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Comparative Phytochemical Screening, Antioxidant and Anti-Inflammatory Evaluation of *In vitro* Developed and Naturally Grown *Geodorum densiflorum* (Lam.) Schltr. of Bangladesh

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Abstract

Geodorum densiflorum (Lam.) Schltr. is an endangered terrestrial orchid of Bangladesh having significant ornamental and medicinal value. To facilitate its conservation and validate its pharmaceutical potential, an efficient *in vitro* propagation protocol was established, followed by a comparative evaluation of phytochemical and biological activities between *in vitro* raised and naturally grown plant parts. Asymbiotic seed germination was evaluated on four basal media *viz*. MS, PM, KC and MVW supplemented with or without PGRs (0.5 mg/l BAP + 0.5 mg/l NAA). The highest seed germination (66.67%) and earliest protocorm development (14.00 ± 0.30 weeks) were recorded on MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. Qualitative phytochemical screening of methanolic extracts revealed alkaloids, flavonoids, tannins and other secondary metabolites in both wild and *in vitro* plantlets. *In vitro* plantlets showed particularly high levels of phlobatannins and phenolics compared to field grown plant parts. In biological assays, *in vitro* developed plantlets exhibited significant antioxidant potential (61.58% DPPH scavenging activity at 250 µg/ml), surpassing the activity observed in natural leaf, root and pseudobulb extracts. However, the anti-inflammatory activity (albumin denaturation inhibition) was higher in natural pseudobulb (81.81%) compared to *in vitro* plantlets (54.55%). This study establishes a reproducible protocol for mass propagation and confirms that *in vitro* derived *G. densiflorum* retains therapeutic properties, offering a sustainable alternative to wild harvesting.

Introduction

Orchidaceae family, is one of the leading and most evolved groups of flowering plants, exhibits incredible diversity in floral morphology and adaptation strategies

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(Dressler 1993). Beyond their aesthetic value in the multi-billion dollar floriculture industry (Hew 1989, De et al. 2014), orchids have a rich history in traditional medicine, particularly in Asia. *Geodorum densiflorum* (Lam.) Schltr., a ground orchid distributed across South and Southeast Asia, including the Chittagong Hill Tracts of Bangladesh. Ethnobotanically, its pseudobulbs and roots are utilized to regulate menstrual cycles (Dash et al. 2008), manage diabetes (Patil and Patil 2005) and treat carbuncles (Nath et al. 2011). Pharmacological studies confirm diverse activities in this species, including antimicrobial, antioxidant, cytotoxic, thrombolytic and analgesic effects. Phytochemical analyses have detected numerous bioactive compounds (e.g. flavonoids, terpenoids, steroids and aromatic acids) in *G. densiflorum* accounting for its therapeutic uses (Hossain et al. 2012).

Despite its importance, *G. densiflorum* populations are declining rapidly due to habitation fragmentation and indiscriminate collection for medicinal trade (Datta et al. 1999, Pant and Raskoti 2013, Chakraborty and Bhattacharjee 2019). In nature, orchid seeds lack endosperm and require specific mycorrhizal symbiosis for germination, resulting in low survival rates (Arditti 1967, Smith and Read 2008). It is necessary to develop a species-specific protocol for *G. densiflorum* (Deb and Imchen 2010, Pant et al. 2011, Roy et al. 2016). Consequently, conventional propagation is insufficient to meet commercial and conservation demands. Plant tissue culture, specifically asymbiotic seed germination, offers a viable solution for mass propagation and protection of threatened orchids (Arditti 1977, Pant et al. 2011).

While *in vitro* propagation protocols exist for various orchids, a critical question remains regarding the biochemical fidelity of tissue cultured plants. It is often hypothesized that *in vitro* plants, grown under controlled, stress free environments, may produce lower quantities of secondary metabolites compared to wild populations exposed to natural stressors (Giri et al. 2012). Validating the phytochemical profile and bioactivity of *in vitro* derived plants is essential to justify their use as alternatives to wild harvested specimens in the pharmaceutical industry. To our knowledge, no previous study has compared phytochemistry, antioxidant and anti-inflammatory activities of wild vs *in vitro* cultures of *G. densiflorum*.

