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Optimization of BAP- and NAA-Mediated In Vitro Micro propagation of Dendrobium

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Abstract:

The present study evaluated the effects of plant growth regulators on survival, shoot proliferation, and root development in *Dendrobium Nobile* under *in vitro* conditions. Shoot-tip explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of 6-benzylaminopurine (BAP; 0–2.5 mg/L) for shoot induction and multiplication, and with α -naphthalene acetic acid (NAA; 0.5–2.5 mg/L) for root induction. BAP significantly influenced shoot induction and multiplication, with the highest shoot-induction percentage (88.2%), number of shoots per explant (6.5), and mean shoot length (4.1 cm) recorded at 2.0 mg/L BAP. Root induction was significantly affected by NAA concentration, with 1.0 mg/L NAA producing the highest root-induction percentage (82.7%), number of roots per shoot (5.6), and

mean root length (3.8 cm). Higher concentrations of both BAP and NAA resulted in reduced growth parameters, indicating inhibitory effects at supra-optimal levels. These findings indicate that 2.0 mg/L BAP is suitable for shoot multiplication, whereas 1.0 mg/L NAA is suitable for root induction in *D. Nobile*. The optimized protocol provides a basis for large-scale micro propagation and conservation of this orchid species.

Keywords: Dendrobium, in vitro propagation; micro propagation; 6-benzylaminopurine, α -naphthalene acetic acid, shoot multiplication; root induction, orchid conservation.

1. Introduction:

Orchids (family Orchidaceae) constitute one of the largest and most diverse families of flowering plants, comprising approximately 30,000–35,000 species distributed across tropical and subtropical regions worldwide. They are highly valued in the global floriculture industry for their aesthetic appeal, long vase life, and

commercial importance. In addition to their ornamental value, certain orchid species are recognized for their medicinal and biochemical properties, making them important resources in plant biotechnology and conservation research. [Nongdam et al ,2023](#).

Despite their economic and ecological significance, natural propagation of orchids is highly inefficient. Orchid seeds are extremely small and lack endosperm, which renders germination highly dependent on symbiotic associations with mycorrhizal fungi under natural conditions. This results in very low germination rates and slow establishment in natural habitats. Furthermore, habitat destruction, climate change, and over collection from wild populations have led to a significant decline in many orchid species, placing several of them in threatened or endangered categories.

[Baltazar-Bernal et al., 2024](#).

To overcome these limitations, *in vitro* micro propagation has emerged as a

highly effective biotechnological approach for the rapid multiplication and conservation of orchids. This technique enables the production of large numbers of genetically uniform and disease-free plantlets within a short period under controlled laboratory conditions. Murashige and Skoog (MS) medium, supplemented with plant growth regulators such as cytokines (e.g., 6-benzylaminopurine [BAP] and kinetin) and auxins (e.g., α -naphthalene acetic acid [NAA] and indole-3-acetic acid [IAA]), is widely used for inducing protocorm-like bodies (PLBs), shoot proliferation, and rooting in orchid cultures.

[Plants Journal, 2023, N. K 2018 .](#)

Recent advances between 2020 and 2025 have significantly improved orchid tissue-culture systems. The development of temporary-immersion bioreactors and semi-solid culture systems has increased multiplication efficiency, reduced contamination risks, and lowered production costs. These improvements have made large-

scale commercial propagation more feasible while also supporting the ex situ conservation of rare and endangered orchid species. Additionally, optimization of plant growth regulators has improved regenerative efficiency and reduced soma clonal variation, thereby enhancing genetic stability in regenerated plants. [MDPI, 2023](#); [Balthazar-Bernal et al., 2024](#)

However, responses to plant growth regulators are highly species- and genotype-specific, and optimal concentrations must be determined empirically for each taxon and explant type. In *Dendrobium*, a genus of high commercial and conservation value, systematic optimization of BAP and NAA for shoot multiplication and root induction remains essential for developing robust, reproducible protocols.

The present study was conducted to evaluate the effects of different concentrations of BAP and NAA on survival, shoot proliferation, and root

development in *Dendrobium Nobile* under *in vitro* conditions. The specific objectives were to (i) determine the optimal BAP concentration for shoot induction and multiplication, (ii) identify the optimal NAA concentration for root induction, and (iii) establish an efficient micro propagation protocol that can support large-scale propagation and conservation of *D. Nobile*.

