

***In Vitro* Propagation of the Endangered Orchid *Dendrobium chryseum* Rolfe from Protocorms Culture**

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Abstract

The immature seeds of *Dendrobium chryseum*, a sympodial epiphytic orchid with yellow flowers, were cultured *in vitro*, and the resultant protocorms were used as explants for seedling development. Protocorms were cultured on ½ M.S. medium fortified with Kinetin (Kn), 6-Benzylaminopurine (BAP), and Gibberellic Acid (GA3) in three concentrations (0.5mg/L, 1.0 mg/L and 2.0 mg/L) both alone and supplemented with 5% and 10% coconut water (C.W.). The highest number of shoots of *D. chryseum* developed on ½ - M.S. medium fortified with 2.0 mg/L of Kn and 10% C.W. and the longest shoots developed on ½ M.S. media fortified with 1.0 mg/L GA3, and 10% C.W. The shoot derived from protocorms were placed in ½ M.S. medium fortified with three different rooting hormones, Indole -3- acetic acid (IAA), Indole -3-butyric acid (IBA) and α -Naphthalene acetic acid (NAA) in different concentrations alone as well as with each 1.0 mg/L hormone combined with 10% C.W. The most effective of these media was ½ M.S. medium fortified with 1.5 mg/L IAA for rooting as well as for the production of longest roots. The present study could be useful for standardizing the protocol for mass propagation of the endangered orchid *Dendrobium chryseum*.

Keywords

Dendrobium, culture, hormone, medium, micropropagation

Introduction

Orchids, which belong to the Orchidaceae family, diverse in morphology and fragrance, are highly valued for cut flower production and decorative purposes because of their attractive rainbow colors. Though orchids are grown primarily as ornamental, many are used as herbal medicine, food, and cosmetics in the different parts of the world. (Blupitt 2007, Pant *et al.*, 2019). Globally 736 genera and 29,000 species of orchid are reported. (Chase *et al.*, 2015). A total of 402 species belonging to 100 genera have been reported from Nepal, including 18 endemic species (Rajbhandari 2015).

In Nepal, the orchid family as a whole is known

as Sunakhari, Sungava, Chadigava, and Jivanti. Nevertheless, there are also a variety of common names, often derived from the color of flower or usage of the plant (Subedi 2013, Pant and Raskoti 2013). All orchids are listed in CITES Appendix II, and most of them are categorized as critically endangered, are therefore legally protected (Koirala *et al.*, 2010; Subedi 2011; Pant, 2013). The conservation of orchid is a matter of global concern, for a variety of reasons, including their low regeneration rate, slow growth, and low germination of seeds. Excessive harvesting and illegal trade, human population pressure, and habitat destruction are the leading causes of reducing their natural populations (Pant *et al.*, 2002; Subedi 2013). Besides, few pods are

developed, and, of the seeds, only 1% of seeds germinate in nature because they need a specific mycorrhizal fungal association to do so (Kumaria & Tandon 2010; Saha *et al.*, 2019).

Dendrobium chryseum is an epiphytic and lithophytic herb found throughout the western and eastern Himalayas. It is also reported from Bangladesh, Bhutan, Nepal, Myanmar, Thailand, Laos, China, Vietnam, and Taiwan. This herb grows in cold climates at the elevations of 1000 m asl to 2150 m asl, is localized, and threatened by deforestation and over-exploitation (Joshi *et al.*, 2017). *D. chryseum* Rolfe is popularly used in traditional Chinese medicine for its antipyretic, and immune-modulatory effects and its benefits for the eyes. Nine chemical compounds, including five bibenzyls, three phenanthrenes, and a coumarin, have been isolated (Yang *et al.*, 2007). The chemical compound isolated from the stem of *Dendrobium chryseum* exhibited antioxidant activity (Yang *et al.*, 2007) and pharmacological experiments showed that polysaccharides from the plant inhibited tumor growth and reduced blood glucose in vivo (Liu *et al.*, 2009). As the species of *Dendrobium* such as *Dendrobium longicornu* and *Dendrobium crepidatum* were found to exhibit antioxidant and cytotoxic activities (Paudel *et al.*, 2017; Paudel *et al.*, 2019), it is expected to have the same type of activities in *D. chryseum*. As this orchid species has great significance in the floriculture industry and also has medicinal value, tissue culture should be promoted for its mass propagation and conservation. Besides, there is a high possibility of the production of secondary metabolites through tissue culture techniques in medicinally important orchids (Pant 2013). Thus, an attempt was made for micropropagation of *D. chryseum* through protocorms culture, which provides a useful way to reestablish plants in the wild for germplasm preservation as well as for commercial propagation.

