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In Vitro Plant Regeneration of *Dendrocalamus stocksii* (Munro) M. Kumar, Remesh & Unnikrisnan, through Somatic Embryogenesis

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Abstract

Dendrocalamus stocksii is fast cultivating economically important forest crop species. National Mission of Bamboo Application (NMBA) of India has been identified in 15 industrially important bamboo species. Traditionally it was propagated through by offset cuttings and rhizome splitting which was not meeting the demand, culm cuttings needed mass material to propagate and rooting percentage mixed. Plant regeneration through somatic embryogenesis was achieved in callus cultures derived from the callus initiated through type of explants *viz.* leaf, leaf sheath, shoot tip, nodal shoot segments, and inter node segments from aseptic cultures. Explants were cultured on Murashige & Skoog basal media supplemented with 2,4 Dichloro diphenyle ethane 0.44 µM/L with additives (Ascorbic acid 8.8 µM/L, citric acid 4.8 µM/L Cysteine 3.02 µM/L and Glutamine 14.6 µM/L) with 3% sucrose and Agar agar 0.6%. Cultures were incubated in the dark at 25˚C ± 1˚C. Out of five types of explants nodal shoot induced callus > 80% followed by leaf sheath (60%) and no callus was induced in leaf. Various nutrient media *viz.* Murashige and Skoog (MS), Woody Plant Media (WP), Gamborg media (B5) and Heller's (HE) media fortified with 2,4 D (0.2 - 1.10 µM/L) and Kinetin 0.10 µM/L were tested for high frequency callus induction. Among four nutrient media tested MS media fortified with 2,4 D (0.55 - 1.1 µM/L) 100% callus induction. Calli multiplication was carried out with various concentrations of PGR's with 10% coconut milk. Out of these MS media 2,4 D 0.55 µM /L and 10% coconut milk concentration were found best for high frequency (80%) calli multiplication. Various combinations of α-naphthalene acetic acid (NAA) with N^6-benzyiaminopurine (BAP) and kinetin were tested for embryo germination, out of which MS media supplemented with NAA 0.55 µM /L and BAP 0.22 µM /L were showed high frequency (80%) germination. Germinated plantlets carefully transferred to polybags containing potting mixture of sand

soil and compost in the ratio of 40:10:50 with 10 Kg/m^3 + 250 gm/m^3 fungicide. Plantlets were kept 4 weeks under poly tunnel inside mist chamber followed by two weeks outside poly tunnel in mist chamber. Plants are lifted to the canopy condition directed to a week before subjected to them in the institute division nursery.

Keywords

Dendrocalamus stocksii, Somatic Embryogenesis, 2,4 D, BAP, Kinetin, NAA

1. Introduction

Bamboo is the most universally useful plant commodities known to man providing food raw material, shelter, and even medicine for the greater part of the world's population. There are 75 genera and 1250 species of the family Poaceae, throughout the world [2]. In India has 125 species in 23 genera spread over in 8.96 million ha in 25 states and union territories, which covered 12.8 % of total forest area [3]. North Eastern states are rich in bamboo Bioresource and represent about 66 percent of the growing stock of bamboo in the country [4]. Bamboo has more than 1500 uses documented in the literature. Apart from commercial applications, bamboos are known for carbon sequestration, checking soil erosion water conservation, wind barrier, biofencing and restoration of degraded land [5]. About one dozen dominant species are commercially valuable and significant. Among the indigenous species, about 10 species are introduced (exotic species) in India. *Dendrocalamus stocksii* (Munro) M. Kumar, Remesh & Unnikrisnan, (Earlier *Pseudoxytenanthera stocksii* Munro/*Oxytenanthera stocksii*) is locally known as Maarihal bamboo/seame bamboo, naturally occurring in the Western Ghats. It is distributed in Karnataka, Goa, Kerala and Maharashtra. *D. stocksii* is a media size species, has stout solid and strong culm. Culm attains height of 9 meters; diameter ranges 2.5 - 5.8 cm and internodes length ranges 15 - 29 cm. This species has great economic and ecological importance and is used in construction, making baskets, umbrella handles and poles [6]. This bamboo species is mostly confined to the banks of streams and requires a well drained deep soil. It is cultivated in coastal belt of Karnataka. *D. stocksii* is considered as an important agroforestry species, also ideal for water shade and coastal region plantations. Due to its multiple uses, NMBA identified this species as the most significant species among 15 industrially important species. Gregarious Flowering has been reported during 1884 and 1889 in North Karnataka twice. Recently, flowering has been observed at silent valley, Kerala [7]. *D. stocksii* is traditionally propagated by the offset cutting, splitting rhizome. Culm cuttings require eight months to produce clonal plant with 90% success rate. Sporadic flowering in *D. stocksii* is common but unfortunately seed setting has not been reported. Vegetative propagation by culm cutting and culm branch cuttings is easy to follow,

