Original Article

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One step pre-hardening micropropagation of *Bambusa balcooa* Roxb.

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ABSTRACT

Bamboo's fast growth and ability to sequester atmospheric carbon consequently mitigate climate change. Abellon's mission is to reduce CO_2 from the environment by growing bamboo on marginal land and using this biomass for bioenergy. *Bambusa balcooa* Roxb. micropropagation protocol is established for a consistent supply of quality plantlets. Surface sterilization of nodal explants using 0.1% mercuric chloride followed by initiation in liquid and solid media with and without Gentamycin (3.0-8.0 mg/L) containing Murashige and Skoog (MS) media supplemented with 0.01% myo-inositol, 3% sugar, 25 mg/L citrate 50 mg/L ascorbate, and 3.5 mg/L 6-benzylaminopurine (BAP) as growth regulator. Shoots were multiplied using MS medium augmented with 3% sugar, 6% agar, 0.01% myo-inositol with 3 mg/L BAP and 0.5 mg/L naphthalene acetic acid (NAA). Shoot clusters were rooted in MS supplemented with 4 mg/L NAA, pre-hardened in half MS, acclimatized using coco peat, and vermicompost while at net house using vermicompost, soil, sand, and vesicular arbuscular mycorrhiza (VAM) culture. 97% bud break was achieved from initiation while overall success ratio for establishment was 85%. Sub-culturing shoots showed proliferation rate of 3.5-fold. Rooting was successfully achieved with 83% rate. Acclimatization rate at primary and secondary hardening was 72%. The uniqueness of the study lies as bamboo confines to tropical region, however; we have described *B. balcooa* mass multiplication protocol using explants from arid region with one step pre-hardening process. The protocol achieved multiplication rate of 3.5-fold, overall survival rate 74.66% using vermicompost and VAM for acclimatization of *B. balcooa*.

KEY WORDS: Acclimatization, bamboo, mass multiplication, micropropagation, tissue culture, vesicular arbuscular mycorrhiza culture

INTRODUCTION

The carbon concentration in the form of carbon dioxide and methane has increased in atmosphere in comparison to its presence in soil and plant which causes global warming. Soil organic pool can be increased by applications of soil reclamation and forestation, organic fertilizers application, water conservation irrigation management, agroforestry practices, and cultivation of energy crops on marginal lands. The challenges caused by climate change are described as mitigation, adaptation, and development (MAD) challenge (http://klima.ku.dk/pdf/professor_h.j._schellnhuber_-_countdown_to_copenhagen.pdf/).

Abellon CleanEnergy Ltd. is an integrated sustainable energy company with a prime focus on Bioenergy – BioPower and Biofuels, sustainable development and other areas of environment friendly energy generation to mitigate global warming. Abellon sees bamboo as a bridge to provide solution to MAD challenges by fixing environmental carbon pool in organic form to the soil. Abellon thrives to generate bioenergy by growing energy crops on waste and marginal arid land thereby improving carbon pool in soil through carbon dioxide sequestration from the environment without conflicting with food, feed, fuel, fiber, and agriculture land.

Bamboo is now receiving a high level of interest to combat global warming due to its exceptional carbon sequestration abilities (Lou *et al*., 2010). Research to date confirms that bamboo has better CO_2 sequestration than other fast growing trees, and it is being seriously considered as a plant to mitigate effects of global warming owing to its fast

growth and high rates of carbon accumulation (Lou *et al*., 2010). However, among the various genera of bamboo, it becomes challenging to identify best species which can serve all potential goal through consistent quality supply of plantlets.

Bambusa balcooa Roxb. (Poaceae: Bambusoideae) is a native Indian subcontinent multipurpose bamboo species. It reaches a height of 12-23 m, diameter of 18-25 cm, and grows up to 600 m altitude in various parts of India (Tewari, 1992). The flowering cycle of *B. balcooa* is 55-60 years, and the plant dies after flowering without seeds setting (Tewari, 1992). Therefore, asexual propagation is the only way for its propagation. Vegetative propagation is difficult through culm, branch or rhizomes due to few and bulky propagules, seasonal variations, and poor rooting potential making this process inefficient for large scale propagation of *B. balcooa* (Pattanaik *et al*., 2004; Hasan, 1977; Seethalakshmi *et al*., 1983).

