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ARTICLE

Influence of plant growth regulators (PGRs) and various additives on *in vitro* plant propagation of *Bambusa arundinacea* (Retz.) Wild: A recalcitrant bamboo species

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Abstract An efficient micropropagation protocol for high frequency plant regeneration was developed using nodal explants derived *in vitro* seedlings of *Bambusa arundinacea* which is an important multipurpose and edible bamboo species and recalcitrant to tissue culture. The nodal explants excised from 20-day-old seedlings were cultured on Murashige and Skoog (MS) medium fortified with various concentrations of 6-benzyl amino purine (BAP) and kinetin (KIN) (0.5–5.0 mg/l) alone and/or in combination with 0.5 mg/l of different auxins [indole-3-butyric acid (IBA) α-naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA)] for shoot bud induction. The combination of BAP (3.0 mg/l) and IBA (0.5 mg/l) was found to be the best for the highest percent of shoot bud initiation (87.2%), with 24.2 shoots/explant. The highest frequency (95.2%) of shoot bud multiplication with maximum number of shoots (90.5 shoots/culture) was noticed on medium containing 4% coconut water with 4% sucrose. The regenerated shoot buds were cultured on MS medium supplemented with various concentrations of auxins alone and/or in combination with $AgNO_3$ (0.5–4.0 mg/l) for *in vitro* rooting. Maximum percent of rooting (85%) was noticed on MS medium augmented with 3.0 mg/l IBA and 2.0 mg/l $AgNO_3$ after 14 days of culture. Well rooted plantlets obtained were established in the field with 92% survival rate. The present plant regeneration protocol could be used for large scale propagation and *ex-situ* conservation of this important bamboo species in the near future.

1. Introduction

Bamboo is the world's strongest and versatile woody plant capable of providing ecological, economic and livelihood security to the people, and also known as 'Green Gold', 'Wonder

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plant' [8,41]. India has the richest bamboo genetic resources with 136 species, including 11 exotic species out of which 58 species belong to 10 genera. The total forest area under bamboo is 8.96 million hectare. This is about 12.8% of total forest area of our country [8]. The use of bamboo, particularly as a substitute for traditional timber species in forms of plywood, particle board, hard board and medium density board is largely found in housing sector, further increasing the demand of bamboo. They also serve as value added product namely bamboo shoots, pickle, candy, chutney, curry, vinegar and food nutrient (mineral and vitamins).

The nutritional value of edible shoots of different bamboo species has been reported by various researchers [37,19,27]. Bamboo is used in fisheries and sericulture as fodder. The bamboo species stabilizes the earth with its roots, preventing erosion [7] and used as activated charcoal. Gasification of bamboo can produce electricity using bamboo biomass as fuel (National Mission on Bamboo Application (NMBA)). Carbon sequestration in bamboo recycles a huge amount of CO_2 (12 tons/hectare) and produces 35% more oxygen than an equivalent stand of trees. They also play a vital role in water conservation, wind barrier, and as bio-fencing [20,16,43].

Bamboo leaf extract has been reported for anticancer properties – In tumor inoculated mouse models (S-180 sarcoma, Meth-A fibrosarcoma, B16-F10 melanoma), the vigorous extracts from *Kumaizasa* bamboo leaves suppressed tumor growth and prolonged survival rate with immunopotentiating and radical scavenging activity significantly [10,38,36]. Anti-inflammatory and antiulcer properties were documented using methanol extract of the leaves of *Bambusa arundinacea* against carrageenin-induced as well as immunologically induced paw edema and also its antiulcer activity in albino rats has been studied and found to be significant when compared to the standard drugs [23]. Antimicrobial activity was reported using fresh leaves of bamboo and evaluated against both gram positive and gram-negative bacterial strains by the disk diffusion method. The results revealed that all extracts showed effective inhibitory action against *Staphylococcus aureas* [42]. *B. arundinacea* seed had showed significant anti-diabetic activity in comparison with the standard glibenclamide [21]. The methanolic extract of *B. arundinacea* had significantly decreased the bone erosion, spleen enlargement and rheumatoid factor [32].

The conventional propagation of bamboos is through seeds and by culms or rhizome:offset cuttings. For bamboo, different propagation techniques are available, such as seed propagation, clump division, rhizome and culm cuttings [4,5]. The habit of unpredictable and gregarious flowering and seeding, followed by death, makes bamboo propagation uncertain through seeds which have short viability, and improvement by inter:intra specific hybridization very difficult. Furthermore, it is necessary to produce minimum of 100,000 seedlings per year to establish bamboo plantation. In order to achieve this goal, *in vitro* micropropagation of nodal explants will offer great potential for commercial scale production of bamboo especially for the most amazing commercially important *B. arundinacea* species.

In vitro propagation has been carried out using seedling explants and there were few reports on field grown plant materials either through axillary shoot proliferation in *Bambusa tulda* [35], *Bambusa vulgaris* [1], *Bambusa nutans* [26] or somatic embryogenesis in *B. nutans* [22], *Bambusa balcooa* [13], *Dendrocalamus giganteus* [29] and *Decalepis hamiltonii* [15]. Recently, immature embryo was used as explants for shoot regeneration in *Phyllostachys violascens* [28]. Though there were reports on shoot bud regeneration from different bamboo species, the regeneration rate was found to be low and still an efficient protocol for *in vitro* propagation is a prerequisite for large scale production of bamboo seedlings, thus paving the way for successful genetic manipulation [46]. An enhanced rate of shoot bud initiation and multiple shoot bud development is necessary for large scale cultivation. Therefore, the present study was focussed to develop an efficient and reliable shoot bud proliferation and rooting protocol through nodal bud proliferation using various plant growth regulators in the presence of different additives for this pharmaceutically important species and to develop large scale propagation in the near future.