but low success rate, availability of right stage of material for short period, bulk requirement of material and low production potential are limiting factors of macro propagation. Biotechnological tools provide scope for rapid and mass clonal propagation of Forestry species, including bamboos (*D. stocksii)*. Genetic improvement work on bamboo species is at beginning stage in India. Macro propagation of bamboo is through offset cutting, rhizome splitting, *D. stocksii* (*Pseudoxytenanthera stocksii/Oxytenanthera stocksii*) [8]. For bamboo, different propagation techniques are used, such as seed based, propagation, offset cuttings, rhizome and culm cuttings [9] [10]. But these suffer from serious drawback for large-scale propagation. Most of the classical techniques for clonal propagation are useful for the small-scale production (up to 10,000 plants/yr). For mass scale propagation (>50,000 plants/yr) classical techniques are largely insufficient and inefficient and tissue culture is the only reliable method [11]. An integrated approach of biotechnology has played a key role in the fast development in the improvement programme of forestry species, including bamboos. Tissue culture based propagation through axillary shoot proliferation and somatic embryogenesis has potential for mass production and improvement [12]. Micropropagation techniques have been employed for propagation of various bamboo species [13] [14] [15]. Most of the studies on micropropagation on bamboo are based on seedling explants, either through axillary shoot proliferation or by somatic embryogenesis [16]. Very few studies deal with *in vitro* cloning from explants of field grown/mature culm [17] [18] [19]. Present study deals with various explants used for high frequency callus induction, effect of PGR's on shoot initiation, effect of PGR's for high frequency embryo induction and germination.

2. Materials and Methods

1) Sample collection: Selection of Candidate Plus Clumps of *Dendrocalamus stocksii* (*Pseudoxytenanthera stocksii*) has been carried out in Gottipura (Hoskote) Karnataka by the Sirsi Forestry College (University of Agricultural Sciences, Dharwad) (E'14˚37'12.00, N'074˚50'60.00).

2) Calli initiation: In order to explore possibility of somatic embryogenesis/callus differentiation for recovery of plantlets in three different experiments viz; a) effect of various types of explants b) effect of various auxins and c) effect of various nutrient media on embryogenic callus induction were carried out. For these experiments, *in vitro* shoot multiplication cultures were used. Callus initiation cultures were incubated at 25˚C ± 1˚C temperature under dark condition in culture room.

3) Effect of various types of explants: In order to induce high frequency callus, various explants *viz.* leaf segment, leaf sheath, shoot tip, nodal segments and inter nodal segments were used in MS agar gelled media fortified with 2,4-D (0.8 - 2.1 μM) alone and with combination of BAP (0.49 μM/L). Each explant in

three treatments with three replicates was tested of each replicate with five explants. Observations were taken after completion of four weeks in response of callus initiation callus intensity and callus texture was recorded.

4) Effect of PGR's with additives: In order to induce high frequency embryogenic callus, various auxins *viz.* IAA, IBA, NAA, NOA (2.65 μM/L) and 2,4-D (0.86 μM/L) were tested either alone or in combination with Kn 0.22 μM/L, coconut milk and additives in the MS media with 18 treatments, each treatment with three replicates of each replicates with five explants. Nodal segment used as an explant. Observations were taken after completion of four weeks in response of callus initiation callus intensity and callus texture was recorded.

5) Effect of nutrient media: Simultaneously, in order to find out requirement of nutrients, various nutrient media *viz.* MS, B5 and WP were also tested with 2,4-D (0.86 - 2.1 μM/L) alone and with Kn (1.12 μM/L) to select the best media for high frequency embryogenic callus induction in ten treatments. Nodal shoot segments were used as an explant for this experiment for callus induction. Cultures were kept under dark condition at 25˚C ± 1˚C temperatures in culture room. Observations were taken after completion of four weeks in response of callus initiation callus intensity and callus texture was recorded.

6) Calli multiplication: In order to further multiply embryogenic callus cultures for somatic embryogenesis. Callus induced from the nodal shoot segment was sub culture on MS media + additives + 2,4-D 0.86 μM/L for three passages and kept under dark condition at 25˚C ± 30˚C temperature in culture room. Greenish white and healthy callus was used from the callus initiation cultures. Brownish and mucilaginous callus was discarded. To standardize PGR's and nutrient media for high frequency further multiplication of embryogenic callus two experiments were conducted *viz.* effect of PGRs and media. Various concentration of 2,4-D were tested with Kn, coconut milk (10% v/v), BAP and Glutamine (154 μM/L - 500 μM/L) in MS media with additives.

7) Effect of PGR's with coconut milk: To standardize PGR's for high frequency multiplication of embryogenic callus, MS media with various concentrations of 2,4-D (0.86 -4.3 μM/L), BAP (0.49 - 0.99 μM/L), Kn (0.47 μM/L), glutamine (100 μM/L) and coconut milk (10% v/v) with additives in ten different treatments were tested. Observations were taken after completion of four weeks.

8) Effect of nutrient media: In order to find out best nutrient media for high frequency embryogenic callus multiplication, various media viz; MS, B5 and WP were used with 2,4-D (0.86 - 4.3 μM/L) either alone or with Kn (1.12 μM/L). Observation was recorded in callus multiplication rate, callus intensity and texture after completion of four weeks sub culturing of embryogenic callus was carried out within 4 - 5 weeks on fresh callus multiplication media. Every time brownish/dead callus mass was removed before sub culturing callus on fresh media.

9) Somatic embryogenesis: In order to differentiate the callus and recover, plants through somatic embryogenesis, embryogenic callus was sub cultured in MS media with various combinations of growth hormones *viz.* 2,4-D (0.21 - 0.43

μM/L), NAA (0.25 - 0.50 μM/L) with Kn (0.22 - 1.12 μM/L), BAP (0.24 - 0.99 μM/L) and additives were tested. Ten treatments of each five replicates were conducted. Somatic embryo induction and germination were recorded after completion of four weeks.