In vitro propagation methods allow large scale production of this species in a significantly short time. There are few reports on use of axillary shoot for *in vitro* propagation of *B. balcooa*. Several limitations like multiplication rates details and frequency of rooting (Das and Pal, 2005a; 2005b), two-step rooting process (Mudoi and Borthakur, 2009; Islam and Rahman, 2005) and tedious acclimatization process by Mudoi and Borthakur (2009) are also reported. Hence far no one has reported micropropagation of *B. balcooa* from arid region ex-plant collection to region specific environmental adoption of the bamboo species.

This study reports an efficient *in-vitro* protocol for micropropagation of *B. balcooa* through axillary shoot proliferation with Abellon's objective of uninterrupted supply of *B. balcooa* for arid marginal land for CO_2 sequestration, land reclamation through bamboo plantation that subsequently helps in rural economy and social forestry programs.

MATERIALS AND METHODS

Ex-plant Collection

Ex-plants of *B. balcooa* containing nodal segments (1.3-2.0 cm length) were collected from 2 to 3 years old *B. balcooa* growing at Abellon plantation site at arid region of Modasa Taluka, Aravalli District in Gujarat, India. Ex-plants were collected between Octobers to January months. Ex-plants were preceded within 2-3 h after its excision. Leaf sheath tissues and part of the upper internodes were removed by making an incision at the base of the leaf sheath using a scalpel.

Aseptic Sterilization

Surface sterilization of nodal segments was performed by Tween 20 for 5 min then treated with 1% Bavistin (Saraswati agro Chemicals (India) Pvt. Ltd., Bari Brahmana, Jammu and Kashmir, India) for 10 min followed by 70% isopropyl alcohol (Finar Chemicals Ltd., Ahmedabad, Gujarat, India) and disinfection with 0.1% mercuric chloride (Finar Chemicals Ltd., Ahmedabad, Gujarat, India) solution for 5 min. The treated ex-plants were rinsed with sterile R.O water for subsequent initiation steps.

Initiation

Initiation was carried out in full Murashige and Skoog (MS) basal media supplemented with 0.01% Myo-inositol (Finar Chemicals Ltd., Ahmedabad, Gujarat, India), 3% sugar (Commercial Grade, Venkateswara Sugar Products, Kolhapur, India), 25 mg/L Citric acid (Finar Chemicals Ltd., Ahmedabad, Gujarat, India), 50 mg/L Ascorbic acid (Finar Chemicals Ltd., Ahmedabad, Gujarat, India) and 3.5 mg/L 6-benzylaminopurine (BAP) (Himedia Laboratory, Mumbai, Maharashtra, India) as a growth regulator. Initiation was done in liquid and solid MS media with and without different concentration of Gentamycin (3.0-8.0 mg/L) (Abbott Helthcare Ltd. Pvt., Pithampur, Madhya Pradesh, India). Each set was consisted of 36 sample size and experiments were performed in triplicates. pH of the medium was set to 5.7 with 1 N NaOH and 1 N HCl before autoclaving it at 121°C for 20 min. All the cultures were stored at 25°C ± 2°C, under 16 h photoperiod with light intensity of 10000-12000 Lux using white fluorescent tubes.

Shoot Multiplication

The shoots were expurgated from nodal segment and cultured in MS media containing 3% sugar, 6% agar as a solidifying agent (Merck Specialties Private Limited, Mumbai, Maharashtra, India), 0.01% myo-inositol with 3 mg/L BAP and 0.5 mg/L naphthalene acetic acid (NAA) as growth promoters (Himedia Laboratory, Mumbai, Maharashtra, India). Newly sprouted axillary shoots containing 5-8 shoots (propagules) were sub-cultured at regular intervals of 3-4 weeks in fresh multiplication media. A number of propagules derived from a cluster of shoots after each cycle were used to calculate the multiplication rates. Decayed shoots or leaves were removed before transferring the cluster to fresh medium.

Rooting

Well grown green propagules having six to eight shoots, measuring approximately 4-5.5 cm were transferred to

rooting media after completion of eight multiplication cycles.

