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RESEARCH ARTICLE

IN VITRO PROPAGATION OF DENDROCALAMUS ASPER AND TESTING THE CLONAL FIDELITY USING RAPD AND ISSR MARKERS

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ABSTRACT

An efficient and reproducible protocol for the large-scale propagation of *Dendrocalamus asper* was achieved using nodal segments as explants. .High frequency of multiple shoots was induced on Murashige and Skoog's (MS) medium supplemented with Benzyl amino purine, BAP (8.86 µM) and adenine sulfate (13.5 µM). Regular sub-culturing carried out every 3 weeks on fresh shoot multiplication medium provided long term shoot cultures. Rooting (up to 90 %) was readily achieved upon transferring the shoot clumps (3-4 shoots) onto MS medium supplemented with Indole butyric acid, IBA (14.76 µM) and Naphthalene acetic acid, NAA (3.67 µM). *In vitro* raised plants were hardened in green house and successfully established in the field conditions, where they exhibited normal growth. Random amplified polymorphic DNA, RAPD and Inter simple sequence repeat (ISSR) markers were used to establish genetic uniformity in micropropagated plants. One hundred thirty three monomorphic fragments derived in 44 (34 RAPD; 14 ISSR) markers based fingerprinting confirmed genetic uniformity in *in-vitro* raised plants and mother plants.

INTRODUCTION

Since the dawn of civilization, bamboos have fascinated the mankind due to their variability, adaptability and versatility in utilization. They not only serve as raw material for various purposes but sustain the livelihood of countless people of the Asian countries (Liese, 1991). Besides being extensively used for meeting the domestic needs, many outstanding industrial products such as quality paper, floor tiles, boards, handicrafts, waxes, activated carbon are made out of bamboos. Moreover, fast growth and high bamboo biomass yields are helpful for mitigating the rise in atmospheric carbon dioxide. Bamboo shoots are treated as delicacies in many countries including China, Japan, Thailand, Malaysia, Singapore, Hong Kong, Indonesia and North–Eastern parts of India (Tripathi, 1998). The shoots are rich in proteins and fiber contents and are known to lower cholesterol (Nirmala *et al*., 2008). Moreover, bamboo leaves act as a fodder for cattle during winters (Sahoo *et al*., 2009). With renewed interest in bamboo propagation in India through Bamboo Mission Programmes, the emphasis is on producing quality bamboo material on large scale and to introduce other economically important bamboo species. Among these *Dendrocalamus asper* attains greater significance. *Dendrocalamus* genus is a tropical giant clumping bamboo with 83 species growing naturally in Indian sub-continent and whole of South-east Asia. But the over exploitation of this bamboo has led to the rapid depletion of natural strands hence, generating a grave concern about conservation as well as to develop efficient propagation methodologies for new plantations and re-establishment of cleared strands. Limitations in traditional propagation methods, such as the use of offsets, branch cuttings together with unpredictable and long flowering cycle (about 100 years), warrants an urgent need for an alternative approach for developing efficient and reproducible protocols for its mass propagation. Given the difficulties of conventional propagation techniques, *in vitro* propagation provides a promising alternative (Nadha *et al*. 2011). Arya *et al*. (2008) raised plantlets of *D.asper* through somatic embryogenesis which carries a higher risk of genetic instability due to somaclonal variations, thereby defeating the purpose of micropropagation. Earlier reports on micropropagation of *D. asper* by the axillary branching method employed seeds/seedlings explants (Arya *et al.,* 1999), which are rarely available due to long flowering cycle (100 years) and short seed viability. Moreover, unknown genetic background and the heterogeneity in seedling populations are the major constraints associated with seed-based micropropagation protocols (Negi and Saxena, 2011). In addition, none of these studies attempted to analyse the genetic fidelity of *in vitro* raised plants of *D. asper*, which is of utmost importance in determining the practicality of any micropropagation protocol. Recently, Singh *et al*. (2012) have established micropropagation protocol of *D. asper* employing nodal explant, but even this report lack description of clonal fidelity analysis.

In this report, we describe an efficient and reproducible micropropagation protocol for generating uniform clones through axillary branch proliferation using vegetative tissue derived from mature field-grown clumps of *D. asper*.

MATERIALS AND METHODS

Culture initiation

Prior selection of the mother plant is a very important step while carrying out micropropagation studies. In the present study, selection of mother plant was done by taking into the consideration important parameters viz; i) height of bamboo culm, ii) girth of culm at third internode from the bottom, iii) number of culms per clump and iv) length of internode. Nodal Segments were taken from the precocious branches of five year old field grown plant.

