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In vitro propagation studies and genetic fidelity assessment of endangered medicinal wild Yam- *Dioscorea prazeri*

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Abstract

A highly efficient *in vitro* regeneration of an indigenous, endangered medicinal plant *Dioscorea prazeri* was achieved using nodal explants and axillary buds on Murashige and Skoog (MS) medium containing sucrose and supplemented with growth regulators Benzyl Amino Purine (BAP) and Naphthalene acetic acid (NAA). Each explant regenerated 21±2 culturable segments after 10 to 12 weeks of inoculation of nodal explants with a regeneration frequency of 98±2% and a survival rate of >96% on field establishment. The plantlets had healthy roots and sprouted tubers *in vitro*; the rooted plantlets were acclimatized and successfully established in soil. The extraction and chromatographic analysis methods were standardized to obtain the maximum yield of diosgenin from plant extracts of *D. prazeri*. The content of diosgenin was observed to vary with age of the plant. An examination of the genetic fidelity of the *in vitro* regenerated explants indicated a genome that was stable. The plants obtained were analyzed further using morphological, molecular and biochemical methods and found to be genetically and metabolically stable at all growth phases. The regenerated *D. prazeri*, using the method developed in this study could be reintroduced into the natural habitat.

Keywords: Micropropagation; Morphological analysis; Steroidal Sapogenin; Extraction; Diosgenin; HPLC; RAPD.

Abbreviations :

2iP 2-isopentanyl adenine
B.O.D. Biological Oxygen Demand
BAP Benzylaminopurine
FYM Farm Yard Manure
GA3 Gibberllic acid
MS Murashige and Skoog
NAA Naphthalene acetic acid
RAPD Random Amplified Polymorphic DNA
TDZ Thiadizuron

Introduction

Yam tubers are staple food for millions in many tropical and sub-tropical countries (Onwueme, 1978). Dioscorea, one of the important genera of yams, comprises of about 600 species that have medicinal and edible importance. The significant species of the genus *Dioscorea* are *D. prazeri* and *D. deltoidia* found at high altitudes in India, *D. composita*, *D. floribunda* and *D. mexicana* in Mexico, *D. elephantipes* (L.) and *D. sylvatica* in South Africa. The long-term availability of many Himalayan herbs has become uncertain due to indiscriminate harvesting resulting in possible threat of extinction (Badoni et al., 2010) and efforts at cultivation have met with limited success (Coursey, 1976). *D prazeri* is enlisted as an endangered species of indigenous medicinal plants of India. Tissue culture offers the means for rapid and mass multiplication of existing stock of germplasm and also a method for conservation of important, elite endangered plants (Razdan, 2003). Diosgenin, one of the most important secondary metabolites present in *D. prazeri* tuber, is a pharmaceutically important steroidal sapogenin. It is a precursor of sex hormones (progesterone), corticosteroids (corticosone) and contraceptives (Onwueme, 1978; Coursey, 1967). Diosgenin is among the ten most important sources of steroids and is also the most often prescribed medicine of plant origin (Fowler, 1984). Diosgenin induces apoptosis in cancerous cells by cyclooxygenase up-regulation and in *HeLa* cells by caspase pathway (Huo et al., 2004). Dioscin, a derivative of diosgenin, has been reported to induce apoptosis in HeLa cells through caspase-9 and caspase-3 (Cai et al., 2002). It leads to inhibition of growth of fibroblast-like synoviocytes in human rheumatoid arthritis with apoptosis induction associated with cylooxygenase-2 up-regulation (Liagre et al., 2004). Diosgenyl saponins also induce apoptosis and mitotic arrest in human leukemia cell lines (Ming-Jie, 2004). Diosgenin has both an antioxidant property and anticholesterolomic activity. For example, it has been reported to have hypocholesterolemic action in rat (Accatino et al., 1998), and antioxidant activity in HIV patients with dementia (Turchan et al., 2003), and

Table 1. The Oligonucleotides that showed clearly identifiable bands for genetic fidelity assessment

SL.No	Primer	Sequence	Primer	Sequence
1	OPP7	5'-GTCCATGCCA-3'	OPF 10	5'-GGAAGCTTGG-3'
2	OPC06	5'-GAACGGACTC-3'	OPK 17	5'-CCCAGCTGTG-3'
3	OPJ 13	5'-CCACACTACC-3'	OPI 08	5'-TTTGCCCGGT-3'
4	OPN18	5'-TCAGAGCGCC-3'	OPL 20	5'-GGAAGCTTGG-3'
5	OPN 8	5'-ACCTCAGCTC-3'	OPO 10	5'-TCAGAGCGCC-3'
6	OPC19	5'-GTTGCCAGCC-3'	OPQ 14	5'-GGACGCTTCA-3'
7	OPD 09	5'-CTCTGGAGAC-3'	OPB 10	5'-CTGCTGGGAC-3'
8	OPH 14	5'-ACCAGGTTGG-3'	OPQ 11	5'-TCTCCGCAAC-3'
9	OPJ 5	5'-CTCCATGGGG-3'	OPL 01	5'-GGCATGACCT-3'
10	OPF 2	5'-GAGGATCCCT-3'	OPG 18	5'-GGCTCATGTG-3'

Primers used for screening micropropagated plants for genetic fidelity. The primers were chosen based on reproducibility and number of bands indicating stability in genomic primer region. The amplified banding patterns of the plants regenerated from nodal explants were identical to those of control plants for all the primers used.

Table 2. Amplification profile of RAPD analysis for *Dioscorea prazeri*

Constituents	Quantity
Template DNA	50 ng (2µL)
DNTP (10 mM)	0.8µL
10x Taq buffer (with $MgCl_2$ of 15mM)	5.0µL
Taq polymerase	2 units (0.8µL)
Primer (10pmoles)	25ng (1.5µL)
$MgCl_2$ (25mM)	0.5µL
HPLC grade water (sterile)	39.4µL
Total Volume	50.0 µL

Table 3. Cycling conditions for amplification

Conditions	Temperature	Time	Cycles
Initial denaturation	94˚ C	4 min	1
Denaturation	94˚ C	1 min	
Annealing	35˚ C	2 min	44
Extension	72˚ C	2 min	
Final extension	72˚ C	10 min	1
	4˚C	Forever	

cyclooxygenase activity in osteosarcoma cells (Molaic et al., 2001). Micropropagation of the *D. prazeri* producing steroidal sapogenin would be most suitable for re-establishing the plant in the natural habitat. Among the different approaches, an *in vitro* culture method provides a means of conserving and rapidly propagating valuable, rare and endangered medicinal plants (Nalawade et al., 2003; Thomas and Shankar, 2009; Rahman et al., 2009). A well-established protocol including plant regeneration from tissue, organ etc. may help plant population from becoming extinct. Another advantage of the technique is improvement in space usage, the improvement of the growth conditions of the plants and the facilitation of international germplasm exchange (Alderete et al., 2006). *In vitro* production of the related species of *D. floribunda* (Sengupta et al., 1984), *D. alata* (Jasik and Mantell, 2000; Martine and Cappadocia, 1991; Mantell and Hugo, 1989) *D. composita* (Alizadeh, 1998) have been reported. Micropropagation of the endangered species of *Dioscorea prazeri*, which is one of the important medicinal yams, threatened and endemic to India have not been carried out. Since the micropropagation protocols are mostly clonal specific in nature, development of these procedures for *D. prazeri* is imperative. The tissue culture technique reported herein demonstrates consistent production and proliferation of shoots and subsequent plant regeneration, important for massive plant propagation studies on *D. prazeri* for utilization of its therapeutic properties and commercial applicability. *In vitro* propagation is a possible means of avoiding loss of embryogenic potential and maintaining genetic stability of highly significant medicinal plant like *Dioscorea prazeri* with the optimised hormonal treatment and micropropagation strategies. The study on morphological, genetic and biochemical stability of the plant under the standardised *in vitro* growth conditions reaffirms the applicability of the method for large scale propagation of this indigenous, endangered medicinal plant.

