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In vitro regeneration of an indigenous bamboo (*Bambusa nutans*) from internode and leaf explant

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Abstract—Organogenic callus was successfully induced from pre-injured single shoots derived from *in vitro* multiplying cultures of *Bambusa nutans*. Plant growth regulator combinations of 2,4-D (5 μM), BAP (2.5 μM) and ABA (1 μM) proved to be more efficient in inducing callus, in 79.93% of cultures with an average of 346.13 mg of fresh weight callus, than auxins used alone or in combination with cytokinins. Induced callus was subsequently proliferated at a faster rate on multiplication medium (MS) supplemented with 2,4-D (5 μM) and BAP (2.5 μM). Organogenic callus was subsequently transferred to shoot regeneration medium. Efficient regeneration of shoot buds and their conversion into shoots was recorded on MS medium supplemented with BAP (5 μM) and NAA (1.25 μM) on which 18.11 buds were induced which proliferated into 10.31 shoots. Spontaneous regeneration of roots on shoots was evidenced on regeneration medium itself.

Key words: *Bambusa nutans*; organogenesis; pre-injury; ABA; shoot bud regeneration; spontaneous rooting.

ABBREVIATIONS

2,4-D	2,4-dichlorophenoxy acetic acid
NAA	α-napthaleneacetic acid
BAP	6-benzylaminopurine
ABA	abscisic acid
MS	Murashige and Skoog (1962)

INTRODUCTION

Bambusa nutans is a naturally occurring bamboo species in sub-Himalayan regions of India from Uttaranchal to Arunachal Pradesh at an altitude of 600–1500 m. It

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is also found in North Western India, Orissa and West Bengal. *B. nutans* is a sympodial bamboo and produces the highest number of culms per clump [1]. Its culms are strong and straight and are used as poles. *B. nutans* plays a significant role in the rural and industrial economies of India. It meets the needs of timber, fuel, and food requirement of villagers. Industries exploit it as a source of fibre and pulp for manufacturing particleboard and paper. However, using conventional methods of propagation (seed and rhizome cutting) it will not be possible to provide a continuous supply of planting material for industrial plantations, afforestation and conservation. *In vitro* propagation techniques can provide alternative means for rapid propagation of this economically important bamboo species.

Organogenesis provides an efficient system for propagation and genetic improvement of plants. Regeneration in bamboos through adventitious shoot formation via callus phase (indirect organogenesis) is limited to a few species such as *B. multiplex* and *Phyllostachys aurea* [2], *Dendrocalamus strictus* [3], *B. flexicosa*, *B. oldhamii*, *D. latiflorus*, *D. asper* [4] and *B. vulgaris* [5]. A variety of explants has been used for inducing organogenic callus, *viz.*, shoot apices [2], leaf and node explants [4] and mature shoots [5]. In the present communication, successful adventitious shoot formation through organogenesis from callus is being reported and is probably the first report on regeneration of *B. nutans* through callus. To date, *in vitro* propagation of *B. nutans* has been achieved using seeds as starting material [6].

MATERIALS AND METHODS

Proliferating shoots from established cultures of *B. nutans* were used as explants. These cultures were raised from nodal segments of mature clumps (40 years old) and maintained on multiplication medium, MS [7], supplemented with 3.75 μM 6-benzylaminopurine (BAP). Explants were given 10–15 incisions along the surface and placed in horizontal orientation on the medium. In all the experiments, MS medium with 3% (w/v) sucrose and 0.8% agar (BDH) was used. The pH of all media was adjusted to 5.8 before autoclaving at 1.02 kg/cm^2 pressure and temperature of 121°C for 20 min and dispensed into pre-sterilized disposable Petri plates (Tarsons).

Callogenesis

To assess the effect of auxin in callus induction, explants were cultured on MS medium supplemented with different concentrations (0, 2.5, 5.0, 7.5, 10.0 or 12.5 μM) of either 2,4-dichlorophenoxyacetic acid (2,4-D) or α-naphthaleneacetic acid (NAA). To improve callogenic response BAP or kinetin (2.5 μM or 5 μM) were added individually to MS medium supplemented with 2,4-D (2.5, 5.0, 7.5 or 10 μM). In order to further augment callogenic response abscisic acid (ABA; 0, 1, 2, 3 μM) was added to the autoclaved medium supplemented with 2,4-D (2.5, 5.0, 7.5 μM) and BAP (2.5 μM) in the laminar air flow chamber. Callus was subcultured on MS medium supplemented with BAP (2.5 μM) in combination with either 2,4-D or NAA (2.5–10 μM) for proliferation.

