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Detection of Albino in Micropropagated Shoots of Bambusa Balcooa Roxb, Using PCR Based Techniques

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***Abstract*— The present work was aimed to detect the albino is micro-propagated shoots of Bambusa balcooa (B. Balcooa). PCR based RAPD and ISSR technique was used to predict the genetic level variation. In this study tissue culture of B. balcooa was carried out in MS medium. Three different CTAB extraction methods are used to isolate DNA. The isolated DNA amplified with 8 ISSR and 3 RAPD primers. MS medium with additives and BAP 5.0 mg/l showed the best shoot initiation of B. balcooa with shoot height of 2.4 cm with 2.5 shoots per explants and 91.6% response. Modified CTAB used to isolate the genomic DNA, Method 1 yielded 88.2 ng/µl with OD of 1.92 and Method 3 yielded 86.2 ng/µl with an OD of 1.87. Both the OD of 1.92 by Method 1 and 1.87 by Method 3 are sufficiently good for amplification of DNA using ISSR and RAPD primers. 7 ISSR primers and 3 RAPD primers showed amplification upon screening with genomic DNA of green shoots of B. balcooa. $Mgcl_2$ of 2.5 mM and 3.0 mM showed the best amplification among the different (1.0-4.0 mM) concentrations of $Mgcl_2$ tested. The present studies carried out to detect the genomic variations at DNA level using PCR technique showed monomorphic bands using 10 primers. The plants produced through auxiliary shoot proliferation showed genetically stable plants.**

Keywords*— Albino, Bambusa balcooa (B. balcooa), Inter Simple Sequence Repeat (ISSR), Plant tissue culture, Polymerase Chain Reaction (PCR) and Random Amplified Polymorphic DNA (RAPD).

I. INTRODUCTION

Bamboo is the vernacular or common term for members of a particular taxonomic group of large woody grasses (family Poaceae, subfamily Bambusoideae). Bamboos encompass 1250 species within 75 genera, which are relatively fast growing, attaining maturity within five years but flowering infrequently. Asia accounts for about 1000 species, covering an area of over 180,000 Km^2, China alone has about 300 species in 44 genera [23], followed by India with 130 species covering 96,000 km^2 or about 13% of the total Indian forest [24].India is one of the leading countries of the world, second only to China, in bamboo production with 32.3 million ton per year. Within India, North Eastern Hill (NEH) region possesses the largest species diversity.

Bambusa balcooa Roxb. (Poaceae: Bambusoideae) is a multi-purpose bamboo species native to the Indian subcontinent. It is a tall bamboo with dull, grayish-green culms measuring 12-23 m in height, 18-25 cm in diameter, 2-2.5 cm thick walled, 30-45 cm long internodes, leaf blade oblong lance late 25±4 cm, Inflorescence compound panicle, with 6-8 spikelet's on nodes. It is distributed in different parts of India up to an altitude of 600 m. It can withstand dry period, suitable for different soils and grows better on heavy clay soil, well drained, they can withstand upto -5°C [26].

B. balcooa is highly valued for its strong culms, regarded as one of the best species for scaffolding and building purposes [19]. Young shoots are edible and bitter in taste. Fermented succulent shoots of *B.balcooa* are rich source of phytosterol, a precursor of many pharmaceutically active steroids. Other uses include paper pulp, handicrafts, and products of the wood chip industry. Due to its various uses, this species has been identified as one of the priority bamboos by the National Bamboo Mission, Government of India [16].

Propagation of bamboo is practiced by the following three methods i.e., Seed propagation, Vegetative propagation and Tissue culture. Due to its long flowering nature and improper seed setting, propagation through seed is inadequate due to lack of availability of seeds. However, vegetative propagation such as culm cuttings, nodal cuttings and rhizomatous cuttings are practiced to some extent but due to their bulkiness and low rooting percentage vegetative propagation also possess disadvantages for large scale propagation. Hence, tissue culture mode of propagation is highly advantages for propagation of bamboo in large scale.

However, somaclonal variation can poses a serious problem in any micropropagation program, where it is important to produce true-to-type plant material [22].

An albino is a plant with white or cream colored leaves or stem which lack chlorophyll and thus lacks photosynthetic pigments and do not photosynthesize sugars.

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The terms albino is also used to indicate a white form of a normally colored flower. The spontaneous appearance of albino regenerates is a frequently occurring phenomenon in bamboo tissue culture [10] and [20]. These morphological variations could be due to high hormonal concentrations, long *in vitro* conditions and long subculturing passage time. These somaclonal variations might be either genetic or epigenetic in nature. Morphological variations can be detected by comparing the variant with the wild type, whereas for the genetic variations, various PCR based molecular marker techniques are utilized to detect the variations at genetic level.

"Polymerase Chain Reaction" is the most important, new scientific technology to come along in the last hundred years [12] (perhaps better known as the Human Genome Project.) has pointed out that, it is far simpler and less expensive than previous techniques for duplicating DNA, PCR has democratized genetic research, putting it within reach of all biologists, even those with no training in molecular biology.

RAPD markers are oligonucleotide fragments used for PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequences, which are able to differentiate between genetically distinct individuals. The RAPD primers are random 10mer nucleotide sequence, which can bind to any part of the genomic DNA during PCR amplification.

