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In vitro propagation of two commercially important bamboo species (*Bambusa tulda* Roxb. and *Dendrocalamus stocksii* Munro.)

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Bamboo plant is an important biodegradable raw material which can play important role in rejuvenation of Indian rural economy through positively impacting agricultural, industrial, energy and in environmental sectors. Traditional methods of vegetative propagation of this plant have insufficient multiplication rate. In the present investigation, *in vitro* bud break and aseptic culture establishment in relation to different seasons of Bihar were evaluated for large scale clonal propagation of two commercially important bamboo species, *Bambusa tulda* Roxb. and *Dendrocalamus stocksii* Munro. The experiment was conducted thrice using a completely randomized design with 10 replicate per treatment. Mean calculation was performed using Duncan's Multiple Range Test (DMRT) at P<0.05. Summer climate (April-June) was the most congenial season for initiation and establishment of aseptic cultures. 6 Benzylaminopurine (BAP) without supplementation of any additive resulted in significant bud breakage in *B. tulda*, however, in case of *D. stocksii,* Thidiazurone (TDZ) with the additives resulted in comparatively better response. For multiplication, BAP (2.5 mg/l) showed maximum number of shoots (24±6.2) in *D. stocksii*. However, in *B. tulda* high multiplication rate with adequate shoot length was observed in semi solid media supplemented with BAP (1 mg/l). For both the species, the survival rate during hardening (primary and secondary) was maximum during monsoon season. The refined *in vitro* regeneration system of two commercially important bamboo species developed is efficient and will be an impetus to raising bamboo nurseries of elite germplasm for bamboo growing areas of Bihar.

Key words: MS media, clonal propagation, *in vitro* regeneration, phytohormones, 6benzylaminopurine, thidiazurone.

INTRODUCTION

Bamboo, belongs to the highest evolved flowering family, Poaceae. It is a natural biodegradable raw material, which can play important role in rejuvenation of Indian rural economy through impacting in agricultural, industrial, energy and environmental sectors. Among the flowering plants, it is one of the highest carbon sequesters, environment friendly and suited to adapt and grow even on degraded lands (Hossain et al., 2015; Singh et al., 2020; Waghmare et al., 2021). This plant can grow very fast (0.9 to 1.2 m/day) within four to five years and can regrow after harvesting, without the need of replanting and thereby, making it a perennial renewable bio-resource. Due to its versatility, this plant is also known as green gold, poor man's timber, friends of the people and also cradle to coffin timber (Sawarkar et al., 2020; Singh et al., 2021).

The green cover in Bihar had reduced to 9% after the bifurcation of Jharkhand from Bihar state in 2000. The population density of this state is quite high (1106 people/km^2) and the average land holdings is quite low, 0.24 ha (2011 census) (India, 2021). In addition, farmers of this state also face extreme causalities both with floods and droughts every year. It was thereby, decided by the state Department of Environment, Forest and Climate Change (DEFCC) to increase the green cover from 15 to 17% and subsequently to 19% through massive development of plantations under ' Haryali Mission'. Bamboo is among one of such plants for integrating it into agriculture by planting on farm boundaries, farm-land (Agro forestry) and non-agricultural land (waste land, degraded lands and in homesteads). Bamboos can also provide farmers a perennial source of income (Hoogendoorn and Benton, 2014; Liese and Kohl, 2015).

Since time immemorial, bamboos have grown naturally but they are now also being cultivated. However, this plant has also witnessed serious depletion as is the case with other plants in the natural habitats. In order to conserve the depleting bamboo resource, the commercially important species need to be selected and propagated. However, the propagation of this plant is impeded by long flowering cycles that can last 40 to120 years, lack of seed availability and also short viability of seeds. Traditional methods of vegetative propagations (rhizome splitting, offset cuttings, branch and branch cuttings) have low multiplication rate. Also, these methods are season specific, being bulky and so inconvenient in handling and transport (Waghmare et al., 2021; Mustafa et al., 2021).

These gaps can be filled by micropropagation. Through tissue culture techniques, large scale mass clonal propagation of high quality germplasms can be made (Goyal and Sen, 2016). India possesses world's largest bamboo resources, next to China, with 136 species and 23 genera spread over sixteen millions hectares of land (Forest Survey of India, 2019).

In this study, two economically important bamboo species (*Bambusa tulda* Roxb. and *Dendrocalamus stocksii Munro.)* were selected due to their adaptability to the local climatic conditions. D. stocksii is thorn less, solid stemmed, loosely spaced, erect bamboo growing upto 9m height with the diameter ranging from 30 to 50 mm. The leaves are lanceolate (10-20 cm long) and are most suited for agroforestry as it has low canopy coverage. It is utilized for furniture, basket making, poles, stakes, crafts and also in construction. *B. tulda* is another commercially important bamboo species, with the characteristics of being tall, quick growing and with the ability to grow up to 13 to 26 m in height. It also prevents soil erosion. This bamboo has high demand as it being one of the fastest growing plants and culms are used to manufacture many handicrafts, furniture, house construction, paper, and pulp production.

Earlier work on the development of protocols for micro clonal propagation of these two species of bamboos showed challenges with rhizogenesis and hardening of the seedlings (Waikhom and Louis, 2014; Sharma and Sharma, 2013; Somashekar et al., 2018; Rajput et al., 2019).