10) Hardening of somatic embryogenic plants: Plantlets were germinated somatic embryos with complete plantlets were transplanted in 600 cc polybags consisted potting mixture (Sand, soil and compost in the ratio 3:1:4). Potting mixture was enriched with 10 Kg/m^3 Neem cake ± 2.5 Kg SSP/m^3 and Bavestin 0.4 kg/m^3 + phorate 0.4 m^3 were add in the potting mixture as prophylactic measures against insect pest attack. Hardening was found essential for 3 - 4 weeks in mist chamber at 30˚C ± 50˚C temperature and 80% ± 50% RH (related humidity) before transplanting in open nursery.

3. Results and Discussion

3.1. Results

1) Effect of explant type and size: Various explants viz; leaf, leaf sheath, internode and nodal segments were tested for callus induction on MS media with 2,4-D (0.86 - 2.1 μM/L) either alone or with BAP 4.99 μM/L under dark at 25˚C ± 1˚C temperature. Among these explants, nodal segment was proved the best in terms of high frequency callus induction as well as intensity of callus. This was followed by the leaf sheath and internode (Table 1). None of the treatments induced callus from the leaf. Callus initiation was observable within 10 days and delaying in sub-culturing (more than five weeks) deteriorated the embryogenic callus growth and turned brown.

2) Effect of growth hormones: Various auxins (IAA, IBA, NAA, NOA and 2,4-D) and their different concentrations were tested and out of which 2,4-D (0.86 - 4.31 μM/L) + MS media with additives were induced callus from the nodal shoot segment. The other auxins substitutes were failed to induce callus, except NAA, which established tiny callusing from nodal region of the explants (Table 2). Higher concentration of 2,4-D did not help in further improvement of callus intensity. Incorporation of Kn/coconut milk along with 2,4-D in the media did not improve the frequency and intensity of the callusing.

3) Effect of nutrient media on callusing: Among the various nutrient media tested, MS media with 2,4-D (0.86 - 4.31 μM/L) only proved the best for callus induction, followed by the B5 media (Gamborg's B5 media). Callusing frequency and intensity was significantly reduced with reduced level of nutrient in the media (Table 3). Precisely slight callus induction was observed on WP (woody plant) media, whereas callus induction was not noticeable on Heller's media.

4) Calli multiplication and effect of PGR's and coconut milk: Callus multiplication was very slow for the initial two sub-cultures. Among the various media and growth hormones tested, MS media with additives 2,4-D 2.1 μM/L with coconut milk 10% proved suitable for callus multiplication. WP and HE media were not found suitable for callus multiplication. Sub culturing of callus on fresh

Table 1. Effect of various explants type on callus initiation from *D. stocksii* on MS media supplemented with additives.

SI. No.	Type of Explant	PGRs				Response%	Callus intensity
		PGRs	µM	PGRs	µM		
1.	Leaf segment	2,4-D	0.86			00	--
2.		2,4-D	2.1			00	--
3.		2,4-D	2.1	BAP	0.49	00	00
4.	Leaf sheath	2,4-D	0.86			36.67	+
5.		2,4-D	2.1			66.67	+++
6.		2,4-D	2.1	BAP	0.49	53.33	++
7.	Shoot tip	2,4-D	0.86			33.33	+
8.		2,4-D	2.1			46.67	++
9.		2,4-D	2.1	BAP	0.49	36.67	+
10.	Nodal segments	2,4-D	0.86			88.33	++++
11.		2,4-D	2.1			100.00	+++++
12.		2,4-D	2.1	BAP	0.49	98.33	+++++
13.	Internode segments	2,4-D	0.86			00	--
14.		2,4-D	2.1			16.67	+
15.		2,4-D	2.1	BAP	0.49	00	---
	SE					26.77	
	CD					54.66	

---; no response, +: Slow growth ++: Moderate growth, +++: Good growth, +++++: Excellent growth.

Table 2. Effect of PGRs & additives on callus initiation in *D. stocksii*.

SI. No.	Treatments								% of response ± SE	Callus intensity
	PGRs	µM	PGRs	µM	PGRs	µM	PGRs	%		
1.	HF	0.00							0.00 ± 00	00
2.	2,4-D	0.86							61.67 ± 0.46	+++
3.	2,4-D	2.1							73.33 ± 0.71	++++
4.	2,4-D	4.31							63.33 ± 0.71	+++
5.	2,4-D	8.6							36.67 ± 0.67	++
6.	2,4-D	0.86	Kn	0.10					31.67 ± 0.16	++
7.	2,4-D	2.1	Kn	0.10					38.33 ± 0.42	++
8.	2,4-D	4.3	Kn	0.10					33.33 ± 0.48	++
9.	2,4-D	2.1	Kn	0.10	Glu	100.00			41.67 ± 0.73	++
10.	2,4-D	2.1	Kn	0.10		249.10			35.00 ± 0.27	++
11.	2,4-D	2.1	Kn	0.10		500.00			26.67 ± 0.24	+
12.	2,4-D	2.1	Kn	0.10			CM	10%	91.67 ± 0.65	+++++
13.	2,4-D	2.1	Kn	0.10	Glu	250.02	CM	10%	21.67 ± 0.52	+
14.	2,4-D	2.1	Kn	0.10	Glu	249.10	CM	10%	18.33 ± 0.61	+
15.	NAA	2.9	Kn	0.10					23.33 ± 0.44	+
16.	NOA	2.7	Kn	0.10					13.33 ± 0.15	+
17.	IAA	3.14	Kn	0.10					00 ± 00	00
18.	IBA	2.7	Kn	0.10					00 ± 00	00

Ascorbic acid 49.9 µM/L, Citric acid 22.8 µM/L, Cysteine 24.98 µM/L. ---; no response, +: Slow growth ++: Moderate growth, +++: Good growth, +++++: Excellent growth.