Another technique called Inter Simple Sequence Repeat (ISSR) reveal numerous microsatellite regions by using primers that may be anchored with one or two nucleotides on either the 5' or 3' end of a repeat region and extend into the flanking region. These methods are widely applicable because they are rapid, inexpensive and simple to perform, do not require prior knowledge of DNA sequence and require every little starting DNA template [6]. [30] studied two markers, the Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) [29] to explore the genetic diversity among 19 Iranian genotypes of *pistachiavera* and were compared for their relative efficiency.

Molecular markers are widely used to detect and characterize somaclonal variation at the DNA level [1], [3] and [7]. Among the available techniques, RAPD is the most useful ones [18], [21], [25], [27] and [28]. Changes in the RAPD pattern may result from the loss or gain of a primer annealing, caused by point mutations or by the insertion or deletion of sequences or transposition elements [17].

Also ISSR markers have proven to be a reliable, reproducible, easy to generate, inexpensive and versatile set of markers that relies on repeatable amplification of DNA sequences using single primers. This is apparent in studies conducted to screen somaclonal variations produced in tissue cultured derived plants such as in *Swertia chirayita* [9].

It has been observed that, regenerants of *B. balcooa* show a very low albino shoot production with variation less than 0.02%. These albino plants generated which lacks chlorophyll does not survive during rooting and hardening process.

To identify the variations is either just a morphological or genetic variation, we evaluate this study to detect the variations of albino in the tissue culture regenerated plants through PCR based RAPD and ISSR techniques.

II. MATERIALS AND METHODS

A. *Shoot Initiation*

Explant collection

The explants *(Bambusa balcooa)* were collected in the month of December 2012 from Genewin Biotech, Hosur. Explants (nodal cuttings) of 2-3 cm with new growth along with the sheath were collected with the help of secateurs.

MS media preparation for shoot initiation

One litre of MS liquid full strength medium [14] was prepared by weighing required quantity of macro and micro nutrients. From the above 1 litre MS medium 120 ml of the medium was taken separately and the pH of the medium was adjusted at 5.8 before autoclaving and labelled it as control.

Again 120 ml of the medium was taken from the remaining 880 ml of MS medium in a separate beaker and BAP at 1.0 mg/l concentration was added and pH of 5.8 was adjusted and poured into 12 test tubes with 10ml each and labelled as Treatment 1. Similarly, all the 6 treatments as given in the table 2 were prepared and labelled. Cotton plugs were applied to the test tubes and placed in test tube rack, packed with newspaper and autoclaved at 120°C at 15psi.

Table 1. Effect of BAP at different concentration for shoot initiation in *B. balcooa.*

T. No.	BAP(mg/l)
1	0.0
2	2.0
3	3.0
4	4.0
5	5.0
6	6.0
7	7.0

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Surface sterilization and inoculation

The nodal shoot segments (explant) of B. balcooa were collected and the explants of 2-3 mm in diameter, 2-3 cm length were made with the help of a secature. The sheath from the explant was removed carefully without damaging the buds. Care was taken to remove any infection in the explants. All the explants were washed with sterile distilled water to remove the dust particles on the surface of the explants in a 500 ml beaker. The above water was discarded and fresh sterile distilled water of 3/4th volume of the beaker is added.

Three to four full spatula scoops of bavistin and two full spatula scoops of streptocyclin was added shake well and keep it for 15 min with intermittent shaking. In the meanwhile, the LAF was cleaned, alcohol, spirit lamp, matchbox, sterile cardboards, sterile cotton cloths, Mercuric chloride and Tween 20,were kept ready for the processing. Mercuric chloride 1200 mg was weighed and dissolved in sterile distilled water in

minimum quantities and made up to 1 liter in sterile reagent bottle.

All the explants were transferred in to LAF for further processing. After thorough shaking, the explants were kept in the bavistin and streptocyclin mixed water, the water was discarded and the explants were washed with fresh sterile distilled water for five times. The explants were then transferred into sterile empty culture bottles and distributed into more bottles so as to lessen the explant number per bottle. To each of the bottle $3/4^{th}$ volume of sterile distilled water was added and around five drops of tween 20 and shake well for 5 min. The above water was discarded and rinsed with sterile distilled water for 5 times to remove all the froth.

70% ethanol was added for each of the bottles and shakes well and incubated it for 30-45 sec and immediately removed. The explants were washed with sterile distilled water three times. The above explants were treated with 0.1% mercuric chloride solution in each of the bottles and sealed with clean wrap tape and incubation for 5 min, with intermittent shaking. The mercuric chloride solution was discarded and the explants were rinsed with sterile distilled water three times. The LAF chamber was cleaned or wiped with 70% ethanol using sterile cotton cloths and all other glass ware used for sterilization were removed from the LAF. Forceps and forceps stand was taken out from the steripot and kept on the working LAF table for cooling it to room temperature.

Test tube rack was wiped with alcohol and kept inside and all the test tubes were also wiped with alcohol and kept in the LAF. Each explant was inoculated vertically into individual test tubes with the help of forceps and the cotton plugs were plugged properly. The inoculated test tubes were kept in dark for 24h followed by incubating in culture room with 16h of light at 25°C for 30 days. The data on percentage response, number of shoots per explant, shoot height was recorded after 30 days of incubation.

Shoot Multiplication

MS media preparation for shoot multiplication

Two litre of MS full strength medium with 3% agar was prepared. To this MS medium, BAP 2.0 mg/l was added as cytokinin source along with NAA 0.2 mg/l as auxin source. pH of 5.8 was adjusted and poured into culture bottles with 50 ml/bottle followed by capping and autoclaved at 120°C at 15psi.