The main objective of the study was to evaluate *in vitro* bud break and aseptic culture establishment in relation to different seasons of Bihar for above two commercially important bamboo species. Effects of different PGRs and additives in various concentrations and combinations to initiate healthy multiple shoots under *in vitro* conditions were monitored. Influence of seasons on hardening (primary and secondary) of tissue culture raised seedlings with survival efficiencies was also observed.

MATERIALS AND METHODS

Source of explants

Explants were collected from actively growing shoots of four years old healthy culms of *D. stocksii* and *B. tulda* which were brought from Institute of Wood Science and Technology (IWST) Bengaluru and were planted and maintained at Plant Tissue Culture (PTC) Lab, Tej Narayan Banaili (TNB) College campus, TilkaManjhi Bhagalpur University (TMBU) Bhagalpur (Figures 3a and 4a). The explants were collected in different seasons (winter, spring, summer and monsoons) from April 2018 to March 2019 following the methods of Saxena (1990) and Choudhary et al. (2016) and were brought to the PTC Lab, TNB College campus Bhagalpur for further processing. The study site, Bhagalpur district is located in the south of Bihar (at the 25.24 latitude and 86.98 longitude) on the bank of the river Ganga. The area is characterized by hot humid summer (36 to 18.7°C) and cool winter (23.4 to 5.2°C).

Surface sterilization

At first the shoots were surface sterilized with the help of cotton swab dipped in 70% ethanol in order to remove the dust and microorganisms. Shoots were then washed in running tap water Explants of the size of 1.5 to 3.0 cm in length were cut into pieces containing one node axillary buds. Surface sterilization procedures. of explants were also observed in order to monitor the bud breakage response and the levels of contamination. In the present investigation, four different procedures were applied for treatment of explants. Those were named as T1, T2, T3, and T4 **(Table 1).**

After each treatment, explants were rinsed with autoclaved distilled water for three to four times. Then the nodal segments were brought to the laminar flow chamber under aseptic conditions and were sterilized by treating buds with 70% ethanol (for 30 to 35 sec) followed by three rinses with autoclaved distilled water. Explants were then treated with 0.1% $HgCl_2$ for 5 min and were subsequently washed with distilled water three to four times.

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Table 1. Steps of different surface sterilization procedures of explants.

Steps	T1	T2	T3	T4
Outside Laminar	Tween2.0+ streptomycin + 0.025% $HgCl_2$ for 10 min followed by treatment with 1% bavistin for 10 min	Tween2.0 + Dettol + 0.025% $HgCl_2$ for 10 min followed by treatment with 1% bavistin for 10 min	Tween2.0+ Dettol for 10 min followed by treatment with 1% bavistin for 10 min	Tween2.0+ Dettol for 10 min followed by treatment with1% bavistin for 10 min
Inside Laminar	70% ethanol treatment for 30 s under laminar air flow and then treatment with 0.1% $HgCl_2$ for 5 min	70% ethanol treatment for 30 s under laminar air flow and then treatment with 0.1% $HgCl_2$ for 5 min	70% ethanol treatment for 30 s under laminar air flow and then treatment with 1% NaOCl for 5 min	70% ethanol treatment for 30 s under laminar air flow and then treatment with 0.1% $HgCl_2$for 5 min

Culture conditions

The modified MS media was used for shoot initiation of *D. stocksii* and *B. tulda*. The MS media (consisting of salt, vitamins and 3% sucrose) was prepared using 100 mg/l myoinositol. Different plant growth regulators (PGRs)-BAP, TDZ and NAA were added at various concentrations to the MS media, before adjustment of pH at 6.0. Then the media was autoclaved at 121°C at 6.80 kg (15 lb) for 20 min. The cultures of all growth stages were incubated under artificial physical conditions 22±2°C, 65 to 70% humidity and 16 h of photoperiod (using white fluorescent tube).

Axillary bud initiation

Following different sterilization treatments (T1, T2, T3 & T4), per cent bud breakage and contamination level in both genus (*B. tulda* and *D. stocksii*) were monitored in different seasons (winter, spring, summer and monsoon) (Table 2). Experiments were conducted thrice for each sterilization procedure for each season. 10 explants were used for one experiment and in each culture tube 15 ml nutrient media (MS+ NAA 0.1 mg/l+ BAP 1.0 mg/l) was employed. Contamination level and bud breakage per cent were calculated after 10 days of inoculation.

For monitoring the influence of various phytohormones on auxiliary bud initiation, explants were inoculated in test tubes containing 15 ml of initiation media supplemented with different concentrations of growth hormones (BAP, TDZ and NAA) alone or with additive. Three concentrations of BAP (1, 2.5 and 5 mg/l) and TDZ (0.1, 0.25 and 0.5 mg/l) were tested. Since TDZ being strong cytokinines and thereby, comparatively less concentration of this cytokinine was taken in our experiments. For observation, 10 tubes were taken for each treatment having one explant (observation unit) in each tube. The experiments were done in triplicate. For each genus a total of 360 experiment units, each time with 120 units were employed. Treatments were made in completely randomized design. After inoculation being made in nutrient media, explants were kept in culture room and observations were made after 21 days (3 weeks) onward till when the auxiliary bud broken and a number of shoots proliferated. Number of shoots was calculated by counting the shoot number in each explant. Shoot length was measured in cm by scale (Table 3).

