IN VITRO ASSESSMENT OF *Bambusa balcooa* Roxb. FOR MICROPROPAGATION

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Abstract : In vitro propagation of Bambusa balcooa Roxb. has been studied which is considered as one of the most significant commercial bamboo species. MS (Murashige and Skoog) basal media supplemented with 5mg/l BAP (6-Benzylaminopurine), 0.8% agar, 3% sucrose and 100mg/l inositol was found to be most suitable for bud break. Maximum number of shoots, 12.67±2.08 was observed after first subculture and 95.67±12.89 after second subculture in MS media which was supplemented with 3mg/l BAP. Subculture was then performed regularly within 3 weeks of time period so as to prevent browning of shoots in media. Optimal rooting was observed on MS media supplemented with 4.5mg/l NAA (Naphthalene acetic acid) with no supply of cytokinin. Maximum number of roots obtained was 236±11.53 with average root length of 13±0.5 after 45-50 days of culture in rooting media. No response was observed when shoot propagules were transferred to rooting media supplemented with IAA (Indole-3-acetic acid) and IBA (Indole-3-butyric acid). Response of explant to different sucrose concentration was also studied where 3% sucrose was the most suitable one where maximum shoot number was 3.33±1.15 with number of leaves 3.66±1.15. 80% success in hardening process was achieved when transferred to greenhouse. During acclimatization, 90% success was achieved when transferred to polybag containing 1:1:2 ratios of soil: ash: vermicompost whereas 70% success in 1:1:2 ratios of soil: sand: vermicompost. Invitro raised plantlets were thus successfully hardened and acclimatized as well. Large scale propagation of B. balcooa species can be regenerated in a time span of 6-7 months which can be time saving as well as economically useful.

IndexTerms - Bambusa balcooa, micropropagation, axillary bud, explant

I. INTRODUCTION

Bamboo is taken as one of the most ecofriendly construction materials and regarded as one of the fastest growing plants in the world which grows from 30cm to 100cm per day. It achieves its maximum size within 60-90 days after shoot sprouting and then can be harvested commercially after 3 to 6 years. There are 81 species (5.2% of the world species) of 23 genera (24% of the world genera) bamboo found in Nepal (Pokhrel, 2008). The entire bamboo coverage area in Nepal is assessed to be around 63,000 hectares among which 60 percent is assessed to be in natural forests. Distribution of bamboo along with their status in Nepal is found to have been studied and reviewed in these papers (Stapleton, 1994; Das, 1988; Shrestha, 1989 and McCracken, 1992).

B.balcooa (Poaceae: Bambusoideae) which is also known as female bamboo is a tropical clumping bamboo that can grow up to a height of 25 m and a thickness of 15 cm. It scatters in several distinct regions having tropical monsoon such in Bangladesh, Nepal together with North-East India, tropics in Asia and Africa (Stapleton, 1994; Ohrnberger, 1999). It is generally referred as 'dhanu bans', 'ban bans', 'harodh bans' in Nepal and is common upto 1600m. This species is used for making poles, house wall, beams, weaving along with erosion control. It is regarded as one of the best species for scaffolding and building purpose (Tewari, 1992). *B. balcooa* has been accredited as a priority bamboo species by (Rao, 1998).

Seed production in *B. balcooa* is generally not recorded after gregarious flowering (Banik, 1987). Moreover, its flowering cycle is reported as 40–100 years and only once during its life time according to Gogoi, 2004. Micropropagation of this species through the use of Tissue Culture Technology is necessary as vegetative propagation of this species through branch, culm cuttings is difficult due to the presence of few bulky propagules. Also, *B. balcooa* which is propagated by culm cuttings, limits the shoot growth including root initiation even after two years of transplanting (Pattanaik, 2004). The initial report on bamboo tissue culture was given by Alexander and Rao, 1968. The primary applications of techniques to plant propagation are to enable rapid multiplication under conditions that maintain freedom from disease, to establish pathogen-free plants and to isolate genetically unique cells or cell lines that can become new plant variants (Hartmann, 1975). Thus, in present study, an attempt is made for the mass propagation of this particular species through nodal segments which is reported to be first in Nepal. This is time friendly as it takes less amount of time to generate large number of plantlets unlike vegetative propagation.

