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In-vitro Propagation of *Dendrobium chryseum* Rolfe

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Abstract

Dendrobium chryseum is an endangered epiphytic and lithophytic orchid species having medicinal and ornamental value. The objective of the present study is to develop micropropagation protocol for *D. chryseum.* The immature seeds of *D. chryseum* were used as an explant for the micropropagation. Protocorms were developed within 90 days in Murashige and Skoog (MS) medium without plant growth regulators. Protocorms were differentiated into micro shoots after 30 days of transfer to MS medium supplemented with 2 mg/L Benzyl amino purine (BAP), 1 mg/L kinetin and 10% coconut water. Among eleven different combinations of plant growth regulators (BAP, á-Naphthalene acetic acid (NAA), Adenine sulphate, Kinetin and coconut water) treated for shoot multiplication, maximum number of shoots were obtained in 0.5 mg/L BAP + 0.5 mg/L NAA (5.8 ± 0.53 SE shoots per explant). Longest shoot length was recorded in MS medium with Adenine sulphate (1 mg/L) (2.54 ± 0.03 cm SE). Root induction was carried out by using MS medium with different concentrations (0.5, 1, 1.5, 2.0 mg/L) of Indole butyric acid (IBA) and NAA. The highest numbers of roots and longest root length, both were observed at 2 mg/L IBA (4.63 ± 0.56 SE and 2.09 ± 0.25 cm SE respectively). 2 mg/L NAA showed poor response for root number (0.94 ± 0.21 SE) and root length (0.43 ± 0.07 cm SE). Successful acclimatization of *in-vitro* grown plantlets was done by wrapping the plantlet with moss kept on fine pine bark and the survival rate of plantlet was about 80% after 30 days. This protocol could be helpful for the effective mass propagation and *ex situ* conservation of *D. chryseum*.

Keywords: *Ex situ* conservation, MS medium, Orchid, Plant growth regulators, Protocorms

Introduction

The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) have listed orchids in the Appendices I and II. They comprise various attractive, beautiful and long lasting flowers. Orchids have high ornamental and medicinal values. A total of 440 orchid species belonging to 95 genera has been reported from Nepal (Shrestha et al., 2022). The diversity of orchids covers more than 7% of total flowering plants of Nepal (Rokaya et al., 2013). More than 100 species of Nepalese orchids have medicinal value (Rajbhandari, 2014).

A huge amount of illegal trade of wild medicinal orchids have been reported from Nepal (Subedi et al., 2013). Due to the over exploitation for trade, consumption and habitat loss, many of these orchids are facing the extreme danger of extinction (Pant et al., 2018). Thus, it is a high time to conserve and seek the alternative means for conservation and sustainable use of orchids, thus reducing the problem of illegal trade. Orchid conservation is a matter of global concern because orchid seeds have only 1% germination in nature due to absence of endosperm and need a specific mycorrhizal fungal association (Saha et al., 2019).

There are 30 species of *Dendrobium* in Nepal (Shrestha et al., 2022). *Dendrobium chryseum* Rolfe is an endangered epiphytic and lithophytic orchid species having ornamental and medicinal value. It is distributed in Bangladesh, China, India, Laos, Myanmar, Nepal, Taiwan, Thailand, Vietnam (https://www.powo.science.kew.org). It usually grows in cold climate at the elevation range of 1000 m asl to 2150 m asl, but due to over exploitation and deforestation it has been confined in limited areas and therefore, its existence is threatened (Joshi et al., 2017). In traditional Chinese medicine, *D. chryseum* is popularly used for its antipyretic and immune-modulatory effects. Five bibenzyls, three phenanthrenes and acoumarin have been

isolated from *D. chryseum* (Yang et al., 2007). The chemical compound isolated from *D. chryseum* exhibited antioxidant activity (Yang et al., 2007). *In-vivo* pharmacological experiments showed that polysaccharides from the plant inhibited tumor growth and reduced blood glucose (Liu et al., 2009).

