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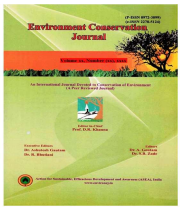

Rapid *in-vitro* micropropagation of Bamboo *(Dendrocalamus strictus)* and its genetic fidelity testing using ISSR markers

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ABSTRACT

Bamboo is a versatile, arborescent, perennial and non-wood forest tree with tremendous commercial importance. For mass scale propagation of bamboo, the micropropagation is an effective way for producing elite, infection free and true-to-type planting material. Here, the nodal explants of *Dendrocalamus strictus* used to develop an effective protocol for micropropagation based on tissue culture technique. In this study, the sterilization treatment of 70% ethanol + Tween 20 + Bavistin + Hgcl2 + PPM was successfully controlled the contamination up to 90 % as compared to other treatments. The shoots were initiated from nodal segments in MS medium supplemented with BAP (4 mg/l) and PPM (500µl/l). Shoot multiplication was found best with BAP (4 mg/l) and kinetin (2 mg/l) by using liquid MS medium. Whereas, rooting in solid MS medium has shown good results when supplemented with NAA (4mg/l). Healthy and disease-free plants were obtained after hardening under greenhouse conditions. Genetic fidelity testing by using ISSR markers reported that there was no variation in plantlets developed through micropropagation.

Introduction

The bamboo (*Dendrocalamus strictus*) is also known as green gold which is an evergreen, perennial, giant and woody plant. Bamboo is an important member of grass family named 'Poaceae'. It is a sustainable, fast-growing and multipurpose plant because its uses in industries, agriculture, medicines, etc. It is a dark green, lightweight plant and also grows very fast with quick maturity (Mohamad and Mohamad, 1994). In the Indian forest area, bamboos occupy about 15.69 million hectares of the country (Tewari *et al.,* 2019). It is majorly found in Madhya Pradesh, Uttar Pradesh, Orissa and Western Ghats (Limaye *et al.,* 1952). Ministry of Agriculture, Government of India, has launched the National Bamboo Mission in 2006 to increase the bamboo plantation areas. In case of Chhattisgarh state, it had more than 59 lakh (59,72,200) hectares of recorded forest area. Of this, 11 lakh (11,06,000) hectares is bamboo-bearing area within the state's forest area (FCCD, 2019). The bamboo culm has 18.5 m height along with12.7 cm thickness and the average length between the internode is 25 to 45 cm (Goyal *et al.,* 2010). The bamboo shoots are rich in proteins, amino acids, saccharides and minerals but low in fat. These shoots are used as vegetables, making pickles, soup, vinegar and salad. It can be propagated by culms cutting or rhizome and seeds. It flowers once in its life and doesn't set fruit properly leading to little seed setting while seeds are highly sterile and infected, hence difficult to propagate a large number of true to type plantlets.

Bamboo plant (*D. strictus)* required the 30-50 years of flowering cycle hence the regeneration by

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using seeds is very problematic and there are several other factors that restrict large-scale multiplication of this species through vegetative or sexual means (Saxena and Bhojwani, 1993). To overcome these problems and fulfill the required demand it has necessitated restoring the productivity of plants by the use of plant tissue culture (Bhattacharjee, 2006), as *in vitro* micropropagation includes the rapid vegetative multiplication of valuable plant material for agriculture and forestry. Thus, tissue culture provides a dependable and effective mass manufacturing option to fulfil worlds demand. Several researchers have been attempted tissue culture technique by using several species of bamboo (Mehta *et al*., 2011; Bejoy *et al*., 2012; Devi *et al*., 2012; Pandey and Singh, 2012).

