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Micropropagation and assessment of genetic stability of *Dendrobium transparens* Wall. Ex Lindl. using RAPD and ISSR markers

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Introduction: *Dendrobium* species have been widely used for many health disorders since ancient times. However, due to unrelenting collection to meet the increasing demand for their use in medication and other health products, the natural habitats of medicinal *Dendrobium transparens* have been devastated and are on the verge of extinction.

Methods: An efficient *in-vitro* propagation protocol for *Dendrobium transparens* using seed derived protocorms was established and genetic homogeneity of the *in-vitro* regenerants and the wild plant was studied.

Results: The maximum seed germination was observed in Full strength Murashige and Skoog medium (FMS). Induction of protocorms were achieved on basal as well as half-strength MS medium. The highest number of shoot (11.9 shoots/explant) was achieved in half MS medium fortified with 100 mL/L coconut water in addition with Benzyl amino purine (BAP) 1 mg/L and Kinetin 2 mg/L. Further, elongated shoots were transferred to full and half strength MS root initiating medium supplemented with different concentration of auxins. However, a maximum of (8.3 ± 0.6, 4.9 ± 0.1 cm) roots were achieved in full MS medium fortified with 100 mL/L coconut water and Napthalene acetic acid (NAA) 1.5 mg/L. Ten rapid Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) primers were used to analyze genetic stability among *in-vitro* and mother plant. RAPD primers produced a total of 23 fragments while ISSR primers produced a total of 16 fragments.

Conclusion: The amplified bands of all the samples of *in-vitro* plants were similar to bands of mother plant. The present research reported here is indicating the applicability of tissue culture for true-to-type plant production and conservation of *D. transperens*.

KEYWORDS

Medicinal plants, *Dendrobium*, Protocorm, Primer, ISSR, RAPD, PCR

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Introduction

Dendrobium is one of the largest genera in Orchidaceae represented by more than 1,100 species in the world and having 30 species in Nepal (Rajbhandari, 2015). These are most prized orchid species for their beautiful long-lasting flowers exhibiting an incredible range of diversity in size, shape and color. Beside ornamental value, *Dendrobium* species are widely used for many health disorders from ancient period of time. The stems of Dendrobium species have been widely used in traditional Chinese medicine (TCM) for improving digestion, promoting and production of body fluids, nourishing, clearing heat, and as tonic (Xu et al., 2013). *Dendrobium* species are sources of large amount of polysaccharides, phenanthrenes, revesteral, polyphenol compounds acting as potent source of antioxidant and cytotoxic effects (Gutiérrez, 2010; Pant, 2013).

D. transparens (Figure 1) is mildly fragment epiphytic sympodial orchid distributing along geographical ranges from Nepal, Bangladesh, eastern Himalayas, India, Bhutan, Myanmar, and Vietnam. These are found in dense forest at elevations of 500 to 2100 meters above sea level. It ranks among the important ornamental orchids with graceful pendulous racemes of medium sized flowers, usually pristine white except purple blemish at tip of the sepals, petals and throat of labellum (efloras, 2020). *D. transparens* based herbal formulations used for geriatric diseases & disorders in Indian traditional healing system. As a result, it has been collected illegally from wild Due to unremitting collection to meet the increasing demand for their use in medication and other health products, the natural habitats of medicinal *D. transparens* has been devastated and are under danger of extinction. Rate of multiplication of *Dendrobium* species in nature is extremely very slow with 2-4 shoots per year as well as rate of seed germination of orchids is very complex as these require specific fungal partner due to lack of endosperm and nutritive materials (Otero et al., 2002; Pant et al., 2017). Thus, micro propagation by tissue culture is particularly useful to conserve their germplasm and for medicinal utilization (Pant et al., 2018). Past couple of decades have magnified the role of *in-vitro* techniques in orchid conservation efforts, it would be one of the most suitable alternative tools to minimize the pressure on natural population of medicinal orchids (Pfab & Scholes, 2004; Pant, 2014) and thus help in their sustainable utilization and conservation. Furthermore, *in-vitro* techniques are highly useful for providing sustainable sources of optimal plant-derived natural products (Bourgaud et al., 2001; Vanisree et al., 2004; Hussain et al., 2012; Pradhan et al., 2014; Ochoa-Villarreal et al., 2016; Espinosa-Leal et al., 2018; Regmi et al., 2017). Orchid species have been cultured with various techniques to synthesize bioactive molecules *in-vitro* (Bourgaud et al., 2001; Vanisree et al., 2004; Giri et al., 2012b; Hussain et al., 2012).

