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Full Length Research Paper

Efficient regeneration of plants from shoot tip explants of *Dendrobium densiflorum* Lindl., a medicinal orchid

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Dendrobium densiflorum **Lindl. is one of the horticulturally important orchids of Nepal due to its beautiful yellowish flower and medicinal properties. The present study was carried out for plant regeneration from shoot tip explants of *D. densiflorum* by tissue culture technique. The shoot tip explants of this species, obtained from *in vitro* grown seedlings were cultured on Murashige and Skoog (MS) media alone and MS medium supplemented with different combination and concentration of 6-benzylaminopurine (BAP) and napthalenacetic acid (NAA). The maximum number of healthy shoot was observed on MS+ BAP (2 mg/l) + NAA (0.5 mg/l) (4 shoots/ culture). The shoot multiplication started after three weeks of culture. The induction of root was observed on all MS medium supplemented with different concentration and combination of plant growth regulators [indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and NAA]. For inducing root, MS media without and with auxins (IAA, IBA and NAA) were used. Among these, the most effective condition for *in vitro* rooting was observed on MS+ IBA (1.5 mg/l). The *in vitro* propagated plantlets were transferred in 2:1:1 ratio of cocopeat, litter and clay containing earthen pot for acclimatization. About 85% plantlets were successfully acclimatized in the greenhouse of Central Department of Botany, Kirtipur.**

Key words: *In vitro,* shoot-tip, explant, multiplication, regeneration.

INTRODUCTION

Orchids are prized for their marvelously long lasting flowers. *Dendrobium* is the second largest genus of orchid family Orchidaceae comprising more than 1100 species in the world. They are widely distributed throughout the world ranging from southern Asia to New Guinea and Australia (Luo et al., 2008; Puchooa, 2004; Xu et al., 2006). In Nepal, 29 species belonging to genus *Dendrobium* has been recognized (Raskoti, 2009). The name '*Dendrobium'* is derived from '*Dendron*' which means tree and "*bios"* means 'life' that is an epiphytic plant that exists by clinging to the branches and trunks of host trees.

Dendrobium densiflorum is one of the endangered epiphytic orchids popularly known as sungava in Nepal due to its golden colour flower. It is distributed in central Nepal and grows an altitude of 900 to 2900 m. The attractive golden flowers of *D. densiflorum* not only gathered considerable interest among horticulturist but also highly used in traditional Chinese medicine as a Yin Tonic to nourish the stomach, promote the secretion of body fluid, prevent the development of cataract, relieve throat inflammation and fatigue, reduce peripheral vascular obstruction and enhance body immunity (Bao et al., 2001). In Nepal pulps of its pseudobulbs are used to treat boils and pimples (Pant and Raskoti, 2013).

At present, the population of this species is disappearing at an alarming rate in natural habitat due to deforestation and indiscriminate collection by local orchid growers to meet the increasing demand for their horticultural and medicinal uses. It is therefore an urgent need to conserve this valuable orchid. Asexual propagation of this species by division of offshoots can be possible but its multiplication rate is extremely low with only two to four

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Abbreviations: MS, Murashige and Skoog medium; **NAA,** napthalenacetic acid; **BAP,** 6-benzylaminopurine; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid.

plants per year (Martin and Madassery, 2006; Nasiruddin et al., 2003). They may be propagated sexually by seeds but this process is also very slow as its seeds lack endosperm and need mycorrhizal association for germination. Hence, *in vitro* micropropagation by using tissue culture techniques will be helpful in mass propagation and conservation of this species.

The present investigation was carried out to standardize the protocol for mass propagation of *D. densiflorum* by using *in vitro* shoot tip explants.

MATERIALS AND METHODS

Young capsules of *Dendrobium densiflorum*Lindl. were collected from ICIMOD Conservation Demonstration Garden, Lalitpur , Nepal.

Surface sterilization of explants

Young capsules of *D. densiflorum* were washed with tap water to remove dust or any other soil particles attached to it. Capsules were than washed thoroughly with running tap water and then with detergent teepol (0.1%). They were surface sterilized in 70% ethanol for 1 to 2 min followed by 1% (w/v) sodium hypochlorite solution for 5 min and were subsequently rinsed with sterile distilled water for three times. The sterilized capsules were first dried on sterilized filter paper and then cut longitudinally with the help of sharp sterilized surgical blade to expose seeds.

