Clonal fidelity assessment of *in vitro* propagated *Bambusa balcooa* plant using DNA marker

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Abstract
Efficient in vitro micropropagation protocol of Bambusa balcooa was established. Nodal segment was used as explant for propagation. Out of the various sterilization treatments tested, S7 treatment supplemented with PPM in culture medium shown minimum contamination percentage i.e.13.33%. Culture initiation in solid Murashige and Skoog (MS) media supplemented with BAP (1,1.5,2,3,4,5,6 mg/l).4 BAP was best for culture initiation with 90% response. Cultures were multiplied using MS medium supplemented with different hormones. M7 treatment containing of Kn (2mg/l) and BAP (4 mg/l) gave best response with ± 6.6 ± 0.24 multiple shoots per explants. Root induction was done in MS medium supplemented with NAA (5 mg/l) has induced 6.0 roots per explant.

Rooted plantlets were successfully acclimatized with soil and coco-peat (1:1; v/v) under primary hardening condition in green house (1 month). After primary hardening, plants were transferred to the polybag for secondary hardening in sand, soil, cocopeat (1:1:1; v/v). Clonal fidelity test for these randomly selected micro propagated plants was done using 14 ISSR (Inter Simple Sequence Repeat) markers, out of which 8 markers were amplified within range of 320-950 bp bands. The result has shown complete uniformity among randomly selected micro propagated plants.

Keywords: Micro propagation, ISSR, Clonal fidelity.

Introduction

Bamboo plants belong to family Poaceae and subfamily Bambusoideae *Bambusa balcooa* Roxb originating from North East India. The flowering cycle of *Bambusa balcooa* is 55-60 years and plant dies after flowering. It reaches a height of 12-22m, diameter of 6-15cm and grows up to 600m altitude in various parts of India. It is tallest, strongest and highly durable and is utilized mostly for structural use and pulping. Micro propagation plays important role for large scale production of bamboo. India is one of the leading countries in the world in bamboo production.

The species is also valued for its edible tender shoots, mainly for food and pickle industry.[5] It is being used in construction, ladders, boats, rickshaw hood frames, to weave mats and baskets, pulp and paper, making handicraft, bio fuels/bio energy and also as bamboo chips. Additionally, tender shoots are edible as vegetable and pickles. Bamboo can form a closely woven mat of roots and rhizome underground, which are effective in holding soil. The soil around bamboo plants is permeated by a mass of intertwining roots.[1]

Young shoots are edible and bitter in taste. Phytosterols present in the fermented succulent shoots of *Bambusa balcooa* are the precursors of many pharmaceutically active steroids.[7] Under Chhattisgarh Bamboo Mission to increase the area and production of bamboo an effective *in vitro* technique is required. So the present study has been undertaken to establish efficient protocol of *Bambusa balcooa* propagation through tissue culture.

Material and Methods

Collection of explants: Healthy nodal segments (1.5-2.0 cm in length) of *Bambusa balcooa* were collected from 3-4 years old and disease-free plant from well-developed forestry field of Godhi Raipur, Chhattisgarh (Fig. 1 A and B). Leaf sheath tissues and dead parts of the upper internodes were removed through scalpel.

Surface sterilization of ex-plant: To sterilize explants, different treatments (S1-S7) were used with different concentration given in table 1. Explants were wiped with 70% ethanol and then washed in 5% Tween 20 solution and treated with bavistin. Rinse the explant with autoclave water. Ex-plant were treated with 0.1% mercuric chloride under laminar air flow and washed (4-5 times) with sterile distilled water. After S7 treatment MS medium supplemented with PPM to reduce the incidence of fungal and bacterial contamination.

Culture Initiation: Culture Initiation was carried out in Murashige and Skoog (MS) solid media supplemented with different hormones concentration from I1-I8 treatment mentioned in table 2. All cultures were kept at 26±2°C under 16 hour's photoperiod and aseptic condition for 2-3 weeks (Fig. 1C and D).