Materials And Methods

Protocorm culture for shoot development

Immature capsules of *D. chryseum* were collected from Godawari, in Lalitpur, Nepal, and used for the establishment of *in vitro* culture. The capsules were first washed with 1-2 drops of TWEEN twenty and with running tap water for at least

half an hour. The capsules were then dipped in a 1% sodium hypochlorite solution for 15 minutes, before being submerged in 70% ethanol. Next, the capsule was held with forceps and flamed rapidly for a few seconds. After they were washed three times in sterile water, surface-sterilized capsules were then cut longitudinally with a sterile scalpel. The exposed seeds were transferred with forceps to a nutrient agar MS medium (0.8% per liter) to germinate. Different strengths of MS media (Murashige & Skoog, 1962) were used for seed germination, and the protocorms formed were used for shoot proliferation.

The seeds started to germinate within three weeks of inoculation on the MS medium and developed into protocorms. For shoot development, eight months old protocorms (measured 0.3 mm - 0.4 mm in diameter) obtained from hormone-free MS medium were cultured (on average 5 per culture), on ½ MS medium supplemented with BAP, Kinetin and GA3 each in different concentrations (0.5 mg/L, 1.0 mg/L and 2.0 mg/L) alone as well as with 5% and 10% C.W. The pH of all the media was maintained at 5.7-5.8 by adding 0.1 N NaOH and 0.1N HCl before adding agar. The medium was autoclaved at 15psi and 121°C for 20 minutes. Cultures were maintained at 1,000 lux with white fluorescent light using a 16/8 hours light/dark regime. The temperature was maintained at 24 ± 1°C. Four replicates of each culture medium were maintained.

Rooting of shoots

After 12 weeks of culture in multiplication media, the shoots which grew *in vitro* from the protocorms, transferred on ½ MS medium fortified with different concentration of each rooting hormones IAA, IBA and NAA (0.5 mg/L, 1.0 mg/L and 2.0 mg/L), and also in combination with 10% CW. The cultures were maintained accordingly, as mentioned in the shoot culture.

Statistical analysis

Data on shooting and rooting are presented as the mean and standard error. The significant difference between the MS medium and MS medium supplemented with different growth hormones were analyzed by using one - way analysis of variance (ANOVA) with F-statistics at a 95% confidence interval using SPSS version 20.

Results

Shoot multiplication of *D. chryseum* using protocorm culture

An average of five protocorms was inoculated on ½ MS medium supplemented with different concentrations of BAP, Kn, and GA3 alone and in conjunction with 5% and 10% CW. The media that was best for shoot multiplication (18.75 ± 0.48 shoots per culture) was ½ MS medium fortified with 2.0 mg/L Kn and 10% CW, whereas the media that supported the most extended shoots (2 ± 0.20 cm) per culture and roots (4.5 ± 0.65) per culture was ½ MS medium fortified with 1.0mg/L GA3 and 10% CW. The longest roots (1.28 ± 0.14 cm) were observed on ½ MS medium supplemented with 0.5 mg/L GA3 and 10% .

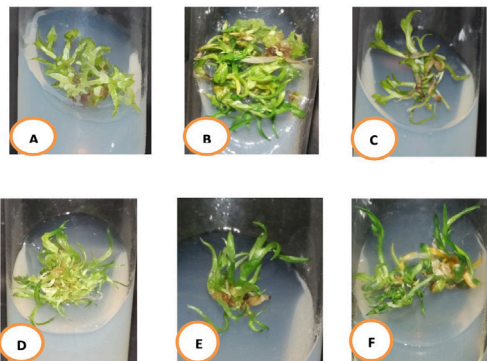


Fig. 1 *In vitro* shoot development through the protocorm culture of *Dendrobium chryseum* ½ MS medium fortified with different concentration of hormones after 12 weeks of culture. Multiplication of shoots: A= 0.5 mg/L BAP +5% CW, B= 0.5 mg/L Kn +5% CW, C= 2.0 Kn +10% CW, D=0.5 mg/L BAP +5% CW, E= 1.0 mg/L GA3 +10% CW and F= 0.5 mg/L GA3 + 10% CW

On ½ MS medium fortified with BAP, the highest number of shoots (7.25 ± 0.25) per culture grew when the concentration was 2.0 mg/L BAP, but the highest number of leaves (10.5 ± 0.29) per culture, the most extended leaves (0.47 ± 0.05 cm) and the longest shoot (0.8 ± 0.0 cm) grew on a BAP concentration of 1.0mg/L (Fig.2 and 3).

