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In vitro Regeneration of Dendrobium terminale, a CITES-Listed Orchid for Sustainable Conservation

Research Article

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Abstract

The present study develops an efficient and reproducible protocol for the rapid in vitro mass propagation of the critically endangered epiphytic orchid *Dendrobium terminale* Par. & Rchb. Juvenile seedlings were produced from seed suspension culture. *Dendrobium terminale* is included in Appendix II of CITES (The Convention on International Trade in Endangered Species of Wild Fauna and Flora), showing its alarming position in natural habitat, ultimately emphasizing the need for its multiplication and conservation. Half MS media alone or supplemented with a combination of various concentrations of growth regulators: 6-Benzyl-aminopurine, BAP (2 and 4mg/L), 2iPA and Indole acetic acid, IAA (2mg/L and 4mg/L), were used, which produced callus, PLB, multiple shoots and leaves. The concentrations and combinations of auxin and cytokinin have a significant impact on plant growth, with certain combinations resulting in synergistic effects. It has been observed that BAP (4mg /L) and IAA (2 mg/L) were found to be most effective in promoting plant growth among the different combinations and concentrations of BAP, 2iPA, with IAA. The BAP (4 mg l^{-1}) and IAA (2 mg l^{-1}) recorded the highest number of PLB formation and significant leaf growth. The combination of IAA (2 mg l^{-1}) and 2iPA (2 mg l^{-1}) also promotes a higher percentage of PLB formation (75%) without necrosis. Whereas higher concentrations of cytokinin (e.g., 2iPA (4 mg l^{-1}) tend to favour more callus formation.

Keywords: *In vitro*; Dendrobium terminale; Callus; PLB; IAA (indole acetic acid); BAP (benzyl aminopurine); 2iPA

Introduction

Orchid plants, which are members of the Orchidaceae family, are renowned for their exquisite flowers and distinctive morphological features. The orchidaceae family is quite large, with 27,000 accepted species and more than 31,000–35,000 species are estimated to exist [1]. Orchids reign throughout the world, in both high alpine and tropical regions. They can be classified as terrestrial, epiphytic, or saprophytic depending on the kind of habitat they grow in, except aquatic systems. Many diverse civilizations and tribes use orchids for their decorative purposes, and others use them as food and as herbal remedies [2].

Among flowering plants, orchids are the most vulnerable species worldwide. Orchid species are severely threatened due to a variety of factors, including over exploitation, illegal trading, land invasion, and climate change [3]. They receive a variety of pollinators, like as insects, tiny birds, and even bats, in addition to more light and air. Over the millennia, this special relationship between orchids and their pollinator has changed, giving the floral parts incredible proportions and shapes[4].e germination rate of orchid seeds in nature is extremely low due to their small size, non-endospermic origin, and reduced embryo encased in a somewhat translucent covering [4].

In addition to their challenging germination process, orchids have an extremely lengthy life cycle when left in their natural habitat. These plants need five to ten years to bloom and set fruitful seeds. Orchid vegetative propagation is likewise a very slow process, so for large-

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scale production can be done by *in vitro* techniques. Nonetheless, a lot of horticultural plants are originally cultivated from tissue culture (rather than seeds) and maintained in highly enriched media, which eliminates the requirement for a symbiotic fungus [5]. It is now acknowledged that the plant tissue culture approach offers a viable substitute for large-scale orchid propagation and conservation of vulnerable, endangered, and threatened species. With these techniques, a large number of identical clones can be grown from a single protocorm or shoot tip explant [6].