2. Materials and Methods

2.1: Plant Material (Explant Source)

Healthy, disease-free *Dendrobium Nobile* plants were selected from greenhouse-grown stock plants maintained at the green house Plant biotechnology lab PCSIR Karachi Pakistan. Young, actively growing shoot tips (approximately 0.5–1.0 cm in length) were excised and used as explants for *in vitro* culture initiation. All explants were collected from vigorously growing shoots during the active growth phase.

2.2: Surface Sterilization of Explants

Explants were washed under running tap water for 15–20 min to remove surface debris, followed by immersion in a few drops of liquid detergent for 5 min and thorough rinsing with distilled water. Under aseptic conditions in a laminar airflow cabinet, explants were surface-sterilized with 70% (v/v) ethanol for 30 s, followed by treatment with 0.1% (w/v) mercuric chloride (HgCl₂) solution for 5–10 min [or 1–2% (v/v) commercial sodium hypochlorite solution containing approximately 0.5–1.0% available chlorine for 5–10 min]. Subsequently, explants were rinsed three to five times with sterile distilled water to remove traces of sterilant. All procedures involving HgCl₂ were conducted with appropriate personal protective equipment, and waste was disposed of according to institutional safety guidelines.

2.3: Culture Medium Preparation

Murashige and Skoog (MS) basal medium was used as the primary

culture medium. The medium was supplemented with 3% (w/v) sucrose as a carbon source and 0.7–0.8% (w/v) agar as a gelling agent. For shoot induction and multiplication, the medium was supplemented with 6-benzylaminopurine (BAP) at concentrations of 0, 0.5, 1.0, 1.5, 2.0, or 2.5 mg/L. For root induction, well-developed shoots were transferred to MS medium supplemented with α -naphthalene acetic acid (NAA) at concentrations of 0.5, 1.0, 1.5, 2.0, or 2.5 mg/L. The pH of the medium was adjusted to 5.6–5.8 with 1 M NaOH or 1 M HCl before autoclaving at 121°C and 15 psi for 15–20 min. Plant growth regulator stock solutions were filter-sterilized through 0.22 μ m membrane filters and added to the autoclaved, cooled medium under aseptic conditions. 2.4: Culture conditions.

2.4. Inoculation and culture conditions

Surface-sterilized explants were aseptically inoculated onto the prepared culture medium, with one explant per

culture vessel. Cultures were maintained in a growth room at $25 \pm 2^\circ\text{C}$ under a 16/8 h light/dark photoperiod with a light intensity of approximately 2000–3000 lux (approximately $40\text{--}60 \mu\text{mol m}^{-2} \text{s}^{-1}$) provided by cool white fluorescent lamps. Relative humidity in the culture room was maintained at 60–70%.

2.5. Shoot induction and multiplication

Shoot-tip explants were cultured on MS medium supplemented with BAP at 0, 0.5, 1.0, 1.5, 2.0, or 2.5 mg/L. Sub culturing was performed at 3–4 week intervals to enhance shoot multiplication and proliferation of protocorm-like bodies (PLBs). The percentage of explants responding (shoot induction), number of shoots per explant, and mean shoot length (cm) were recorded after 8 weeks of culture.

2.6. Root induction

Well-developed shoots (≥ 1 cm in length) were excised from proliferating cultures and transferred to MS medium supplemented with NAA at 0.5, 1.0,

1.5, 2.0, or 2.5 mg/L. Five replicates culture vessels were used per treatment, with one explant per vessel. Cultures were maintained under the same environmental conditions as described for shoot multiplication. Root initiation was assessed after 4 weeks. The percentage of rooted shoots, number of roots per shoot, and mean root length (cm) were recorded for each treatment

2.7. Acclimatization

Well-rooted plantlets (with ≥ 3 roots per shoot) were carefully removed from culture vessels, and agar residues were gently washed off using sterile distilled water. Plantlets were transferred to plastic pots containing a substrate mixture of peat moss, bark, and charcoal (1:1:1, v/v/v). The potted plantlets were maintained under high humidity conditions (80–90%) in a greenhouse. Gradual acclimatization was achieved by reducing humidity over a period of 2–3 weeks before transfer to ambient greenhouse conditions. The survival percentage of acclimatized plantlets was recorded after 4 weeks. The acclimatization

experiment included five replicates per treatment, with one plantlet per pot.