Shoot multiplication: For shooting media, different treatment were used in solid as well as liquid MS medium with concentration of cytokinin (BAP and Kn) as given in table 3. Newly developed axillary shoots containing 2-3 shoot propagules were sub-cultured at regular intervals of 3-4 weeks in fresh multiplication media for induction of multiple shoots for 7-8 multiplication cycles (Fig. 1 E and F).

Rooting: Developed shoots were cultured in solid as well liquid MS basal medium which is supplemented with different auxin concentration. To develop root, different

treatments (R1-R17) were used as given in table 4. Well shooted propagules were transferred to rooting media.

Hardening: Rooted plantlets were removed from culture bottles, washed thoroughly under running water to remove traces of medium from roots. Plantlets were transferred to pots containing sterile soil /coco-peat (1:1; v/v) for primary hardening in green house (25-27°C) for one month. Four weeks after primary hardening plantlet were shifted to polythene packets each filled with sand, soil, cocopeat (1:1:1; v/v) for their secondary hardening in net house. (Fig. 1, I). The plantlets were acclimatized in green house maintaining temperature for next four weeks.[6]

Table 1
Treatments for sterilization

S.N.	Treatment no.	Contamination %
S1	Cotton swab (70% ethanol), Tween20 (15 min), Bavistin 0.5%(30min) + $Hgcl_2$ (5min	37.5%
S2	Cotton swab (70% ethanol) +Tween20 (15 min) +Bavistin1% (30min) + $Hgcl_2$ (5min)	**25%**
S3	Cotton swab (70% ethanol) +Tween20(15min) + $Hgcl_2$ (5min) +Bavistin1%(30min)	62.5
S4	Cotton swab (70% ethanol) + Tween20 (15min) + Bavistin1% (30min), $Hgcl_2$ (10min)	100
S5	Cotton swab (70% ethanol) + Tween20 (15 min) + Bavistin (0.5%) (30min) + $Hgcl_2$ (5min) +gentamycin (15 min)	75
S6	Cotton swab (70% ethanol+ Tween20 (10 min) + Bavistin (1%) (30min) + $Hgcl_2$ (5min) +gentamycin(5min)	62.5
S7	Cotton swab (70% ethanol + Tween20 (20 min) + Bavistin 1% (30min) + $Hgcl_2$ (5min) + PPM in culture media	13.33

Table 2
Culture Initiation supplemented with BAP

Treatment	Media	No. of inoculated bottle	Response %
I1	MS	10	30
I2	1BAP	10	30
I3	1.5BAP	10	50
I4	2BAP	10	50
I5	3BAP	10	70
I6	**4BAP**	**10**	90
I7	5BAP	10	80
I8	6BAP	10	80

Table 3
Shoot Multiplication media

Treatment	Growth Regulator		Multiple shoots per explants
In solid medium			
	BAP	Kn	Mean ±SE
M1	1	0	2.2±0.2
M2	2	0	2.8±0.37
M3	3	0	3.4±0.24
M4	4	0	4.6±0.24
M5	5	0	3.0±0.31
M6	4	1	2.6±0.4
M7	**4**	**2**	**6.6±0.24**
M8	4	3	4.0±0.31
In liquid medium			
M9	4	0	9±0.31
M10	4	1	10.8±0.37
M11	**4**	**2**	**13.4±0.24**
M12	4	3	10±0.31

Table 4
Root initiation media

Treatment	Growth regulator			
In solid medium				
	IBA	**NAA**	**No. of primary roots per explant (ag)**	**Root length in cm.**
R1	0	0	0	0.0
R2	1	-	1.0	0.6
R3	2	-	1.2	0.6
R4	3	-	1.8	1.7
R5	4	-	3.0	1.9
R6	5	-	2.8	1.2
R7	-	1	1.2	0.7
R8	-	2	1.4	0.3
R9	-	3	2.0	1.5
R10	**-**	**4**	**5.6**	**3.5**
R11	**-**	**5**	**6.0**	**3.6**
R12	4	4	3.6	3.0
R13	4	5	3.8	3.1
In liquid medium				
R14	3	3	4.0	2.9
R15	-	3	3.8	3.0
R16	**-**	**4**	**5.2**	**3.5**
R17	-	5	4.2	3.2

