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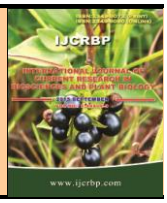

Original Research Article

In Vitro Multiple Shoot Induction from the Nodal and Shoot Tip Explants of *Dysolobium pilosum* (Fabaceae)

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Abstract

The present study has successfully demonstrated a reproducible protocol for multiple shoot induction in *Dysolobium pilosum* (Klein ex Willd.) Maréchal. As there are no earlier reports on the plant propagation, for the first time, an attempt was made to develop an efficient protocol for *in vitro* micropropagation using nodal and shoot tip explants. Maximum numbers of multiple shoots were induced in MS medium, supplemented with N^6-Benzyladenine (BA). Nodal explants developed multiple shoots on 8.90 µMBA supplemented MS medium and produced a good number (26.92 ± 0.29) of multiple shots with an average shoot length of 6.64 ± 0.44. Shoot tip explants at 13.3 µM of BA induced 18.20±0.28 shoots, with a mean length of 5.61±0.23. The numbers of shoots induced on BA were significantly high when compared to other growth regulators (TDZ and Kn). Isolated shoots placed on rooting medium induced multiple roots on half MS and 4.90 µMIBA. Genetic fidelity was assessed and confirmed by using ISSR primers, and no variation has been observed in successive subcultures. The banding patterns of *in vitro* and hardened plants were monomorphic and similar to that of the mother plant.

Keywords

Acclimatization
Dysolobium pilosum
Inter simple sequence repeat
Micropropagation
Nodal and shoot tip explants

Introduction

India, being one of the richest biodiversity centers for medicinal plants, is known for utilizing them in medicinal formulations and in organized health care regimes since 5000 years (Patwardhan 2000). Medicinal plants are moving from marginal to main stream use since people are seeking cure free from side-effects caused by synthetic drugs (Flower, 1983; Sahoo et al., 1997). Employing eco- and biofriendly plant-based products for the prevention and curing of several human ailments are in progress. Almost 80% of the population living in developing countries still implement traditional practices derived from plants

for their primary health care prerequisites (Cunningham, 1993; De Silva, 1997). India's active participation in the global economy sector for marketing medicinal plants has made it as the world's leading supplier of raw materials and is designated as '*the botanical garden of the world*'. Prior reports indicate that about 3000 plants with medicinal potential are used in traditional/ folk practicing and more than 6000 are used for their medicinal properties for treating several disorders (Dubey et al., 2004).

Rapid globalization and exploitation for human requirements has resulted in loss of valuable natural resources at an alarming rate. Factors such as deforestation for agricultural development and urbanization have resulted in the loss of valuable medicinal plants from the wild. Micropropagation is employed for rapid and *ex situ* conservation of RET (Rare, Endemic and Threatened) medicinal plant species (Arora and Bhojwani, 1989; Purohit et al., 1994; Sudha and Seeni, 1994). Medicinal plants have an array of biochemicals with varied biological activities (Buckingham, 1999) and are used as constituents in several drug formulations. The *in vitro* propagation of pharmaceutically important plants are produced on a large scale for their medicinal properties (Murch et al., 2000). Maintaining stability in the environment, propagation of endangered and threatened species has become a prerequisite for ensuring the survival of plant species. The plant kingdom entails diverse plant species that encompass compounds of medicinal significance and several are yet to be discovered from the wild.

Dysolobium pilosum (Klein ex Willd.) Maréchal [Syn. *Dolichos pilosus* Willd., *Dolichovigna formosana* Hayata, *Dolichovigna pilosa* (Willd.) Niyomdham, *Phaseolus difformis* Wall., *Vigna pilosa* (Willd.) Baker] of Fabaceae, is a perennial herbaceous twiner found mostly in the marshy areas of the forest (Babu et al., 1985; Polhill, 1990; Pullaiah and Murthy, 2001; Welzen and Den Hengst, 1985). The family comprises of 600 genera and 12,000 species and is considered to be the third largest seed plants and several of them are used in tribal medicine (Sharma and Kumar, 2013).