Culture medium

Murashige and Skoog (MS) medium (1962), was used as the basal medium for inoculation of seed and other explants. MS medium alone and supplemented with different concentration and combination of hormones [6-benzylaminopurine (BAP) and napthalenacetic acid (NAA)] were used for this investigation. Media were prepared by adding different concentration and combination of cytokinin (BAP) and auxin (NAA) as given in Table 1 .The pH of medium was adjusted to 5.8 before gelling with agar (0.8% w/v). Agar was dissolved by boiling the medium. Molten medium was dispensed about 20 ml into sterile culture tubes (150 × 25 mm) and covered with aluminum foil. The tubes were autoclaved at 120°C for 20 min at 1.05 kg/cm^2 pressure. Cultures were maintained at 25°C (± 2°C) and 350 to 500 lux under 16/8 h photoperiod.

Seed culture

Sterile young capsule of *D. densiflorum* was kept on sterile Petri dish containing sterile filter paper and cut longitudinally with the help of sterile surgical blade to expose the tiny seeds. Seeds were scooped out by sterile spatula and spread over the surface of MS medium alone and supplemented with different combination and concentration of BAP and NAA. Seeds underwent germination and finally give rise to seedling in culture room at 25°C (± 2°C) under 16/8 h (day/light) photoperiod. Microshoots about 5 mm in length excised from seedlings were used as explants for plant regeneration.

Plant regeneration

In vitro shoot tips were inoculated on hormone free MS medium and MS medium supplemented with different combination and concentration of BAP and NAA ranged between 0.5 to 2.0 mg/l to observe their multiplication rate. After multiplication, microshoot with an average height of about 3 cm was again taken as explants for *in vitro* rooting. They were individually grown on agar solidified three different types of rooting media viz. IAA, NAA and BAP at the range of 0.5 to 2.0 mg/l either alone or in different combination and concentration for the development of better root. *In vitro* grown plants with well developed roots were used for acclimatization.

Acclimatization

Culture tube containing rooted plants were opened and kept in room temperature for one week before transferring to earthen pot. After that, these plants were taken out from culture tube containing medium and washed thoroughly under running water to remove traces of nutrient medium completely without causing damage to the roots. Plantlets were then treated with 1% fungicide (Bavistine) for 2 to 5 min and transferred to earthen pot containing a mixture of cocopeat, litter and clay in the ratio of 2:1:1 with the sphagnum moss topping and covered with holed transparent poly bag for one week to decrease humidity. The potted plants were watered daily and fertilized with nitrogen, phosphorous and potassium (NPK) in ratio 20:10:10 at weekly interval with the help of spray. The potted plants were exposed to normal day light for 2 to 3 h per day for initial one week and increased the exposure period subsequently for 1 to 2 h from the next week. After one month, the plantlets were finally left in normal full day light condition in green house.

Statistical analysis

Statistical analysis was done by using analysis of variance (ANOVA) one way classifiction system. The data obtained were analysed using application software-microsoft excel. The significant difference between the MS medium and MS medium supplemented with different growth hormones were analysed and means were compared by Duncan's multiple range test at $P \leq 0.05$ using SPSS version 11.5 (SPSS Inc. USA).

RESULTS AND DISCUSSION

Shoot tips about 5 mm length were excised from *in vitro* shoot developed from seeds and inoculated on MS basal medium with or without supplement with various combination and concentration of BAP and NAA for inducing multiple shoots. Almost all conditions favoured the shoot multiplication but showed varied response. These micro shoots were differentiated into callus, protocorm-like bodies and shoot bud which later developed into multiple shoot.

Microshoot produced multiple shoot only after intervening callus on MS + BAP (1.5 mg/l) + NAA (0.5 mg/l). Here, shoot multiplication was started only after four weeks of culture. The average number of shoot was found to be 3.25 per culture. This condition was also followed by MS + BAP (0.5 mg/l) + NAA (0.5 mg/l) (Figure 1).