Results and discussion

Micropropagation

High regeneration frequency of 98±2% was obtained on MS media with BAP and NAA, and the latter were found to be the most suitable combination of hormones for shoot initiate on and regeneration for *Dioscorea prazeri*; explants grown in MS plain media were used as controls. Explants grown in media with 0.5 mgL^{-1} BAP and 0.01mgL^{-1} NAA showed the highest rate of multiplication and survival as compared with explants in media with growth regulators like Thiadizuron (TDZ), Zeatin, Kinetin and 2iP (Table 4&5). The combination of hormones with BAP and NAA resulted in the initiation of growth < 3 days (Fig 1A). A notable growth pattern was observed 4 to 5 weeks post-inoculation, (Table. 5). High multiplication rate of 21 segments that can be cultured were obtained in 10 to 12 weeks of inoculation from nodal segments. Multiple shoots were noticed (Fig 1B and C) and up to 9 shoots were observed from single node without callus formation. Morphogenic callus were formed while culturing for a longer period that resembled shoot primordium, and adventitious buds were formed on its

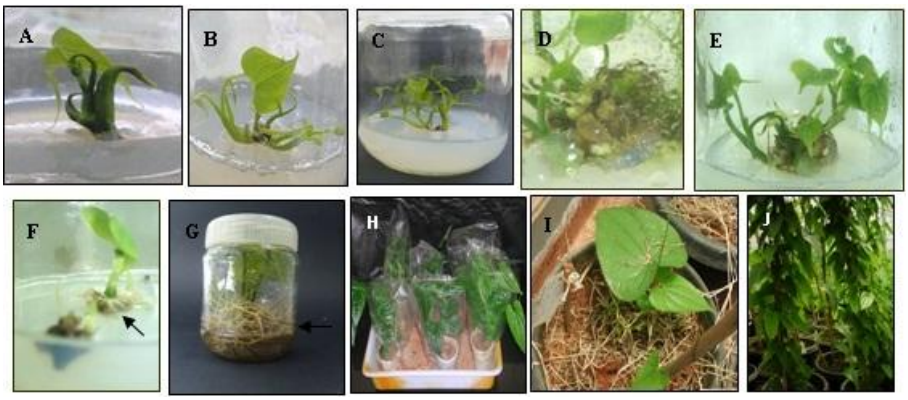
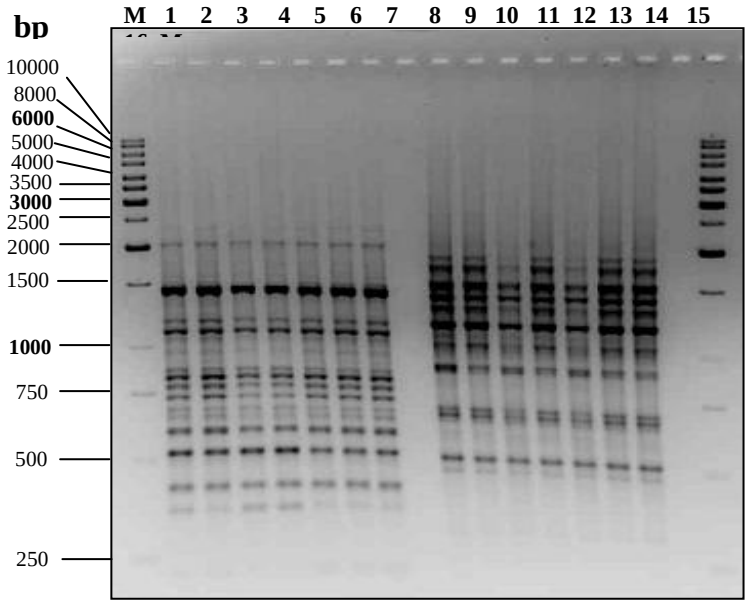


Fig 1. Regeneration and micropropagation of *D. prazeri* **(A)** Initiation of shoot regeneration in MS medium with 0.5 mgL^{-1} BAP and 0.01mgL^{-1} NAA +30gL^{-1} sucrose; **(B)** Shoot regeneration and multiple shooting of *D. prazeri* from the nodal explants on two weeks of culturing on MS medium supplemented with 0.5 mgL^{-1} BAP and 0.01mgL^{-1} NAA with 30gL^{-1} sucrose; **(C)** Multiple shooting of *D. prazeri* from the shoot buds; **(D)** Growth initiation of adventitious shoots from *in vitro* regenerated callus of *D. prazeri* from nodal segments; **(E)** Culturable segments formed out of adventitious shoots;**(F)** *In vitro* regenerated tubers from the nodal explants of *D. prazeri*; **(G)** Rooting of *in vitro* regenerated plantlets of *D. prazeri*; **(H)** Hardening of rooted plantlets of *D. prazeri* (15 days) in Coco peat mix to red soil in 3:1 ratio and the plantlets were covered with polythene cover to maintain humidity; **(I)** Hardened plantlet transferred to the pots contained 1:1:1 ratio of Farm yard manure, sand to soil for further establishment; **(J)** *D. prazeri*- acclimatized *in vitro* raised plants in Greenhouse



*M Molecular marker

Fig 2. The RAPD gel profile of donor wild plants and *in vitro* regenerated plantlets: Lane 1-2: Amplification profile of *D. prazeri* donor plant using primer OPJ 05; Lane 3-7: *D. prazeri in vitro* regenerated plants using primer OPJ 05; Lane 9-10: Amplification profile of *D. prazeri* donor plant using primer OPN 08; Lane 11-15: Amplification profile of *D. prazeri in-vitro* regenerated plants using primer OPN 08; Lane 8 and 16: Negative control Lane M: Molecular marker

surface. It was removed and cultured on MS medium with 0.5 mgL^{-1} BAP and 0.01mgL^{-1} NAA, and regenerated in a large number of plantlets (Fig 1D - F). Profuse root induction was observed with this combination of hormones resulting in 12.5 to 14.5 cm in root length in 8 to 10 weeks (Fig 1G) after inoculation of shoots and *in vitro* tubers were also obtained in 14 to 16 weeks of culturing. The sprouted healthy tubers obtained from nodal explants were used for propagation. Explants were regenerated in MS medium with BAP and NAA and compared to those grown in medium with BAP, NAA and 0.01 mgL^{-1} GA3. The results showed that a combination of BAP, NAA and 0.01 mgL^{-1} GA3 was useful in elongation of shoots in initial stages of explant growth. Continued sustenance in the medium resulted in fragile shoots due to extensive elongation (Table 6) attributed to the activity of GA3. Increased concentration of BAP resulted in delay of the initiation in growth (8-12 d) and the resultant plants were stunted. With further increase in concentration of NAA and GA3, plantlets and shoots turned brown and dried very fast. The rooted plantlets were hardened (Fig 1H and I) for 15 days and then acclimatized in greenhouse. The acclimatized plantlets showed a frequency of regeneration up to 98±2% in media supplemented with BAP and NAA as compared to the tested set of hormones. It was observed with *D. prazeri* that BAP and NAA were showing highest regeneration frequency with healthy growth in comparison with other growth regulators like Kinetin (1.5 mgL-1 with 40% regeneration frequency; 6.8 cm of shoot length in 3