Several medicinal *Dendrobium* species has been *in-vitro* propagated through asymbiotic seed germination or direct shoot regeneration or protocorm formation from different explants including *D. candidum* (Shiau et al., 2005), *D. chrysotoxum* (Xu et al., 2001), *D. fimbriatum* (Roy and Banerjee, 2003; Sharma et al., 2005), *D. nobile* (Nayak et al., 2002; Malabadi et al., 2005), *D. tosaense* (Lo et al., 2004), *D. chrysanthum* (Rao & Barman, 2014), *D. longicornu* (Dohling et al., 2012), (Teixeira da Silva et al., 2015b). So that these important species can be conserved and sustainably utilized for different medicinal use (Teixeira da Silva et al., 2014). Somaclonal variations are often observed within the *in-vitro* raised plants. To ascertain the genetic similarity between the *in-vitro*

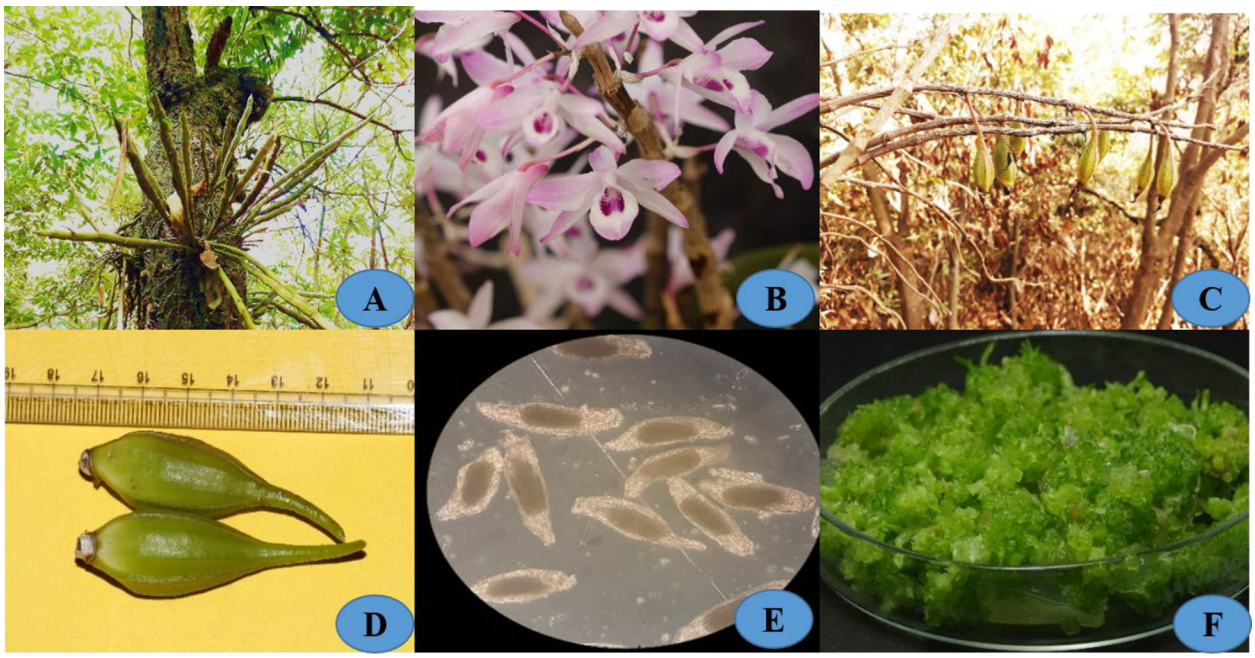

FIGURE 1
(A) Habitat; **(B)** Close up flower; **(C)** Seed pods in nature; **(D)** Seed pods of length 4-5 cm long; **(E)** seeds of **(D)** transparens; **(F)** Protocorms developed earlier most of 4 weeks in FMS medium.

grown plantlets and the mother plant, molecular markers are often used to study the variation within the germplasm (Kumar et al., 2014). Traditionally, various conventional molecular markers like random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), and simple sequence repeats (SSR) are used extensively in the assessment of clonal fidelity in a wide range of plants species including orchids (Devi et al., 2013; Bhattacharyya et al., 2014; Devi et al., 2014; Bhattacharyya et al., 2015; Pandey et al., 2020; Oliya et al., 2021). Moreover, use of more than one molecular marker to evaluate clonal fidelity of tissue culture-raised plants is significant (Palombi and Damiano, 2002).

Because of this, in the current study, RAPD and ISSR were used to assess the clonal fidelity of the regenerates. The former method uses a single primer to detect nucleotide sequence polymorphism while the latter allows the detection of polymorphism in inter-microsatellite loci using a primer made from dinucleotide or trinucleotide simple sequence repeats (Parab and Krishnan, 2008). The wild population of the medicinal and ornamentally significant orchid *D. transparens* is being exploited as a result of random collection. Because micropropagation of *D. transparens* can help scale up several fold through *in-vitro* propagation, the present investigation was carried out on *D. transparens* to examine the effects of various plant growth regulators on seed germination, protocorm induction, and shoot development.

Material and methods

Plant material and surface sterilization

Immature capsules of *D. transparens* were collected during June and July of 2018 from Bhaktapur district (27.6451°N, 85.4427°E) of central Nepal. They were used as explant to produce protocorms *in vitro*. A voucher specimen of this plant was deposited in the Tribhuvan University Central Herbarium (TUCH) (voucher number P08), Kathmandu, Nepal. The capsules were surface sterilized with 1-2 drops of TWEEN-20 and washed under running tap water for at least half an hour. The capsules were then dipped in 1% sodium hypochlorite solution for 15 minutes. After that they were submerged in 70% ethanol with the help of long forceps and were rapidly set a flame for a few seconds. The capsules were rinsed in sterile water intermittently. The explants were then blot dried using a Whatman filter paper placed onto sterile petri plates and inoculated in test tubes containing Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium containing 3% sucrose (w/v), 0.1% myoinositol supplemented with coconut water and plant growth hormones The pH of the medium was adjusted to 5.8 with 1N NaOH or 1N HCL and solidified with 0.8% plant tissue culture tested agar-agar (w/v) (Himedia, India), prior to autoclaving (121°C, 20 min), The cultures were maintained at 25°C ± 2°C and 1000 flux lux under 16/8 h photoperiod with 75% relative humidity (RH). These surface sterilized capsules were then cut longitudinally with a sterile scalpel and the exposed seeds were transferred to an agar gel (8gm/L) nutrient medium for seed germination.