Micropropagation is the most important practical application of plant biotechnology (da Silva et al., 2015). Orchid pods contain millions of tiny seeds having naked undifferentiated embryos without any functional endosperm. Green pod culture technique is one of the advancements in orchid seed culture for micropropagation, in which immature seeds within the green pod obtained from the plant after fertilization but prior to dehiscence are cultured on nutrient medium (Sharma et al., 2005). *In-vitro* propagation of immature seeds of many species of *Dendrobium* species through direct regeneration of protocorms has been carried out by Nayak et al. (1997) and Nayak et al. (2002). *In-vitro* propagation of this endangered orchid *D. chryseum* from protocorms culture has previously been done by Maharjan et al. (2020). In the present study, green immature pod of *D. chryseum* was cultured *in-vitro* to develop protocorms which were further used for *in vitro* propagation. The main objective of the present study was to develop micropropagation protocol for *D. chryseum* from seed which could be a useful approach for germplasm conservation as well as mass propagation.

Materials and Methods

Plant materials and its sterilization

Immature capsule of *Dendrobium chryseum* was collected from Maipokhari Botanical Garden (27°007'N, 87°93'E, 2100 m asl), Ilam district during February 2021. The fresh capsule was air dried for 10 days. The capsule was rinsed with detergent Tween-20 under the running water for 45 min. followed by rinsing with distilled water thrice to completely remove the traces of Tween-20 from the surface of the capsule. The capsule was surface sterilized with 70% ethanol for 2 min. followed by 0.1% mercuric chloride for 5 min. and then rinsed with sterile distilled water thrice.

Nutrient medium for culture

Murashige and Skoog (MS) was used as the basal nutrient medium as it has been considered as the most common basal media for *Dendrobium* (da Silva et al., 2015). Different concentration of 6-benzylaminopurine (BAP), α-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA), kinetin and adenine sulphate were added in the nutrient medium. As a source of carbon and gelling agent, 3% (w/v) sucrose and 0.8% (w/v) agar were added. Thereafter, before autoclaving, pH of entire medium was adjusted to 5.8 using 0.1N NaOH or HCl. Finally, the media were autoclaved at 121°C and 15 psi for 20 min.

Seeds inoculation and its germination

The surface sterilized capsule was longitudinally cut on sterilized petridish with a sterile blade and then seeds were spread thinly over the surface of solidified full strength MS medium. After 90 days, the seeds developed into the protocorms on the MS medium without plant growth regulators. Protocorms were transfer to the MS medium supplemented with 2 mg/L BAP, 1 mg/L kinetin and 10% coconut water.

Multiplication and elongation of shoots

About 0.5 to 0.8 cm long shoot tips were used as explants for shoot multiplication. MS medium with different concentration of BAP (0.5, 1, 1.5 and 2 mg/L) with or without 0.5 mg/L NAA and Adenine sulphate 1mg/L were used for the multiplication and elongation of shoots.

Root formation on shoots

The single shoot was cultured on MS medium supplemented with different concentrations of rooting hormone (IBA and NAA at 0.5, 1, 1.5 and 2 mg/L respectively). All the culture were maintained at 25 ± 2°C under a 16/8 hrs. light/dark photoperiod cycle using white fluorescent lamps.

Acclimatization

In-vitro rooted plantlets were acclimatized in the room temperature for 15 days. The individual plantlet was rinsed thoroughly with running tap

water to remove all the media residue from it. The individual plantlet was wrapped with moss and then kept on fine pine bark. Watering was done thoroughly to make the moss moist.

Data analysis

For each treatment, all the data were presented as a mean ± standard error of the mean. The significant difference between the MS medium and MS medium with different growth hormones were analyzed by one way ANOVA with F-statistics followed by Tukey multiple comparisons of mean test at 95% confidence interval using RStudio (RStudio Team, 2016) in R platform and all the figures were drawn in R program version 3.6.1 (R Core Team, 2020).