Table 4. Comparative study of effect on plant growth using MS media supplemented with different growth regulators

MS+ Growth regulators	Media MS+ *GR mgL^{-1}	Frequency of Regn.	No. Of shoots/ Explants regenerated	Shoot length/ Culture (cm)
BAP	0.5	90	90	18.2
	1.0	90	69	13.2
	1.5	81	58	11.3
Kinetin	0.5	39	7	6.0
	1.0	40	7	6.3
	1.5	40	5	6.8
TDZ	0.5	78	40	6.4
	1.0	70	30	8.1
	1.5	60	24	6.9
Zeatin	0.5	60	57	5.4
	1.0	35	34	6.8
	1.5	25	21	7.1
2-isopentanyladenine (2iP)	0.5	60	30	7.3
	1.0	40	24	8.1
	1.5	30	13	7.9

*GR Growth regulator. The regeneration frequency and growth pattern in MS medium supplemented with different growth regulators are given. Explants treated with MS+BAP of 0.5 mgL^{-1} resulted in higher rate of multiplication and survival compared to explants regenerated in media (MS) supplemented with other growth regulators like Thiadizuron (TDZ), Zeatin, Kinetin and 2iP.

Table 5. Comparative study of effect on plant growth (10 to 12 Weeks) using MS media supplemented with combination of growth regulators

Combination	Media MS+ *GR mgL^{-1}	Frequency (%)	No. Of Shoots regenerated/ Explant	Shoot length/ Culture[1] (cm)
	0.5 BAP+0.01 NAA	100	117	23.1
BAP and NAA	0.5 BAP+0.03 NAA	60	31	11.6
	0.5 BAP+0.05 NAA	50	00	4.4
TDZ and NAA	0.5 TDZ+0.01 NAA	86	48	7.9
	0.5 TDZ+0.03NAA	70	31	8.6
	0.5 TDZ+0.05 NAA	40	04	6.9
Zeatin and NAA	0.5 Zeatin+0.01 NAA	65	64	6.2
	0.5 Zeatin +0.03 NAA	35	28	7.4
	0.5 Zeatin +0.05 NAA	20	12	7.8
2iPand NAA	0.5 2iP +0.01 NAA	60	47	8.2
	0.5 2iP +0.03 NAA	20	26	10.4
	0.5 2iP +0.05 NAA	8	15	7.8

*GR: growth regulator supplementation in mgL^{-1}. [1] Shoot length is the average length of main shoots and axillary branches. MS media supplemented with combination of growth regulators and its effects showed that the highest regeneration frequency of 98±2% was achieved in MS+0.5 mgL^{-1} BAP and 0.01mgL^{-1} NAA.

weeks), Zeatin (0.5 mgL-1 with 60% regeneration frequency with 5.3 cm shoot length in 3 weeks) and 2iP (60% with 7.3 cm of shoot length). The explants were regenerated at frequency of 78% with 6.4 cm shoot length in MS+ TDZ medium in case of *D. prazeri.* The combination of hormones enhanced the regeneration frequency as well as healthy elongation of shoots. BAP along with NAA, than BAP alone, showed an enhancement of 10% in frequency of regeneration and 5cm more growth in shoot at same time period of observation. TDZ with NAA showed 8% enhancement of regeneration frequency with 1.5 cm more of shoot length, Zeatin and NAA with 5% more frequency of regeneration with 0.8 cm of more shoot length but 2iP did not show any variation in frequency of regeneration but an enhancement of 0.9 cm elongation showed on observation and characterisation. Previous studies with other plant species *Dioscorea prazeri* have shown multiple shoot formation with BAP (Sharma et al., 2006; Alam et al., 2010b). In the present study on *D. prazeri* we demonstrate that additon of BAP resulted in multiple shoots with a shoot length of 18.2±1cm and with a frequency of regeneration as high as 90 %. Addition of growth regulator Naphthalene acetic acid (0.01mgL^{-1}) along with BAP enhanced the multiple shoot formation as well as frequency of regeneration of explants as high as 100% and shoot length of 23.1±1 cm. It also facilitated root formation of *in vitro* regenerated plants and tubers in the same regeneration media. A further increase in the concentration of NAA (0.5 mgL^{-1}) resulted in decreasing the regeneration frequency to 50% with stunted growth. Several studies support the role of GA3 or combination of Kin and GA3 for elongation of shoot prior to rooting (Baskaran and Jayabalan, 2005; Mohamed et al., 2006). In our study, we observed that although GA3 in combination with BAP and NAA resulted in elongation of the explants, high concentrations of GA3 (0.02 mgL-1) in combination with growth regulators produced weak shoots when compared to other hormonal treatments. Even though lower concentration of GA3 (0.01mgL^{-1}) produced healthy plantlets on micropropagation, BAP and NAA alone showed efficacy in regeneration with high shoot length and root formation. The previous reports on other monocots indicate

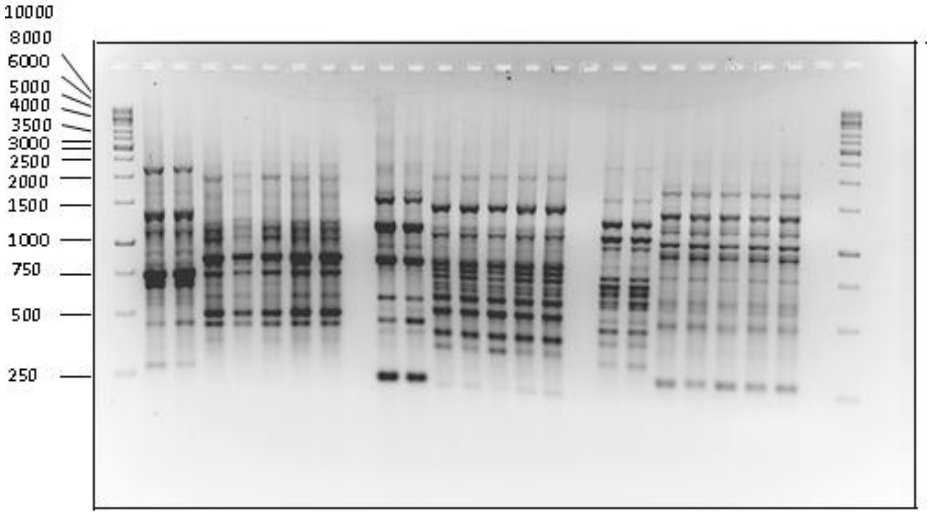


Fig 3. The RAPD gel profile of donor plant and the *in vitro* regenerated plants : Lane 2-3: *D. allata* using primer OPJ-13, Lane 4-5: *D. prazeri* donor plant using primer OPJ-13, Lane 6-8: *D. prazeri in vitro* regenerated plants using primer OPJ-13, Lane 10-11: *D. allata* using primer OPJ-18, Lane 12-13: *D. prazeri* donor plant using primer OPJ-18, Lane 14-16: *D. prazeri in vitro* regenerated plants using primer OPJ-18, Lane 18-19: *D. allata* using primer OPD-09, Lane 20-21: *D. prazeri* donor plant using primer OPD-09, Lane 22-24: *D. prazeri in vitro* regenerated plants using primer OPD-09, Lane 9, 17,25: Negative control for primers OPJ-13,OPJ-18 and OPD-09 respectively; Lane 1 and 26: Molecular marker (1 kb)