Table 5
The Inter Simple Sequence Repeats (ISSR) markers for fidelity testing

Primers	5'-3' motif	Tm ºC	Scorable Bands	Monomorphic Bands	Polymorphic Bands	Range of Amplification (bp)
UBC857	(AC)8YG	54	2	2	0	520,700
UBC812	(GA)8A	45	NA			
UBC848	(CA)8RG	51	2	2	0	550,800
UBC813	(CT)8T	45	NA			
UBC850	(GT)8YC	54	4	4	0	530-800
UBC842	(GA)8YG	47	NA			
UBC860	(TG)8RA	45	NA			
UBC830	(TG)8G	53	1	1	0	600
UBC840	(GA)8YT	53	2	2	0	810,950
UBC843	(CT)8RG	50	1	1	0	600
UBC814	(CT)8A	45	NA			
UBC852	(TC)8RA	50	2	2	0	500,700
UBC836	(AG)8YA	45	NA			
UBC841	(GA)8YC	54	2	2	0	320,720

Fidelity testing: To test the clonal fidelity among the tissue culture raised plant, DNA of mother plant and 10 randomly selected tissue culture raised plant was extracted from young leaves by using CTAB (Cetyl Trimethyl Bromide Method).[2] ISSR primers were used in the study to confirm the clonal fidelity. The total fourteen ISSR markers were screened. PCR (Applied Biosystem) amplification was carried out by EmeraldAmp GT PCR Master Mix. The master mix includes an optimized buffer, PCR enzyme, dNTP mixture, gel loading dyes(green) and a density reagent in primers and DNA template. PCR was performed in a volume of 10 ul PCR reaction containing Master mix 5 ul, H_2O (3ul), 50 (ng) of genomic DNA.

DNA amplification was performed in PCR (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 ™ mp Imaging system Bio-RAD). The fragment size was estimated through 100 bp ladders.

Results and Discussion

Explants collected from 3-4 year old *Bambusa balcooa* plant were surface sterilize. Different concentration of chemical were used for sterilization process. The sterilization treatment S7 (cotton swab (70% ethanol + Tween20 for 20 min + Bavistin 1% for 30min + $Hgcl_2$ for 5 minutes + PPM in culture media) has given better response with minimum contamination 13.33% (Table 1).

The sterilized explants was inoculated in MS basal medium which contain different concentration of BAP. It was observed that after 7 days of inoculation, the axillary bud break was noticed. Initiation treatment (I6) with BAP (4 mg/l) has given best response with 90% culture initiation (Table 2). Bud break percentage was found to be good in medium type of explant.[5] After 12-15 days of bud break, the elongated shoots were separated from nodes by using sterilized scalpel and transferred in the fresh solid (Fig. 1E) as well as liquid medium supplemented with different concentration of cytokinin (Fig. 1F). It was observed that in solid and liquid media (M7 and M11) treatment (4BAP+2Kn), maximum shooting response was obtained with an average 6.6±0.24 in solid and 13.04±0.3 liquid media (Table 3).

Sub culturing MS medium at lower concentration of BAP (0.5-1 mg/l) raised unhealthy growth of the shoots, leading to death of the cultures. All explants did not produce equal number of new shoots within the same period of time and same media. Delaying of sub-culturing period resulted in gradual browning of the shoots.[4] After 7-8 multiplication cycle of shoot culture, it was transferred to the rooting medium with different concentration of auxin (Table 4).

The rooting treatment (R_{11}) in solid medium consists of NAA (5 mg/l) which gave highest root length of 3.6 cm as well as highest number roots per explants with an average 6.0 root per explant (Fig. 1 G). However, in liquid medium R_{16} consists of NAA (4 mg/l) which gave an average 5.2 roots per explants with 3.5 cm root length. (Fig. 1 H) Higher concentration of IBA shows gradual deterioration of growth of the shoots and resulted in dryness of plans.

