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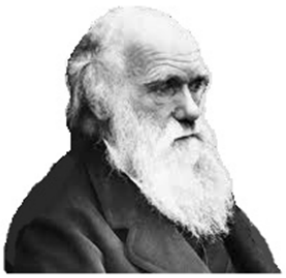

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An Improved Micropropagation Protocol for Manga Bamboo - *Pseudoxytenanthera stocksii* (Munro) T.Q. Nguyen

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ABSTRACT

The present report illustrates an improved and reproducible micropropagation system for economically valuable bamboo species *Pseudoxytenanthera stocksii* using nodal segments. Direct organogenesis from nodal segments was accomplished by culturing on Murashige and Skoog (MS) medium supplemented with 6.0 mg/L 6-benzylaminopurine (BAP). On this medium combination 9.0±0.25 shoots per explant were induced. Shoot multiplication was subjective to the combination of auxins and cytokinins concentrations used and number of repeated transfer of mother explants or subculturing of *in vitro* regenerated shoot clumps on fresh medium. Maximum 41.9±1.00 shoots were observed on MS medium supplemented with 4.0 mg/L of BAP and 0.25 mg/L of naphthalene acetic acid (NAA). Half strength MS medium containing various concentrations of auxins was used for *in vitro* root formation. Successful rooting with maximum response was achieved on half strength MS medium + 50% sucrose and 1.0 mg/L IBA (24.3±0.27 roots). The rooted plantlets were planted the soilrite and hardened in the greenhouse for 6-8 weeks. Completely acclimatized plantlets exhibited 96% survivability. The present propagation protocol could satisfy the demand of *P. stocksii* and could be explored for the commercial propagation of this valuable manga bamboo.

***Keywords*:** *In vitro* propagation, Plant growth regulators, *Dendrocalamus stocksii*, Acclimatization

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1. INTRODUCTION

Bamboos belong to the grass family Poaceae, subfamily Bambusoideae and tribe Bambuseae. Bamboos stand as a valuable crop for the welfare of human as raw material for range of products and various industrial applications from time immemorial. Ecologically, bamboos support various forms of life and are preferred as important species in soil and water conservation, carbon sequestration, balancing O_2 and CO_2 in the atmosphere, barrier of ultra violet rays by reducing light intensity (Venkatachalam *et al*., 2015). Cultivation/ production of bamboo are foremost in India among the Asian countries. Across the globe, around 75 genera with approximately 1500 species were reported (FAO, 2001; Qing *et al*., 2008). In India, about 18 genera with 128 species have been reported and among them 20 species are under commercial cultivation (Seethalakshmi and Kumar, 1998). The bamboo dependent economy of India was estimated to be Rs. 26000 Crore by 2015 (Mahapatra and Shrivastava, 2013). The demand for bamboo is increasing than its production and Govt. of India has emphasized on the cultivation of bamboo in large scale on priority to support the developing industries with sufficient raw material.

Conventionally, bamboo species are cultivated through macroproliferation through rhizomes, branch cutting or rooting of culms, which could not meet the huge demand (Adarsh Kumar, 1991; Banik, 1994). Commercially exploited woody bamboos are reported to be gregarious flowering group, hence the species/plant led to death followed by flowering, which leads to the extinction of various commercially important bamboo species. Lack of seed and/or other types of propagules could be a limiting factor for establishment of large-scale commercial plantations of the desired species. Hence, attention is needed to overcome the conventional propagation problems and tissue culture protocols could be necessary measures to satisfy the demand of propagules preparation in a short period of time (Banik, 2015).

Several micropropagation protocols were developed in number of bamboo species all over the world viz., *Gigantochloa atroviolaceae* (Bisht *et al*., 2010), *Dendrocalamus giganteus* (Devi *et al*., 2012), *Bambusa tulda, Melcocanna baccifera* (Waikhom and Louis, 2014), *Drepanostachyum falcatum* (Saini *et al*., 2016), *Bambusa bambos* (Raju and Roy, 2016), *Bambusa balcooa* (Ansari *et al*., 2017), *Dendrocalamus strictus* (Rajput *et al*., 2019) etc.

