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In vitro Propagation and Genetic Homogeneity Assessment of *Dendrobium crepidatum* Lindley & Paxton

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Key words: Dendrobium crepidatum, Genetic homogeneity, *In vitro* culture, RAPD marker

Abstract
In vitro propagation is one of the most dependable methods to develop true-to-type plants for the commercial and conservation aspects. This study aims to produce the genetically identical plantlets of *Dendrobium crepidatum* Lindley and Paxton using *in vitro*-developed shoot explants. Coconut water (5 - 10%) supplemented Murashige and Skoog (MS) medium responded towards effective shoot formation with BAP (1.0 - 2.0 mg/l) and NAA (0.1 - 0.5 mg/l). NAA (0.5 - 1.0 mg/l) alone or in combination with BAP was found to be effective for root initiation in MS medium. Four RAPD (Random Amplified Polymorphic DNA) primers viz., G04, G10, G17 and G18 showed 100% genetic homogeneity in plants obtained from *in vitro*-developed shoot explant. However, G10, G17 and G18 RAPD primers showed 60, 25 and 100% genetic polymorphisms respectively in plants obtained from callus. Plants obtained from callus were genetically polymorphic where somaclonal variation might occur. This study reported the successful genetic homogeneity of shoot explant-derived plants of *D. crepidatum*. Hence, the present study has validated the establishment of standard *in vitro* protocol for significant propagation of superior genetically indistinguishable *D. crepidatum* for viable conservation and commercialization.

Introduction
Dendrobium crepidatum Lindley & Paxton, commonly called the shoe-lip Dendrobium, is an epiphyte on tree trunks in open forests. It is native to southern China (Guizhou, Yunnan), the eastern Himalayas (India, Assam, Sikkim, Bhutan, Arunachal Pradesh, Nepal, Bangladesh), and northern Indochina (Laos, Myanmar, Thailand, Vietnam) at an elevation of 600 - 2100 m (Rajbhandari 2014). *D. crepidatum* is one of the pioneer's orchids

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with medicinal and horticultural values (De and Pathak 2015). The stem paste of this orchid species has often been used in traditional medicine to cure bone fractures (Rajbhandari 2014). It has many bioactive secondary metabolites which have antioxidant, anticancer and antimicrobial activities (Paudel et al. 2018, 2019). *D. crepidatum* is largely being transported to foreign countries for its floriculture and aesthetic values (Subedi et al. 2013).

However, uncontrolled collection for illegal trade to accomplish the demand-supply and natural habitat destruction led to a sharp decline in its population (Fay 2018). *D. crepidatum* has been inducted in Appendix-II of nearly threatened species under CITES (Convention of International Trade in Endangered Species) (Koirala et al. 2011, Fay 2018). Its threatened category is due to a low rate of vegetative propagation and seed germination (2-5%) in nature (Piria et al. 2008, da Silva 2013). Low seeds germination in nature is due to the very minute non-endospermic seeds and the requirement of association with specific mycorrhiza for effective germination (Rasmussen 2002; Cameron et al. 2006). There is an urgent need for the vegetative propagation of *D. crepidatum* for the commercial and conservation purpose. *In vitro* culture is one of the alternative methods for vegetative propagation without mycorrhizal association (Pant 2013, Yeung 2017, Pant et al. 2019). The maintenance of genetic homogeneity of *in vitro* propagated plantlets is an important facet of the conservation and commercial aspects of *D. crepidatum*. The use of explants and tissue culture techniques might change the genetic makeup of *in vitro* plantlets which may concern the production of true-to-type plants (Ferreira et al. 2006, Tikendra et al. 2019). It was proved that genetically varied plantlets were produced in tissue culture through the seeds (da Silva et al. 2015, 2016). Therefore, the assessment of genetic homogeneity between *in vitro* plantlets and mother plants is becoming mandatory. There are many PCR based molecular markers *viz.,* RAPD, RFLP, AFLP and ISSR have successfully been applied for the assessment of genetic homogeneity (Tingey and del Tufo 1993, Ferreira et al. 2006, Posselt et al. 2006, Bhattacharyya et al. 2014). So, the objectives of this research were *in vitro* propagation of *D. crepidatum* in the different additives containing MS medium and assessment of their genetic homogeneity using the RAPD marker.

Materials and Methods

In vitro developed shoots obtained from germinated seeds of *Dendrobium crepidatum* were taken as explants. About 0.1 - 0.2 cm long shoot was transferred onto the MS medium with the supplement of different concentrations of hormones/additives; such as GA_3, BAP, NAA and coconut water (CW). The cultures of explant were transferred to the culture room facilitated with 16/8 hrs of light and dark cycle at 25 ± 2°C.