Inoculation in culture medium and shoot proliferation, elongation and root formation

Immature seeds were placed on MS solid medium supplemented with 6-benzylaminopurine (BAP) 0.5–2.0 mg/L, in combination with α-naphthalene acetic acid (NAA) 0.5–1.0 mg/L for the formation of protocorms. The Protocorms were transferred to the basal MS medium for proliferation. After 2 months of maturation, protocorms were cultured on growth regulator-free MS medium as control and the media containing NAA, BAP or Kin with a concentration range from 0.5 to 2.0 mg/L in various combinations for shoot development. Healthy shoots with 2–3 leaves were sub cultured on the basal MS medium for further development. For root initiation, the basal MS medium was supplemented with NAA, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) each at 1–1.5 mg/L and coconut water (100 mL/L) in different combination. The frequency and number of shoots formed were evaluated after 4 weeks of inoculation. Morphological changes were recorded on the basis of visual observations.

Genetic stability study of *in-vitro* developed plants compared with mother plants

Selection of *in-vitro* samples for DNA isolation

For the genetic profiling, leaves of *in vivo* mother plants were used. The rooted plants and protocorms of *in-vitro* materials were selected based on medium that provided the optimal growth responses. Leaves of four *in-vitro* plant samples grown on FMS medium containing 2 mg/L BAP+1 mg/LNAA, 10% CW and protocorm developed on HMS medium were used for the genetic fidelity studies. The genetic profiling experiment was performed in Annapurna Research Center, Maitighar, Kathmandu, Nepal.

Extraction of DNA by CTAB method

Genomic DNA from mother plants and *in-vitro* grown plant samples were extracted using the CTAB (hexadecyltrimethyl ammonium bromide) method of Doyle (1991) with some modifications. Briefly, 0.2 g of *in vivo* (leaf) and *in-vitro* (leaf and protocorm) samples were taken and grinded to a fine powder in liquid nitrogen using mortar and pestle. To this, 1 mL of CTAB buffer (2% CTAB, 0.5 M EDTA, 5 M NaCl, 1 M Tris HCl, Ph 8, 0.2% β-mercaptoethanol) was added and mixed to a fine paste. The paste was transferred into a clean sterilized micro-centrifuge tube (vol. 1.5 mL) and incubated in water bath at 65°C for 30 min. with a gentle inversion every 10 min. After incubation samples were allowed to cool for 3– 5 min. and then centrifuged at 12,000 rpm for 8 min. to spin down cell debris. The resultant supernatant was transferred to a clean sterilized microcentrifuge tube and an equal volume of chloroform: isoamyl alcohol in the ratio of 24: 1 was added and mixed gently by inversion for 5– 8 min. It was again centrifuged at 13,000 rpm for 5 min. and the upper aqueous phase was transferred to sterilized microcentrifuge tube. Then 1/10th volume of ammonium

acetate (7.5 M) was added to each sample followed by the addition of 500 µL chilled ice-cold absolute ethanol. The tubes were slowly inverted several times (3– 5 min.) to precipitate the DNA. After that, the DNA was centrifuged at 13, 000 rpm for 2 min. to form the pellet. The supernatant was discarded and the pellet was washed with ice-cold 70% ethanol (Vol. 500 µL). Again, it was centrifuged at 13,000 rpm for 1 min. to get rid of salt. The remaining ethanol was then pipetted out and the pellet was allowed to dry inside a Laminar Flow Cabinet for 30 min. Finally, the extracted DNA was diluted in 1X TE buffer and the quality of the extracted DNA was evaluated using 1% agarose gel (w/v) electrophoresis. The DNA was stained by ethidium bromide for 45 min. with a continuous supply of 70 voltage power. After that, it was visualized by UV gel documentation system (UVITEC Cambridge). The quantity of the DNA was evaluated using a fluorimeter (Thermo Fisher scientific, USA). The concentration of extracted DNA was adjusted to 30 ng and stored at – 20 °C.

DNA amplification and RAPD, ISSR analysis

A total of ten RAPD and ISSR markers were screened and markers with clear and reproducible bands were considered for the analysis. The PCR amplification was performed on a final volume of 15 µL containing 30 ng of genomic DNA, 6.5 µL of Dream Taq PCR master mix (Thermo Fisher Scientific, USA) and 1 µM each primer. The cycling condition was carried out in a Pro Flex PCR (Thermo Fisher Scientific, USA) programmed at 94 °C for 5 min. followed by 40 cycles of denaturing at 94 °C for 45 s, annealing at 40-45°C for 1min for RAPD and 50-58°C for 1 min for ISSR, extension at 72 °C for 30 s, and then a final extension at 72 °C for 5 min. To test the utility of the primers, PCR products were detected on 1% agarose gels with a constant voltage of 70 V for 1.5 h. The size of the amplification product was determined by comparison with the 100 bp DNA ladder marker (Thermo Fisher Scientific, USA). The clear and reproducible bands were considered for counting alleles.