Green small protocorm-like bodies was also observed from shoot tip culture which may or may not be differentiated into shoots on MS + BAP (1.0 mg/l), MS + BAP (1.0mg/l) + NAA (0.5 mg/l) and MS + BAP (2.0 mg/l) + NAA (0.5 mg/l).

Development of shoot buds and formation of multiple shoots from shoot tip explants were noticed in all the experimental conditions. The increase in concentration of

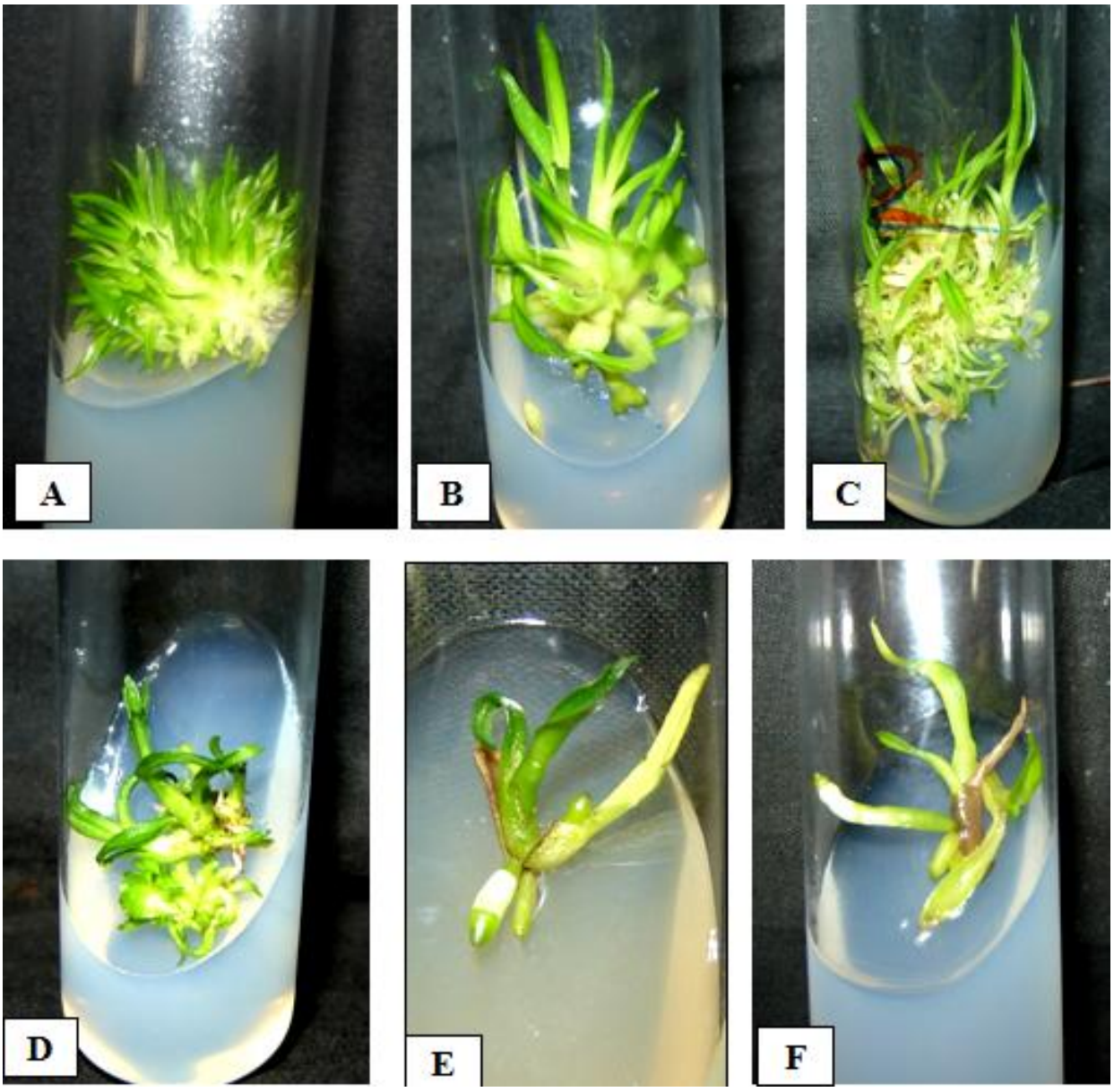

Figure 1. Shoot multiplication and development of root from shoot tip explants of *Dendrobiumdensiflorum*. **A.** Development of micro shoots on MS medium. **B.** Multiplication of shoot on MS + BAP (1.5 mg/l) + NAA (0.5 mg/l). **C.** Shoot proliferation on MS + BAP (2 mg/l) + NAA (0.5 mg/l), **D.** Development of shoot on MS + BAP (1mg/l) + NAA (0.5 mg/l) from shoot tip explant after 12 weeks of culture, **E-F.** Shoot with healthy root developed on MS + IBA (1.5 mg/l).