Results and Discussion

Seed germination and protocorm formation

Immature seeds of *D. chryseum* germinated and developed into protocorm in MS medium without plant growth regulators within 90 days of seed culture. MS medium showed good response for the seed germination of *D. chryseum.* MS medium is highly enriched with macro and microelements with different vitamins and the result is similar to the previous study in *Esmeralda clarkei* (Paudel et al., 2012).

Protocorms differentiated into micro shoots after 30 days of transfer to MS medium supplemented with 2 mg/L BAP, 1 mg/L kinetin and 10% coconut water (Figure 1A and 1B). Luo et al. (2008) also found the differentiation of protocorms of *D. densiflorum* into micro shoots within one to two months. The development of micro shoots from germinating seeds was suppressed in MS medium. Pant et al. (2022) observed that MS medium supplemented with 15% coconut water was found suitable for highest number of shoot formation from the protocorms in *Dendrobium densiflorum.* Goswami et al. (2015) observed that ½ MS medium supplemented with 0.5 mg/L NAA and 0.5 mg/L BAP stimulated the increased number of shoots from protocorm-like bodies (PLBs) of *Dendrobium* species.

Multiplication and elongation of shoots

The shoot tips about 0.5-0.6 cm were inoculated on MS medium fortified with different concentration of BAP, NAA, adenine sulphate, kinetin and coconut water. In the present research work, it was found that MS medium alone was not effective for multiple shoot induction. Similar result was obtained in *Dendrobium* species in which plant growth regulators with the nutrient medium was found to be essential for further growth, development and proliferation of shoot tip explants (Yasugi et al., 1994). The maximum number of shoots were obtained at 0.5 mg/L BAP + 0.5 mg/L NAA (5.8 ± 0.53 SE shoots per explant) (Figures 1 C & 2). The longest shoot length was recorded in MS medium with adenine sulphate (1 mg/L) (2.54 ± 0.03 cm SE) (Figures 1D & 3). The shortest shoot length was recorded in MS medium with BAP (1.5 mg/L) (0.78 ± 0.17 cm SE). The minimum number of shoots were obtained at 1 mg/L BAP + 0.5 mg/L NAA (2.06 ± 0.56 SE) and MS medium without hormone (2.37 ± 0.3SE shoots per explant). da Silva et al. (2015) and da Silva & Acharya (2014) reported that multiple shoot production was observed in the combination of BAP and NAA in *Dendrobium* species. According to Ahmed (1996), Sheelavantmath et al. (2000) and Malabadi et al.(2005), shoot multiplication in *Rhynchostylis retusa* was obtained on MS + 2 mg/L IAA + 0.5 mg/L Kin. In *Geodorum* species, multiple shoot formation was obtained in the combination of NAA (2.0μM) + BAP (5.0 μM) (Seeni & Latha, 2000). Multiple shoot development from shoot tip section was significantly promoted by concentrations of BAP (0.5 - 2.0 mg/L) in combination with NAA (0.5 mg/L) in *Esmeralda clarkei* (Paudel et al., 2012). Maximum number of rootless healthy shoots (4.5/culture) on MS medium fortified with BAP (1.5 mg/L) and NAA (0.5 mg/L) (Pant et al., 2012). Similar result was obtained by Dhungana et al. (2022) where the MS medium with BAP (1.5 mg/L) and NAA (0.5 mg/L) were most effective for the shoot multiplication in *Dendrobium crepidatum*.