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Explants were subjected to repeated washings under running tap water for 30 minutes to remove all the adhering dust particles and microbes from the surface. The explants were then cleaned with liquid detergent (Tween20-HIMEDIA) with the help of a sable hair brush and then washed properly with distilled water to remove the traces of detergent. After that the explants were treated with a suitable fungicide like bavistin and an antibiotic (Streptomycin sulphate) for another 20 minutes to remove fungus and bacteria respectively. Different concentrations of bavistin (0.1-1%) and streptomycin sulfate (0.02-0.1%) were tried for surface sterilization but 0.1% bavistin and 0.04% streptomycin sulfate were found to be the most effective. Under sterile conditions in a laminar air flow cabinet, the explants were treated with 70% alcohol for one minute followed by a treatment with $HgCl_2$ solution (0.04% w/v) for 6 minutes. The explants were then inoculated onto MS basal medium containing 2% (w/v) sucrose for culture initiation.Micropropagation work was carried out by exploiting forced axillary branching technique from nodal explants. Various factors like initial response of explants, contamination rate, bud break and survival percentage were considered for initiation of cultures. Nodal explants were collected from January, 2008 to December, 2008 on a regular basis to study the effect of season on the desired response of the explants. Twenty four explants were inoculated after every 15 days. Nodal explants were inoculated onto MS basal medium or MS medium supplemented with BAP, 2% sucrose and 0.8% agar for initial screening. Percent survival was tested on tenth day basically for asepsis and bud break.

Shoot multiplication

The sprouts were excised from the explants and transferred to media supplemented with different concentrations and combinations of cytokinins for shoot proliferation. Regular sub-culturing was resorted to every three weeks. Dead decaying tissue was removed during each sub-culturing.After first cycle of multiplication, the shoots were divided into clumps of 3, 6, 9 and 12 shoots and the effect of number of shoots per propagule was evaluated by inoculating shoot clumps onto the medium for further multiplication. To study the effect of carbon source on shoot multiplication, the clumps of shoots were also inoculated onto multiplication medium supplemented with 2% sucrose, glucose and table sugar respectively. Best carbon source was selected on the basis of number of shoots induced and length of shoots achieved**.** Effect of various gelling agents like phytagel, agar and liquid medium on multiple shoot proliferation was also studied.

Rooting of microshoots

To achieve rooting of microshoots, different concentration of various auxins were tested either singly or in different combinations. Cut ends of the microshoots in bunches of 3-4 were placed in MS medium supplemented with auxins.

Acclimatization

Rooted plantlets were washed thoroughly in lukewarm water with the help of a soft, sable-hair brush for removal of agar adhering to the plantlets growing on the gelled medium. These were transferred to plastic pots (4" dia.) containing wet sand and kept in a poly/greenhouse, covered initially with inverted glass beakers for 8–10 days for acclimatization. These were then transferred to the different potting mixture (PM1 - sand:soil:manure :: 1:1:1; PM2 - sand:soil:manure :: 1:1:2 PM3 - sand:soil:manure :: 1:0:0; PM4 - sand:soil:manure :: 0:0:1).

Statistical analysis

The effect of different treatments was determined by analysis of variance (ANOVA) using STATISTICA data analysis software v7 (StatSoft Inc., Tulsa, OK). Significance differences between the means were assessed by Duncan's Multiple Range Test (DMRT) at p=0.05.

Clonal fidelity

To test the clonal fidelity, the *in vitro* raised plants along with various organogenesis stages selected during the subcultures and hardened plants were chosen randomly. Total genomic DNA of the mother plant and *in vitro* raised plants was extracted from young leaf tissue by using modified Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1990) with minor modifications. Sixty seven (40 RAPD; 27 ISSR) markers (Sigma-Aldirch, Bangalore, India) were used for initial screening. PCR amplifications were carried out in total volume of 25 µl containing 20 ng of genomic DNA. The single reaction of RAPD and ISSR consisted of 10 x assay buffer (100 mM Tris-HCl; pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.1 % gelatin), 200 µM dNTPs, 15 ng primer, 0.3 U Taq DNA polymerase. PCR amplification was performed in a Bio Rad I cycler from Bio Rad Laboratories (India) Pvt. Ltd. RAPD PCR amplification reactions were carried out at initial denaturation at 94^0C for 5 minutes, followed by 45 cycles of 1 minute denaturation at 94^0C, 1 minute annealing at 37^0C and 2 minutes extension at 72^0C, with a final extension at 72^0C for 7 minutes. While, ISSR was programmed for initial DNA denaturation at 94^0C for 4 minute, followed by 44 cycles of 1 minute denaturation at 94^0C, 1 minute annealing (temperature specific to the primer) and 1 minute extension at 72^0C, with a final extension at 72^0C for 7 minutes. The amplified fragments were electrophoresed in 1.8% agarose gel matrix in an electrophoresis system (Bio Rad Subsell 96) for atleast 2 hours or until the bands were clearly separated and visible.