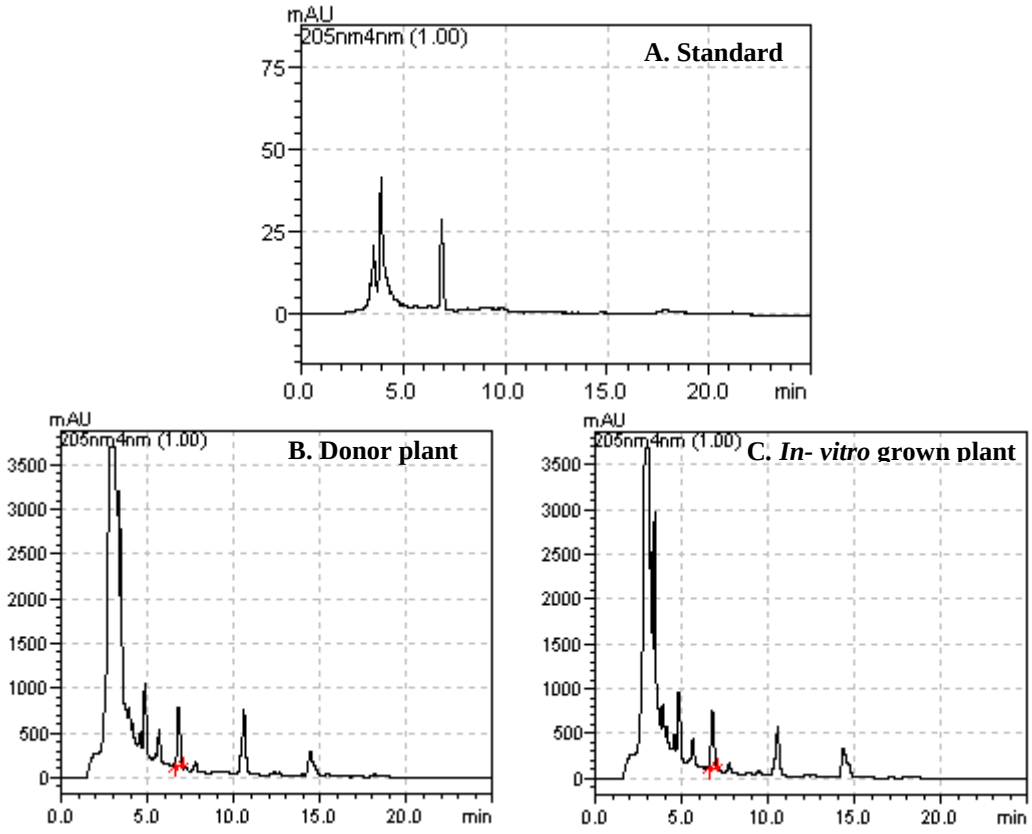


Fig 4. Chromatogram obtained from HPLC analysis of the petroleum ether extract with Soxhlet for contents of steroidal sapogenin, Diosgenin of donor (wild) plant and *in vitro* regenerated plants of *D. prazeri* **(A)** The chromatogram showing the concentration of 1µg of diosgenin; **(B)** The Soxhlet extraction for steroidal sapogenin, Diogenin of donor plants of *D. prazeri*; **(C)** The chromatogram of extract from *in vitro* regenerated plants of *D. prazeri*. The retention time of the required peak was at 6.7 minute with methanol as mobile phase

Table 6. Comparative study of growth pattern of *D. prazeri* in media containing BAP, NAA and GA_3 in 4 to 5 Weeks

Media MS+ Growth regulators (mgL^{-1})	Shoot Length[1](Cm)	No. of Nodes[2]	Nodal Length[3](Cm)	No. of roots	Root length(Cm)
Basal MS	0.0	0.0	0.0	0.0	0.0
0.5 BAP+0.01 NAA	13.3 ± 1	8 ± 1	2.5 ±0.4	11 ± 2	10 ± 0.5
0.5 BAP+0.01NAA+0.01GA3	11.1±0.3	7.0±1	2.5 ± 0.4	10±2	10 ± 0.5
0.5 BAP+0.01NAA+0.02GA3	7.3±0.3	7.0±1	1.5 ± 0.5	10±2	6.8 ± 0.5
0.5 BAP+0.02 NAA+0.02GA3	*4.5±1.0	2.0±1	1.0 ± 0.2	2.0±1	0.3±0.2
1BAP+0.02NAA+0.02GA3	*----	----	----	----	----

Values are the average of 5 replicates of 30 explants each ± standard deviation. [1].Shoot length is the average of length of main shoots and axillary branches. [2]. Number of nodes is the number of culturable segments on sub-culturing. [3]. Length of culturable segments obtained on sub-culturing. *----: While increasing the concentration of NAA shoots were turning brown very soon than responding positively and resulted in stunted growth. Comparative study of growth pattern on media containing BAP, NAA and GA3 resulted that explants grown in MS+0.5 BAP+0.01NAA+0.01GA3 has given highest regeneration frequency and survival rate. It resulted in a higher multiplication rate and regeneration of healthy explants with higher shoot, root and nodal length, with more number of nodes and roots.

Table 7 (A). Compound table view of concentration obtained on HPLC analysis with *in vitro* grown plant extract

Name	Concentration	Rt	Channel	Area	Height
In vitro *grown 1*	28.861	7.0	Ch2 205 nm	8770718	665978
In vitro grown 2	28.861	7.0	Ch2 205 nm	8770717	665976
In vitro grown 3	28.861	7.0	Ch2 205 nm	8770716	665975

Table 7 (B). Compound table view of concentration obtained on HPLC analysis with Donor plant extract

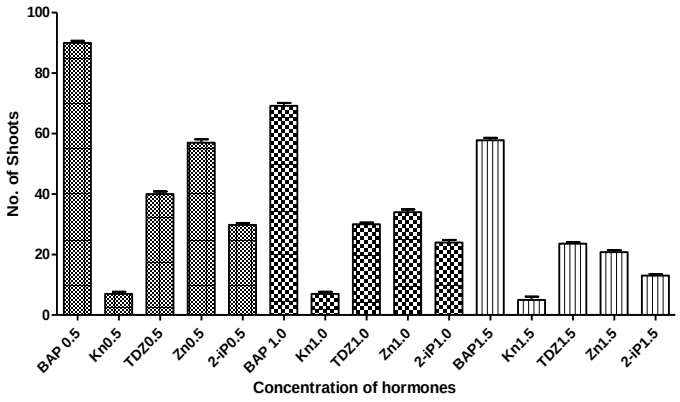
Name	Concentration	Rt	Channel	Area	Height
Donor plant 1	28.065	7.0	Ch2 205 nm	8528347	664038
Donor plant 2	28.065	7.0	Ch2 205 nm	8528349	664037
Donor plant 3	28.065	7.0	Ch2 205 nm	8528346	664038

Table 7 (C). Compound table view of concentration obtained on HPLC analysis with Standard (Diosgenin)

Name	Concentration	Rt	Channel	Area
Std Dg 1	1.000	7.0	Ch2 205 nm	235935
Std Dg 2	2.000	7.0	Ch2 205 nm	497542
Std Dg 3	3.000	7.0	Ch2 205 nm	785119
Std Dg 4	4.000	7.0	Ch2 205 nm	1019160
Std Dg 5	5.000	7.0	Ch2 205 nm	1359612

*R2: 0.9970856; R: 0.9985418; Mean RF: 3.936323e-006; Dg: Diosgenin. The concentration of steroidal sapogenin, Diosgenin obtained from donor plant as well as *in vitro* regenerated plants on HPLC analysis exhibited genetic integrity on analysis.

