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Development Of An Efficient Technique For *In Vitro* Clonal Propagation Of *Bambusa Tulda*

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Abstract

***Bambusa tulda* is a highly preferred bamboo species in North East India for its various utilities. To meet the demand of large number of quality propagules, an attempt was undertaken to develop an effecient technique for *in-vitro* clonal propagation of superior germplasm of *B. tulda*. Highest induction of multiple shoot from nodal explants was observed in liquid MS medium modified with BAP (2 mg/L) and Kinetin (0.5 mg/L). After detachment of mother node from the regenerated multiple shoots, BAP (1 mg/L) along with Kinetin (0.25 mg/L) produced maximum number of shoots per clump.100% root induction was achieved in ½ strength MS supplemented with 4 mg/L IBA, 10 mg/L coumarin and 75 mg/L putrescine. The plants were successfully hardened and more than 95% survivability was recorded in polybags after three months.**

Key words: *Bambusa tulda*; $HgCl_2$; Coumarin; Putrescine; vermiculite

Introduction

Bambusa tulda is one of the most economically important multipurpose bamboos in India. It is used for construction, pulping, handicrafts and numerous other traditional purposes. In recent days, *B. tulda* has become one of the most sought after species of bamboo for plantation in different parts of India. The escalated demand for quality propagules of *B. tulda* cannot be met using conventional propagation techniques alone. In this paper, a highly efficient and cost effective micropropagation technique is reported. Though, few reports on micropropagation of *B. tulda* from mature clump exist [Saxena, 1990; Mishra *et al.*, 2008; Sharma and Sarma, 2013; Mishra *et al*, 2017], this is the first report where 100% rooting has been achieved.

Materials and Methods

Explant collection, preparation and surface sterilization

Actively growing lateral branches of a selected superior clump of *B. tulda* with unsprouted buds were collected from the germplasm bank of Rain Forest Research Institute, Assam. After removing the leaves and leaf sheaths, the branches were wiped with cotton swab dipped in 70% Ethanol. The branches were cut into 3-4 cm long pieces, each containing a single node, to use as explants and were then washed with 1% (V/V) solution of Tween 20 for five minutes in a bottle. Thereafter, explants were pre-trated in a solution containing Bavistin and Indofil M – 45 (0.5% each) for one hour and then washed with sterile distilled water. Finally, explants were surface sterilized with 0.1% $HgCl_2$ solution for 10 minutes followed by rinsing 5 times with sterile distilled water. The inoculated culture materials were kept in growth room in 25±2°C temperature with 16-hour light period of 2000 Lux.

Experimnts On Shoot Multiplication

Effect of cytokinins for multiple shoot induction from nodal explant

To optimize an efficient shoot initiation medium, nodal explants were inoculated in 16 different treatments of MS liquid medium fortified with 8 doses of Benzylaminopurine *i.e*. BAP (0.5 to 4.0 mg/L) alone and in combination of 0.25 mg/L

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kinetin. Hormone free liquid MS medium was kept as control. Observation on number of shoot per explants and shoot length was recorded.

Effect of cytokinins on shoot multiplication in shoot clumps without node

After 6 weeks of culture, the initiated shoots from axillary buds were detached from the mother nodes. The shoots thus produced were separated into 2-3 number of clusters and transferred to the medium supplemented with 8 different doses of BAP (1.0-4.0 mg/L) alone and in combination with Kinetin (0.25 mg/L) to assess multiplication rate of healthy shoots.

Experiments on in vitro rooting:

For rooting induction, shoot clumps of 3-4 shoots were isolated from shoot clumps by excision at the base. Clumps of 3-4 shoots were used for different experiments. For optimization of rooting process, experiments to study the effect of different auxins, use of additives, strength of media and concentration of agar were assessed. For all the experiments, data on number of roots generated per clump and their length were recorded after three weeks.