2. Materials and methods

2.1. Seed sterilization

B. arundinacea (Retz.) Wild seeds were purchased commercially from Forestry Network market, Bangalore. The seeds have been collected from the Sringeri area, Chikmagalur District, Karnataka (Western Ghats region) and were authentically confirmed to be *B. arundinacea* (Retz.) Wild by The Forest College and Research Institute, Tamil Nadu Agricultural University (TNAU), Mettupalayam, Coimbatore. Seeds were dehusked by removing the seed coat by using fine razor blade and were soaked overnight with Bavistin (Carbendazim Fungicide) to prevent fungal contamination. After soaking, seeds were treated with 10% (v/v) commercial surfactant (Tween 20) followed by washing with sterile distilled water. Subsequently, the seeds were treated with 0.1% (w/v) mercuric chloride solution for 15 min, finally washed with sterile distilled water for 5 times to remove the mercuric chloride traces. All the experiments were carried out in the Laminar Air Flow Chamber under sterile conditions. The seeds were dried for 2 min on sterile Whatmann No. 1 filter paper and placed on to basal Murashige and Skoog [24] medium for germination in the dark condition.

2.2. Media and culture conditions

The explants were cultured on MS medium prepared in culture tubes (25 × 150 mm) and plugged tightly with non-absorbent cotton. In the present study, MS salts containing 3% (w/v) sucrose were used as the basal medium with different plant growth regulators. The pH of the medium was adjusted to 5.7 with 0.1 N NaOH or HCl prior to adding 7.0 g/L agar and autoclaved at 121 °C for 20 min at 15 psi. All the cultures were maintained under 16/8 h (light/dark) photoperiod with a light intensity of 60μ E mol m^{-2} s^{-1} (cool white fluorescent light) and maintained at controlled temperature (25 ± 2 °C).

2.3. Shoot bud initiation

The seeds were placed on basal MS medium after sterilization and maintained under dark condition. The nodal explants excised from 20-day-old (>4–5 cm length) *B. arundinacea* seedlings were used for *in vitro* shoot bud initiation. The

excised nodal explants were cultured on MS medium supplemented with different concentrations of 6-benzyl amino purine (BAP; 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) and kinetin (KIN; 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) along with 0.5 mg/l of various auxins namely indole-3-acetic acid(IAA), α-naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) for shoot bud induction.

2.4. Shoot bud multiplication

Proliferated *in vitro* shoot buds were separated into 3–4 shoots and used for further shoot bud multiplication. The regenerated shoot buds were cultured on MS medium fortified with optimized concentration of BAP (3.0 mg/l) and/or KIN (4.0 mg/l) in combination with various concentrations of different additives such as CW (2, 4, 6, 8 and 10% v/v), sucrose (2, 3, 4, 5 and 6% w/v) and $AgNO_3$ (0.5, 1.0, 2.0, 3.0 and 4.0 mg/l). Subculture was performed at 3 week interval for further shoot bud proliferation. The shoot multiplication cycles were carried out in solid medium due to easy handling of cultures and to avoid vitrification of shoots when placed in liquid medium.

2.5. Effect of coconut water (CW) on shoot multiplication

Coconut water (coconut liquid endosperm) was used as a growth supplement in plant tissue culture. The regenerated shoot buds were subcultured onto MS medium supplemented with sucrose (3.0%) and different concentrations of coconut water (2, 4, 6, 8 and 10% v/v) along with either BAP (3.0 mg/l) or KIN (4.0 mg/l) for shoot bud multiplication.

2.6. Effect of sucrose on multiple shoot regeneration

The most commonly used carbon source for *in vitro* propagation usually varies from 2.0–5.0% for efficient shoot proliferation. The composition of culture medium is a major determinant of *in vitro* growth of plants. The mineral salts sugar as carbon source and water are the main components for most plant tissue culture media. Generally the growth and development increase with sugar concentration, until an optimum is reached and then decrease at high concentrations. Therefore, this study was performed to find out the effect of different concentrations of sucrose (2.0, 3.0, 4.0, 5.0 and 6.0% w/v) in combination with either BAP (3.0 mg/l) or KIN (4.0 mg/l) in the presence of optimized concentration of CW (4.0%) for *in vitro* shoot bud multiplication.

2.7. Effect of silver nitrate ($AgNO_3$) on multiple shoot regeneration

An experiment was carried out to identify the most effective concentration of $AgNO_3$ for *in vitro* shoot multiplication. The regenerated shoots were cultured on MS medium augmented with either BAP (3.0 mg/l) or KIN (4.0 mg/l) in the presence of various concentrations of $AgNO_3$ (0.5–4.0 mg/l) to enhance the shoot multiplication rate.

2.8. Effect of adenine sulfate (ADS) on shoot bud elongation

In order to induce shoot bud elongation, the regenerated shoot buds were cultured on MS medium fortified with different concentrations of $AdSO_4$ (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) along with optimized dose of BAP (3.0 mg/l) and/or KIN (4.0 mg/l).

2.9. Effect of auxins and $AgNO_3$ on in vitro rooting

For *in vitro* rooting shoot clusters (3–5 shoots) were cultured on MS medium supplemented with different concentrations of IBA, NAA and IAA (0.5–5.0 mg/l). The effect of different concentrations of $AgNO_3$ (0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) in combination with optimized auxin concentrations of IBA (3.0 mg/l), NAA (2.0 mg/l) and IAA (3.0 mg/l) was also studied.