Dendrobium Sw. is one of the three largest genera in the family Orchidaceae, with about 800-1500 species worldwide. The majority of *Dendrobium* species are terrestrial, very few are epiphytes in primary forests, and are less frequent lithophytes with worldwide distribution, except the coldest and driest areas of the world [7] . About 30 species have shown common distributional patterns and are present in almost all the states of North-East India [8]. Among them, *Dendrobium terminale* are is included in Appendix II of CITES (The Convention on International Trade in Endangered Species of Wild Fauna and Flora), where its status is now threatened with extinction, which means strict regulation in trading is required for their survival [9]. The attractive flowers are 10-12 mm tall, white or pink with many longitudinal pink stripes on the lip, and are borne singly or in pairs close to the apex of the stem [10]. For many reasons, including their slow development, low seed germination rate, and low regeneration rate, orchid conservation is an international concern. The main factors affecting their natural population decline include habitat damage, overharvesting and illegal trafficking, and pressure from human population growth [11]. The most economically successful tissue culture technique is the alternative means of plant vegetative propagation known as micropropagation. The *in vitro* approach through the application of plant tissue culture technology provides an excellent opportunity for effective conservation by mass propagating orchids in a short period [12]. The significant advantage offered by micropropagation over conventional methods is that a large number of plants can be produced from a single individual, independent of the seasons [13]. Several valuable species of *Dendrobium* have been reported to be propagated through asymbiotic germination of immature seeds or direct shoot regeneration of PLBs from different explants. [14] . The objective of the current study is to establish of suitable protocol for successful in vitro culture of *Dendrobium terminale* for PLB and callus formation.

Materials and Methods

Two-month-old juvenile seedlings of *Dendrobium terminale* Par. & Rchb has been used as plant material, which has been developed from seed suspension culture. The work was carried out in the Cytogenetics and Plant Tissue Culture Laboratory, Department of Botany, Siksha Bhavana, Visva Bharati, Santiniketan, during 2023 and 2024. Murashige and Skoog [15] (1962), a medium in half concentration were used for culturing the planting materials. The MS medium was supplemented with different plant growth regulators (BAP, 2iPA, and IAA) in different concentrations. The culture is maintained under 16±5°C temperature and 8 hours of photoperiod in four replications for 60 days.

The 1 L stalk solution was prepared by adding 2.3 g of half MS media in 200ml of distilled water, then 30 g of sucrose was added. The solution was supplemented with various concentrations of the growth regulators listed in (Table 1) and properly mixed. Then, by adding distilled water, the mixture volume is made up to 1 L. By adding either 0.1 N NaOH or 0.1 M HCl, the medium's pH was adjusted to 5.5. After that, 8gms of Agar powder was added to the mixture and dissolved by heating. The prepared media were poured into different culture tubes, then covered with a cotton plug and autoclaved at 121°C for 20 minutes under 15 psi pressure. After cooling at room temperature, the medium was used for inoculation and incubation. A plant growth regulator-free medium was used as a control.

Results and Discussion

The studies recorded that seed suspension growth of *Dendrobium terminale* is successful in ½ MS medium. Thus, ½ MS medium supplemented with combination of IAA with BAP, 2iPA and 2iPA alone was inoculated with a single seedling. After 60 days of growth, every change in growth was carefully observed and recorded (Figure 1). In control, very little growth has been observed. The combination of IAA (2 mg l^{-1}) + BAP (mg l^{-1}) showed relatively high PLB formation and leaf growth. Whereas IAA (2 mg l^{-1}) + BAP (4 mg l^{-1}) has resulted in significantly increased PLB formation, leaf growth, and

Table 1: Different concentrations of growth regulators supplemented with ½ MS medium

1. ½ MS(CONTROL)
2. ½ MS + IAA (2 mg. l^{-1}) +BAP (2 mg. l^{-1})
3. ½ MS + IAA (2 mg. l^{-1}) +BAP (4 mg. l^{-1})
4. ½ MS + IAA (4 mg. l^{-1}) +BAP (2 mg. l^{-1})
5. ½ MS + IAA (2 mg. l^{-1}) +2iPA (2 mg. l^{-1})
6. ½ MS + IAA (2 mg. l^{-1}) + 2iPA (4 mg. l^{-1})
7. ½ MS + IAA (4 mg. l^{-1}) +2iPA (2 mg. l^{-1})
8. ½ MS + 2iPA (2 mg. l^{-1})

