

## Full Length Research Paper

# Refinement of protocol for rapid clonal regeneration of economical bamboo, *Bambusa balcooa* in the agroclimatic conditions of Bihar, India

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*Bambusa balcooa* is a commercially important bamboo. The present study was undertaken for refinement of protocol for the rapid and mass production of this species of bamboo in agroclimatic condition of Bihar. Micro-clonal propagation techniques have been employed for the study. This technique is the only method for the large scale production of *B. balcooa*. Explants were collected from Bamboo setum from TNB College campus. Combined effect of 6-benzylaminopurine (BAP) and Kinetin (Kn) (BAP 2 mg/l + Kn 0.5 mg/l) resulted in 85% bud breakage during initiation of cultures. One remarkable feature that was observed is that microshoots in medium without supplementation of naphthalene acetic acid (NAA) had necrotic shoots and they had less multiplication rate in liquid media. High multiplication rate with comparatively longer shoots were observed in liquid media. NAA (2.5 mg/l) along with half strength of MS showed 100% rooting but it was less than 50% when Murashige and Skoog (MS) media was supplemented with indole 3-butyric acid (IBA). Remarkably, in half strength of MS, clumps dried at first within 7 days in rooting media, however, new green healthy shoots proliferated later (after 21 days). The data obtained from this study has set a refined protocol for the clonal regeneration of *B. balcooa* for the state of Bihar. The current study will help to produce this species of bamboo on large scale, and thereby help to boost rural economy of Bihar.

**Key words:** *In vitro* regeneration, clonal propagation, bamboo, *Bambusa balcooa*, micropropagation.

## INTRODUCTION

Bamboos are one of the fastest growing monocotyledonous plants which have multipurpose utility. They produce huge amounts of biomass. They are universal in occurrence except in European continent (Nadgauda et al., 1993). Being a substitute of timber, they have

attracted the growers and scientists widely throughout the world. They are preferred due to their high agroclimatic suitability, as renewable source of energy and also as they do help to prevent soil erosion (Gantait et al., 2016).

After the creation of Jharkhand state, Bihar is left with a

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6.87% green cover only causing a drastic change in climate due to human consumption (India State of Forest report, 2009). Earlier, Bihar used to receive an annual rainfall of 1200 to 1500 mm annually which has now come down to 800 to 1000 mm only for last 11 years. It is believed that bamboos can help man to mitigate global warming as they are one of the fastest growing plants and also they have comparatively better CO<sub>2</sub> sequestration abilities (Nasreen et. al., 2015). They can fix atmospheric carbon in organic form to soil. Under the "Hariyali Mission (a mission for Green Cover)", Department of Environment and Forest, Bihar Government is striving to increase green cover by 19% consequent upon which there is a target to plant massive quality bamboo plantlets in wastelands, marginal lands and also in agroforestry. Among the 136 species of bamboos in India, *Bambusa balcooa* is the strongest and multipurpose bamboo (Sharma and Sarma, 2011). Some of the morphologically distinguishable features of this species are like they have short internodes (20 to 24 cm), nodes being swollen (8 to 15 cm in diameter), at the lower nodal region prominent brown hairs remain present as well as white rings mostly present at the nodal rings of young culms. On leaf sheaths mostly brownish hairs remain present; however, culm sheath auricles may remain present or absent. Flowering cycle of this species is of 35 to 45 years and after that plants die without seed setting. It is being used in construction, ladders, boats, rickshaw hood frames, to weave mats and baskets, pulp and paper, making handicraft, biofuels/bioenergy and also as bamboo chips. Additionally, tender shoots are edible as vegetable and pickles.

Considering the versatile use of *B. balcooa*, it is an important desirable species for cultivation. Plant tissue culture is the only technique which can be utilized to solve the challenges of rapid and mass regeneration of this species (Kaur et. al., 2014). The offset and rhizome cuttings used for vegetative propagation being bulky are troublesome to handle (Singh et. al., 2013). They have very low rooting frequency and also culms/rhizome is susceptible of getting desiccated, which thereby restricts their large scale production (Vishwanath et al., 2012). They also show high season specificity owing to the morpho-physiological state of the plant. This method can thus be tried only to a limited scale. In addition, *in vivo* technique requires large number of bamboo culms for micropropagation in order to meet high demand of the species. Hence, *in vitro* propagation is the only option for bulk production of *B. balcooa*. There are few earlier reports on clonal propagation of *B. balcooa* through modal shoot proliferations, however, all such studies have either one or other limitations like lack of information about explant treatments, suitable season for initiation, frequency of multiplication/rooting or discussed with complex hardening procedures (Das and Pal, 2005a, b; Mudoi and Borthakur, 2009; Negi and Saxena, 2011, Wei et al., 2015).



Figure 1. Clump of *Bambusa balcooa*.

Our study demonstrates the various critical factors effecting *in vitro* propagation and also the comparative measures to overcome those factors as well as an efficient method for micropropagation of *B. balcooa* in agroclimatic conditions of Bihar. Explants were excised every month to evaluate the influence of seasons on micropropagation. The various sizes of explants were selected to find out the most suitable for the climate of Bihar. Mean and standard deviations of the data were analysed.

## MATERIALS AND METHODS

### Aseptic culture establishment

The newly grown culm and culm branches (2 to 3 years old) were collected periodically from actively growing branches of *B. balcooa* planted in the PTC lab premises, TNB College campus, Bhagalpur, Bihar, India (Figure 1). Collections were made during four seasons (spring, summer, rainy and winter).

Surfaces of shoot segments were swabbed with 70% ethanol.

**Table 1.** Seasonal variations in bud initiation for establishment of aseptic culture (Oct 2015 to Sept 2016).

Season	Date of explant collection	Number of shoots	Shoot length (cm)	Initiation of bud (%)	Contamination (%)
Autumn + Winter	08-Oct	3.2 ± 0.03	1.33 ± 0.06	45	41
	15-Oct	2.8 ± 0.05	1.9 ± 0.01	42	31
	10-Nov	2.3 ± 0.02	2 ± 0.4	35	25
	25-Nov	2.1 ± 0.04	1.2 ± 0.05	20	36
	09-Dec	1.1 ± 0.05	1.5 ± 0.03	10	33
	23-Dec	1.5 ± 0.09	1.2 ± 0.06	12	41
	07-Jan	1.8 ± 0.02	1.6 ± 0.03	15	39
	16-Jan	1.9 ± 0.01	1.1 ± 0.07	18	44
Spring + Summer	03-Feb	3.2 ± 1.0	2.3 ± 0.03	48	35
	17-Feb	7.2 ± 0.09	3.8 ± 0.04	72	25
	05-Mar	7.8 ± 0.06	6.9 ± 0.02	89	15
	25-Mar	11.3 ± 0.01	7.2 ± 0.02	85	10
	20-Apr	14.6 ± 0.03	5.6 ± 0.05	91	12
	07-May	20.1 ± 0.8	3.9 ± 0.07	92	18
	21-May	25.1 ± 0.7	4.2 ± 0.06	78	13
	06-Jun	30.6 ± 0.4	3.5 ± 0.04	75	20
Rainy season	23-Jun	15.6 ± 0.7	3.2 ± 0.04	72	15
	16-Jul	14.1 ± 0.9	5.8 ± 0.06	74	83
	29-Jul	15.2 ± 1.0	5.4 ± 0.05	79	90
	07-Aug	10.7 ± 2.0	5.2 ± 0.03	81	92
	23-Aug	9.3 ± 0.5	5.7 ± 0.05	89	85
	06-Sep	8.4 ± 0.8	5.9 ± 0.04	69	87
	25-Sep	8.9 ± 0.9	5.3 ± 0.03	65	81