Experimental design and statistical analysis

Experiment was carried out in a randomized design and replicated with repetition at least three times. Visual observations were made every week. Explants forming protocorms and number of protocorms per explant were recorded after 4 weeks of protocorms induction. Protocorms number developing into shoots was counted after 4 weeks of shoot induction. The percentage of shoots producing roots and number of roots per plantlet were recorded after 25 days of root initiation. Data were statistically analyzed for significance using one way analysis of variance (ANOVA) and the differences were contrasted *via* Duncan's test at $P \leq 0.05$ using the SPSS version 20

Result

Seed germination and protocorms induction

The seeds of *D. transparens* were cultured *in-vitro* on FMS alone and full MS supplemented with BAP (0.5 to 2.0) mg/L and NAA (1 mg/L) (Table 1). The quick response on seed germination of *D. transparens* was observed on hormone free FMS medium that took 4 weeks of culture (Table 1; Figure 1). Protocorms were initiated earlier (4 weeks of seed culture) with longest span life on FMS medium. Thus, protocorms developed in full MS medium were used for phytochemical extraction. The full MS medium supplemented with BAP 2 mg/L and NAA 1 mg/L initiated protocorms after 6 weeks of seed culture however, the primary shoot and root were premature within 8 weeks and 11 weeks of culture respectively (Table 1; Figure 2).

This condition was followed by full MS supplemented with BAP 1.5 mg/L and NAA 1 mg/L which took 10 weeks of culture for shoot initiation and 14 weeks of culture for root initiation. It was found that the lower concentration of BAP in addition with NAA were not effective for the development of shoot and root until 16 weeks of culture.

Shoot multiplication using protocorms

For the shoot multiplication, MS medium with full (FMS) and half strength (HMS) MS medium supplemented with or without hormone and coconut water were used. During study, the maximum shoot multiplication of *D. transparens* was observed on half MS medium fortified with 100 mL/L coconut water in addition with BAP 1 mg/L and Kinetin 2 mg/L with highest number of shoot (9.0 shoots/plant) with shoot length (4.5 ± 0.1 cm) (Figure 2).

TABLE 1 Effect of different strength of MS medium with/out BAP and NAA for *in-vitro* culture of *D. transparens*.

S.N.	Media	PGRs		Initiation of events observed in a Week			
		BAP	NAA	Germination	Protocorms	Shoot	Root
1.	HMS	0	0	5 ± 0.3	7 ± 0.6	10 ± 0.5	12 ± 0.7
2	FMS	0	0	4 ± 0.4	6 ± 0.5	9 ± 0.7	0 ± 0
3	FMS	0.5	1.0	7 ± 0.5	9 ± 0.3	0 ± 0	0 ± 0
4	FMS	1.0	1.0	6 ± 1.6	7 ± 0.5	0 ± 0	0 ± 0
5	FMS	1.5	1.0	6 ± 1.1	7 ± 0.7	10 ± 0.2	14 ± 0.7
6	FMS	2.0	1.0	5 ± 0.4	6 ± 1.5	8 ± 0.3	11 ± 0.2

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TABLE 2 *In-vitro* shoot development of *D. transparens* in various media supplements.

S.N.	Media	Organic Additive	PGRs			Observation after 5 wks. of protocorms culture	
		CW	BAP	Kin	NAA	Shoot No.	Shoot Length
1.	HMS	100	1.0	0	0	4.6 ± 0.6	1.6 ± 0.2
2.	HMS	100	1.0	1.0	0	5.3 ± 0.6	2.9 ± 0.6
3.	HMS	100	1.0	2.0	0	9.0 ± 1.0	4.5 ± 0.1
4.	HMS	100	1.0	0	1.0	4.0 ± 1.0	2.5 ± 0.1
5.	HMS	100	1.0	0	2.0	1.6 ± 1.2	1.4 ± 0.2
6.	FMS	100	1.0	0	0	4.0 ± 0.0	4.1 ± 0.3
7.	FMS	100	1.0	1.0	0	5.0 ± 1.0	5.0 ± 0.4
8.	FMS	100	1.0	2.0	0	3.3 ± 0.6	3.3 ± 0.5
9.	FMS	100	1.0	0	1.0	2.3 ± 0.6	2.3 ± 0.9
10.	FMS	100	1.0	0	2.0	4.3 ± 0.6	4.1 ± 0.3

[Means within the column are not significantly different by Dunkan test ($P \leq 0.05$)].

In-vitro root initiation

The maximum numbers and length of root was observed on full MS medium fortified with 100 mL/L coconut water and NAA 1.5 mg/L (8.3 ± 0.6 no, 4.9 ± 0.1 cm) (Table 2; Figure 2). In the present study, full strength of MS medium was found very effective for proper germination. For better growth, germinating seedlings were transferred to half strength medium supplemented with 100 mL/L coconut water after formation of protocorms after 6 weeks of germination. The efficacy of multiple shoot formation differed with concentrations and combinations of BAP, Kinetin, and NAA and coconut water. Multiple shoots were derived from the protocorms like bodies. Explants cultured on half-strength MS medium supplemented with BAP 1 mg/L, Kinetin 2 mg/L with 100 mL/L coconut water developed multiple shoots (Figure 2). Well-developed roots were induced in all the shoots derived from protocorms with different auxins within 15 d of culture. In control treatment, where full and half strength of MS medium was not supplemented with any auxin, the rooting of plants developed from shoots delayed and roots appeared after 25 to 30 days of culture. Rooting efficiency under such condition when analyzed among the auxin treatments, IBA with 100 mL/L coconut water deliberated the highest number of roots developed (10 roots/plant), differing significantly from other treatments. However, development

TABLE 3 *In-vitro* root development of *D. transparens* in various media supplements.