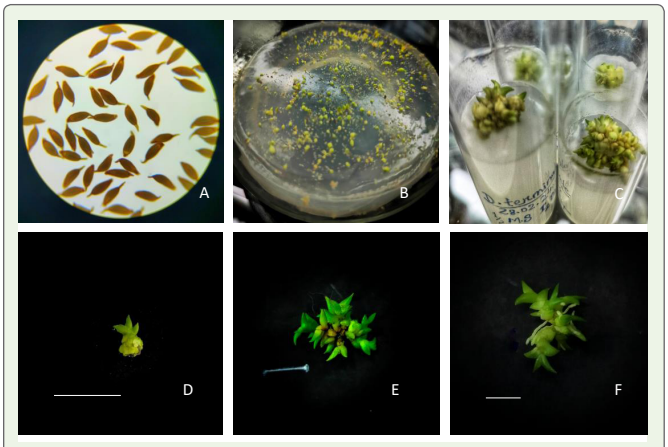

Figure 1: Multiple shoots and root formation *in vitro* culture of *Dendrobium terminale* (A) Microscopic seeds of *D. terminale* obtain from manual pollination (10X compound microscope), (B) Developing globular PLB, (C) mature PLB and Callus, (D) Mature PLB with leaf primordia, (E) Multiple shoots, (F) Well rooted multiple shoots on ½ MS medium supplemented with IAA (2 mg l^{-1}) and BAP (1 mg l^{-1})

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Table 2: Effect of IAA, BAP and 2iPA on multiple shoots and root development in *Dendrobium terminal*

Treatment (mg. l^{-1})	Number of multiple shoots (±SE)	Number of roots (±SE)
CONTROL	2.75±1.65^{ns}	0
I0.5/B0.5	3.5±1.32^{ns}	0
I0.5/B1	3.5±1.66^{ns}	1.2±1.32^{ns}
I 0.5/B2	3.75±1.89^{ns}	0.8±1.52^{ns}
I1/B0.5	8.0±1.35^{ns}	2.5±1.21^{ns}
I1/B1	7.25±2.06^{ns}	2.1±0.8*
I1/B2	12.25±1.55**	3.2±0.68**
I2/B0.5	4.75±0.85*	2.6±0.75^{ns}
I2/B1	5.5±3.8^{ns}	3.6±1.02***
I2/B2	4.75±1.60^{ns}	2.4±1.56^{ns}
I 0.5/2iPA0.5	4.25±0.75*	1.4±1.11^{ns}
I 0.5/2iPA1	2.0±1.68^{ns}	1.2±1.26^{ns}
I 0.5/2iPA2	3.5±1.47^{ns}	1.8±0.98*
I1/2iPA0.5	5.0±1.22^{ns}	0.6±1.42^{ns}
I1/2iPA1	3.5±0.50*	0
I1/2iPA2	4.25±1.11^{ns}	0
I2/2iPA0.5	3.0±1.22^{ns}	0
I2/2iPA1	6.5±1.71^{ns}	1.2±1.32^{ns}
I2/2iPA2	2.0±1.47^{ns}	1.12±1.52^{ns}

(Here I denote IAA, B denotes BAP)

(Values are expressed as mean ± standard error (SE). Asterisks indicate statistically significant differences (*Tukey's test*, $p < 0.05$). Statistical analysis was performed using GraphPad Prism version 9.0).

root development compared to the control and other combinations. However, IAA (4 mg l^{-1}) + BAP (2 mg l^{-1}) leads to a decrease in PLB formation (25%) and an increase in callus (50%), without necrosis. The combination of IAA with 2iPA responded differently in different combinations. The IAA (2 mg l^{-1}) + 2iPA (2 mg l^{-1}) showed moderate PLB formation and leaf growth than IAA(2mg/L) + 2iPA (4 mg l^{-1}) and control. The combination of IAA (2 mg l^{-1}) + 2iPA (4 mg l^{-1}) induced higher PLB and callus formation. The combination of IAA (4 mg l^{-1}) + 2iPA (2 mg l^{-1}) equals the proportion of PLB and callus formation. The addition of 2iPA (2 mg l^{-1}) alone in culture medium lowers the PLB but induces callus formation.