When different concentrations of BAP in ½ MS medium were supplemented with 5% and 10% CW, it was ½ MS medium supplemented with 0.5 mg/L BAP and 5% CW that produced the longest

shoots (1.03 ± 0.03 cm) and the ½ MS medium fortified with 0.5 mg/L BAP and 10% CW that produced the highest number of shoots (9.25 ± 0.75) per culture, leaves (15.2 ± 0.63) per culture and longest leaves (0.43 ± 0.05 cm) (Fig.2 and 3). Roots grew only on ½ MS medium supplemented with 1.0 mg/L BAP and 10% CW. There were 0.25 ± 0.25 roots per culture, and they measured 0.13 ± 0.13 cm long.

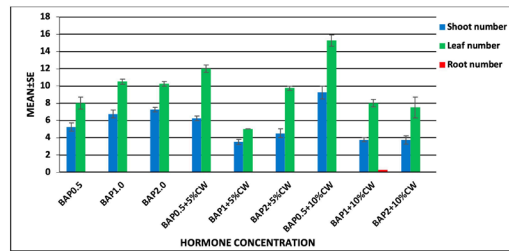


Fig. 2 Average numbers of shoots, leaves, and roots produced through the protocorms culture of *Dendrobium chryseum* ½ MS medium supplemented with BAP at different concentrations alone and in combination with 5% and 10% C.W.

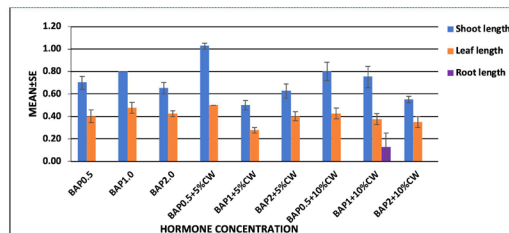


Fig. 3 Average lengths of shoots, leaves, and roots through the protocorms culture of *Dendrobium chryseum* on ½ MS medium supplemented with BAP at different concentrations both alone and in combination with 5% and 10% CW

In the case of ½ MS medium fortified with different concentrations of Kn (0.5 mg/L, 1.0 mg/L, and 2.0 mg/L), only a few responses were observed. On ½ MS medium supplemented with 1.0mg/L Kn and 2.0 mg/L Kn, protocorms did grow but became necrotic within seven weeks. The highest number of shoots (5.25 ± 0.75) per culture and roots (0.5 ± 0.29) per culture were observed on ½ MS medium fortified with 0.5 mg/L Kn (Fig. 4).

Of the MS medium fortified with different concentrations of Kn (0.5 mg/L, 1.0 mg/L and 2.0 mg/L) alone and with 5% and 10% C.W., it

was the ½ MS medium supplemented with 0.5 mg/L Kn and 5% CW that was the most effective for promoting the most considerable number of roots (1.25 ± 0.25 roots per culture) and the most extended shoots (1.45 ± 0.25 cm). However, ½ - MS medium fortified with 2.0 mg/L Kn and 10% C.W. produces the most significant number of shoots (18.75 ± 0.478 shoots per culture) and leaves (24.25 ± 0.25 leaves per shoot culture), and most extended leaves (0.55 ± 0.028 cm per shoot) (Fig.4 and Fig. 5). However, Maharjan *et al.*, (2019) found that 1.0mg/L Kn fortified with 10% CW was the best medium for shoot multiplication in *Vanda pumila*. Kalpona *et al.*, (2000) observed that Vacin and Went (V.W.) medium supplemented with a combination of 3% banana pulp and 10% CW enhanced the production of *Dendrobium* orchid. The present study found that ½ MS medium supplemented with 0.5 mg/L Kn and 10% CW was effective in promoting the maximum number roots (1 ± 0.0) per culture, and the longest roots (1.13 ± 0.13 cm) and that roots grew in all concentrations of Kn supplemented with 10% CW In terms of the highest number of shoots and leaves, 2.0 mg/L Kn with 10% CW was the most effective of the Kinetin supplemented media. Chyuam and Saleh (2011) reported that ½ MS medium fortified with 2.0 µM Kn resulted in the highest proliferation of the PLBs of *Paphiopedilum* and that ½ MS medium fortified with 20% CW was favorable for tertiary PLBs proliferation in *Paphiopedilum*.