BAP alone from 0.5 to 2.0 mg/l in the MS medium enhanced the shoot proliferation; the concentrations had less significant effect on multiple shoot formation in comparison to the combine effect of BAP and NAA. MS medium supplemented with only NAA was also not effective for shoot development in comparison to different concentrations of BAP alone as well as different combination treatment of BAP and NAA.

Among different concentration of BAP alone, MS + BAP (1.5 mg/l) was most effective for shoot proliferation which took only four weeks of culture to start multiplication. Here, the average number of shoot was found to be 2.75 per culture. This condition was also followed by MS + BAP (0.5 mg/l) and MS + BAP (2.0 mg/l) which took five weeks of culture for proliferation. On MS + BAP (1 mg/l), the shoot multiplication was retarded though it took five week of culture for multiplication but the average number of shoot was found to be 1.5 per culture which was the second most least condition for multiplication.

In the present investigation, combine treatment of BAP with NAA on MS medium gave synergistic effect on shoot growth and multiplication. Maximum number of shoots

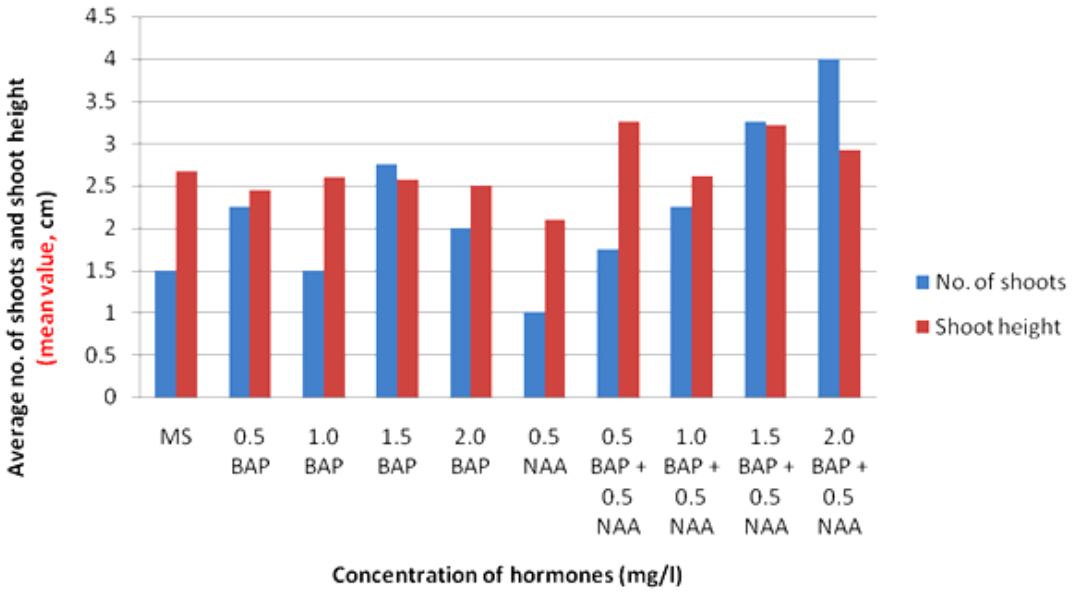

Figure 2. Average number of shoots and shoot height after 12 weeks of culture of shoot tip of *Dendrobium densiflorum* Lindl.

per culture was found on MS medium supplemented with BAP (2 mg/l) and NAA (0.5 mg/l). It was followed by MS + BAP (1.5 mg/l) + NAA (0. 5 mg/l), MS + BAP (1.0 mg/l) + NAA (0. 5 mg/l) and MS + BAP (0.5 mg/l) + NAA (0. 5 mg/l). The least multiplication of shoot was found on MS medium supplemented with NAA (0.5 mg/l).