The major goal of the micropropagation is to develop true to type plants in order to preserve the original characteristics of parent, however there is a potential of somaclonal alterations during the *in-vitro* process. As a result, the clonal fidelity of plantlets created during micropropagation must be determined. However, there are just a few papers on bamboo that have used DNA markers to evaluate the clonal fidelity of micropropagated plantlets (Mehta *et al*., 2011; Singh *et al*., 2013; Nadha *et al*., 2011). Hence, the goal of the current study was to develop an effective method for development of genetically uniform plantlets as parent and also applied ISSR marker system to determine whether genetically uniform plants were produced or not.

Material and Methods

Micropropagation

In-vitro micropropagation of bamboo was carried out at R.H. Richharia Research Laboratory, Department of Plant Molecular Biology and Biotechnology, IGKV, Raipur (Chhattisgarh). The explants were collected from the forest nursery of the Forest Department, Government of Chhattisgarh, Raipur. The basal sheath covering of bamboo explant was removed and wiped with 70% ethanol in laboratory conditions and cut into single-node segments in the size of 3-4 cm. After that, all nodal explants were taken in bottles and washed under running tap water to remove the dust particles. For surface sterilization, eight different treatments were used (table 1) (Liu *et al.,* 2011; Hu *et al*., 2011). After the sterilization procedure, the explants were inoculated in MS medium containing Sucrose (3%), Myo-inositol (0.1%), agar (0.8%) and PPM (500 µl/l) along with eight different concentrations of hormones for culture initiation (table 3) (Zamora, 1994; Yasodha *et al.,* 1997). Stock solutions of different hormones and medium were prepared and pH was adjusted to 5.8. The culture initiation was done in a growth chamber with 28± 2°C along with 1000 lux light and 75% humidity under completely sterile conditions. The shoots were separated by dissecting them after 15-20 days of culture initiation. Multiplication of shoots was done in liquid as well as solid medium by using combinations of 12 different treatments of hormone (table 5) to get the maximum number of shoots per explants (Bejoy *et al*., 2012; Devi *et al*., 2012). Rooting was done with MS medium containing different concentrations of hormone in solid and liquid medium (table 8) (Kumaria *et al*., 2012; Goyal *et al*., 2015). Plants produced in culture medium were taken out and washed thoroughly with distilled water and treated with bavistin solution (1%) for 10 minutes followed by hardening with sand, soil and cocopeat (1:1:1 ratio) for 1 month inside the greenhouse (28±2°C). The experiments were performed in replicates and carried out at separate times. All data on sterilization, culture initiation and shoot multiplication were analysed using completely randomized design (CRD) design where the statistical analysis was performed in IBM SPSS statistical software v.19.0.

Fidelity test by using PCR

In the present study, a total of nine ISSR markers (table 9) were used for assessment of genetic fidelity of twelve plantlets along with parent. DNA extraction was carried out by CTAB method (Doyle, 1990) and quantification was done using nanodrop spectrophotometer (ND-1000), whereas quality test of genomic DNA was done on 1 % agarose gel. DNA amplification was performed in thermal cycler (verity 96 well Thermal cycler) for amplification for initial DNA denaturation at 98ºC for 10s followed by 35 cycle of 10s denaturation at 98ºC, 30s annealing at 52ºC, extension at 72ºC for 1 minute and final extension at 72ºC for 5 minutes. The amplified product was resolved by electrophoresis on 2% agarose gel in Tris-borate EDTA (TBE) buffer stained with ethidium

bromide. Electrophoresis was carried out at 70(mV) for 40 minutes and gel was visualised and photographed using a gel documentation system (ChemicDoc TMmp Imaging system Bio-RAD). The fragment size was estimated through 100 bp ladders.