Therefore, the present study was design to establish an efficient protocol for *in vitro* seed germination and seedling development of *G. densiflorum* using various basal media and PGRs combinations. Comparative phytochemical screening, antioxidant and anti-inflammatory activities both *in vitro* derived plantlets and naturally grown plant parts used to determine pharmaceutical potential.

Materials and Methods

Mature green capsules of *G. densiflorum* (Lam.) Schltr. were collected from Bangchari, Kaptai, Rangamati, Bangladesh. The capsules were washed under running tap water for 10 minutes to remove surface debris. Surface sterilization was performed in a laminar air flow cabinet. Capsules were treated with 70% (v/v) ethanol for 30 seconds, followed by

immersion in 3% (w/v) sodium hypochlorite (NaOCl) for 10 min. Finally, they were submerged in a 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 10 min and rinsed three times with sterile double distilled water. For comparative phytochemical analysis, naturally grown plant parts (leaves, pseudobulbs and roots) were collected from the same habitat, while *in vitro* whole plantlets were harvested from the culture room.

Four basal media were evaluated for seed germination *viz*. MS; PM (Phytamax, Arditti 1977); KC (Knudson 1946) and MVW (Modified Vacin and Went 1949). The media were used at full strength, with or without PGRs. Cytokinin (0.5 mg/l BAP) and auxin (0.5 mg/l NAA) were used. All media were supplemented with 2-3% (w/v) sucrose and gelled with 0.8% (w/v) agar (Fluka). The pH was adjusted to 5.0-5.8 depending on the medium prior to autoclaving at 121°C and 1.05 kg/cm² pressure for 20 min. Cultures were maintained at 25 ± 2°C under a 14/10h light/dark photoperiod (3000-4000 lux).

Sterilized capsules were dissected longitudinally and the seeds were inoculated onto the prepared media. Observations were recorded on germination initiation, protocorm formation and the differentiation of leaf and root primordia. Germination percentage was determined based on the proportion of culture vessels exhibiting a positive response.

Methanolic extracts of natural plant parts (leaf, pseudobulb, root) and *in vitro* whole plantlets were prepared. Qualitative screening for secondary metabolites (alkaloids, flavonoids, phlobatannins, terpenoids, steroids, glycosides, saponins, tannins, etc.) was conducted using standard protocols described by Cromwell (1955), Sofowara (1993), Trease and Evans (1989) and Harborne (1973). Specific reagents included Dragendroff's, Mayer's, Hager's and Wagner's reagents for alkaloids.

Methanolic extracts were prepared from leaves, pseudobulbs, roots and *in vitro* plantlets. Extracts were dilute to concentrations of 50, 100, 150, 200 and 250 µg/ml in methanol. The free radical scavenging activity was determined using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method (Brand-Williams et al. 1995). Absorbance was measured at 517 nm against ascorbic acid as the standard. Ascorbic acid served as positive control. All tests were performed in triplicate. Data were expressed as % inhibition of DPPH.

$$\text{Scavenging activity } (\%) = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100$$

The inhibition of heat-induced albumin denaturation was measured and albumin denaturation inhibition assay was performed following Mizushima et al. (1968). Acetyl Salicylic Acid (ASA) served as the standard. Absorbance was measured at 660 nm. Each assay was performed in triplicate.

$$\text{Inhibition } (\%) = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100$$

Data were analyzed using standard statistical methods by Microsoft Excel 2013 software. Means ± Standard Error (SE) were calculate for growth parameters.

Results and Discussion

The seeds of the indigenous ground orchid *Geodorum densiflorum* were aseptically germinated on 0.8% (w/v) agar solidified KC, MS, PM and MVW media with or without PGRs (Table 1). The poor percentage of seed germination of cultured vessel (16.67%) was recorded on PGRs free KC medium (Fig. 1a). The percentage of culture vessels with germinated seeds were highest on PGRs (0.5 mg/l BAP and 0.5 mg/l NAA) fortified MS (66.67%, Fig. 1b) medium followed by PGRs supplemented full strength PM (58.34%). PGRs supplemented MS medium showed the early initiation of seed germination within the minimum time (10.07 ± 0.22 weeks); whereas, the highest time (20.23 ± 0.27 weeks) required on KC medium for seed germination. Development of protocorm (Fig. 1c), differentiation of first leaf primordial (Fig. 1d-e), differentiation of first root primordia and development of seedlings (Fig. 1f) were first noted on PGRs supplemented MS medium within minimum time 14.00 ± 0.30, 18.57 ± 0.38, 23.63 ± 0.27 and 28.23 ± 0.25 weeks accordingly. But the maximum time for development of protocorm, differentiation of first leaf primordia, differentiation of first root primordia and development of seedlings was recorded on PGRs free KC medium within 20.23 ± 0.27, 26.13 ± 0.34, 32.20 ± 0.28, 40.20 ± 0.30 and 46.17 ± 0.28 weeks respectively. It is noted that PGRs supplemented MS medium was better than other three medium subsequently PM, MVW and KC medium for better seed germination and differentiation.