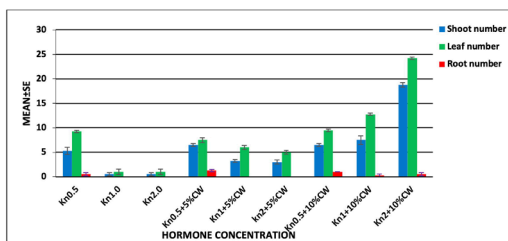


Fig. 4 Average numbers of shoots, leaves, and roots through the protocorm culture of *Dendrobium chryseum* on ½ MS medium supplemented with Kn at different concentrations both alone and in combination with 5% and 10% CW

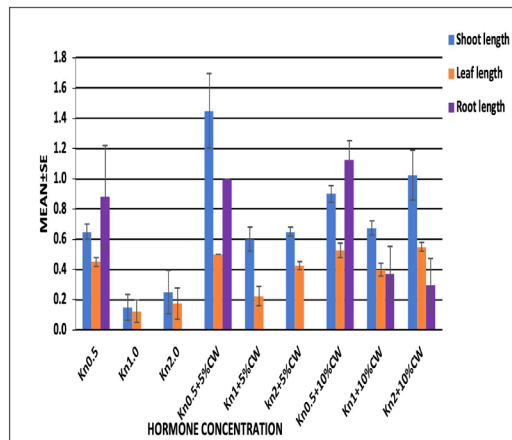


Fig. 5 Average lengths of shoots, leaves, and roots through the protocorm of *Dendrobium chryseum* on ½ MS medium supplemented with Kn at different concentrations both alone and in combination with 5% and 10% CW

Of ½ MS medium fortified only with different concentrations of GA3 (0.5 mg/L, 1.0 mg/L and 2.0 mg/L), 2.0 mg/L GA3 produced the highest number of shoots (4.00 ± 0.040) and leaves (8.50 ± 0.288) per culture. Shoot multiplication was not better in a medium with the only GA3. Dohling *et al.*, (2008) reported that seed germination and seedling development in *Dendrobium* species were inhibited when GA3 was added to a medium. On ½ MS medium fortified with different concentrations of GA3 (0.5 mg/L, 1.0 mg/L and 2.0 mg/L) and 5% and 10% CW, 0.5 mg/L GA3 and 10% CW produced the maximum number of shoots (14.7 ± 0.25) per culture and leaves (24.50 ± 0.5) per culture, but the longest shoots (2 ± 0.20 cm) and leaves (1.05 ± 0.23 cm) were found on ½ MS medium supplemented with 1.0 mg/L GA3 and 10% CW (Fig. 6 and Fig. 7), and it was on ½ MS medium fortified with 0.5 mg/L GA3 and 10% CW that the highest number of roots (4.5 ± 0.65) per culture and the longest roots (1.28 ± 0.14 cm) were observed. However, no roots grew on any concentration of GA3 alone, and 5% C.W. Tao *et al.*, (2011) reported that ½ MS medium supplemented with different concentrations of GA3 (0.5 mg/L – 2.0 mg/L) did not promote the PLBs proliferation of *Cymbidium faberi*.

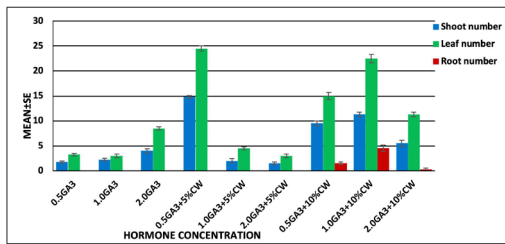


Fig. 6 Average numbers of shoots, leaves, and roots in the protocorm culture of *Dendrobium chryseum* on ½ MS medium supplemented with GA3 at different concentrations both alone and in combination with 5% and 10% CW

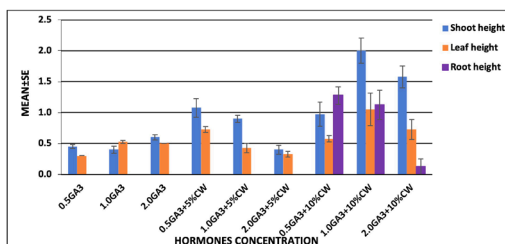


Fig. 7 Average lengths of shoots, leaves, and roots produced through the protocorm culture of *Dendrobium chryseum* on ½ MS medium supplemented with GA3 at different concentrations both alone and in combination with 5% and 10% CW