The other species of the genus include *Dysolobium apioides*, *D. dolichoides*, *D. grande*, *D. lucens*, and *D. tetragonum*. The family members yield nutritious food, fiber, shelter, valuable medicines and also virulent poisons (Datta and Mukherji, 1952). The seeds are said to possess anti-periodic and the root anthelmintic activity (Sharma and Kumar, 2013). The products such as resins (amylaceous and oleaginous); balsams and dyes. A few are astringent, some are acrid and bitter, and some are poisonous, emetic and purging, while few others are used as formulations in tonics and restoratives. *Dysolobium pilosum* was first time reported by Suthari et al. (2014) from Warangal district as an addition to Telangana State. Parts of the plants are used as food, feed and in drinking formulations (Jain, 1991), cattle straw feed or as green fodder and manure (Suthari et al., 2014). The seeds have nutritive value and so are consumed at times of scarcity (Ambasta et al., 1986).

Plant tissue culture offers a potential solution for the propagation of endangered and superior genotypes. Development of protocol for tissue culture is one of the important requisitions toward clonal mass multiplication for conservation of plant species. Regeneration studies have been successfully reported in the cotyledonary node explants of*Vigna radiata* (Gulati and Jaiwal, 1994), Asiatic *Vigna species* (Avenido and Hattori, 1999) and African cowpea variety *Vigna unguiculata* (L.) Walp. (Diallo et al., 2008). Plantlets of *Vigna mungo* were regenerated from cotyledon and embryonal axis (Ignacimuthu et al., 1997) and immature embryos of *V. unguiculata* and *V. vexillata* were successfully rescued (Pellegrineschi, 1997).

The present study was aimed to develop a rapid clonal propagation protocol by using nodal and shoot tip explants of *D. pilosum* and establish uniform plants.

Materials and methods

Plant material and culture conditions

Seeds were obtained from plants growing in the Malluru forest, Telangana, India (latitude: 18° 12' 01.9" N, longitude: 80° 34' 22.4" E). The overnight soaked seeds were sterilized with 0.1% mercuric chloride for 120 sec with continuous shaking and washed thrice with sterile distilled water. The seeds were inoculated into bottles containing 50 ml of autoclaved hormonal free Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The seeds were germinated at temperature of 24±1°C and a relative humidity of 60-65%. Photoperiod of 16 h (photon flux intensity of 80 µmol m^{-2} s^{-1}) was maintained throughout the experimental procedure.

Nodal multiplication

Nodal explants were obtained from *in vitro* grown 3-4 week old plants. Nodes of 1.0 - 1.5 cm were sliced from *in vitro* grown plantlets and were cultured on MS medium which was augmented with various concentrations of growth regulators containing TDZ (2.27-18.16 µM), BA (2.22-17.80 µM) singly or in combinations with 1.14 and 2.85 µM IAA. The concentrations were tested to induce shoot multiplication from nodal explants. After 5-7 days of root initiation, the induced shoots were isolated and transferred onto rooting medium.

Shoot multiplication

Healthy and juvenile shoots tips of 1.5–2.0 cm long explants were excised from 3-4 weeks old *in vitro* grown plants. The shoot tips were inoculated on MS medium supplemented with different concentrations of 6-benzyladenine (BA; 4.40 - 22.20 µM) or Kinetin (Kn, 4.60 - 23.20 µM) alone, or in combinations of BA (4.40 - 22.2 µM) and Kn (9.30 and 13.9 µM).

Statistical analysis

For multiple shoot induction from shoot tips and nodal explants, 20 explants were used for each treatment and replicated twice. The experiments were repeated twice to reproduce the same results. The data pertaining to number of shoots and shoot length from *in vitro*-derived shoots was recorded and analyzed (Pillai and Sinha 1968). The number of shoots per culture, shoot regeneration percentage and mean shoot length were recorded after 45 days of culture, and are expressed as the mean ± standard error (SE).

