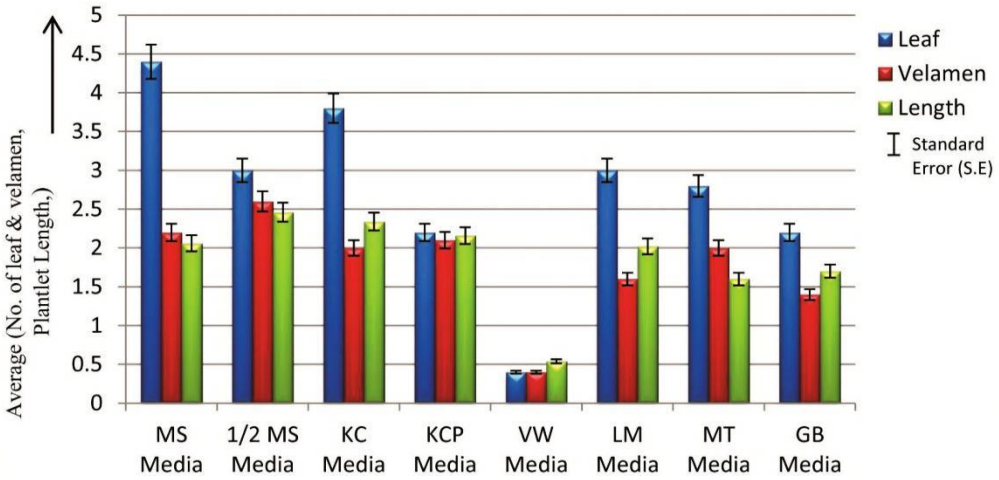

Fig. 2. Plantlet growth analysis of *Doritis pulcherrima* following four weeks of culture.

These varying developmental outcomes highlight key physiological principles regarding plant development. Primarily, they demonstrate that root structural evolution exhibits high nutritional plasticity being heavily regulated by mineral balances rather than rigid genetic templates and relies on critical nutrient synergies, such as the interaction between phosphorus and zinc required to drive auxin mediated root apical meristem development. Ultimately, the consolidated data indicates that KC and ½MS are the most effective nutrient formulations for the *in vitro* cultivation of *D. pulcherrima*. While MS and GB serve as favourable secondary options for early-stage growth, the VW medium is fundamentally toxic and unsuited for this species under these controlled conditions.

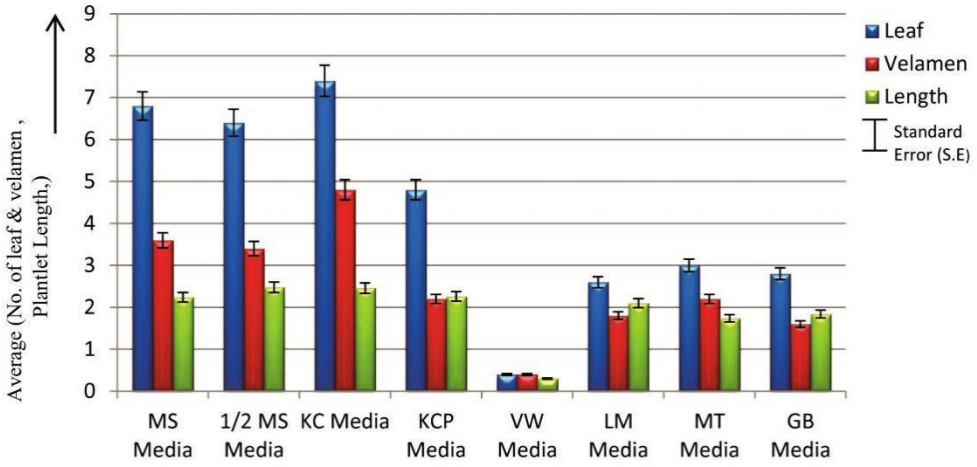

Fig. 3. Plantlet growth analysis of *Doritis pulcherrima following* eight weeks of culture.