RESULTS AND DISCUSSION

Culture initiation

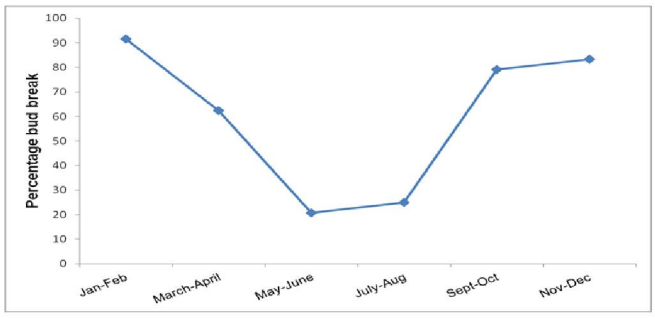
Due to the considerable variations in the environmental conditions during different periods of the year, maturity status of the explants varied with season, hence, the response of explants to the culture initiation also varied. During active growth from June to August, the explants were found to be tender and unable to bear the harsh sterilization treatments. On the other hand, during the periods of slow growth, appropriately hardened explants were available which could endure the sterilization treatment. Further, breaking the dormancy of buds varied with their position on the plant and season of the year as midculm nodes of tertiary branches were found to be the best explants for axillary shoot proliferation. In this case, best period for initiating aseptic cultures were January and February when maximum bud break was achieved (Figure 1a). The rate of contamination in the cultures also varied with season. Rainfall had a direct influence on contamination rates and survival percentage of explants. March-April and November-December were observed to be the best months for initiating aseptic cultures because of low rates of contamination. In contrast, the highest rate of contamination was observed during the period of maximum rainfall (June-August). The prior screening of explants by inoculating them on half strength MS medium supplemented with 2% sucrose for 7-10 days proved to be beneficial. Variation in contamination percentage with rainfall from January to December (2008) is shown in Figure 1b.

Shoot multiplication

For inducing sprouting, nodal explants were inoculated on MS medium with or without any cytokinins. Nodal explants cultured on MS basal medium without any cytokinin took more time to sprout (25 days) and that too with low efficiency (30%). However, the nodal explants sprouted within 15 days of inoculation on MS medium supplemented with BAP (8.86 µM) in 90% of the cultures (Figure 2a). The sprouting response declined with increase (13.29 µM) as well as decrease (2.26 to 4.43 µM) in the concentration of BAP. Nodal explants failed to respond on kinetin supplemented MS medium.The sprouted buds were excised from the nodal segments and transferred onto MS medium containing different concentrations and combinations of cytokinins like BAP(2.26-22.15 µM), Kn (2.32-13.95 µM) and adenine sulfate (1.35-27 µM) for inducing axillary

shoot proliferation. The number of sprouted buds at each node was treated as individual shoot during present investigation. Preliminary experiments on MS medium supplemented with 2% sucrose led to necrosis and death of shoots. This necessitated the inclusion of cytokinins in the shoot multiplication medium either alone or in combinations. Among the various cytokinins employed for shoot proliferation, BAP (8.86 µM) in conjunction with adenine sulfate (13.5 µM) was found to be the best where an average of 48.66 shoots were formed after 60 days. Higher concentrations of BAP or adenine sulfate not only lowered the multiplication rate but also resulted in the formation of stunted shoots. Addition of kinetin alone did not result in any shoot proliferation. The shoots remained dormant for some time and ultimately died. The details of the effect of different cytokinins on shoot proliferation are depicted in Table 1. Once the best combination of cytokinins for shoot multiplication was found, the cytokinin-supplemented medium was tested with the addition of different auxins such as IBA (1.23-9.84 µM), IAA (1.42-11.42 µM) and NAA (1.83-14.7 µM). It was observed that the addition of any auxin into the multiplication medium considerably reduced the proliferation rate, although they increased the length of shoots. Hence, their addition to the multiplication medium was discontinued. After the optimization of various combinations and concentrations of cytokinins for shoot proliferation (Table 1), the effect of various gelling agent such as agar (0.8%) and phytagel (0.25%) was also studied. In the present study, replacement of the solidified medium with liquid medium during multiple shoot proliferation did not prove effective. Use of phytagel instead of agar was found to be more beneficial for promoting shoot multiplication.

Propagule used for recurrent multiplication of shoots is a critical factor during *in vitro* studies. In the present study, shoot clumps rather than single shoots were observed to be effective for multiplication of bamboo plants. The *in vitro* raised shoots were divided into clumps of 3, 6, 9 and 12 shoots each. The effect of number of shoots (3,6,9,12) per propagule inoculated on multiplication medium was evaluated by culturing the clumps on MS medium supplemented with BAP(8.86 µM) and adenine sulfate (13.5 µM). Six shoots per propagule were found most effective for further multiplication where 27.2 shoots were obtained after 4 weeks of culture. However, use of more number of shoots per propagule (9,12) reduced multiplication rate as well as length of shoots formed. Effect of the number of shoot/clump on shoot multiplication in *D. asper* are shown in Figure 1c. To study the effect of different carbon sources on shoot multiplication, six shoots per propagule were cultured on MS medium containing BAP (8.86 µM) and adenine sulfate (13.5 µM) supplemented with either 2% sucrose, glucose or Table sugar respectively. Sucrose was found to be most suitable carbon source, as on an average 48.2 shoots were obtained after 6 weeks of culture. Length of shoots was also maximum (3.22 cm) in sucrose supplemented medium. However, replacement of sugar with less expensive table sugar did not affect multiplication rate significantly, instead it reduced cost of production considerably. Hence, table sugar was preferred over sucrose for carrying out micropropagation studies presently.