MS basal media with 3% sugar 0.01% myo-inositol gelled with 2% BioM Gel (Merck Specialties Private Limited, Mumbai, Maharashtra, India) supplemented with different concentration of root inducing growth regulators were tried in different combinations and concentrations in two experiments design each in triplicate with 100 experimental plants in each group.

One Step Process

The first set of experiment was carried out in solid media consisted of 3 mg/L BAP while another set without BAP. In both the sets, different concentration of Auxins (Himedia Laboratory, Mumbai, Maharashtra, India), NAA, indolebutyric acid (IBA) (Himedia Laboratory, Mumbai, Maharashtra, India) and indoleacetic acid (IAA) (Himedia Laboratory, Mumbai, Maharashtra, India) in a range of 1-4 mg/L were kept as variable to observe effect of auxins with and without BAP (Table 1).

Two Steps Process

This experiment was of two steps process where plants were first induced for rooting in liquid media and then transferred to solid media. All the three growth regulators were mixed in different concentrations (0.5-2 mg/L) along with 3 mg/L BAP in liquid media (Table 2) for 3 weeks and then transferred to solid multiplication media with 3 mg/L BAP and 0.5 mg/L NAA for 3 weeks. Percentage of rooting was calculated as total rooted cultures divided by total number of bamboo cultures at experimental rooting stage. Average roots length was calculated taking mean of root length of rooted cultures.

Pre-hardening

Pre-hardening is the process to harden tissue culture grown bamboo cultures in lab for 20-30 days before transferred to greenhouse. 3-4 weeks old rooted bamboo cultures were transferred to full strength MS media containing BAP (3.5-4.5 mg/L) and without BAP. Another set was processed using half strength MS media containing BAP (3.5-4.5 mg/L) and without BAP. All experiments were performed in triplicate, each set with 100 numbers of plants.

Primary Hardening

The *in vitro* plantlets raised after pre-hardening were removed from culture jars, washed thoroughly under R.O. water to remove traces of medium from the roots.

Table 1: Effect of growth hormones and BAP on root induction

Auxins (mg/L)	With BAP 3 (mg/L)			Without BAP		
	Number of roots	Root length (cm)	% Rooting	Number of roots	Root length (cm)	% Rooting
NAA						
1	0.5	0.3	23	2.1	1.5	20
2	2.1	0.6	20	1.1	3	35
3	0.6	0.3	28	6.7	6.5	55
4	3.7	1.2	46	9.6	8	83
IAA						
1	-	-	-	-	-	-
2	0.8	0.6	21	-	-	-
3	-	-	-	1.2	0.4	14
4	-	-	-	-	-	-
IBA						
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	-	-	-	-	-	-

Each experiment was carried out with 100 plants in each set. Experiment was run in triplicate. Results are expressed as mean of three sets of experiments. BAP: 6-benzylaminopurine, NAA: Naphthalene acetic acid, IBA: Indolebutyric acid, IAA: Indole acetic acid

Table 2: Effect of growth hormones in rooting with 3 mg/L BAP

NAA (mg/L)	IBA (mg/L)	IAA (mg/L)	Number of roots	Root length (cm)	% Rooting
1	1	1	5.3	4.2	29
1	2	2	5.1	4.1	32
2	2	1	10.2	9.1	62
2	1	2	8.6	7.8	49
2	2	2	8.2	7.4	42
2	0.5	0.5	6.2	5.9	39
2	0.5	1	4.1	3.6	22
2	1	0.5	2.4	1.8	19
0.5	0.5	0.5	1.3	0.7	20
1	0.5	0.5	2.2	1.4	18
1	-	2	3.1	2.0	22
1	2	-	-	-	-

Each experiment was carried out with 100 plants in each set. Experiment was run in triplicate. Results are expressed as mean of three sets of experiments. BAP: 6-benzylaminopurine, NAA: Naphthalene acetic acid, IBA: Indolebutyric acid, IAA: Indole acetic acid

Plantlets were transferred to hardening trays (98 wells). One set of hardening tray was consist of 3:1 ratio of coco peat and vermicompost (vermicomposting was prepared at Abellon's plantation site at Modasa, Aravalli district in Gujarat, India), while another set was grown by planting in coco peat only. The plants were grown under greenhouse conditions maintained at 27-31°C and relative humidity 70-75% for 3-4 weeks. The mortality rate was calculated as total dry cultures divided by a total number of bamboo cultures transferred. All experiments were performed in triplicate, each set with 150 numbers of plants.