***In vitro* shoot multiplication**

The above regenerated shoots from shoot initiation were inoculated vertically on the shoot multiplication medium for further multiplication of shoots. The culture bottles were kept at 25±2°C at 16h photoperiod for 3 weeks for shoot multiplication. After 3 weeks of period, the new shoots regenerated were further sub cultured in fresh shoot multiplication medium for increase in further shoot number.

Green shoots regenerated through auxiliary shoot proliferation were used for DNA extraction. Approximately 0.02% of the shoots showed albino growth only after 6 weeks of inoculation and these shoots were used for DNA extraction.

The *in vitro* regenerated shoots as explained above were used for DNA extraction for detection of genetic variation in albino shoots.

Chlorophyll Estimation

300 mg of fresh leaves were collected from 4 week old saplings. If the leaves have high water content, partially dehydrate them by soaking them in 95% ethanol for 2 to 5 min. The leaves were made into small pieces, placed in mortar and grinded with absolute alcohol using pestle.5 ml of 80% acetone was added into 15 ml Falcon tube and transferred the powder into the tube and mixed them in dark for 15 to 30 min (note: chlorophylls degrade under light).

The falcon tubes were centrifuged at 4°C for 15 min at 3,000 rpm and the supernatant was transfer to a new centrifuge tube and keep in dark. Again 5ml of acetone was added and it was repeated followed by transferring the supernatant to the centrifuge tube. It was repeated another two times and the supernatant was combined. Mix the tube thoroughly and measure the absorbance at 645 nm and 663 nm using spectrophotometry. The chlorophyll concentrations are calculated as follows (use 80% acetone as a blank control).

Ca (mg/g) = [12.7xA663-2.69xA645)xV/1000xW

Cb (mg/g) = [22.9xA645-4.86xA663)xV/1000xW

Ca+b (mg/g) = [8.02xA663 + 20.20 x A645)xV/1000xW (1)

B. DNA EXTRACTION AND PURIFICATION

Method 1- Extraction

One gram of leaf sample was taken and cut into small pieces using sterile scissors, ground well into powder by using absolute alcohol in mortar and pestle. 1% β mercaptoethanol was added to the extraction buffer and it was warmed at 65ºC for 15 min.10 to 20 ml warmed extraction buffer was added to the ground sample.

The sample was thoroughly mixed to from slurry and transferred to a screw capped 30 ml centrifuge. 100 to 200 mg of polyvinyl pyrollidone (PVP) was added. The tube were incubated at 65ºC for 30 min with occasional mixing and cooled to room temperature. Equal volume of chloroform: octanol (24:1) was added to slurry. The slurry was centrifuged at 6000 rpm for 5 min at 4ºC. The supernatant was transferred to the fresh tube and second extraction can be performed, if the aqueous phase was cloudy in appearance.

0.5 volumes of 5M NaCl was added and mixed well. Equal volume of ice cold absolute alcohol was added and refrigerated for 15 to 20 min. The sample was centrifuged at 3000 rpm for 3 min and then increased to 5000 rpm for additional 3 min at room temperature. This differential spinning will help to keep the DNA at the bottom of the centrifuge. The supernatant was poured off and the pellets were washed in 80% ethanol.

The ethanol was evaporated by leaving the tube at 37ºC for 10 to 15 min. The pellets were re-suspended in 0.5ml of 1X TE buffer.

RNAse TREATMENT

2 µl of RNAse stock solution was added to the nucleic acid mixture in the eppendorf tube and incubated at 55ºC for 10 min or 37ºC for 1h. Equal volume of (0.5ml) phenol: chloroform (24:1) was added and centrifuged at 10000 rpm for 5 min. The upper aqueous layer was collected, transferred into fresh tube and more than double volume of 100% ethanol and 50 µl of 3M sodium acetate was added. The tube is kept at -20ºC overnight for precipitate. The precipitated

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sample was centrifuged at 12,000 rpm for 15 min at 4ºC. The pellet was collected and washes with 0.5 ml of 70% ethanol.

The sample was centrifuged at 10,000 rpm for 5 min at 4ºC. The pellet was re-suspended in 100 µl of 1X TE buffer. The re-suspended pellets were collected and stored at -20ºC.

Method 2

30 ml of the CTAB buffer was heated on water bath at 60°C before use. One gram of leaf sample was taken in a clean mortar and pestle and 5 ml of pre heated CTAB extraction buffer along with 10mg of PVP and 10 µl of β-Mercaptoethanol was added and grind them properly.

The mixture was transferred into clean 30 ml centrifuge tube. The sample was incubated in water bath at 60°C for one hour with occasional mixing by gentle swirling. Equal volume of chloroform: isoamylalcohol (24:1) was added and mixed properly by inverting the tube. The solution was centrifuged at 10,000 rpm for 20 min at RT.

The top aqueous phase was collected carefully without disturbing the bottom phase in a clean 30 ml tube and to this 2/3 volume of chilled absolute ethanol was added. The tube was mixed gently by inverting the tube. The DNA appeared flocculants and the tube was centrifuged at 5,000 rpm for 5 min and the supernatant was discarded gently.

5 ml of 70% ethanol was added to the precipitated DNA sample and centrifuged at 5,000 rpm for 5 min and the supernatant was discarded and this step was repeated once again. The pellet was air dry at RT to remove the ethanol. 50 µl of TE buffer was added to the pellet and incubated overnight at 37°C or one hour at 60°C to dissolve the DNA.