Table 1. Effects of full strength KC, MS, PM and MVW media with or without PGRs combination on *in vitro* seed germination, differentiation and seedling development of *Geodorum densiflorum* (Lam.) Schltr.

Medium	Culture condition	Time taken in weeks					% of culture vessel germinated	Remarks
		Initiation of germination (Mean ± SE)	Development of protocorm (Mean ± SE)	Differentiation of 1st leaf primodia (Mean ± SE)	Differentiation of 1st root primodia (Mean ± SE)	Development of seedling (Mean ± SE)		
KC	PGRs free	20.23 ± 0.27	26.13 ± 0.34	32.20 ± 0.28	40.20 ± 0.30	46.17 ± 0.28	16.67	+
	With PGRs	16.63 ± 0.28	23.70 ± 0.41	29.17 ± 0.34	35.07 ± 0.34	40.30 ± 0.30	33.34	+
MS	PGRs free	14.73 ± 0.26	18.80 ± 0.33	24.53 ± 0.33	29.47 ± 0.30	35.50 ± 0.31	50.00	++
	With PGRs	10.07 ± 0.22	14.00 ± 0.30	18.57 ± 0.38	23.63 ± 0.27	28.23 ± 0.25	66.67	++
PM	PGRs free	16.43 ± 0.34	21.67 ± 0.37	27.47 ± 0.30	31.23 ± 0.26	36.37 ± 0.34	41.67	+
	With PGRs	12.20 ± 0.29	15.70 ± 0.27	21.53 ± 0.33	26.57 ± 0.35	31.07 ± 0.30	58.34	++
MVW	PGRs free	18.30 ± 0.38	22.63 ± 0.31	28.17 ± 0.40	34.03 ± 0.32	40.10 ± 0.22	25.00	+
	With PGRs	14.27 ± 0.32	20.43 ± 0.40	25.30 ± 0.31	31.57 ± 0.35	38.73 ± 0.27	41.67	+

Half strength without PGRs, **Full strength without PGRs, *** Full strength with PGRs (0.5mg/l BAP + 0.5mg/l NAA); + = Minimum germination (0% ≤ + ≤ 49%), ++ = Medium germination (50% ≤ ++ ≤ 74%), +++ = Maximum germination (75% ≤ +++ ≤ 100%). Values represent mean ± SE of each experiment consist of 12 replicates.

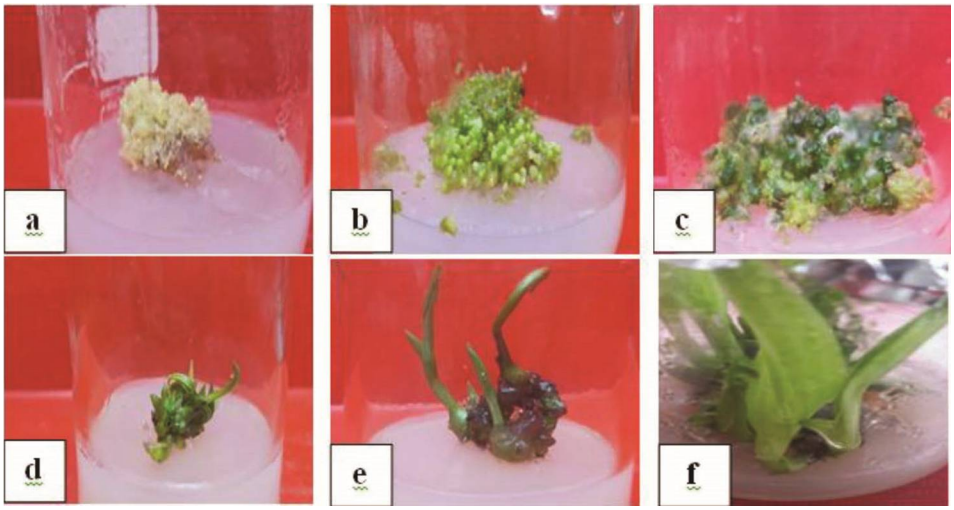

Fig. 1(a-f). Seed germination and plantlets development of *G. densiflorum*: (a) seed germination on KC medium, (b) seed germination on PGRs fortified MS medium, (c) protocorm development, (d) differentiation of leaf primordia, (e) development of microshoots, and (f) seedlings regeneration.