Secondary Hardening

Bamboo plants with well-developed root balls were then transferred to shade net house in two sets of

polythene bags (6 cm × 6 cm) containing potting mixer of vermicompost:soil:sand in 1:1:1 ratio. Second set was consisted of vermicompost:soil:sand:vesicular arbuscular mycorrhiza (VAM) in a 1:1:1:0.5 ratios (VAM culture was purchase from ICAR, New Delhi and Established at Abellon R&D center at Ahmedabad, Gujarat, India, and mass production at Abellon hardening center at Modasa, Aravalli district in Gujarat, India.)The temperature of shade net house was kept between 35°C and 38°C and humidity between 50% and 60%. Mortality rate was calculated as total dry cultures divided by total number bamboo of cultures transferred. All experiments were performed in triplicate, each set with 150 numbers of plants.

RESULTS

Aseptic Initiation

Bamboo ex-plants collected between Octobers to January were preceded for aseptic sterilization. Gentamicin was added to the liquid and solid media (0-8 mg/L) to identify best contamination control protocol. After 10-15 days of initiation, 3-5 Sprouted axillary shoots were observed in both liquid and solid media. Axillary shoots sprouting was seen 3-5 days earlier in liquid media than the solid media with 3 mg/L, 5 mg/L, and 7 mg/L of gentamicin (Table 3).

Sprouting was observed 2 days late in 8 mg/L gentamicin treated liquid media than same concentration in solid media (Table 3). We observed the best result in liquid media with darker green shoots and leaves (Figure 1a) as compared to solid media with yellow leaves/shoots (Figure 1b) at 7 mg/L gentamicin concentration.

Highest sprouting (97.22% liquid vs. 91.13% solid), shoot number (4.79 liquid vs. 3.58% solid), and aseptic culture (46.7% liquid vs. 40.4% solid) were noted. Contamination was higher (81.1%) in solid media without gentamicin as compared to liquid media without gentamicin (76.3%) (Table 3).

Shoot Multiplication

Different combinations of BAP and NAA were tried for shoot multiplication. The most effective was NAA 0.5 mg/L and BAP 3 mg/L with good culture condition and clusters with 12-15 shoots at the end of 3 weeks (Figure 2 shooting) with an average multiplication rate of 3.5-fold.

Figure 1: *Bambusa balcooa* initiation in liquid and solid media, (a) Liquid media, (b) solid media in 0.6% agar

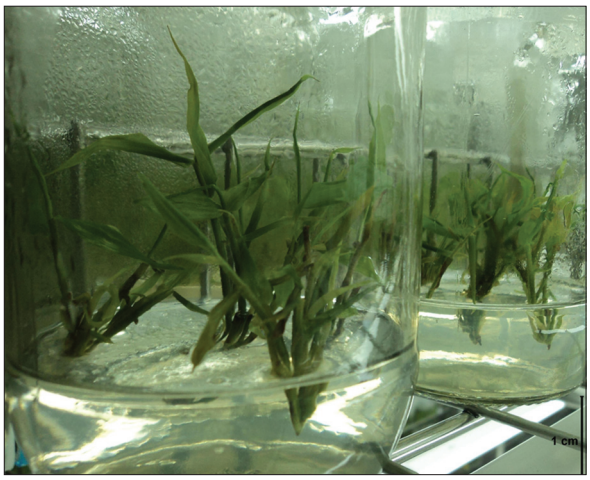

Figure 2: *Bambusa balcooa* shoot multiplication in solid media

Table 3: Effect of gentamicin on sprouting and contamination

Gentamicin (mg/L)	Liquid				Solid			
	Shoot* No/Clump	Sprouting# (%)	Contamination# (%)	Success# (%)	Shoot* No/Clump	Sprouting# (%)	Contamination# (%)	Success# (%)
0	2.08±1.4	81.23	76.3	23.7	1.29±1.1	69.12	81.1	18.9
3	3.84±1.7	92.44	62.5	37.5	1.72±0.9	86.18	68.3	31.7
4	2.15±0.5	89.66	63.8	36.2	1.98±1.3	82.13	68.1	31.9
5	1.93±0.4	91.66	68.4	31.6	2.14±0.9	90.21	66.4	33.6
6	2.36±0.6	88.84	61.8	38.2	1.89±0.7	86.62	63.8	36.2
7	4.79±2.1	97.22	53.3	46.7	3.58±1.6	91.13	59.6	40.4
8	3.12±1.3	79.52	69.6	30.4	2.18±1.8	81.29	64.8	35.2