Using scalpel and leaf sheath were removed carefully without giving any injury to the buds.

### Seasonal variation and explant collection

Collections of explants were made throughout the year from October 15 to September 16 in order to ascertain the most suitable season/period for establishment of cultures. The initiation and contamination percentage in different seasons were also monitored (Table 1).

### Processing and surface sterilization of explants

Single node shoot segments of variable length and diameter were fine cut at both ends using sharp blade secateurs. For surface sterilization of explants disinfectants were used in variable combinations (Table 2).

### Procedure I

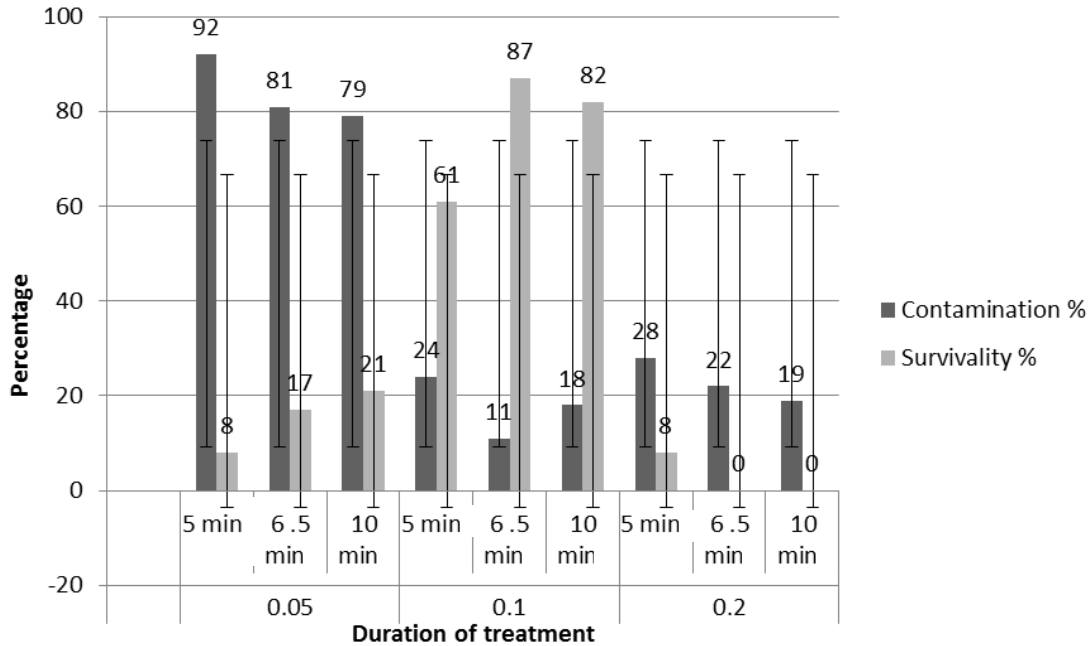
Explants (nodal segments) were first dipped in 0.1% (v/v) Tween 20 solution (5 to 10 min) and they were shaken continuously to remove dust and dirt particles from the surface. Then after, they were washed 5 to 6 times with distilled water. The explants were subsequently treated with fungicide (Bavistin) (1%) for 10 min

**Table 2.** Explant treatment procedure and percentage survival.

Treatment No.	Contamination (%)	Survivality (%) of explant
Treatment 1	11	83
Treatment 2	10	88
Treatment 3	13	81
Treatment 4	68	45
Treatment 5	90	10
Treatment 6	87	15

Treatment 1: Tween 20 (5-10') -> Bavistin 0.1% (10') -> Ethanol 70% (30 sec.) -> 0.1 % mercuric chloride (5'); Treatment 2: Bavistin 0.1% + Indofil 0.1% (1 hour) -> Ethanol 70% (35 sec.) -> Tween 20 + mercuric chloride 0.1% (6'); Treatment 3: Tween 20 (5-10') -> Bavistin 0.1% + Indofil 0.1% (1 hour) + Ethanol 70% (30 sec.) -> mercuric chloride 0.1% (6'); Treatment 4: Tween 20 (5-10') -> Bavistin 0.1% (10') -> Ethanol 70% (35 sec.) -> NaOCl, 0.1% (6'); Treatment 5: 5 - 10 drops Savlon (5-10') -> Ethanol 70% (35 sec.) -> 0.1 % mercuric chloride (6'); Treatment 6: 5-10 drops Teapol (5-10') -> Ethanol 70% (30 sec.) + 0.1% mercuric chloride (6').

followed by 70% ethanol (35 s) and surface sterilization was made with different concentrations of HgCl<sub>2</sub> (0.05, 0.1, and 0.2%) at different intervals (5 to 10 min) (Graph 1).



**Graph 1.** Effect of duration of mercuric chloride treatment on contamination and survival rate of explants (0.05, 0.1 and 0.2 are expressed in %).

#### Procedure II

In this case explants were first dipped in (0.1%, v/v) Bavistin and (0.1%, v/v) Indofil solution for 1 h followed by treatments with (70%) ethanol for 35 s and (0.1%) mercuric chloride along with (0.1%, v/v) Tween 20 solutions for 6 min.

#### Procedure III

Explants were first washed with (0.1%, v/v) Tween 20 for 5 to 10 min and subsequent treatments were same as mentioned earlier followed in Procedure II.

#### Procedure IV

In this case explants were treated with sodium hypochlorite (0.1%) instead of mercuric chloride for 6 min. However, other processes were the same as in Procedure I.

#### Procedure V and VI

Savlon and Teepol (10 drops each for 5 to 10 min) for washing of explants were used.

Different surface sterilization techniques as mentioned earlier were compared in order to evaluate the successful establishment of cultures under aseptic conditions. The percentage contamination following different sterilization procedures was also examined. Percentage survival rate of explants was also monitored.