Table 2. Plantlet growth analysis of *Doritis pulcherrima* following Anova for four weeks of culture using various media.

Name of the Media	Weeks	No. of Leaves (Mean ± SD)	No. of Velamen (Mean ± SD)	Plantlet Length (cm) (Mean ± SD)*
KC Media	Four weeks	3.8 ± 0.748^{a}	2.0 ± 0.894^{ab}	2.34 ± 0.475^{a}
	Eight weeks	7.4 ± 1.356^{a}	4.8 ± 1.469^{a}	2.46 ± 0.454^{a}
MS Media	Four weeks	3.4 ± 0.800^{a}	2.2 ± 0.748^{ab}	2.06 ± 0.387^{ab}
	Eight weeks	6.8 ± 1.166^{a}	3.6 ± 0.489^{ab}	2.24 ± 0.475^{a}
½MS Media	Four weeks	3.0 ± 0.632^{ab}	2.6 ± 0.489^{a}	2.46 ± 0.538^{a}
	Eight weeks	6.4 ± 1.200^{a}	3.4 ± 1.019^{ab}	2.48 ± 0.699^{a}
LM Media	Four weeks	3.0 ± 0.632^{ab}	1.6 ± 0.489^{bc}	2.02 ± 0.416^{ab}
	Eight weeks	3.2 ± 1.019^{c}	1.8 ± 0.748^{c}	2.10 ± 0.509^{a}
MT Media	Four weeks	2.8 ± 0.748^{b}	2.0 ± 0.632^{ab}	1.60 ± 0.209^{b}
	Eight weeks	3.0 ± 0.632^{bc}	2.2 ± 0.748^{bc}	1.74 ± 0.634^{a}
KCP Media	Four weeks	2.2 ± 0.748^{bc}	2.2 ± 0.748^{ab}	2.16 ± 0.516^{ab}
	Eight weeks	4.8 ± 0.748^{b}	2.2 ± 0.748^{bc}	2.26 ± 0.516^{a}
GB Media	Four weeks	2.2 ± 0.748^{bc}	1.4 ± 0.489^{c}	1.70 ± 0.126^{b}
	Eight weeks	2.8 ± 0.748^{bc}	1.6 ± 0.489^{c}	1.84 ± 0.332^{a}
VW Media	Four weeks	0.4 ± 0.489^{c}	0.4 ± 0.489^{d}	0.54 ± 0.355^{c}
	Eight weeks	0.4 ± 0.489^{d}	0.4 ± 0.489^{d}	0.58 ± 0.200^{b}

*Based on α= 0.05 significance level the mean values followed by the same letter within a column are not significantly different at P ≤0.05 according to Tukey's HSD test for four weeks and eight weeks growth results.

Preliminary normality testing using the *Shapiro-Wilk* test in JAMOVI (The jamovi project 2022, R Core Team 2022) indicated that data (Tables 3A and 3B) for the mean number of leaves (W=0.940W=0.940, p=0.613 p=0.613) and mean number of velamen (W=0.892 W=0.892, p=0.243 p=0.243) were normally distributed (i.e., p>0.05 p>0.05), supporting the use of parametric methods for these variables (Rouder et al. 2009, Morey

et al. 2024). The analysis shows highly significant growth at the four weeks of interval. These observations clearly indicate that in four-week period MS and KC media are most conducive to the early-stage micropropagation of *D. pulcherrima* and are preferable for supporting robust vegetative growth, leaf initiation, and root adaptation under *in vitro* conditions.

Table 3A. T-Test analysis for the four and eight weeks of different growth parameters.