Shoot regeneration

The *in vitro* multiplied callus was cultured on regeneration medium supplemented with varying concentrations (2.5, 5.0, 7.5, 10.0 or 12.5 μM) of BAP or kinetin. To enhance shoot regeneration NAA (0, 1.25, 2.5 μM) was supplemented to medium with BAP (2.5, 5.0 and 7.5 μM). All the cultures were maintained at 25 $\pm$ 1°C and given long day (16 h/8 h day/night) condition under a light intensity of 1600 lux. Microscopic studies were carried out using a Nikon stereozoom microscope (Model SMZ-U) to determine the type of callus and weight of the callus was recorded using a balance (Afcoset) in a laminar air flow chamber.

Data recording

Percentage of responding explants, growth of callus (fresh weight of callus induced per responding explant) and the type of callus induced (friable or compact) were recorded to evaluate callus induction. For callus multiplication, 500 mg callus (per unit callus) was cultured on multiplication medium. Growth of callus was recorded as the increase in fresh weight of callus and multiplication rate of the callus was assessed by subtracting the initial amount of cultured callus (500 mg) from the amount of multiplied callus and dividing by 500 mg. Data on shoot regeneration was recorded as average number of buds induced per unit callus (500 mg) and average number of shoots proliferated. Forty-eight explants per treatment were maintained for all the experiments thrice. Standard deviation was calculated for each treatment of the experiment. Observations of each experiment were recorded after six weeks for callus induction and three weeks for callus multiplication and shoot regeneration. Data analysis (one-way ANOVA) was performed using the SPSS 8.0 package and post hoc analysis was performed using Scheffé's test.

RESULTS AND DISCUSSION

Callogenesis

In the present study, *in vitro* callus induction was obtained using single shoots that were incised (10–15 incisions) all along their surface. These pre-injured shoots were cultured horizontally on MS medium supplemented with either 2,4-D or NAA with concentrations ranging from 0 to 12.5 μM. Observations from the study revealed that explants cultured on basal medium remained unresponsive towards callogenesis and maintained their green texture upto 2 weeks and then senesced. On the other hand, explants cultured on medium supplemented with growth regulators exhibited rapid yellowing and necrosis after one week of culture.

Amongst the auxins tested, 2,4-D was more effective in eliciting callogenic response from explants compared to NAA (Table 1). The highest callogenic response was recorded at 5.0 μM 2,4-D. At this concentration, 42.9% of explants responded towards callusing and yielded an average of 249.3 mg of callus. Induction period

Table 1.
Callogenic response of shoot explants of *Bambusa nutans* after 6 weeks of culture on MS medium supplemented with auxin

Auxin	Concentration (μM)	Percentage of explant response (mean±SD)	Weight of callus (mg) (mean±SD)
Control	0	0.00 ± 0.00^{f}	0.00 ± 0.00^{c}
2,4-D	2.5	$29.2 \pm 5.51^{a,b,c}$	$200.1 \pm 32.31^{a,b}$
	5.0	43.1 ± 4.33^{a}	249.3 ± 30.65^{a}
	7.5	$36.8 \pm 6.36^{a,b}$	$205.1 \pm 46.74^{a,b}$
	10	$27.1 \pm 5.51^{b,c,d}$	$184.0 \pm 40.93^{a,b}$
	12.5	$20.8 \pm 4.17^{c,d,e}$	$173.9 \pm 27.40^{a,b}$
NAA	2.5	$9.7 \pm 1.20^{e,f}$	129.3 ± 20.03^{b}
	5.0	$22.2 \pm 3.18^{b,c,d,e}$	158.5 ± 28.62^{b}
	7.5	$27.8 \pm 4.81^{a,b,c,d}$	$185.5 \pm 35.96^{a,b}$
	10	$17.4 \pm 2.41^{c,d,e}$	148.0 ± 18.31^{b}
	12.5	$12.5 \pm 2.08^{d,e,f}$	132.5 ± 25.20^{b}
CD at 0.05		15.93	78.82

Note for all tables: the letters in superscript mean that values with similar superscripts do not differ significantly; values with different superscripts do differ significantly.

of callus varied with growth regulator type and its concentration. Callus was induced earlier (9–17 days) on explants cultured on 2,4-D compared to NAA (13–20 days). Efficacy of 2,4-D as a callogenic agent has been elicited in earlier works on *P. viridis* [8], *Sesbania* spp. [9] and *D. latiflorus* [10].