Effect of auxins on root induction:

Three auxins 1-Naphthaleneacetic acid (NAA), Indole-3-butyric acid (IBA) & Indole-3-acetic acid (IAA) were used singly in MS media (semi solid) to assess their effect on rooting. Each of the auxins was used in 4 different concentrations (1, 2, 3 & 4 mg/L). Observations on response (percentage of rooting), number of root produced and length of root were recorded.

Effect of additives on enhancement of rooting:

Some additives are known to have synergistic effect on rooting. Therefore, two additives coumarin (5, 10, 15 & 20 mg/L) and putrescine (25, 50 & 75 mg/L) were used in 16 different concentrations and combinations to assess their effect. MS media fortified with 4.0 mg/L IBA was used.

Effect of various media strength on rooting:

Effect of different concentration of agar on rooting in four different strength (Full, 1/2, 1/3 and 1/4) of MS media fortified with 4.0 mg/L IBA were experimented to ascertain the optimum strength of the media for rooting.

Effect of agar concentration on rooting:

In order to find out the best concentration of agar for rooting medium five different doses (4-8 gm/L) were used in the 1/2 MS medium supplemented with 4.0 mg/L IBA, coumarin (10 mg/L) and putrescine (75 mg/L).

Hardening

Healthy plants with well developed roots were taken out from the culture vessels and washed thoroughly in running tap water to remove traces of agar. These plantlets were planted in plastic trays (50x30x20 cm^3) filled with vermiculite and kept under shade covered with polythene sheets to maintain the humidity around 80% for initial 7 days. The plants were watered at 3-4 days interval. 1/4 strength liquid MS (Macro and Micro salts without organics) was applied to the plants at 15 days interval. After one month, the plants were taken out of the trays and transplanted in pots containing sand: soil: FYM (farm yard manure) in 1:1:1 ratio and kept under agro shade net for another three months for secondary hardening. Regular watering was done and mortality percentage was recorded.

Experimental Design and data analysis:

Experiments with nodal explants were carried out in culture tubes with one explant each (one replicate). Total 18 replicates were used in the studies of surface sterilization and shoot multiplication in nodal explants. Experiment for shoot multiplication studies after removal of the mother node were carried out in 400 ml culture jam bottles (one replicate) each carrying 4 shoot clumps (4-5 shoots/clump). 6 replications were kept against each treatment. For *in vitro* rooting studies, four culture bottles (replications) each with four shoot clumps (3-4 shoots/clump) were used.

The data were analyzed with 2-way ANOVA and the best treatments medium were determined using Student-Newman-Keuls test at 5% probability level.

Results and Discussion

Effect of cytokinins for multiple shoot induction from nodal explant

All the treatments including control induced multiple shoots (Table 1). However, among the different treatments of cytokinin (BAP& Kinetin), 2 mg/L BAP in combination with kinetin (0.25mg/L) was found to be the most effective in producing highest number of shoots per node (5.33) with maximum shoot length (4.70 cm). It was also observed that BAP in combination with kinetin produced better result than BAP alone in all treatments.

Synergistic effect of two cytokinins in *Bambusa tulda* has been demonstrated earlier by Saxena and Bhojwani, [1991]. Combined effect of two cytokinins were also found to be more effective in inducing multiple buds from nodal explants in some other species of Bamboo *viz. B. balcooa* [Das and Pal, 2005], *D. longispathus, B. vulgaris,* [Saxena and Bhojwani, 1993] and *Dendrocalamus giganteus* [Ramanayake and Yakandawala, 1997]. But Mishra *et al.* [2008] carried out bud breaking and shoot multiplication of *B. tulda* in MS liquid medium fortified with both cytokinin (10 µM BA) and auxin (0.1 µM IAA).