Rooting and acclimatization

Acclimatization of *in vitro* grown plants is the most critical step of micropropagation studies. For root induction, the plantlets with 4-6 leaves were harvested from each culture passage and transferred to full and half-strength MS medium supplemented with 0.98 – 9.80 µM IBA. Percentage of responding cultures for root induction, length and their number per shoot were recorded. Shoots with well-developed roots were removed from the culture bottles, rinsed gently with sterile water to remove medium adhering the roots and planted in plastic containers with a mixture of sterile soil, sand and manure (6:2:1). Plants were maintained under natural shade by covering the plant individually with small polybags. Frequent watering was done to maintain soil moisture. Healthy plants were potted and transferred to the green house for acclimatization.

DNA extraction and PCR amplification

Total genomic DNA was isolated from the leaf tissue of *Dysolobium pilosum* mother plant, *in vitro* 8th and 12th subcultures and from the green house hardened plants (Doyle and Doyel, 1990). The genetic fidelity was assessed using inter simple sequence repeat (ISSR) markers and confirmed by using agarose gel electrophoresis. A total reaction volume of 25 µl containing 2 µl of DNA (25 ng), 10 µl of 10X PCR buffer, 2 µl 50 mM $MgCl_2$, 2 µl of 2.5mM dNTPs, 1 µl of 1 U *Taq* DNA polymerase, and 8 µl of autoclaved distilled water, using a thermocycler. An initial denaturation step of 94°C for 3 min was followed by 60 sec (94°C denaturation step) for 30 cycles, template annealing at 45 sec at 50°C, extension step of 120 sec at 72°C, followed by final extension of 7 min at 72°C, and final temperature of 12°C was maintained. The amplified DNA was separated by electrophoresis on 1% agarose gel (35 mV, 3 h). The sizes of the amplicons were analyzed using a 1kb ladder. The gels were visualized and photographed using a gel documentation system. All PCR reactions were repeated at least twice to ensure reproducibility.

Results and discussion

Seeds germinated on the fifth day of inoculation and subsequently developed into a complete plant in 4-5 weeks. About 80-85% germination rate was recorded after 45 days of seed inoculation. The shoot tip and nodal explants of 4-5 weeks old *in vitro* grown seedlings were used in regeneration studies.

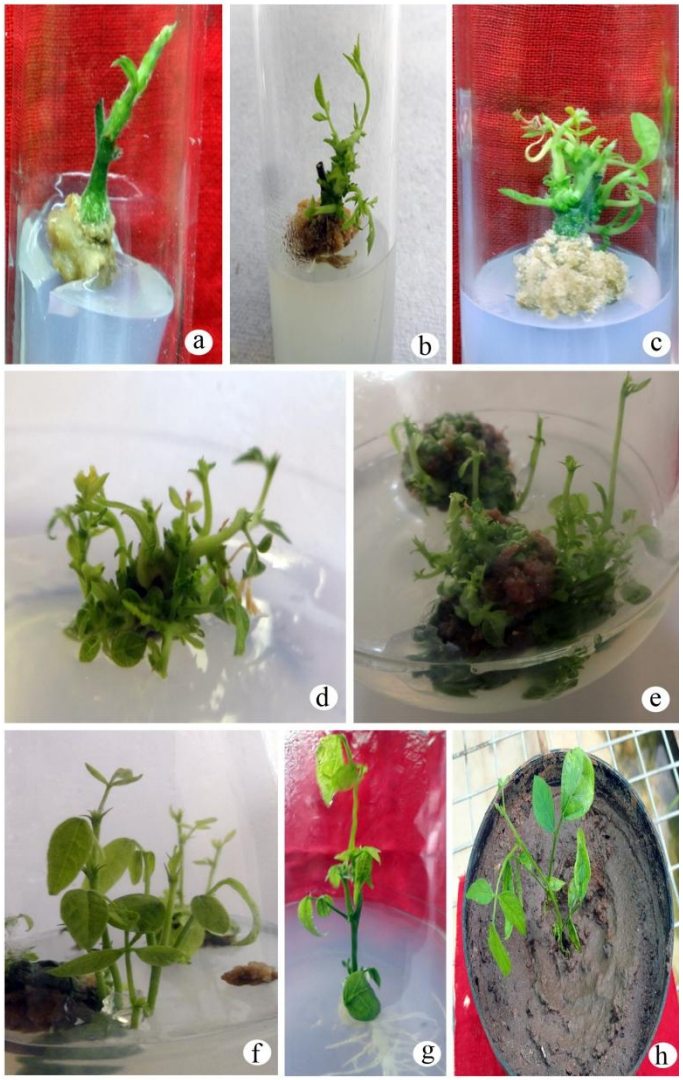
In the present investigation, attempts were made to standardize a reproducible protocol for *in vitro* clonal propagation of *D. pilosum.* Nodal explants, serving as source of pre-existing meristems, easily develop into shoots and clonal fidelity is maintained in successive generations. The regeneration efficiency of nodal plants varied depending on the type of growth hormones used for propagation. Nodal explants on MS medium fortified with different concentrations of BA and TDZ were tested singly or in combinations with IAA. Multiple shoots were induced after 3-4 weeks of inoculation (Fig. 1). Cytokinin BA favored best response with a