*Values are expressed as mean±SE; #Mean of three sets of experiments. SE: Standard error

NAA 0.8 mg/L and BAP 3 mg/L also showed good growth of cultures after the end of 3 weeks with yellowish leaves, while in other combinations of BAP and NAA, the culture condition and proliferation rate was in the range of 5-10 shoots/clump with necrotic leaves in most of shoots.

Rooting

We wanted to know qualitative and quantitative effect of individual auxins with and without the addition of BAP (Table 1), as well as combinations of all 3 auxins together with BAP (Table 2).

One Step Process

The best rooting were observed within 15 days in NAA 4 mg/L without addition of BAP (Figure 3a) showing average 9.6 root numbers, root length 8 cm and 83% rooting as compared to NAA (1-4 mg/L) with addition of BAP (3 mg/L) (Figure 3b) (Table 1). In the case of IAA (2 mg/L) and BAP (3 mg/L), 21% rooting was observed. While, 3 mg/L IAA without the addition of BAP showed 14% rooting (Table 1). No rooting was observed in IBA (1-4 mg/L) either in addition with BAP (3 mg/L) or without BAP (Table 1). The plants were kept in rooting stage for 3 weeks.

Two Step Process

The second set of rooting experiment was consisted of BAP concentration of 3 mg/L with combination of various concentrations of all three auxins in liquid media for 3 weeks. Later the plants were transferred to solid multiplication media with 3 mg/L BAP and 0.5 mg/L NAA for three weeks. Sixty-two percentage rooting, 10.2 average number of roots and 9.1 cm average root length was observed in IAA + IBA + NAA combination at 1:2:2 mg/L concentrations respectively (Table 2, Figure 3b liquid media, and Figure 3c solid media).

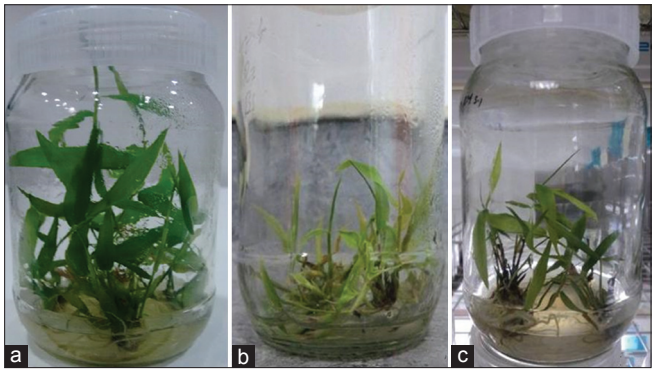

Figure 3: One step and two steps root induction in *Bambusa balcooa,* (a) One step root induction with 4 mg/L naphthalene acetic acid without 6-benzylaminopurine in solid medium, (b) root induction for 3 weeks in liquid media, (c) root induced plants (b) transferred to solid media for 3 weeks

Pre-hardening

The bamboo shoot elongation was highest at half strength of MS medium with 3 mg/L BAP showing 6.8 ± 2.2 cm shoot length and 12.4 ± 2.1 cm root length (Figure 4). The quality of green shoots and leaves were better than the full strength of MS medium. Overall survival rates of plants were 68% in half strength MS media as compared to 37% in full strength MS medium.

Primary Hardening

Primary hardening of bamboo plants were completed in 2 different potting mixtures. One set with coco peat alone and another set in a combination of coco peat + vermicompost (3:1). The best results were observed in coco peat + vermicompost mixture with shoot length 6.85 ± 0.04 cm and root length 14.70 ± 0.1 cm. While bamboo plants in only coco peat showed shoot length 5.45 ± 0.09 cm and root length 11.55 ± 0.08 cm (Figure 5).

Figure 6 shows comparative growth of coco peat alone (Figure 6a) and coco peat + vermicompost mixture (Figure 6b). Survival rate of plants was 72% in vermicompost added plants vs. coco peat alone (61%).