II. RESEARCH METHODOLOGY

Initiation of culture:

For this experiment, one and half year *B. balcooa* plants were brought from Sarlahi district (26.9627° N, 85.5612° E) carefully in truck and kept in green house at Dhulikhel district (27.61° latitude, 85.55 ° longitude) for about 3 months. Single node segments of 1.5-2.5cm with auxiliary bud were then excised from those plants. Leaf sheath were then thoroughly removed and dipped in 70% ethanol for 30 second. They were then dipped in 0.1% $HgCl_2$ for 2 minutes followed by treatment in 5% Tween 20 solution for 2 hour. The explants were then kept in running tap water for 45 minutes which was then rinsed with double distilled water thoroughly for 4 times. Further sterilization processes were performed inside Laminar Air Flow. Explants were then dipped in 0.1% bavistin for 25 minutes with proper shaking followed by 3-4 times rinsing with double distilled water. Nodal segments were then treated with 0.1% $HgCl_2$ for 9 minutes followed by rinsing with distilled water for 4 times. Finally, inoculation was done on MS media after trimming the surface sterilized nodal explants with sterilized sharp scalpel. MS medium was supplemented with cytokinins of various concentrations, 3% sucrose, 0.8% agar, 100mg/l inositol. The pH of the medium was maintained 5.8 with the help of 0.1% NaOH and 0.1% HCl. The jam jars with media were then autoclaved at 121^0C for 15 minutes. All cultures after inoculation were maintained at 25 ± 2^0C under 16 hour photoperiod with a light intensity of 47.29 µmol m^{-2} s^{-1}. This light was maintained by using white fluorescent tubes and humidity of growth chamber was maintained 65-75%.

Bud break was observed within a week of culture. Subculture was then performed on MS basal media when further shoots were developed after 3-4 weeks. Inoculation of clump containing 1-2 shoot yielded less result than inoculation with more than 2 shoots. During subculture, browning of shoots were observed so as to avoid this, frequent subculture within 3 weeks was performed which helped in reduction of browning. More number of shoots and leaves were observed during second subculture which was then transferred to rooting media which was fortified with various concentrations of BAP and NAA.

Shoot multiplication:
For further shoot multiplication, shoot clumps grown in media of initial culture were used. Shoot clump containing 3-4 invitro formed shoots were transferred to media containing cytokinin BAP in various concentrations, 0.8% agar, 3% sucrose and 100mg/l inositol. Shoots which were brown and black were discarded and only healthy green shoots were transferred. Among the various concentrations of BAP used, most suitable concentration was chosen which was found to be 3mg/l. Also, effect of sucrose to this suitable concentration of BAP was further studied and analysed. In order to avoid browning, regular subculture was performed within 3-4 weeks period of time.

Rooting:
Shoots which multiplied in subculture media were transferred to rooting media containing various concentrations of cytokinin along with auxin. For this, clump containing 6-10 shoots were selected after 3-4 weeks of subculture and transferred to rooting media. Rooting media was supplemented with 3% sucrose, 0.8% agar and 100mg/l inositol. For this also, best concentration of hormone was selected and was varied by differing concentration of sucrose and agar.

Hardening/Acclimatization:
Roots were formed in rooting media after 40-45 days of culture and shoots were also found to grow longer in length. These were then taken in green house where roots were washed with tap water in order to remove attached agar and planted in tray of sand for sand rooting. Sand was washed properly prior to plantation of in-vitro grown plant in order to remove dust particles. Tray was then kept within enclosed rooting chamber which was sprayed with water between 2-3 days of time. Humidity was maintained 80-90% inside rooting chamber. These plantlets were then transferred to polybag containing soil: sand/ash: vermicompost in 1:1:2 ratios after 3 weeks of sand rooting. In sand rooting, new leaves were formed and other secondary shoots were also found to develop. These polybag were kept within rooting chamber for 1 week completely. After 1 week, these plantlets were exposed to greenhouse light at daytime and again kept within rooting chamber at night for further 1 week. Finally, these plantlets were taken out or rooting chamber and kept within greenhouse for further growth. After about six months, these plants were planted at Kathmandu University premises and their growth was observed (Figure 1xi).

III. RESULTS AND DISCUSSION

Initial Culture:
For the bud break of explant, different surface sterilization techniques were applied. When $HgCl_2$ was used alone as disinfectant inside Laminar Airflow hood, contamination was above 70%. When $HgCl_2$ treatment (9min) was given after treating with bavistin (25 min), contamination was reduced by about 30% and bud-break was achieved in about 75% of the inoculated culture. Bud-break was observed after 1 week (Figure 1i) and found to be more efficient in solid media unlike Negi et.al, 2011 who found liquid media to be more efficient. When surface sterilized explants were cut by sharp scalpel in both ends inside Laminar Airflow hood, it reduced contamination by about 25% than the explants which were not trimmed. However, in some cases the explants were rigid and hard to trim and wounds appeared while trimming. In such cases, although bud break appeared, it later turned to brown or else contamination appeared. Here, two cytokinins BAP and kinetin were used in MS media alone and in combination. Kinetin alone in combination with BAP failed to produce desired result and optimal hormone concentration was found to be 5mg/l of BAP **(Table 1)** with 3.4±0.53 shoot length and 3.33±0.57 shoot number. These plantlets were then further subcultured twice (**Table 2).**