Table 1: Effect of Cytokinin on shoot multiplication from nodal explants of *Bambusa tulda* after two weeks of culture

Treatment No.	Cytokinins (mg/L)	Shoot No. per Node	Shoot Length (cm)
T1	Control (Hormone free)	0.50^{f}	0.47^{e}
T2	0.5 BAP	0.67^{f}	0.69^{e}
T3	1.0 BAP	1.50^{ef}	1.24^{de}
T4	1.5 BAP	1.78^{de}	1.97^{cd}
T5	2.0 BAP	4.28^{b}	2.04^{cd}
T6	2.5 BAP	3.78^{bc}	2.08^{cd}
T7	3.0 BAP	2.72^{cde}	3.17^{bc}
T8	3.5 BAP	2.61^{cde}	3.16^{bc}
T9	4.0 BAP	2.39^{cde}	2.68^{bc}
T10	0.5 BAP + 0.25 Kinetin	2.61^{cde}	2.91^{bc}
T11	1.0 BAP + 0.25 Kinetin	2.89^{bcd}	2.97^{bc}
T12	1.5 BAP + 0.25 Kinetin	3.33^{bc}	2.52^{bc}
T13	2.0 BAP + 0.25 Kinetin	5.33^{a}	4.70^{a}
T14	2.5 BAP + 0.25 Kinetin	3.39^{bc}	3.01^{bc}
T15	3.0 BAP + 0.25 Kinetin	2.72^{cde}	3.42^{bc}
T16	3.5 BAP + 0.25 Kinetin	2.56^{cde}	3.60^{b}
T17	4.0 BAP + 0.25 Kinetin	2.39^{cde}	3.04^{bc}
SEd		0.43	0.46
CD		0.71	0.76
P- value		3.18E-30	

Treatments followed by different letters are significantly different from each other.
Numbers of replicates for each treatment were 18 and each replicate consisted of single nodal explants.
SEd: Standard Error of Difference at 5% probability level & CD: Critical Difference at 5% probability level.

Effect of cytokinins on shoot multiplication in the shoot clumps

Among the different hormonal combinations BAP (1 mg/L) along with Kinetin (0.25 mg/L) produced maximum number of shoots per clump (13.83) with highest shoot length (6.13 cm). It was also observed that all the treatments with BAP in combination with kinetin produced better result than treatments with BAP alone in terms of both shoot number and shoot length **(Table 2**).

Saxena and Bhojwani [1991] reported better shoot multiplication of *B. tulda* from shoot clumps in media containing BAP and IBA whereas Mishra *et al.,* [2008] reported best shoot multiplication in MS liquid medium supplemented with 100 µM glutamine +0.1 µM IAA + 12 µM BA. This variation may be due to use of different clones or ecotypes in the studies.

Table 2: Effect of cytokinin on shoot multiplication in the shoot clumps (4-5 shoots) after detachment of the node

Treatment No.	Cytokinin (mg/L)	Shoot number./clump	Shoot length (cm)
T1	Control (Hormone free)	7.08^{e}	3.00^{e}
T2	1.0 BAP	11.29^{b}	5.15^{b}
T3	2.0 BAP	9.67^{c}	4.86^{c}
T4	3.0 BAP	8.38^{d}	4.78^{c}
T5	4.0 BAP	8.67^{d}	4.64^{d}
T6	1.0 BAP + 0.25 Kinetin	13.83^{a}	6.13^{a}
T7	2.0 BAP + 0.25 Kinetin	10.96^{b}	5.24^{b}
T8	3.0 BAP + 0.25 Kinetin	9.83^{c}	4.95^{c}
T9	4.0 BAP + 0.25 Kinetin	8.96^{cd}	4.78^{c}
SEd		0.36202	0.0604
CD		0.60964	0.1018
P - value		8.4E-20	5E-37

Treatments followed by different letters are significantly different from each other.
Numbers of replicates for each treatment were 6 and each replicate consisted of 4 shoot clumps.
SEd: Standard Error of difference at 5% probability level & CD: Critical Difference at 5% probability level

Effect of auxins on root induction

In our study efficacy of three root promoting auxins *viz.* IBA, NAA and IAA were evaluated. All these three hormones at all different doses induced roots under *in vitro* condition. However, maximum root number was produced in IBA (4 mg/L). However, this treatment was statistically at par with 6 other treatments in terms of root length (**Table 3**).