Genomic DNA of plant samples (seed-derived shoots, callus-derived shoots and wild leaf) was extracted by using an extraction kit (Promega, USA). About 50 mg of plant samples were grinded in the liquid nitrogen to obtain fine powder and transferred to the

sterile micro-centrifuges tube after the addition of 600 µl nuclei lysis solution. They were incubated in the water bath at 65°C for 15 minutes. About 3 µl of RNase solution was added to the sample solution after its incubation and the solution was gently swirled and incubated again in the water bath at 37°C for 15 minutes. After incubation, the micro-centrifuge tubes containing the sample solution were allowed to cool at room temperature, and a 200 µl protein precipitation solution was added to each tube before the vortex. The samples were centrifuged at 13000-16000 x g for 3 minutes. The supernatant was shifted to clean sterile micro-centrifuge tubes, containing 600 µl isopropanol, and the samples were centrifuged again at 13000-16000 x g for 1 minute. The pellets were washed with 70% ethanol before centrifuge at 13000-16000 x g for 1 minute. The supernatant was aspired out from the tube and pellets were dried at room temperature for 15 minutes. About 100 µl of DNA rehydration solution was added to the pellet after drying it and the pellet was stored at 4°C overnight.

Four different RAPD primers namely G04 (5′ ACGACCGACA 3′), G10 (5′ AGGGCCGTCT 3′), G17 (5′ ACGACCGAC 3′) and G18 (5′ GGCTCATGTG 3′) used for the DNA amplification were purchased from Macrogen, Korea. DNA amplification reaction mixture was contained 1µl of 50 ng DNA (concentration of DNA sample was estimated by gel electrophoresis and using a spectrophotometer at 260 nm), 12.5 µl of master mix (Thermo Scientific, USA), 20 piCD mol/µl of 1 µl primer, and 10.5 µl of nuclease-free water. Amplification of DNA was performed in a thermal cycler (GeNei, India) programmed as a pre-heat lid at 106°C for 5 min, initial denaturation of DNA at 94°C for 3 min, denaturation of DNA at 94°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 2 min followed by 40 cycle and final extension was performed by at 72°C for 10 minutes.

Amplified DNA products were fractionated by agarose gel electrophoresis in 1X TBE buffer stained by 1µl ethidium bromide (10 mg/ml) at 70 volts for 55 minutes using an EMBI Tec gel tank (Santiago, CA). The gel was then viewed under ultraviolet light and photographed in a UV transilluminator (UVITEC, Cambridge). The data of micropropagation was expressed in mean ± SD of six replicates. Duncan test at $p < 0.05$ was used to compare the mean value of each nutrient medium using SPSS version 20. Molecular data were observed directly using gel documented photographs.

Results and Discussion

In vitro-developed shoots of about 0.1 - 0.2 cm were inoculated onto the different concentrations of GA_3, BAP, NAA and CW supplemented MS medium for their growth and multiplication. Shoot proliferation was observed in all the treatments however, the rate of shoot development was varied in different treatments (Table 1). The maximum number of shoots was obtained on the MS medium supplemented with a concentration of BAP ranging from 1.0 - 2.0 mg/l along with the concentration of NAA ranging from 0.1 - 0.5 mg/l alone or in combination with 5 - 10% of CW (Fig. 1). A concentration of BAP

less than 1.0 mg/l in the medium gradually reduced the number of shoot production. In the combination of BAP and NAA, yellow-greenish callus was observed after 16 weeks of culture. However, GA_3 supplemented medium was found to be effective for the elongation of regenerated shoots but the drastic change in the multiplication of shoots was not noticed in this medium. Among all the treatments, the length of shoots was found almost similar however, MS medium supplemented with 10% CW, BAP and NAA were enhanced with more elongated shoots. Similarly, a concentration of NAA (0.5 – 1.0 mg/l) added to MS medium with or without BAP was found to be effective for the formation of a greater number of roots with similar lengths (Table 1).