Table 3. Effect of various nutrient media on callus initiation from nodal shoot segment of *D. stocksii.*

SI. No	Media	PGRs				Response%
		PGRs	µM	PGRs	µM	
1.	MS	2,4-D	0.86			65.47
2.	MS	2,4-D	2.1			100.00
3.	MS	2,4-D	4.3			100.00
4.	MS	2,4-D	4.3	Kn	1.12	95.67
5.	B5	2,4-D	0.86			00
6.	B5	2,4-D	2.1			00
7.	B5	2,4-D	4.3			00
8.	B5	2,4-D	2.1	Kn	1.12	00
9.	WP	2,4-D	0.86			56.67
10.	WP	2,4-D	2.1			80.00
11.	WP	2,4-D	4.3			85.00
12.	WP	2,4-D	2.1	Kn	1.12	75.00

Additives: Ascorbic acid 49.9 µM/L, citric acid 22.8 µM/L, Cysteine 24.9 µM/L and Glutamine 100 µM/L. ---; no response.

media for further multiplication was found essential within 4-5 weeks period. Embryogenic callus was maintained for a year by sub-culturing on fresh media. Dark incubation condition at 25˚C ± 1˚C proved better than light for embryogenic callus multiplication. Out of various concentrations of 2,4-D 0.86 - 4.31 µM/L with kinetin (0.47 µM/L) and BAP 2.22 - 4.44 µM/L and coconut milk 10% tested, MS media fortified with 2,4-D 2.1 µM/L with coconut milk 10% proved the best for further multiplication of callus. On this callus was healthy, whitish in colour and organized and compact. Callus produced on media consisted 2,4-D 0.86 - 4.31 µM/L alone was whitish brown in colour and soft. Incorporation of BAP/CM with 2,4-D resulted in nodular and compact callusing (**Table 4**). Higher concentration of 2,4-D (4.31 µM/L) in the media was not suitable and callus turned brown at the end of 4 weeks.

5) Effect of nutrient media: Various nutrient media viz.; MS, B5 and WP fortified with additives, 2,4-D (0.86-4.31µM/L) alone with kan (1.12 µM/L) callus multiplication, MS media fortified with additives + 2,4-D 0.86 µM/L proved the best for high frequency callus multiplication which was healthy, whitish in colour and organized (**Table 5**). This was followed by [20] media with 2,4-D 2.1 µM/L and exhibited slow callus multiplication. Comparing B5 and WP media, callus was whitish and organized on WP media with 2,4-D 0.86 µM/L.

6) Somatic embryogenesis: Somatic embryogenesis was observed on MS hormone free media as well as on media consist NAA (0.25 - 0.50 µM/L) + BAP (0.24 - 0.99 µM/L). MS Medium fortified by NAA 0.25 µM/L with BAP 0.99 µM/L (85%) were found the best for somatic embryo germination followed by

Table 4. Effect of various concentrations of 2,4-D, kinetin and coconut milk on callus Multiplication of *D. stocksii* on MS media.

SI. No.	PGRs						Response%	Callus intensity
	PGRs	µM	PGRs	µM	PGRs	µM		
1.	HF	--		--			12.67	+
2.	2,4-D	0.86					23.67	+
3.	2,4-D	2.1					38.67	++
4.	2,4-D	4.3	Kn	0.22			33.67	++
5.	2,4-D	0.86			CM	10%	66.33	+++
6.	2,4-D	2.1			CM	10%	88.33	++++
7.	2,4-D	4.3			CM	10%	66.67	+++
8.	2,4-D	0.86	BA	0.49			26.67	+
9.	2,4-D	2.1	BA	0.99			33.33	++
10.	2,4-D	4.3	Kn	0.22	Gl.	685	41.67	++

Additives: Ascorbic acid 49.9 µM/L, citric acid 22.8 µM/L, Cysteine 24.9 µM/L and Glutamine 100 µM/L. ---; no response +: Slow growth ++: Moderate growth, +++: Good growth, ++++: Excellent growth.