Shoot multiplication:

For the purpose of shoot multiplication, various media with varying concentration of cytokinins BAP and kinetin was used. Of the various hormone used, 3mg/l BAP was found to be the most efficient one (Figure 1ii). When higher concentration of BAP was used, it generated greater number of shoots but the length of shoots and number of leaves was reasonably less. Regular subculture was preferred as it reduced browning and dying of plantlets. Kinetin when used alone or in combination with BAP was not preferred as it was not able to provide good result. Similar result was obtained by Sharma and Sarma, 2011. Although the subcultures plantlet did not die, it failed to produce more number of shoots. Also, the shoots generated were short in length and leaves were also less in number so kinetin was not preferred. In shoot multiplication, we tried to study the effect in number of shoots and leaves when sucrose was varied in different concentration. It was found that 2.5 – 3% sucrose were most effective with shoot length 3.93±0.25 and shoot length 3.33±1.15. Less than 2.5% sucrose generated less number of shoots (**Table 2**). When plantlets were subcultured, best result was obtained in 3mg/l BAP in both first and second subculture (Figure 1c and 1d). Shoot length of 2.9±0.56 and 6.83±0.30 along with shoot number of 12.67±2.08 and 95.67±12.89 were achieved after first and second subculture respectively **(Table 3**). When hormone concentration exceeded this range, although there was not much difference in shoot length, there was decrease in the total number of shoots. So less hormone concentration was required than initial culture in subculture stage. This result was similar to Mudoi and Borthakur, 2009 who also found that less hormonal concentration was required in subculture stage.

Rooting:

Shoot clump having more than 5 shoots were transferred to rooting media containing various amount of cytokinins and auxins. Among these, no growth was seen in media containing IAA and IBA which is similar to Mudoi and Borthakur, 2009; Sharma and Sarma 2011. Maximum number of roots obtained was 236±11.53 with average root length of 13±0.5 after 45-50 days of culture in rooting media containing 4.5mg/l of NAA (Figure 1v). Least number of rooting was 3.66±1.57 when 0.5mg/ml of NAA alone was used (**Table 4).** Similarly, although there was more number of roots when BAP and NAA were used in combination; root length was found to be comparatively smaller than the results shown by media containing NAA alone. Root length was measured with the help of ruler (Figure 1vi). Thus, plantlets grown on rooting media containing NAA alone was further selected for hardening and acclimatization process.

Hardening/Acclimatization:

For the hardening process, in-vitro raised plantlets were transferred to sand tray (Figure 1vii) and kept in rooting chamber where humidity was maintained. There was 80% success in hardening and during this period of time, numbers of leaves were found to increase rapidly along with the generation of new shoots. After there was seen growth of some new leaves, 20 each plantlets were transferred to polybag containing soil: sand: vermicompost and soil: ash: vermicompost in the ratios 1:1:2 respectively (Figure 1viii). There was rapid growth on the number of shoots and leaves after transferring to polybag (Figure 1ix, 1x). Wood ash is found to contain calcium carbonate as its major component by representing 25 or even 45 percent, potash is less than 10 percent and phosphate is less than 1 percent and there is also presence of trace elements of iron, manganese, zinc, copper and some heavy metals as well (Lerner, 2000). It is thus used as an organic fertilizer which is used to enhance soil nutrition. Soil: ash: vermicompost ratio gave 90% success whereas soil: ash: vermicompost gave 70% result in hardening. Using ash in polybag is not practiced by many but it gave comparatively better result in our case so it can be a new source of nutrition to *in-vitro* raised plantlets. In our study, rooting was comparatively more difficult in comparision to transfer of plantlets to growth chamber. This is similar to Murashige, 1974 where rooting and transplantation of plantlets to the field is taken as the most important and difficult task.

IV. CONCLUSION

Micropropagation of *B. balcooa* was thus performed successfully. Our findings showed the best shoot development in MS supplemented with BAP whereas MS supplemented NAA was found to be best for root induction. The use of ash as a component in hardening process was a different initiative which was found to be much successful than old school methodology. Thus, mass propagation of *B. balcooa* is no longer a process to be amazed of. Following the protocol for bud breaking, shoot induction, root induction, acclimatization and transfer to field we can successfully propagate the bamboo in short time, higher number and greater efficiency year around. However, there can still be made modifications in the composition of media in order to achieve better results and reduce the total time of production of plantlets in vitro.