that BAP (2.0 - 2.5 mgL^{-1}) supplemented media is more effective towards induction of multiple shoot buds. Similar results were also reported (Sheelavantmath et al., 2000) in combination with BAP and NAA at higher concentrations in case of monocots. *Dioscorea prazeri* also showed high frequency of regeneration in combination with the hormones reported but at a lower concentration of 0.05 mgL^{-1} that resulted in the highest frequency of regeneration with strong shoots (23.2 cm, 12 weeks) when compared with 1.0 mg/L (90% regeneration frequency with 13.2 as average shoot length) and 1.5 mgL^{-1}(81% with 11.3cm shoot length). At concentrations exceeding 1.5 mg/ L there was significant inhibition of growth of the explants. It has been shown in other species of Dioscorea that BAP in combination with auxins leads to generation of healthy tubers *in vitro* (Bhadra and Hussein, 2003) albeit at different concentrations. The microtuber production by the combined effect of BAP (4-8 mgL^{-1}) and Kinetin (1-2 mgL^{-1}) has been reported in other species like *D. zingiberensis* (Li et al., 2000) and *D. oppositofolia* (Behera, 2009). It was also observed with *D prazeri* that BAP and NAA were showing highest regeneration frequency with healthy growth in comparison with other growth regulators like Kinetin, Zeatin and 2iP and TDZ. In this study it was noticed that rather than individual hormone treatment the combination of hormones enhances the frequency of regeneration as well as elongation of shoots in a healthy manner. BAP was more responsive than kinetin in inducing multiple shoot formation in case of *Dioscorea prazeri*, as reported earlier with *D. wighitti* (Poornima and Rai, 2007) shoot formation was found be high with BAP (Asha and Nair, 2007). This study showed that the combination of cytokinins and auxins were the significant rate limiting factor for the organogenesis as explained by Skoog and Miller (1957) for the first time. But the results obtain from this study confirms that considerable variability exists among genera, species and even cultivars and optimisation of the concentration is highly essential for induction of morphogenesis. The plantlets produced could be successfully transplanted to the field with high establishment and with 96% survivability (Fig 1J). *D. prazeri* was propagated by multiple shoots and healthy tubers obtained



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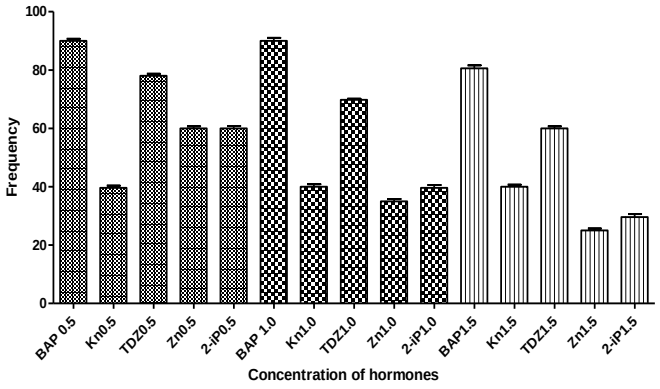

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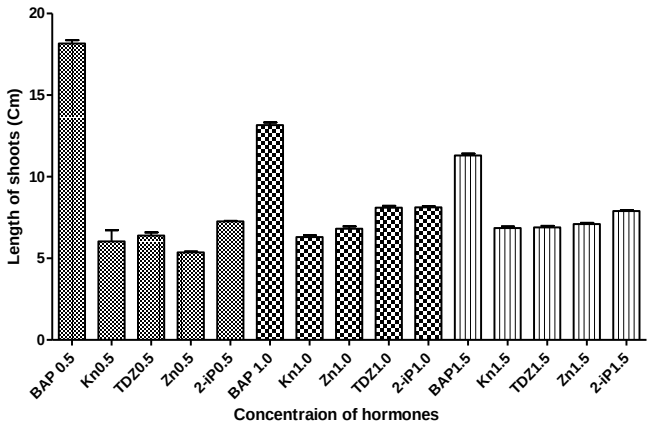
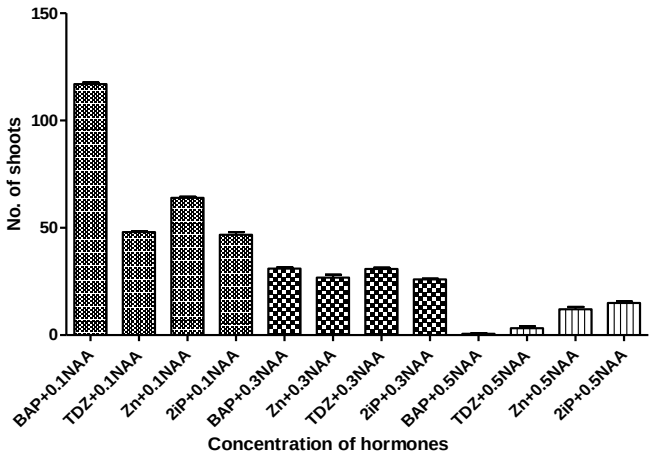
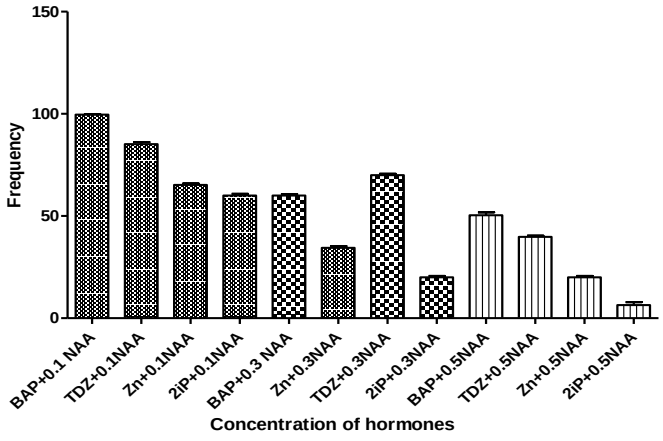


(c)

Fig 5. The graphical representation of statistical analysis for individual hormonal treatment **(A)** Number Of Shoots formed on micropropagated *D.prazeri* with MS medium + Growth regulators; **(B)** Frequency of *in vitro* regeneration of *D. prazeri* with MS medium + Growth regulators; (**C)** Length of Shoots of *in vitro* regenerated *D. prazeri* with MS medium+ Growth regulators



(A)



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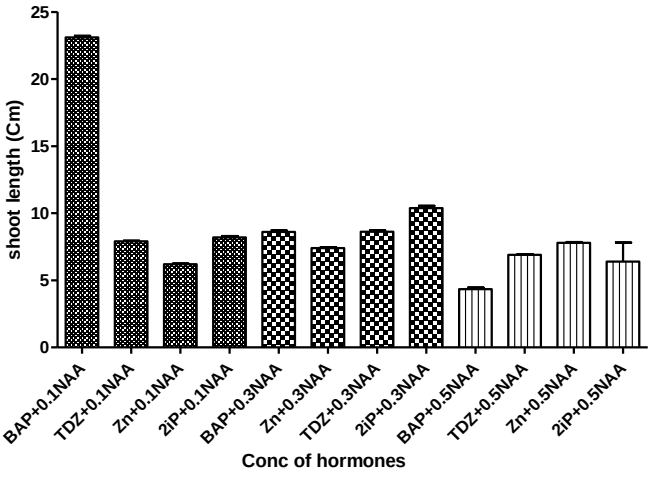

(C)

Fig 6. The graphical representation of statistical analysis for different combination of hormone treatment **(A)** Number of shoots formed on micropropagated *D. prazeri*; **(B)** Frequency of *in vitro* regeneration of *D. prazeri*; (**C)** Length of Shoots formed on *in vitro* regenerated *D.prazeri*

Table 8. Comparative studies of morphological characters of *D.prazeri*

Morphological characters	*In-vitro* grown plants	Wild plants
Length/breath ratio of mature leaf	10.7±3.6:8.2±2.1	10.3±3.2:7.8±2.25
Number of primary stems	8.0±2.0	7.0±2.0
Petiole length	4.69±1.8	4.28±2.01
Internodal length	9.4±2.0	8.3±0.7
Lamina/petiole length ratio	10.7±3.6: 4.69±1.8	10.3±3.2: 4.28±2.01

Comparative studies of morphological characters of *in vitro* grown and the wild type plants grown in greenhouse did not show any significant difference in growth pattern and found to be morphologically stable.