2.10. Hardening and acclimatization

After 3 weeks of culture on rooting medium, the *in vitro* derived shoots produced well developed roots and plantlets were removed successfully from the culture tubes and the roots were washed thoroughly with tap water to remove the adhering agar medium. Following washing, these plantlets were transferred into plastic cups containing autoclaved soil and sand (3:1). Transparent polythene bags were covered over these plantlets in plastic cups to maintain the humidity and watered on alternate days. Initially, plastic cups were kept inside a culture room (25 ± 2 °C, 16/8 h light/dark) with 65% relative humidity for 15 days and gradually moved to the greenhouse for further growth. After 15 days, the plantlets were transferred to polythene bags containing equal proportion of soil and farm yard manure (1:1) and placed in the greenhouse for hardening. After 2 months, the plantlets were transferred to pots containing soil and organic manure and kept in the green house. Finally, the *in vitro* hardened plantlets were acclimatized and successfully transferred to the field condition.

2.11. Experimental design and statistical analysis

All experiments were conducted using a completely randomized block design (CRBD) and each experiment had 10 replications and it was repeated at least 3 times. Data on percent of shoot bud regeneration, shoot bud multiplication, shoot length and rooting were recorded and used for analysis of variance (ANOVA) using IBM SPSS version 20 program. The differences in mean were analyzed by Duncan's Test at the $P \leqslant 0.05$ significance level.

3. Results

3.1. Shoot bud initiation

In the first experiment, BAP along with three auxins were tested for shoot bud regeneration. Shoot bud development was achieved on MS medium supplemented with different concentrations of BAP (0.5–5.0 mg/l) in combination with 0.5 mg/l IBA, NAA and IAA (Table 1). Among the three combinations, BAP (3.0 mg/l) and IBA (0.5 mg/l) combination was found to be more efficient for the highest frequency of shoot bud induction (87.2%) with the maximum number of shoots (24 shoots/explant) (Fig. 1A) followed by BAP (2.0 mg/l)

and NAA (0.5 mg/l) combination in which the highest percentage of shoot bud initiation noticed was 76.75% along with 11.16 shoots/explant. When BAP and IAA combinations were tried, the BAP (3.0 mg/l) and IAA (2.0 mg/l) combination produced maximum rate of shoot bud induction (69%) with 10.2 shoots/explant (Table 1).

In the second experiment, different doses of KIN (0.5–5.0 mg/l) in combination with three auxins (IBA, NAA and IAA) were examined to determine the optimum concentration for shoot bud initiation (Table 2). The highest frequency of shoot bud regeneration (81.25%) with 11.4 shoots/explant was noticed on MS medium fortified with the combination of KIN (4.0 mg/l) and IBA (0.5 mg/l) (Fig. 1B) followed by the combination of KIN (4.0 mg/l) and NAA (0.5 mg/l) where the percentage of shoot bud initiation obtained was 79.5% with more number of shoot buds (10.4 shoots/explant). In the case of KIN and IAA combinations, the maximum shoot bud induction rate (69.25%) with 6.43 shoots/explant, was noticed on MS medium supplemented with 4.0 mg/l KIN and 0.5 mg/l IAA. The number of shoots buds was decreased with KIN in combination with NAA and/or IAA (Table 2). It was observed that the combination of KIN (4.0 mg/l) and IBA (0.5 mg/l) was optimum for shoot bud induction.

Table 1 Effect of different concentrations of BAP along with 0.5 mg/l of three auxins on shoot bud initiation from nodal explants collected from *in vitro* grown seedlings of *Bambusa arundinacea*.

Growth regulator concentration (mg/l)				Shoot regeneration (%) (Mean ± SE)*	No. of shoots/explant (Mean ± SE)*
BAP	IBA	NAA	IAA		
0.5	0.5	–	–	71.00 ± 0.80^{c,d*}	10.6 ± 0.51^{b,c,d*}
1.0	0.5	–	–	76.70 ± 1.00^{b}	11.8 ± 0.80^{b,c}
2.0	0.5	–	–	79.10 ± 0.40^{b}	14.0 ± 1.41^{b}
3.0	0.5	–	–	87.20 ± 0.63^{a}	24.2 ± 0.58^{a}
4.0	0.5	–	–	68.40 ± 0.50^{c,d,e}	9.6 ± 0.87^{c,d,e}
5.0	0.5	–	–	59.20 ± 0.32^{g}	7.2 ± 0.59^{d,e,f,g}
0.5	–	0.5	–	67.60 ± 1.00^{d,e,f}	9.0 ± 0.70^{c,d,e}
1.0	–	0.5	–	71.50 ± 1.00^{c}	10.4 ± 1.70^{b,c,d}
2.0	–	0.5	–	76.75 ± 1.75^{b}	11.1 ± 1.31^{b,c,d}
3.0	–	0.5	–	70.50 ± 0.88^{c,d}	9.1 ± 0.33^{c,d,e}
4.0	–	0.5	–	64.50 ± 0.54^{f}	8.0 ± 1.10^{c,d,e,f}
5.0	–	0.5	–	59.20 ± 0.80^{g}	6.4 ± 0.50^{e,f,g}
0.5	–	–	0.5	56.70 ± 1.00^{g}	5.0 ± 1.51^{f,g}
1.0	–	–	0.5	61.00 ± 0.30^{g}	8.1 ± 0.73^{c,d,e,f}
2.0	–	–	0.5	66.40 ± 0.40^{e,f}	9.4 ± 1.72^{c,d,e}
3.0	–	–	0.5	69.50 ± 0.63^{c,d,e}	10.2 ± 0.80^{c,d,e}
4.0	–	–	0.5	53.40 ± 0.60^{h}	4.6 ± 1.24^{f,g}
5.0	–	–	0.5	50.60 ± 0.20^{h}	4.0 ± 0.15^{g}

* Means followed by same letter within a column are not significantly different at ($P \leqslant 0$ .05).