One such significant domesticated bamboo species explored enormously and being in demand is *Pseudoxytenanthera stocksii*, commonly known as Manga Bamboo, endemic to central Western Ghats (Singhal and Gangopadhyay, 1999). It is an economically important and multipurpose bamboo, recommended by National Bamboo Mission (NBM) amongst the top 20 bamboo species of India. It grows up to 25 m, which is used extensively as building material for making house construction, scaffolding, basketry, furniture making, crafts, stakes and agri tools (Nath and Das, 2008; Viswanath *et al*., 2013). As per the report by Rane *et al*. (2016), In India, the potential revenue estimated about Rs. 4.5 lakhs (USD 6705)/year/ha from culms. Farmers earn Rs. 50/- to 60/- from each stick and in general, 10 to 15 sticks used to be harvested from each clump, so there is massive need of planting material.

P. stocksii are characterized by the presence of glabrous, solid, grey-green stem with a pubescent ring. Its leaves varies in size (10-20 x 1.2-2 cm), petiolate with setaceous tip, rounded base, narrow midrib and toothed ligule. Spikelets range 1 cm long, glabrous, flower glumes 2 ovate, sub-acute and dorsally mucronate. Ovary is ovoid with long and slender style, grains elongate, beaked and smooths (Rane, 2015). The nutritional analysis of *P. stocksii* reveals the presence of protein, fat, carbohydrate, fibre and the cyanogenic glucosides, which causes

bitterness is comparatively less in *P. stocksii* (AOAC, 1998, 2005; Rane *et al*., 2016). Its leaf was found to have allelopathic effect on *Arachis hypogaea* (Rawat *et al*., 2018).

The various synonyms of *Pseudoxytenanthera stocksii* are *Dendrocalamus stocksii* (Munro) M.Kumar, Remesh & Unnikrishnan, *Gigantochloa stocksii* (Munro) T.Q. Nguyen, *Oxytenanthera stocksii* Munro, *Pseudotenanthera stocksii* (Munro) R.B. Majumdar and *Pseudoxytenanthera stocksii* (Munro) H.B. Naithani (The Plant List, 2019).

P. stocksii is commonly propagated by seeds and culm cuttings, but the major hindrance to the species is the production of sterile flowers and absence of viable seeds. Due to the growing concern in this species, the prime objective of the study was to evaluate various factors affecting shoot proliferation and successful establishment of *P. stocksii* to be adapted for commercial propagation.

2. MATERIALS AND METHODS

2. 1. Selection of plant material and disinfection

Freshly emerged nodal shoot segments from 6 years old physically healthy plants of *P. stocksii* were collected from the Vadia Palace Forest Campus, Rajpipla during June 2018 to February 2019. The nodal segments measuring 2.0-3.0 cm in length were used as explants. The explants were treated with 0.1% (w/v) solution of systemic fungicide (Bavistin) for 5-10 min and washed with autoclaved distilled water. The explants were disinfected using 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5-10 min and thereafter washed with autoclaved distilled water for 6-10 times to remove the traces of $HgCl_2$ from the explants.

2. 2. Culture medium and *in vitro* culture conditions

Cultures were established with MS basal medium (Murashige and Skoog, 1962) supplemented with 0.8% agar and additives (50 mg/L ascorbic acid, 25 mg/L each of adenine sulphate, L-arginine and citric acid). The pH of nutrient medium was attuned to 5.8 ±0.02 using 0.1 N NaOH or HCl prior to steam sterilization at 1.06 kg cm^{-2} pressure for 15 min at 121 ºC temperature. All the cultures were incubated at 16/8 h/d light/dark period in a growth room maintained with 40–50 µmol $m^{-2}s^{-1}$ Spectral Flux Photon Density (SFPD) light intensity, provided by cool and white fluorescent tubes (Philips, India) at 28 ±2 ºC temperature and 60-70% relative humidity (RH).