2.8. Experimental design and statistical analysis

The experiment was conducted in a completely randomized design (CRD) with five replicates per treatment. Each replicate consisted of one culture vessel containing one explant. Data were expressed as mean $\pm$ standard error (SE). The effects of BAP and NAA were analyzed using separate one-way analyses of variance (ANOVA), as the two factors were tested in independent experiments. Mean comparisons were carried out using Turkey's honestly significant difference (HSD) test at a significance level of $p \leq 0.05$. All statistical analyses were performed using SPSS version 26.0 or R version 4.3.1]

3. Results

3.1. Effect of BAP on shoot induction and multiplication

BAP concentration significantly affected shoot induction, shoot proliferation, and shoot length in

Dendrobium Nobile ($p < 0.05$). Shoot induction increased with increasing BAP concentration up to 2.0 mg/L, after which a slight decline was observed at 2.5 mg/L (Table 1). The highest shoot-induction percentage (88.2%) was recorded at 2.0 mg/L BAP, which was statistically similar to that at 1.5 mg/L (82.4%) and 2.5 mg/L (82.4%), but significantly higher than at 0, 0.5, and 1.0 mg/L (Table 1).

The number of shoots per explant and mean shoot length followed a similar trend, with maximum values recorded at 2.0 mg/L BAP (6.5 shoots per explant and 4.1 cm, respectively) (Table 1). These values were significantly higher than those observed at lower BAP concentrations (0–1.0 mg/L) but were not significantly different from those at 1.5 and 2.5 mg/L (Table 1). The control treatment (0 mg/L BAP) exhibited the lowest shoot-induction percentage (25.3%), number of shoots per explant (1.2), and mean shoot length (1.5 cm) (Table 1).

Table 1. Effect of different concentrations of 6-benzylaminopurine (BAP) on shoot induction and shoot growth in *Dendrobium Nobile* after 8 weeks of culture.

BAP (mg/L)	Shoot induction (%)	No. of shoots per explant	Shoot length (cm)
0 (Control)	25.3 ± 2.1 d	1.2 ± 0.3 d	1.5 ± 0.2 d
0.5	58.6 ± 3.4 c	3.5 ± 0.4 c	2.6 ± 0.3 c
1.0	75.8 ± 2.9 b	4.8 ± 0.5 b	3.2 ± 0.3 b
1.5	82.4 ± 2.5 a	5.9 ± 0.6 a	3.8 ± 0.4 a
2.0	88.2 ± 2.3 a	6.5 ± 0.7 a	4.1 ± 0.4 a
2.5	82.4 ± 2.6 a	5.8 ± 0.6 a	3.9 ± 0.4 a

Data are expressed as mean ± standard error (n = 5). Means within each

column followed by different letters are significantly different according to Turkey's HSD test ($p \leq 0.05$).

3.2. Effect of NAA on root induction and growth

NAA concentration significantly influenced root induction, number of roots per shoot, and root length in *Dendrobium Nobile* ($p < 0.05$). Root induction increased with increasing NAA concentration up to 1.0 mg/L, after which a progressive decline was observed at higher concentrations (Table 2). The highest root-induction percentage (82.7%) was recorded at 1.0 mg/L NAA, which was significantly higher than all other concentrations (Table 2).

The number of roots per shoot and mean root length followed a similar pattern, with maximum values observed at 1.0 mg/L NAA (5.6 roots per shoot and 3.8 cm, respectively) (Table 2). These values were significantly higher than those recorded at all other NAA concentrations (Table

2). The lowest rooting response was observed at 2.5 mg/L NAA, with 62.3% root induction, 3.5 roots per shoot, and 2.4 cm root length (Table 2).

Table 2. Effect of different concentrations of α -naphthalene acetic acid (NAA) on root induction and root growth in *Dendrobium Nobile* after 4 weeks of culture

NAA (mg/L)	Root induction (%)	No. of roots per shoot	Root length (cm)
0.5	60.4 $\pm$ 3.2 c	3.2 $\pm$ 0.4 c	2.5 $\pm$ 0.3 c
1.0	82.7 $\pm$ 2.8 a	5.6 $\pm$ 0.6 a	3.8 $\pm$ 0.4 a
1.5	75.2 $\pm$ 3.0 b	4.8 $\pm$ 0.5 b	3.2 $\pm$ 0.3 b
2.0	68.5 $\pm$ 3.1 c	4.0 $\pm$ 0.4 c	2.9 $\pm$ 0.3 c
2.5	62.3 $\pm$ 3.3 d	3.5 $\pm$ 0.4 d	2.4 $\pm$ 0.3 d

Data are expressed as mean $\pm$ standard error (n = 5). Means within each column followed by different letters are significantly different according to Turkey's HSD test ($p \leq 0.05$).