3.2. Shoot bud multiplication

3.2.1. Coconut water (CW) on shoot bud multiplication

In the present study, the influence of different concentrations of CW (2–10%) along with the optimum dose of BAP (3.0 mg/l) and/or KIN (3.0 mg/l) on multiple shoot bud regeneration was examined. When MS medium was supplemented with BAP (3.0 mg/l), CW (4.0%) and sucrose (3.0%), the number of multiple shoot buds was enhanced to 3-fold within 2 wk of culture. The highest frequency (89.5%) of shoot bud multiplication rate was recorded with maximum number of shoots (86.9 shoots/culture) (Table 3) (Fig. 1D). Among the KIN and CW concentrations tested, MS medium fortified with 4.0 mg/l KIN and 4.0% CW combination produced the highest percent of shoot bud multiplication rate (74.6%) along with 71.8 shoots/culture (Fig. 1C).

3.2.2. Sucrose concentrations on shoot bud proliferation

In order to test the effect of sucrose, various concentrations of sucrose (2–6%) were added into MS medium containing BAP (3.0 mg/l)/KIN (4.0 mg/l) in combination with CW (4%). When the regenerated shoot buds were subcultured onto MS medium augmented with sucrose (4.0%), it was found to be the best dose for maximum rate of shoot bud multiplication. When MS medium was fortified with BAP (3.0 mg/l) in the presence of sucrose (4.0%) and CW (4.0%), the highest percent of shoot multiplication (95.2%) as well as maximum number of shoots (90.5 shoots) was achieved. Among the KIN (4.0 mg/l), sucrose (2–6%) and CW (4.0%) combinations tested, maximum shoot regeneration rate noticed was 80.65% with 70.6 shoots/culture in the presence of 3.0 (%) sucrose containing medium (Table 3).

3.2.3. $AgNO_3$ on shoot multiplication

To study the effect of $AgNO_3$ on multiple shoot bud induction, the regenerated shoot buds were subcultured onto MS medium supplemented with either BAP (3.0 mg/l) or KIN (4.0 mg/l) in the presence of various concentrations of $AgNO_3$ (0.5–4.0 mg/l). In the case of $AgNO_3$ and BAP combination, the shoot bud multiplication percent obtained was 64.45% with 25.64 shoots/culture, while the $AgNO_3$ and KIN combination produced only 56.84% of shoot bud multiplication rate (Table 3).

3.2.4. ADS on elongation of shoot buds

The determine the effect of ADS on elongation of shoot buds, the regenerated shoot buds were subcultured onto MS medium containing various concentrations of ADS (0.5–5.0 mg/l) in combination with the optimized dose of BAP (3.0 mg/l) and/or KIN (4.0 mg/l). Among the BAP and ADS concentrations tested, a maximum shoot elongation was achieved on MS medium containing BAP (3.0 mg/l) + ADS (5.0 mg/l) with 5.82 cm of shoot length. In the case of KIN and ADS combinations, maximum shoot length recorded was 5.4 cm on medium fortified with KIN (4.0 mg/l) and ADS (3.0 mg/l) (Table 4).

3.2.5. $AgNO_3$ on in vitro rooting

Various concentrations of auxins each of IBA, NAA, IAA from 0.5 mg/l to 5.0 mg/l were used for rooting. Among the different concentrations of IBA tested, maximum percent of root induction (72.4%) with more number of roots (9.25 roots/shoot) was observed on medium containing IBA (3.0 mg/l) (Fig. 1E, F and G) followed by NAA (2.0 mg/l) that produced the highest frequency of root induction (70.5%) with 8.65 roots/shoot. In the case of IAA, the rooting frequency noticed was 68.4% with 6.43 roots/shoot on MS medium containing 3.0 mg/l IAA (Table 5).

In another experiment, the effect of different concentrations of $AgNO_3$ (0.5–4.0 mg/l) was also studied along with the optimum dose of IBA (3.0 mg/l), NAA (2.0 mg/l) and/or