Table 5. Effect of nutrient media with additives on callus multiplication in *D. stocksii.*

SI. No.	Media	PGRs				Response%	Callus intensity
		PGRs	µM	PGRs	µM		
1.		2,4-D	0.86			11.67	+
2.		2,4-D	2.1			86.67	++++
3.	MS	2,4-D	4.3			76.67	++++
4.		2,4-D	2.1	Kn	1.12	48.33	++
5.		NAA	5.00	Kn	1.12	26.67	+
6.		2,4-D	0.86			8.33	+
7.		2,4-D	2.1			31.67	++
9.	B5	2,4-D	4.3			53.33	+++
10.		2,4-D	2.1	Kn	1.12	48.33	++
11.		NAA	5.00	Kn	1.12	33.33	++
12.		2,4-D	0.86			16.67	+
13.		2,4-D	2.1			46.67	++
14.	WP	2,4-D	4.3			58.33	+++
15.		2,4-D	2.1	Kn	1.12	43.33	++
16.		NAA	5.00	Kn	1.12	28.33	+

Additives: Ascorbic acid 49.9 µM/L, citric acid 22.8 µM/L, Cysteine 24.9 µM/L and Glutamine 100 µM/L. ---; no response +: Slow growth ++: Moderate growth, +++: Good growth, ++++: Excellent growth.

medium contains NAA 0.500 - BAP 0.99 µM/L (83.33%) Cultures incubated under light condition developed root as well as shoot from the embryoids. In

some treatments *in vitro* flowering was observed directly from the somatic embryo cultures (Table 6).

3.2. Discussion

1) Callus initiation: Selection of suitable explant, plant growth hormones, nutrient media and incubation conditions are important factors, which have influence on frequency of callusing, intensity of callusing and quality of callus. It was revealed from the results of the effect of various explants on callus induction, nodal shoot segments obtained from the *in vitro* grown shoot cultures proved the best for induction of high frequency embryogenic callus in both the species on MS media supplemented with additives (ascorbic acid 8.8 µM/L + citric acid 4.8 µM/L + Cysteine 3.0 µM/L) + 2,4-D (0.86 - 4.31 µM/L) under dark conditions at 25˚C ± 1˚C temperature within 4 weeks period. It was revealed from the results of PGRs, media, explants and additives experiments that MS media supplemented with additives + 2,4-D, 2.1 µM + Kn 0.22 µM + CM 10% exhibited high frequency (70%) and high intensity of embryogenic callus from the nodal segment under dark condition. 2,4-D alone was less effective at all the concentrations (0.86 - 4.31 µM/L) tested. Incorporation of glutamine with 2,4-D in the media has not improved frequency, intensity and quality of the callus. Nutrient media have direct effect on callus frequency and intensity. Among the MS, B5 and WP media tested with 2,4-D, MS media with 2,4-D 4.31 µM/L proved the most suitable media for high rate of callus induction (100%) from the nodal shoot segment under dark condition at 25˚C ± 1˚C temperature (Figure 1). Though frequency of callusing was quite high (80%) in WP media but callus growth was poor. Similar to our results, [21] reported callus induction from nodal explants of *in vitro* grown seedling and excised mature zygotic embryo of

Table 6. Effect of PGR'S in MS media with additives on Somatic embryos germination in *D. stocksii.*

SI. No.	PGRs				Frequency of Embryoids	Germination%
	PGRs	µM	PGRs	µM		
1	HF	--	--	--	++	38.33
2	2,4-D	0.20	Kn	0.22	++	51.67
3	2,4-D	0.43	Kn	0.22	+++	76.67
4	NAA	0.25	BA	0.24	++	41.67
5	NAA	0.50	BA	2.22	+++	78.33
6	NAA	0.25	BA	0.49	++++	85.00
7	NAA	0.25	BA	0.99	++++	83.33
8	NAA	0.25	Kn	0.56	++	56.67
9	NAA	0.50	Kn	1.12	++	53.33

Additives: Ascorbic acid 49.9 µM/L, citric acid 22.8 µM/L, Cysteine 24.9 µM/L and Glutamine 100 µM/L. +: Slow growth ++: Moderate growth, +++: Good growth, ++++: Excellent growth.

Figure 1. (A) Various explants for callus initiation; (B) Developing callus.

three bamboo species on MS media supplemented with 2,4-D (0.94 μM/L) + Kn (0.42 μM/L) and adenine sulphate (3.68 μM/L). Similarly [22] used nodal and internodes tissues from the *in vitro* grown plants on MS media with 2,4-D (0.65 μm/L) and kinetin (0.42 μm/L) with coconut milk (0.1%) and 6.0% sucrose for embryogenic callus in *B. edulis*. [23] obtained friable and compact callus from the embryogenic ends of the seeds (mesocotyl area of the zygotic embryo) of *D. strictus* on B5 media supplemented with 2,4-D (0.66 μm/L) + sucrose 2%. In another study, [24] established callus from mature embryo and explants from aseptically grown seedling on B5 media with 2,4-D in *D. strictus* and *B. bambos*. In the current study leaf, leaf sheath and root segment were not found suitable for induction of embryogenic callus whereas nodal shoot segments, other type of explants either remained healthy or induced tiny callusing. This may be attributed to the origin of explants from the mature plants and its recalcitrant nature. Contrary to our finding [24] reported induction of callus from inflorescence, zygotic embryo, rhizome, nodes and leaf sheath of juvenile plants of *B. bambos* and *D. strictus*on B5 media with 2,4-D. Similarly, [17] reported callus induction from leaf (inner most part) explant of *P. viridis* on media consisted macronutrients of MS media and micronutrient of Nitsch media with 2,4-D (9×10^{-6} M) and 2% sucrose within 4 - 5 weeks period. Whereas, [18] obtained callus from shoot apices of *Bambusa*, *Phyllostachys* and *Sesa*on MS media with 2,4-D. [25] observed callus induction from leaf section of *B. glacescens* on MS media with 2,4-D (0.87 μM/L) under dark condition and callus proliferation started after one week from epidermal layer of leaf. [26] observed callus initiation from young florets and adventitious roots (obtained from the florets) on MS media with high concentration of 2,4-D (0.44 μM/L) + Kn (0.96 μM/L) and 6% sucrose. [26] obtained callus from mature zygotic embryo of *Sinocalamus latifolia* on MS media with 2,4-D (63.8 μM/L) + Kn (0.31 μM/L) + sucrose 5% and PVP (625 μM/L) under dark condition. They encountered phenolics problem, which was overcome by the addition of PVP in the media. [27] obtained embryogenic callus from seeds of *D. strictus* on MS media supplemented with 2,4-D. They observed two types of callus *viz*. a) yellow and compact-embryogenic and b) white and friable which was non-embryogenic. Rarely, third type of callus, which was translucent and jelly like, also produced. There is no previous report on the ef-