Secondary Hardening

150 plantlets in triplicate were transferred to two potting mixtures to optimize the hardening procedure and transfer of regenerated plants to soil containing different ratios of vermicompost:soil:sand:VAM and another mixture of vermicompost:soil:sand as described in materials and methods. Vermicompost:soil:sand: VAM culture showed best shoot and root length 8.25 ± 0.4 cm and 18.60 ± 0.1 cm, respectively, (Figure 7).

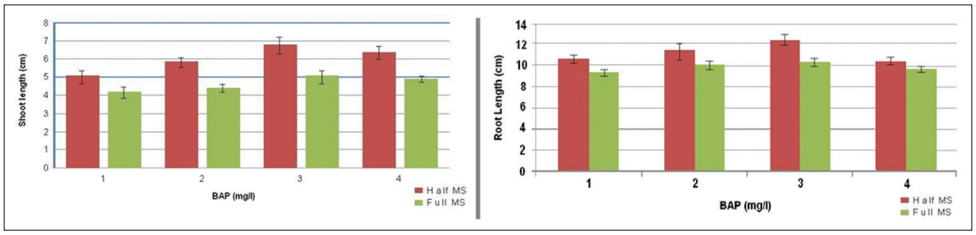

Figure 4: Growth characteristics at pre-hardening of *Bambusa balcooa* in full and half Murashige and Skoog with and without 6-benzylaminopurine

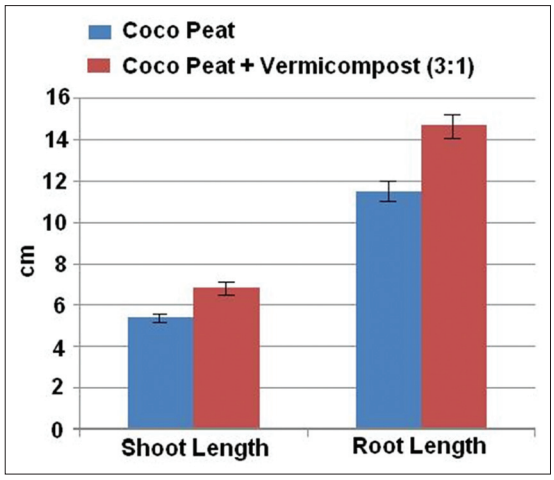

Figure 5: Growth characteristics of *Bambusa balcooa* in 3:1 ratio of coco peat and vermicompost versus coco peat alone

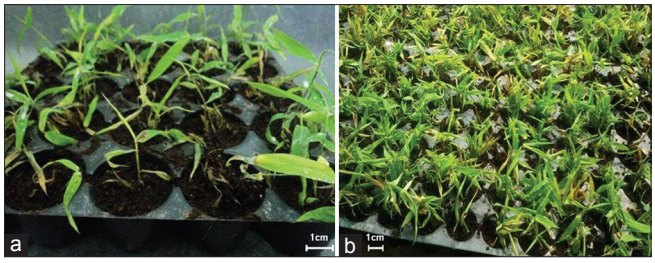

Figure 6: *Bambusa balcooa* growth with and without vermicompost in primary, (a) Coco peat alone, (b) coco peat + vermicompost: 3:1 ratio

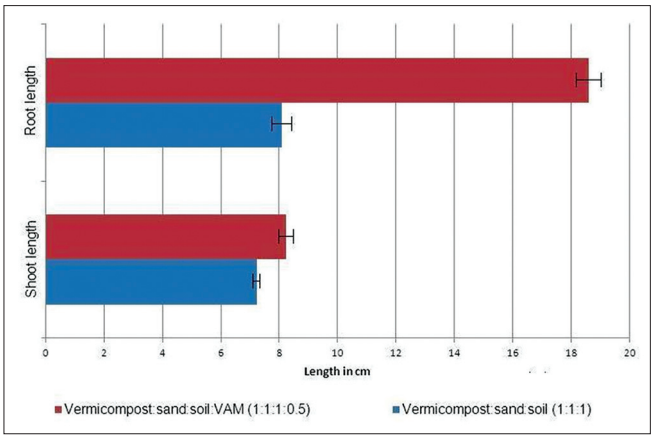

Figure 7: Effect on growth characteristics of vesicular arbuscular mycorrhiza culture at secondary hardening of *Bambusa balcooa*