maximum number (26.92 ± 0.29) of shoots (Table 1) with an average shoot length of 6.64 ± 0.44 at 8.90µM BA. Maximum shoot efficiency of 85% was obtained at the aforesaid concentration. Multiple shoots were induced at all the concentrations of BA and their number elevated with an increase in BA concentration. The maximum number of multiple shoots were induced at 8.90 µM BA, followed by 11.12 µM BA (22.14 ± 0.23). Earlier reports indicate that the superiority of BA over other cytokines in shoot induction of *Andrographis paniculata* (Purkayastha et al., 2008) and the efficiency of BA in multiple shoot induction in several aromatic and medicinal plants such as *Ocimum basilicum* (Sahoo et al., 1997), *Withania somnifera* (Sen and Sharma, 1991) and *Hippophae rehnoides* (Purohit et al., 2009).

Table 1. Effect of BA and TDZ on multiple shoot induction from nodal explants of *Dysolium pilosum*.

BA (µM)	TDZ (µM)	% of Responding culture	Mean no. of shoots/explant ± S.E	Mean length of shoots/explant ± S.E
2.22		53	6.88 ± 0.29	3.37 ± 0.24
4.40		62	11.68 ± 0.24	4.69 ± 0.32
6.62		75	18.44 ± 0.21	5.64 ± 0.37
8.90		85	26.92 ± 0.29	6.64 ± 0.44
11.12		79	22.14 ± 0.23	5.58 ± 0.37
13.30		76	18.76 ± 0.29	4.61 ± 0.28
15.52		65	12.24 ± 0.29	3.73 ± 0.26
17.80		56	8.48 ± 0.23	2.53 ± 0.25
BAP+ IAA (µM)				
8.90+1.14		80	6.58±0.75	5.32±0.69
11.12+1.14		76	4.32±0.58	3.86±0.61
8.90+2.85		72	2.56±0.36	2.32±0.32
11.12+2.85		68	1.85±0.12	2.02±0.84
	2.27	58	3.54±0.37	1.40±0.64
	4.54	65	5.65±0.58	2.65±0.88
	6.81	72	10.69±0.69	4.58±0.45
	9.08	68	7.25±0.23	3.86±0.21
	11.35	62	5.12±0.84	2.65±0.36
	13.62	54	2.35±0.65	2.12±0.19
	15.89	52	1.84±0.42	1.69±0.66
	18.16	50	0.9±0.36	1.02±0.47
TDZ+IAA (µM)				
6.81+1.14		69	4.28±0.59	3.22±0.91
9.08+1.14		65	2.45±0.65	2.15±0.68
6.81+2.85		58	1.23±0.41	1.95±0.45
9.08+2.85		53	0.68±0.58	1.02±0.47

Fig. 1: Micropropagation of *D. pilosum* on MS medium from nodal explants. a & b. Initiation of shoots from *in vitro* derived nodal explants on 8.90 µM BA.; c & d. Proliferation of shoots; e. & f. Elongation of multiple shoots; g. Rooting of micro shoots on half MS fortified with 4.90 µM IBA; h. Hardened plant in green house.