2. 3. Culture induction and multiplication of shoots

The surface sterilized explants dissected to an appropriate size (2-3.5×0.5-1.5 cm) were inoculated horizontally and vertically on MS medium enriched with additives (50 mg L^{-1} ascorbic acid and 25 mg L^{-1} each of adenine sulfate, citric acid and arginine), 3% (w/v) sucrose, gelled with 0.8% (w/v) agar and supplemented with various concentrations (2.0-10.0 mg L^{-1}) of cytokinins (6-benzylaminopurine [BAP] and 6-furfurylaminopurine [Kn]). The shoots emerged from the nodes were periodically subcultured after every two-four weeks time interval on MS medium augmented with same concentration of cytokinins. The MS medium devoid of PGRs treated as control. Shoots were multiplied by repeated transfer of mother explants with freshly regenerated shoots on the fresh MS medium. These were also multiplied by the transfer of *in vitro* raised shoot clumps on the fresh medium and cultured on MS medium augmented

with different concentrations and combinations of cytokinins and auxins for 4 weeks in culture vessels and incubated at 25±2 ºC temperature under 12 h/d photo period with 50 µmol/m^2/s PPFD light intensity.

2. 4. *In vitro* rooting of the shoots and Acclimatization of plantlets

The actively growing shoots from the multiplication stage were separated from the culture cluster and inoculated on different strengths of MS medium (full, half and one-fourth) incorporated with various concentrations of auxins (Indole-3-acetic acid [IAA], indole-3-butyric acid [IBA] and 1-Naphthaleneacetic acid [NAA]) (0.5-3.0 mg/L) for rooting under *in vitro* conditions. The rooted shoots were transferred to nursery pro trays (diameter 10 cm) containing sterile soilrite®, moistened with one-fourth aqueous MS salts and maintained in the greenhouse for 4 week. The entire set up was maintained in the greenhouse, where low temperature (26-28±2 °C) and high humidity (70-80 %) were maintained. After two months, the plantlets were shifted to high temperature (30-32±2 °C) and low humidity (55-65 %) zone of the greenhouse for a month. The acclimatized and hardened plants were then shifted to nursery and finally transfer to the field.

2. 5. Data scoring and statistical analysis

All the experiments were conducted thrice with ten replicate per treatment. Data for different concentrations and combinations of cytokinins and auxins with MS basal medium (response of explants, percentage of shoot regeneration response, number of shoots and shoot length, number of roots and root length) were recorded after 4-5 weeks of culture. Data collected in the experiments were analyzed using SPSS version 16.0. The mean values and the differences within the treatments were compared using one-way analysis of variance (ANOVA). Duncan's Multiple Range Test (DMRT) was also performed at $P \leq 0.05$.

3. RESULT AND DISCUSSION

3. 1. Culture initiation in *P. stocksii*

Surface sterilization with 0.1% (w/v) $HgCl_2$ for 7 min and 0.1% of Bavistin for 9 min was found most effective for the establishment of cultures with low incidence of contaminants (both bacteria and fungi). A number of reports state that mercuric chloride and sodium hypochlorite (NaOCl) are generally used as sterilants in bamboo micropropagation (Jiménez and Guevara, 2007). Disinfection of bamboo explants were discussed by Thakur and Sood (2006) and Ray *et al*. (2017). Ali *et al*. (2009) reported that a combination of antibiotics with bavistin successfully reduced the contamination in the cultures of *Bambusa tulda, B. wamin, B. balcooa, B. bambus* and *Dendrocalamus asper*.

The sterilized explants cultured on MS medium supplemented with different concentrations of cytokinins induced organogenesis from the explants studied but the percent of culture induction response and regeneration of multiple shoots from nodal meristems varied with the concentration of cytokinins tested. Of the two cytokinins studied, BAP was reported more effective than Kinetin (Kn) for the development of multiple shoots (Table 1). Comparatively the nodal segments cultured on MS medium with Kn responded with less number of shoots. Maximum response (92%) was reported on MS medium + 6.0 mg/L BAP

with the formation of 9.0±0.25 shoots per explant and 5.3 cm average shoot length were observed (Fig. 1A). The number and length of shoots decreased gradually further than 6.0 mg/L BAP. Shoots on Kinetin containing medium responded maximum (84 %) of culture initiation and 4.3 shoots with 3.0 cm shoot length was reported. Very few shoots were regenerated on control experiments (MS medium + growth regulator free). Significant effect of BAP in healthy and multiple shoots formation from axillary buds were also reported in *Drepanostachyum falacatum* and *Bambusa balcooa* (Arya *et al*., 2008), *Arundinaria callosa* (Waikhom and Sharma, 2009), *D. hamiltonii* (Agnihotri and Nandi, 2009) and *Gigantochloa atroviolacea* (Bisht *et al*., 2010).