#### Shoot initiation

For shoot initiation of *B. balcooa*, MS liquid medium supplemented with different concentrations of hormones, additives (ascorbic acid

50 mg/l + citric acid 25 mg/l + cysteine 25 mg/l) with 3% sucrose (Table 3) were used and the pH of medium was adjusted to 6.2 prior to autoclaving. In order to obtain high frequency of multiple shoot induction, explants of different sizes (2 to 5 cm) and diameter (2 to 4 mm) were cultured (Graph 2) in different combinations of growth regulators utilizing solid and liquid media. Percentage shoot induction and number of shoots per explants were calculated after three weeks duration.

#### Effects of plant growth regulators (PGRs)

To determine the most suitable hormonal combinations for explant establishment and bud proliferation, sterilized explants were cultured on MS media supplemented with different cytokinins (6-benzylaminopurine (BAP), Kinetin (Kn) and thidiazuron (TDZ)) of different concentrations (1, 2, and 5 mg/l) along with naphthalene acetic acid (NAA; 0.25 mg/l) (Graph 3) (Wei et. al., 2015). Each set was of 30 sample size and the experiment was performed in triplicate.

All the cultures were grown under a photoperiod of 16 h a day (illuminated by 40 watt cool white fluorescent tubes of 1200 lux). The explants were regularly transferred to fresh media within 12 days in order to avoid any browning of explants.

#### Shoot multiplication

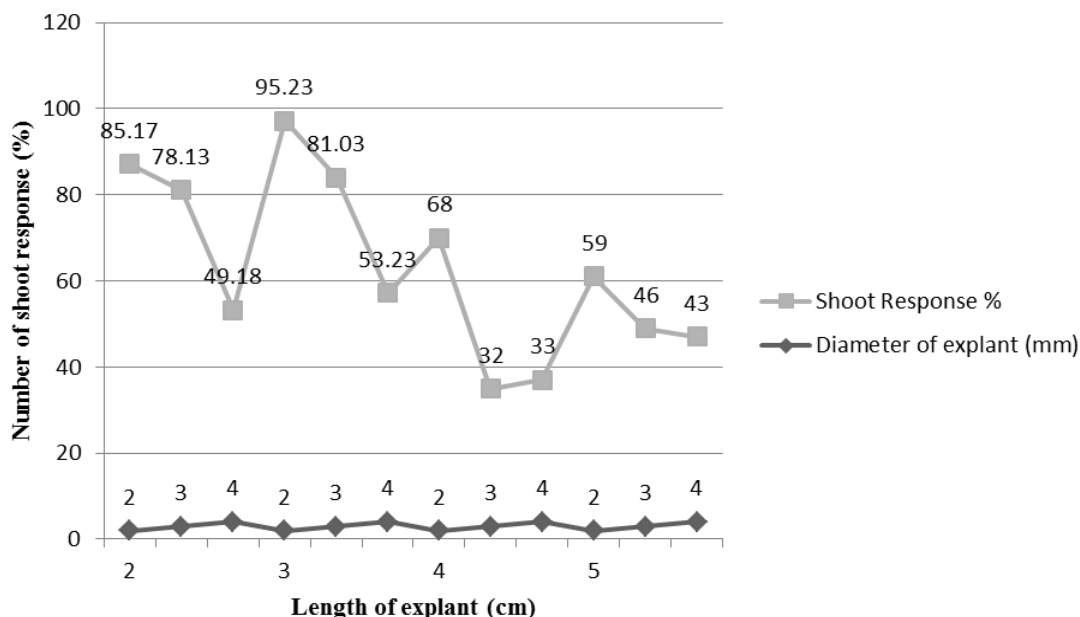
For multiplication of shoots and shoot clumps were excised from the explants and were subsequently sub-cultured on fresh medium along with additives and different combinations of phytohormones. 5 to 8 clumps of shoots were taken in each cycle. Each time sub-culturing was made after 10 days.

#### Effect of PGRs

In order to obtain high multiplication rate and better shoot length,

**Table 3.** Number of shoots, shoot length and percentage response of multiplication in liquid and agar gelled media at different phytohormonal concentrations.

Combination of PGRs	Number of shoots		Shoot length (cm)		Response % for bud sprouting	
	Solid	Liquid	Solid	Liquid	Solid	Liquid
MS + BAP (1 mg/l)	5	12	3.4 ± 0.02	5.6 ± 0.06	50	55
MS + BAP (2.5 mg/l)	8	18	2.5 ± 0.9	4.5 ± 0.05	41	48
MS + BAP (5 mg/l)	6	10	1.5 ± 0.02	2 ± 0.01	40	50
MS + BAP (2 mg/l) + Kn (0.5 mg/l)	6	8	1.4 ± 0.01	2 ± 0.03	35	30
MS + NAA (0.1 mg/l) + BAP (1 mg/l)	11	20	4.1 ± 0.3	6.2 ± 0.07	65	67
MS + NAA (0.1 mg/l) + BAP (2.5 mg/l)	16	25	3.5 ± 0.04	5.2 ± 0.03	70	79
MS + NAA (0.25 mg/l) + BAP (1 mg/l)	8	13	3 ± 0.3	5 ± 0.1	60	64
MS + NAA (0.25 mg/l) + BAP (2.5 mg/l)	10	18	1.9 ± 0.9	2.5 ± 0.3	48	41
MS + Add + BAP (1 mg/l)	13	22	2.1 ± 0.07	5.1 ± 0.4	80	81
MS + Add + BAP (2.5 mg/l)	14	26	3.4 ± 0.9	4.7 ± 0.3	82	84
MS + Add + NAA (0.1 mg/l) + BAP (1 mg/l)	21	30	4.2 ± 0.02	6.8 ± 0.8	92	95
MS + Add + NAA (0.1 mg/l) + BAP (2.5 mg/l)	29	35	3.9 ± 0.08	6.4 ± 0.3	90	96
MS + Add + NAA (0.25 mg/l) + BAP (1 mg/l)	16	19	3.5 ± 0.3	5.6 ± 0.5	72	78
MS + Add + NAA (0.25 mg/l) + BAP (0.25 mg/l)	12	15	2.6 ± 0.7	3.9 ± 0.3	69	72
MS + Add + Glutamine + NAA (0.25 mg/l) + BAP (2.5 mg/l)	25	14	2.1 ± 0.2	2.1 ± 0.2	82	64