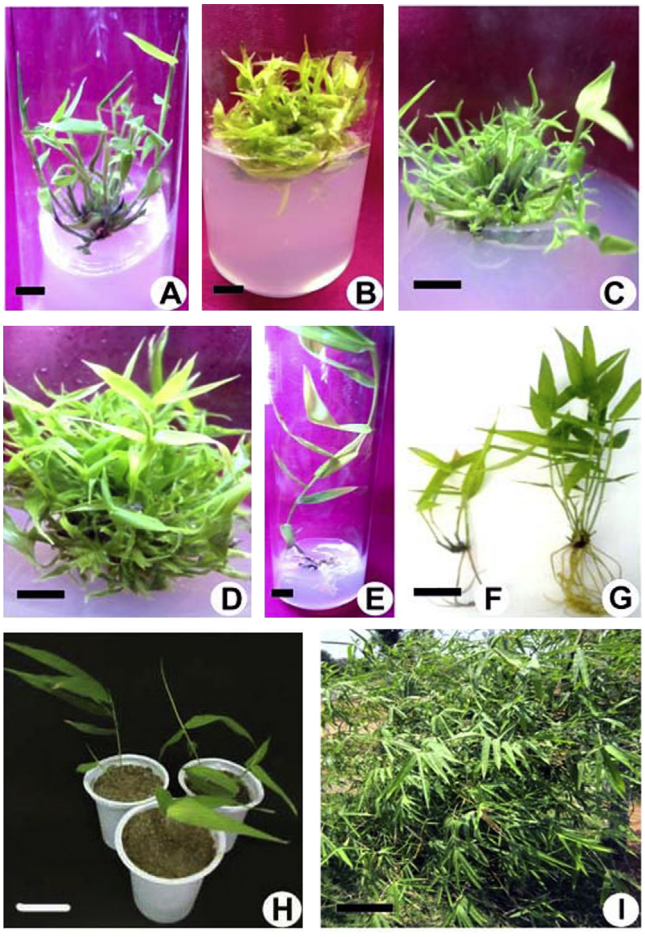

Figure 1 Plant regeneration from nodal explants of *in vitro* grown *Bambusa arundinacea* seedlings. (A) Shoot bud development on MS medium with 3.0 mg/l BAP and 0.5 mg/l IBA;(B) Shoot regeneration on MS medium containing 4.0 mg/l KIN and 0.5 mg/l IBA; (C) Multiple shoot buds growing on MS medium with 4.0 mg/l KIN and 4% (*v*/*v*) coconut water; (D) Regeneration of multiple shoots obtained on MS medium containing 3.0 mg/l BAP and 4% (*w*/*v*) coconut water + sucrose 4 (*w*/*v*); (E) Rooting of *in vitro* regenerated shoots; (F) Rooted (thin root) *in vitro* bamboo plantlets produced on MS medium with 3.0 mg/l IBA; (G) Rooted (thick root) *in vitro* bamboo plantlets developed on MS medium with 3.0 mg/l IBA and 2.0 mg/l $AgNO_3$; (H) *In vitro* regenerated bamboo plantlets growing in plastic cups; (I) A tissue culture bamboo tree growing in the Biodiversity Garden, Periyar University (*Bar line* = 1 cm).

IAA (2.0 mg/l). The highest percentage of root induction (85.15%) with maximum number of roots (9.34 roots/shoot) was noticed on MS medium augmented with IBA (3.0 mg/l) + $AgNO_3$ (2.0 mg/l) combination. Among the NAA and $AgNO_3$ combination tested, NAA (2.0 mg/l) and $AgNO_3$ (1.0 mg/l) combination was found to be the best for the highest frequency (80%) of root induction with 8.93 roots/shoot followed by IAA (3.0 mg/l) and $AgNO_3$ (2.0 mg/l) combination which produced maximum percent of root induction (71.16%) with 6.73 roots/shoot (Table 6).

Table 2 Effect of different concentrations of KIN along with 0.5 mg/l of three auxins on shoot bud initiation from nodal explants collected from *in vitro* grown seedlings of *Bambusa arundinacea*.

Growth regulator concentration (mg/l)				Shoot regeneration (%) (Mean ± SE)*	No. of shoots/explant (Mean ± SE)*
KIN	IBA	NAA	IAA		
0.5	0.5	–	–	64.25 ± 1.25^{d,e*}	4.2 ± 0.60^{d,e,f,g*}
1.0	0.5	–	–	70.53 ± 1.50^{c}	4.0 ± 1.50^{d,e,f,g}
2.0	0.5	–	–	76.30 ± 1.00^{b}	6.6 ± 1.67^{c,d,e}
3.0	0.5	–	–	77.10 ± 1.25^{b}	5.2 ± 1.39^{c,d,e,f,g}
4.0	0.5	–	–	81.25 ± 1.25^{a}	11.4 ± 2.15^{a}
5.0	0.5	–	–	61.25 ± 1.75^{e,f}	2.4 ± 0.86^{g}
0.5	–	0.5	–	54.50 ± 2.50^{h,i}	4.1 ± 0.31^{d,e,f,g}
1.0	–	0.5	–	58.75 ± 1.25^{f,g}	5.4 ± 0.70^{c,d,e,f,g}
2.0	–	0.5	–	65.25 ± 2.75^{d}	7.6 ± 0.40^{b,c,d}
3.0	–	0.5	–	70.20 ± 1.00^{c}	8.1 ± 0.50^{a,b,c}
4.0	–	0.5	–	79.51 ± 1.50^{a,b}	10.4 ± 0.50^{a,b}
5.0	–	0.5	–	54.75 ± 2.75^{h,i}	5.0 ± 0.58^{c,d,e,f,g}
0.5	–	–	0.5	57.51 ± 2.50^{g}	2.5 ± 0.50^{f,g}
1.0	–	–	0.5	62.50 ± 2.25^{d,e}	4.0 ± 0.61^{d,e,f,g}
2.0	–	–	0.5	65.34 ± 2.95^{d,e}	5.7 ± 0.52^{c,d,e,f,g}
3.0	–	–	0.5	69.25 ± 2.75^{c}	6.4 ± 1.13^{c,d,e,f}
4.0	–	–	0.5	61.75 ± 2.75^{d,e,f}	4.5 ± 0.75^{c,d,e,f,g}
5.0	–	–	0.5	53.50 ± 1.50^{i}	3.0 ± 0.62^{e,f,g}

* Means followed by same letter within a column are not significantly different at ($P \leqslant 0$ .05).

4. Discussion

The major objective of this study was to evaluate various factors that are influencing the shoot bud regeneration and rooting in *B. arundinacea*. In the present study, two experiments were conducted in the presence of cytokinins and auxins combination for shoot bud initiation. Maximum shoot bud initiation was achieved in the presence of BAP at 3.0 mg/l and IBA 0.5 mg/l combination followed by MS medium augmented with KIN (4.0 mg/l) and IBA (0.5 mg/l) combination (Tables 1 and 2). Effect of cytokinins (BAP and KIN) and auxins (IBA and IAA) for shoot bud regeneration using meristem explants of *B. arundinacea* was studied by Nayak et al. [25]. The results of the present study suggested that the combination of BAP (3.0 mg/l) and IBA (0.5 mg/l) was found to be the best for induction of the maximum number of multiple shoots (24 shoots/explant) from the nodal explants of *B. arundinacea*.