Sharma and Sarma [2013] reported the best rooting was obtained in NAA but no root was initiated in IBA and IAA. This may be due to other factors like genotype, type of shoot selected for rooting etc. Suitability of IBA for *in vitro* rhizogenesis is well established in many other bamboo species. Bag *et al.,* (2000) have obtained 100% rooting in microshoots of *Thamnocalamus spathiflorus* on IBA supplemented medium. Similarly, Ravikumar *et al.* [1998] and Arya *et al.,* [2001] have obtained about 90% and 95% respectively rooting with addition of IBA in *Dendrocalcmus strictus* and *D. asper* respectively.

Table 3: Effect of auxins on root induction from *in vitro* multiplied shoots

Treatment No.	Auxins (mg/L)	Response (%)	Root number/clump	Root length (cm)
T1	Control	0.00	0.00^{e}	0.00^{d}
T2	1.0 NAA	50.00	1.25^{d}	1.30^{bc}
T3	2.0 NAA	56.25	1.50^{cd}	1.47^{bc}
T4	3.0 NAA	62.50	1.75^{cd}	1.62^{abc}
T5	4.0 NAA	62.50	2.31^{bc}	1.74^{abc}
T6	1.0 IBA	50.00	1.25^{d}	1.50^{bc}
T7	2.0 IBA	62.50	1.81^{cd}	1.76^{abc}
T8	3.0 IBA	68.75	2.81^{b}	2.03^{ab}
T9	4.0 IBA	75.00	5.06^{a}	2.29^{a}
T10	1.0 IAA	56.25	1.12^{d}	1.15^{c}
T11	2.0 IAA	56.25	1.62^{cd}	1.54^{bc}
T12	3.0 IAA	68.75	1.37^{cd}	1.63^{abc}
T13	4.0 IAA	62.50	1.93^{cd}	1.74^{abc}
SEd			0.3	0.22
CD			0.509	0.37
P value			3.55E-15	3E-09

Treatments followed by different letters are significantly different from each other.
Numbers of replicates for each treatment were 4 and each replicate consisted of 4 shoot clumps.
SEd: Standard Error of difference at 5% probability level & CD: Critical Difference at 5% probability level.

Effect of additives on rooting

Supplementation of medium with additional substance *i.e.* additives sometimes yield synergistic influence in plant tissue culture. We have tested two additives coumarin and putrescine in our study to assess their root promoting effects. It was found that coumarin and putrescine had a significantly better root promoting effect in combination with 4 mg/L IBA than 4 mg/L IBA alone (**Table 4**).

In *Bambusa tulda,* Mishra *et al.* [2008] reported 98% rooting in liquid MS supplemented with 40 µM coumarin alone and Saxena [1990] reported more than 90% rooting in MS media with IAA ($1x10^{-5}$M) and coumarin ($6.8x10^{-5}$M). Ramanayake and Yakandawala, [1997] reported 71.5% rooting on 1/2 MS with 3 mg/L IBA and 10 mg/L coumarin in 27 days in *Dendrocalamus giganteus.* Saxena and Bhojwani, [1993] reported rooting (73%) in *D. longispathus* on 1/2 MS+IAA (10 µM) + coumarin (68 µM) within 14 – 21 days.