***In vitro* shoot multiplication**

Initiated axillary buds were separated and subsequently cultured for multiple shoot proliferations. Established aseptic cultures of proliferated clumps of shoots were propagated in semi-solid (clerigel used as solidifying agent) and liquid MS media under different cytokinins concentrations BAP (1, 2.5, and 5 mg/l) and TDZ (0.1, 0.25 and 0.5 mg/l). The clumps of 5 to 10 shoots were used for *in vitro* shoots multiplication. The sub-culturing of *in vitro* raised shoots was carried out by transferring in fresh MS media at regular intervals. For liquid media, sub-culturing time intervals were kept 8 to 10 days, however, for solid media it was in between 15 and 21 days. During sub-culturing, dead parts of the plantlets were removed carefully. Also, periodical transfers were made in order to avoid browning and leaching and to maintain healthy cultures. The performance of multiplication in different concentrations of growth hormones was calculated by observing the number of shoots and shoot length.

The shoot length and shoot number were also observed when media were supplemented with different concentrations of additives, Ascorbic acid (AA), Cysteine (Cys) and Citric acid (CA) applied alone or in combinations (50 mg/l AA, 50 mg/l AA + 50 mg/l Cys, 50 mg/l AA + 25 mg/lCys. AA + 50 mg/l CA, 50 mg/l AA + 25 mg/l CA, 50 mg/l AA + 25 mg/l Cys + 25 mg/l CA).

In vitro rooting

After 5 to 6 successful passages of cycles, the well-developed clump with 3 to 4 shoots was inoculated into the MS rooting media. The *in vitro* raised shoots of adequate heights were transferred to full strength and half strength of MS media containing different concentrations of auxins such as IBA (1.0, 2.5 and 5.0 mg/l), NAA (1.0, 2.5 and 5.0 mg/l) in order to access their response on rooting. The performance of *in vitro* rooting was calculated after 4 weeks (30 days) of incubation by observing root per cent and the mean number of roots in each clump.

$$\text{Rooting (\%)} = \frac{\text{Number of clumps in which roots were present}}{\text{Number of clumps used in rooting experiment}} \times 100$$

Acclimatization

For acclimatization, *in vitro* raised bamboo plantlets seedlings were transferred into portrays containing different potting mixtures (**Figure** 1) and then they were subsequently allowed to grow inside the closed tunnel of green house for three weeks. Then the partially acclimatized plantlets were kept in green house (outside of closed tunnel) for 3 weeks. Primary hardened plantlets were subsequently transferred to the propagation bed of the Net/Shade house for a period of 45 days. The survival rate during hardening processes was observed in different seasons (winter, spring, summer and monsoons).

Experimental design and statistical analysis

The experiment was conducted thrice using a completely

Table 2. Initiation of *B. tulda* and *D. stocksii* in different seasons after following different sterilization procedures of explants.

Sterilization techniques	Contamination and bud breakage %	Winter		Spring		Summer		Monsoon	
		B. tulda	***D. stocksii***	***B. tulda***	***D. stocksii***	***B. tulda***	***D. stocksii***	***B. tulda***	***D. stocksii***
T1	Contamination%	40.3±.088	32.0±7.52	23.0±3.61	21.1±3.50	26.3±0.89	12.4±1.60	74.0±4.00	65.5±2.21
	Bud breakage%	44.3±5.17	62.7±7.48	72.3±0.88	64±0.71	76.0±1.15	82.3±1.86	45.3±1.86	72.3±3.18
T2	Contamination%	15.0±2.52	16.6±3.59	10.0±1.53	9.09±0.23	8.0±0.58	7.33±0.69	36.0±2.08	22.5±3.39
	Bud breakage%	33.3±7.54	66±7.47	80.7±2.85	70±2.00	91.7±0.88	87.33±0.98	59.3±2.73	72.6±3.17
T3	Contamination%	71.7±0.88	73.4±5.27	60.7±4.67	58.8±6.2	51.7±4.10	49.06±3.75	80.3±4.91	83.5±1.36
	Bud breakage%	19.7±0.89	44.0±2.16	45.7±2.87	63.5±4.5	53.3±7.26	62.3±5.04	30.0±4.16	54.3±6.36
T4	Contamination%	77.7±2.19	74.5±4.49	38.0±4.16	58.3±2.0	34.7±2.33	43.63±5.61	85.0±1.73	81.5±1.64
	Bud breakage%	16.3±3.28	41.2±1.88	58.7±2.03	61±8.00	62.7±5.59	56±4.72	50.0±3.06	37.3±1.86

T1, T2, T3 and T4 are the surface sterilization techniques adopted for different explants.

Table 3. Initiation in *B. tulda* and *D. stocksii*: Influence of different concentrations of cytokinins in relation to shoot number and shoot length.