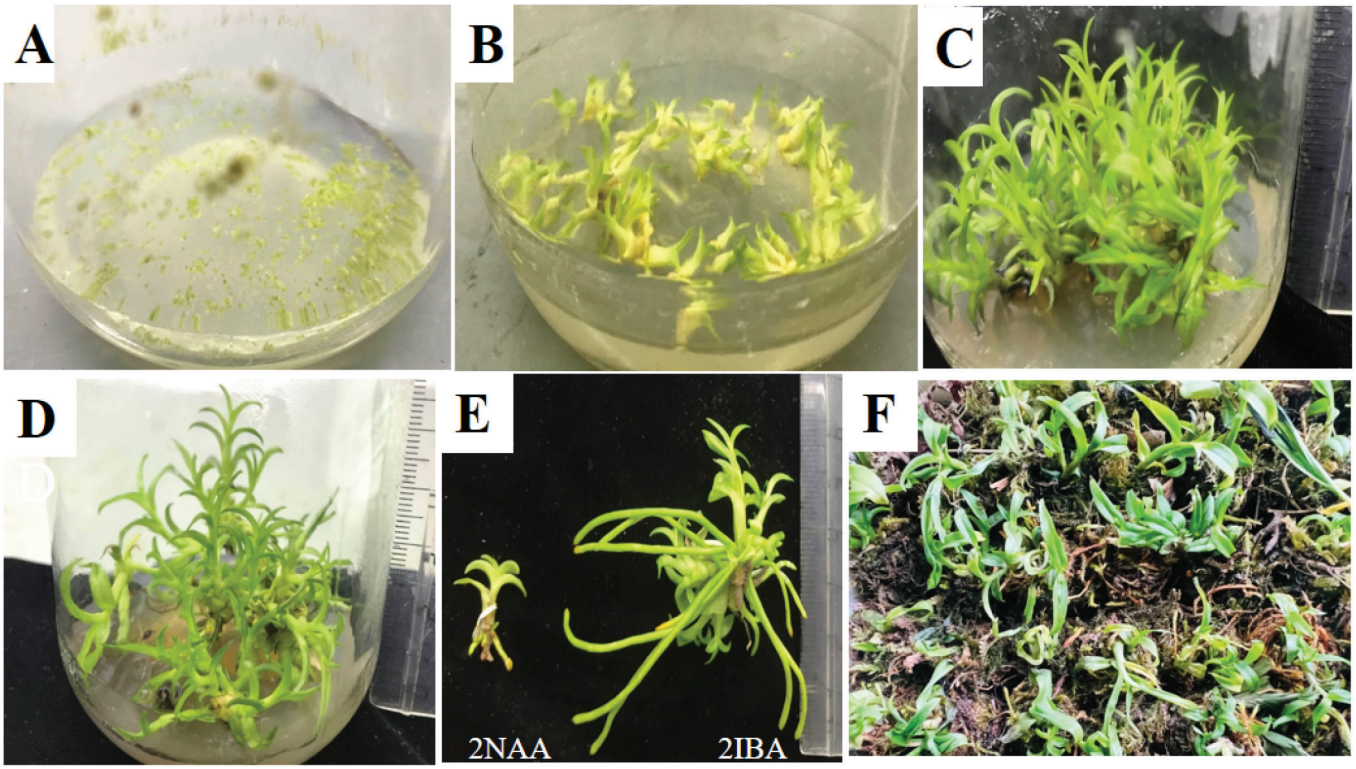

Figure 1: *In-vitro* propagation of *D. chryseum,* **A.** Development of protocorm of *D. chryseum* in MS medium within 90 days of seed culture, **B.** Protocorms were differentiated into micro shoots on MS medium with 2 mg/L BAP, 1 mg/L kinetin and 10% coconut water after 30 days, **C.** Development of multiple shoots on MS medium with 0.5 mg/L BAP + 0.5 mg/L NAA after 16 weeks of culture, **D.** Longest shoot length on MS medium with 1 mg/L Adenine sulphate after 16 weeks of culture, **E.** Minimum and maximum number of roots formation on MS medium with 2 mg/L NAA and with 2 mg/L IBA respectively, after 8 weeks of culture, **F.** Transplanted plantlets wrapped with moss and kept on fine pine bark