The observations revealed that the number of shoots gradually decreased with increase of BA concentration. Reduction in the number of shoots generated from each node at BA concentrations higher than the optimal level was also reported for several medicinal plants (Sen and Sharma, 1991; Vincent et al., 1992). On the contrary, the nodal explants cultured on TDZ induced 10.69±0.69 shoots at a shoot length of 4.58±0.45 at 6.81 µM TDZ. These results obtained were higher than that of the mean number of shoots induced on TDZ in *Vigna* species (Sen and Guha-Mukherjee, 1998). BA (8.90 and 11.12 µM) and TDZ (6.81 and 9.08 µM) in combination with IAA were tested for multiple shoot induction, but the number of shoots in combinations were found to be far more less than BA and TDZ individually, indicating the hormonal combinations on the nodal explants showed little effect on multiple shoot formation. The nodal explants at 8.90 µM BA and 1.14 µM IAA produced microshoots of 6.58±0.75 when compared to 6.81 µM TDZ +1.14 µM IAA (4.28±0.59). In another set of experiment, the nodal explants from *in vitro* gown seedlings were cultured on BA 2.22 – 17.80 µM in combination with 0.58 µM GA_3. A maximum shoot length of 9.23±0.33 (Table 2) was induced at 8.90 µM BA, combined with 0.58 µM GA_3. Shoot length of 2.58±0.89 cm difference was observed when compared to BA individually (6.64±0.44).

Table 2. Influence of GA3 on shoot elongation of nodal explants.

BA + GA 3 (µM)	% of responding culture	Mean length of shoots/explant ± S.E
2.22 ± 0.58	66	4.65±0.78
4.40 ± 0.58	75	5.32±0.35
6.62 ± 0.58	78	7.59±0.42
8.90 ± 0.58	80	9.23±0.33
11.12 ± 0.58	76	7.02±0.12
13.30 ± 0.58	72	5.15±0.53
15.52 ± 0.58	69	4.82±0.46
17.80 ± 0.58	62	3.21±0.14

Fig. 2: Plant regeneration from shoot tip explants of *D. pilosum*. a & b. Shoot induction from *in vitro* derived shoot tip explants on 13.3 µM BA; c & d. Multiple shoots induction and elongation after 2 weeks of culture; e. Adventitious root formation on half MS and 4.90 µM IBA; f. Plants shifted to green house.

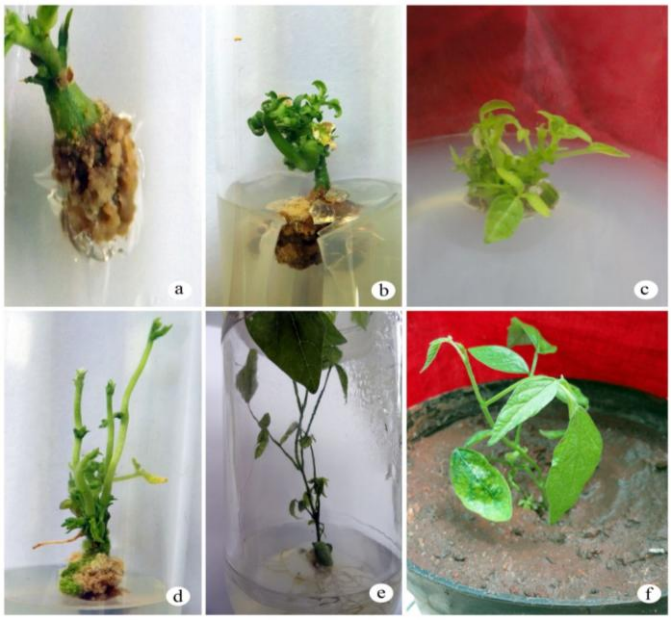


Table 3. Effect of cytokines on multiple shoot induction from shoot tip explants.