S.N.	Media	Organic Add.	PGRs			Observation after 8 wks. of shoot culture	
		CW	NAA	IAA	IBA	Root No.	Root Length
1.	HMS	0	0	0	0	2.7 ± 1.2	0.4 ± 0.4
2.	HMS	100	1.0	0	0	7.3 ± 0.6	2.2 ± 0.3
3.	HMS	100	1.5	0	0	5.3 ± 0.6	1.1 ± 0.1
4.	HMS	100	0	1.0	0	0.7 ± 0.6	0.2 ± 0.4
5.	HMS	100	0	1.5	0	1.3 ± 0.6	0.4 ± 0.1
6.	HMS	100	0	0	1.0	0.7 ± 0.6	0.8 ± 0.6
7.	HMS	100	0	0	1.5	0.7 ± 0.6	0.7 ± 0.6
8.	FMS	0	0	0	0	0.7 ± 0.6	0.1 ± 0.1
9.	FMS	100	1.0	0	0	7.7 ± 0.6	2.9 ± 0.1
10.	FMS	100	1.5	0	0	8.3 ± 0.6	4.9 ± 0.1
11.	FMS	100	0	1.0	0	0.3 ± 0.6	0.4 ± 0.3
12.	FMS	100	0	1.5	0	3.0 ± 0.0	0.3 ± 0.3
13.	FMS	100	0	0	1.0	4.7 ± 0.6	2.2 ± 0.2
14.	FMS	100	0	0	1.5	6.7 ± 0.6	2.8 ± 0.3

[Means within the column are not significantly different by Dunkan test ($P \leq 0.05$)].

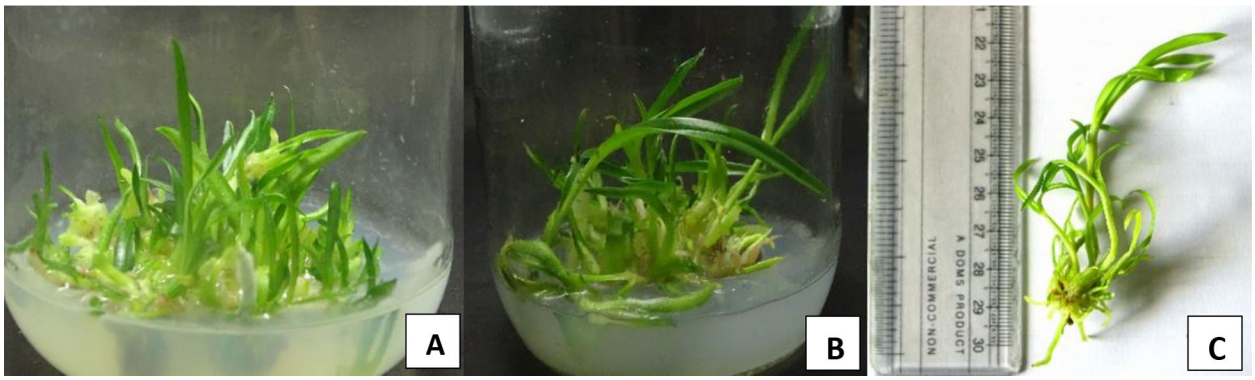

FIGURE 2
(A) From left: shoot development on HMS+10%CW +BAP (1mg/L) + KIN(2mg/L); **(B)** Root development on FMS+10% CW+NAA (1.5 mg/L); **(C)** Plantlet developed in HMS+ 7NAA.

of maximum mean root length (4 cm) was favored by full MS medium supplemented with NAA 1.5 mg/L.

However, best response of rooting in the present study was found on full strength MS medium supplemented with NAA (1.5 mg/L) and coconut water, resulting in higher number of early rooting than when cultured on an auxin free medium. The auxin, NAA exerted better response on longer root length. The well shooted and rooted plantlet were transferred in various concentration of NAA before acclimatization from 3mg/l to 10mg/l where we found that high concentration of NAA (7mg/l) was best for plantlet development before acclimatization. During the study it was observed that that FMS supplemented with 1.5 mg/L NAA was found best for the robust root and shoot elongation in case of D. transparens (Table 3).

Genetic fidelity analysis of *in-vitro* plantlets

Somaclonal variations causes genetic defects which can limit the use of the *in-vitro* propagation system (Salvi et al., 2001). However, for large-scale propagation, efficiency of propagation methods along with genetic stability of the regenerated plants is of paramount importance (Haisel et al., 2001). In the present study, out of the 10 RAPD primers tested only eight primers (OPA 10, OPA 03, OPA 06, OPA 07, OPC 15, OPC19, OPC 20, OPA 01) gave similar and reproducible banding patterns between the *in-vitro* raised plantlets and protocorm and the mother plant (Table 4; Figure 3). A total of 18 bands were scored from *in-vitro* plants of *D. transparens*. The number of scorable bands varied from 2 to 5 within the approximate size range of 200-1300 bp while compared to ladder marker (1000bp sized). Similarly, five ISSR markers (UBC 880, UBC 834, UBC 826, UBC 844, UBC 807) showed clear and reproducible band. A total of 17 band were scored from *in-vitro* and mother plants of *D. transparens* which varied from two to three within the approximate size range of 500-700 bp while compared to ladder marker (1000bp sized) (Table 5; Figure 4).