One Sample T-Test for the four and eight weeks growth							
Parameters	Weeks		Statistic	± %	df	p	Mean difference
No. of Leaves (mean)	Four weeks	Student's t	6.42		7.00	<.001	2.73
		Bayes factor$_{10}$	95.3	3.93e-6			
		Wilcoxon W	36.0			0.014	2.86
	Eight weeks	Student's t	4.90		7.00	0.002	4.28
		Bayes factor$_{10}$	26.3	3.49e-7			
		Wilcoxon W	36.0			0.008	4.55
No. of Velamen (mean)	Four weeks	Student's t	7.53		7.00	<.001	1.80
		Bayes factor$_{10}$	215.0	2.95e-8			
		Wilcoxon W	36.0			0.014	1.90
	Eight weeks	Student's t	5.15		7.00	0.001	2.50
		Bayes factor$_{10}$	32.8	2.46e-6			
		Wilcoxon W	36.0			0.014	2.54
Plantlet Length (cm) (mean)	Four weeks	Student's t	8.52		7.00	<.001	2.00
		Bayes factor$_{10}$	415.6	1.38e-9			
		Wilcoxon W	36.0			0.014	2.15
	Eight weeks	Student's t	7.69		7.00	< .001	1.93
		Bayes factor$_{10}$	240.1	9.17e-9			
		Wilcoxon W	36.0			0.008	2.10

Note. H_a $\mu \neq 0$

Data shown are the mean of five replicates. The mean values are used Student's t test, Bayes factor$_{10}$, Wilcoxon W in JAMOVI project, 2022. Where the p <0.001, shows highly significant data distributions.

Table 3B. Normality Test *(Shapiro-Wilk)* for four and eight weeks of different growth parameters.

Normality Test (*Shapiro-Wilk*) for four and eight weeks of growth			
		W	P
No. of Leaves (mean)	Four weeks	0.940	0.613
	Eight weeks	0.931	0.530
No. of Velamen (mean)	Four weeks	0.892	0.243
	Eight weeks	0.968	0.884
Plantlet Length (cm) (mean)	Four weeks	0.761	0.011
	Eight weeks	0.754	0.009

The *Shapiro-Wilk* test's W statistic and P-value determines significance value where the W, p >0.05. Shows highly significant data distribution.

Shapiro-Wilk test in JAMOVI (The jamovi project 2022 , R Core Team 2022) confirmed normality tests yielded statistically significant results, indicating that the data (Table 3A-3B) population means for these traits are reliably greater than zero (number of leaves: t=4.90 t=4.90, p=0.002 p=0.002; number of velamen: t=5.15 t=5.15, p=0.001 p=0.001; plantlet length: t=7.69 t=7.69, p<0.001 p<0.001). Bayesian factors ranging from 26.3 to 240.1 further substantiate strong evidence against the null hypothesis. The analysis shows highly significant growth at the eight weeks of interval (Rouder et al. 2009, Morey et al. 2024).

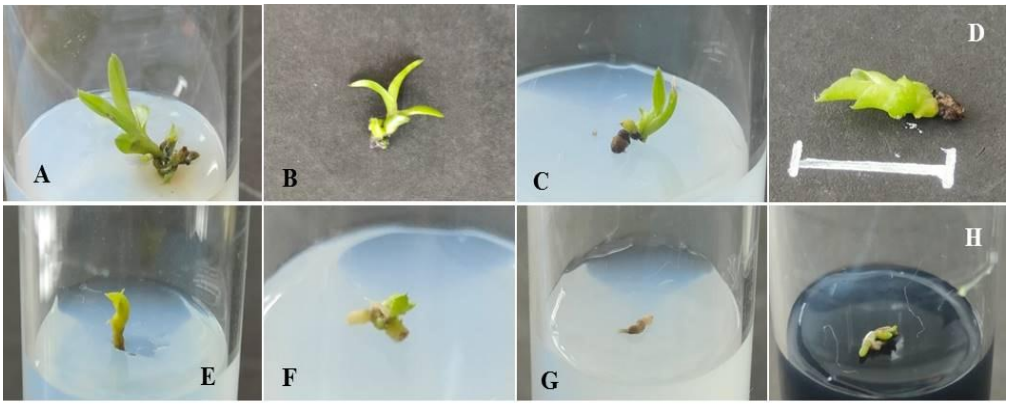

Fig. 4(A-H). Growth of *Doritis pulcherrima in* various culture media in four weeks of culture: (A) KC media, (B) ½MS media, (C) KCP media, (D) MS media, (E) LM media, (F) MT media, (G) VW media and, (H) GB media. (Bar Scale = 1 cm).