Results of the above experiment provided the base for investigating the role of BAP and kinetin (2.5 and 5.0 μM) in combination with 2,4-D (2.5–10.0 μM). Addition of cytokinins in combination with auxins in the induction medium enhanced the callus induction potential of explants and reduced the rate of necrosis in comparison to those cultured on auxins alone but there was no variation in time taken for callus induction and callus type. Higher cytokinin concentration (5.0 μM) decreased the responsiveness of explants towards callogenesis. This corresponds with results of a study on *Acacia auriculiformis* [11]. Explants cultured on combination of 2,4-D (5 μM) and BAP (2.5 μM) exhibited the highest responsiveness (58.0%) to callus induction and induced maximum amount of callus (320.3 mg) compared to other combinations (Table 2).

In an attempt to improve percentage of explants exhibiting callogenic response as well as callus yield, ABA (0–3.0 μM) was also added to the medium supplemented with 2,4-D (2.5–10.0 μM) and 2.5 μM BAP. Explants cultured on media supplemented with 1.0 μM ABA exhibited enhanced callogenic response and callus yield over explants cultured on control medium without ABA or with higher ABA concentration (2.0–3.0 μM) (Table 3). The lower percentage of explants responding towards callusing at comparatively higher ABA concentration (2.0–3.0 μM) is possibly due to the enhanced rate of senescence of explants as ABA tends to

Table 2.
Effect of auxin–cytokinin interaction on callus induction from shoot explants of *Bambusa nutans* after six weeks of culture

2,4-D (μM)	Cytokinin (μM)	Percentage of explant response (mean±SD)	Weight of callus (mg) (mean±SD)
2.5	2.5 μM BAP	$42.3 \pm 3.17^{a,b,c}$	$235.1 \pm 24.54^{a,b}$
5		58.0 ± 7.30^{a}	320.3 ± 21.64^{a}
7.5		$55.5 \pm 3.18^{a,b}$	$276.7 \pm 16.31^{a,b}$
10		$47.8 \pm 4.16^{a,b,c}$	$258.2 \pm 43.90^{a,b}$
2.5	5.0 μM BAP	$40.2 \pm 4.32^{a,b,c}$	$233.3 \pm 36.24^{a,b}$
5		$54.8 \pm 5.23^{a,b}$	$264.9 \pm 30.34^{a,b}$
7.5		$42.9 \pm 6.35^{a,b,c}$	$216.6 \pm 37.51^{a,b}$
10		$35.4 \pm 3.60^{b,c}$	198.5 ± 18.56^{b}
2.5	2.5 μM kinetin	$38.8 \pm 4.32^{b,c}$	$219.8 \pm 29.02^{a,b}$
5		$47.8 \pm 4.80^{a,b,c}$	$265.8 \pm 22.72^{a,b}$
7.5		$41.6 \pm 5.50^{a,b,c}$	$238.5 \pm 31.38^{a,b}$
10		32.6 ± 5.23^{c}	$233.7 \pm 23.11^{a,b}$
2.5	5.0 μM kinetin	$36.1 \pm 4.32^{b,c}$	$213.5 \pm 17.06^{a,b}$
5		$40.9 \pm 6.35^{a,b,c}$	$257.9 \pm 25.80^{a,b}$
7.5		$39.5 \pm 5.08^{a,b,c}$	$231.2 \pm 29.86^{a,b}$
10		31.9 ± 4.33^{c}	194.9 ± 14.08^{b}
CD at 0.05		21.91	114.30

Table 3.
Interactive effect of ABA, 2,4-D and BAP (2.5 μM) on callus induction from shoot explants of *B. nutans* cultured on MS medium after six weeks of culture