V. TABLES AND FIGURES

Table 1: Average shoot length and number in the MS media after first inoculation micropropagation represented as Mean ± SD

BAP(mg/l)	Kinetin(mg/l)	Shoot length(cm)	Shoot number
1	0	1.33±0.41	1.33±0.57
2	0	1.76±0.25	1.66±0.57
3	0	2.33±0.45	1.66±0.57
4	0	2.86±0.35	2.66±1.15

5	**0**	**3.4±0.53**	**3.33±0.57**
6	0	3.1±0.36	3.66±1.15
0	1	0.9±0.26	1±0
0	2	1.33±0.58	1±0
0	3	1.4±0.2	1±0
1	0.5	1.76±0.68	1.66±0.57
1	1	1.93±0.30	2±0

Table 2: Alteration in media for the optimization for micropropagation represented as Mean ± SD

Sucrose	Shoot length(cm)	Shoot number	Number of leaves
2%	2.16±0.15	1.33±0.57	1.66±0.58
2.50%	3.33±0.30	1.67±0.57	3.66±1.15
3%	**3.93±0.25**	**3.33±1.15**	**3.66±1.15**
3.50%	2.7±0.26	2.33±0.57	2.75±0.95
4%	2.3±0.40	2±0	1.66±0.57

Table 3: Average shoot length and number in the MS media after first and second subculture represented as Mean ± SD

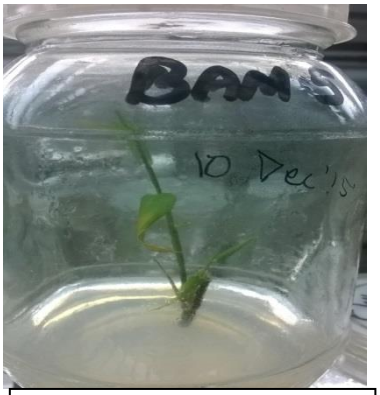
BAP (mg/l)	After first subculture		After second subculture	
	Shoot length (cm)	Shoot number	Shoot length (cm)	Shoot number
1	1.6±0.2	6.33±0.57	1.83±0.25	10±2.64
2	2.23±0.30	7.0±1.0	3.16±0.75	34±7.81
3	**2.9±0.56**	**12.67±2.08**	**6.83±0.30**	**95.67±12.89**
4	2.46±0.35	8.33±2.08	5.83±0.41	72.66±3.51
5	2.93±0.15	5.66±1.15	6.7±0.52	69.66±5.03

Table 4: Different concentration of root induction hormone optimization represented as Mean±SD

BAP(mg/l)	NAA(mg/l)	Number of roots	Root length(cm)
0	0.5	3.66±1.57	2.2±0.34
0	1	11.5±2.12	3.25±0.35
0	1.5	17.5±3.53	4.1±0.3
0	2	36±3.6	5.0±0.3
0	2.5	66.33±5.68	5.2±0.84
0	3	94.66±10.01	9.06±0.60
0	3.5	138.5±2.12	11.4±0.56
0	4	150.33±6.11	12.8±0.29
0	**4.5**	**236±11.53**	**13±0.5**
0	5	207.5±10.60	13.3±0.42
1	1	18.5±2.12	3.4±0.62
1	2	80.67±5.03	8.2±0.7
1	3	114±6.02	13.43±1.88
1	4	103.5±7.77	11.99±0.75
2	2	96.33±1.15	8.16±0.73
2	3	130.5±4.94	12.35±0.35
2	4	120.5±6.36	11.06±0.30

Figure 1: Stages of micropropagation

i: Bud break in initial explant after 15 days of culture (effect of 5mg/l BAP)



ii: Less number of shoot before 1st subculture (effect of 3mg/l BAP)

iii: Increase of shoots after 1st subculture (effect of 3mg/l BAP)

iv: Clump of shoots and leaves after 2nd subculture (effect of 3mg/l BAP)

v: Increase in length and appearance of roots in rooting media (effect of 4.5 mg/l NAA)

vi:Measurement of length of plantlet and roots with the help of ruler

vii: Sand rooting in tray in growth chamber

viii:Transfer to polybag after leaves and shoots increased (soil:sand/ash: vermicompost)

ix:Rapid growth in number of shoots after transferred to polybag

x: Plantlets after 3 months of transfer to polybag

xi: Tissue cultured plant after about 2 year

xii: Tissue cultured plant at University premises

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