Method 3

Young and fresh leaves were collected and washed with running tap water to remove dust particles.

The leaves weighing 250 mg were separately ground to fine powder in absolute alcohol using clean and sterilized pre-chilled pestle and mortar. While grounding, 10 mg PVP was added.

The ground powder was scrapped and transferred to a 15 ml centrifuge tube containing 5 ml of pre-heated extraction buffer [consisting of 3% (w/v) CTAB, 20mM EDTA, 1.4M NaCl, 100mM Tris-HCL (pH 8.0)], and 0.2% (v/v) β-mercaptoethanol, Which was added separately.

The homogenate was incubated at 65^{0}C temperature for one hour with periodic shaking to breakdown cellular membranes, followed by extraction with an equal volume of Phenol: Chloroform: isoamylalcohol (25:24:1) and centrifuge at 10,000 rpm for 10 min. The supernatant was transferred to new centrifuge tube using sterile micropipette and further extracted by equal volume of Chloroform: isoamylalcohol (24:1) and again centrifuged at 10,000 rpm for 10 min. The above supernatant was carefully transferred using micropipette to new vial without disturbing the organic phase containing phenol and other contaminants. DNA was precipitated from the aqueous phase by mixing with 1/4th volume of 5M NaCl and equal volume of ice chilled isopropanol by 2h incubation at -20^{0}C temperature. This was followed by centrifuge at 13,000 rpm for 15 min at 4^{0}C temperature.

The DNA pellet was washed twice with 70% ethanol, air dried and re-suspended in TE buffer and stored at -20^{0}C temperature for further purification.

PURIFICATION OF DNA

To the extracted DNA samples, 10 µg/ml of RNAse and 10 µg/ml Protinase-K were added and mixed gently by inverting the microcentrifuge tubes and incubate at 37^{0}C temperature for 1h in a water bath.

After incubation, DNA was extracted by Chloroform: iso-amyl alcohol (24:1) and centrifuged at 10,000 rpm for 10 min. The above supernatant was carefully transferred using micropipette to new vial without disturbing the organic phase.

DNA was precipitated from the aqueous phase by mixing with an equal volume of absolute alcohol. After centrifugation at 10,000 rpm for 10 min, DNA pellet was washed twice with 70% ethanol, air dried and pellet was finally dissolved in 100 µl of TE buffer (pH 8.0).

Genomic DNA samples isolated by adopting the above protocol were labelled individually and stored in 1.5 ml microcentrifuge tube and kept at -20^{0}C temperature in deep freezer for further use.

C. ESTIMATION OF DNA

10 µl of genomic DNA was diluted with 1.90 ml of 1X TE buffer. The above 2 ml of the diluted DNA was transferred into a quartz cuvette and baseline correction was done. The absorbance was noted at 260 nm and 280 nm, ratio was calculated.

An absorbance of A260 of 1.0 corresponds to 50 µg double stranded DNA per ml of the solution and 40 µg of RNA/ml respectively. From this the concentration of DNA and RNA in the test sample can be calculated. The result were tabulated and recorded.

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II. SCREENING OF RAPD AND ISSR PRIMERS

Materials

RAPD primers, ISSR primers, PCR grade distilled water, PCR buffer, Taq polymerase, dNTP's, $MgCl_2$, Micropipettes and PCR tube. Based on earlier studies in genetic analysis of *B. balcooa* 8 ISSR primers and 3 RAPD primers were selected for the present study. These primers were custom synthesized from Amnion Biosciences Pvt. Ltd.,. ISSR primer sequence of 800 series were designed by University of British, Columbia and hence called UBC-800 series. 3 RAPD and 8 ISSR primers (2.5 µl) are taken and added individually to the PCR tubes followed by 22.5 µl of the master mix. The DNA was amplified using PCR machine.

The amplified products are run in 1.5% agarose gel electrophoresis and the gels were analyzed.

Table 2. Master Mix for PCR

S. No	Items	Volume	Concentration
1	PCR grade water	13.7µl	-
2	PCR buffer	2.5µl	1X
3	dNTP's	1.0µl	10mM
4	$MgCl_2$	2.5µl	3.0mM
5	Genomic DNA	2.5µl	30ng
6	Primer	2.5µl	10mM
7	Taq polymerase	0.3µl	1.2U
	Total	25µl	-

Table – 3 List of ISSR primers

S. No	UBC code	ISSR Primer Sequences	Length
1	UBC-812	GAGAGAGAGAGAGAGAA	17
2	UBC-813	CTCTCTCTCTCTCTCTT	17
3	UBC-814	CTCTCTCTCTCTCTCTA	17
4	UBC-818	CACACACACACACACAG	17
5	UBC-830	TGTGTGTGTGTGTGTGG	17
6	UBC-836	AGAGAGAGAGAGAGAGYA	18
7	UBC-840	GAGAGAGAGAGAGAGAYA	18
8	UBC-841	GAGAGAGAGAGAGAGAYC	18

Table - 4. List of RAPD primers

S. No	Seq.Id	Seq. Name	RAPD Primer sequences	Length of Primers
1	AB12879	RAPD 1	GGTGACGCAG	10
2	AB12881	RAPD 3	AGGTGACCGT	10
3	AB12882	RAPD 4	TGAGCGGACA	10

A. DNA Amplification using PCR machine

PCR amplification cycles for RAPD primer was carried out with following cycles profiles.