The best condition of shoot multiplication was recorded on the basis of time taken and number of shoots developed on different experimental conditions. MS medium supplemented with BAP (2.0 mg/l) and NAA (0.5 mg/l) was the most effective condition for shoot proliferation in *D. desnsiflorum* which took three weeks for induction of shoots. In this condition, the average number of shoots was found 4 per culture. Though highest number of shoot was observed on MS + BAP (2.0 mg/l) + NAA (0. 5 mg/l), the elongation of shoot was found to be 2.92 cm per culture in this condition which was average growth in comparison to other conditions.

In present investigation, the maximum elongation of shoot was observed on MS medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l) which was found to be 3.25 cm/culture. It was followed by MS + BAP (1.5 mg/l) + NAA (0. 5 mg/l) and MS + BAP (2.0 mg/l) + NAA (0. 5 mg/l) which was found to be 3.22 and 2.92 cm/culture, respectively. The least growth rate of shoot was found on MS + NAA (0.5 mg/l) which was found to be 2.1 cm/ culture in comparison to other combinations.

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MS medium without plant growth regulators also gave significant result on shoot proliferation as well as development of shoot. In this condition, shoot multiplication started after six weeks of culture and shoot height was observed as 2.67 cm per culture. Here, the time taken for proliferation of shoot was quite high in comparison to other conditions except MS + NAA (0.5 mg/l).

Here, MS medium supplemented with NAA (0.5 mg/l) was less effective in both cases either in elongation or multiplication of shoot (Figure 2). Hence, it was found that the rate of growth and multiplication of shoot was dependent upon the different combinations of plant growth regulators (BAP and NAA) used in media. Use of high concentration of BAP than NAA was most efficient in this species for multiplication of shoot.

These findings were supported by the work of different researchers. Swar and Pant (2004) obtained maximum number of shoots on MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l) in *Coelogyne cristata* Lindl. and Shrestha and Rajbhandary (1993) developed protocorms from the shoot tip explants and established the clonal propagation of *D. densiflorum* Lindl. on MS medium supplemented with BAP (2.5 mg/l) and NAA (1 mg/l). 15% coconut milk and 1 g/l casein hydrolysate developed protocorms from the shoot tip explants. Talukder et al. (2003) found highest shoot proliferation (1.90 shoots / explant) on MS + BAP (2.5 mg/l) + NAA (0.5 mg/l) in *Dendrobium* orchid.

The elongated shoots with an average height of 3 cm length were individually grown on 0.8% (w/v) agar solidified MS medium supplemented with three different auxins *viz*. IAA, IBA and NAA with the range of 0.5 to 2.0 mg/l for rooting. MS medium supplemented with IBA (1.5 mg/l) was the best condition for induction of thick, healthy and long root in comparison with other tested rooting medium. In this concentration, the average number of root was found as 4.5 per culture and root length ranged from