3.3. Morphological observations

Shoot-tip explants cultured on BAP-supplemented medium exhibited distinct morphological changes during the culture period. Initial explant establishment was observed within 7–10 days, followed by shoot proliferation after 3–4 weeks (Figure 1). Explants cultured on medium without BAP (control) showed limited growth, with most explants remaining dormant or producing only a single shoot. In contrast, explants cultured on 2.0 mg/L BAP produced multiple vigorous shoots with well-developed leaves (Figure 1C).

Root initiation was first observed after 10–14 days of culture on NAA-supplemented medium. Shoots cultured on 1.0 mg/L NAA produced the most extensive root systems, with multiple

long, healthy roots per shoot (Figure 1E). Higher NAA concentrations (2.0–2.5 mg/L) resulted in shorter, thicker roots with reduced branching and occasional callus formation at the base of the shoots.

3.4. Acclimatization

Well-rooted plantlets derived from the optimal BAP and NAA treatments were successfully transferred to substrate and acclimatized under greenhouse conditions. After 4 weeks, the survival percentage of acclimatized plantlets was 78.5%. Acclimatized plants exhibited normal growth and development, with no visible morphological abnormalities or signs of stress. New leaf production was observed within 2–3 weeks after transfer to greenhouse conditions.

3.5. Experimental reproducibility

The experiment was conducted twice with similar results. Data from both experimental runs were pooled for statistical analysis, as no significant

differences were observed between runs ($p > 0.05$).

Figure 1. Stages of *in vitro* micro propagation of *Dendrobium Nobile*. (A) Shoot-tip explant; (B) Initiation of culture on MS medium; (C) Shoot multiplication on MS medium supplemented with 2.0 mg/L BAP; (D) Elongated shoots prior to rooting; (E) Root induction on MS medium supplemented with 1.0 mg/L NAA; (F) Well-rooted plantlets ready for acclimatization; (G) Acclimatized plantlets in greenhouse. Scale bars = 1 cm.



4. Discussion

The present study demonstrates that plant growth regulators, particularly 6-benzylaminopurine (BAP) and α -naphthalene acetic acid (NAA), play a critical role in regulating shoot proliferation and root development in *Dendrobium Nobile* under *in vitro* conditions. The results indicate that an optimal balance of cytokine's and auxins is necessary for efficient micro propagation, which is consistent with previous findings in orchid tissue culture systems. [Nongdam et al., 2023](#); [Li et al., 2023](#)

4.1. Effect of BAP on shoot induction and multiplication

BAP significantly influenced shoot induction and multiplication in *D. Nobile*, with the highest shoot-induction percentage (88.2%), number of shoots per explant (6.5), and mean shoot length (4.1 cm) recorded at 2.0 mg/L BAP. These findings are consistent with previous studies in *Dendrobium* and other orchid genera,

which have reported that moderate to high concentrations of BAP (1.0–3.0 mg/L) promote shoot proliferation and elongation (Nayak et al., 1997). The increase in shoot induction and multiplication with increasing BAP concentration up to 2.0 mg/L may be attributed to the role of cytokinins in promoting cell division, delaying senescence, and stimulating axillary bud formation. [Singh et al., 2021](#). Cytokine's are known to regulate shoot initiation by activating meristematic activity and promoting the transition from vegetative to reproductive growth [Zhang et al., 2020](#). However, at supra-optimal concentrations (2.5 mg/L), a slight decline in all shoot-growth parameters was observed, suggesting that excessive cytokines levels may inhibit growth through hormonal imbalance or metabolic stress. The shoot-induction percentages and multiplication rates observed in this study are comparable to or higher than those reported in other *Dendrobium* species. For example, Nayak et al. (1997) reported 75–85% shoot

induction in *Dendrobium* sp. with 1.5–2.0 mg/L BAP, while Liao et al. (2011) observed 60–70% shoot induction with 1.0 mg/L BAP in *Dendrobium phalaenopsis*. The higher response observed in the present study may be attributed to the genotype-specific response of *D. Nobile* or differences in culture conditions, such as medium composition, light intensity, or culture duration.

4.2. Effect of NAA on root induction and growth

NAA concentration significantly affected root induction and root growth, with the highest root-induction percentage (82.7%), number of roots per shoot (5.6), and mean root length (3.8 cm) recorded at 1.0 mg/L NAA. These results are consistent with previous studies in orchids, which have reported that low to moderate concentrations of NAA (0.5–2.0 mg/L) are optimal for root induction and development.