Coconut water also appears to have growth regulatory properties, e.g., cytokinin-type activity. CW appears to be an essential additive required for cell division, and thus promotes rapid growth during shoot bud multiplication. Therefore, in the present study, BAP (3.0 mg/l), CW (4.0%) and sucrose (3.0%) CW proved to be highly essential for massive multiplication. But at the same time, the higher concentrations (8 and 10%) were noticed with inhibitory effect and often caused vitrification of shoots. The other concentrations of KIN and CW combinations proved to be less effective for shoot bud multiplication. Rani and Nair [31] reported that medium containing 8% coconut water produced maximum number of shoots in *Vitex negundo* L. It has been reported that CW was found to be essential for shoot bud multiplication in *D. giganteus* [30].

Table 3 Effect of different concentrations of additives along with 3.0 mg/l BAP or 4.0 mg/l KIN on multiple shoot bud regeneration from nodal explants of *Bambusa arundinacea*.

Growth regulator concentration (mg/l)		Additives concentration			Shoot regeneration (%) (Mean ± S.E)*	No. of shoots/explant (Mean ± S.E)*
BAP (v/v)	KIN (w/v)	CW %	Sucrose %	$AgNO_3$		
3.0	–	2	3.0	–	80.6 ± 1.32^{e,f*}	73.8 ± 1.88^{e,f,g*}
3.0	–	4	3.0	–	89.50 ± 1.20^{b}	86.9 ± 1.40^{b}
3.0	–	6	3.0	–	85.71 ± 1.54^{c,d}	82.5 ± 1.32^{c}
3.0	–	8	3.0	–	83.33 ± 1.43^{d,e}	76.4 ± 1.20^{d,e}
3.0	–	10	3.0	–	73.8 ± 1.69^{g}	65.9 ± 1.19^{i,j}
–	4.0	2	3.0	–	72.3 ± 1.65^{g,h}	63.8 ± 2.09^{j}
–	4.0	4	3.0	–	74.6 ± 1.43^{g}	71.8 ± 1.67^{g,h}
–	4.0	6	3.0	–	61.5 ± 1.77^{h}	35.6 ± 2.42^{l}
–	4.0	8	3.0	–	45.1 ± 2.87^{l}	23.75 ± 1.9^{m,n}
–	4.0	10	3.0	–	33.4 ± 2.57^{n}	20.80 ± 1.22^{n,o}
3.0	–	4.0	2.0	–	88.5 ± 0.80^{b,c}	85.80 ± 1.21^{b}
3.0	–	4.0	3.0	–	90.8 ± 0.90^{b}	88.9 ± 1.40^{a,b}
3.0	–	4.0	4.0	–	95.2 ± 0.56^{a}	90.5 ± 2.09^{a}
3.0	–	4.0	5.0	–	83.33 ± 1.43^{d,e}	77.8 ± 2.21^{d}
3.0	–	4.0	6.0	–	81.46 ± 1.32^{e,f}	75.4 ± 1.20^{d,e,f}
–	4.0	4.0	2.0	–	78.56 ± 1.72^{f,g}	68.8 ± 2.20^{h,i}
–	4.0	4.0	3.0	–	80.65 ± 1.54^{e,f}	70.6 ± 1.19^{g,h}
–	4.0	4.0	4.0	–	75.50 ± 1.89^{f,g}	72.8 ± 0.80^{f,g}
–	4.0	4.0	5.0	–	69.75 ± 1.75^{h}	66.5 ± 1.80^{i,j}
–	4.0	4.0	6.0	–	62.46 ± 1.63^{i,j}	39.4 ± 1.42^{k}
3.0	–	–	3.0	0.5	51.20 ± 1.12^{k}	20.90 ± 3.56^{n,o}
3.0	–	–	3.0	1.0	64.45 ± 0.68^{i}	25.64 ± 2.11^{m}
3.0	–	–	3.0	2.0	59.11 ± 0.25^{j,h}	21.43 ± 1.78^{n,o}
3.0	–	–	3.0	3.0	48.32 ± 1.23^{k,l}	18.20 ± 2.34^{o,p}
3.0	–	–	3.0	4.0	33.76 ± 2.03^{n}	12.45 ± 2.87^{q,r}
–	4.0	–	3.0	0.5	45.23 ± 1.10^{l}	14.32 ± 3.75^{q,r}
–	4.0	–	3.0	1.0	49.65 ± 1.45^{k}	15.15 ± 2.78^{p,q}
–	4.0	–	3.0	2.0	56.84 ± 0.65^{j}	19.54 ± 2.65^{o}
–	4.0	–	3.0	3.0	39.75 ± 0.43^{m}	13.49 ± 1.54^{q,r}
–	4.0	–	3.0	4.0	32.46 ± 0.75^{n}	11.26 ± 1.49^{r}

* Means followed by same letter within a column are not significantly different at ($P \leqslant 0.05$).

It is reported that the addition of sucrose as carbon source to the MS medium showed enhanced shoot bud multiplication rate. Since CW (4.0%) was found to the best concentration to produce more number of multiple shoots, it was selected as optimum dose for multiple shoot regeneration. At lower concentration of sucrose (1–2%) strength, pale yellow leaves and thin shoots were developed with reduced shoot bud multiplication rate. The number of shoots decreased with increasing the concentrations of sucrose above 4.0 mg/l. Thus, the present results suggested that optimum concentration of sucrose at 4.0% (w/v) was found to be effective for multiple shoot bud regeneration in *B. arundinacea* (Table 3). The above results for sucrose corroborated with the findings of Beena et al. [6] that described that sucrose has induced bud break and multiple shoots in *Bambusa pallida*.