Root development of *Dendrobium chryseum*

When the shoots grown in vitro from protocorms were about 0.5-0.8 cm long, they were transferred on ½ MS medium supplemented with different concentrations (0.5 mg/L, 1.0 mg/L, 1.5 mg/L and 2.0 mg/L) of three rooting hormones IAA, IBA, NAA as well as on 1.0 mg/L concentrations of all three hormones in combination with 10% CW

Of these media, ½ MS medium - fortified with 1.5 mg/L IAA produced the highest number of roots (5 ± 0.0) per culture and the longest roots (1.7 ± 0.17 cm) (Fig.10). Thapa and Pant (2012) found that for *Dendrobium primulinum*, the highest number of roots grew in full - strength MS medium supplemented with 0.5 mg/L IAA. Bhadra and Hossain (2003) reported that full - strength MS medium supplemented with 1.0 mg/L IAA and 0.1% (w/v) activated charcoal (A.C.) the most effective of several media in promoting the production of roots by *Geodorium densiflorum*.

The dissimilarity of the finding on IAA may be due to the differences in species or the fact that the MS medium used in the earlier studies was twice as durable.

Root development on ½ MS medium supplemented with IBA was observed only on 1.0 mg/L IBA, and the number of roots was limited (0.75 ± 0.25) per culture. This finding was supported by the findings of Rafique *et al.*, (2012) and Aktar *et al.*, (2007) on *Dendrobium orchids* (which produced 0.20 roots per plantlet when the same hormone was used) and by Riva *et al.*, (2016), who studied *Dendrobium benosinae* and found that IBA - supplemented media produced the highest number of roots. On NAA-supplemented media, the highest number of roots was recorded for the concentration 0.5 mg/L (2 ± 0.0) per culture and the longest roots 1.0 mg/L NAA (1.2 ± 0.17 cm). This finding is similar to that of Paudel and Pant (2012), who found that for *Esmeralda clarkei*, the highest number of roots and the longest roots were produced on full - strength MS medium fortified with 0.5 mg/L NAA. The present result was partially supported by the findings of Parvin *et al.*, (2009), who reported that *Dendrobium orchid* produced the highest number of roots and the longest roots when an MS medium supplemented with 0.2 mg/L NAA was used.

In C.W. supplemented media, ½ MS medium fortified with 1.0 mg/L NAA and 10% C.W. was produced the most significant number of roots (3.25 ± 0.25) per culture and the longest roots (1.62 ± 0.23 cm). Although shoots were the most prolonged when 1.0 mg/L IAA and 10% C.W. were used, this medium produced very few roots (Fig.9).

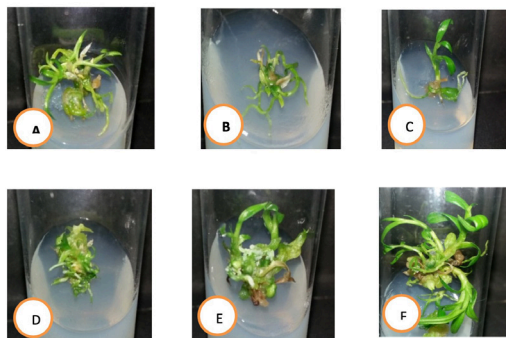


Fig. 8. The in vitro rooting of *Dendrobium chryseum* on ½ MS medium fortified with different concentration of IAA, IBA and NAA. A= 0.5 mg/L NAA, B=1.0 mg/L NAA, C=1.5 mg/L IAA, D=2.0 mg/L IAA, E=1.0mg/LNAA and 10% CW and F=1.0 IAA+10% CW after 12 weeks of culture.

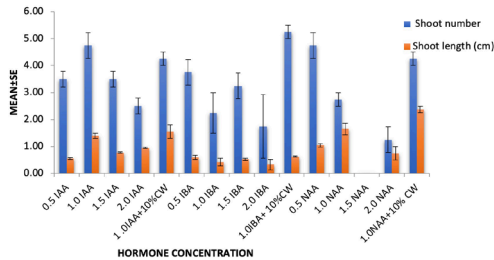


Fig. 9 Average numbers and lengths of shoots produced by the shoot culture of *Dendrobium chryseum* on ½ MS medium supplemented by IAA, IBA, and NAA at different concentrations.

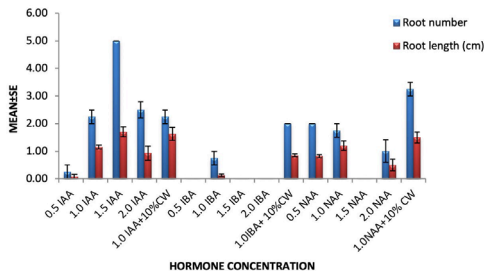


Fig. 10 Average numbers and lengths of roots through the shoot culture of *Dendrobium chryseum* on ½ MS medium supplemented with IAA, IBA, and NAA at different concentrations.