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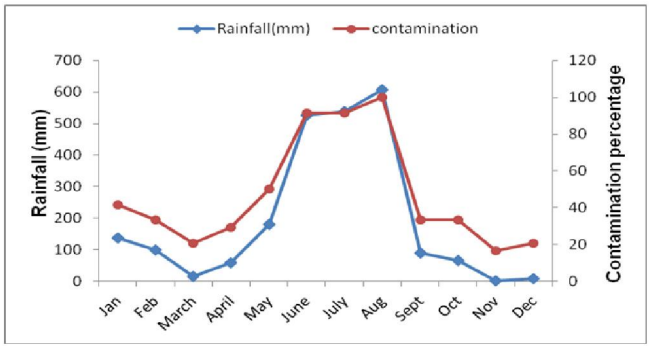

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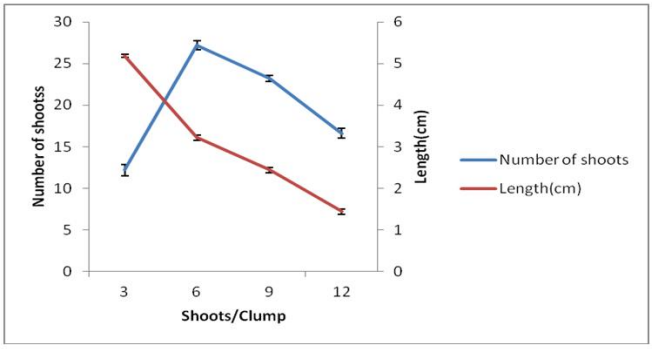


c

Figure 1. (a) Variation in bud-break in nodal segments from January to December (2008) (b) Variation in contamination percentage with rainfall from January to December (2008) (c) Effect of the number of shoot/clump on shoot multiplication in *D. asper*.

Rooting

Rooting is a major bottleneck while carrying out *in vitro* multiplication of bamboos. It was observed that rooting was more effectively induced when clusters of shoots rather than individual shoot were used. The clumps of 3 to 4 shoots were inoculated onto MS medium supplemented with different concentrations and combinations of auxins. Shoots failed to root even after 45 days of

Table 1. Effect of different cytokinins on shoot proliferation

S.No	Treatment	No. of shoots after 45 days	No. of shoots after 60 days
1	BAP(4.43 µM)	6.66 ± 0.88 ^{no}	9.33 ± 0.66 ^{m}
2	BAP(8.86 µM)	10.66 ± 0.66 ^{lm}	13.66 ± 0.33 ^{jk}
3	BAP(13.29 µM)	12.33 ± 0.88 ^{kl}	14.66 ± 0.66 ^{j}
4	BAP(22.15 µM)	5.00 ± 0.57 ^{op}	6.33 ± 0.33 ^{op}
5	BAP(8.86 µM)+AS(2.7 µM)	20.33 ± 0.33 ^{i}	24.66 ± 0.33 ^{h}
6	BAP(8.86 µM)+AS(6.75 µM)	25.66 ± 0.66 ^{h}	33.66 ± 0.33 ^{f}
7	BAP(8.86 µM)+AS(10.8 µM)	29.33 ± 0.88 ^{g}	39.33± 0.88 ^{cd}
8	BAP(8.86 µM)+AS(13.5 µM)	37.66 ± 1.45 ^{de}	48.66 ± 1.33 ^{a}
9	BAP(8.86 µM)+AS(16.20 µM)	37.00 ± 1.52 ^{e}	45.66 ± 0.88 ^{b}
10	BAP(13.29 µM)+AS(16.20 µM)	33.66 ± 0.88 ^{f}	41.00 ± 0.57 ^{c}
11	BAP(8.86 µM)+Kn(2.32 µM)	7.00 ± 0.57 ^{no}	8.66 ± 0.66 ^{mn}
12	BAP(8.86 µM)+K(4.65 µM)	4.33 ± 0.33 ^{p}	5.66 ± 0.33 ^{op}
13	BAP(8.86 µM)+Kn(9.3 µM)	0.00 ± 0.00 ^{q}	0.00 ± 0.00 ^{q}
14	Kn(2.32 µM)	0.00± 0.00 ^{q}	0.00 ± 0.00 ^{q}
15	Kn(4.65 µM)	0.00 ± 0.00 ^{q}	0.00 ± 0.00 ^{q}

Duncan test; p=0.05; Values followed by the same letters in superscript within the column are not significantly different.

culture on MS medium when supplemented with auxins like IAA and NAA. Although, rooting was observed on IBA supplemented medium but response to rooting was delayed. Moreover, only a few propagules rooted. On lower concentration of IBA (2.46 µM) only 10% rooting was observed. Rooting percentage improved with increase in concentration of IBA with maximum rooting (50%) on 14.76 µM IBA supplemented medium. With further increase in the concentration of IBA, development of roots declined and propagules did not survive. Addition of NAA (3.67 µM) to IBA (14.76 µM) supplemented medium proved to be very effective as maximum rooting response of 90% was observed with an average of 5.66 roots per propagule and mean root length of 3.06 cm after 45 days of culture (Figure 2d). After 15 days of transfer to root induction medium, the propagules turned brown in all treatments. Hence, a regular transfer to the fresh media was done to ensure development of healthy shoots and roots. Effects of various auxins on rooting are shown in Table 2.