fect of additives like ascorbic acid, citric acid, cysteine to minimize leaching and browning problem in bamboo. In the present study addition of the above cited minimized additives in the media, reduced the leaching and browning but also showed auxiliary effect on callus intensity.

2) Embryogenic callus multiplication and induction of somatic embryos: In order to maintain embryogenic callus, it is essential to optimize growth hormones and nutrient media. Results of the effect of PGRs on callus multiplication revealed that MS media with 2,4-D 0.86 - 4.31 μM/L with or without coconut water proved most suitable for multiplication of embryogenic callus as well as induction of somatic embryos under dark condition at 25˚C temperature **Figure 2**. Delaying in sub culturing of callus (more than 4 weeks) resulted in induction of somatic embryoids. Other auxins like IAA, NAA and NOA did not indulgence of callus multiplication. Similar to our findings [26], multiplied embryogenic callus

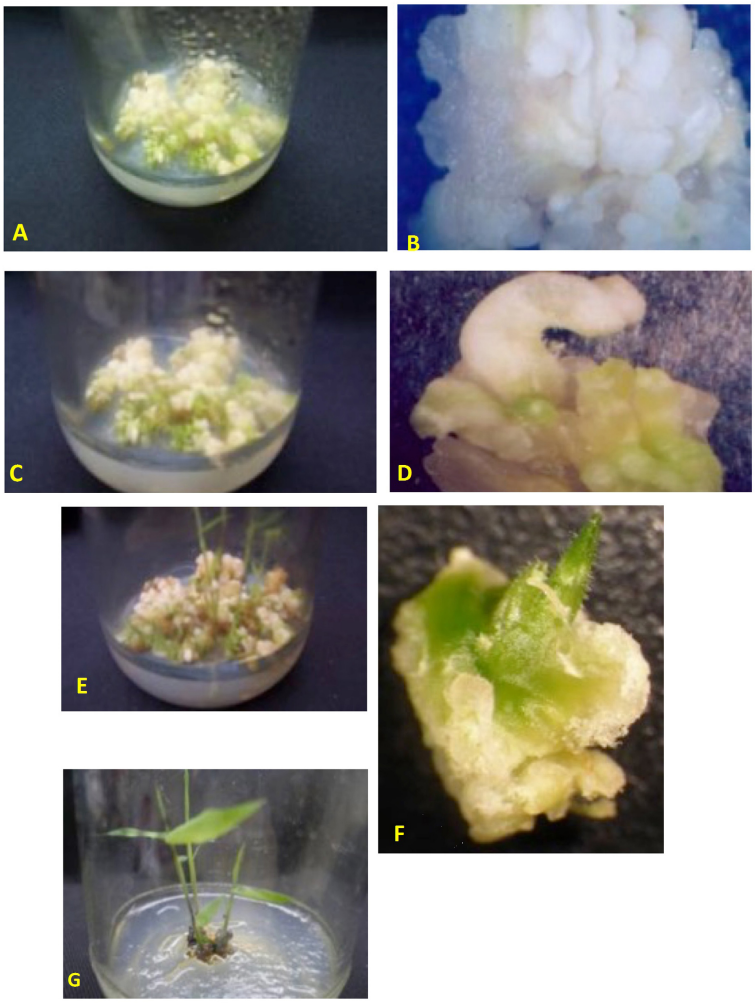

Figure 2. Regeneration of plantlet from young shoots of mature bamboo: (A) Callus was induced from the shoots of Ma bamboo. (B) The callus grown on the callus multiplication media. (C) Callus turns green on shoot induction media. (D) The induced shoots on shoot induction media. (E) The adventurous shoots grow in cluster. (F) Root was induced on root induction media. (G) Germinated somatic embryogenic plant.

of *Sinocalamus latifolia* by the sub-culturing on callus multiplication media (MS + 2,4-D + Kn + PVP) in 3 weeks interval, which resulted in white compact callus formation. They observed that prolong culture on the callus multiplication media, resulted in embryoids formation and also found that no other auxins viz; IAA, NAA can substituted 2,4-D. Among the various media *viz.*; MS, B5 and WP tested with 2,4-D (0.22– 1.1 µM/L), MS media with 2,4-D 0.55 µM/L proved the best followed by WP for multiplication of embryogenic callus and subsequent development of somatic embryos. Whitish, compact and slow growing callus after 3 weeks of sub-culturing produced embryoids of various stages on the MS media with 2,4-D 022 - 0.55 µM/L (**Figure 3**). [26] reported that further multiplication of embryogenic callus was on the same media as of callus initiation. They found that MS media with 2,4-D + Kn favoured in multiplication of embryogenic callus of *B. beechayana.* *[26]* observed three types of callus colonies from the leaf tissues of *B. glaucescens* on MS media with 2,4-D. Only slow growing compact callus was embryogenic. Lower concentration of 2,4-D with

Figure 3. (A) Germinated embryos. (B) Shoot of germination embryos. (C) Later stage of germination shoot. (D) Shoot and root of germinating embryos. (E) Elongated root germinated plant.