BA (µM)	Kn (µM)	% of Responding culture	Mean no. of shoots/explant ± S.E	Mean length of shoots/explant ± S.E
4.4		68	6.48±0.35	3.86±0.12
8.9		76	14.88±0.70	4.82±0.56
13.3		80	18.20±0.28	5.61±0.23
17.8		78	15.28±0.68	5.26±0.69
22.2		70	10.24±0.52	4.25±0.85
	4.6	60	1.68±0.19	1.26±0.36
	9.3	72	3.76±0.32	2.18±0.15
	13.9	66	2.40±0.23	1.54±0.24
	18.5	63	1.72±0.21	1.14±0.93
	23.2	58	1.12±0.15	1.02±0.65
4.4	9.3	66	4.16±0.33	2.52±0.65
8.9	9.3	70	8.12±0.71	3.25±0.48
13.3	9.3	75	11.8±0.93	4.51±0.69
17.8	9.3	60	6.84±0.40	3.12±0.43
22.2	9.3	56	3.88±0.35	2.15±0.58
4.4	13.9	65	3.12±0.31	2.11±0.47
8.9	13.9	70	4.04±0.30	2.53±0.96
13.3	13.9	74	5.12±0.45	2.82±0.28
17.8	13.9	67	2.60±0.25	1.53±0.32
22.2	13.9	62	1.36±0.22	1.12±0.14

In vitro multiple shoots were induced on various concentrations of BA and Kn singly or in combinations from shoot tip explants. BA at 13.3 µM induced maximum number of 18.20±0.28 shoots (Table 3) and a mean length of 5.61±0.23 with 80% of responding cultures (Fig. 2). About 15.28±0.68 shoots were induced at 17.8 µM BA with an average of 5.26±0.69 shoot length on MS medium. The medium supplemented with 9.3 µM Kn induced 3.76±0.32 shoots on an average length of 2.18±0.15 shoots. BA in combination with Kn produced 11.8±0.93 shoots with a mean shoot length of 4.51±0.69. Similar results were reported for *Clitoria ternatea* and mint (Ismail et al., 2012). Maximum numbers of shoots were initiated on MS medium supplemented with BA individually when compared to Kn (singly) and in combination with BA.

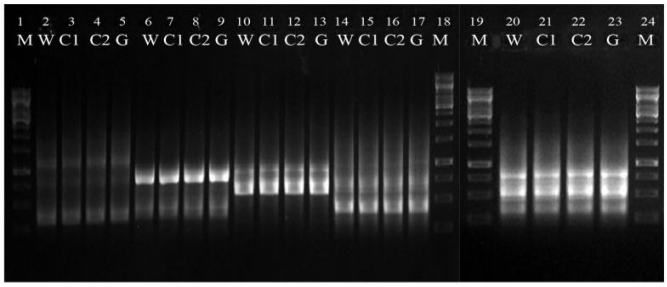
Table 4. Effect of auxin (IBA) on rooting of *in vitro* derived nodal explants on half and full MS.

MS medium	IBA (µM)	% of roots producing cultures	Mean no. of roots/explant ± S.E	Mean root length of roots/explant ± S.E
Full	0.98	60	4.84±0.37	2.06±0.14
Full	2.46	66	5.88±0.38	2.93±0.53
Full	4.90	72	7.48±0.50	3.84±0.29
Full	7.36	67	5.48±0.42	2.86±0.36
Full	9.80	59	4.20±0.39	1.98±0.15
Half	0.98	70	6.88±0.51	3.12±0.69
Half	2.46	78	10.56±0.67	4.05±0.52
Half	4.90	82	12.28±0.81	4.98±0.14
Half	7.36	80	11.08±0.78	4.62±0.35
Half	9.80	75	8.32±0.71	3.96±0.23