Seeds, shoot apices and nodal shoot segments from adult clumps were used as explants for micropropagation of bamboo species. Seeds were used as explants in micropropagation of *Dendrocalamus giganteus* (Devi *et al*., 2012), *D. hamiltonii* (Jha *et al*., 2013) and *Bambusa arundinacea* (Venkatachalam *et al*., 2015). Shoot apices were reported in *B. oldhamii* (Huang *et al*., 1989) and *B. ventricosa* by Huang and Huang (1995).

Table 1. Effect of cytokinins on multiple shoot regeneration from nodal segment explants of *Dendrocalamus stocksii*.

Conc. of Cytokinins (mg/L) BAP	Kn	Response (%)	Shoot Number (Mean ± SD)	Shoot Length (cm) (Mean± SD)
0.0	0.0	23	1.3 ± 0.00	1.7 ± 0.00
1.0	-	47	3.0 ± 0.11	2.5 ± 0.45
2.0	-	75	5.0 ± 0.27	3.4 ± 0.30
4.0	-	87	8.2 ± 0.13	4.0 ± 0.29
6.0	-	92	9.0 ± 0.25	5.3 ± 0.15
8.0	-	86	5.2 ± 0.16	4.1 ± 0.32
10.0		79	2.5 ± 0.20	3.8 ± 0.19
-	1.0	31	1.2 ± 0.27	1.2 ± 0.31
-	2.0	68	2.7 ± 0.20	2.0 ± 0.20
-	4.0	77	3.0 ± 0.11	2.8 ± 0.22
-	6.0	84	4.3 ± 0.24	3.0 ± 0.27
	8.0	70	3.0 ± 0.33	2.7 ± 0.16
	10.0	63	2.0 ± 0.17	2.3 ± 0.24

Note: MS – full strength; culture period - 4 weeks of incubation.

Season of explants collection, diameter of explants determines the physiological state of the tissue (Bag *et al*., 2000; Singh *et al*., 2012), which supports culture response and production of cultures free of contamination (Sandhu *et al*., 2018). The explants selected to the size of 2-3.5 length × 0.5-1.5 cm diameters were resulted with multiple number of shoots production. The present finding is relatively agreed with the earlier reports in micropropagation of *Pseudoxytenanthera stocksii* (Sanjaya and Rai, 2005). Somashekar *et al*. (2008) reported that explants with single nodes ranging 2–3 mm in diameter with 2.5–3.5 cm in length from mature clump responded in highest shoot regeneration in *P. stocksii*. Culture initiation through axillary bud break was found effective in multiple shoot production of *Dendrocalamus asper* (Arya *et al*., 2002), *D. hamiltonii* (Bag *et al*., 2002), *Guadua angustifolia* (Rathore *et al*., 2009), *Bambusa balcooa* (Sharma and Sarma, 2011), *B. tulda* and *Melcocanna baccifera* (Waikhom and Louis, 2014) etc.