Qualitative tests of secondary metabolites in wild and *in vitro* derived *G. densiflorum* are presented in Tables 2 and 3. All major groups (alkaloids, flavonoids, tannins, terpenoids, steroids, glycosides, coumarins, phenols) were detected to some degree in various plant parts. In alkaloid tests (Table 2), both wild tissues and *in vitro* plantlets gave positive reactions with Dragendorff's, Mayer's, Wagner's and other reagents (signs from + to +++). Wild root and *in vitro* shoot buds gave the strongest responses (in Dragendorff's and Wagner's tests, indicating abundant alkaloids. Overall, natural roots and *in vitro* shoot buds appeared richest in alkaloids, whereas wild leaf and pseudobulb showed moderate levels. For other metabolites (Table 3), wild leaf gave the highest phlobatannins and moderate terpinoids and coumarins, but lacked glycosides, quinines and phenols. Wild pseudobulb showed highest flavonoids and glycosides, plus moderate tannins, anthroquinone and phenols. Wild root had moderate phlobatannins, flavonoids, terpenoids and glycosides. *In vitro* plantlets had the highest phlobatannin and phenol levels (moderate tannins, terpenoids, steroids and proteins and only trace glycosides or coumarins. In summary, *in vitro* plants were especially rich in phlobatannins and phenolics but lacked many other wild constituents; notably flavonoids, saponins, anthroquinone and quinine. The presence of multiple metabolites in both sources verifies reports of *G. densiflorum* as a phytochemically diverse medicinal orchid.

The DPPH radical scavenging activities of methanolic extracts and ascorbic acid (control) showed very high scavenging (95.43-98.59%) even at 50-250 µg/ml (Table 4 and Fig. 2). Among plant extracts, the *in vitro* derived whole plantlet extract exhibited the highest activity 58.67% at 50 µg/ml rising slightly to 61.58% at 250 µg/ml. Leaf extracts of wild plant parts showed intermediate activity, peaking at 49.79% at 100 µg/ml and

declining at higher dose (40.25% at 250 µg/ml). Wild pseudobulb extract was weakest at low dose (35.76% at 50 µg/ml) but increased to 51.95% at 200 µg/ml. Root extract showed 43.82-48.46% activity across doses. Thus, at the highest dose (250 µg/ml), per cent scavenging was: *in vitro* plantlets 61.58%, wild pseudobulb 51.03%, root 47.72%, leaf 40.25%. All extracts activities were significantly lower than ascorbic acid at the same concentrations (98.59%).

Table 2. Qualitative test for alkaloids of the plant parts of *G. densiflorum.*

Sl. No.	Name of Reagents	Plants part used					
		Natural			*In vitro*		
		Leaf	Pseudobulb	Root	Callus	SPSs	Shoot Bud
1.	Dragendroff's Reagent	+	++	+++	++	+	+++
2.	Hager's Reagent	+++	++	+++	+	+	++
3.	Mayer's Reagent	++	+	+++	+	++	+++
4.	Tannic Acid Reagents	++	+++	++	+	++	+++
5.	Wagner's Reagent	+++	+++	+++	++	+	+++

Here, "+++" means highest response, "++" means medium response and "+" means lowest response.

Table 3. Qualitative test of secondary metabolites of natural and *in vitro* developed plantlets of *G. densiflorum*.