Discussion

Since mycorrhizal fungus association is necessary for orchid seed germination in nature, ex situ conservation of threatened medicinal

TABLE 4 List of RAPD primers used to confirm the genetic fidelity of *D. transparens*.

Primer Name	Sequence (5′-3′)	Length (bp)	Annealing Temperature (˚C)	No. of Fragments
OPA-1	CAGGCCCTTC	10	44.2	1
OPA-3	AGTCAGCCAC	10	44.2	3
OPA-6	GGTCCCTGAC	10	44.2	2
OPA-10	GTGATCGCAG	10	44.2	3
OPC-11	AAAGCTGCGG	10	44.2	0
OPC19	GTTGCCAGCC	10	44.2	2
OPC 20	ACTTCGCCAC	10	41.4	2
OPC 15	GACGGATCAG	10	44.8	4
OPC 18	TGAGTGGGTG	10	40.4	0
OPA 07	GAAACGGGTG	10	40.7	1

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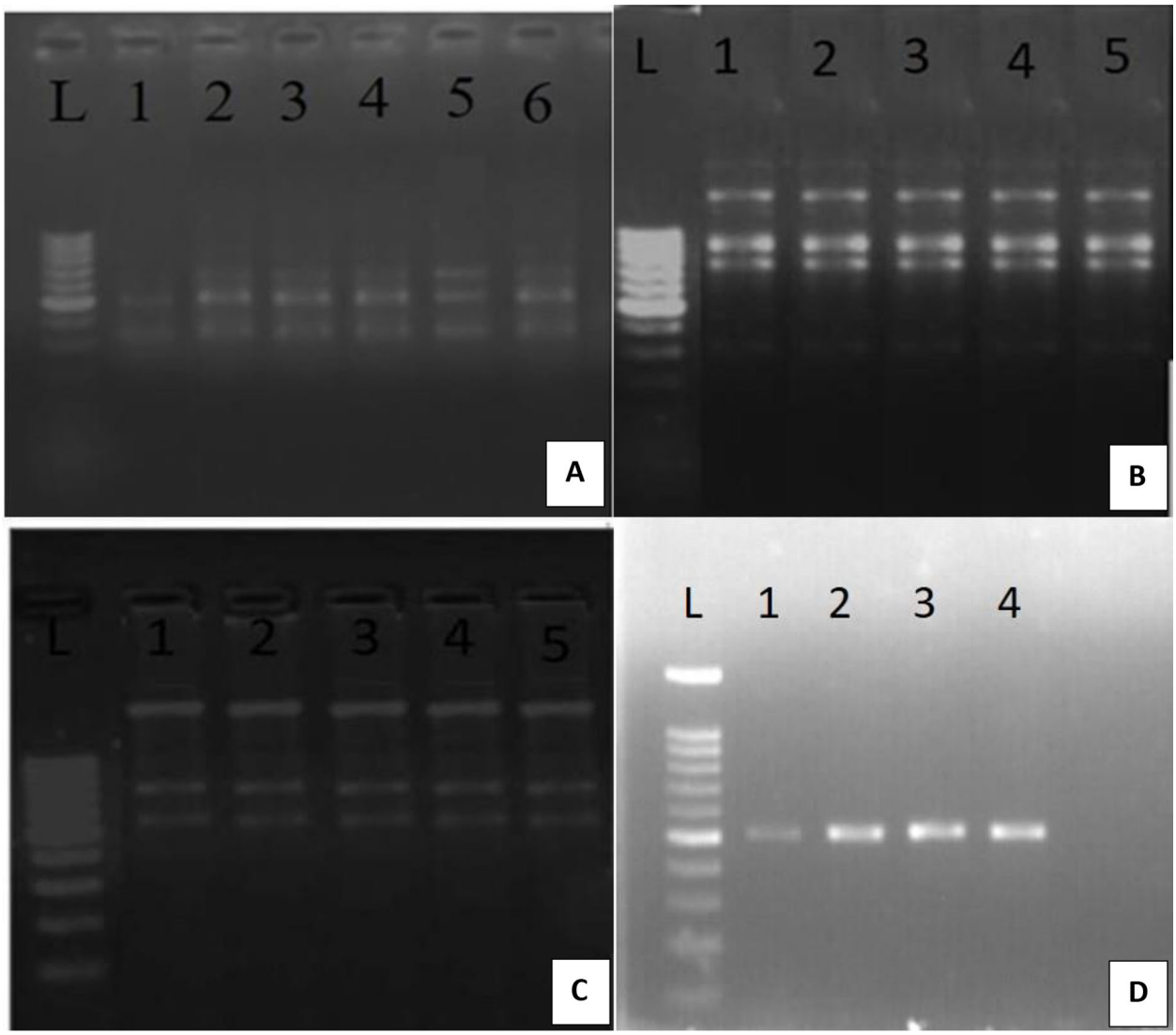