ABA (μM)	2,4-D (μM)	Percentage of explant response (mean±SD)	Weight of callus (mg) (mean±SD)
0	2.5	39.5 ± 6.36^{d}	243.0 ± 19.47^{b}
	5.0	$57.5 \pm 4.23^{a,b,c,d}$	$285.3 \pm 28.64^{a,b}$
	7.5	$56.2 \pm 4.16^{b,c,d}$	$274.9 \pm 31.80^{a,b}$
1	2.5	$51.3 \pm 6.01^{b,c,d}$	$285.3 \pm 27.84^{a,b}$
	5.0	79.7 ± 8.40^{a}	346.1 ± 12.50^{a}
	7.5	$67.9 \pm 5.23^{a,b}$	$304.7 \pm 14.82^{a,b}$
2	2.5	$42.3 \pm 4.17^{c,d}$	$270.7 \pm 20.67^{a,b}$
	5.0	$63.8 \pm 6.04^{a,b,c}$	$331.7 \pm 18.40^{a,b}$
	7.5	$56.8 \pm 7.30^{b,c,d}$	$282.1 \pm 45.10^{a,b}$
3	2.5	38.1 ± 5.23^{d}	$265.6 \pm 16.92^{a,b}$
	5.0	$49.9 \pm 6.24^{b,c,d}$	$286.6 \pm 35.98^{a,b}$
	7.5	$46.4 \pm 4.33^{b,c,d}$	$256.2 \pm 16.30^{a,b}$
CD at 0.05		22.61	94.36

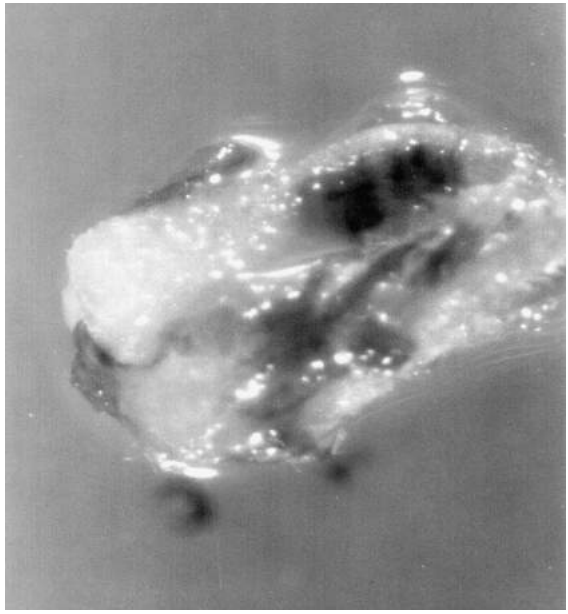

Figure 1. Induction of callus from shoot explant on MS medium supplemented with ABA (1 μM), BAP (2.5 μM) and 2,4-D (5 μM) with 3% sucrose.

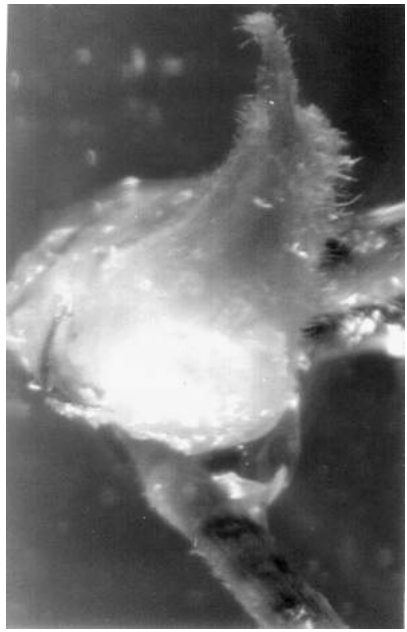

Figure 2. Subcultured explant on MS medium with 2,4-D (5 μM) and BAP (2.5 μM).

promote senescence. The most effective media combination was 2,4-D (5.0 μM), ABA (1.0 μM) and BAP (2.5 μM), on which 79.7% explants induced an average of

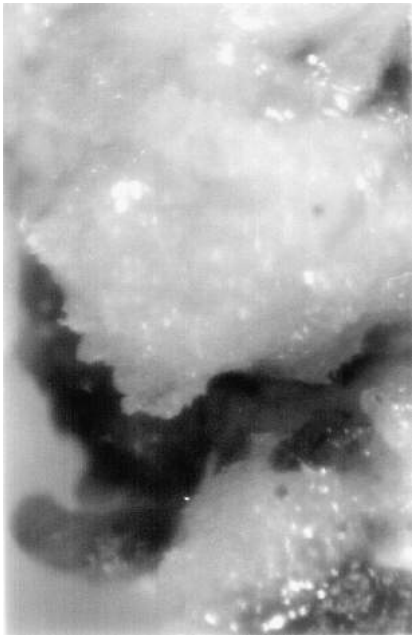

Figure 3. Proliferation of callus on MS medium supplemented with 5 μM 2,4-D.