Well developed roots were observed after the 10th day of culture in all the concentrations of IBA. On an average, a maximum number of 12.28±0.81 roots (Table 4) and a mean root length of 4.98 developed on ½ MS supplemented with 4.90 µM IBA. Rooting on full strength MS supplemented with 4.90 µM IBA produced 7.48±0.50 roots with a mean root length of 3.84±0.29 per explant. The results indicate that the percentage of rooting is best on ½ MS with 4.90 µM IBA compared to full MS with IBA. Rooted plantlets (with more than 3-5 roots and average root length exceeding 1 cm) were isolated from the culture bottles, rinsed with sterile distilled

water for removing traces of the medium and transferred to plastic containers containing a mixture of sterile soil + garden soil + sand (2:2:1; v/v/v) kept in a growth chamber and maintained at 24±2°C and 70% humidity under a 14 h photoperiod. After 2 weeks they were transferred to greenhouse for further growth. The acclimatized plants showed normal growth and were morphologically similar to wild mother plant.

Fig. 3: Establishing clonal fidelity by ISSR analysis. M-1 Kb Marker; W- Wild; C1 & C2 – 8th and 12 sub cultures; G-Green house plant; Amplification pattern of primer 1: Lanes 2, 3, 4 and 5; Primer 2: Lanes 6, 7, 8 and 9; Primer 3: Lanes 10, 11, 12 and 13; Primer 4: Lanes 14, 15, 16 and 17; Primer 5: Lanes 20, 21, 22 and 23.



Fifteen individual ISSR primers were tested and on the basis of their suitability while 5 primers exhibited a clear banding pattern (Fig. 3) and the ISSR analysis was performed (Williams et al., 1990). Establishment of genetic fidelity among the regenerants using various molecular markers is yet another significant parameter towards clonal propagation. Variations prompted in *in vitro* plants can be screened by marker systems (Phillips et al., 1994). Banding pattern results indicate that the plantlets were regenerated directly from nodal explants of the field grown wild *D. pilosum*. ISSR analysis can reinforce highly complex and reproducible banding profiles (Fang and Roose, 1997).

Amplicons on screening confirmed similar banding pattern among the successive subculture and the mother plant, thereby confirming the genetic fidelity. As there were no polymorphic bands, the results confirm that micropropagated *D. pilosum* maintains genetic integrity even after prolonged periods of maintenance in aseptic conditions. The current report validates that the nodal multiplication is an innocuous method for producing true-to-type plants. Prior reports indicate similar results for detection of genetic fidelity using ISSR markers in different crops and medicinal plants such as *Dioscorea bulbifera* (Narula et al., 2007), *Swertia chirayita* (Joshi and Dhawan, 2007), *Saccharum officinarum* (Tawar et al., 2008) and *Cymbopogon martinii* (Bhattacharya et al., 2010).The current protocol appears to be highly effective, as it does not allow induction of variations resulting genetic stability essential for germplasm conservation, and to the best of our knowledge it is the first report on genetic fidelity testing in micropropagated plants of *D. pilosum.*

Table 5. ISSR primers used for detecting the genetic stability in micropropagated plants of *D. pilosum*.

S. No	Primer code	Primer sequence (5'-3')	Range of amplification (bp)
1.	K 1: (GA) 8 T	GAGAGAGAGAGAGAGAT	250-1500
2.	K 2: (TC) 8 C	TCTCTCTCTCTCTCTCC	250-1000
3.	K 3: (AG) 8 YT	AGAGAGAGAGAGAGAGYT	500-1000
4.	K 4: (GA)8 YT	GAGAGAGAGAGAGAGAYT	250-500
5.	K 5: GCC(GA)7	GCCGAGAGAGAGAGAGA	250-750
(Y=T/C)			

Conclusion

In the present study, an efficient protocol for micropropagation of *D. pilosum* was developed by testing various concentrations of growth regulators and culture conditions. A maximum number of multiple shoot produced from nodal explants than shoot tips when cultured on BA individually than its combination with IAA. ISSR analysis reveals genetic fidelity among the regenerants and maintenance of true-to-type plants. The current protocol will facilitate the propagation and conservation of *D. pilosum* in an efficient way.

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