FIGURE 3
Genetic fidelity analysis of *in-vitro* and mother plants of *D transparens* using RAPD marker **(A)** OPA 10 Primer L- Lader, 1 to 5 *in-vitro* plants; 6 mother plant; **(B)** OPA03 Primer L- Lader, 1 to 4 *in-vitro* plants; 5 mother plant. **(C)** OPA06 Primer L- Lader, 1 to 4 *in-vitro* plants, 5 mother plant, **(D)** OPA07 Primer L- Lader, 1 to 3 *in-vitro* plants 4 mother plant; (Size of ladder- 1000 bp).

orchid species is crucial. The most popular method for multiplying various orchid species is the *in-vitro* seed germination technique, which has been developed for a number of species. The most trustworthy method for preserving and using plant materials sustainably is the production of true-to-type plants using plant tissue culture (Teixeira da Silva et al., 2015a; Teixeira da Silva et al., 2015b). In the current study, the micropropagation procedure for *D. transparens*, a threatened orchid species, was standardized using protocorm culture. FMS showed the best seed germination, and the same holds true for protocorm development. *D. transparens* protocorms were transplanted into medium that also contained plant growth regulators like BAP, KIN, NAA by itself or fortified with 10% C.W and the shooting and rooting of the plants were also evaluated (Table 1). The development of *D. transparens* protocorm and plantlets was successful under every test condition. Recent study on the asymbiotic germination of the immature embryos of *D. transparens* on full-strength MS medium, it was found that the maximum shoot multiplication of *D. transparens* occurred on this medium when it was fortified with 100 mL/L of coconut water along with 1 mg/L of BAP, and 2 mg/L of kinetin (Alam et al., 2002; Sunitibala et al., 2009). Study on *D. transparens*, which was similar, it was observed that BAP in conjunction with NAA induced best shoots. Contrary to our study, the use of NAA in the initial condition was not found to be active in the proliferation of the shoots, but the use of HMS along with NAA and CW was found to be significant to be helpful in elongation of the shoots. Similar findings were seen in *D. densiflorum*, where shoot development was improved by FMS fortified with coconut (Pant et al., 2022). According to other studies, a few *Dendrobium* orchids needed nutrient media with organic additives to induce multiple shoots. To encourage multiple shoots in *D. antennathum*, peptone was added to the medium (Kukulczanka and Wojciechowska, 1982), whereas yeast extract or urea was used for *D. chrysanthum* (Vij and Pathak, 1989). The most effective method for inducing multiple shoot and root

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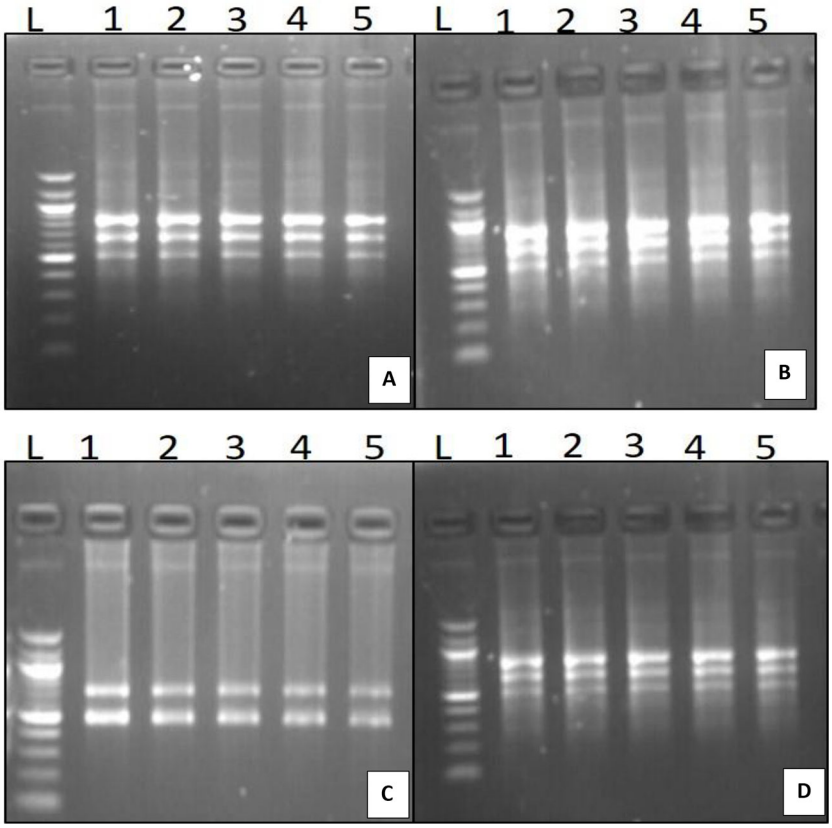

FIGURE 4
Genetic fidelity analysis of *in-vitro* and mother of *D.transparens* using ISSR marker **(A)** UBC 826 Primer L- Lader, 1 to 4 *in-vitro* plants; 5 mother plant; **(B)** UBC 834 Primer L- Lader, 1 to 4 *in-vitro* plants; 5 mother plant. **(C)** UBC 844 Primer L- Lader, 1 to 4 *in-vitro* plants, 5 mother plant, **(D)** UBC 880 Primer L- Lader, 1 to 4 *in-vitro* plants 4, 5mother plant; (Size of ladder- 1000 bp).