346.1 mg callus (Fig. 1). Addition of ABA in the medium did not influence the duration taken for callus induction or callus type. Investigations on the role ABA on *in vitro* cultures have revealed that it induces a wide array of responses [12–14]. Nevertheless, its role in influencing organogenesis has been restricted to a few reports. However, in this study, ABA at low concentrations (less than 2 μM) enhanced induction of organogenic callus. Similar enhancement of organogenic response at low concentrations was reported in callus raised from hypocotyls of *Cucumis melo* [15], conifers [14, 16, 17] and cotyledon explants of *C. sativus* [18].

The induced callus along with initial explant was subcultured on the induction medium for another three weeks to maximize the callus induced before transferring it to multiplication medium (Fig. 2). Microscopic studies of the induced callus revealed two types of induced calli embryogenic and organogenic. Embryogenic calli appeared to be globular and white and showed smooth torpedo shaped structures. Organogenic callus on the other hand appeared to be hard, nodular, greenish and translucent.

In order to proliferate the induced callus, it was isolated from the original explant and transferred onto multiplication medium (2,4-D or NAA in combination with 2.5 μM BAP) after six weeks (Fig. 3). Rate of callus proliferation was higher when 2,4-D was used compared to NAA (Table 4). The rate of callus multiplication was fastest (2.48) on medium supplemented with 5.0 μM 2,4-D and 2.5 μM BAP. Our observations are in close conformity with those on callus multiplication in *Sesbania* spp. [9].

Table 4.
Multiplication rate of shoot callus (500 mg) cultured on medium supplemented with auxin and BAP (2.5 μM) after three weeks of culture

Auxin	Concentration (μM)	Increase in callus fresh weight (mg) (mean±SD)	Multiplication rate
2,4-D	2.5	$816.7 \pm 67.19^{c,d}$	1.83
	5.0	1244.3 ± 31.91^{a}	2.48
	7.5	$1174.8 \pm 67.47^{a,b}$	2.34
	10.0	$987.7 \pm 91.31^{a,b,c}$	1.97
NAA	2.5	561.5 ± 48.89^{d}	1.12
	5.0	$772.2 \pm 76.00^{c,d}$	1.54
	7.5	$1003.5 \pm 73.57^{a,b,c}$	2.07
	10.0	$914.7 \pm 42.12^{b,c}$	1.82
CD at 0.05		263.44	

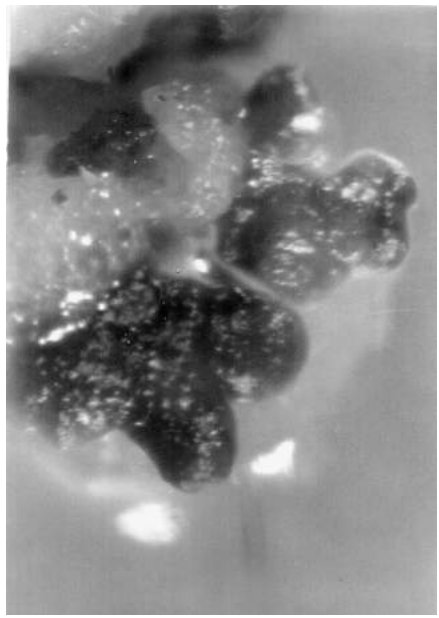

Figure 4. Development of bud primordia on auxin-enriched media.

Microscopic observations of callus cultured on multiplication medium supplemented with auxins (2,4-D or NAA), revealed change in the texture of these calli from colourless to dark green after their transfer from induction medium to the multiplication medium. These green areas were sites where adventitious bud primordia developed (Fig. 4). Some of these adventitious buds developed into complete shoots on the callus multiplication medium itself (Fig. 5). Shoots that developed on the NAA enriched medium were green, whereas on 2,4-D they were greenish-yellow.