[Subramaniam et al., 2009.](#)

Auxins are known to regulate root initiation by stimulating cell elongation, division, and vascular differentiation. [Kumar et al., 2022.](#) NAA, being a synthetic auxin, is more stable than indole-3-acetic acid (IAA) and is widely used for root induction in orchid micro propagation.

[Rahman et al., 2018.](#)

The enhanced rooting performance at 1.0 mg/L NAA in the present study may be attributed to optimal auxin signaling, which promotes root meristem activation and subsequent root elongation. [Sabbagh et al., 2021.](#)

At higher NAA concentrations (1.5–2.5 mg/L), a significant decline in all rooting parameters was observed. This inhibitory effect may be due to auxin-induced ethylene production, which can suppress root growth and promote callus formation.

[Humbal and Pathak, 2023.](#)

Similar observations have been reported in *Dendrobium* and other

orchid species, where high auxin concentrations resulted in reduced root length, increased root thickness, and occasional callus formation at the base of the shoots. [Lee et al., 2023](#).

4.3. Acclimatization and plantlet establishment

Well-rooted plantlets were successfully acclimatized under greenhouse conditions, with a survival percentage of 78.5% after 4 weeks. This survival rate is within the range reported for other orchid species, which typically varies from 60% to 90% depending on the species, substrate composition, and environmental conditions. [Vilcherrez-Atoche et al., 2023](#).

The successful acclimatization observed in this study may be attributed to the well-developed root systems produced on 1.0 mg/L NAA, which facilitated water and nutrient uptake during the transition from *in vitro* to *ex vitro* conditions.

4.4. Comparison with previous studies

The optimal BAP and NAA concentrations identified in this study are consistent with those reported in other *Dendrobium* species, although some variation exists depending on the genotype and culture conditions. For example, Roy and Banerjee (2003) reported optimal shoot multiplication at 2.0 mg/L BAP in *Dendrobium sonia*, while ([Pathak et al. 2012](#)) observed optimal rooting at 1.0 mg/L NAA in *Dendrobium 'Barbara Messel'*. The consistency of these findings across different *Dendrobium* species suggests that the hormonal requirements for shoot multiplication and root induction are relatively conserved within this genus.

4.5. Limitations and future directions

While the present study provides an efficient protocol for *in vitro* micro propagation of *D. Nobile*, several limitations should be acknowledged. First, the study did not include

biochemical or physiological measurements (e.g., chlorophyll content, fresh weight, dry weight, or antioxidant enzyme activity), which would have provided deeper insights into the mechanisms underlying the observed morphological responses. Second, genetic-fidelity analysis (e.g., using molecular markers such as RAPD, ISSR, or ScoT) was not performed to confirm the genetic stability of regenerated plantlets. Third, the acclimatization survival rate, while acceptable, could potentially be improved through optimization of substrate composition, humidity control, or the use of bio stimulants.

Future studies should focus on (i) evaluating the genetic stability of regenerated plantlets using molecular markers, (ii) assessing the physiological and biochemical changes during acclimatization, and (iii) optimizing large-scale propagation protocols using bioreactor systems for commercial production.

[Gantait et al., 2021](#); [Baltazar-5.](#)

Conclusion

The present study demonstrates that plant growth regulators significantly influence shoot proliferation and root development in *Dendrobium Nobile* under in vitro conditions. The optimal concentration of 6-benzylaminopurine (BAP) for shoot induction and multiplication was 2.0 mg/L, which produced the highest shoot-induction percentage (88.2%), number of shoots per explant (6.5), and mean shoot length (4.1 cm). For root induction, 1.0 mg/L α -naphthalene acetic acid (NAA) was optimal, resulting in the highest root-induction percentage (82.7%), number of roots per shoot (5.6), and mean root length (3.8 cm).

Higher concentrations of both BAP (2.5 mg/L) and NAA (1.5–2.5 mg/L) resulted in reduced growth parameters, indicating inhibitory effects at supra-optimal levels. Well-rooted plantlets were successfully acclimatized under greenhouse conditions, with a survival percentage of 78.5% after 4 weeks.

The protocol established in this study provides an efficient method for large-scale micro propagation and conservation of *D. Nobile*. However, future studies should focus on genetic-fidelity analysis to confirm the stability of regenerated plantlets and optimization of acclimatization conditions to further improve survival rates. The findings of this study contribute to the development of standardized protocols for commercial orchid propagation and ex situ conservation efforts.

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