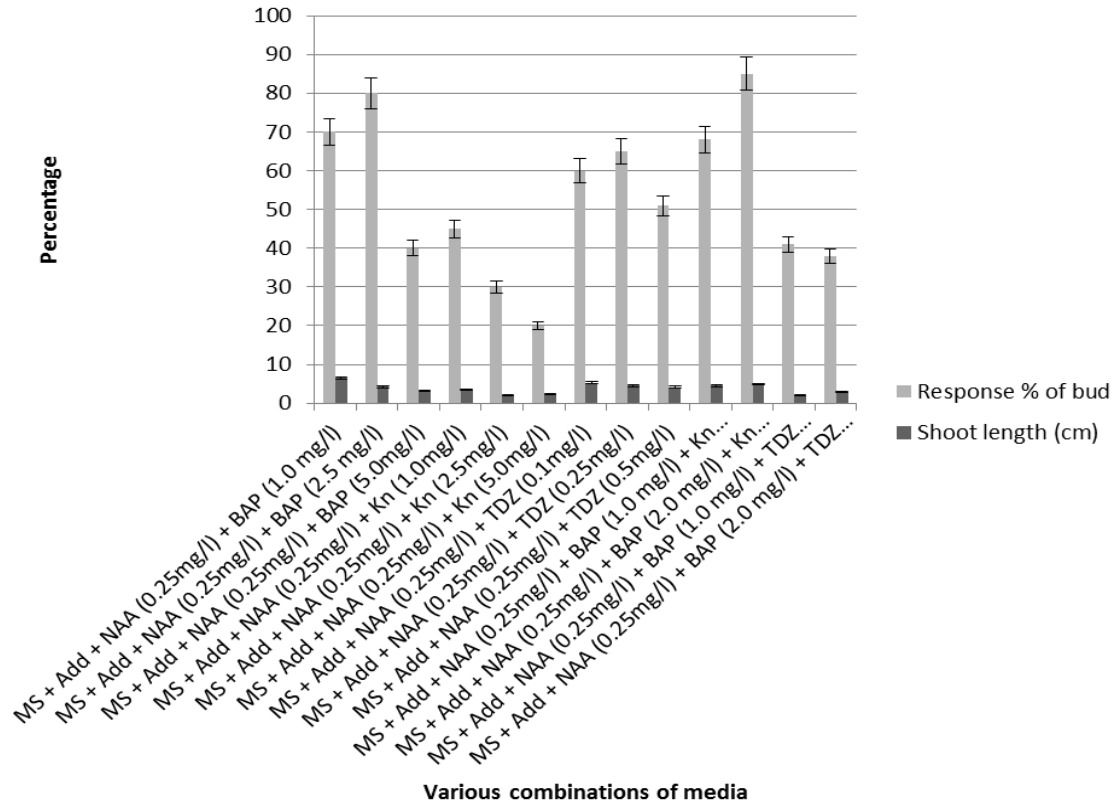
**Graph 2.** Effect of diameter and length of explants on percentage response and contamination.

various cytokinins BAP (1, 2, and 5 mg/l), Kn (1, 2.5, and 5 mg/l) and TDZ (0.1, 0.25, and 0.5 mg/l) in combination with NAA (0.1 mg/l) were tested in MS liquid medium supplemented with additives (ascorbic acid, citric acid and cysteine) (Graph 3). Altogether 13 treatments were made and each set consisted of 30 samples and the experiment was made in triplicate. Sub-culturing of *in vitro* shoots was carried out at an interval of 10 days or at least before the medium started turning brown (browning problems appeared due to phenolic exudates). The rate of multiplication was calculated

by counting the number of shoots produced after each cycle divided by the number of shoots involved in regeneration.

#### Effect of liquid vs. agar gelled medium

To compare the liquid vs. agar gelled medium for shoots multiplication and subsequent growth, MS liquid and agar gelled media (0.6% w/v) with hormones (NAA 0.1 mg/l and BAP 2.5 mg/l)



**Graph 3.** Effect of different concentrations and combinations of growth regulators on shoot induction.

supplemented with or without additives were tested (Table 3). Influence of NAA in combination with BAP on both the media (solid or liquid) was also examined.

#### Effect of glutamine

Effect of glutamine in solid and liquid media was also tested. Glutamine (25 mg/l) was added to MS media to determine effect on shoot multiplication.

#### In vitro rooting

Five to eight shoot clumps of *B. balcooa* were inoculated in nutrient MS media (half strength) for *in vitro* rooting. Multiplied shoot cultures after fifth cycle onwards were tested for the *in vitro* rooting.

#### Effect of various auxins on in vitro rooting

Clumps bearing 5 to 8 shoots were transferred to MS solid media containing different concentrations (1 and 2.5 mg/l) of auxins, namely, indole 3-acetic acid (IAA), indole 3-butyric acid (IBA) and NAA (Graph 4). Each hormone concentration was tested in triplicate with 100 experimental plants. Rooting response and rooting percentage on supplied medium were observed.

#### Effect of nutrient media

Full and half strength of MS nutrient media for *in vitro* rooting were also tested. Observations were made after 7 and 21 days for

calculation of rooting percentage.