Different combinations of growth hormones	***B. tulda***		***D. stocksii***	
	Number of shoots (Mean±SE)	**Shoot length (cm) (Mean±SE)**	**Number of shoots (Mean±SE)**	**Shoot length (cm) (Mean±SE)**
MS + BAP 1 mg/l + NAA 0.1 mg/l	$5.5±0.63^{ab}$	$7.16±0.27^{a}$	$2.0±0.30^{ghijk}$	$7.14±0.19^{bc}$
MS + BAP 2.5 mg/l + NAA 0.1 mg/l	$5.9±0.64^{a}$	$6.43±0.31^{ab}$	$3.8±0.25^{abcde}$	$5.56±0.26^{de}$
MS + BAP 5 mg/l + NAA 0.1 mg/l	$4.6±0.37^{abc}$	$4.1±0.30^{cde}$	$4.2±0.20^{abc}$	$4.48±0.16^{gh}$
MS + TDZ 0.1 mg/l + NAA 0.1 mg/l	$1.5±0.22^{efghi}$	$4.67±0.30^{c}$	$1.5±0.17^{jk}$	$7.19±0.29^{ab}$
MS + TDZ 0.25 mg/l + NAA 0.1 mg/l	$2.4±0.33^{def}$	$3.67±0.33^{def}$	$2.9±0.28^{cdefghi}$	$5.27±0.33^{ef}$
MS + TDZ 0.5 mg/l + NAA 0.1 mg/l	$2.1±0.59^{efg}$	$1.91±0.19^{ejk}$	$4.1±0.38^{abcd}$	$2.61±0.22^{ij}$
MS + BAP 1 mg/l + NAA 0.1 mg/l + Additives	$3.8±0.48^{cd}$	$4.53±0.36^{cd}$	$2.1±0.23^{ghij}$	$6.53±0.31^{bcd}$
MS + BAP 2.5 mg/l + NAA 0.1 mg/l + Additives	$2.9±0.62^{de}$	$3.36±0.37^{efgh}$	$4.9±0.66^{ab}$	$5.11±0.19^{efg}$
MS + BAP 5 mg/l + NAA 0.1 mg/l + Additives	$2±0.49^{efgh}$	$2.13±0.20^{ij}$	$3.3±0.54^{cdefg}$	$2.33±0.16^{ijk}$
MS + TDZ 0.1 mg/l + NAA 0.1 mg/l + Additives	$1.8±0.24^{efghi}$	$3.47±0.22^{efg}$	$3.7±0.53^{bcdef}$	$7.93±0.24^{a}$
MS + TDZ 0.25 mg/l + NAA 0.1 mg/l + Additives	$1.7±0.15^{efghij}$	$2.75±0.2^{ghi}$	$5.1±0.41^{a}$	$3.10±0.12^{l}$
MS + TDZ 5 mg/l + NAA 0.1 mg/l + Additives	$1.1±0.31^{efghijk}$	$1.95±0.13^{ijk}$	$3.1±0.55^{cdefgh}$	$2.40±0.21^{ijk}$

Values are given in mean (n =10). Values followed by different letters in superscript within the column are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

randomized design with 10 replicates per treatment. The effects of PGRs and additives on initiation and multiplication of both the species were calculated and the level of significance was determined through analysis of variance (ANOVA). Mean calculations were performed using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$.

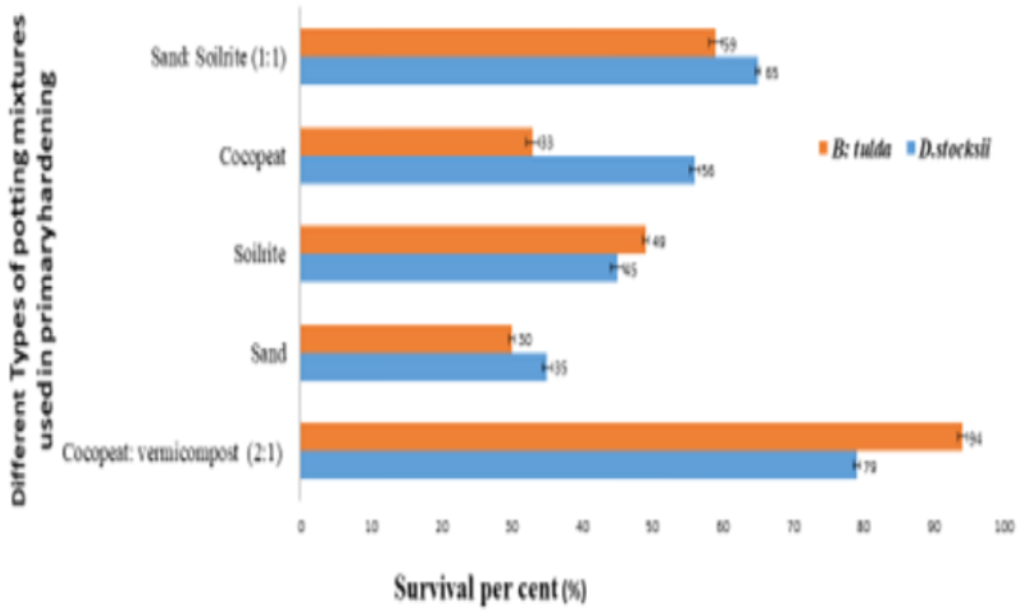

Figure 1. Primary hardening of *B. tulda* and *D. stocksii* in different potting mixture.

Evaluation of efficient sterilization techniques, axillary bud initiation and rooting per cent was made through the experimental designs as previously discussed.

RESULTS AND DISCUSSION

Initiation and establishment of *in vitro* aseptic culture

Initiation in different seasons

Seasons had marked impact on the establishment of aseptic cultures and on multiple bud breaks. In T2 treatment, there was maximum bud proliferation with least microbial contamination in all the seasons followed by treatment T1 (with the application of antibiotic streptomycin) (Tables 1 and 2). Comparatively less bud break was observed in explant treated with antibiotic as surface sterilants. Microbial contamination was the maximum during the monsoons in treatment T4 (85%) and T3 (80.3%). Earlier workers have also reported the retarded growth of explants with the applications of antibiotics (Yasodha et al., 2008; Negi and Saxena, 2011). Moreover, the treatment with antibiotics is not only cost effective but also time consuming. Negative effects of antibiotics at higher concentrations have been attributed to their toxic effects on morphogenesis (Mathias and Mukasa, 1987; Venkatasalam et al., 2013; Buckseth et al., 2017).