Certain researchers have reported the healthy growth of orchid protocorms in media having a balanced supply of organic and inorganic nutrients. According to research on *D. aphyllum* tissue culture on MS media, the seeds of the plant responded progressively to varying kinetin concentrations [16]. The combination of IAA (2 mg l^{-1}) and BAP (4 mg l^{-1}) seems to be the most effective in promoting plant growth, as it leads to the highest number of PLBs, significant leaf growth. A simple and efficient growth protocol was developed for *Dendrobium lowii*, an endangered and Borneo's endemic epiphyte orchid, using four-month-old protocorms as explant sources produced by asymbiotic seeds germination [17]. *Dendrobium lowii* protocorms were cultivated on Knudson C (KC) medium supplemented with plant growth regulators (NAA, Zeatin, and BAP) at varying doses. But our study showed that ½ MS supplemented with IAA, BAP, and 2iPA has a significant impact on plant growth with synergistic effects. The plant growth regulators BAP, 2iPA and IAA are very suitable for quick micropropagation because they have significant role in cell division and root-shoot formation, leading correct direction of plant developmental pathway under in vitro conditions mimicking natural growth hormones.The combination of IAA (2 mg l-1) and 2iPA (2 mg l-1) promotes the highest percentage of direct somatic embryogenesis (75%) without inducing necrosis. Higher concentrations of cytokinin 2iPA (4 mg l-1) tend to favour the formation of callus over PLB.

The results indicate that the combination of IAA (1 mg/L) with BAP (2 mg/L) (I1/B2) was the most effective treatment, significantly enhancing both multiple shoot formation (12.25 ± 1.55) and root induction (3.2 ± 0.68), marked by a high level of statistical significance (**$p < 0.01$**). This suggests a strong synergistic effect between these concentrations of auxin and cytokinin. Another effective combination was I2/B1, which yielded the highest number of roots (3.6 ± 1.02) with high significance (***$p < 0.001$***), although its shoot number was comparatively moderate (5.5 ± 3.8) and not statistically significant. Other combinations, such as I1/B0.5 and I1/B1, also showed improved shoot (8.0 ± 1.35 and 7.25 ± 2.06, respectively) and root numbers, although without consistent significance. In contrast, treatments involving 2iP generally resulted in lower shoot and root counts, with no combination showing comparable performance to the BAP + IAA treatments. The control and low concentrations of IAA and cytokinins (e.g., I0.5/B0.5 or I0.5/B1) resulted in poor shoot proliferation and little to no rooting. Overall, the combination of **IAA 1 mg/L and BAP 2 mg/L** emerged as the optimal condition for simultaneous shoot and root development in *Dendrobium terminale*, underlining the greater efficacy of BAP over 2iP in this orchid species. The superior response observed with the combination of BAP (6-benzylaminopurine) and IAA (indole-3-acetic acid) in the micropropagation of *Dendrobium terminale* can be attributed to their synergistic roles in promoting protocorm-like body (PLB) formation and shoot induction. BAP, a potent cytokinin, effectively stimulates cell division and shoot bud initiation, while IAA, a natural auxin, supports cell elongation and root meristem development. When applied together in an optimal ratio (BAP 2 **mg. l^{-1}**+ IAA 1 **mg. l^{-1}**), they create a hormonal balance that favours organized tissue development over callusing, enhancing both shoot proliferation and early root induction. Compared to other plant growth regulator combinations

Conclusion

In conclusion, this study successfully established an *in vitro* culture protocol for *Dendrobium terminale*, a threatened and conservation-priority orchid species. After 60 days of culture, the maximum protocorm-like body (PLB) formation occurred on MS medium supplemented with BAP (4 mg l^{-1}) and IAA (2 mg l^{-1}). This was followed by a combination of 2iPA (4 mg l^{-1}) and IAA (2 mg l^{-1}), while the lowest PLB induction was observed with IAA (2 mg l^{-1}) and 2iPA (2 mg l^{-1}). The medium containing 2iPA (2 mg l^{-1}) was found to promote greater leaf development. For root induction, the most effective response was obtained from MS medium fortified with IAA (2 mg l^{-1}) and BAP (1 mg l^{-1}).

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