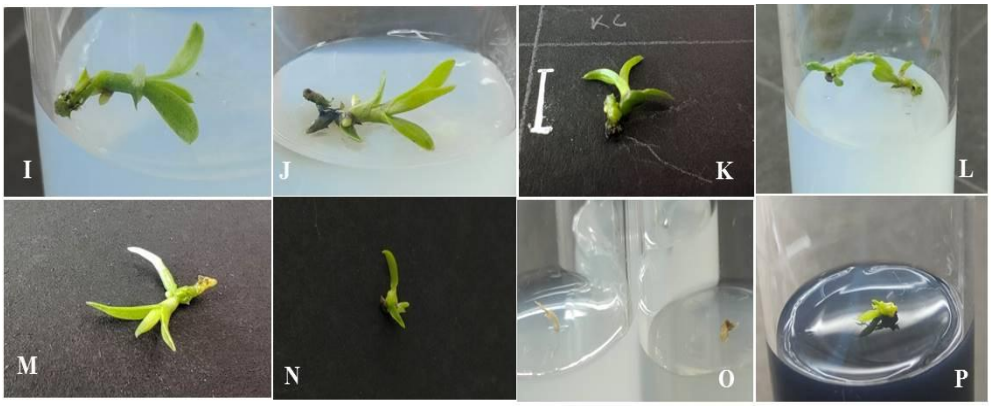

Fig. 5(I-P). Growth of *Doritis pulcherrima in* various culture media after eight weeks: (I) KC media, (J) ½MS media, (K) KCP media, (L) MS media, (M) LM media, (N) MT media, (O) VW media, and (P) GB media. (Bar Scale = 1 cm).

To address the severe population declines of *D. pulcherrima* caused by habitat loss, overharvesting, and intrinsic reproductive barriers, an experiment was conducted to optimize *in vitro* micropropagation by evaluating various basal media for seedling development. The findings demonstrated that the Knudson C (KC) medium, maintained

under a 16/8 hrs light/dark photoperiod, consistently supported the most vigorous and statistically significant seedling growth. Specifically, seedlings cultivated on KC medium yielded average leaf counts, velamen numbers, and plantlet lengths of 3.8 ± 0.748, 2 ± 0.894, and 2.42 ± 0.722 cm at four weeks (Table 2, Fig. 2 and Fig. 4), which progressed to 7.4 ± 1.356, 4.8 ± 1.469, and 2.46 ± 0.454 cm at eight weeks (Table 2, Fig. 3 and Fig. 5), respectively. While ½MS medium also exhibited significant development and serves as a highly viable secondary option, supplementing the KC medium with 0.1% peptone reduced overall growth compared to the baseline. Furthermore, media such as LM, MT, GB, and particularly VW which caused extensive tissue necrosis failed to support substantial development. The Student's t-test and Analysis of Variance (ANOVA) are fundamental statistical methods used to determine whether observed differences in group averages are statistically significant or merely due to random chance. Table 3A-3B and shows the four and eight weeks T-test data which denotes the data are significant. The data (Table 2) also determines that the results are significant. These results successfully establish an effective asymbiotic seed culture protocol for producing robust seedlings. Similarly, *Dendrobium terminale* also show better growth KC medium (Dutta and Roy 2025). Such approaches may further enhance shoot proliferation, plantlet growth, and the potential for successful *ex situ* conservation and eco-restoration of this ornamentally important orchid. Future research may investigate supplementing the optimal KC baseline with various abiotic factors, such as phytohormones, amino acids, and vitamins, alongside biotic factors like plant growth-promoting rhizobacteria (PGPR).

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