Table 9. One-way analysis of variance for individual hormonal treatment

One-way Analysis of Variance	No. of shoots	Frequency	Length of shoots
P-value	<0.0001	<0.0001	<0.0001
P value summary	***	***	***
[*2]MSD	P< 0.05	P< 0.05	P< 0.05
No. Of groups	15	15	15
F	990	710	260
R^2	1	0.99	0.98

The data of tables 8 and 9 were graphically represented by Fig. 4 [*2] Mean significant difference

from nodal explants. The growth and morphology of the re-established plantlets were evaluated and assessed with donor plants for genetic integrity using molecular markers.

Genetic fidelity assessment by RAPD analysis

The genomic DNA of *D. prazeri* from *in vitro* grown plants, donor plants (wild), and control plant *D. allata* (obtained from same family but different species) was amplified to assess the genetic stability of regenerated plantlets from donor germplasm .The RAPD fragment patterns of *in vitro* regenerated plantlets was compared with that of donor plants (wild). The assessment of genetic stability of *in vitro* regenerated plantlets is highly significant for further studies. The amplified banding pattern was analysed for genetic integrity based on number of fragments and reproducibility. RAPD profile revealed identity between donor and *in vitro* regenerated plants indicated stability in genome (Fig 2). The gels were scored for bands obtained from amplification of genomic DNA with 20 oligonucleotides and the RAPD gel profile was analysed for genetic stability because it is significant to use it as a tool for biotechnological application for any plant material. RAPD profile showed identical fragments indicated stability in genomic primer region. Out of 20 primers screened, 11 showed an amplification pattern with major bands with uniform staining intensity. The oligonucleotides sequenced are OPK 17: 5'-CCCAGCTGTG-3', OPJ 13: 5'-CCACACTACC-3', OPI 08: 5'-TTTGCCCGGT-3', OPN18: 5'-TCAGAGCGCC-3', OPO 10: 5'-TCAGAGCGCC-3', OPD 09: 5'-CTCTGGAGAC-3', OPN 8: 5'-ACCTCAGCTC-3', OPQ 11: 5'-TCTCCGCAAC-3', OPJ 5: 5'-CTCCATGGGG-3', OPL 01: 5'-GGCATGACCT-3', OPG 18: 5'-GGCTCATGTG were scored for 16 to 24 amplified DNA fragments; remainder of the primers studied resulted in an amplification with 9 to 11 bands. RAPD analysis was carried out in triplicates for fingerprinting analysis and these bands showed high reproducibility. The size of the fragments obtained ranged from 250 base pair to 2250 base pair. The primer generated monomorphic profile for the donor plants as well as the *in vitro* generated plantlets. The patterns derived from *in vitro* regenerated plants showed high similarity to donor plants and no genetic changes were apparent between these two. *D. allata*, which was used as one of the control for the study on polymorphism, from same family but different species *(D. allata)* showed polymorphism on the electrophoresis pattern (Fig 3) with respect to *D. prazeri* fragmentation. This indicated that RAPD analysis can play a significant role in establishing the genetic fidelity. The amplified banding pattern of the plants micropropagated from nodal explants/shoot tips were identical to those of control plants for all the primers used and this *in vitro* propagation technique can be followed for conserving the germplasm of endangered *Dioscorea prazeri*, and for related species like *D. allata*. The minor fragments that were unstable in staining intensity were not considered. A molecular based approach for the genetic fidelity of the micropropagtion experiment is highly essential for further research and for confirming stability of plants that have therapeutic properties. In some instances micropropagtion was reported to induce somoclonal variation mainly with leaf disc culture (Jain, 1997; Khoddamzadeh, 2010) and hence it is highly essential to confirm that the conditions optimsed for the *in vitro* propagation maintained the genetic integrity of the micropropagted plants. A marker assisted genetic fidelity assessment tool is essential for *in vitro* studies to develop optimised conditions for the plants. In this study on *D. prazeri* the primers used demonstarted the genetic stability of *vitro* regenerated plants in comparison with donor plants. RAPD was reported as an important tool for detection of polymorphism with the fragment analysis and was used as a genetic marker system for this study for the detection of polymorphism if any [Dixit, 2003]. A control plant DNA (*D. allata*) from the same family but a different species was used for the confirmation of RAPD analysis showing integrity and exhibits polymorphism if there is any variation, clearly showed polymorphism with *D. prazeri*. It was reported as a well defined approach for genetic evaluation and characterisation with operon primers (Punia et al., 2009). The monomorphic fragments of DNA obtained on amplification with 20 primers showed that the conditions optimised to generate the *in vitro* propagated plants of *D. prazeri* are reproducible and do not compromise the genetic integrity of the plant. RAPD analysis is essential to verify genetic stability and RAPD patterns found stable on evaluation of *D. prazeri* as previously reported in the study of *Solanum*