Discussions

Ex-situ conservation of threatened medicinal orchid species is essential, as their rates of propagation in natural habitat are meager. In the present study, protocorm culture was used to standardize the micropropagation protocol in *D. chryseum*, a threatened orchid species.

In the present investigation, plant growth regulators such as BAP, K.N. or GA3 alone or fortified with 5% and 10% C.W. were tested for the proliferation of protocorms. All the tested conditions responded positively to protocorm and

plantlet development of *D. chryseum*. Protocorms were able to proliferate on all the tested conditions. However, the synergistic effect of BAP with coconut water on 1/2 MS media (0.5 mg/L BAP and 10% C.W.) that produced the highest number of shoots (9.25 ± 0.75) per culture from protocols. The requirement of exogenous cytokinins for the regeneration of protocorms or shoot buds and plantlet development has been reported for many orchid species. However, the combinations, concentrations, and the ratio between them are usually critically important. The concentration of cytokinin for shoot multiplication and plantlet development formation varies from species to species in different orchid genera. (Pradhan *et al.*, 2013; Pant *et al.*, 2019). Gnasekaran *et al.*, (2010) reported that ½ MS supplemented with 10% C.W. stimulated the proliferation of *Phalaenopsis violacea* protocorm - like bodies (PLBs), but that 20% C.W. and 30% C.W. tended to inhibit PLBs proliferation. Coconut water plays a vital role in protocorm proliferation. According to Gnasekaran *et al.*, (2010), coconut water is added to tissue culture medium because it contains diphenyl urea, a growth factor that exhibits cytokinin-like responses. Saiprasad *et al.*, (2003) reported that 0.5 mg/L Kn supplemented with 10% C.W. is ideal for protocorm multiplication. Similarly, Pyati *et al.*, (2002) reported that MS medium fortified with 10% C.W. produced more shoots in the nodal explant of *Dendrobium macrostachyum* than did MS medium supplemented with either Kn or B.A.

Multiplication of shoots from protocorms was active when the media was supplemented with BAP. This result was partially supported by Luo *et al.*, (2008), who found that 2.0 mg/L BAP was more effective than other concentrations to achieve 100% PLBs conversion of *Dendrobium densiflorum* shoots. Similarly, 1.0 mg/L BAP was found better in shoot multiplication (6 shoots per culture) in *Vanda Pumila* (Maharjan *et al.*, 2019). Other researchers also found that BAP stimulates growth: David *et al.*, (2008) reported that the *Vanda helvola* produced the highest number of shoots on a Knudson C medium supplemented with 1.0 mg/L BAP, while Goswami *et al.*, (2015) observed that ½ MS medium plus 0.5 mg/L NAA and 0.5 mg/L BAP fostered the highest number of shoots from PLBs of *Dendrobium* species.

In this study, Kn alone was less effective for shoot multiplication than BAP. This result is similar to that of the result of which Luo *et al.*, (2008) in *Dendrobium densiflorum* but contrasts with the result of Martin and Madassery, (2006) in *Dendrobium* hybrids, who found ½ MS medium supplemented with 6.97 µM Kn produced maximum shoots. They also reported that a higher concentration of Kinetin in the medium increase in the number of roots, a finding partially similar to the present investigation. The present investigation found the development of roots on Kinetin alone (0.5 mg/L) and media with Kinetin fortified with C.W.

Auxins are essential for root initiation in tissue culture media. Different concentrations of IAA, NAA, and IBA either alone or in combination with coconut water were tested for rooting in the present investigation. Overall, ½ MS medium supplemented with 1.5 mg/L IAA generated longer roots in *Dendrobium chryseum* than any other concentrations of IAA or any other rooting hormones did so with or without C.W.

Conclusions

To achieve the ultimate goal of conservation and sustainable use of medicinal orchids, an alternative propagation technique, which is plant tissue culture, is desirable. In this regard, an efficient protocol for the development of a large number of plantlets from the protocorms of *Dendrobium chryseum* has been developed. This protocol could be useful for the mass propagation and ex-situ conservation of this threatened and medicinally important orchid species. However, effort on cultivation techniques of medicinal orchids is yet to be perfected. Such efforts could minimize the collections of natural plant populations for trade and consumption and secure the wild orchid species.

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