Plant Part Used		Secondary metabolites (0% of coloration)											
		Phl	Flv	Sap	Tan	Ter	Str	Gly	Ant	Qui	Cou	Phe	Pro
Natural	Leaf	+++	+	+	+	++	+	-	+	-	++	-	+
	Pseudobulb	+	+++	-	++	+	+	+++	++	-	+	++	-
	Root	++	++	-	+	++	-	++	-	-	+	+	-
In vitro	Plantlets	+++	-	-	++	++	++	+	-	-	+	+++	++

Phl. = Phlobatannins, Flv. = Flavonoids, Sap. = Saponins, Tan. = Tanins, Ter. = Terpinoids, Str. = Steroids, Gly. = Glycosides, Ant. = Anthroquinone, Qui. = Quinine, Cou. = Coumarin, Phe. = Phenol and Pro. = Protein. Here, "+++" means highest response, "++" means medium response, "+" means lowest response and "-" means absent.

Table 4. DPPH free radical scavenging assay of methanolic crude extract of naturally grown leaf, pseudobulb, root and *in vitro* developed plantlets of *G. densiflorum* and standard.

Con. (µg/ml)	Naturally grown			*In vitro*	Ascorbic Acid
	Leaf	Pseudobulb	Root		
50	47.80 ± 0.01	35.76 ± 0.01	45.39 ± 0.02	58.67 ± 0.01	95.43 ± 0.04
100	49.79 ± 0.03	42.24 ± 0.03	43.82 ± 0.02	60.58 ± 0.03	96.93 ± 0.02
150	45.31 ± 0.06	42.41 ± 0.04	48.46 ± 0.05	60.91 ± 0.03	97.84 ± 0.01
200	47.97 ± 0.02	51.95 ± 0.04	45.39 ± 0.03	61.33 ± 0.02	98.09 ± 0.01
250	40.25 ± 0.02	51.03 ± 0.01	47.72 ± 0.02	61.58 ± 0.02	98.59 ± 0.04

Results are presented as Mean ± SEM (n = 2).

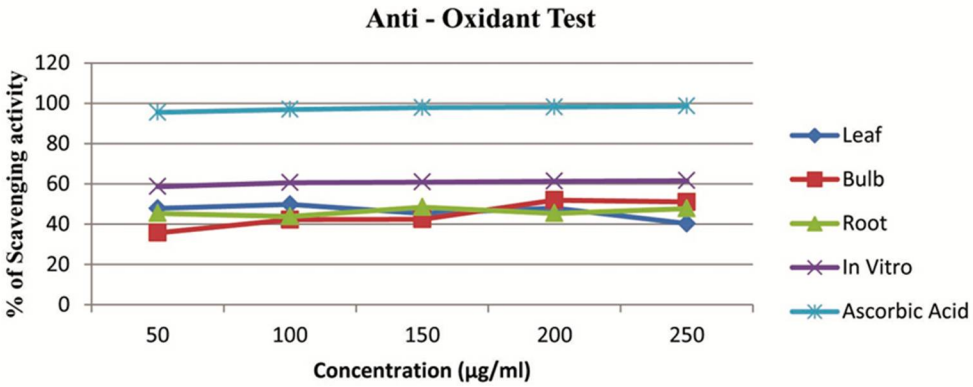

Fig. 2. Relative % of scavenging activity of standard antioxidant ascorbic acid and methanolic crude extracts of leaf, pseudobulb, root and *in vitro* development *G. densiflorum*.

Pseudobulb extract showed the highest activity (81.81%) of albumin denaturation inhibition at 250 µg/ml (Table 5 and Fig. 3) followed by leaf (72.73%), root (63.64%), *in vitro* plantlets (54.55%) and compared to the standard Acetyl Salicylic Acid (91.30%).

Table 5. Albumin protein denaturation assay of anti-inflammatory activity for methanolic crude extract of naturally grown Leaf, pseudobulb, root and *in vitro* developed plantlets of *G. densiflorum* and standard.

Inhibition (%) of albumin denaturation of *G. densiflorum*					
Con. µl/ml	Naturally grown			*In vitro*	ASA (Standard)
	Leaf	Pseudobulb	Root		
250 µl/ml	72.73	81.81	63.64	54.55	91.30

ASA = Acetyl Salicylic Acid.