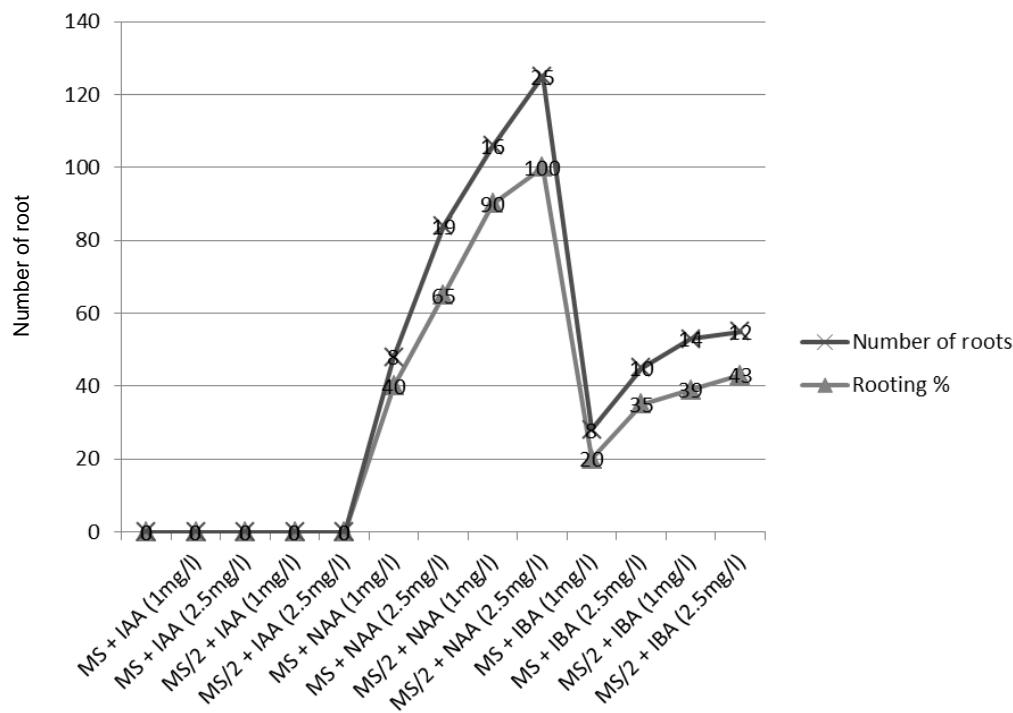
#### Hardening

*In vitro* rooted shoots were carefully taken out from culture bottles and were washed with water. They were then transferred to seedlings trays containing different types of transplanting media, namely, sand: soilrite (1:1), sand: soilrite: cocopit (1:1:1), cocopit, cocopit: vermicompost (2:1) as well as vermicompost. Initially, plantlets were kept under poly-tunnel for 3 weeks in green house at high temperature ( $30 \pm 5^\circ\text{C}$ ) and relative humidity ( $80 \pm 5\%$ ). Later on, plants were transferred to mother bed (sand: soil: FYM, 1:1:1) in agro shade net house for 3 to 4 weeks and then they were transferred to polybags containing soil and FYM (1:1). Plantlets were irrigated on alternate day and sprayed with MS/4 basal salts at an interval of 15 days.

## RESULTS AND DISCUSSION

### Aseptic culture establishment

Seasons had pronounced effect on *in vitro* shoot proliferation of *B. balcooa* (Agnihotri and Ansari 2000 in *B. vulgaris*). The establishment of pure cultures was influenced with the season of explant collections. In our study, spring and summer season (February to June) were comparatively better period for collection of explants, when percentage of successful initiation of



**Graph 4.** Effect of various auxins in different strength of MS media on *in vitro* rooting.

cultures was high (48 to 92%) (Table 1). Initiation response of explants in rainy seasons had also marked effect (65 to 89%), however, the levels of contamination were comparatively high (81 to 92%). Shoot length in average was high (5.2 to 5.9 cm) in rainy season followed by spring/summer (2.3 to 7.2 cm). Due to high levels of contamination in rainy season, there were difficulties in establishment of pure cultures. It has been reported that during the summer bamboo outgrowths pathogen which in turn results in viable bud response and culture establishment. Successful establishment of aseptic cultures and bud response depend on the level of contamination, physiological state of explants and also the season of collection (Saxena and Bhojwani, 1993; Ramanayake and Yakandwala, 1997; Sanjaya et al., 2005; Mishra et al., 2008; Singh et. al., 2012, Nadha et. al., 2013).

During the growing season of bamboo there is higher secretion of endogenous phytohormones which result in corresponding higher growth of the developing seedling (Cinivora and Sladsky, 1990). Earlier, Negi and Saxena (2011) have observed high aseptic cultures along with 90% bud response during July to October. However, in Bihar, July to September is the rainy season. Cultures established during those periods remain prone to microbial contamination, thereby, diminished the chances to procure aseptic cultures (Msogoya et. al., 2012; Torres et. al., 2016)). Higher level of contaminated axillary bud was also observed by Mishra et al. (2008) during the

rainy season. Influence of seasons on bud breakage in *Dendrocalamus giganteus* and *Berberis vulgaris* has also been reported (Ramanayake et al., 1995). They observed seasonal effect on bud initiation and found that February to March was the congenial period for obtaining axillary buds for cultures development (Mudoi et. al., 2009).

### Surface sterilization

In the present studies, six different procedures for treatment of explants prior to initiation (Table 2) were used. In procedure I, explants were treated with Bavistin (0.1%) for 10 min; however, in procedures II and III, explants were treated with Bavistin (0.1%) for 1 h. In procedures II and III instead of Bavistin, we used Indofil fungicide (0.1%). The percentage contamination was quite less (10%), however, the survival rates of explants were maximum (88%) when the explants were treated with Bavistin (0.1%) and Indofil (0.1%) together for 1 h (as in procedure II) (Table 2). While utilizing procedure IV, we used sodium hypochlorite instead of mercuric chloride, but in that case the percentage survival was quite less (45%) (Shroti et. al., 2012). Following procedures IV and V, we treated explants with Savlon and Teapol but in those cases microbial contaminations were quite high (87 to 90%).

It was observed, out of six ways of surface sterilization, explants first treated with (0.1%) Bavistin and (0.1%)



Indofil for 1 h followed by surface sterilization with ethanol 70% (35 s) and mercuric chloride 0.1% (6 min) along with Tween 20, were the most suitable way for aseptic sterilization and establishment of cultures (as in procedure II) resulting in 88% of uncontaminated explants.

Different procedures for surface sterilization of explants have been reported by earlier workers. Negi and Saxena (2011) utilized savlon and teepol for sterilization of explants and establishment of aseptic culture. In another experiment while culturing the nodal segments of *B. balcooa*, washing with Cetavelon (5 min) followed by 3 to 4 washing with water and subsequent treatment with 1% Bavistin (fungicide) for 5 to 7 min to avoid the problem of fungal contamination, has been reported by Arya et al. (2008). Another worker treated explants of *B. balcooa* with fungicide (Bavistin 1%) for 2 to 3 min and then the surface sterilization was made with 0.1% mercuric chloride (12 to 15 min) (Arya et al., 2006).

Different concentrations (0.05, 0.1 and 0.2%) have been observed of  $\text{HgCl}_2$  for different time durations of sterilization of explants (Graph 1). With lower concentrations (0.05%) of  $\text{HgCl}_2$ , there were comparatively very high levels of microbial load. At higher levels (0.2%) of mercuric chloride treatments, the explants became black, dried and also their survival rate was negligible. High percentage of  $\text{HgCl}_2$  treatment for more duration (10 min) had adverse impact on bud proliferation. However, 0.1%  $\text{HgCl}_2$  concentration proved to be comparatively better responsive for establishing aseptic cultures as in that case chances of survival of cultures were more (87%). Previous workers have also recommended the similar concentrations of  $\text{HgCl}_2$  as an effective sterilizing agent for bamboo species (Saxena and Bhojwani, 1991; Arya et al., 2001).