Table 4: Effect of additives on root induction from *in vitro* multiplied shoots

Treatment No.	Additives (mg/L)	Response (%)	Root No./clump	Root Length (cm)
T1	4 IBA	75.00	5.06^{b}	1.61^{bc}
T2	4 IBA+5 C	75.00	5.38^{b}	1.57^{c}
T3	4 IBA+10 C	75.00	5.63^{b}	1.75^{abc}
T4	4 IBA+15 C	75.00	6.13^{b}	1.89^{abc}
T5	4 IBA+20 C	81.25	6.81^{b}	2.17^{abc}
T6	4 IBA+5 C+25 P	75.00	6.44^{b}	2.09^{abc}
T7	4 IBA+10 C + 25 P	81.25	7.06^{b}	2.35^{abc}
T8	4 IBA + 15 C+ 25 P	75.00	7.06^{b}	2.24^{abc}
T9	4 IBA + 20 C + 25 P	81.25	7.69^{b}	2.43^{abc}
T10	4 IBA + 5 C + 50 P	75.00	7.25^{b}	2.34^{abc}
T11	4 IBA + 10 C + 50 P	81.25	8.00^{b}	2.61^{abc}
T12	4 IBA + 15 C + 50 P	81.25	8.06^{b}	2.65^{ab}
T13	4 IBA + 20 C + 50 P	81.25	8.31^{b}	2.70^{a}
T14	4 IBA + 5 C + 75 P	75.00	8.13^{b}	2.58^{abc}
T15	4 IBA + 10 C + 75 P	87.50	11.00^{a}	2.77^{a}
T16	4 IBA + 15 C + 75 P	81.25	7.69^{b}	2.23^{abc}
T17	4 IBA + 20 C + 75 P	81.25	6.69^{b}	2.21^{abc}
SEd			0.9034	0.3013
CD			1.514	0.505
P-value			4.4E-29	3.38E-37

C = Coumarin & P = Putrescine

Treatments followed by different letters are significantly different from each other.

Numbers of replicates for each treatment were 4 and each replicate consisted of 4 explants.

SEd: Standard Error of difference at 5% probability level & CD: Critical Difference at 5% probability level.

Effect of various strength of MS medium on rooting

In our comparative study, rooting was achieved in all the concentration. However, highest percentage was achieved in ½ strength MS supplemented with 4mg/L IBA, 10 mg/L coumarin and 75 mg/L putrescine (**Table 5**).

There are several report, where different species of bamboo has been rooted in ½ strength MS successfully. In contrast several species have been rooted in full MS strength successfully. Under field conditions, reduced nutrient strength signals the cuttings to induce roots so that the plant is equipped to survive. Reduced ionic concentration in ½ strength MS, might have simulated similar condition and attributed towards better rooting performance. However, further reduction of ionic strength of salts in 1/3 and 1/4 MS did not yield good result probably because of reduction of nutrient level beyond optimum level.

Table 5: Effect of different strength of media on root induction from *in vitro* multiplied shoots

Treatment No.	MS Salt Strength	Response (%)	Root No./clump	Root Length (cm)
T1	1/4 Strength	81.25	3.69^{c}	2.69^{c}
T2	1/3 Strength	87.50	5.19^{bc}	3.27^{b}
T3	1/2 Strength	100.00	7.44^{a}	4.11^{a}
T4	Full Strength	93.75	6.50^{ab}	3.05^{bc}
SEd			0.71	0.19
CD			1.299	0.341
P-Value			.00261	0.00021

Treatments followed by different letters are significantly different from each other.
Numbers of replicates for each treatment were 4 and each replicate consisted of 4 explants.
SEd: Standard Error of difference at 5% probability level & CD: Critical Difference at 5% probability level

Effect of gel strength on rooting

Gel strength has a correlation with rooting process. Though 100% rooting was observed in all the levels, better root growth in terms of root number and length was observed at 6 gm/L compared to the normal rate of 8 gm/L. Probably, lowering the agar might have provided better root aeration due to which better growth was observed (**Table 6**).