The rate of contamination and bud initiation percentage of both the species varied in different seasons. Summer climate (April-June) was the most congenial season for initiation and establishment of aseptic cultures. In case of *D. stocksii* the per cent bud break response was maximum (87.33%) during summer season followed by monsoons (72.6%), when they were treated as T2 sterilization technique. During the monsoons, the microbial contamination percent was comparatively high (22.5%). Microbial load was also maximum during rainy seasons (65.5, 83.5 and 81.5%) compared to other seasons. This might be due to prolonged high humidity coupled with high temperature prevailing in this locality during monsoons. In case of *B. tulda* also, the summer was the most suitable season for bud initiation due to high percent (91.7%) of bud breakage with minimal contamination level (8.0%), followed by spring season with 80.7% bud breakage and 10.0% microbial contamination (Table 2). Previous workers have also reported the influence of climatological factors on the levels of microbe contamination and establishment of aseptic cultures (Saxena and Dhawan, 1999; Kiran and Ansari, 2000; Choudhary et al., 2017; Sandhu et al., 2018). In an investigation, working on *Dendrocalamus asper*, Nadha et al. (2013) observed that the rainfall had the direct influence on contamination rate and survival per cent of explants. In *D. stocksii,* the contamination level was moderately low during winters and spring, 16.6 and 9.09%, respectively, when treated with T2 procedure. However, in the same sterilization procedure (T2), the bud proliferation percentage (66.1%) was comparatively lesser in winters compared to other seasons. It might be due to high phenol production during this season (Banerjee et al., 2011). In *B. tulda* also bud breakage percentage was less (33.3%) during winter, even after following the same sterilization technique. Browning and death are the common problems associated with the

culture of woody plants which might be attributed to the oxidation of phenolic compounds in explants tissues (Choudhary et al., 2004; Arya et al., 2008; Suwal et al., 2020).

Influence of PGR

The influence of PGRs combinations varied in both the species of bamboos. In case of *D. stocksii*, TDZ when applied along with additives resulted in high number of shoots and also high shoot length (Table 3). The maximum number (5.1±0.41) of shoots was observed in the combinations of MS + TDZ 0.25 mg/l+ NAA .1 mg/l+ additive, followed by the combinations of MS+ BAP 2.5 mg/l + NAA 0.1 mg/l + additives, with 4.9 ± 0.66 shoots. The shoot length was high (7.93±0.24) in TDZ concentration of 0.1 mg/l in combination with additives. However, *B. tulda*, TDZ as cytokinine was not very effective for bud proliferation. In this case, BAP alone at the concentration of 2.5 mg/l and 1 mg/l was found as the most suitable phytohormones for bud breakage (Table 3). The number of shoots was 5.9±0.64 at concentration of 2.5 mg/l, followed by 5.5 ± 0.63 at the concentration of 1.0 mg/l. The shoot length was adversely affected with the increase in the concentrations of the PGR. At low concentration of PGR, there was high shoot length; however, at considerably higher concentrations there was minimum length. Similar findings were also observed in case of *D. stocksii*. The high level of BAP or cytokinine has been reported to induce programmed cell death in cultures (Singh et al., 2012). Moreover, cytokinine at higher level is also known to promote ethylene biosynthesis and thereby, adversely affecting the growth in culture (Suwal et al., 2020).

Different cytokinines (BAP and TDZ) had varied responses towards both the species. The BAP without additive resulted in significant numbers of bud breakage in *B. tulda.* Contrary to this, TDZ with additives had better response in *D. stocksii*. This might be due to the endogenous level of growth hormones in the tissues of the culture as well as the extent of phenolic which might vary in different strains of the same species (Sandhu et al., 2017; Jiménez, 2005).

Previous researchers working on different bamboo species have also reported high percentage of bud breakage, when BAP was applied without additives as in *B. tulda* (Choudhary et al., 2020), *Dendrocalamus hamiltonii* (Agnihotri and Nandi, 2009; Sood et al., 2002), *Bambusa bambos* (Arya and Sharma, 1998) and *Guadua angustifolia* (Jiménez et al., 2006). However, Kabade (2009) and Beena et al. (2012) observed multiple shoot induction when TDZ was applied in combination with NAA. Somesekhar et al. (2008) working on *D. stocksii* reported the synergistic effect of BAP with NAA for high shoot induction with adequate shoot length. The endogenous auxins and the added auxins in the media might have resulted in increased total auxin levels of seedlings in cultures resulting thereby in high apical dominance and subsequent suppression of shoot proliferation. However, interaction with cytokinine might promote shoot proliferation (Rasool et al., 2009; Bin Azizan, 2017).