Another important aspect for establishment of aseptic culture was the diameter and length of explants. Graph 2 showed shoot initiation response, while utilizing different lengths and diameters of explants. Explants size of 3 cm with diameter of 2 mm revealed better response (95%) in shoot initiation. Higher length and diameter (5 cm and 3 to 4 mm) were least responsive (32 to 46%). This might be attributed to high rate of endogenous phenolics secreted in the medium resulting thereby in inhibition in shoot proliferation and shoot length. High rate of response in culture initiation with medium sized explants might be due to minimum leaching of phenolics in the medium (Mudoi et al., 2013). Explants of 2 to 3 mm diameter and 2.5 to 3.5 cm in length in case of *Pseudoxytenanthera stocksii* (Sanjaya et al., 2005; Somashekhar et al., 2008) and 2 to 3 cm in length in case of *B. balcooa* (Arya et al., 2006; Arya et al., 2008) have been reported by previous workers as desirable size for initiation of cultures.

Duration of ethyl alcohol (70%) treatment also affected the degree of aseptic culture establishment. When treated with 70% ethyl alcohol for 30 s, explants showed comparatively high level of contamination. Treatment of explants with ethyl alcohol (70%) for 35 s was found most



Figure 2. Initiation of *B. balcooa*.

responsive.

### Shoot initiation

Among the various concentrations and combinations of phytohormones (growth regulators) tried in our experiment (Graph 3), the combined effect of BAP and Kn (MS + Add + NAA 0.25 mg/l + BAP 2 mg/l + Kn 0.5 mg/l) resulted in 85% bud breakage (Venkatachalam et al., 2015). BAP alone showed 80% bud response (Figure 2).

A similar combined effect of two cytokinins (BAP and Kn) has been demonstrated previously in *B. balcooa* (Negi and Saxena, 2011; Das and Pal, 2005a, b), *Bambusa tulda* (Saxena, 1990), *D. giganteus* (Arya et al., 2006), *Dendrocalamus strictus* (Chowdhury et al., 2004) and *Bambusa nutans* (Choudhary et al., 2016). Cytokinin BAP was found to be most effective for shoot initiation through axillary bud proliferation. In shoot initiation experiment, we used auxin (NAA 0.25 mg/l) along with different combinations of cytokinins (BAP, Kn, and TDZ). However, Arya et al. (2008), while working on *Dendrocalamus asper* observed that there was no significant increase in shoot proliferation if auxin either NAA or IAA were added along with BAP. Increased levels of BAP, Kin and TDZ showed poor response in bud



initiation. High level of cytokinins perhaps induce programmed cell death in cell cultures resulting in yellowing of leaves and reduced root mass in intact plants (Carimi et al., 2003).

Due to exudation of phenolic compounds, it was required to transfer proliferated bud into new fresh media (after 8 to 12 days) in order to avoid the browning and leaching problems (Das and Pal, 2005a, b; Arya et al., 2008; Saxena and Bhojwani, 1993; Negi and Saxena, 2011).

Uses of Kn (1, 2 and 2.5 mg/l) showed higher number of shoots, however, growth of seedling was stunted. Contrary to other cytokinins in TDZ, there was less number of shoots, but their size was longer. Combined effect of BAP and TDZ had non-significant response.

### Solid and liquid media

MS agar gelled media resulted in dwarf and lower number of shoots (2 to 3 shoots per explant). This may be due to leaching and browning problems (Somshekhar et al., 2008). Arya and Sharma (1998) also observed leaching of exudates (phenolic) and poor growth of shoot in agar gelled medium.

However, Nadgir et al. (1984) while working on *D. strictus* found agar gelled medium comparatively better media for multiple shoot induction from nodal segments of mature plants and they observed shoots (2 to 3) within 3 to 4 weeks. High rate of shoot initiation in liquid medium compared to agar gelled medium may be attributed to easy availability and faster uptake of nutrients in liquid medium (Figure 3). Liquid cultures generally grow faster, required less hardening time (Bonga and Vongvaleskar, 1992).

Liquid media showed 90% response within 3 to 4 days, however, in semisolid media cultures responded 70% within 6 to 7 days.

### Shoot multiplication

The initiated microshoot clumps were separated from the explant and transferred to the semisolid and liquid media supplemented with different concentrations of phytohormonal compositions. As obvious (Table 3), variable effects in shoot multiplication were observed, while using different concentrations and combinations of growth regulators. BAP was found most important cytokinins for shoot multiplication in case of *B. balcooa*. High rate multiplication of shoot of *B. balcooa* in MS media (with BAP) has also been reported by previous workers (Das and Pal, 2005a, b; Mudoi and Borthakur, 2009; Arya et al., 2006). Kn alone did not show significant response in shoot multiplication (Kumar and Banerjee, 2014). When Kn (alone) was used in multiplication media, the clumps turned dry and brown. High efficacy BAP over Kn in relation to shoot



**Figure 3.** Shoot proliferation in *B. balcooa*.

multiplication has also been reported by previous workers (Bonga and Von Aderkas, 1992; Saini et al., 2016). In case of shoot multiplication, combined effect of BAP in combination with Kn and BAP in combination with TDZ did not show significant effect. However, the strong cytokinin, TDZ was found suitable for shoot multiplication.

The media supplemented with growth regulator as 1 and 2 mg/l of BAP showed remarkable effect. In case of NAA (0.1 mg/l) + BAP (1 mg/l), the shoot length was longer (6.2 cm) as compared to BAP (2 mg/l) + Kin (0.5 mg/l), where the average length was 4.9 cm. MS media was also observed with and without additives to minimize the cost of production. The media lacking additive turned brown after five days due to phenolic secretion and later showed the symptoms of necrosis of shoot clump. On the other hand, MS supplemented with additive (MS + Add + NAA 0.1 mg/l + BAP 2.5 mg/l or 1 mg/l) showed better response (Table 3). MS media supplemented with additives (Ascorbic acid, Cysteine, Citric acid) showed positive response in shoot multiplication. This might have been due to minimized phenolic exudation (Negi and Saxena, 2011; Devi and Sharma, 2009). Glutamine supplemented either in liquid or solid media had no effect on proliferation of microshoots. One remarkable feature that was observed was that microshoots in medium without supplement of NAA showed necrotized shoots and they had less multiplication rate in liquid media.



**Figure 4.** Multiplication stage of *B. balcooa*.

A comparative study was done for shoot multiplication rate in liquid and semi-solid media. High multiplication rate with comparatively longer shoots were observed in liquid media (Figure 4). However, in semi-solid media, low multiplication rate with dwarf shoots were developed. After 2 to 3 cycles of multiplication, there was drastic reduction in multiplication rate as well as in shoot length. Our findings are in conformity with the previous works (Negi and Saxena, 2011; Arya et al., 2008).

BAP (2 mg/l) in combination with Kn (0.5 mg/l) had high response in the axillary bud breakage, however, in the same combination, cytokinins were least effective for shoot multiplication. After 2 to 3 cycles in the same media, the cultures started drying of leaves and turned pale brown.