Results and Discussion

Micropropagation of *Dendrocalamus strictus*

In-vitro propagation has been a reliable and routine approach for large-scale rapid plant multiplication, which is based on plant cell, tissue and organ culture on defined tissue culture medium under aseptic conditions. Nodal explant of *D. strictus* with medium thickness (0.5-0.7cm) had given better initiation in culture medium as compared to very thick (>0.7cm) and thin (<0.5cm) segments. The contamination rate is often high because of different kinds of contaminants on explants at field conditions. To reduce the incidence of fungal and bacterial contamination, various sterilization treatments were used during the experiment (table 1). Recently several researchers successfully disinfected bamboo explants using mercuric chloride and 70 percent ethanol (Arshad *et al*., 2005; Hu *et al*., 2011). On the basis of analysis of variance a wide range of variation was present for the treatments used for sterilization (table 2). Out of the eight treatments tested, T8 (cotton swab 70% ethanol + Tween 20 (20 min) + Bavistin (30min) + Hgcl2 (5min) + PPM (500 µl/l) was successfully controlled the contamination up to 90 % as compared to other treatments. Aside from these, numerous chemicals have been utilised by different tissue culture scientists to remove contaminations in a variety of bamboo species from various genera (Bejoy *et al*., 2012; Devi *et al*., 2012). In this experiment for sterilization, we have applied combination ethanol, Tween 20, Bavistin, Hgcl2 and PPM that showed better results than earlier researchers (Liu *et al.,* 2011; Hu *et al*., 2011).

For *in vitro* shoot initiation MS medium was used. The use of MS basal medium for micro propagation of *D. strictus* has been reported in earlier reports (Reddy, 2006; Pandey and Singh, 2012). According to the previous studies, for micropropagation of bamboo the suitable medium was MS medium which give best response for regeneration (Bejoy *et al*., 2012; Pandey and Singh, 2012; Devi *et al*., 2012). Thus, all the following study on *D. strictus* was undertaken in MS medium with constant additives (ascorbic acid 50 mg/l + citric acid 25

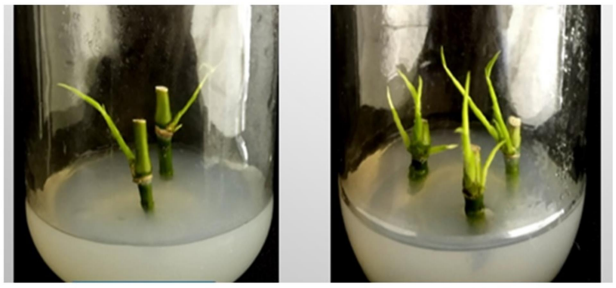

Figure 1: Culture initiation at different concentration BAP

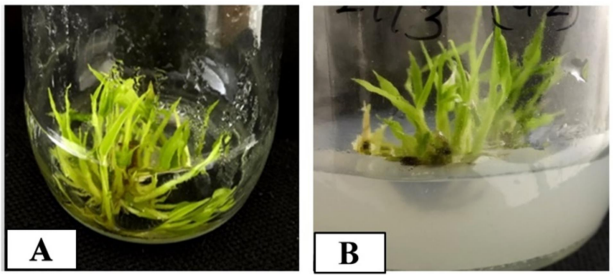

Figure 2: Shoot multiplication with BAP+ kinetin in MS medium (A - Shooting in liquid culture, B - Shooting in solid culture)

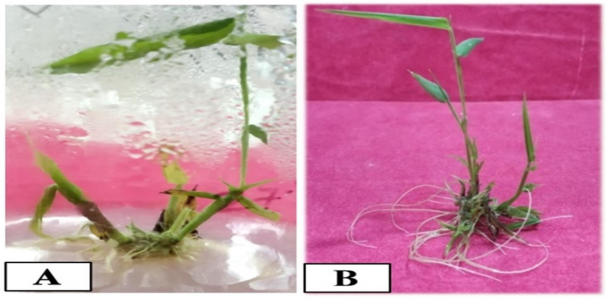

Figure 3: Rooting response of tissue culture raised plants medium (A - Rooting in solid culture, B - Rooting in liquid culture)