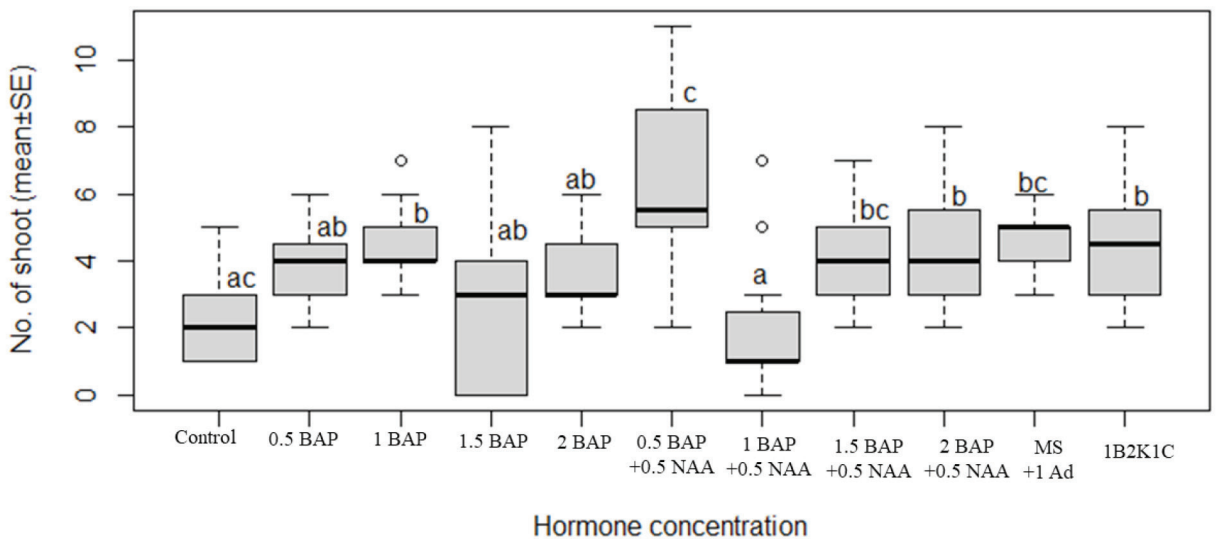

Figure 2: Average number of shoots through shoot tip culture on MS medium with BAP at different concentrations both alone and in combination with NAA, adenine, kinetin and 10% coconut water after 16 weeks of culture
Note: Values are means ± SE, n≥12. Different letters indicate statistically significant difference between different treatments at $p<0.05$

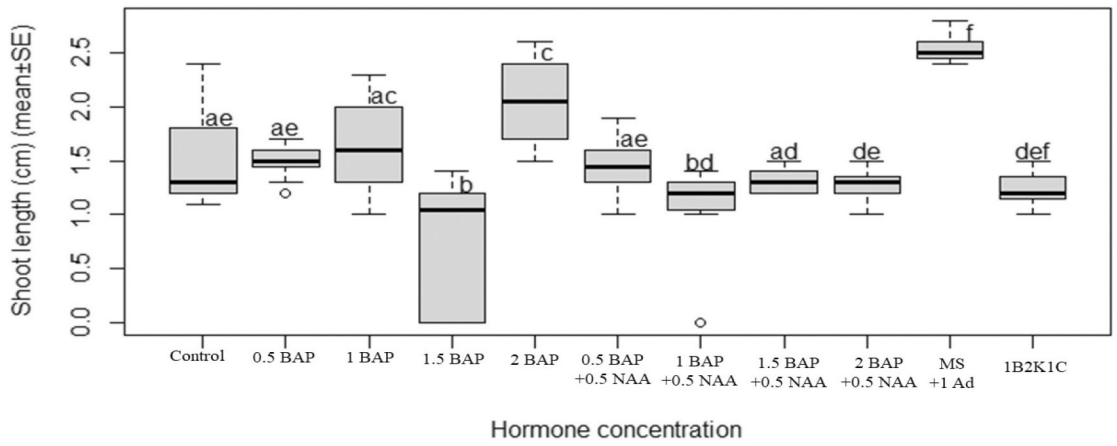

Figure 3: Average shoot length through shoot tip culture on MS medium with BAP at different concentrations both alone and in combination with NAA, adenine, kinetin and 10% coconut water after 16 weeks of culture
Note: Values are means ± SE, n≥12. Different letters indicate statistically significant difference between different treatments at $p<0.05$