Figure 8: *Bambusa balcooa* growth with and without vesicular arbuscular mycorrhiza (VAM) in secondary hardening, (a) Vermicompost:soil:sand in 1:1:1 ratio , (b) Vermicompost:soil:sand:VAM in a 1:1:1:0.5 ratio

While only vermicompost:soil:sand mixture (without VAM) showed shoot and root length 7.25 ± 0.08 cm and 8.10 ± 0.08 cm, respectively. Effect of bamboo growth without VAM (Figure 8a) and with VAM culture (Figure 8b) is shown in Figure 8. Survival of the plants was 84% in both the cases, however, quality of shoot, leaves, and overall growth was better in VAM nourished bamboo plants.

DISCUSSION

Micropropagation techniques offer the remarkable potential for rapid multiplication of bamboo species. *In vitro* multiplication of this species reported by other workers lack some valuable information of contamination rate with certain associated limitations (Das and Pal, 2005a; 2015b; Islam and Rahman, 2005; Gillis *et al*., 2007; Mudoi and Borthakur, 2009).

The sources of culture contamination are usually difficult to determine (Leifert and Waites, 1994). Bacteria which contaminate plant cultures may originate from explants, laboratory environments, operators, mites, and trips or ineffective sterilization techniques. Bacteria are associated with plants as epiphytes or endophytes (Sigee, 1993; Gunson and Spencer-Phillips, 1994). Explants from field-grown plants, diseased specimens, from plant parts which are located close to or below the soil may be difficult or impossible to disinfect due to both endophytic and epiphytic microbes (Leifert el al., 1994).

Endophytic bacterial contamination is one of the major problems in plan tissue culture (Kneifel and Leonhardt, 1992) and cannot be eliminated with any surface sterilization techniques, thus requires antibiotic therapy (Mathias *et al*., 1987). Barbara and Piyarak (1995) noted endophytic bacterial contamination at Bamboo initiation process which could not be eliminated by any surface sterilization technique. In this study, initiation was established in liquid and solid MS media with and without addition of different concentration of Gentamycin (3.0-8.0 mg/L). Contamination was higher (81.1%) in solid media without gentamicin as compared to liquid media without gentamicin (76.3%). We observed that 7 mg/L gentamicin concentration in liquid media controls contamination (53.3%), with shoot number/clump 4.79 ± 2.1, sprouting rate 97.22%, with overall success rate 46.7%. Rudin *et al*. (1970) also observed that gentamicin is more effective than penicillin or streptomycin to suppress contamination in tissue culture media. However, Divya and

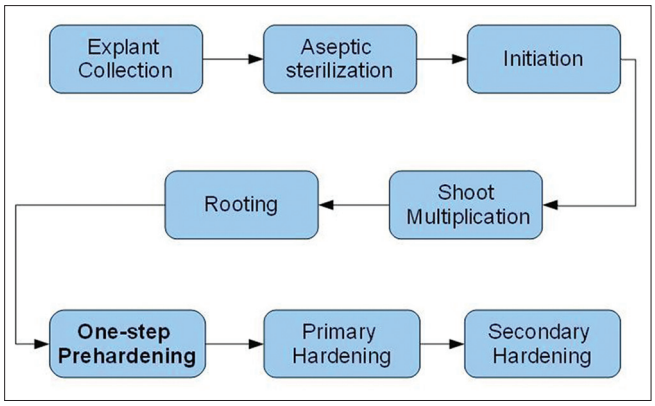

Figure 9: Flow chart for plant tissue culture of *Bambusa balcooa*

Sanjay (2011) reported 85% aseptic cultures with 90% bud break without using antibiotics in the sterilization procedure, when initiation was done during July and October. While in the present study, we found the best ex-plant collection seasons from arid region of Gujarat is October to January months that fulfill prerequisites for bamboo micropropagation technique with good ex-plant health resulting into overall success rate.

Propagule size has major impect on shoot proliferation and *in vitro* rooting. Das and Pal (2005b) and Mudoi and Borthakur (2009) used propagule consisting of more than three shoots for shoot multiplication. While in the present study, we have used 5-8 propagules for shoot multiplication.