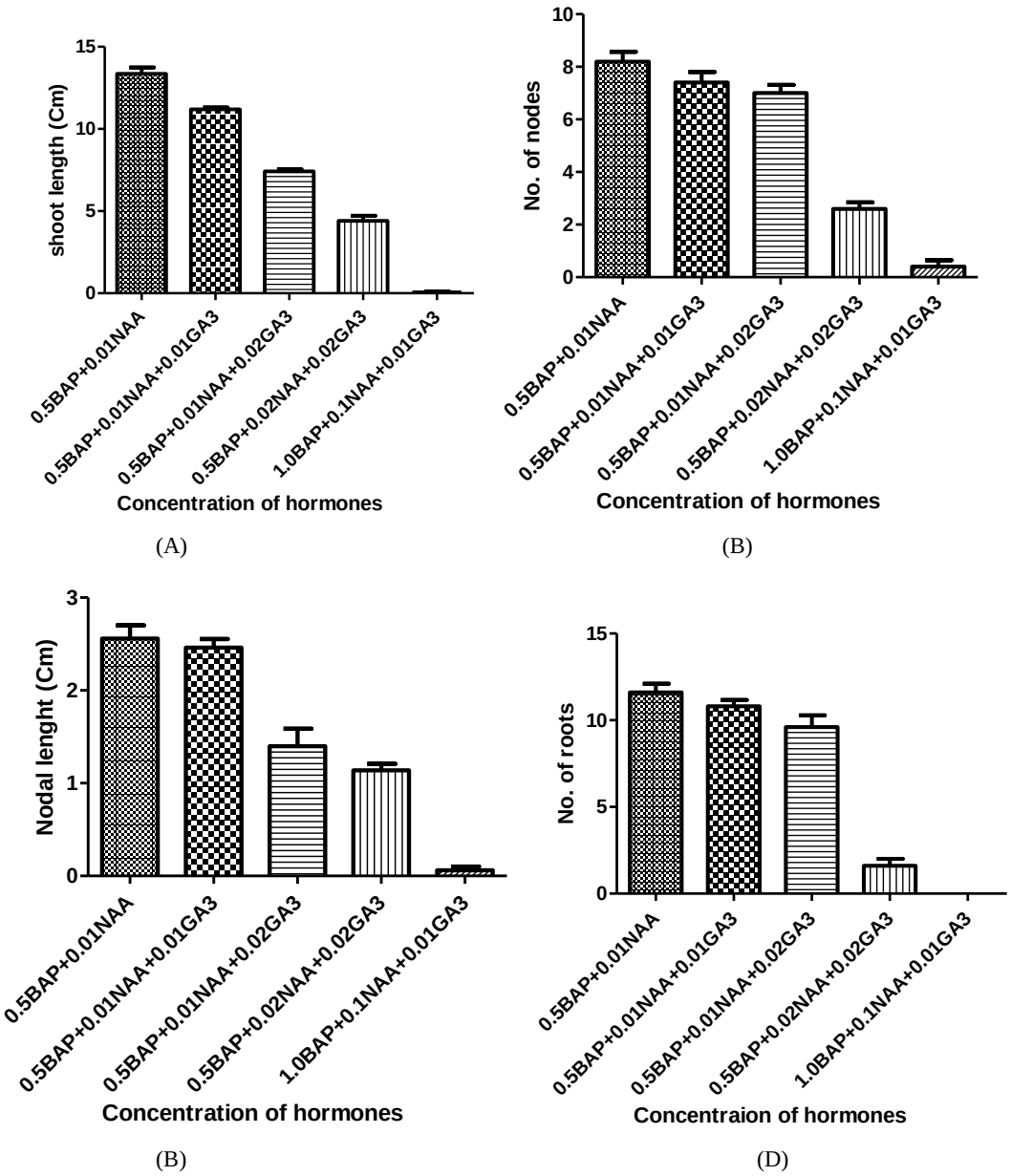

Fig 7. The graphical representation of statistical analysis for multiple combinations of BAP, NAA and GA3 with MS media

tuberosum L (Hirai and Sakai, 2000). *In vitro* propagated *D. prazeri* plants with monomorphic bands with donor plant ranging from 250 base pair to 2250 base pair used to analyse the genetic stability. Molecular analysis confirmed the genetic stability of the *in vitro* regenerated plantlets, as reported (Alizadeh and Singh, 2009). In another study, the plantlets regenerated were assessed with molecular markers and monomorphic bands were well characterised in determining genetic integrity as reported in dicot as well as monocot plants (Gupta, 2009). In this study it was shown that the plants propagated in highly favourable medium optimised for growth will not result in any polymorphism on micropropagation based on the electophoretic monomorphic pattern of DNA fragments obtained using RAPD markers, which is well documented for determining genetic integrity in a number of plant species (Devarumath, 2002; Rahman, 2001;Rout, 2002). Furthemore the use of a molecular based assessment on stability of genome of clonal material and to certify stability throughout the micropropagated plant system of is sorely needed (Rout, 1998).

HPLC analysis: Diosgenin assay

HPLC analysis was carried out to determine the content of the secondary metabolite, diosgenin, from *D. prazeri*. Various absorption spectra ranging from 195 to 235 nm to obtain the absorption maximum for steroidal sapogenin, diosgenin from *D. prazeri* were sampled, and 205nm was found to be optimal. The column was developed with methanol as mobile phase to resolve diosgenin. Among various solvents used for analysis methanol was showed maximum yield and prominent chromatographic peak. The biochemical analysis showed the diosgenin content was the same in regenerated plantlets as that observed in donor (wild) plants. Diosgenin content of the *in vitro* regenerated explants and the donor plants were analyzed with HPLC, wherein *D.*

Table 10. One-way analysis of variance for combination of hormonal treatment

One-way ANOVA for combination of hormones	Frequency	No. of shoots	Length of shoots
P-value	<0.0001	<0.0001	<0.0001
P value summary	***	***	***
*MSD	P< 0.05	P< 0.05	P< 0.05
No. Of groups	12	12	12
F	1200	1600	130
R^2	1	1	0.97

The data was graphically represented by Fig. 5 *2 Mean significant difference

Table 11. One-way analysis of variance for factorial combination of BAP, NAA and GA_3

One-way Analysis of Variance for MS+ BAP with NAA and GA3	
P-value	<0.0001
P value summary	***
*2MSD	P< 0.05
No. Of groups	5
R^2	0.99

*1 The data was graphically represented by Fig. 6 *2 Mean significant difference. The statistical analysis on various effects of hormonal treatment showed high significance.

allata, used as a negative control for the assay, did not indicate the presence of diosgenin. The retention time was confirmed using the calibration curve obtained with diosgenin standard (Sigma). The diosgenin content of tubers obtained according to the age of the plants was investigated. Even though *D. prazeri* produced diosgenin at younger stages, the yield was very low in comparison with the older plants. It varied from 0.7±0.2% to 2.6±0.2% (w/v) according to the growth phases of the plant from 12 weeks to 3 years old plants. The maximum level of diosgenin was observed in plants at 2.5 - 3.0 years of growth and found to be 2.2±0.2 %(w/v) on average. When compared to the biochemical analysis data obtained from the donor plant (wild), the level of diosgenin content of *in vitro* regenerated plants did not show significant variation in terms of steroidal sapogenin secondary metabolite product pattern (Fig 4A-C) (Table 7(A, B, C). Saponins are reported to have a wide range of biological activities (Francis et al., 2002) and isolation techniques enabling the characterization of saponins have been reported (Gurfinkel and Rao, 2003). Since *Dioscorea prazeri* is an endangered medicinal plant that has been subjected to microporpagation the stability of diosgenin, an important metabolite, was one of the main concerns in this study. The extraction conditions were standardised for *Dioscorea prazeri* species using pulverized tubers for the extraction and estimation of diosgenin and to study the stability of steroidal sapogenin, of *in vitro* raised plants. The pure fraction of diosgenin obtained was compared with known concentration of the standard. The diosgenin content of the donor plant and the *in vitro* propagated plants showed significant stability on analysis. The procedure developed in this study is highly efficient with respect to time and yield of diosgenin obtained, even from very limited amounts (0.15 g) of dried material.

Morphological analysis

The regenerated plantlets proliferated as vigorously as control plantlets and morphological abnormality was not detected. The ability to produce microtubers remained unaltered as both regenerated plantlets and control plantlets produced healthy leaves, shoots and roots on propagation. The analysis on morphological characters between donor and *in vitro* propagated plants did not show any significant difference and the morphology was found to be stable (Table 8). The morphology of the plants regenerated were analysed with the donor plant with respect to mature leaf, primary stems, petiole length and intermodal length and its ratio and found to highly stable. The *in vitro* regeneration in noramlised conditions were found be stable in previous studies on other species of Dioscorea (Dixit, 2003 and Ahuja, 2002). The stability in morphology on micropropagation and healthy growth observed in *D. prazeri* in this study was similar to that seen in other plant species (Annarita, 2009).