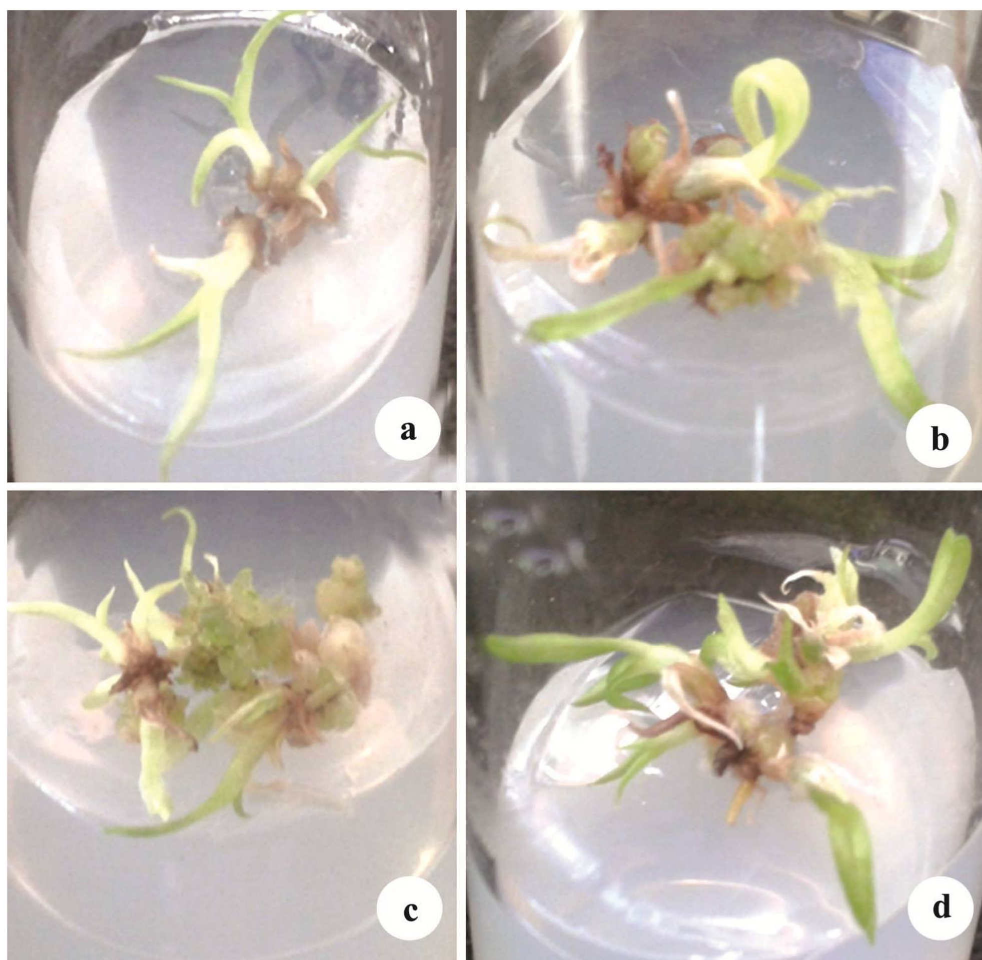

Fig. 1. Development of shoots from *in vitro*-derived micro-shoot of *D. crepidatum* in different medium. **a.** 1.0 mg/l BAP, 0.5 mg/l NAA and 10% CW containing MS medium. **b.** 1.5 mg/l BAP, 0.5 mg/l NAA and 10% CW containing MS medium. **c.** 2.0 mg/l BAP, 0.5 mg/l NAA and 10% CW containing MS medium. **d.** 2.0 mg/l BAP, 0.5 mg/l NAA and 5% CW containing MS medium.

Table 1. Growth and development of shoots and roots obtained from the inoculated *in vitro*-derived micro-shoots of *D. crepidatum*.