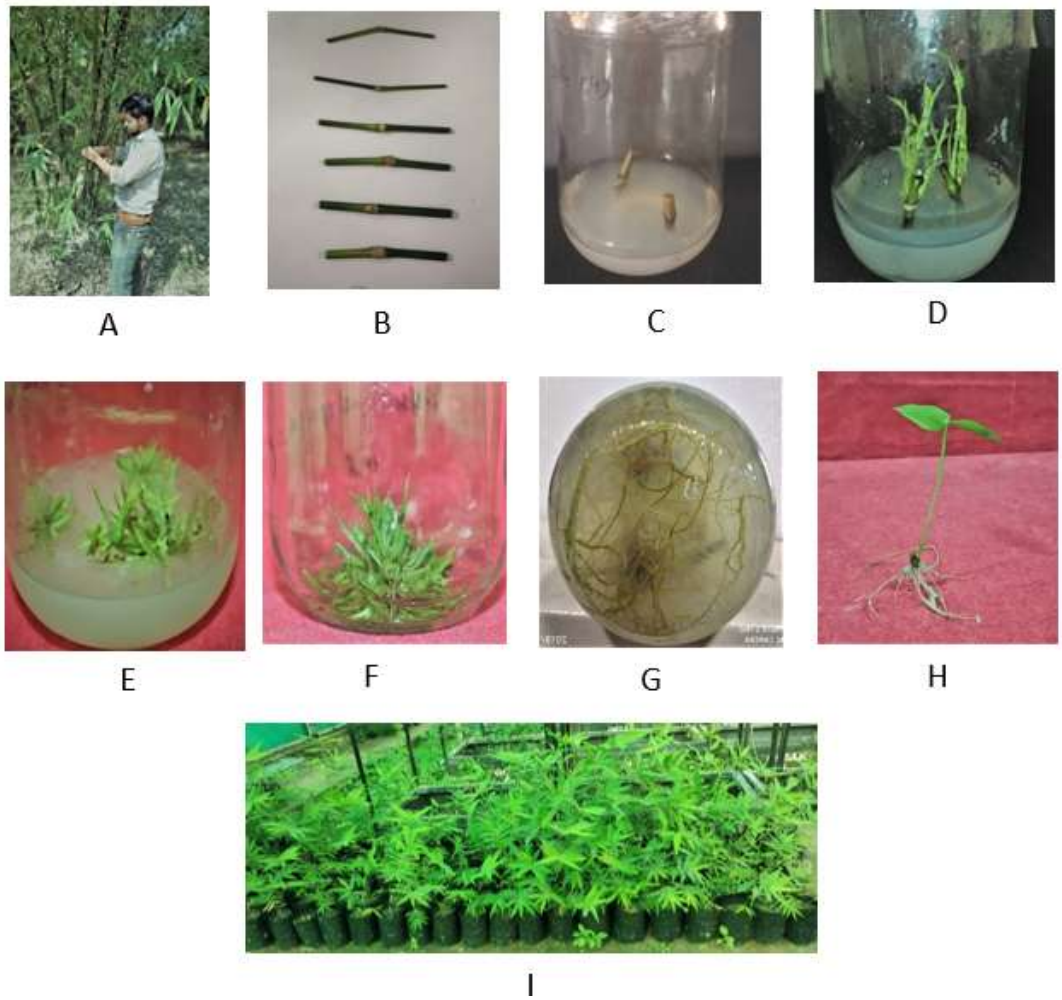

Fig. 1: Different stages of the *Bambusa balcooa* under in *vitro* propagation
A-mother plant, B- Explants (*Bambusa balcooa*), C-inoculation, D-shoot initiation, E-Multiplication (Solid), F- Multiplication (Liquid), G-Rooting (Solid), H- Rooting (Liquid), I-Hardening