Statistical data analysis

The data obtained by treating *D. prazeri* explants using multiple growth regulators were analysed statistically. For every treatment level, 5 replicates having 30 explants each were used and showed a mean significant difference of $P<0.05$ and the coefficient of variation (R^2) of 0.99. The summary of data obtained with hormonal treatments and the regeneration of explants showed high significance. The treatment conditions were analysed with one way analysis of variance and Bonferroni's multiple comparison test. Graphical representations based on statistical analysis showed that MS with 0.5 mgL^{-1} BAP and 0.01mgL^{-1} NAA was the most suitable media for the micropropagation experiments to obtain healthy plants **(**Table 9, 10 & 11). The analysis on regeneration efficiency of treatment with individual hormones (Fig 5), various combinations of hormones (Fig 6) and factorial combination of BAP, NAA and GA_3 (Fig 7) are reported. This is with reference to various parameters such as number of shoots, frequency of regeneration, length of shoots, number of nodes, and length of nodes.

Materials and methods

Plant material

Dioscorea prazeri is a native of North-Eastern Himalayas. The tubers obtained from West Bengal (Darjeeling, Mungpoo) were established and maintained in greenhouse under controlled conditions for healthy growth of the plant.

Tubers were planted in 1:1:1 proportion of farmyard manure, soil and sand, maintained at a temperature below 25±2 ºC and watered to sustain moisture level, for the study. It was deposited in Herbarium of Avesthagen Limited, India; under the voucher specimen number 35(A).

***In vitro* regeneration and micropropagation**

Explants of leaves, internodes, nodes, petiole and shoot tips of *D. prazeri* were collected from actively growing *D. prazeri* plants in the greenhouse. The explants were surface sterilized with Tween 20 (1.0%), Cetrimide (1000 ppm), Bavistin (1000 ppm) and with 0.1% Mercuric Chloride. After each treatment the explants were rinsed thoroughly with sterile water. The explants were excised into 1.5-2 cm of length for inoculation. The culture medium consisted of the mineral salts and organic nutrients of Murashige and Skoog medium (Murashigue and Skoog, 1962), 3% sucrose and 0.8% agar. The basal medium was supplemented with different combinations of growth regulators such BAP, NAA, GA3, Thiadizuron (TDZ), Zeatin, Kinetin and 2-isopentanyl adenine (2iP). The plants were grown at 25 ± 2 ºC under a photoperiod of 16 hrs light/8 hrs dark with a photon dose of 36-mmol $m^{-2}s^{-1}$. Sub-culturing of the plantlets was performed every 15-17 days. The healthy plantlets were acclimatized with 1:1:1 ratio of farmyard manure, sand and soil. Acclimatised plants were transferred to the field for further establishment. The regenerated plants were taken for morphological, biochemical and genetic evaluation.

Random amplified polymorphic DNA (RAPD) analysis

The genomic DNA was isolated from *in vitro* grown plants of *D. prazeri*, donor plants of *D. prazeri*. *Dioscorea allata*, a plant from the same family but different species, was used as one of the control explants for RAPD analysis. The lithium chloride (LiCl) based method for aromatic and medicinal plants, was used for isolation of DNA (Pirttila et al., 2001). DNA was quantified by gel electrophoresis (1% agarose gel) and quality of the DNA was checked using Nanodrop spectrophotometer (ND-1000). RAPD analysis was carried out by amplification of 50 ng template DNA using polymerase chain reaction with 20 oligonucleotides like OPP7, OPC06, OPF 10, OPJ 13, OPK 17, OPN18, OPI 08, OPN 8, OPL 20, OPC19, OPO 10, OPD 09, OPQ 14, OPH 14, OPB 10, OPJ 5, OPQ 11, OPF 2, OPL 01 and OPG 18, which were decamers (Microsynth, Singapore) (Table 1). . Each amplification reaction (50 µL) (Table 2) contained template DNA (50 ng), dNTP (1mM), Taq DNA polymerase buffer (1x), Taq polymerase enzyme (2 units), Primer (1 picomole), $MgCl_2$ (1 mM) and sterile HPLC grade water was subjected to PCR using conditions shown in Table 3. The PCR amplified sample was electrophoresed on a 1.5% agarose gel, stained with Ethidium Bromide, and photographed using a phosphoimager (Bio-Rad). The gel was scored for clearly identifiable bands along with the donor plants and with the respective control for the genetic fidelity assessment for confirming the genetic stability. DNA fragments obtained from amplification with low visual intensities and those that could not be readily distinguishable were not scored.

Morphological analysis

The *in vitro* grown rooted plantlets were washed and transferred to vermiculate, coco peat mix, to red soil in 3:1:1 ratio for 15 days for hardening. The pots were covered with polythene bags to maintain humidity and watered regularly. The plants were drenched once in three days with Bavistin (0.1%) to avoid collar rot. The plantlets with well-developed root system were transferred to the greenhouse containing farm yard manure, vermiculate, soil in 1:1:1 ratio at regulated temperature of 25±2ºC. The morphological characters were analyzed from different set of experiments and the average value was calculated.

HPLC analysis of in vitro raised plantlets of D. prazeri

The samples for HPLC analysis was prepared by drying the tubers of *in vitro* raised plantlets and wild plants of *D. prazeri* at 45ºC for two days and subsequent hydrolysis at 95ºC for 3½ hours in 2N hydrochloric acid. The extract was neutralized with 2N sodium hydroxide to pH7.0, centrifuged and the residue was dried at 55 ºC for 36 hours in hot air oven. A second extraction was carried out by distillation with methanol, petroleum ether and hexane for the comparative study on yield of steroidal sapogenin (Biosox Unit, Techno Reach). The distillate, 5mL, was dried using roto-evaporator at 50ºC for 20 minutes with pressure of 300per square inch. The dried extract was subsequently dissolved in various solvents and subjected to chromatographic analysis using a C18 column (Atlantis® d C18 5µm 4.6x250mm column) using a Shimadzu Series LC-20 AT system, and a diode array detector SPD-M20A. All data were processed using LC-Solution software (Shimadzu, Japan). The chromatographic peak of the samples was compared with the standard (Diosgenin (98%), Sigma) to determine the yield of diosgenin in the extract. The tuber extract of *Dioscorea allata*, which does not express diosgenin, was used as a negative control.

Statistical analysis

Values were expressed as the average of 5 replicates ± standard deviation of the experiments. Statistically, the data was analyzed with one-way analysis of variance using Bonferroni's Multiple Comparison Test. The results were obtained from independent experiments with 5 replicates with 30 explants each. The mean significant value was calculated for the frequency of regeneration and for various hormonal treatments and the growth pattern obtained from the experiments.

Conclusion

Dioscorea prazeri, an endangered, indigenous medicinal plant was efficiently regenerated through *in vitro* culture of nodal explants and propagated by multiple shoots and healthy tubers resulting from it. In the present study, plantlets were successfully induced that could be successfully transplanted to the field with high field establishment. Using the optimized protocol this medicinally important threatened plant can be multiplied *in vitro* on a large scale and re-introduced into the natural habitat. Young shoot tips obtained from *in vitro* raised plantlets, precultured in B.O.D in dark for a very short period showed the best survival rate. The growth, morphology and the genetic integrity of the re-established plantlets did not show any variation among the micropropagated plants in comparison with the donor plant. The plants regenerated were found to be biochemically similar to the donor plant. The regeneration and

micropropagation of *D. prazeri* was achieved successfully with a high frequency (98±2%) and a survival rate of >96% on field establishment. The micropropagation of *Dioscorea prazeri* was successfully established with genetic fidelity, biochemical stability and morphological integrity. The propagation techniques and the analysis were subsequently useful for germplasm conservation and for genetic transformation studies. This enables the potential in exploring generation of the bioactive compound, diosgenin, which has significant medicinal value. Sustainable management and utilization of this valuable medicinal yam is now feasible through reinstating the healthy plants into their natural habitat.

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