GA_3 (mg/l)	BAP (mg/l)	NAA (mg/l)	CW %	Number of shoot ± SD	Length of Shoot (cm) ± SD	Number of root ± SD	Length of root (cm) ± SD
-	-	-	-	1.00 ± 0.00[a]	0.43 ± 0.05[a]	-	-
0.1	-	-	-	1.00 ± 0.00[a]	0.44 ± 0.02[a]	-	-
0.2	-	-	-	1.00 ± 0.00[a]	0.47 ± 0.04[b]	-	-
0.25	-	-	-	1.00 ± 0.00[a]	0.46 ± 0.03[b]	-	-
0.5	-	-	-	1.02 ± 0.12[a]	0.55 ± 0.03[b]	-	-
1.0	-	-	-	1.30 ± 0.28[b]	0.42 ± 0.05[a]	-	-
-	0.1	0.1	-	1.33 ± 0.52[b]	0.36 ± 0.07[a]	-	-
-	0.2	0.1	-	1.38 ± 0.34[b]	0.44 ± 0.05[a]	-	-
-	0.25	0.1	-	1.56 ± 0.77[b]	0.41 ± 0.08[a]	-	-
-	0.5	0.1	-	1.94 ± 0.57[c]	0.40 ± 0.02[a]	-	-
-	1.0	0.1	-	2.11 ± 0.32[c]	0.40 ± 0.02[a]	-	-
-	0.1	0.2	-	1.04 ± 0.46[a]	0.40 ± 0.12[a]	0.50 ± 0.54[a]	0.05 ± 0.05[a]
-	0.2	0.2	-	1.11 ± 0.19[a]	0.40 ± 0.06[a]	0.66 ± 0.51[a]	0.06 ± 0.05[a]
-	0.25	0.2	-	1.77 ± 0.75[b]	0.38 ± 0.07[a]	0.50 ± 0.54[a]	0.05 ± 0.05[a]
-	0.5	0.2	-	1.59 ± 0.45[b]	0.40 ± 0.03[a]	0.50 ± 0.54[a]	0.05 ± 0.05[a]
-	1.0	0.2	-	2.00 ± 0.09[c]	0.38 ± 0.03[a]	0.66 ± 0.52[a]	0.06 ± 0.05[a]
-	0.5	0.5	-	1.27 ± 0.23[a]	0.51 ± 0.10[a]	0.66 ± 0.52[a]	0.06 ± 0.05[a]
-	1.0	0.5	-	1.63 ± 0.43[b]	0.54 ± 0.03[b]	0.83 ± 0.41[b]	0.83 ± 0.04[c]
-	1.5	0.5	-	2.19 ± 0.31[c]	0.62 ± 0.15[b]	1.10 ± 0.13[c]	0.16 ± 0.04[b]
-	2.0	0.5	-	2.99 ± 0.95[c]	0.59 ± 0.08[b]	1.00 ± 0.00[c]	0.13 ± 0.04[b]
-	0.5	1.0	-	1.16 ± 0.30[a]	0.56 ± 0.08[b]	1.83 ± 0.41[c]	0.18 ± 0.05[b]
-	1.0	1.0	-	1.16 ± 0.42[a]	0.54 ± 0.09[b]	0.83 ± 0.41[b]	0.11 ± 0.06[b]
-	1.5	1.0	-	1.51 ± 0.30[b]	0.47 ± 0.05[b]	0.50 ± 0.55[a]	0.05 ± 0.05[a]
-	2.0	1.0	-	1.32 ± 0.15[b]	0.46 ± 0.04[b]	1.16 ± 0.75[c]	0.09 ± 0.05[b]
-	0.5	-	-	1.42 ± 0.59[b]	0.51 ± 0.12[b]	0.66 ± 0.52[a]	0.06 ± 0.05[a]
-	1.0	-	-	1.58 ± 0.07[b]	0.49 ± 0.09[b]	0.83 ± 0.41[b]	0.09 ± 0.00[b]
-	1.5	-	-	1.24 ± 0.21[a]	0.56 ± 0.06[b]	0.50 ± 0.55[a]	0.05 ± 0.05[a]
-	2.0	-	-	1.56 ± 0.06[b]	0.54 ± 0.07[b]	0.50 ± 0.55[a]	0.05 ± 0.05[a]
-	-	0.5	-	1.16 ± 0.41[a]	0.62 ± 0.02[b]	1.00 ± 0.00[c]	0.15 ± 0.05[b]
-	-	1.0	-	1.28 ± 0.36[a]	0.56 ± 0.06[b]	1.18 ± 0.22[c]	0.20 ± 0.04[b]
-	1.0	0.5	5.0	2.35 ± 0.26[c]	0.64 ± 0.07[b]	-	-
-	1.5	0.5	5.0	2.30 ± 0.40[c]	0.67 ± 0.12[b]	-	-
-	2.0	0.5	5.0	2.30 ± 0.21[c]	0.72 ± 0.07[b]	-	-
-	1.0	0.5	10.0	2.00 ± 0.34[c]	1.35 ± 0.13[c]	-	-
-	1.5	0.5	10.0	2.53 ± 0.30[c]	1.13 ± 0.19[c]	-	-
-	2.0	0.5	10.0	3.08 ± 0.23[c]	1.10 ± 0.15[c]	-	-

Culture condition: 25 ± 2ºC, 24 weeks of culture, 6 replicates were used in each combination. The values with the same superscript in each column are not significantly different at $p \leq 0.05$.

The development of plantlets from shoot-tip explant has been relied on the nutrient medium supplemented with BAP, NAA and CW in several *Dendrobium* species (da Silva et al. 2015). GA_3 is another necessary growth-promoting phytohormone that helps with the elongation of shoots by increasing the rate of cell division (DeMason 2005). A low concentration of GA_3 was more effective to increase the shoot and root length of the orchid, *Guarianthe skinneri* (Coello et al. 2010). However, in the present study, the employment of GA_3 (0.1 mg/l to 1.0 mg/l) in the MS medium was less favourable in the proliferation of long shoots from shoot-tip culture as compared to the other combined effect of BAP, NAA and CW. CW is a high source of cytokinin (Yong et al. 2009) which is effective for the multiplication and differentiation of tissues. In distinction, in the present study, MS medium supplemented with 2.0 mg/l BAP, 0.5 mg/l NAA and CW (10%) was more effective for the development of shoots. This means that the nutritional requirements for shoot multiplication might vary among the species. The increased concentration of CW (5-10%) in the medium promoted shoot multiplication and elongation. The present result was supported by the findings of the researchers who discovered the development of *Dendrobium* shoots on the MS medium supplemented with cytokinin and/or CW (Nasiruddin et al. 2003, Sheela et al. 2006, Zhao et al. 2008, Nuraini et al. 2010, Dutta et al. 2011, Park et al. 2014). Similarly, Baque et al. (2011) found that the increased concentration of CW (10 - 50 ml/l) in the culture medium was helpful for *in vitro* propagation of *Calanthe* hybrid. In a previous study, the highest number of shoots of *D. densiflorum* was developed on MS medium in combination with 2.0 mg/l BAP and 0.5 mg/l NAA (Pradhan et al. 2013). Pant and Thapa (2012) reported that MS medium supplemented with 1.5 mg/l BAP was active for shoot development in *D. primulinum*. Thus, the results presented in this paper further substantiate the significant growth-promoting role of BAP and CW in the proliferation of shoot in *D. crepidatum*. Similarly, MS medium added with NAA (0.5 - 1.0 mg/l) has induced more roots on the shoots which were similar to the previous studies in *Dendrobium* (Parvin et al. 2009; Parthibhan et al. 2015) and other orchids such as *Coelogyne* (Hartati et al. 2017), *Rhynchostylis retusa* (Oliya et al. 2021).