The most effective shoot multiplication was observed using NAA 0.5 mg/L and BAP 3 mg/L with good culture condition and a cluster (propagule) with 12-15 shoots at the end of 3 weeks. Mutual effect of BAP and NAA stimulated growth and multiplication of shoots. A similar synergistic effect of these two cytokinins has been reported in *B. balcooa* by Das and Pal (2005a), in *Dendrocalamus giganteus* by Arya *et al*. (2006), and in *Dendrocalamus strictus* by Chowdhury *et al*. (2004). Myo-inositol works as an organic supplement in shoot multiplication medium which stimulates the growth of *in vitro* cultures and also acts as an alternative for coconut water. Pollard *et al*. (1961) reported that myo-inositol content is also responsible for the growth promoting property of coconut milk.

Huang *et al* (2002) reported culture browning phenomenon that refracts oxidation of phenolic compounds released during tissue excision and injury of woody plant material. Das and Pal (2005b) reported that an increase of 0.04-0.08 g% in total phenolic content found to be harmful for the successful *in vitro* propagation of *B. balcooa*. The problem of browning of the medium can be addressed by regulart transfer to fresh medium (Divya and Sanjay, 2011). We have minimized tissue necrosis and browning by frequent transfer of culture to fresh medium.

The best rooting were observed within 15 days in 4 mg/L NAA without addition of BAP (Figure 3a) showing average 9.6 root numbers, root length 8 cm and 86% rooting as compared to NAA (1-4 mg/L) with addition of BAP (3 mg/L). This one step method is time and cost saving as compared to two steps method we and others followed (Mudoi and Borthakur, 2009). Pratibha and Sarma (2011) reported maximum number of root formation from 15 to 21 days of transfer with 85-90% root formation for 3-4 mg/L NAA. Pratibha and Sarma (2011) were analogous with our findings that IBA did not show any root induction in *B. balcooa*. Arya *et al*. (2002) also reported favorable response of NAA for *in vitro* rooting of *Bambusa arundianceae* species.

We followed one step pre-hardening process to harden tissue culture grown bamboo plants in lab for 20-30 days before transferred to greenhouse. Rooted plants were kept in full strength MS and half strength MS with and without the addition of BAP. The bamboo shoot elongation was highest in half strength MS with 3 mg/L BAP with 6.8 ± 2.2 cm of shoot length and 12.4 ± 2.1 cm of root length. Mudoi and Borthakur (2009) followed two steps pre-hardening process wherein the rooted plants were transferred to MS liquid medium for root elongation for 15-20 days, which was followed by transfer of rooted shoots in half-strength MS liquid medium for another 15 days. Afterward, the plantlets were soaked in unsterilized filtered water for 15 days in culture room followed by another 15 days at ambient room temperature ($28°C \pm 2°C$). It is important to note that during one step pre-hardening stage, white new secondary roots developed were responsible for highest survival frequency when transferred for acclimatization to greenhouse.

The tissue culture plants, following heterotrophic mode of nutrition cannot withstand the environmental changes without proper hardening and adaptation. In the present study, mixture of coco peat and vermicompost (3:1) in primary hardening stage showed best results with 93% survival as compared to coco peat alone. Pratibha and Sarma (2011) reported 100% survival using the same practice. Pre-harden plants were subjected to secondary hardening after 3-4 week wherein poly bags containing vermicompost:soil:sand:VAM showed good shoot, leaves and overall growth of plants as compared to VAM deficient plants. The VAM fungi are best known for their phosphate solubilizing activity which has an

important role in the improvement of plant growth Other benefits of VAM colonization includes increased absorption of nitrogen, potassium, magnesium, copper, zinc, boron, sulfur, molybdenum, and other elements and making them available to the plants. It helped in retaining moisture around root zone of plants. Therefore, bamboo plants nourished with VAM culture has additional advantage of good plant health through minerals and nutrition supply. Bamboo plants in poly bags containing Vermicompost: Soil: Sand: VAM when supplied to farmers would also improve fertility, nutrition and health of bamboo at the site of plantation with comprehensive survival benefits The protocol developed is unique in process where pre-hardening process has been developed in house for overall plant adaptation and survival in hard and arid environment as mentioned process flow chart in Figure 9.

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