TABLE 5 List of ISSR primers used to confirm the genetic fidelity of *D. transparens*.

Primer Name	Sequence (5′- 3′)	Length	Annealing Temperature (˚C)	No of Fragments
HB9	GTGTGTGTGTGTGG	14	58	0
UBC 815	CTCTCTCTCTCTCTCTG	17	58	0
UBC 880	GGAGAGGAGAGGAGA	15	56	3
HB 10	GAGAGAGAGAGACC	14	52.3	0
UBC 834	AGAGAGAGAGAGAGAGYT	18	55	3
UBC 814	CTCTCTCTCTCTCTCTA	17	53	0
UBC 826	ACACACACACACACACC	17	56	3
UBC 843	CTCTCTCTCTCTCTCTRA	18	56	0
UBC 844	CTCTCTCTCTCTCTCTRC	18	56	2
UBC 807	AGAGAGAGAGAGAGAGT	17	53	6

micropropagation of *D. transparens*, according to the most recent results, was CW. The present study was supported by the observation that HMS and CW were used in the micropropagation of *D. chryseum* for multiple shoot regeneration. (Maharjan et al., 2020; Teixeira da Silva et al., 2015a). Similar findings showed that FMS supplemented with 1.5 mg/L NAA was best for *D. transparens* robust root and shoot elongation (Figure 2; Table 3). These findings were further proved by (Aktar et al., 2007), who discovered that *Dendrobium* sp. roots with the longest lengths were grown on MS medium supplemented with 1 mg/L IBA. The positive effect of auxins on root initials as seen by these responses was explained by (De Klerk et al., 1997). Shiau et al. (2005) reported that half-strength MS medium supplemented with

0.2 mg/L NAA was effective for the development of roots on *D. candidum*, supporting the current finding. The shoots of *Dendrobium* hybrids showed rooting on Went medium (VW) medium supplemented with 2 mg/L IBA and IAA in the earlier study (Dodds, 1991). *Geodorum densiflorum* shoots were established *in-vitro* on MS medium containing 1.0 M NAA (Sheelavantmath et al., 2000). According to a review of dendrobium micropropagation by (Teixeira da Silva et al., 2015b), BAP and NAA, either together or separately in the culture medium, are the most frequently used plant growth regulators (PGRs) in *Dendrobium* micropropagation. According to (Li et al., 2013), different species have different standardized media for inducing shoots and roots. For *D. pendulum* and *D. primulinum*, the appropriate media for shoot induction were HMS 0.5 mg/l BA + 0.1 mg/l NAA + 100 ml/l coconut water (CW), and for *D. heterocarpum*, HMS 0.25 mg/l BA + 0.1 mg/l NAA + 100 ml/l CW.

Compared to Ladder marker, there are 1 to 6 scorable bands that are roughly 1000 bp in size (100bp sized). More polymorphisms are a sign of more genetic diversity. However, the current study found that *D. transperens* had more genetic similarity than polymorphism (Figures 3, 4). The *in-vitro* cultured plants displayed 100% monomorphism between and within them, according to RAPD and ISSR markers. The current finding is corroborated by (Giri et al., 2012a), who discovered no differences in ISSR profiles between plantlets of *Habenaria edgeworthii* (Terrestrial orchids) that were *in-vitro* regenerated and derived from 6-benzyladenine (BAP) and a-naphthalene acetic acid (NAA) treatments. Inter-retrotransposon amplified polymorphism (IRAP) and start codon targeted (SCoT) profiles among the *in-vitro* regenerated plantlets of *Ansellia africana* (Leopard orchid) derived through TDZ and NAA treatments revealed a high level of genetic homogeneity (Bhattacharyya et al., 2018). Similar to this, (Roy et al., 2012) discovered a 5.81% variation in RAPD profiles among *Cymbidium giganteum in-vitro* regenerants produced as a result of thidiazuron (TDZ) treatment. Direct somatic embryo regenerated plants (94.22%) have higher genetic homogeneity than indirect organogenesis, according to (Sherif et al., 2018). In our study no genetic variation was observed between the *in-vitro* and wild *D. transperens* when analyzed using RAPD and ISSR markers.

Conclusion

The technique of *in- vitro* propagation can be effectively used to propagate medicinally important and commercially valuable plant species. In this study a highly reproducible, rapid and efficient protocol was established for high-frequency immature seed germination of *D. transparens*. The genetic fidelity analysis using two different markers to assess the similarity with the mother plant was carried out and no variations were detected in both tissue culture derived plantlets and the subsequently mother plant of *D. transparens*. When compared to their mother plant, the *in-vitro* regenerated plants displayed a high degree of genetic stability. The current technology aims to be a promising one for conservation and sustainable utilization of germplasm and producing *D. transparens* plantlets that are true to type in large scale. This technology can be economical and highly benefitted for long term *in- vitro* conservation of other orchids as orchid germination in nature is very difficult. To our knowledge, this is the first report on *D. transparens in- vitro* propagation and also assessment of genetic fidelity in the *in-vitro* generated plants of orchids.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

PJ designed, executed the experiments and prepared the manuscript. SP helped in the experimental analysis and assisted in manuscript preparation. LM helped in the experimental analysis. BP overall supervised the entire research work and critically evaluated the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcosc.2022.1083933/full#supplementary-material

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