For shoot bud multiplication, regenerated shoot buds were cultured on medium fortified with different concentrations of $AgNO_3$ (0.5–4.0 mg/l) along with two cytokinins (BAP and KIN) (Table 3). Of the two combinations used, BAP and $AgNO_3$ combination was found to be the best for maximum percent of shoot bud multiplication. When shoots were cultured on $AgNO_3$ augmented medium, they showed stunted growth but the shoot elongation was noticed on medium containing coconut water. It is reported that the silver nitrate

Table 4 Effect of different concentrations of ADS along with 3.0 mg/l BAP or 4.0 mg/l KIN on shoot elongation from nodal explants of *Bambusa arundinacea*.

Hormone conc. (mg/l)			Shoot length (cm) (Mean ± S.E)*
BAP	KIN	ADS	
3.0	–	0.5	2.72 ± 0.146^{c,d}
3.0	–	1.0	3.08 ± 0.124^{c,d}
3.0	–	1.5	3.88 ± 0.11^{b,c,d}
3.0	–	2.0	4.54 ± 0.16^{a,b,c}
3.0	–	3.0	5.22 ± 0.06^{a,b}
3.0	–	5.0	5.82 ± 0.292^{a}
3.0	–	10.0	3.82 ± 0.213^{b,c,d}
–	4.0	0.5	2.36 ± 0.12^{d}
–	4.0	1.0	3.52 ± 0.086^{b,c,d}
–	4.0	1.5	3.76 ± 0.081^{b,c,d}
–	4.0	2.0	4.2 ± 0.124^{a,b,c,d}
–	4.0	3.0	5.4 ± 0.130^{a,b}
–	4.0	5.0	4.62 ± 0.17^{a,b,c}
–	4.0	10.0	3.64 ± 0.211^{b,c,d}

* Means followed by same letter within a column are not significantly different at ($P \leqslant 0.05$).

Table 5 Effect of various auxins on rooting from elongated shoots of *Bambusa arundinacea*.

Growth regulator concentration (mg/l)			Root induction (%) (Mean ± SE)*	No. of roots/culture (Mean ± SE)*	Root length (cm) (Mean ± SE)*
IBA	NAA	IAA			
0.5	–	–	0.0	0.0	0.0
1.0	–	–	$54.50 \pm 1.00^{f,g*}$	$2.41 \pm 0.73^{h*}$	$1.42 \pm 0.30^{f*}$
2.0	–	–	63.65 ± 0.15^{c}	$5.35 \pm 0.65^{c,d,e,f}$	$3.86 \pm 0.25^{b,c,d}$
3.0	–	–	72.40 ± 0.60^{a}	9.25 ± 0.85^{a}	$5.33 \pm 0.11^{a,b}$
4.0	–	–	$62.45 \pm 0.51^{c,d}$	$5.10 \pm 0.48^{c,d,e,f}$	$4.02 \pm 0.15^{a,b,c,d}$
5.0	–	–	47.75 ± 0.25^{i}	2.32 ± 0.24^{h}	1.39 ± 0.56^{f}
–	0.5	–	0.0	0.0	0.0
–	1.0	–	$57.50 \pm 3.10^{e,f}$	$6.75 \pm 0.47^{c,d}$	$4.56 \pm 0.13^{a,b,c}$
–	2.0	–	$70.50 \pm 0.81^{a,b}$	$8.65 \pm 0.37^{a,b}$	5.80 ± 0.10^{a}
–	3.0	–	68.13 ± 0.75^{b}	$7.20 \pm 0.73^{b,c}$	$5.12 \pm 0.11^{a,b}$
–	4.0	–	$59.45 \pm 0.15^{d,e}$	$5.20 \pm 0.52^{c,d,e,f}$	$4.14 \pm 0.30^{a,b,c,d}$
–	5.0	–	$48.32 \pm 0.35^{h,i}$	$4.60 \pm 0.24^{e,f,g}$	$3.04 \pm 0.42^{c,d,e,f}$
–	–	0.5	0.0	0.0	0.0
–	–	1.0	$51.50 \pm 1.52^{g,h}$	2.45 ± 0.26^{h}	1.95 ± 0.18^{f}
–	–	2.0	64.21 ± 0.45^{c}	$5.60 \pm 0.21^{c,d,e,f}$	$3.67 \pm 0.49^{b,c,d,e}$
–	–	3.0	68.40 ± 0.37^{b}	$6.43 \pm 0.14^{c,d,e}$	$4.32 \pm 0.63^{a,b,c,d}$
–	–	4.0	52.45 ± 0.71^{g}	$4.10 \pm 0.33^{f,g,h}$	$2.48 \pm 0.13^{d,e,f}$
–	–	5.0	43.75 ± 0.32^{j}	$3.50 \pm 0.22^{g,h}$	1.64 ± 0.20^{f}

* Means followed by same letter within a column are not significantly different at ($P \leqslant 0.05$).

Table 6 Effect of $AgNO_3$ along with auxins on rooting from elongated shoots of *Bambusa arundinacea*.