Table 6: Effect of different concentrations of Agar on root induction from *in vitro* multiplied shoots

Treatment No.	Agar (gm/L)	Response (%)	Root number / clump	Root Length (cm)
T1	5.00	100	3.87^{c}	3.05^{b}
T2	6.00	100	7.68^{a}	4.10^{a}
T3	7.00	100	6.75^{b}	3.81^{a}
T4	8.00	100	6.75^{b}	3.62^{ab}
SEd			0.132	0.264
CD			0.243	0.485
P-value			2.09E-09	0.019

Treatments followed by different letters are significantly different from each other.
Numbers of replicates for each treatment were 4and each replicate consisted of 4 shoot clumps.
SEd: Standard Error of difference at 5% probability level & CD: Critical Difference at 5% probability level

Hardening

Primary hardening done in vermiculite (**Fig.1; Plate I**) promotes maximum root growth since it is well aerated and retains moisture and nutrients. Plantlets were healthy and there was no symptom of deficiency during primary hardening and survivability was 100%. After one month, the plantlets were transplanted to polypots containing soil as secondary hardening. During this period average height was increased from 11.8 cm (at transplanting) to 53.7 cm after three months (average of 20 plantlets). New tillers were observed emerging after 15 days of transplanting. Average tiller number also increased from initial 1.7 to 3.5 per propagules in three months. In pots more than 95% survivability was observed after 3 months of transplanting.

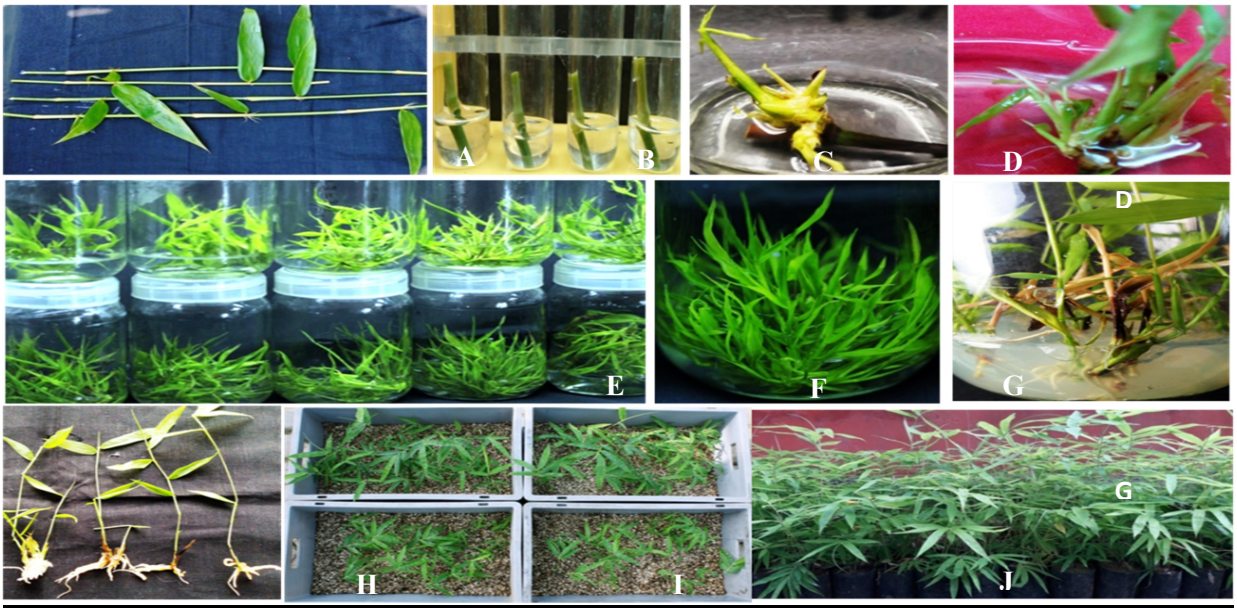

Figure 1: (Plate: A-B) Initiation of axillary bud of *B. tulda*; **(Plate: C-D)** Induction of multiple shoots from nodal explants in liquid Medium; **(Plate: E-F)** Multiplication of shoots in liquid Medium; **(Plate: G-H)** *In-vitro* root induction in semi-solid media; **(Plate: I)** Primary hardening of *in vitro* regenerated plantlets in vermiculite; **(Plate: J)** Secondary hardening in pots.

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