Root formation on shoots

Root induction was carried out by using MS medium with different concentrations (0.5, 1, 1.5, 2.0 mg/L) of IBA and NAA. During this research work, the highest numbers of roots and the longest root length, both were observed at 1.5 and 2 mg/L IBA. However, the average number of roots and root length was higher in 2 mg/L IBA (4.63 ± 0.56 SE and 2.09 ± 0.25 cm SE respectively). 2 mg/L NAA showed poor response for root number (2.09 ± 0.25SE) (Figures 1E, 4 & 5). The shortest root length was observed in 2 mg/L NAA (0.43 ± 0.07 cm SE) and MS media without hormone (0.56 ± 0.12 cm SE) respectively. But, Maharjan et al. (2020) reported 1.5 mg/L IAA as the most effective for rooting in *D. chryseum*, besides, NAA performed better response in rooting compared to IBA. In some species of *Dendrobium,* rooting has been highly induced by IBA (Nayak et al., 1997; 2002). Aktar et al. (2007) observed that 1.0 mg/L IBA increased the number and length of root. Asghar et al. (2011) reported that IBA (2 mg/L) increased the rooting percentage by 97.5%, number

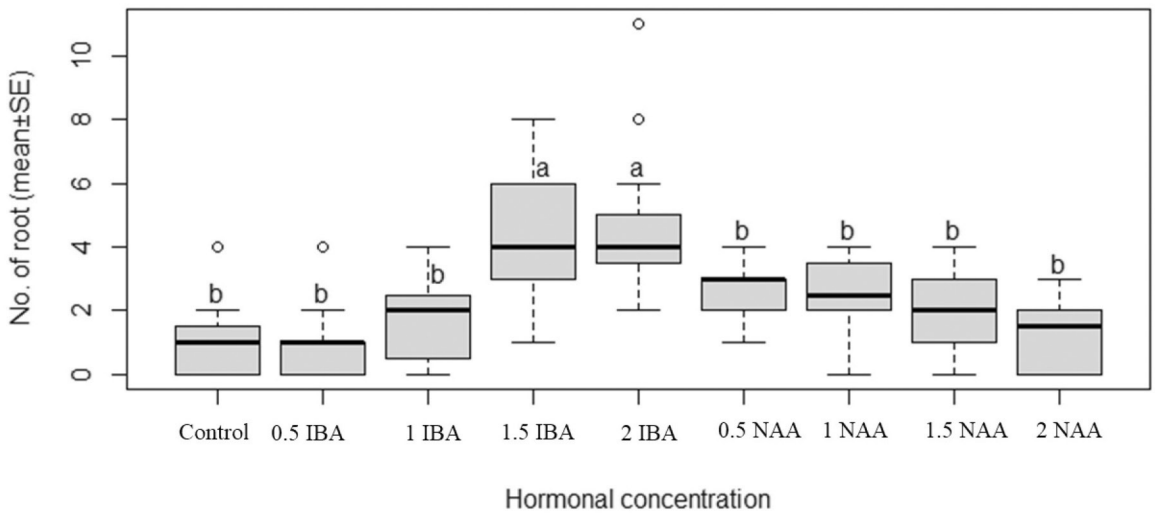

Figure 4: Average number of roots formation on MS medium alone and MS medium with IBA and NAA at different concentrations after 8 weeks of culture
Note: Values are means ± SE, n≥12. Different letters indicate statistically significant difference between different treatments at $p<0.05$