Acclimatization

Plantlets were taken out of flasks and washed with luke warm water to remove agar sticking to them. These were then transferred to plastic pots containing river bed sand and covered with plastic jars for maintaining high relative humidity (80-85%). After 25 days, the plantlets were transferred into potting mixture containing sand:soil:farmyard manure in different ratios (v/v;1:1:1; 1:1:2; 1:0:0; 0:0:1) and kept in green house. Under green house conditions, foliage was sprayed with Hoagland solution after every 7 days. After one month of transplantation, the percentage survival was 95% in the 1:1:1 sand:soil:manure mixure, 90% in 1:1:2 sand:soil:manure mixture, 80% in sand and 60% in manure respectively . Hardened plants were finally transplanted in the field to pits (2ft×2ft×2ft) at a plant to plant and row to row distance of 6 meters. They were successfully established under field conditions and plants showed well developed root and shoot systems and all the plants are thriving

Figure 2 (a) Axillary bud break on BAP (8.86 µM) after 15 days of inoculation (b) Multiple shoot proliferation on BAP (8.86 µM) and adenine sulphate (13.5 µM) after 45 days of culture (c) Formation of roots on the combination of IBA (14.76 µM) and NAA (3.67 µM) (d) Complete plantlets formation before acclimatization (e) Acclimatized plants of *D. asper* in the green house.

Table 2. Effect of different auxins on root induction and length of roots

S.No	Treatments	No. of roots formed	Length of roots(cm)
1	IBA (2.46 µM)	0.33 ±0.33 ^{e}	0.06±0.66 ^{g}
2	IBA (4.92 µM)	1.66±0.33 ^{de}	0.45±0.02 ^{f}
3	IBA (9.84 µM)	2.66±0.33 ^{cd}	0.47±0.01 ^{f}
4	IBA (14.76 µM)	3.00±0.00 ^{cd}	0.54±0.04 ^{f}
5	IBA (4.92 µM)+ NAA (3.67 µM)	3.00±0.00 ^{bc}	1.23±0.01 ^{e}
6	IBA (9.84 µM)+ NAA (3.67 µM)	4.00±0.57 ^{a}	2.17±0.05 ^{c}
7	IBA (14.76 µM)+ NAA (3.67 µM)	5.66±0.33 ^{d}	3.06±0.02 ^{a}
8	IBA (24.60 µM)+ NAA (3.67 µM)	5.33±0.33 ^{ab}	1.65±0.02 ^{d}
9	IBA (14.76 µM)+ NAA (7.35 µM)	5.33±0.33 ^{ab}	2.77±0.01 ^{b}

Duncan test, p=0.05; Values followed by the same letters in superscript within the column are not significantly different

very well in field conditions with no phenotypic variations observed when compared to the mother plant.

Clonal fidelity

DNA samples from *in vitro*-grown shoots under various stages of subculture, hardened plants growing in the greenhouse, plants growing in the field and the mother plant were subjected to RAPD and ISSR analysis. A total of 67 (40 RAPD; 27 ISSR) markers were screened. Out of these 44 (30 RAPD; 14 ISSR) markers having detected multiple and reproducible amplifications in repeated reactions were selected for genetic fidelity testing. in the present study. The 14 ISSR primers produced 40 distinct and scorable bands in the size range of 250 bp to 2400 bp (Table 3). Likewise, 30 RAPD primers produced 93 distinct and scorable bands in the size range of 200 bp to 2000 bp with an average 3.1 bands per primer (Table 4). In total, 133 bands were amplified across tissue culture raised samples and mother plants. The banding profiles from micropropagated plants were found to be monomorphic and similar to those of mother plant hence confirming their true to type nature (Figure 3a; 3b). These results confirmed that *D. asper* plants obtained by axillary branching method under *in vitro* conditions retained their clonal fidelity.

DISCUSSION

Over exploitation of natural bamboo cover coupled with growing demands by rising population and inefficient conventional method for propagation present an urgent need for developing methods for large scale propagation of bamboos. In this regard, an efficient *in vitro* propagation technique serves as a viable alternative for large scale establishment of bamboo cover. Taking into account the problems associated with conventional propagation techniques, it will be useful to develop efficient aseptic method of propagation of *D. asper* using nodal explants. For the initiation of cultures, judicious selection of the explant is fundamental because different tissue types within the same plant differ in their response to a particular culture condition and the season of the year. In *Dendrocalamus asper*, among the various explants tested, unexpanded buds from tertiary branches were found to be the best for raising cultures as they responded favourably to different media combinations besides being easier to handle. Till date, micropropagation protocols for many bamboo species have been successfully worked out employing both seed/seedling and mature explants. Unfortunately, very limited success has been achieved with adult tissues because of several technical problems such as seasonal constraints in initiating the cultures, microbial contamination, slow