3. 2. Proliferation of shoot clumps *in vitro*

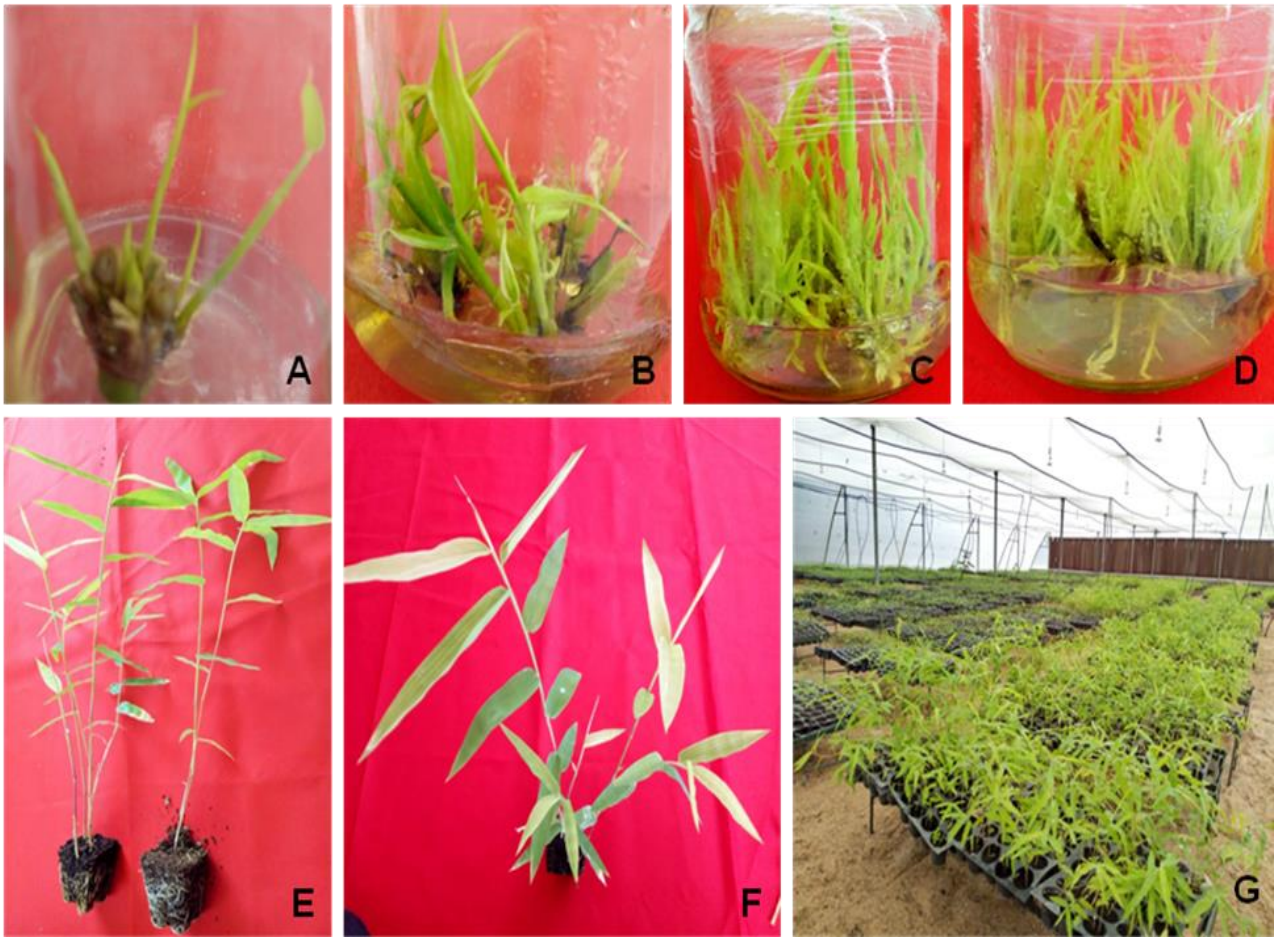

Fig. 1. A. Emergence of shoot buds from explants on full strength MS + 6.0 mg/L BAP. **B** and **C.** Proliferation of shoots in *P. stocksii* on MS + 4.0 mg/L BAP + 0.25 mg/L NAA + additives. **D.** *In vitro* rooted plantlets on half strength MS + 1.0 mg/L IBA. **E** and **F.** Primary hardening of *in vitro* raised *P. stocksii* plantlets. **G.** A view of hardening of *P. stocksii* plantlets in greenhouse.

Shoots were multiplied by repetitive transfer of mother explants with regenerated shoots on the fresh nutrient medium. The MS medium containing various concentrations of BAP in combinations with auxins produced multiple shoots within four weeks. Addition of auxins with BAP in MS medium resulted in enormous shoot multiplication. The maximum number of shoots was regenerated by repetitive transfer of mother explants with regenerated shoots. The addition of additives into the multiplication medium considerably improved the shoot multiplication. A combination of 4.0 mg/L of BAP and 0.25 mg/L NAA in MS medium produced highest number (41.9±1.00) of shoots with 8.13±0.50 cm shoot length (Figs. 1B and C). The rate of shoots multiplication was optimized at 0.25 mg/L NAA, beyond that, number of shoots were decreased (Table 2).