Figure 4: Hardening of tissue culture raised plants

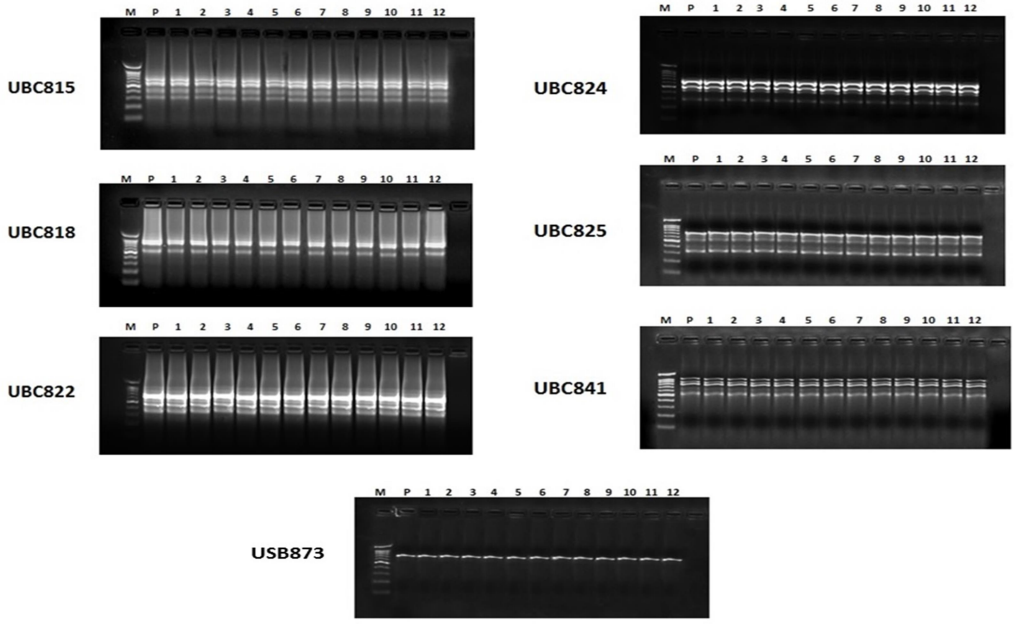

Figure 5: Amplifications produced by using ISSR marker UBC 815, UBC 818, UBC 822, UBC824, UBC825, UBC841 and UBC873. Lane M represent ladder, lane P is the mother plant and lane 1-12 represent the tissue culture (TC) – raised plant

Table 1: Nodal explant response with different sterilization treatment

Treatments	Treatment for sterilization of explant	Fresh shoot outcome in 6 Replication (5 explant for each replication)						Mean ± SE	Contamination %
		R1	R2	R3	R4	R5	R6		
1	Tween20 (20 min) + Bavistin (10min) + $Hgcl_2$ (5 min)	1	1	0	2	1	2	1.17	76.66667
2	Tween20 (10 min) +Bavistin (30min)+ $Hgcl_2$ (5min)	0	1	1	0	2	2	1	80
3	cotton swab 70% ethanol + $Hgcl_2$ (5min)	1	0	0	1	1	0	0.5	90
4	cotton swab 70% ethanol + Tween20 (2hour) + Bavistin 0.5%(30min) + gentamycin(15 min)	2	1	1	0	2	1	1.17	76.66667
5	cotton swab 70% ethanol + Tween20 (15 min) + $Hgcl_2$ (5min)	2	2	1	1	2	2	1.67	66.66667
6	cotton swab 70% ethanol + Tween20 (20 min) + Bavistin (30min) + $Hgcl_2$ (5min)	3	2	3	2	2	3	2.5	50
7	cotton swab 70% ethanol + Tween20 (20 min) + Bavistin (30min) + gentamycin(15 min) + $Hgcl_2$ (5min)	1	2	0	2	1	2	1.34	73.33
8	cotton swab 70% ethanol + Tween20 (20 min) + Bavistin (30min) + $Hgcl_2$ (5min) + PPM in culture medium	5	5	3	4	5	4	4.34	13.33