Initial denaturation 94^{0}C for 3 min, followed by repeated 40 cycles. Denaturation at 94^{0}C for 30 sec. Annealing at 45^{0}C for 30 sec. Extension at 72^{0}C for 1 min and Final extension at 72^{0}C for 10 min.

B. AGAROSE GEL ELECTROPHORESIS OF AMPLIFIED DNA

Agarose

It is linear polysaccharide made up of basic repeat unit agarobiose, which comprises alternating units of galactose and 3, 6-anhydrogalactose.

Agarose gels were formed by suspending agarose in aqueous buffer, then boiling the mixture until a clear solution forms. This is poured and allowed to cool at room temperature to form rigid gel. The gelling properties are attributed to both inter and intra molecular hydrogen bonding within and between the long agarose chains. This cross linked structure gives the gel good anti convectional properties. The pore size in the gel is controlled by the initial concentration of agarose. Larger pore sizes are formed from low concentrations and smaller pore sizes are formed from the higher concentrations.

Electrophoresis buffer

The electrophoretic mobility of DNA was affected by the composition and ionic strength of the electrophoretic buffer. The most commonly used buffer for duplex DNA is TAE (Tris-acetate-EDTA) and TBE (Tris-borate-EDTA).

Gel loading buffer

Used to increase the density of the sample ensuring that the DNA drops evenly into the well, they gives color to the sample, thereby simplifying the loading process and contain dyes that in an electric field move towards the anode at predictable rate. Bromophenol blue migrates through agarose gel, run in 1 X TAE at approximately the same rate as linear double stranded DNA 100bp in length.

Visualization

Ethidiumbromide is a fluorescent dye used to detect nucleic acid in agarose. This dye intercalates between bases of DNA and RNA. To visualize DNA or RNA, the gel is placed on ultraviolet transilluminator and bands are seen in orange color.

Procedure-Preparation of 1.5% agarose gel

The gel tray was placed in the gel casting tray along with the comb. Weighed 0.750 g of agarose and dissolved in a small conical flask containing 50 ml of 1X TAE. The agarose was boiled using microwave oven until clear solution formed. The agarose was cooled to 40^{0}C and 10 µl of ethidiumbromide (10 mg/ml) was added and slowly mixed by shaking.

The above agarose gel was poured in the gel tray and kept aside without disturbing for 45 min for complete solidification (it should appear milky white). After solidification the combs were carefully removed from gel tray without damaging the well. 100 ml of 1X TAE buffer were poured in horizontal electrophoresis reservoir tank.

Buffer level should be maintained just above the gel (3-5 mm of buffer over the gel). 5 µl of gel loading dye was placed on to a piece of parafilm. 20 µl of DNA sample was added, mixed well and then loaded into the well carefully.

The electrophoresis unit was connected to the power pack such that the negative position is placed towards the well and the other side with positive end.

Electrophoresis was performed at 50 Volts for 2h. The gel was examined under UV transilluminator to observe the bands and the results were recorded.

Standardization of $MgCl_2$ Concentration

$MgCl_2$ concentration plays a very important role in the Taq polymerase activity. Based on the concentration of $MgCl_2$, the activity of the Taq changed. Hence, to optimize the reaction, optimization of $MgCl_2$ concentration is a very important step in polymerase chain reaction.

In order to identify the best $MgCl_2$ concentration for optimum amplification of DNA, various concentrations of $MgCl_2$ ranging from 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0mM were tested along with the master mix.

The above discussed in the screening of primers, the same concentration of master mix and the amplification reaction cycles of ISSR primers were used for standardization of $MgCl_2$ concentration.

Master mix for seven reactions was prepared and each reaction mixture consisted of different concentration of $MgCl_2$. Genomic DNA of the normal green shoot was added to the master mix and amplified in the ISSR PCR cycles.

The amplified products were analyzed using agarose gel electrophoresis as followed earlier in the screening of RAPD and ISSR primers.

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Detection of genetic variation in albino plants

Based on the above results obtained from the screening of primers and $MgCl_2$ concentration, total of 10 primers (7 ISSR and 3 RAPD primers) were used to detect genetic variations in the genomic DNA of albino regenerated shoots in comparison with the genomic DNA of normal green shoots.

A total of 20 reaction master mix was prepared. The master mix was added to individual 200 µl of PCR tube followed by 2.5 µl of genomic DNA of albino in 10 sets of PCR tubes and another 10 sets consisted of genomic DNA of normal green shoots.

2.5 µl of individual primer was added individually to a pair of PCR tube (i.e., 1 tube consisted of genomic DNA of normal green shoot and another consisted of genomic DNA of albino shoots). Similarly, for all the 20 PCR tubes, 10 pairs were made to detect the genetic variation in albino plants for comparison with the normal green shoots.

The above mentioned master mixes were subjected to DNA amplification as mentioned in the screening of RAPD and ISSR section. The amplified products were subjected agarose gel electrophoresis as mentioned in earlier section (Screening of RAPD and ISSR markers).