## Rooting

*In vitro* root initiation depends upon various auxin concentrations in the media. Rooting was maximum in half strength of MS media supplemented with NAA (2.5 to 5 mg/l). The root induction experiment was carried out in two strengths of MS media (MS, MS/2) along with variable concentrations of auxins (Graph 4). In full strength MS media, the percentage of root induction was low compared to MS/2 strength.

While considering different type of auxins (IBA, NAA and IAA), pronounced root induction frequency was observed in media containing different concentrations of NAA (1 and 2.5 mg/l). Auxins (NAA and IBA) were responsive in root induction; however, in IAA there was no root induction either in full strength or half strength of MS. NAA (2.5 mg/l) along with half strength of MS showed 100% rooting (Figure 5), but it was less than



**Figure 5.** *In vitro* rooting in MS containing NAA (2.5 mg/l).

50% when MS media was supplemented with IBA. Remarkably, clump become dry after seven days when MS strength was lower (half), however, after 21 days new green healthy shoots proliferated from old clumps with profuse growth of roots. In full strength of MS media,





**Figure 6.** Profuse root growth.

clumps were healthy in appearance even after 7 days, but the rooting response was poor. This might be due to sufficient uptake of nutrients by cultures in full strength MS media (Negi and Saxena, 2011).

It was observed that 28 days was enough for procuring well-developed rooted clump (Figure 6), however, earlier workers have reported 40 days duration for sufficient rooting in cultures.

Varied rooting experiments have also been done by earlier workers using different auxins. Saxena (1990) working on *B. tulda* suggested to supplement Coumarin in rooting media. Similarly, Ramanayake and Yakandwala (1997) working on *D. giganteus* and Sood et al. (2009) on *Drosera hamiltonii* reported high frequency of rooting when IBA was used in combination with Coumarin. In case of *D. strictus*, up to 90% rooting was reported in medium containing IBA (Mishra et al., 2008; Arya et al., 2001). However, well-developed roots with



**Figure 7.** Plantlets in green house.

healthy shoots were observed in half strength MS medium containing NAA (3.0 mg/l) (Goyal and Sen, 2016). IBA supplemented medium showed only 40% rooting frequency with poorly developed roots. Thus, it was studied that IAA was ineffective in root initiation.

### Hardening

After attaining the height of 5 cm, rooted plantlets were needed to be hardened and acclimatized. Among the various rooting mixtures with different ratios of sand, soil, cocopit, vermicompost, and soilrite tested (Ray and Ali, 2016), we observed suitably well-developed healthy plants in cocopit and vermicompost (2:1) and also there was low rate of mortality. Healthy rooted plantlets were transferred to seedling trays containing different types of transplanting media like sand (1:0), soilrite (1:0), sand: soil (1:1), cocopeat: vermicompost (2:1) and cocopit (1:0). Root trainers containing potting media were sprayed with MS/4 nutrient media and were maintained in the mist chamber for 2 to 3 weeks. During hardening, use of medium with reduced mineral salts forced the regenerated plantlets to rely on their own photosynthetic apparatus for nutrition (Kozai et al., 1988). Combination of cocopit and vermicompost was found congenial for survival and growth of plantlets (Figure 7). Singh et al. (2012) have reported high survival rate in combination of Dune sand + vermicompost for hardening of *D. hamiltonii*. Soilrite, perlite, vermiculite and compost as potting mixture in seedling trays were used by earlier



**Figure 8.** Seedlings in polybag in net house.

workers (Mishra et al., 2011) and soilrite was found most suitable for hardening of *B. tulda* seedlings.

Initial application of MS/2 minerals to the plantlets was found essential for better hardening and acclimatization. After 3 weeks, plants from the trays were transferred to mother bed made of the mixture of sand: soil: cow dung (Figure 8).

#### Conflicts of Interests

The authors have not declared any conflict of interests.

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#### Abbreviations

**PGR**, Plant growth regulators; **MS**, Murashige and

Skoog; **BAP**, 6-benzylaminopurine; **Kn**, kinetin; **TDZ**, thidiazurone; **IAA**, indole 3-acetic acid; **IBA**, indole 3-butyric acid; **NAA**, naphthalene acetic acid.

#### REFERENCES

- Agnihotri K, Ansari SA (2000). Adventitious rhizogenesis in relation to seasonal variation, size of culm branch, cuttings and IAA treatment in bamboo, Indian For. 126: 971-982.
- Arya S, Sharma S (1998). Micropropagation technology of Bambusa bambos through shoot proliferation. Indian For. 124:725-731
- Arya ID, Satsangi R, Arya S (2001). Rapid Micropropagation of Edible Bamboo *Dendrocalamus asper*, J. Sustain For. 14(2-3):103-114.
- Arya ID, Sharma R, Arya S, Sharma D, Chauhan S (2006). *In vitro* shoots proliferation in *Bambusa balcooa* Roxb. from nodal explants, Indian J Trop. Biodiv. 14(1):14-18.
- Arya S, Satsangi R, Arya ID(2008). Direct regeneration of shoots from immature inflorescences in *Dendrocalamus asper* (edible bamboo) leading to mass propagation. The J of Amer Bamb Soc. 21(1):14-20.
- Bonga J, Von P (1992). *In vitro* cultures of trees. Kluwer Academic Publishers, Dordrecht, 43 p.
- Choudhary AK, Ranjan A, Kumari P (2016). *In vitro* shoot proliferation for rapid and mass production of quality planting materials of *Bambusa nutans* in the climatic conditions of Bihar, India. Ind. J. Energy 5(2):1-11.
- Chowdhury P, Das M, Sikdar S, Pal A (2004). Influence of the physiological age and position of the nodal explants on