IAA + BAP produced nodular structures [25]. MS media with 2,4-D (2.99 μM/L)) and TDZ (0.010 μM/L) with 3.0% sucrose for callus proliferation in *B. edulis.* Sub-culturing was carried out in 2 months on fresh media for callus multiplication. [17] observed multiplication of callus on MS media with 2,4-D and recorded two types of callus *viz.* embryogenic and non-embryogenic callus bin *P. viridis*. Huang *et al.* (1989) observed that callus developed on 2,4-D media differentiated into shoot apices on media supplemented with BAP under light condition in bamboo viz.; *Bambus, Phyllostachys* and *Sesa* Spp. In the present study, MS media supplemented with additives + 2,4-D 0.022 - 0055 μM/L + coconut milk, 10% induced somatic embryos (**Figure 4(A)**). Alternatively MS media supplemented with NAA 0.093 μM/L + BAP 0.56 μM/L also induced high frequency of somatic embryos. Embryogenic callus was maintained for one year on MS media with additives + 2,4-D 0.22 μM/L at 25˚C ± 1˚C temperature under dark condition. [28] observed that MS media was better than B5 media for embryogenesis and MS media with 2% sucrose + 2,4-D (0.66 μM/L) + BA (0.11 μM/L) was suitable for

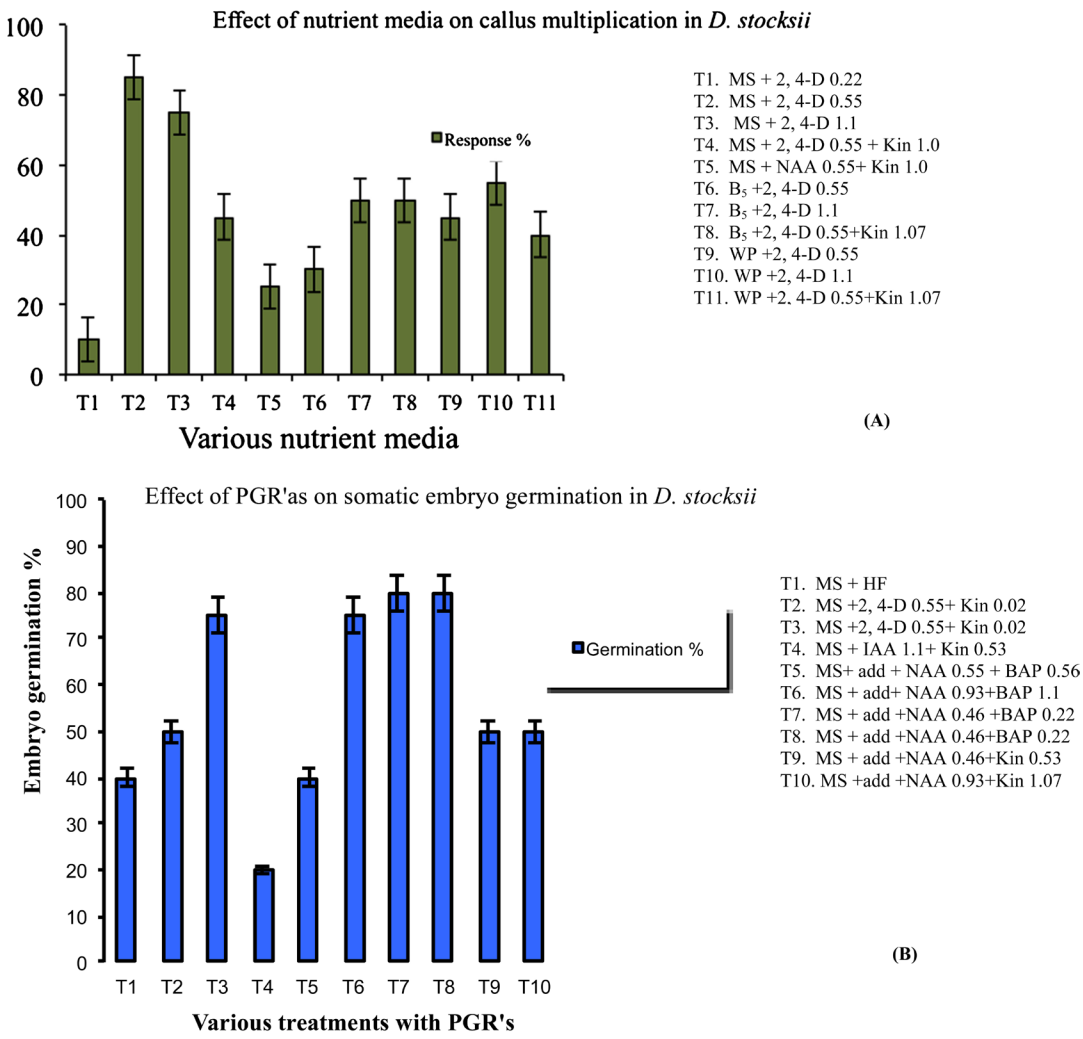

Figure 4. (A) Graph profiling of nutrients on callus multiplication. (B) Graph profiling of PGR's on Somatic embryos germination.