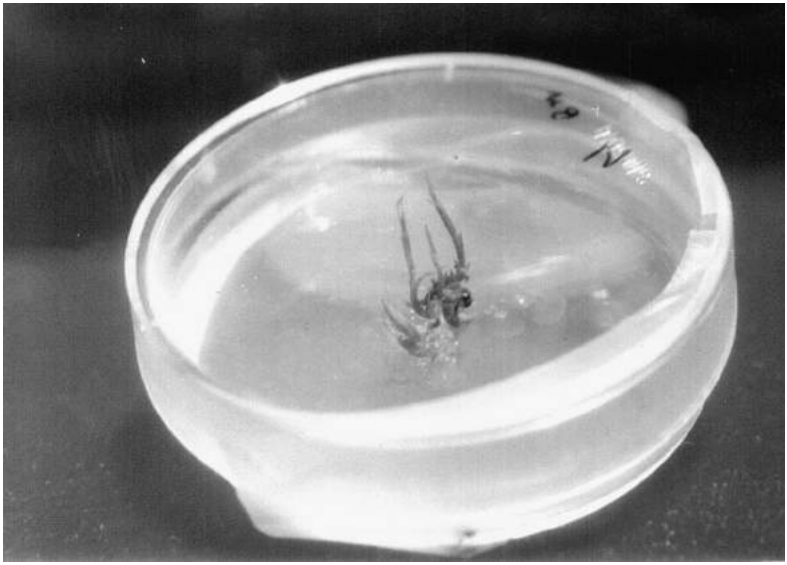

Figure 5. Regeneration of shoot from callus on MS medium supplemented with 5 μM BAP and 2.5 μM NAA.

Callus multiplied on both 2,4-D and NAA maintained its regenerative potential, even after 20 subculture cycles (three weeks per subculture).

Shoot regeneration

In order to enhance shoot regeneration, callus multiplied on 5.0 μM 2,4-D and 2.5 μM BAP was transferred to medium supplemented with cytokinins (BAP or kinetin). Shoot regeneration from callus was more efficient when BAP was used compared to kinetin (Table 5). The highest shoot regeneration (9.11 shoots) was achieved on medium supplemented with BAP (7.5 μM). The number of buds formed was higher with BAP concentrations greater than 7.5 μM, but their conversion into shoots was low and they needed to be subcultured on the basal medium to develop shoots.

Microscopic observations of calli cultured on cytokinin-enriched regeneration media revealed that shoot primordia developed earlier (7–8 days after culture) on medium containing BAP than with kinetin (10–14 days). Shoot bud primordia were sparsely distributed at lower concentration than at higher concentration. Shoot buds induced on 2.5–5.0 μM BAP developed into shoots earlier (10–12 days) as compared to higher cytokinin concentrations (10.0–12.5 μM BAP) (Table 5). This delayed elongation of shoot buds into shoots at higher concentration of BAP may be due to clustering effect where several buds compete for nutrients. Increased adventitious bud/shoot regeneration efficiency (either through direct or indirect organogenesis) on media supplemented with BAP has been mentioned in several reports [2, 10].

Table 5.
Regeneration efficiency of shoot callus (500 mg) cultured on varying concentrations of cytokinin

Cytokinin	Concentration (μM)	Average No. of buds induced (mean±SD)	Average No. of shoots induced (mean±SD)	No. of days for shoot proliferation
BAP	2.5	$10.3 \pm 2.04^{c,d}$	4.9 ± 0.38^{c}	10–12
	5.0	$14.4 \pm 0.71^{a,b,c}$	$6.2 \pm 0.63^{b,c}$	10–12
	7.5	17.9 ± 1.58^{a}	9.1 ± 1.01^{a}	11–15
	10.0	19.6 ± 0.96^{a}	$6.9 \pm 0.50^{a,b,c}$	12–17
	12.5	18.7 ± 1.92^{a}	5.2 ± 0.71^{c}	13–20
Kinetin	2.5	7.2 ± 0.96^{d}	4.6 ± 0.65^{c}	8–10
	5.0	9.5 ± 0.95^{d}	$5.9 \pm 0.36^{b,c}$	8–10
	7.5	$11.8 \pm 0.92^{b,c,d}$	$6.2 \pm 0.90^{b,c}$	11–13
	10.0	$15.1 \pm 1.11^{a,b,c}$	$6.8 \pm 0.52^{a,b,c}$	11–13
	12.5	$15.7 \pm 1.08^{a,b}$	$7.7 \pm 0.54^{a,b}$	12–15
CD at 0.05		4.92	2.47	