Four RAPD primers namely; G04, G10, G17 and G18 were used to amplify the genomic DNA extracted from the *in vitro* and *in vivo* plant samples of *D. crepidatum*. The leaf of the naturally growing mother plant, *in vitro* shoots obtained from shoot explant and *in vitro* shoots obtained through callus, were used for the extraction of their genomic DNA. The total number of amplified gene bands, homogeneity bands and polymorphic bands observed on the genomic DNA of different plant samples formed by the RAPD primers was shown in Table 2. G04 RAPD primers gave a single band in all the *in vitro* regenerants obtained from both shoot and callus culture which was genetically similar to that of the mother plant (Fig. 2a). G10 primer gave five bands from the DNA of each sample derived shoot explant that is genetically homogeneity to the DNA of the mother plant. This primer also amplified five separate bands from the DNA of callus derived shoots, where 60% bands showed genetically polymorphic and 40% bands showed

genetically homogeneity to mother plants (Fig. 2b). G17 primer amplified the genomic DNA of all the samples. It amplified five separate DNA bands observed in each sample of shoot derived from shoot culture, shoots derived from callus culture and mother plant. All the five bands obtained from the *in vitro* regenerants derived from the shoottip culture were genetically similar to that of the mother plant. However, out of five bands obtained from the *in vitro* regenerants derived from the callus culture, one band showed polymorphism and four bands showed genetically homogeneity to that of the mother plant (Fig. 2c). Similarly, G18 primer generated four genetically homogeneity bands among each sample of shoots derived from shoot culture and the mother plant, whereas shoots derived from callus showed one polymorphic band in each sample used (100% polymorphism) as compared to DNA bands of the mother plant (Fig. 2d).

Table 2. The number of genetic homogeneity and polymorphic bands was observed after the amplification of genomic DNA of different samples of *D. crepidatum* using RAPD primers.

Different plant samples used	Total number of bands				Homogeneity band compared with the mother plant				Polymorphic band compared with the mother plant			
Primers →	G04	G10	G17	G18	G04	G10	G17	G18	G04	G10	G17	G18
In vitro shoot-derived shoot tip-1	1	5	5	4	1	4	5	4	-	-	-	-
In vitro shoot-derived shoot tip-2	1	5	5	-	1	4	5	-	-	-	-	-
In vitro shoot-derived shoot tip-3	1	5	5	4	1	4	5	4	-	-	-	-
In vitro shoot-derived shoot tip-4	1	5	5	4	1	4	5	4	-	-	-	-
In vitro callus-derived shoot tips-1	1	5	5	1	1	2	4	-	-	3	1	1
In vitro callus-derived shoot tips-2	-	5	-	1	1	2	-	-	-	3	-	1
Mother plant's leaf sample	1	5	5	4	1	4	5	4	-	-	-	-

Various studies demonstrated that *in vitro* propagation technique is useful for the production of a large number of plantlets under the aseptic condition. At the same time, there may be a high risk of production of true-to-type plantlets depending on the culture media formulations (Yadav et al. 2013; Bhattacharyya et al. 2014, Lin et al. 2020). According to da Silva et al. (2015), shoot development that occurs via the callus may result in the somaclonal variation which produced the polymorphic plants. The usage of

a higher concentration of phytohormones might induce genetic variability which is key to failure in the production of true-to-type plants (Palama et al. 2010). Thus, the assessment of genetic homogeneity between *in vitro*-developed plants and mother plant is essential. In the present study, the RAPD marker has assessed the genetic homogeneity between the six arbitrarily selected *in vitro*-developed plantlets of *D. crepidatum* and the mother plant. All the four RAPD primers viz., G04, G10, G17 and G18 showed 100% genetic homogeneity among the *in vitro* regenerants of shoot-tip-derived plantlets with the mother plant. However, three RAPD primers viz., G10, G17 and G18 showed polymorphism in callus derived plantlets. This finding is following genetic polymorphism among the *in vitro* propagated plantlets of *Dendrobium* using RAPD and