Shoot multiplication

Influence of PGR concentrations

After 45 days of inoculation, shoots developed well in a group of 5-20, then the clumps of 5-10 micro-shoots were transferred to different types of nutrient media (MS semi solid and liquid media) containing different concentrations of cytokinins (BAP and TDZ). The number of shoots as well as shoot lengths of *D. stocksii* and *B. tulda* cultures was evaluated subsequently after 15 to 21 days of sub culturing (Table 4). In case of *D. stocksii,* the number of shoots was maximum (24.87±0.62) in liquid media supplemented with BAP 2.5 mg/l, followed by 20.37±0.27 in the semisolid media; however, with same concentration (2.5 mg/l) of BAP. Shoot length was maximum (7.0±0.14) at the BAP concentration of 1.0 mg/l followed by 5.23±0.11 at the concentration of 2.5 mg/l of BAP. Moreover, for *D. stocksii* the liquid media supplemented with 2.5 mg/l BAP was most congenial as it resulted in maximum shoot number with adequate length (5.23±0.11). Another cytokinine (TDZ) was not very effective due to slow rate of multiplication along with dwarf shoots.

In *B. tulda,* the multiplication rate and shoot length were high in semi-solid media. The number of shoots was observed maximum (24.44±0.50) in semi solid media supplemented with BAP at 1.0 mg/l, followed by 13.44±83 shoots at BAP concentration of 2.5 mg/l.

Physical state of media (liquid/semisolid) requirements depends upon the nature of the explant used for the micropropagation. In case of *D. stocksii*, MS liquid media resulted in better response than semi solid media. Browning and leaching were comparatively lower in this media. This might be due to easier uptake of nutrients and regulators without any restrictions compared to gelling agents (Negi and Saxena, 2011). Earlier, Saxena (1990) reported multiplication of *B. tulda* in liquid media. However, in case of *B. tulda* we found semisolid media (with clerigel) as most suited for mass propagation. In liquid media, there was slower rate of multiplication after 2 to 3 cycles, which might be due to hyperhydricity (Saxena and Bhojwani, 1993; Arshad et al., 2005). Among the different cytokinines, BAP at the concentrations of 1 to 2.5 mg/l was found most suitable phytohormones for *in vitro* multiplication of bamboo shoots (Choudhary et al., 2016; Mane et al., 2020). However, concentrations above this range influenced multiplication but resulted in stunted shoot growth. Previous workers have also confirmed it while working on different species of bamboos (Arya and Arya, 1997; Bag et al., 2000; Bhadrawale et al., 2018).

Table 4. Multiplication of *B. tulda* and *D. stocksii* in different combinations of growth hormones.

Concentration of growth hormones	Types of media	*B. tulda*		*D. stocksii*	
		Number of shoots (Mean±SE)	Shoot length (cm) (Mean±SE)	Number of shoots (Mean±SE)	Shoot length (cm) (Mean±SE)
BAP 1 mg/l	Semi solid	24.44±0.50^{a}	4.72±0.46^{b}	15.23±0.17^{c}	3.35±0.17^{def}
	Liquid	11.67±0.28^{bc}	5.66±0.51^{a}	12.56±0.11^{ef}	7.01±0.14^{a}
BAP 2.5 mg/l	Semi solid	11.33±0.70^{cd}	3.02±0.18^{cdef}	20.37±0.27^{b}	2.49±0.18^{ghi}
	Liquid	13.44±0.83^{b}	3.36±0.19^{cd}	24.87±0.62^{a}	5.23±0.11^{b}
BAP 5 mg/l	Semi solid	8.22±0.90^{ef}	2.52±0.17^{fghi}	12.8±0.79^{e}	1.79±0.07^{k}
	Liquid	6.33±0.33^{ghi}	2.33±0.12^{fgh}	14.7±0.52^{cd}	2.46±0.08^{ghij}
TDZ 0.1 mg/l	Semi solid	6.86±0.35^{fgh}	2.87±0.27^{cdefg}	7.61±0.25^{ij}	3.58±0.30^{de}
	Liquid	8.78±0.618^{e}	3.49±0.05^{c}	9.7±0.29^{h}	5.04±0.18^{bc}
TDZ 0.25 mg/l	Semi solid	7.89±0.26^{efg}	2.09±0.23^{ghij}	8.1±0.23^{i}	2.96±0.19^{fg}
	Liquid	5.89±0.63^{hij}	3.23±0.17^{cde}	11.88±0.42^{efg}	3.61±0.13^{ed}
TDZ 5 mg/l	Semi solid	5.22±0.27^{hijk}	1.47±0.13^{ijk}	5.771±0.20^{k}	1.82±0.14^{k}
	Liquid	4.33±0.23^{jk}	1.68±0.06^{hijk}	6.55±0.29^{jk}	2.59±0.08^{gh}

Values are given in mean (n=10). Values followed by different letters in superscript within the column are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

Influence of additives

Influence of different types of additives and their combined effects was also evaluated. After 1 to 2 sub-culturing, cultures of *D. stocksii* became brown and died due to secretion of phenolic compounds. In order to overcome the browning and leaching of those cultured plants, the effect of various combinations of additives in different concentrations was monitored. Additives in combination of 50 mg AA + 25 mg CA + 25 Cys. mg/l resulted in maximum (13.3±0.67) shoot multiplication with high (6.69±0.18 cm) shoot length (Table 5). Our findings are in conformity with the observations of previous workers (Rathore and Ravishankar Rai, 2005; Somashekar et al., 2008; Rajput et al., 2020). However, in case of *B. tulda,* MS basal media without the addition of any additives resulted in maximum number of shoots (21.44±7.15) and shoot length (4.64±1.55 cm) (Table 5). Earlier, Thorpe et al. (1991) and Prutpongse and Gavinlertvatana (1992) have also reported micropropagation of some of the bamboo species in MS media supplemented either with cytokinine or BAP.