III. RESULTS

Table 5. Effect of different BAP concentration on shoot initiation in *B. balcooa*

Table No.	BAP conc.	(%) response	Shoot number	Shoot length (cm)
1	0.0	-	-	-
2	3.0	58.3	3.0	1.8
3	4.0	75.0	2.5	1.9
4	5.0	91.65	2.5	2.4
5	6.0	66.6	2.33	1.74
6	7.0	91.6	2.9	1.7

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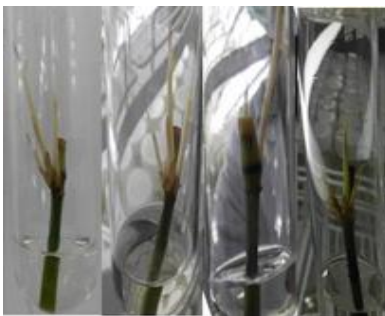

Figure. 1 Shoot initiation of *B. Balcooa* through auxiliary shoot proliferation

Figure. 2. A. shoot multiplication of *B. Balcooa* through auxiliary shoot proliferation (Normal).

Figure. 2. B. shoot multiplication of *B. Balcooa* through auxiliary shoot proliferation (Albino).

Table – 6 Chlorophyll estimation in normal green shoots and albino white shoots

	Normal shoots	Albino shoots
Chlorophyll A	0.65mg/g	0.011mg/g
Chlorophyll B	0.002mg/g	0.184mg/g
Chlorophyll A+B	0.417mg/g	0.072mg/g

Table – 7 DNA extraction

S. No.	Sample ID	ng/µL	A260	A260/A280
1	METHOD 1	88.2	0.204	1.92
2	METHOD 2	501.1	1.269	2.10
3	METHOD 3	86.2	0.922	1.87

The data obtained were analyzed using ALPHA EASE software for comparison of bands generated between amplified products of normal green shoots and albino white shoots.

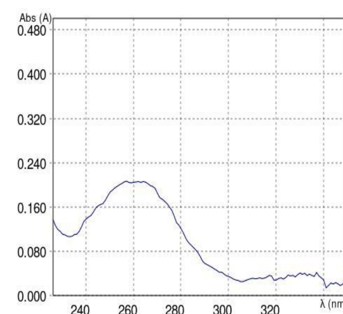

Figure. 3.A. Graphical representation of concentration of DNA by method 1

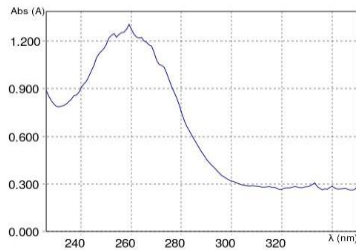

Figure 3.B. Graphical representation of concentration of DNA by method 2.

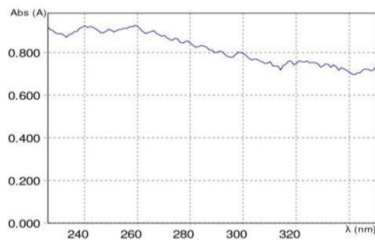

Figure: 3.C. Graphical representation of concentration of DNA by method 3

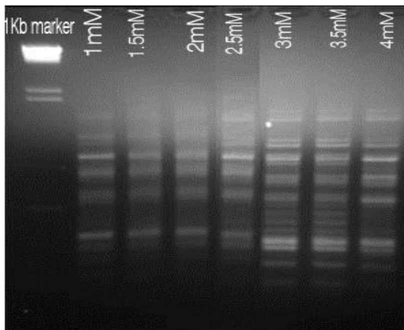

Figure: 4. Electrophoresis image for Standardization of $MgCl_2$ Concentration

Table – 8 Details of primers used to detect variation in albino regenerated plants and its amplified products.

Primer number	No. of bands amplified	No. of monomorphic bands	No. of polymorphic bands	Mol. Wt.
UBC- 812	6	6	0	1475-384
UBC-813	3	3	0	1003-726
UBC-814	3	3	0	1348-752
UBC-818	6	6	0	1563-631
UBC-830	5	5	0	1610-556
UBC-836	6	6	0	1475-567
UBC-840	6	6	0	1460-480
UBC-841	-	-	-	-
RAPD1	6	6	0	778-400
RAPD3	8	8	0	987-385
RAPD4	8	8	0	997-385

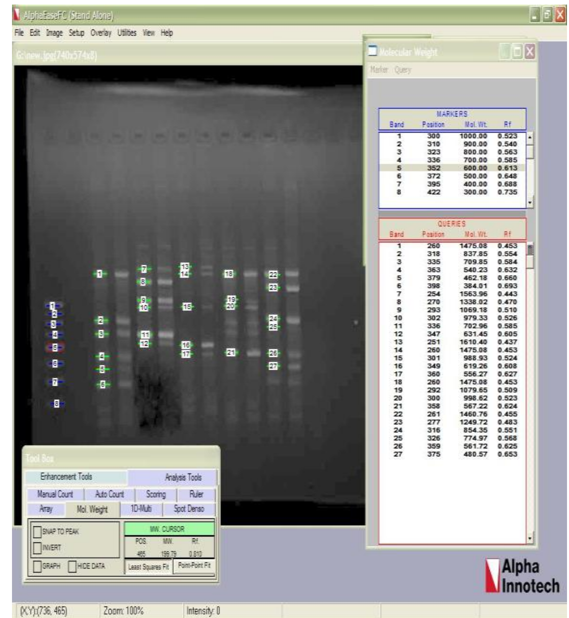

Figure. 5 Molecular weight prediction for the amplified DNA with ISSR primers by the software AlphaEase