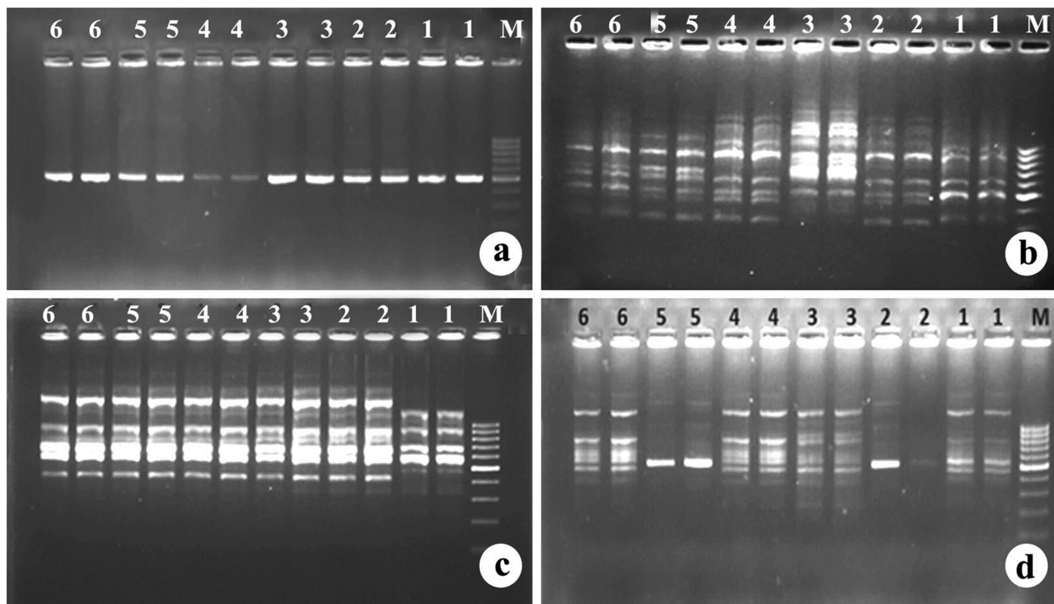

Fig. 2. Amplification of genomic DNA of different samples of *in vitro*-developed plants and mother plant of *D. crepidatum* by using four different RAPD primers G04 (a), G10 (b), G17 (c), and G18 (d). **a.** RAPD primer G04: 100 bp DNA ladder (M), DNA from *in vitro* shoot-derived shoot tips (1-4), DNA from mother plant's leaf (5) and DNA from callus-derived shoot tips (6). **b.** RAPD primer G10: 100 bp DNA ladder (M), DNA from *in vitro* shoot-derived shoot tips (1, 2, 4, 6), DNA from callus-derived shoot tips (3) and DNA from mother plant's leaf (5). **c.** RAPD primer G17: 100 bp DNA ladder (M), DNA from callus-derived shoot tips (1), DNA from *in vitro* shoot-derived shoot tips (2-5) and DNA from the mother plant's leaf (6). **d.** RAPD primer G18: 100 bp DNA ladder (M), DNA from *in vitro* shoot-derived shoot tips (1, 3, 4), DNA from callus-derived shoot tips (2, 5) and DNA from mother plant's leaf (6).

AFLP markers (Xiang et al. 2003, Ferreira et al. 2006) and low polymorphism reported in *Dendrobium moschatum* and *D. chrysotoxum* by using RAPD markers (Tikendra et al. 2019, Tikendra et al. 2019). Similarly, Bhattacharyya et al. (2014) reported 97% of genetic fidelity among the *in vitro* regenerants of *D. nobile*. Cerasela and Lazar (2009) also reported the efficiency of RAPD markers in identifying somaclonal variation in

Cymbidium species. In the present study, the result of the RAPD analysis was considered to be more reproducible and informative.

The genetic homogeneity between *in vitro* plantlets and mother plants was confirmed by RAPD markers. However, the plantlets developed from the shoot-tip explant through callus were genetically different from mother plants. This research paved out the application of *in vitro* culture for the development of true-to-type plants of *D. crepidatum* for the worth of conservation and horticulture.

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References

Baque A, Shin YK, Elshmari T, Lee EJ and **Paek KY** (2011) Effect of light quality, sucrose and coconut water concentration on the microporpagation of *Calanthe* hybrids ('Bukduseong' × 'Hyesung' and 'Chunkwang' × 'Hyesung'). Aust. J. Crop Sci. **5**: 1247-1254.