*** $Hgcl_2$- Mercuric chloride, PPM- Plant preservative mixture**

mg/l + cysteine 25 mg/l + glutamine 100 mg/l). The nodal explant of *D. strictus* was used for shoot initiation where within 4-5 days culture was initiated (figure 1). For micropropagation most of the workers have used nodal segments from seedlings (Ansari *et al.*, 1996; Maity and Ghosh, 1997). Seedling explant was also used for multiple shoot induction in *Thamnocalamus spathiflorus* (Zamora, 1994) and *B. nutans* (Yasodha *et al.,* 1997). The response of nodal shoot segments to the eight concentrations of hormones is given in Table 3. The treatment I5 (BAP-4 mg/l) showed best response for shoot initiation (90%), whereas the lowest response was observed in treatment number I1 (20%). These findings are in accordance with the earlier work on *in vitro* propagation of different bamboo species like *B. glaucescens* (Shirin and Rana, 2007), *B. balcooa* (Mudoi and Borthakur, 2009), and *D. hamiltonii* (Arya *et al*., 2012) where BAP has been widely used and was found to be effective. The results of analysis of variance (ANOVA) for shoot initiation treatments shown significant difference (table 4). Initiated shoots were multiplied on MS solid and liquid MS medium fortified with additives (ascorbic acid 50mg/l, citric acid 25mg/l, cysteine 25mg/l, and glutamine 100mg/l) in order to strengthen the shoots to withstand stress conditions (figure 2).

For shoot multiplication twelve different treatments was used in solid MS medium supplemented 6-Benzyl amino purine (BAP) and kinetin, among these the treatment M10 (BAP - 4 mg/l + kinetin - 2mg/l) was found best with average of 6.2 shoots/explant and the minimum shoot multiplication was found in treatments M1 and M5 with 2.6 shoots/explant (table 5). In case of shoot multiplication the analysis of variance showed a significant difference among treatments (table 6). The effect of growth hormones on shoots multiplication of *D. strictus* has been studied by different scientists. Induction of shoot proliferation in several bamboos *viz.* in *B. arundinacea* (Nadguada *et al.,* 1990) and in *D. asper* (Banerjee *et al.,* 2011) using cytokinins, especially 6-benzylaminopurine (BAP) either singly or in combination with auxin and complex additives has been reported. Further, M10 treatment (BAP - 4 mg/l + kinetin - 2mg/l) also performed for shoot multiplication in MS liquid medium (without agar) and it has been showed better result *i.e.* average of 11.2 shoots /explant (table 7 and figure 2). Therefore it has been proved that the liquid MS medium without agar was best than solid MS medium with same hormone composition. For shoot multiplication, different concentration of BAP was also used for earlier research work in different bamboo species (Arya and Arya, 1997; Arya *et al.,* 2012; Mudoi and Borthakur, 2009) where BAP was effective.

Among the thirteen different treatment of root initiation with solid MS medium, the treatment R10 (4 mg/l NAA) had good rooting response (7.2 roots/explant) while lowest root response was found in treatment R1 (0.2 roots/explant) (table 8). During the experiment MS liquid medium (without agar) was also applied for root induction. Where, among the four treatments of MS liquid medium R16 was given a better response with average of 6.6 roots/explant (table 8, figure 3). Hence, it has been indicate that the solid MS medium with agar was best than the liquid MS medium in case of root induction. In last decade the similar result was also obtained by researchers (Kumaria *et al*., 2012; Goyal *et al*., 2015). For Primary hardening, well-rooted shoots were thoroughly washed under running tap water. Then transferred into plastic cups containing the mixture of sand, soil and cocopeat with the ratio of 1:1:1 and maintained in greenhouse (figure 4). Secondary hardening was performed in polythene bag which were filled with soil, cocopeat and FYM manure in the ratio of 1:1:1 (Goyal *et al*., 2015). In our experiment, we have applied combination of solid and liquid culture along with same concentrations of BAP and Kinetin where it has been found that the liquid MS medium without agar was best than solid MS medium for shoot multiplication. In case of root induction the solid MS medium with agar was best than the liquid MS medium with same concentration of NAA. This finding was superior than earlier reports where researchers used solid media for shoot multiplication and liquid media for root initiation (Nadguada *et al.,* 1990; Singh *et al.,* 2000; Mudoi and Borthakur, 2009; Arya *et al.,* 2012).