Table 2. Combined effects of cytokinins and auxins on shoot multiplication of *P. stocksii*.

BAP concentration (mg/L)	NAA concentration (mg/L)	Shoot Number (Mean ± SD)	Shoot Length (cm) (Mean± SD)
1.0	0.1	12.6 ± 0.26	2.20 ± 0.13
1.0	0.25	19.0 ± 0.41	2.54 ± 0.30
1.0	0.5	15.7 ± 0.49	2.27 ± 0.16
1.0	1.0	13.2 ± 0.50	2.00 ± 0.24
2.0	0.1	23.0 ± 0.56	4.30 ± 0.20
2.0	0.25	39.4 ± 0.68	4.99 ± 0.13
2.0	0.5	32.3 ± 0.91	4.21 ± 0.29
2.0	1.0	25.9 ± 0.70	3.80 ± 0.15
4.0	0.1	32.3 ± 0.92	6.10 ± 0.23
4.0	0.25	41.9 ± 1.00	8.13 ± 0.39
4.0	0.5	35.0 ± 1.13	5.00 ± 0.21
4.0	1.0	29.2 ± 0.85	4.96 ± 0.50
60	0.1	20.8 ± 0.75	3.11 ± 0.17
6.0	0.25	36.3 ± 0.69	5.12 ± 0.31
6.0	0.5	30.0 ± 1.00	4.59 ± 0.26
6.0	1.0	22.6 ± 0.77	4.00 ± 0.10

Note: MS – full strength; culture period - 4 weeks of incubation.

Combined effect of BAP and NAA in shoot proliferation was reported in *Oxtyenanthera abyssinica* (Diab and Mohamed, 2008), *Dendrocalamus hamiltonii* (Agnihotri and Nandi, 2009), *B. balcooa* (Sharma and Sarma, 2011) and *B. nutans* (Sharma *et al*., 2012).

Promotary effect of additives in micropropagation of bamboo species were investigated enormously. Ascorbic acid, citric acid and cysteine were reported for multiple shoots production of *P. stocksii* in the earlier reports by Sanjaya and Rai (2005) and Somashekar *et al*. (2008). Coconut milk was used for *in vitro* propagation of *D. brandisii* (Nadgauda *et al*. 1990), *B. arundinacea* (Venkatachalam *et al*., 2015), *D. longispathus* (Saxena and Bhojwani, 1993). The impact of adenine sulphate was reported in *D. strictus* (Chaturvedi *et al*., 1993), *B. oldhamii* (Thiruvengadam *et al*., 2011) and *B. nutans* (Sharma *et al*., 2012).

The present findings were contrary with the report of Somashekar *et al*. (2008), they reported that dwarf and less number of shoots were observed in *P. stocksii* when cultured on MS agar-gelled medium with leaching and browning. But in present study, the reported problems were not encountered.

In vitro propagation via somatic embryogenesis was also reported in *Bambusa balcooa* (Gillis *et al*., 2007), *Bambusa ventricosa* (Cheah and Chaille, 2011), *Dendrocalamus hamiltonii, Dendrocalamus asper, Drepenostachyum falcatum, Melocalamus compactiflorus, Melocalamus buccifera, Phyllostachys edulis, Phyllostachys violascens* and *Phyllostachys heterocycla* var. *pubescens*, (Godbole *et al*., 2002; Lin *et al*., 2012; Jin-Ling *et al*., 2013; Arya and Arya, 2015). Somashekar *et al.* (2018) reported somatic embryogenesis in *Dendrocalamus stocksii* by culturing leaf sheath and nodal shoot segments on MS medium + 2,4-D. The somatic embryos were germinated on MS medium supplemented with BAP and NAA.