Bhattacharyya P, Kumaria S, Diengdoh R and **Tandon P** (2014) Genetic stability and phytochemical analysis of the *in vitro* regenerated plants of *Dendrobium nobile* Lindl., an endangered medicinal orchid. Meta Gene **2**: 489-504.

Cameron DD, Leake JR and **Read DJ** (2006) Mutualistic mycorrhiza in orchids: evidence from plant-fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. New Phytol. **171**: 405–416.

Cerasela P and **Lazar A** (2009) *In vitro* variability of *Cymbidium* sp. based on random amplified polymorphic DNA (RAPD) markers. J. Hortic. Biotechnol. **13**: 475-477.

Coello CY, Miceli CL, Orantes C, Dendooven L and **Gutierrez FA** (2010) Plant growth regulators optimization for *in vitro* cultivation of the orchid *Gaurianthe skinneri* (Bateman) Dressier & W.E. Higgins. Gayana Bot. **67**: 19-26.

De LC and **Pathak P** (2015) Value addition in orchids. J. Orchid Soc. India **29**: 31-37.

DeMason DA (2005) Auxin-cytokinin and auxin-gibberellin interactions during morphogenesis of the compound leaves of pea (*Pisum sativum*). Planta **222**: 151-166.

Dutta S, Chowdhury A, Bhattacharjee B, Nath PK and **Dutta BK** (2011) *In vitro* multiplication and protocorm development of *Dendrobium aphyllum* (Roxb.) CEC Fisher. Assam Univ. J. Sci. Technol. **7**: 57-62.

Fay MF (2018) Orchid conservation: how can we meet the challenges in the twenty-first century? Bot. Stud. **59**: 16.

Ferreira WDM, Kerbauy GB and **Costa APP** (2006) Micropropagation and genetic stability of a *Dendrobium* hybrid (Orchidaceae). In Vitro Cell. Dev. Biol. - Plant **42**: 568-571.

Hartati S, Arniputri RB, Soliah LA and **Cahyono O** (2017) Effects of organic additives and naphthalene acetic acid (NAA) application on the *in vitro* growth of Black orchid hybrid (*Coelogyne pandurata* Lindley). Bulg. J. Agric. Sci. **23**: 951-957.

Koirala P, Pyakurel D and **Gurung K** (2011) Orchids in Rolpa district of Western Nepal: Documentation, stock, trade and conservation. Banko Janakari **20**: 3-13.

Lin W, Wang J, Xu X, Wu Y, Qiu D, He B, Sarsaiya S, Ma X and **Chen J** (2020) Rapid propagation *in vitro* and accumulation of active substances of endangered *Dendrobium cariniferum* Rchb. f. Bioengineered **11**: 386-396.

Nasiruddin KM, Begum R and **Yasmin S** (2003) Protocorm like Bodies and Plantlet Regeneration from *Dendrobium formosum* Leaf Callus. Asian J. Plant Sci. **2**: 955-957.

Nuraini A, Heriliya W, Suminar E and **Marliana E** (2010) Responses of protocorm like bodies hybrid *Dendrobium* orchid on various types and concentration of cytokinin and auxin on Murashige and Skoog (MS) medium. *International Seminar on Horticulture to Support Food Security*. Bandar Lampung, Indonesia, 130-135.

Oliya BK, Chand K, Thakuri LS, Baniya MK, Sah AK and **Pant B** (2021) Assessment of genetic stability of micropropagated plants of *Rhynchostylis retusa* (L.) using RAPD markers. Sci. Hortic. **281**: 110008.

Palama TL, Menard P, Fock I, Choi YH, Bourdon E, Govinden-Soulange J, Bahut M, Payet B, Verpoorte R and **Kodja H** (2010) Shoot differentiation from protocorm callus cultures of *Vanilla planifolia* (Orchidaceae): proteomic and metabolic responses at early stage. BMC Plant Biol. **10**: 82.

Pant B (2013) Medicinal orchids and their uses: Tissue culture a potential alternative for conservation. African J. Plant Sci. **7**: 448-467.

Pant B, Pradhan S, Paudel MR, Shah S, Pandey S and **Joshi PR** (2019) Various culture techniques for the mass propagation of medicinal orchids from Nepal. J Kim, YJ Kim, and SJ Park (eds). Acta Hortic. **1262**: 109-124.

Pant B and **Thapa D** (2012) *In vitro* mass propagation of an epiphytic orchid, *Dendrobium primulinum* Lindl. through shoot tip culture. African J. Biotechnol. **11**: 9970-9974.

Park CM, Kwon JC, Han NK, Joung MS, Paek KY and **Choi JW** (2014) Comparative Study of Protocorm-like body and Multiple Shoots from *Dendrobium Candidum* on Biological Activities. J. Soc. Cosmet. Sci. Korea **40**: 29-36.