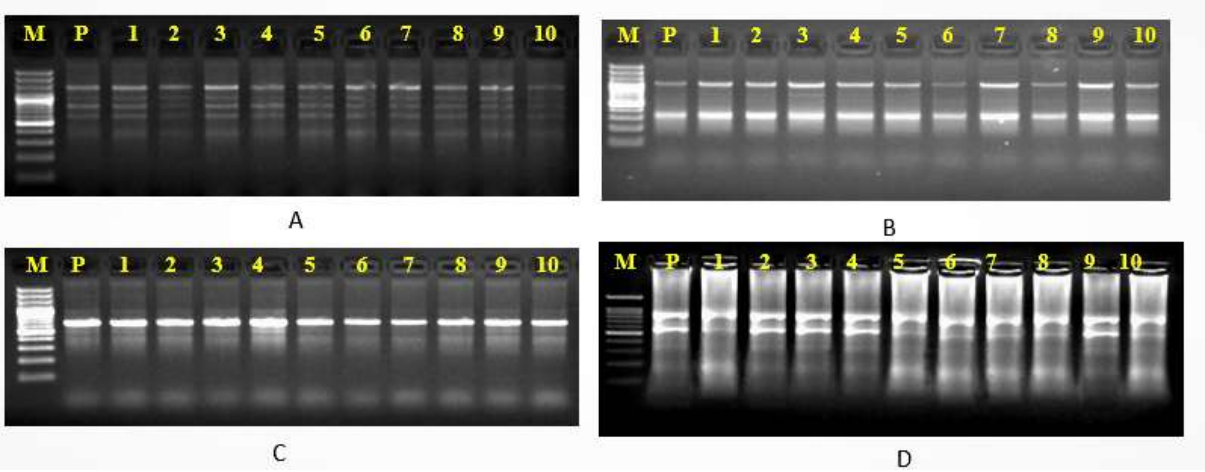

Fig. 2: Amplification with ISSR marker (A) UBC 850, (B) UBC 841, (C) UBC 843, (D) UBC 848 Lane M present 100 bp ladder, Lane 1-10 Tissue culture raised plant, Lane P represents the parent plant of *Bambusa balcooa*.

Clonal fidelity testing using ISSR molecular markers: The scarcity of reports on ascertaining the genetic fidelity of tissue culture raised plants can jeopardise the quality of micro propagated plants especially in perennial like bamboo where any undesirable variant would last for several years, therefore screening of the regenerates for the occurrence of any variation is required.

Conclusion

10 randomly selected plants were subjected to ISSR analyses. Out of fourteen markers screened, only 8 primers produced amplicon as shown in table 5. Amplified products is in the range of 320 bp to 950 bp (Fig. 2). Optimum temperature for ISSR falls between 50-54°C. For ISSR analysis UBC 850 gave the maximum amplified products in the range from 530 bp -800 bp. All the 8 primers has shown uniform pattern of banding as similar to mother plant. Hence clonal fidelity was established with no epigenetic and 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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References

1. Ben-Zhi Z., Mao-Yi F., Jin-Zhong X., Xiao Sheng Y. and Zheng-Cai L., Ecological functions of bamboo forest: research and application, *Journal of Forestry Research*, **16(2),** 143-147 **(2005)**

2. Negi D. and Saxena S., Micropropagtion of *Bambusa balcooa* Roxb. Through axillary shoot proliferation, *In vitro Celluar and Developmental Biology Plant*, **47(5),** 604-610 **(2011)**

3. Nirmala C. and Bisht M.S., Molecular markers in bamboo systematic and germplasm screening, *Plant Cell Biotechnology and Molecular Biology*, **13 (3/4)**, 73-82 **(2012)**

4. Patel B., Gami B., Patel N. and Bariya V., One step pre-hardening micro propagation of *Bambusa balcooa* Roxb., *Journal of Phytology*, **7**, 1-9 **(2015)**

5. Pratibha S. and Sarma K.P., In vitro propagation of Bambusa tulda: An important plant for better environment, *Journal of Environmental Research and Development*, **7**, 1216 1223 **(2013)**

6. Gantait S., Pramanik B.R. and Banerjee M., Optimization of planting materials for large scale plantation of Bambusa balcooa Roxb.: Influence of propagation methods, *Journal of the Saudi Society of Agricultural Sciences*, **17(1)**, 79-87 **(2018)**

7. Sarangthem K. and Singh T.N., Microbial bioconversion of metabolites from fermented succulent bamboo shoots into phytosterols, *Current Science*, 1544-1547 **(2003)**.

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