Growth regulator concentration (mg/l)				Root induction (%) (Mean ± SE)*	No. of roots/culture (Mean ± SE)*	Root length (cm) (Mean ± SE)*
IBA	NAA	IAA	$AgNO_3$			
3.0	–	–	0.5	$69.50 \pm 1.60^{d*}$	$5.10 \pm 0.33^{e,f,g,h,i*}$	$4.70 \pm 0.05^{b,c,d,e,f*}$
3.0	–	–	1.0	$70.50 \pm 0.40^{c,d}$	$6.88 \pm 0.48^{c,d,e}$	$5.43 \pm 0.15^{b,c}$
3.0	–	–	2.0	85.15 ± 0.23^{a}	9.34 ± 0.63^{a}	7.40 ± 0.18^{a}
3.0	–	–	3.0	71.45 ± 0.50^{c}	$5.50 \pm 0.53^{e,f,g,h}$	$5.23 \pm 0.08^{b,c,d}$
3.0	–	–	4.0	$62.13 \pm 0.80^{f,g}$	$4.90 \pm 0.21^{f,g,h,i}$	$3.97 \pm 0.75^{c,d,e,f,g}$
–	2.0	–	0.5	71.50 ± 0.80^{c}	$8.40 \pm 0.12^{a,b,c}$	$5.22 \pm 0.06^{b,c,d}$
–	2.0	–	1.0	80.00 ± 0.50^{b}	$8.93 \pm 0.89^{a,b}$	$6.17 \pm 0.72^{a,b}$
–	2.0	–	2.0	67.00 ± 0.40^{e}	$7.40 \pm 0.45^{b,c,d}$	$4.08 \pm 0.54^{c,d,e,f,g}$
–	2.0	–	3.0	$60.50 \pm 0.50^{g,h}$	$6.10 \pm 0.37^{d,e,f,g}$	$3.45 \pm 0.50^{d,e,f,g}$
–	2.0	–	4.0	57.45 ± 0.30^{i}	$4.60 \pm 0.62^{g,h,i}$	$3.66 \pm 0.34^{c,d,e,f,g}$
–	–	2.0	0.5	60.30 ± 0.80^{h}	$5.57 \pm 0.29^{d,e,f,g,h}$	$4.90 \pm 0.50^{b,c,d,e}$
–	–	2.0	1.0	63.70 ± 0.40^{f}	$5.85 \pm 0.34^{d,e,f,g,h}$	$3.05 \pm 0.37^{e,f,g}$
–	–	2.0	2.0	$71.16 \pm 0.23^{c,d}$	$6.73 \pm 0.56^{c,d,e,f}$	$5.46 \pm 0.11^{b,c}$
–	–	2.0	3.0	56.24 ± 0.50^{i}	$4.14 \pm 1.42^{h,i}$	$2.85 \pm 0.04^{f,g}$
–	–	2.0	4.0	45.18 ± 0.25^{j}	3.42 ± 0.20^{i}	2.21 ± 0.43^{g}

* Means followed by same letter within a column are not significantly different at ($P \leqslant 0$ .05).

showed improved callus proliferation [11], enhanced shoot regeneration Khan et al. [18], and promoted root formation [17] in various plant species. Similarly Sridhar et al. [39] described that medium supplemented with $AgNO_3$ inhibited the shoot morphogenesis in *Solanum nigrum*. Results clearly suggest that $AgNO_3$ showed inhibitory effect on shoot bud multiplication in *B. arundinacea*.

The presence of adenine sulfate in the culture medium was beneficial in combination with cytokinin for shoot bud elongation. Among the BAP and ADS concentrations tested, a maximum shoot elongation was achieved on MS medium containing BAP (3.0 mg/l) + ADS (5.0 mg/l) (Table 4). Similarly, Garima and Amla [12] described the promotive effect of ADS in combination with BAP on shoot bud elongation.

In the present investigation, two experiments were adopted for rooting. In the first experiment, three auxins were tried for *in vitro* root induction from elongated shoots of *B. arundinacea*. The percent of rooting was increased with increasing the concentrations of IBA up to 3.0 mg/l, then it was declined beyond these concentrations. Among the three auxins tried for *in vitro* rooting, IBA was found to be the best auxin for rooting of the cultured shoots of *B. arundinacea* followed by NAA (Table 5). Similar results were also reported in *Dendrocalamus asper* and *Bambusa bamboo* [2], *B. nutans* [44], *D. giganteus* [45], *B. balcooa* [9], and in *Pseudooxytenanthera stocksii* [34].

In the second experiment, various concentrations of $AgNO_3$ (0.5–4.0 mg/l) were used along with formulated combinations of various auxin concentrations for rooting.

The frequency of rooting and number of roots were inhibited at higher concentrations of $AgNO_3$ above 2.0 mg/l. The present results suggested that IBA and $AgNO_3$ combination was found to be the best for rooting response compared to the auxin alone (Table 6). The present results are in agreement with earlier reports as in *Rotula aquatica* Lour. [40], *Vanilla planifolia* [14], and in *D. hamiltonii* [3,33].

The rooted shoots with 4 to 5 leaves were successfully transplanted from culture tubes to plastic cups containing soil and sand (3:1) and placed in the growth room for acclimatization. After 15 days, they were shifted to the greenhouse condition before being transferred to field. In the present study, the survival rate noticed was 92% however, the earlier study showed only 85% survival rate in bamboo while earlier studies reported up to 80–85% of survival rate [2]. The field established plants were morphologically uniform with normal leaves, shape, and growth patterns (Fig. 1H and I).

5. Conclusion

In summary, the present study describes an efficient protocol for induction of high frequency multiple shoots directly from the nodal explants of *B. arundinacea* by using different additives such as CW, sucrose, $AdSO_4$ and $AgNO_3$. Of the two cytokinins tested, BAP was found to be the best for shoot bud initiation. Maximum percent of shoot bud initiation was achieved with 3.0 mg/l BAP + 0.5 mg/l IBA combination. The highest frequency of shoot bud multiplication with maximum number of shoots was obtained by the addition of 3.0 mg/l BAP + 4.0% CW + 4.0% sucrose. Maximum percent of rooting was achieved on MS medium supplemented with 3.0 mg/l IBA followed by NAA and IAA. It is interesting to note that the addition of 2.0 mg/l $AgNO_3$ along with 3.0 mg/l IBA has enhanced the rooting frequency as well as length of the roots. The rooted plantlets were acclimatized in the green house and subsequently established in the field. The present study suggests that CW has the potential to enhance the percent of multiple shoot bud regeneration, and silver nitrate produced thick and long healthy roots in *B. arundinacea*.

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