Fidelity assessment by using ISSR markers

Genetic uniformity studies are important in micropropagation of plants in order to identify true to type individuals. Uniform genetic fidelity within

Table 2: ANOVA for sterilization treatment

Source of Variation	Sum of Squares	Degree of freedom	Mean Sum of Squares	F calculated	F tabulated
Treatment	7	61.25	8.75	16.93548*	2.249024
Error	40	20.66667	0.516667	-	-
Total	47	81.91667	-	-	-

*** = Significant at 1% level.**

Table 3: Treatments for Culture initiation

Treatment	MS Medium with diff. conc. of BAP(mg/l)	Culture initiation (10 replication and one explant per replication)										Mean	Initiation %
		R1	R2	R3	R4	R5	R6	R7	R8	R9	R10		
I1	(control)	0	0	0	0	1	0	0	0	1	0	0.2	20
I2	1BAP	1	1	1	0	1	0	0	1	1	1	0.7	70
I3	2BAP	1	1	0	1	1	1	1	1	0	1	0.8	80
I4	3BAP	1	1	1	1	0	1	1	0	1	1	0.8	80
I5	4BAP	1	1	1	1	1	1	0	1	1	1	0.9	90
I6	5BAP	0	1	1	1	1	0	1	1	1	1	0.8	80
I7	6BAP	1	1	0	1	1	1	1	1	0	1	0.8	80
I8	7BAP	1	1	1	0	1	1	1	1	1	0	0.8	80

*** 0- Not response of the nodal explant, 1- Response of the nodal explant, BAP- 6-Benzyl amino purine.**

Table 4: ANOVA for Culture initiation

Source of Variation	Sum of Squares	Degree of freedom	Mean Sum of Squares	F calculated	F tabulated
Between Groups	3.35	7	0.48	2.734694*	2.139656
Within Groups	12.6	72	0.17	-	-
Total	15.95	79	-	-	-

*** = Significant at 1% level.**

Table 5: Effect of kinetin on shoot multiplication with BAP on MS medium

Treatments	BAP mg/l	Kinetin mg/l	Shoot no. per explant					Mean
			R1	R2	R3	R4	R5	
M1	1	0	3	2	3	2	3	2.6
M2	2	0	3	4	2	3	4	3.2
M3	3	0	4	3	3	4	4	3.6
M4	4	0	4	5	3	5	4	4.2
M5	0	1	3	2	2	3	3	2.6
M6	0	2	4	3	3	5	2	3.4
M7	0	3	4	5	3	4	3	3.8
M8	0	4	3	4	4	6	4	4.2
M9	4	1	4	2	3	3	2	2.8
M10	4	2	7	5	6	7	6	6.2
M11	4	3	4	5	5	6	5	5
M12	4	4	5	4	4	5	4	4.4

Table 6: ANOVA for shoot multiplication

Source of Variation	Sum of Squares	Degree of freedom	Mean Sum of Squares	F calculated	F tabulated
Between Groups	61.53333	11	5.593939	8.717828*	1.99458
Within Groups	30.8	48	0.641667	-	-
Total	92.33333	59	-	-	-

*** = Significant at 1% level**

Table 7: Difference between Solid and liquid culture for shoot multiplication

Growth regulator		Solid culture	Liquid culture
BAP mg/l	Kn mg/l	Mean	Mean
4	2	6.2	11.2
	3	5	10.6
	4	4.4	10