- micropropagation of the field-grown *Dendrocalamus strictus* Nees. Plant Cell Biotech and Mol Biol. 5(1&2): 45-50.
- Cinivora B, Sladky Z (1990). Stimulation of the regeneration capacity of tree shoot segment explants *in vitro*. Biol. Plant. 32:407-413.
- Das M, Pal A (2005a). *In vitro* regeneration of *Bambusa balcooa* Roxb.: Factors affecting changes of morphogenetic competence in the axillary buds. Plant Cell Tissue Organ Cult. 81(1):109-112.
- Das M, Pal A (2005b). Clonal propagation and production of genetically uniform regenerants from axillary meristem of adult bamboo. J. Plant Biochem. Biotechnol. 14:185-188.
- Devi WS, Sharma GJ (2009). *In vitro* Propagation of *Arundinaria callosa* Munro- an Edible Bamboo from Nodal Explants of Mature Plants. Open Plant Sci. J.3:35-39.
- Gantait S, Pramanik B, Banerjee M (2016). Optimization of planting materials for large scale plantation of *Bambusa balcooa* Roxb.: Influence of propagation methods. J. Saudi Soc. Agric. Sci. In Press.
- Goyal A, Sen A (2016). *In vitro* regeneration of bamboos, the "Green Gold": An overview. Ind. J. Biotechnol. 15:9-16.
- India State of Forest Report (2009). Land use statistics, Ministry of Agriculture, Govt. of India.
- Kozai, Koyama Y, Matunabe I (1988). Multiplication of potato plantlets *In vitro* with sugar free medium under high photosynthetic photon pulse. Acta Hort. 2301:121-127.
- Kumar V, Banerjee M (2014). Albino regenerants proliferation of *Dendrocalamus asper in vitro*. World J. Agric. Sci. 10(1):9-13.
- Mishra Y, Patel P, Ansari SA (2011). Acclimatization and macro proliferation of micro propagated plants of *Bambusa tulda* Roxb, Asian J. Exp. Biol. Sci. 2:498-501.
- Mishra Y, Patel PK, Yadav S, Shirin F, Ansari SA (2008). A micropropagation system for cloning of *Bambusa tulda* Roxb. Sci. Hortic. 115(3): 315-318.
- Msoyoga T, Kaniagha H, Mutigitu J, Kulebelwa M, Mamiro D (2012). Identification and management of microbial contaminations of banana *in vitro* cultures. J. Appl. Biol. 55:3987-3994.
- Mudoi Dutta K, Borthakur M (2009). *In vitro* micropropagation of *Bambusa balcooa* Roxb. through nodal explants from field grown clumps and scope for upscaling. Curr. Sci. 96(1):962-966.
- Mudoi KD, Saikia SP, Goswami A, Gogoi A, Bora D, Borthakur M (2013). Micropropagation of important bamboos: A review. Afr. J. Biotechnol. 12(20):2770-2785.
- Nadgauda RS, John CK, Mascarenhas AF (1993). Floral biology and breeding behaviour in bamboo: *Dendrocalamus strictus* Nees. Tree Physiol. 13:401-408.
- Nadha H, Kumar R, Sharma R, Anand M, Sood A (2013). *In vitro* propagation of *Dendrocalamus asper* and testing the clonal fidelity using RAPD and ISSR markers. Int. J. Curr. Res. 5(8):2060-2067.
- Nasreen S, Ananthlakshmi R, Simla G, Muthu M, Subhasini J (2015). *In vitro* standardization of explant sterilization and shoot initiation in three bamboo species *Bambusa balcooa*, *Dendrocalamus stocksii* & *Dendrocalamus asper*. Life Sci. Arch. 1(15):287-292.
- Negi D, Saxena S (2011). Micropropagation of *Bambusabalcooa* Roxb. through axillary shoot proliferation. In Vitro Cell. Dev. Biol. Plant 47(5):604-610.
- Ramanayake SMSD, Yakandawala K (1997). Micropropagation of the giant bamboo (*Dendrocalamus giganteus* Munro) from nodal explants of field grown culms. Plant Sci. 129(2):213-223.
- Ramanayake SMSD, Yakandawala K, Nilmini Deepika P and Iqbal M (1995). Studies on micropropagation of *Dendrocalamus giganteus* and *Bambusa vulgaris* var. *striata*. Bamboo, people and the Environment. Vol. I, Propagation and management INBAR Technical Report No. pp. 8: 75-85.
- Ray S, Ali N (2016). Factors influencing micropropagation of bamboo species using nodal explants: A review. Res. J. Pharm. Biol. Chem. Sci. 7(5):2877-2889.
- Saini H, Arya I, Arya S, Sharma R (2016). *In vitro* micropropagation of himalayan weeping bamboo, *Drepanostachyum falcatum*. Am. J. Plant Sci. 7:1317-1324.
- Sanjaya, Rathore TS, Ravishankar Rai V (2005). Micropropagation of *Pseudoxytenanthera stocksii* Munro. *In vitro* Cell. Dev. Biol. Plant 41(3):333-337.
- Saxena S, Bhojwani S.S. (1993). *In vitro* clonal multiplication of 4-year-old plants of the bamboo *Dendrocalamus longispathus* Kurz. In vitro Cell. Dev. Biol. Plant 29:135-142.
- Saxena S, Bhojwani SS (1991). Towards regeneration and mass propagation of bamboos through tissue culture, Bamboo in Asia and the Pacific. In: Proceedings of the fourth International Bamboo workshop, pp. 157-164.
- Sharma P, Sarma K (2011). *In vitro* propagation of *Bambusa balcooa* for a better environment. International Conference on Advances in Biotechnology and Pharmaceutical Sciences, pp. 248-252.
- Shrotri RK, Upadhyay R, Niratkar C, Singh M (2012). Micropropagation of *Dendrocalamus asper* through Inter Nodal Segment. Bull. Environ. Pharmacol. Life Sci. 1(3):58-60.
- Singh S, Dalal S, Singh R, Dhawan A, Kalia R (2012). Micropropagation of *Dendrocalamus asper* {Schult. & Schult. F.} Backer ex k. Heyne): an exotic edible bamboo. J. Plant Biochem. Biotechnol. 21(2):220-228
- Singh S, Singh R, Kalia S, Dalal S, Dhawan A, Kalia R (2013). Limitations, progress and prospects of application of biotechnological tools in improvement of bamboo - a part with extraordinary qualities. Physiol. Mol. Biol. Plants 19(1):21-41.
- Somashekhar PV, Rathore TS, Shashidhar KS (2008). Rapid and simplified method of micropropagation of *Pseudoxytenanthera stocksii*. In: Forest Biotechnology in India, Ansari S.A., Narayan C and Mandal A.K. (eds), Satish serial publishing house Delhi. Pp. 165-182.
- Torres G, Houllou L, Souza R (2016). Control of contaminants during introducing and establishment of *Bambusa vulgaris in vitro*. Res. Biotechnol. 7:58-67.
- Venkatachalam P, Kalairasi K, Sreemanan S (2015). Influence of plant growth regulators (PGRs) and various additives on *in vitro* plant propagation of *Bambusa arundinacea* (Retz.) wild: A recalcitrant bamboo species. J. Gen Eng. Biotechnol. 13(2):193-200.
- Vishwanath S, Joshi G, Somashekhar P, Rane A, Sowmya C, Joshi S (2012). *Dendrocalamus stocksii* (Munro): A potential multipurpose bamboo species for peninsular India. A Publication of Institute of Wood Science & Technology, Bangalore. IWST Technical Bulletin No. 10.
- Wei Q, Cao J, Qian W, Xu M, Li Z, Ding Y (2015). Establishment of an efficient micropropagation and callus regeneration system from the axillary buds of *Bambusa ventricosa*. Plant Cell. Tissue Org. Cult. 122(1):1-8.