Table 3. The inter-simple sequence repeat (ISSR) primers utilized to verify *D.asper* clones

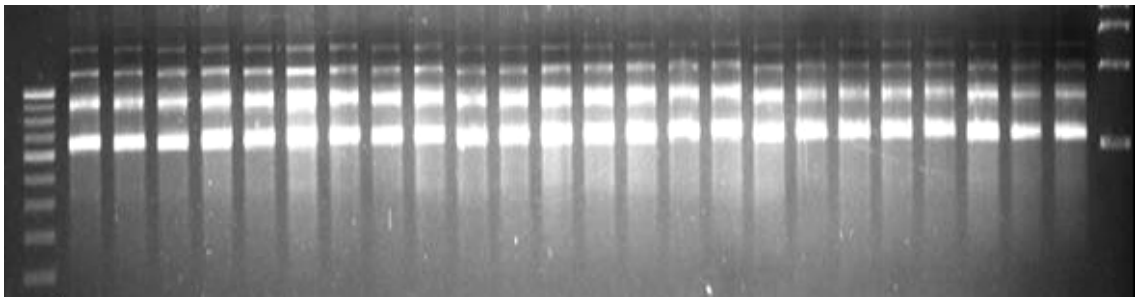
Primers	5'-3' motif	Annealing Temperature(°C)	No. of scorable bands	Range of amplification (bp)
UBC 807	AGAGAGAGAGAGAGAGT	37.5	5	250-700
UBC 811	GAGAGAGAGAGAGAGAC	38.3	4	1200-2400
UBC 812	GAGAGAGAGAGAGAGAA	40.7	3	500-1100
UBC 815	CTCTCTCTCTCTCTCTG	41.8	4	600-1400
UBC 818	CACACACACACACACAG	47.1	1	1700
UBC 836	AGAGAGAGAGAGAGAGYA	48.5	1	600
UBC 841	GAGAGAGAGAGAGAGAYC	41	1	600
UBC 842	GAGAGAGAGAGAGAGAYG	43.8	2	400-500
UBC 843	CTCTCTCTCTCTCTCTRA	42.1	2	400-650
UBC 848	CACACACACACACACARG	50.5	3	550-2100
UBC 873	GACAGACAGACAGACA	42.4	5	600-2000
UBC 888	BDB CAC ACA CAC ACACA	47.3	4	900-1800
UBC 889	DBDACACACACACACAC	42	1	2000
UBC 891	HVHTGTGTGTGTGTGTG	46.8	4	250-600

R=A+G; H=A+G; Y=C+T; V=G+A+C; B=G+T+C; D=G+A+T

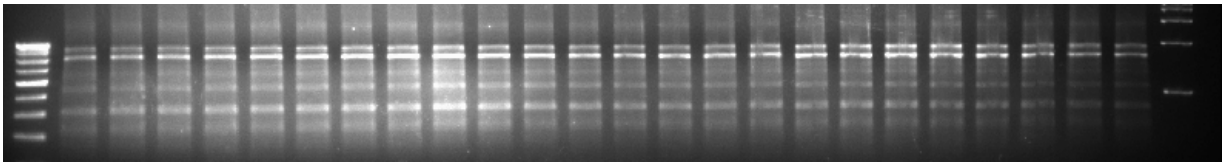
Table 4. The random amplified polymorphic DNA (RAPD) primers utilized to verify *D. asper* clones

S.No.	Primers	5'-3' motif	No. of scorable bands	Range of amplification (bp)
1	OPA 03	AGTCAGCCAC	1	700
2	OPA 04	AATCGGGCTG	2	550-650
3	OPA05	AGGGGTCTTG	2	700-900
4	OPA 07	GAAACGGGTG	4	450-2000
5	OPA 08	GTGACGTAGG	4	400-1300
6	OPA 09	GGGTAACGCC	2	300-800
7	OPA 10	GTGATCGCAG	2	450-550
8	OPA 12	TCGGCGATAG	1	600
9	OPC 06	GAACGGACTC	5	600-1500
10	OPC 09	CTCACCGTCC	2	1200-1300
11	OPC 10	TGTCTGGGTG	3	600-1200
12	OPC 12	TGTCATCCCC	2	650-800
13	OPC 13	AAGCCTCGTC	3	800-1500
14	OPC 14	TGCGTGCTTG	6	350-900
15	OPC 15	GACGGATCAG	7	300-1500
16	OPC 16	CACACTCCAG	3	500-1200
17	OPC 17	TTCCCCCCAG	2	200-500
18	OPC 18	TGACTGGGTG	5	550-1400
19	OPC 19	GTTGCCAGCC	3	600-1000
20	OPC 20	ACTTCGCCAC	5	500-1300
21	OPS 01	CTACTGCGCT	3	450-1500
22	OPS 02	CCTCTGACTG	1	650
23	OPS 03	CAGAGGTCCC	5	400-1200
24	OPS 04	CACCCCCTTG	4	500-950
25	OPS 05	TTTGGGGCCT	2	700-1400
26	OPS 08	TTCAGGGTGG	2	600-900
27	OPS 09	TCCTGGTCCC	1	450
28	OPS 11	AGTCGGGTGG	3	300-850
29	OPS 12	CTGGGTGAGT	6	200-1200