Table 8: Rooting response on different concentration IBA and NAA

Treatments	Rooting Hormone mg/l IBA	Rooting Hormone mg/l NAA	No. of primary roots per explant	Root length in cm
Solid culture				
R1	0	0	0.2	0.2
R2	1	-	0.6	0.8
R3	2	-	2	0.5
R4	3	-	2.4	1.3
R5	4	-	2.6	0.3
R6	5	-	2.6	0.3
R7	-	1	1.8	0.7
R8	-	2	3.4	0.3
R9	-	3	5.6	1.3
R10	-	4	7.2	0.7
R11	-	5	7	0.5
R12	4	4	2.4	0.3
R13	4	5	3	0.5
Liquid culture				
R14	3	3	4.8	2.06
R15	-	3	3.6	4.16
R16	-	4	6.6	2.98
R17	-	5	5.8	3.54

***IBA-Indole-3-butyric acid, NAA- Nephthalene acetic acid**

Table 9: ISSR markers for fidelity testing.

Primers	5'-3' motif	Tm ºC	Scorable Bands	Monomorphic Bands	Polymorphic Bands	Range of Amplification (bp)
UBC815	(CT)8G	45	4	4	0	310-715
UBC818	(CA)8G	52	2	2	0	532-850
UBC822	(TC)8A	46	3	3	0	352-680
UBC824	(TC)8G	49	4	4	0	295-690
UBC825	(AC)8T	49	5	5	0	320-715
UBC841	(GA)8YC	46	5	5	0	545-900
UBC873	(GACA)4	45	1	1	0	720
UBC810	(GA)8T	49	-	-	-	-
UCB856	(AC)8YA	50	-	-	-	-

the tissue culture derived genotypes is essential for the successful selection of superior identical individuals. Although it is assumed that the *in-vitro* produced plants would be genetically identical to the mother plant, the chance of certain genetic differences cannot be ruled out. Somaclonal variation, which includes a variety of genetic and epigenetic variants, is assumed to be a typical occurrence in *in-vitro* produced plants (Goyal *et al*., 2015). Using ISSR markers, an attempt was undertaken to screen *in-vitro* produced *D. strictus* plantlets for somaclonal variation. Out of 100 plants, 12 random tissue culture plants were taken to check the fidelity. Selected plants were subjected to ISSR molecular marker analysis where seven markers produced scorable bands shown in table 9. A total of seven ISSR markers amplified its products with the ranges 295 to 900 bp as shown in Figure 5. The optimum annealing temperature for ISSR markers ranges from 45 to 52 ºC. For ISSR analysis, UBC 825 and UBC 841 gave the maximum number of bands in the range of 320 to 900 bp. We found that all banding profiles from the micropropagated plants were monomorphic with each primer and similar to those of the mother plant. Therefore, assessment of genetic uniformity of regenerants was developed with no somaclonal variations. Some researchers also reported the clonal fidelity testing among the tissue culture derived plantlets of bamboo using DNA markers to assess the genetic uniformity (Das and Pal, 2005; Goyal *et al*., 2010). In the last few decades, the researcher successfully assessed the clonal fidelity of *in-vitro* raised plants of *D.hamiltonii* using RAPD markers (Godbole *et al.,* 2002). However, there are several reports available on the comparative genetic stability of regenerants and mother plant of bamboo using the ISSR marker system. In present study we found that there was no polymorphisms between *in-vitro* raised plants and parent based on ISSR marker system indicating the true to type nature which is essential for micropropagation.

Conclusion

This research led to the development of a standard *in-vitro* methodology for *D. strictus* regeneration**.** ISSR studies were performed on twelve plants chosen at random. Amplicon was created using a set of seven primers. The frequency range of amplified products is 320 bp to 950 bp. All seven primers showed a similar banding pattern to the mother plant. As a result, clonal fidelity was achieved without any epigenetic or genetic variation. Therefore it can be concluded that the established protocol for micropropagation can be well utilized for large scale of production of bamboo without any variation.

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