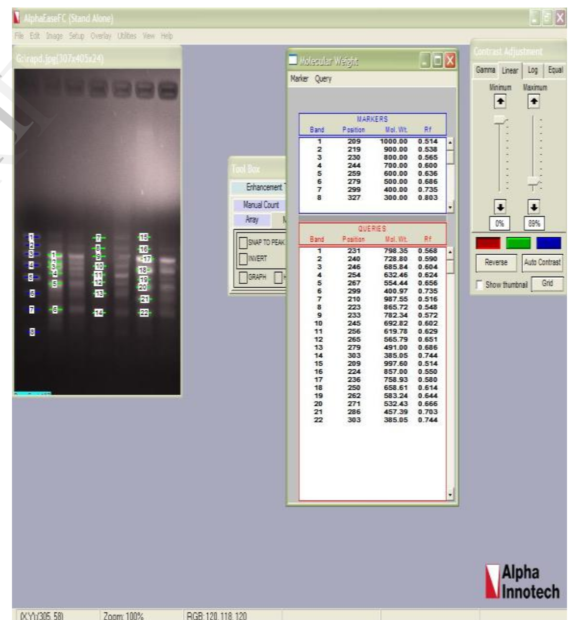

Figure: 6. Molecular weight analysis or the amplified DNA with RAPD primers by the software AlphaEase

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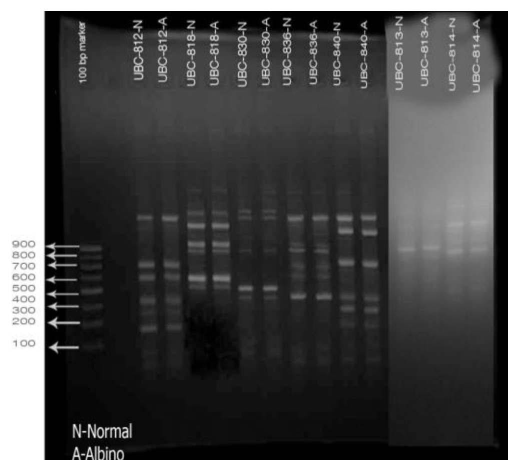

Figure: 7. Gel image of amplified PCR products from ISSR primers for detection of albino variation in *Bambusa balcooa.*

III. DISCUSSION

Shoot initiation

Different concentrations of BAP were tested for identification of best concentration for *in vitro* regeneration of shoots in MS medium with additives (ascorbic acid 50 mg/l + citric acid 25 mg/l). Among the 5 different concentrations of BAP tested, MS medium, additives with BAP 5.0 mg/l, showed the best results in terms of percentage response of 91.65, 2.5 shoots per explant with highest 2.4 cm shoot length. This was followed by MS medium containing BAP 7 mg/l, which also showed 91.6% response with 2.9 shoots with 1.7 shoot length. The blank devoid of any cytokinin did not responded for any shoot initiation and dried after 3 weeks period. Though, MS medium containing 3 mg/l showed the highest shoot number of 3 shoots per explant, but the percentage response was low (58.3%) from Table 5 represented in Figure.1.

However, [5] reported the best results of shoot initiation in MS medium supplemented with 2.5 mg/l BAP with 1.0 mg/l Kinetin. Similarly, [15] reported use of 1.0 mg/l BAP along with 0.5 mg/l Kinetin for shoot initiation. They observed 90% response in bud break. [13] also reported use of 1.0 mg/l BAP in MS medium for shoot initiation in *B. balcooa*, which showed 8-10 shoots/explant.

In our present studies, it was observed that BAP at 5.0 mg/l showed the best concentration for multiple shoot initiation with optimum number of shoots and shoot length.

Shoot multiplication

The *in vitro* regenerated shoots of *B. balcooa* were further multiplied on MS agar gelled medium with additives (ascorbic acid 50 mg/l + citric acid 25 mg/l) along with BAP 2.0 mg/l and NAA 0.2 mg/l. A multiplication of 3 fold was observed, after 6 weeks of shoot multiplication few shoots produced albino shoots, which were used for detection of genetic variation using PCR based ISSR and RAPD techniques Shown in Fig. 2A and Fig. 2B.

Chlorophyll Assay

Chlorophyll was estimated by acetone extraction method and using UV spectrophotometry. It was observed that, normal shoots which exhibited chlorophyll content and appeared green showed chlorophyll a of 0.65 mg/g, chlorophyll B 0.002 mg/g and chlorophyll A+B 0.417 mg/g calculated using Formula (1). However, the albino shoots showed very less chlorophyll content with chlorophyll A of 0.011 mg/g, chlorophyll B 0.184 mg/g and Chlorophyll A+B 0.072 mg/g. the results from this estimation reflects the absence of chlorophyll pigment in the albino shoots shown in Table.6.

Similarly, [11] reported that two albino mutants (ab1 and ab2) were derived from long-term shoot proliferation of *Bambusa edulis*. They reported that the results of transmission electronic microscopy data showed that, the chloroplasts of these mutants were abnormal.

DNA EXTRACTION AND PURIFICATION

Three different modified CTAB DNA extraction methods were used for extraction of genomic DNA of *in vitro* regenerated shoots of *B.balcooa*. Among the three methods used, method 1 and 3 showed yield of equal quality of genomic DNA both in quantity and quality. Method 1 yielded 88.2 ng/µl with OD of 1.92 and Method 3 yielded 86.2 ng/µl with an OD of 1.87. Both the OD of 1.92 by Method 1 and 1.87 by Method 3 are sufficiently good for amplification of DNA using ISSR and RAPD primers. The method 2 though yielded the highest (501.1 ng/µl) amount of genomic DNA, but the purity was very less as observed with an OD of 2.10 at 260/280 nm shown in table 7.