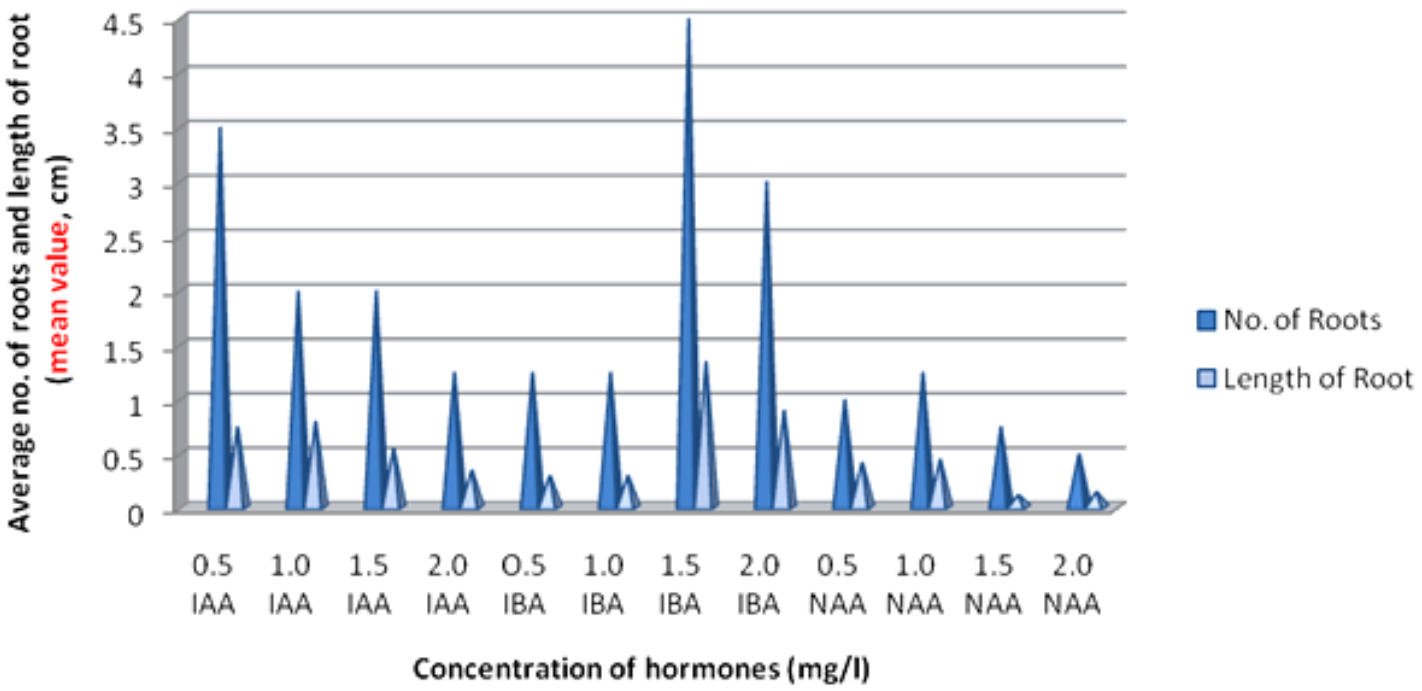

Figure 3. Average number of root and root length after 12 weeks of rooting of shoot tip explant of *Dendrobium densiflorum* Lindl.

0.3 to 1.35 cm after 10 weeks of culture (Figure 3). The present result is consistent with the findings made by Nayak et al. (1997) who obtained rooted shoots on MS medium containing 1.0 mg/l IBA in *Acampe praemorsa*. Swar and Pant (2004) also found best rooting on MS medium supplemented with 1 ppm IBA in *Cymbidium iridiodes* D. Don and *Coelogyne cristata* Lindl. Shrestha (2005) reported that MS + 2 ppm IBA was the most effective condition for rooting of *Coelogyne ovalis* Lindl. Aktar et al. (2007) reported the 1.81 per explants root on *Dendrobium* sp. in 1 mg/l IBA. The result was also partially supported by Talukder et al. (2002), where they found 1.62 roots plantlet^{-1} from 2 mg/l IBA with MS media at 30 day after inoculation (DAI). The quality, quantity and nature of growth regulators have foremost effect on the regeneration capacity of the shoot tip (Pant et al., 2012). BAP has a marked effect in stimulating the growth of axillary and adventitious buds and foliar development of shoot tip cultures (Abeyaratne and Lathiff, 2002; Buah et al., 2010).

In the present investigation, different concentration of IAA and NAA also showed somehow response on rooting but MS medium alone did not give any satisfactory result of rooting (Figure 3).

In vitro rooted shoots measuring about 4 to 5 cm were transferred in earthen pot containing mixture of coco-peat, litter and clay by topping sphagnum moss in 2:1:1 ratio for acclimatization. About 85% plantlets successfully survived in this potting mixture. They were finally established in Garden of Central Department of Botany, T.U., Kirtipur for their further development.

Conclusions

From this above experimental results, it was concluded that MS medium supplemented with BAP (2 mg/l) and NAA (0.5 mg/l) was the best condition for efficient regeneration of *D. densiflorum* from shoot tip explants. Here, the regeneration potential of shoot tip explants is markedly influenced by their physiological status and active ingredients present in the nutrient pool. Hence, this protocol is very effective for mass propagation, conservation and cultivation of this species by using *in vitro* shoot-tip explants.

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