Rooting

In vitro rooting was observed in both the species of bamboos by adding different concentrations of auxin either in full or half strength of MS basal media. In *D. stocksii*, the half strength of MS media was suffice for *in vitro* rooting, however, in case of *B. tulda* full strength resulted comparatively in better rooting percentage (Table 6). In *D. stocksii*, rooting percentage was maximum (81%) with 4.2±0.39 roots when half strength of MS media was supplemented with NAA 2.5 mg/l (Figure 4f and g). However, in *B. tulda*, full strength of MS media containing NAA 5 mg/l resulted in maximum percent (97%) of rooting with 6.44±2.15 roots in average (Figure 3f and g). Among different auxins, NAA was the most effective hormone for root induction and growth for both species. However, previous workers reported high efficiency of rooting in IBA alone in case of *Bomarea glaucescens* (Shirin and Rana, 2007) or by supplementing combinations of auxins in *Dendrocalamus strictus* (Chaturvedi et al., 1993). Responses of phytohormones on cultures might be influenced with the nature of strains of different species. There are several reports, suggesting half MS strength as most appropriate for rooting, however, in case of *B. tulda* we found MS full strength as most suitable for *in vitro* rooting. In half strength of MS, there was profuse rooting but the mortality rate was comparatively high due to dryness and browning. This might be due to nutrient deficiency. Root induction is the high energy demanding process and thereby, the endogenous requirements of metabolic substrates might vary in different species (Yasodha et al., 2008; Sandhu et al., 2018).

In conformity with the previous reports on different species of bamboos, we observed half strength of MS media as more effective in root induction than MS full strength for *D. stocksii.* Singh et al. (2012) on *D. asper* and *D. hamiltonii*, Ramanayake and Yakandwall (1997)

Table 5. Effect of additives on shoot multiplication of *B.tulda* and *D. stocksii*.

Different concentration and combination of additives	*B. tulda*		*D. stocksii*	
	Number of shoots (Mean±SE)	Shoot length (cm) (Mean±SE)	Number of shoots (Mean±SE)	Shoot length (cm) (Mean±SE)
Without Additives	21.44±7.15^{a}	4.64±1.55^{a}	11.8±1.01^{ab}	4.93±0.16^{b}
50 mg Ascorbic acid	9.56±3.19^{b}	2.91±0.97^{b}	4.4±0.27^{ef}	3.369±0.09^{de}
50 mg Ascorbic acid + 50mg Cysteine	4.33±1.44^{defg}	2.31±0.77^{bc}	5.2±0.42^{e}	2.627±0.08^{f}
50 mg Ascorbic acid + 25mg Cysteine	8.22±2.74^{bc}	2.26±0.75^{bcde}	9.5±0.43^{cd}	3.44±0.17^{d}
50 mg Ascorbic acid + 50 mg Citricacid	4.67±1.56^{def}	2.28±0.76^{bcd}	4.9±0.28^{ef}	2.6±0.10^{f}
50 mg ascorbic acid + 25 mg citric acid	5.78±1.93^{cde}	1.79±0.66^{cdef}	10.78±1.23^{bc}	4.4±0.09^{c}
50 mg Ascorbic acid + 25 mg Cysteine+ 25 mg Citric acid	6.33±2.11^{cd}	1.60±0.53^{f}	13.3±0.67^{a}	6.69±0.18^{a}

Values are given in mean (n=10). Values followed by different letters in superscript within the column are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

Table 6. Effect of different auxins in rooting (%) and numbers of roots of *B. tulda* and *D. stocksii*.

Auxins concentration	*B. tulda*		*D. stocksii*	
	Rooting (%)	Number of roots in clump (Mean±SE)	Rooting (%)	Number of roots in clump (Mean±SE)
MS + NAA (1.0 mg/l)	43	3±0.17^{d}	42	0.5±0.17^{efghij}
MS + NAA (2.5 mg/l)	75	4.11±0.20^{b}	76	1.5±0.27^{c}
MS + NAA (5.0 mg/l)	97	6.44±0.53^{c}	65	1.3±0.26^{cde}
MS + IBA (1.0 mg/l)	11	0.78±0.22^{ghijk}	17	0.4±0.16^{ghijk}
MS + IBA (2.5 mg/l)	41	1.56±0.24^{fg}	54	1.4±0.22^{cd}
MS + IBA (5.0 mg/l)	45	1.89 ±0.11^{f}	40	1±0.30^{cdefg}
MS/2 + NAA (1.0 mg/l)	33	1.44±0.18^{fgh}	72	0.9±0.10^{defgh}
MS/2 + NAA (2.5 mg/l)	41	2.67±0.25^{de}	81	4.2±0.39^{a}
MS/2 + NAA (5.0 mg/l)	68	3.56±0.24^{bc}	73	2.5±0.34^{b}
MS/2 + IBA (1.0 mg/l)	12	0.44±0.24^{jk}	23	0.3±0.15^{hijk}
MS/2+ IBA(2.5 mg/l)	24	1.11±0.35^{fghij}	60	1.2±0.20^{cdef}
MS/2+ IBA(5.0 mg/l)	30	1.33±0.17^{fghi}	40	0.8±0.36^{cdefghi}

Values are given in mean (n=10). Values followed by different letters in superscript within the column are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

on *Dendrocalamu giganteus*, Rathore and Ravishankar Rai (2005) on *D. stocksii*. Somasheker et al. (2008) found rooting in one fourth strength of MS media. The low strength of MS media create partial nutrient stress (Singh et al., 2012) and also provide low osmotic potential resulting thereby, plantlets to produce more roots, early adaptation during acclimatization and to induce them to become autotrophic (Arab et al., 2018).