Screening of primers

Based on earlier studies in genetic analysis of *B. balcooa* 8 ISSR primers and 3 RAPD primers were selected for the present study. These primers were custom synthesized from Amnion Biosciences Pvt. Ltd., ISSR primers designed by University of British, Columbia, of 800 series were selected for screening of DNA. The primers UBC-812, 813, 814, 818, 830, 836, 840 and 841 were used for screening and it was observed that all the primers showed amplification except UBC-841. Primer 812 and 813 showed the least number of bands of 3 number followed by UBC 830 (5 bands) and other UBC 814, 818, 836, 840 showed 6 amplified bands. Similarly, [15] observed that UBC 812, 813, 814, 818, 830, 836, 840 and 841 primers showed amplification of genomic DNA for testing of genetic fidelity in micropropagated plants of *B. balcooa*.

Among the 3 RAPD primers used for screening, all the primers showed amplification with genomic DNA of *B. balcooa*. Primer RAPD 1 showed the amplification with 6 amplified bands and RAPD 3 amplified 8 bands and RAPD 4 amplified 6 bands.

Standardization of $MgCl_2$ Concentration.

After identification of the primers after screening the concentration of $MgCl_2$, was varied for optimization of concentration of $MgCl_2$ for amplification of genomic DNA. Among the different concentrations of $MgCl_2$ tested with varying concentrations (1.0mM, 1.5mM, 2.0mM, 2.5mM, 3.0mM, 3.5mM and 4.0mM) along with the master mix, it was observed that $MgCl_2$ concentration of 2.5 and 3.0mM showed optimum bands and hence for further all experiment concentration of 2.75mM was used for amplification of DNA indicated in Fig. 4.

Detection of albino using RAPD and ISSR primers.

ISSR primer UBC-812 amplified six bands from the genomic DNA isolated from both normal green and albino white shoots. The amplification of bands ranged from 1475-384 bp. Both UBC-813 and 814 produced three bands on

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amplification with genomic DNA of both normal green and albino white shoots. UBC-818 amplified six bands similar to UBC-812 with various molecular size, UBC-830 amplified 5 bands, UBC-836 and UBC-840 both were amplified and gave six bands with various molecular sizes. All the primers were gave same number, molecular weight bands for both normal and albino. There are no polymorphic bands in normal and albino which is clearly indicated in table. 8.

The RAPD primers RAPD-1, RAPD-3 and RAPD-4 gave 6, 8 and 8 bands respectively the number of bands and molecular weight for each band amplified by every primer was same this also show there was no polymorphism.

It was observed that among all the 3 RAPD and 7 ISSR primers used for detection of genetic variation in albino shoots, showed monomorphic banding pattern. All the amplified products did not able to detect any genetic variations in the genomic DNA.

Use of two marker systems i.e., RAPD and ISSR would help in amplifying different part of the genome. The RAPD primer binds randomly in any part of the genome and amplifies it as the length of the primers is also short (10mer). Whereas, the ISSR markers binds only in between the simple sequence repeats and amplify. The binding of ISSR primers is more precise as the number of nucleotide is more as compared to RAPD. Along with the primer size and the higher annealing temperature of the ISSR primers (50^0C) as compared to RAPD (45^0C) used in the present study, the binding of the primers stringency increase and hence produces more stable bands. It was observed in our studies that the amplified products of RAPD showed molecular weight of 385-997, whereas, ISSR primers amplified bands in the range of 480-1475. It was also observed from the studies that, use of more number of markers would yield more amounts of details of the genome and chances of detection of any variation.

The morphological pattern of albino shoots would be due to non-expression of chlorophyll pigment and would not be due to genetic variations, this was also reported by [11], where he observed albino mutants in *B. edulis*, but not all mutants showed deletions in DNA. As the morphological variations were as low as 0.02% and no genetic variations were detected, the micro propagation protocol of *B. balcooa* can be carried out without any somaclonal variations.

As discussed earlier, [11] observed albino mutants in *B. edulis* and reported that not all albino mutants have a deletion in the chloroplast genome. They also identified three albino mutants in *Bambusa oldhamii* from 2, 7, and 20 years age and found that was no difference between the mutants and the wild type with respect to the 33gene copy number was observed. Because these albino plants could not process the products of photosynthesis, however they could not survive in *in vivo* conditions. The regeneration of albino mutants during tissue culture is a costly waste of resources with respect to time, effect, and expenses. While such a system as that used by [8] may reduce the percentage of albino regeneration; it remains very difficult to separate albino and wild type callus.

However, [4] found that one Random Amplified Polymorphic DNA (RAPD) product was missing from the dwarf off-type banana; sequencing revealed that this DNA is chloroplast-encoded. Similarly, [11] demonstrate that the albino mutants derived from multiple shoots of *B.edulis* have major deletions in their chloroplast genome.

By gaining an understanding of the mutation(s) of albino regeneration, we may be able to develop technology for detecting albino cells and, consequently, adapt the technology for reducing albino regeneration in tissue culture. In addition, there are many ornamental plants having leaves with undesirable albino stripes or spots. Our results may be one step in the direction of developing such techniques.

IV. CONCLUSION

From the present study it was concluded that during the micropropagation of *B.balcooa* few albino shoot formation was observed. The PCR based markers such as RAPD and ISSR were useful techniques for detection of variation at genetic level. The present protocol used for large scale production of *B.balcooa* through axillary shoot proliferation produce genetically stable plants and can be used without any problem.

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