Table 6.
Interactive effect of BAP-NAA on regeneration of shoots from per unit callus (500 mg)

BAP (μM)	NAA (μM)	Average No. of buds induced (mean±SD)	Average No. of shoots proliferated (mean±SD)	No. of days for shoot proliferation
2.5	0	8.5 ± 0.88^{c}	$5.9 \pm 0.67^{c,d}$	10–12
5.0		$15.5 \pm 0.93^{a,b}$	$6.2 \pm 0.64^{c,d}$	10–12
7.5		19.4 ± 0.87^{a}	$9.3 \pm 1.06^{a,b}$	11–15
2.5	1.25	9.2 ± 1.02^{c}	$6.0 \pm 0.61^{c,d}$	8–9
5.0		18.1 ± 1.29^{a}	10.3 ± 1.04^{a}	8–9
7.5		$16.4 \pm 0.71^{a,b}$	$8.4 \pm 0.73^{a,b,c}$	8–11
2.5	2.5	8.4 ± 1.02^{c}	3.7 ± 1.01^{d}	11–13
5.0		$12.6 \pm 1.14^{b,c}$	$6.3 \pm 1.06^{b,c,d}$	11–14
7.5		$12.1 \pm 0.24^{b,c}$	4.0 ± 0.23^{d}	13–17
CD at 0.05		4.97	3.04	

A synergistic effect of NAA (1.25 μM) with BAP (2.5–10.0 μM) was observed. Regeneration efficiency was lower with the medium without NAA or with higher concentration (2.5 μM) NAA (Table 6). On medium supplemented with 5.0 μM BAP and 1.25 μM NAA the best shoot regeneration was achieved (Fig. 6). This combination produced an average of 18.1 buds, which proliferated into 10.3 shoots fastest (8–9 days). The above results confirm the importance of the cytokinin/auxin ratio in shoot organogenesis and are in line with similar trends reported earlier [2, 5]. Regeneration of roots spontaneously on the shoot regeneration medium was at the frequency of 75%, a pattern observed in adventitious organogenesis of *B. vulgaris* [5].

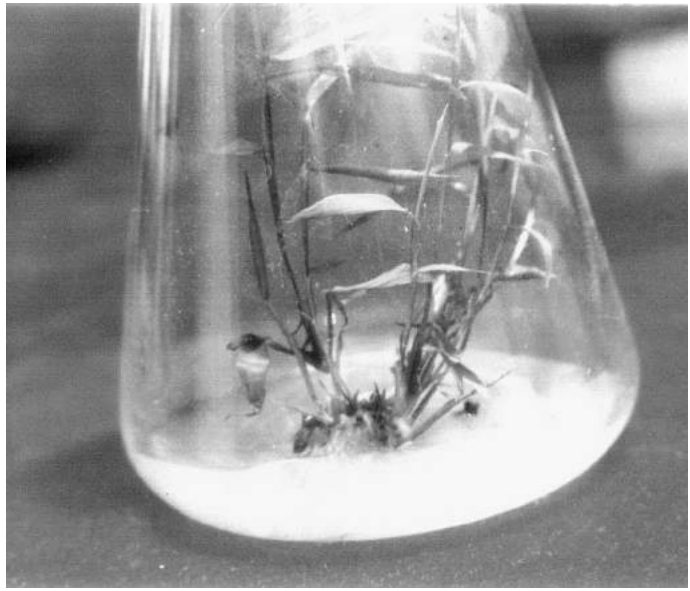

Figure 6. Complete plantlet regeneration.

Organogenesis offers a vast potential for bamboo improvement and multiplication programme. An efficient regenerative methodology via callus phase is a pre-requisite for a successful genetic modification study. The study offers a speedy method of regeneration of this bamboo with shortened regeneration procedure into complete plantlets as roots were directly induced on base of shoots on regeneration medium itself. Thus, the present study has opened long desired, new vistas in the genetic improvement programmes of bamboo.

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