Acclimatization and field transfer

Primary hardening

Well developed *in vitro* rooted plantlets were transferred to greenhouse under closed tunnel. The development and growth of plants were monitored in five different potting mixtures (cocopeat + vermicompost; sand; soilrite; cocopeat; sand + soilrite) for primary hardening. The use of 2:1 ratio of cocopeat and vermicompost was found most suitable potting mixture with high survival rate (79% in *D. stocksii* and 94% in *B. tulda*) for both species (Figure 1). The vermicompost being used in potting mixture was effective in providing high porosity and better aeration for root growth (Singh et al., 2012).

Secondary hardening and field transfer

Influence of seasons on survival of seedlings in hardening

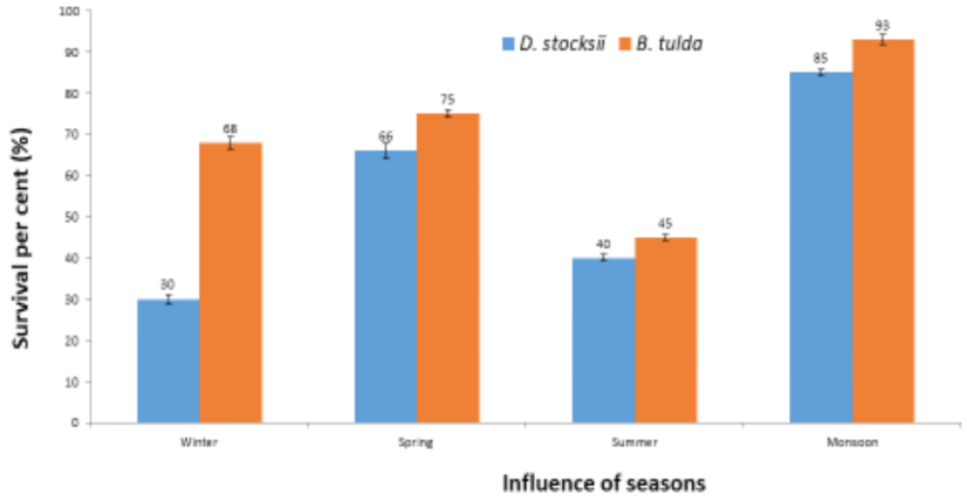

Figure 2. Secondary hardening of *B. tulda* and *D. stocksii* in different seasons.

Figure 3. *In vitro* propagation of *B. tulda* using axillary node explants: (a) Source of explants, (b) Explants used for initiation, (c) One explant inoculated in MS media (MS+NAA 0.1 mg/l+BAP 1.0 mg/l), (d) Bud proliferation, (e) Multiple shoot regeneration, (f &g) Regenerated plantlets with well-developed roots, (h) Partially acclimatized plants in green house, (i) Fully acclimatized plant in shade house, and (j) One years old tissue culture raised plants in field.

was also monitored. After 30 to 45 days of primary hardening, plants were transferred to the Net or Shade house on mother bed constituting of sand and cow dung (1:1). For both species of bamboos, survival rate was maximum during monsoons (93% in *B. tulda* and 85 % *D. stocksii*) (Figure 2). High survival rate in hardening during July and August has also been reported earlier on different bamboos (Mishra et al., 2008; Singh et al., 2012,

Figure 4. *In vitro* propagation of *D. stocksii* using axillary node explants: (a) Source of explants, (b) Explants used for initiation, (c) One explant inoculated in MS media (MS+NAA 0.1 mg/l+BAP 1.0 mg/l), (d) Bud proliferation, (e) Multiple shoot regeneration, (f & g) Regenerated plantlets with well-developed roots, (h) Partially acclimatized plants in green house, (i) Fully acclimatized plant in shade house, and (j) One years old tissue culture raised plants in field.

2021). With the important variables taken into account, the study reveals about the large scale mass clonal propagation of two important bamboo species (*B. tulda* and *D. stocksii*). These findings can be helpful for industrial adoption of *in vitro* propagation technology for large scale commercial production.

However, in tissue culture raised plants, heterogeneity to some extent limit the purpose of *in vitro* propagation system. Therefore, it is advisable to test clonal fidelity after 10 sub culturing cycles. In our investigations, we followed up to 9-10 cycles of sub-culturing during multiplication. Axillary branching is, however, least susceptible to soma clonal variations (Negi and Sexena, 2011).

Conclusion

Because of the long flowering cycles of *B. tulda* and *D. stocksii* and the limitations of vegetative propagation, the difficulties are confronted with regards to the supply and the growing demand of these two commercially important bamboo species. In the present investigation, an efficient refined protocol for large scale mass clonal propagation, multiplication with high rooting and acclimatization in the soil with abundant growth performance has been developed. These findings can be helpful for industrial adoption of *in vitro* propagation technology for large scale production of high quality planting materials (true to the type). These two bamboo species have tremendous potential to develop agro industrialization in rural areas and to bring marginal lands into use.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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