This study demonstrates a reliable protocol for propagating *G. densiflorum via* asymbiotic seed culture and provides novel comparative data on phytochemical screening and bioactivity. The study highlights the efficacy of MS medium over PM, MVW and KC for the *in* vitro germination of *G. densiflorum*. The superiority of MS medium, particularly when supplemented with cytokinins (BAP) and auxins (NAA), may be attributed to its high nitrate and ammonium content, which is crucial for nitrogen uptake in orchid protocorms (Arditti 1977, Pant et al. 2011). Similar enhancement of germination using auxin-cytokinin combinations has been reported in *Cymbidium aloifolium* (Bhowmik et al. 2017), *Dendrobium chrysotoxum* (Bhowmik and Rahman 2020) and *Vanda helvola* (David et al. 2015). Banerjee (2001) reported >90% germination of *G. densiflorum* in several media but noted that NAA enhanced protocorm growth while BAP alone could inhibit development; our results agree that NAA contributes to growth, though the combination with low BAP was beneficial.

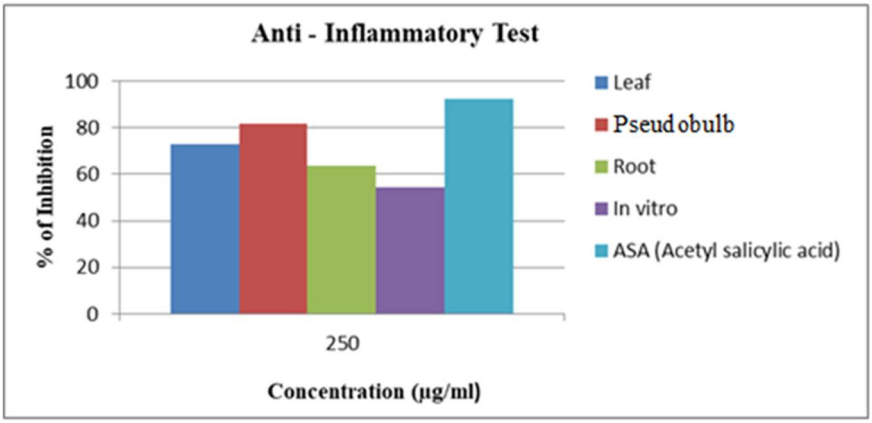

Fig. 3. Variation of percentage of inhibition in leaf, pseudobulb, root and *in vitro* development *G. densiflorum* compare to standard ASA (Acetyl Salicylic Acid).

A essential aspect of this research was the comparative phytochemical evaluation. The *in vitro* derived plantlets synthesized significant quantities of alkaloids, phenols and phlobatannins. This contradicts the assumption that field conditions are strictly required for secondary metabolite production. The presence of these compounds in tissue cultured plants has been supported by studies on *Dendrobium nobile* (Bhattacharyya et al. 2016) and *Ansellia africana* (Chinsamy et al. 2014), indicating that genetic control often supersedes environmental influence for specific metabolite pathways.

The antioxidant activity of *in* vitro plantlets (61.58%) was surprisingly higher than natural vegetative plant parts. This could be attributed to the younger, actively dividing tissues in culture which often have higher metabolic rates and phenolic content (Chao et al. 2014). This parallels findings in other species *Salvia miltiorrhiza* had higher antioxidant activity and phenolic levels than seed grown plants was noted by Liu et al. (2011). However, the anti-inflammatory activity was higher in natural pseudobulbs. This variance suggests that while antioxidant compounds; likely phenols are stable *in vitro*, specific anti-inflammatory compounds perhaps specific glycosides or flavonoids abundant in natural pseudobulbs may require ontogenic maturity or environmental elicitors found in the wild (Akter et al. 2020). Nevertheless, the presence of substantial bioactivity in *in vitro* plantlets validates their potential as a sustainable source for herbal formulations, reducing pressure on wild populations.

These findings contribute to the ethnopharmacological knowledge of *G. densiflorum*. This species contains bioactives with multiple medicinal effects. The higher anti-inflammatory response of pseudobulb could reflect accumulation of such compounds. Overall, *in vitro* propagation not only provides a means of conservation and mass production but also yields plant material with a complement of valuable phytochemicals. Future studies should quantify key metabolites and test biological activities of individual extracts or isolated compounds.

The present study established a robust protocol for the rapid mass propagation of the endangered orchid *G. densiflorum* using MS medium supplemented with BAP and NAA. Furthermore, the comparative analysis confirms that *in vitro* raised plantlets possess comparable and in some cases superior (antioxidant), bioactive properties to naturally grown plant parts. *In vitro* developed plantlets can effectively substitute wild specimens in traditional medicine and pharmaceutical applications, thereby aiding in the conservation of this species in its natural habitat.

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