somatic embryos multiplication. Embryogenic callus maintained more than two years in *Otateaacuminata aztecorum*. Average 10.7 cultures of embryos recovered per culture. Casein hydrolysate did not help in embryo induction, but incorporation of 2,4-D was found essential in the media. [22] maintained embryogenic callus on B5 media with 2,4-D at 27˚C ± 2˚C temperature under the 2500 Lux light condition. Secondary embryoids were developed from the primary embryos. About 67% of callus cultures developed somatic embryos. [24] maintained embryogenic cultures on B5 with 2,4-D in *B. bambos* and *D. strictus.* [21] observed that MS media with 2,4-D + Kn favoured callus multiplication in *B. vulgaris, D. strictus* and *D. gigenteus.* Sub-culturing was carried out routinely at the interval of 4 weeks. [27] reported the multiplication of embryogenic callus cultures of *D. strictus* on MS media with 2,4-D. Sub-culturing of somatic embryos was carried out in 5 - 6 weeks. During sub-culture, non-embryogenic callus and dead tissues were removed. Two types of media *viz.* a) MS + 2,4-D (1×10^{-5} M) + Kn (5×10^{-6} M) + IBA (1×10^{-5} M) and b) MS + 2,4-D (10^{-5} M) + BAP (10^{-5} M) were used for the multiplication of somatic embryos. Comparing these two, former proved better for 70% - 80% genotypes. Multiplication rate was 2 - 5 fold in 5 weeks period and browning problem was reduced by the addition of PVP and ascorbic acid in the media.

3) Germination of somatic embryos: Somatic embryo produced on MS media with 2,4-D media with or without coconut milk exhibited high frequency germination on hormone free MS media with 4 weeks period. Germination frequency was improved by adding NAA 0.500 µM/L + BAP 0.24 - 0.99 µM/L in the MS media (**Figure 4(B)**). In this media about 80% somatic embryos germinated. In the present study frequency of albino plants was less than 1%. [21] reported more than 80% somatic embryo germination of MS media supplemented with TDZ (0.455 µm/L) in *B. edulis.* They found that NAA inhibited germination. There were no albino plants from the callus cultures. Well-developed plantlets were successfully transferred to soil. [20] observed regeneration of somatic embryo and germination on MS/2 media with Kn (0.24 µM/L). They revealed that for initial three weeks greenish white embryogenic callus which subsequently develop into plantlets in bamboo species viz.; *B. vulgaris, D. giganteus* and *D. strictus.* They were also obtained few numbers of albino plants. [25] found that somatic embryoids developed on 2,4-D + Kn media developed plantlets, when transferred to a media containing lower concentration of 2,4-D (0.66 µM/L) and kinetin (2 mg/L). [26] observed spontaneously germination of somatic embryos on the embryogenic callus multiplication media *i.e.* MS media with 2,4-D + Kn. [17] reported germination of somatic embryos on hormone free MS media and plantlets developed within two weeks of *P. vividis*. [29] tested IBA and NAA (10^{-6} - 10^{-5} M) either alone or in combination with BAP or Kn for somatic embryo germination of *D. strictus.* They found that MS media with NAA (5×10^{-6} M) + Kn (5×10^{-6} M) proved to be optimal for somatic embryo germination and germination frequency was 90% and nearly 85% genotypes produced plantlets. Media solidification with gelrite (0.2%) proved better than agar (0.7%) and ger-

mination frequency reduced 30% - 50% in agar gelled media. Incorporation of PVP in the germination not only reduced browning state whereas it increased the germination frequency. They observed inhibitory role of ABA in the germination media. Rao *et al.,* (1985) reported somatic embryo germination on B5 media with IBA and NAA and 40% embryos developed plantlets. Further development of plantlets occurred on B5 liquid media (half strength) + sucrose (1%) + IBA (5×10^{-7} M) + NAA (10^{-7} M). [15] observed more than 95% of the germination somatic embryos developed shoots and roots on B5 media transferred to soil with 85% success.

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Author Contributions

1st author contributed 50% and others contributed 50%.

Author Statement

All authors read, reviewed, agree and approved the final manuscript.

Conflicts of Interest

None declared.

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Abbreviations

BAP: N^6-benzyiaminopurine
IAA: Indole-3-acetic acid
IBA: Indole-3-butyric acid
Kn: 6-furfurylaminopurine
MS media**:** Murashige and Skoog [1] media
NAA: α-naphthalene acetic acid
NOA: Napthoxy acetic acid
B_5 media**:** Gamborg *et al.* (1968) media
HE media**:** Heller, (1953) media
WP: Woody Plant Media, (Lloyd and McCown, 1980)
HF: Hormone free media
CM: Coconut milk
PGR's: Plant growth regulators