3. 3. *In vitro* rooting of shoots

In vitro raised shoots were successfully rooted on half strength MS medium augmented with various concentrations of auxins. The percentage of rooting response and development of healthy and sturdy roots with root number and length were varied with the corresponding type and concentration of auxins. Rooting was not observed on the control experiments within four weeks. Hence, auxins are essential for the timely rooting of *in vitro* raised shoots of *P. stocksii*. Maximum 24.3±0.27 roots with 12.0±0.15 cm length were observed on half strength MS medium augmented with half strength sucrose (15 g/L) and 1.0 mg/L IBA (Table 3 and Fig. 1D). Comparatively 9.11 and 7.53 roots were resulted with 1.0 mg/L NAA and IAA respectively.

Table 3. Effect of auxins on *in vitro* root induction of *P. stocksii*.

PGR and its concentration (mg/L)	Response (%)	Root Number (Mean ± SD)	Root Length (cm) (Mean± SD)
0	0	0.00 ± 0.00	0.00 ± 0.00
IAA			
0.5	59	5.10 ± 0.22	4.3 ± 0.19
1.0	63	7.53 ± 0.20	5.0 ± 0.25

2.0	69	7.00 ± 0.31	4.8 ± 0.20
3.0	70	6.82 ± 0.26	4.0 ± 0.14
IBA			
0.5	96	20.1 ± 0.39	10.7 ± 0.22
1.0	100	24.3 ± 0.27	12.0 ± 0.15
2.0	89	19.6 ± 0.20	11.4 ± 0.36
3.0	79	14.0 ± 0.43	9.3 ± 0.10
NAA			
0.5	68	7.00 ± 0.19	6.5 ± 0.29
1.0	76	9.11 ± 0.35	7.9 ± 0.31
2.0	70	8.93 ± 0.22	6.0 ± 0.30
3.0	64	8.20 ± 0.30	5.4 ± 0.25

Note: MS – half strength; sucrose – half strength, culture period - 4 weeks of incubation.

The strength of rooting medium was found to have significant effect in rooting efficiency of *in vitro* shoots. Full strength MS medium with auxins found unsuitable for *in vitro* roots formation and 1/4th strength of MS medium developed poor number and thin roots. In general, the half strength of MS medium improves roots initiation and elongation due to the scarcity of the nutrients in the medium (Shekhawat and Shekhawat, 2011).

Half strength MS medium augmented with IBA was found effective in rooting of *Dendrocalamus hamiltonii* and *D. asper* (Agnihotri and Nandi, 2009; Arya and Arya, 2009; Singh *et al*., 2012), *Bambusa tulda, Melcocanna baccifera* (Waikhom and Louis, 2014), *Bambusa balcooa* (Das and Pal, 2005), *B. nutans* (Yasodha *et al*., 2008), *B. giganteus* (Yasodha *et al*., 2010) etc. The previous report on *P. stocksii* by Somashekhar *et al*. (2008) also revealed that half strength MS media was found suitable for rooting.

3. 4. Hardening and field transfer of bamboo plantlets

Hardening of bamboo is one of the most import stage in successful plant establishment. The *in vitro* rooted shoots were carefully removed from the culture vessels and transplanted in the nursery pro trays filled with sterile soilrite®, moistened with one-fourth strength aqueous MS salts solution and maintained in the greenhouse. The plantlets hardened in the greenhouse for 2 months produced fresh leaves and increased in fresh weight (Figs. 1E to 1G). After 2 months, the acclimatized plantlets were shifted to nursery polybags and irrigated sporadically. Fresh growth observed in the plantlets implies the *in vivo* adaptation of plantlets and 96% of survival success was achieved.

4. CONCLUSION

The present study describes, the clonal propagation of *Pseudoxytenanthera stocksii* using nodal shoot segments as explants. Full strength MS medium with BAP was found better for multiple shoot regeneration. MS medium supplemented with BAP and NAA was best for proliferation of *in vitro* raised shoots. The rooting of shoots was aided by half strength MS salts, half sucrose and IBA. The rooted shoots were hardened in the greenhouse and 96 % of survival success was achieved after secondary hardening. This propagation protocol could be exploited for commercial cultivation of Manga Bamboo in Indian climatic conditions.

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