R=A+G; H=A+G; Y=C+T; V=G+A+C; B=G+T+C; D=G+A+T



(a)



(b)

Figure 3. (a) ISSR products generated from 23 *in vitro* regenerated plants and mother plants of *D.asper* amplified with primer UBC 815 showing monomorphic pattern. Lane L1 represents 100-bp ladder, Lane M represents mother plant, Lane 1-23 represent *in vitro* raised clones of *D .asper* and Lane L2 represents 500-bp ladder (b) Polymerase chain reaction (PCR) amplification products obtained with a random amplified polymorphic DNA (RAPD) primer OPC 14. Lane L1 represents 100-bp ladder, Lane M represents mother plant, Lane 1-23 represent *in vitro* raised clones of *D.asper* and Lane L2 represents 500-bp ladder

growth, poor multiplication rates and low rooting frequency etc. However, multiplication of superior bamboo clumps with desirable traits is possible only with the use of adult tissues than with seed/seedling explants. However, success with explants taken from mature plants of bamboos for propagation has been limited to a few cases only. McClure (1966) observed that in nature, dormancy and breaking of dormancy in buds of bamboos varied with their position on the plant, the season of the year and the species. In *D. giganteus*, the mid-culm nodes of secondary branches have been reported to be the best explants for axillary shoot initiation (Ramanayake and Yakandawala, 1997). In our study, mid culm nodes of tertiary branches proved better. In *Bambusa vulgaris*, bud-break was induced more frequently throughout the year and was strong and positively correlated with rainfall (Kumari and Ramanayake, 1996). Saxena and Bhojwani (1993) found that *in vitro* bud-break in *Dendrocalamus longispathus* took place during the monsoon. According to Paranjothy *et al*. (1990), meristems in bamboos are most active only during rainy season and fresh cultures should be initiated during this period. In tropical species including bamboos, changes in the environment such as those caused by rainfall or its onset may trigger the synthesis or breakdown of endogenous substances that control growth responses. But in our study involving *D. asper*, best period for initiating aseptic cultures was January and February when maximum bud break was achieved.

According to Hirimburegama and Gamage (1995), cytokinins play an important role in inducing bud break. This study has been supported by many researchers later on. Among different cytokinins, BAP has been very effective in inducing sprouting of axillary buds in several bamboo species belonging to different genera (Prutpongse and Gavinlertvatana, 1992; Sood *et al*., 1992; Ramanayake and Yakandawala, 1997; Ravikumar *et al*., 1998; Bag *et al*., 2000). In *Guadua angustifolia*, in the absence of BAP, only 13% of the explants sprouted while highest sprouting rates (37.5%) were obtained with the addition of BAP (Jimenez *et al*. 2006). Presently, in *D .asper*, nodal explants cultured on MS basal medium without any cytokinin took more time to sprout (25 days) and that too with low efficiency (30%). On the other hand, the nodal explants sprouted within 15 days of inoculation on MS medium supplemented with BAP (8.86 µM) in 90% of the cultures. We observed that the sprouting response declined with increase as well as decrease in the concentration of BAP.

In *D. asper*, during preliminary experiments on MS basal medium, it led to necrosis and death of shoots in first passage. This necessitated the inclusion of cytokinins. On MS medium containing BAP (8.86 µM) in conjunction with adenine sulfate (13.5 µM), 48.66 shoots were formed after 60 days. Initially rate of multiplication was slow but it gradually increased during subsequent subcultures. The effectiveness of cytokinins especially BAP in promoting axillary shoot proliferation in forest trees as well as bamboos is well documented (Godbole *et al*., 2002; Arya *et al*., 2006; Kalia *et al*., 2007; Devi and Sharma, 2009; Bisht *et al*., 2010). In our study with *D. asper*, use of higher concentrations of BAP not only lowered the multiplication rate but also resulted in stunted shoots. Addition of kinetin alone did not result in any shoot proliferation. Instead, shoots remained dormant for some time and ultimately died. Negi and Saxena (2011) also reported the similar findings that higher concentration of BAP inhibited the shoot proliferation and addition of Kn did not promote shoot proliferation. Godbole (2003) reported that at higher concentration of BAP, the leaves remained curled up and failed to expand. During *in vitro* studies, we observed that the type of propagule (number of shoots per clump) used for shoot multiplication is a critical factor. Shoot clumps rather than single shoots were found to be effective for multiplication of bamboo plants (Arya *et al*.1999; Ramanayake *et al*., 2001, Sood *et al*., 1992). Bag *et al*. (2000) highlighted that propagules containing a minimum of three to four shoots proliferated at a maximum rate whereas single shoot would proliferate at a much slower rate. During micropropagation studies in *D. strictus*, Ravikumar *et al*. (1998) recommended that for further sustained growth and multiplication, the shoots should be transferred in groups of 5-7 during subculturing. In *D.asper*, we observed that six shoots per propagule were found most effective for multiplication where 27.2 shoots were obtained after 4 weeks of culture.