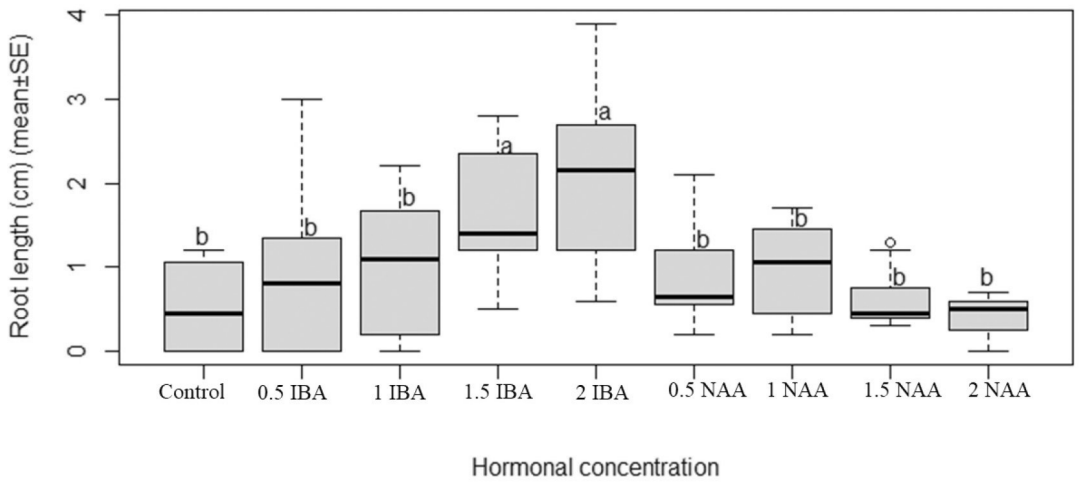

Figure 5: Average root length on MS medium alone and MS medium with IBA and NAA at different concentrations after 8 weeks of culture

Note: Values are means ± SE, n≥12. Different letters indicate statistically significant difference between different treatments at $p<0.05$

of roots by 4.7 and root length by 3.47 cm more efficiently than NAA in *Dendrobium nobile*. Riva et al. (2016) reported that 90% of root induction was found at 1.0 mg/L BA +1.5 mg/L IBA. The various concentrations of IAA and IBA were found to be effective hormone for rooting of *D. primulinum* in comparison to NAA (Pant et al., 2012).

Physiologically, IBA is considered as more active auxin and stimulate rooting more efficiently than other auxins due to its weak toxicity and greater stability for induction of roots (Han et al., 2009; Liu et al., 2002). Asghar et al. (2011) reported that 2 mg/L BAP produced maximum number of shoots and IBA (2 mg/L) increased rooting percentage by 97.5% and as more efficient than NAA in *Dendrobium nobile*. In *Dendrobium densiflorum*, maximum number of roots were developed on micro shoots grown on the full-strength MS medium supplemented with 1.5 mg/L IBA (Pant et al., 2022).

In-vitro grown plantlets wrapped with moss and kept on the fine pine bark were successfully acclimatized and the survival rate of plantlet was about 80% after 30 days (Figure 1F).

Conclusion

The present paper reported the micropropagation of *D. chryseum*. The initiation of protocorms was efficient and subsequently their conversions to shoots followed by shoot multiplication by the combined effect of BAP and NAA. IBA was found to be the best for root induction in *D. chryseum*. The protocol might be simple and effective for mass propagation and *ex situ* conservation of *D. chryseum.*

Author Contributions

J. Pathak, S. Maharjan and M.S. Thapa Magar were involved in concept development, research designing and literature review. J. Pathak, S. Maharjan, G. Rijal and A. Maharjan were involved in lab work. J. Pathak and S. Maharjan collected data, analyzed data and prepared the manuscript. M.S. Thapa Magar edited and finalized the manuscript.

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