Parthibhan S, Rao MV and **Kumar TS** (2015) *In vitro* regeneration from protocorms in *Dendrobium aqueum* Lindley-An imperiled orchid. J. Genet. Eng. Biotechnol. **13**: 227-233.

Parvin MS, Haque ME, Moniruzzaman FA and **Khaldun ABM** (2009) Effect of different levels of NAA on in vitro growth and development of shoots of *Dendrobium* orchid. Bangladesh J. Agric. Res. **34**: 411-416.

Paudel MR, Chand MB, Pant B and **Pant B** (2019) Assessment of antioxidant and cytotoxic activities of extracts of *Dendrobium crepidatum*. Biomolecules **9**: 478.

Paudel MR, Rajbanshi N, Sah AK, Acharya S and **Pant B** (2018) Antibacterial activity of selected *Dendrobium* species against clinically isolated multiple drug resistant bacteria. African J. Microbiol. Res. **12**: 426-432.

Piria RS, Rajmohan K and **Suresh S** (2008) *In vitro* production of protocorms and protocorm like bodies in orchids–A review. Agric. Rev. **29**: 40-47.

Posselt UK, Barre P, Brazauskas G and **Turner LB** (2006) Comparative analysis of genetic similarity between perennial ryegrass genotypes investigated with AFLPs, ISSRs, RAPDs and SSRs. Czech J. Genet. Plant Breed. **42**: 87-94.

Pradhan S, Paudel YP and **Pant B** (2013) Efficient regeneration of plants from shoot tip explants of *Dendrobium densiflorum* Lindl., a medicinal orchid. African J. Biotechnol. **12**: 1378-1383.

Rajbhandari KR (2014) Orchids of Nepal: Status, threat and conservation. *Proceedings of National Workshop on NTFP/MAPs Sector Action Plant Development: Orchid*. Department of Plant Resources, Ministry of Forest and Soil Conservation and Central Department of Botany, Tribhuvan University, Kathmandu, Nepal, 1-40.

Rasmussen HN (2002) Recent developments in the study of orchid mycorrhiza. Plant Soil **244**: 149-163.

Sheela VL, Sarada S and **Anita S** (2006) Development of protocorm-like bodies and shoots in *Dendrobium* cv. Sonia following gamma irradiation. J. Trop. Agric. **44**: 86-87.

da Silva JAT (2013) Orchids: Advances in tissue culture, genetics, phytochemistry and transgenic biotechnology. Floric. Ornam. Biotechnol. **7**: 1-52.

da Silva JAT, Cardoso JC, Dobránszki J and **Zeng SJ** (2015) *Dendrobium* micropropagation: a review. Plant Cell Rep. **34**: 671-704.

da Silva JAT, Winarto B, Dobranszki J, Cardoso JC and **Zeng SJ** (2016) Tissue disinfection for preparation of *Dendrobium in vitro* culture. Folia Hortic. **28**: 57-75.

Subedi A, Kunwar B, Choi Y, Dai Y, van Andel T, Chaudhary RP, de Boer HJ and **Gravendeel B** (2013) Collection and trade of wild-harvested orchids in Nepal. J. Ethnobiol. Ethnomed. **9**: 64.

Tikendra L, Amom T and **Nongdam P** (2019) Molecular genetic homogeneity assessment of micropropagated *Dendrobium moschatum* Sw. - A rare medicinal orchid, using RAPD and ISSR markers. Plant Gene **19**: 100196.

Tikendra L, Koijam AS and **Nongdam P** (2019) Molecular markers based genetic fidelity assessment of micropropagated *Dendrobium chrysotoxum* Lindl. Meta Gene **20**: 100562.

Tingey SV and **del Tufo JP** (1993) Genetic Analysis with Random Amplified Polymorphic DNA Markers. Plant Physiol. **101**: 349-352.

Xiang N, Hong Y and **Lam-Chan LT** (2003) Genetic analysis of tropical orchid hybrids (*Dendrobium*) with fluorescence amplified fragment-length polymorphism (AFLP). J. Am. Soc. Hortic. Sci. **128**: 731-735.

Yadav K, Aggarwal A and **Singh N** (2013) Evaluation of genetic fidelity among micropropagated plants of *Gloriosa superba* L. using DNA-based markers - a potential medicinal plant. Fitoterapia **89**: 265-270.

Yeung EC (2017) A perspective on orchid seed and protocorm development. Bot. Stud. **58**:33.

Yong JWH, Ge L, Ng YF and **Tan SN** (2009) The Chemical Composition and Biological Properties of Coconut (*Cocos nucifera* L.) Water. Molecules **14**: 5144-5164.

Zhao P, Wu F, Feng FS and **Wang WJ** (2008) Protocorm-like body (PLB) formation and plant regeneration from the callus culture of *Dendrobium candidum* Wall ex Lindl. In Vitro Cell. Dev. Biol. - Plant **44**: 178-185.

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