During propagation of *D. asper*, replacement of the solidified medium with liquid medium during multiple shoot proliferation did not prove effective. Use of phytagel instead of agar was found to be beneficial for promoting shoot multiplication. The use of agar lowered multiplication rate and reduced the length of shoots as compared to phytagel solidified medium. Bag *et al*. (2000) pointed out that culturing in agar gelled medium resulted in secretion of phenolics by explants and was associated with browning of medium and reduced multiplication. They recommended the use of medium gelled with phytagel which resulted in comparable multiplication efficiency. Similarly, Ramanayake and Yakandwala (1997), Zamora *et al*. (1988), Agnihotri *et al*. (2009) recommended the use of phytagel for

healthy growth of plants. Effect of carbon source (sucrose, table sugar and glucose) on shoot multiplication was also studied. In *D. asper,* although sucrose (3%) was found to be most suitable carbon source as 48.2 shoots were obtained with 3.22 cm shoot length after 6 weeks of culture but replacement of sugar with less expensive table sugar did not affect multiplication rate significantly, although it reduced cost of production considerably. Hence, table sugar was preferred over sucrose for carrying out micropropagation studies. For the development of any successful micropropagation protocol, root induction from excised shoots and subsequent survival of plantlets in the soil are crucial. Rooting is a major bottleneck while carrying out *in vitro* studies in bamboos. It was observed that rooting was more effectively induced when cluster of shoots rather than individual shoot were used. In *D. asper*, on lower concentration of IBA (2.46 µM) only 10% rooting was observed while on IBA (14.76 µM) maximum rooting response (50%) was discerned. With further increase in concentration of IBA development of roots declined and propagules failed to survive. Addition of NAA to the IBA supplemented medium proved effective as maximum rooting (90%) was achieved with an average of 5.66 roots per propagule and mean root length of 3.06 cm after 45 days of culture.Auxins when used in combination are known to enhance rooting frequency significantly. Many reports are available in which different combinations and permutations of auxins were employed to induce rooting in bamboos (Negi and Saxena, 2011; Islam and Rahman, 2005; Arya *et al*., 2006).

After the plantlets were established *in vitro,* these were subjected to hardening under green house conditions. These plantlets were transferred to pots containing river bed sand and covered with jars to maintain high humidity (80-85%) for 25-30 days before transferring to potting mixture. In *D. asper*, the plants were transferred into potting mixture containing sand:soil:manure in different ratios (v/v;1:1:1; 1:1:2; 1:0:0; 0:0:1). The plants transplanted in pots containing 1:1:1 (sand:soil:manure) potting mixture showed a successful hardening rate of 95%. The plants when shifted to field conditions exhibited 100% survival. Arya *et al*. (1999) obtained 95% survival rate after transplantation of *D. asper* plants raised through seeds. Although the i*n vitro* germplasm conservation depends on micropropagation technique, the phenotypic and genetic variations may occur during *in vitro* propagation and subsequently may give rise to somaclonal variants (Kaeppler *et al.,* 2000). Therefore, it is essential to assess the clonal stability of *in vitro* derived plants to obtain genetically pure elites rather than having indifferent populations (Eshraghi *et al*. 2005; Chandrika and Rai, 2008; Mehta *et al*., 2010). Among various methods of *in vitro* propagation, the axillary shoot proliferation is least susceptible to genetic modification (Shenoy and Vasil, 1992). However, the possibility of somaclonal variations cannot be ruled out even with this method. There are very few reports which have confirmed the clonal fidelity of bamboo plantlets derived from axillary bud proliferation. The scarcity of reports on ascertaining the genetic fidelity of tissue culture raised plantlets can jeopardise the quality of micropropogated plants, especially in perennials like bamboo where any undesirable variant would last for several years (Negi and Saxena, 2010). Therefore, it is pertinent to screen the regenerants at regular intervals for the occurrence of any somaclonal variation. Earlier, Das and Pal (2005) established the clonal fidelity of regenerants of *Bambusa tulda* and *B. balcooa* using only four markers to assess their genetic uniformity among the regenerants. Godbole (2003) assessed the clonal fidelity of *in vitro* raised plants of *D. hamiltonii* using 18 RAPD markers. Later, Negi and Saxena (2010) employed 15 ISSR markers to validate the clonal fidelity of *in vitro* raised *B. balcooa* plantlets through axillary bud proliferation. However, there is no report available on the comparative genetic stability of regenerants and mother plant of *D. asper* by using RAPD and ISSR markers. In the present study, we did not find any polymorphism during the RAPD and ISSR analysis of *in vitro* raised clones, thus confirming true to type nature of *in vitro* raised plants. Similar results have been reported in *Bambusa balcooa* (Negi and Saxena, 2010), *Bambusa nutans* (Mehta *et al*., 2010; Negi and Saxena, 2011), gerbera (Bhatia *et al*., 2011).

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