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In vitro propagation of *som* plant (*Persea bombycina* king): A primary food plant of muga silkworm

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Abstract
The study was conducted to optimize different compositions of modified MS media as treatments for explant establishment, shoot initiation, shoot proliferation and root induction. Establishment of the cultures (58%) and shoot initiation rate (75%) increased significantly at P=0.05 on T_1C, MS supplemented with Kinetin (0.5mg/l), BAP (3mg/l), GA_3 (0.5mg/l) and fenbendazole (100mg/l). It was also observed that although kinetin stimulates shoot initiation and proliferation but at the same time it induced slight callusing at the base of the explants. Shoot proliferation showed best result in T_2C, MS supplemented with Kinetin (1.5mg/l), BAP (4mg/l), GA_3 (0.5mg/l) and fenbndazole (100mg/l). Development of calli was observed at the base of the shoots and failed in regenerating roots when cultured on rooting media (T_3 and T_4) Thus, it was inferred that treatment T_1C and T_2C were most effective for the establishment, shoot initiation and multiple shoot regeneration of the *som* explant.

Keywords: Host plants, *In vitro* propagation, muga silkworm, propagation, som, tissue culture

Introduction
The culture of muga silkworm, *Antheraea assama* Westwood (Saturniidae: Lepidoptera) and production of golden shimmering muga silk is prerogative to North-Eastern region of India, particularly confined to Brahmaputra valley of Assam and the adjoining hills. Muga silk industry is depend on host plants available in the natural habitat and organized manmade farms [1]. Muga silkworm is a semi domestic, polyphagous insect feeding on various endemic plants (15 different host plant species) – mostly of family Lauraceae. It is reared mainly on the aromatic leaves of Som, *Persea bombycina* King and Soalu, *Litsea monopetala* Juss [2]. Som is evergreen, Monoecious, medium sized tree with spreading branches. Due to evergreen nature of the plant, muga silkworm can be reared on it throughout the year [1].
The vegetative propagation of som, (*Persea bombycina* King) though showed some success, is not fully suitable for large scale production of saplings because the techniques involved are either expensive, requiring special skill or limited to a certain season only. Considering these constrains, Problems faced by the plant breeders in propagating this plant by conventional methods can be overcome by using the applications of tissue culture technology. Axillary buds are widely used for micropropagation as they have entire rudimentary vegetative shoot and can be induced to develop into new plants easily, which are true-to-type to the parental type. *In vitro* plant regeneration from the apical/axillary shoot buds and nodal explants has been reported in a number of species of mulberry [3, 4, 5, 6, 7, 8, 9, 10] which is a sole food plant of mulberry silkworm. Preliminary study on tissue culture of *Persea bombycina* was reported by Bhagawati in the year 1992 [11], Yadav and Goswami in the year 1993 [12]. Since then there is no report exist on *in vitro* regeneration of *Persea bombycina* King the primary food plant of muga silkworm, which is very essential to optimize an *in vitro* propagation of som plants for large scale production of disease-free quality planting material [13]. Therefore, the present work has been planned for optimization of *in vitro* regeneration protocol for *som* plant (*Persea bombycina* king).

Materials and method
The proposed investigation was carried out in the plant transformation laboratory, Department of Agricultural Biotechnology, Assam Agricultural University (AAU), Jorhat. The materials and methods used and applied during the experiments are discussed below:

i) Washing and sterilization of glassware

At first glassware were soaked in saturated chromic acid solutions for 4-6 hours followed by washing with running tap water. After that glassware and plasticwares were cleaned with dilute detergent (5% Teepol) solution using cleaning brush followed by thoroughly washing with running tap water and finally rinsed with distilled water. After washing dried in hot air oven at about 55^0C followed by autoclaving at 15psi pressure (121^0C) for 20 minutes.

ii) Culture media preparation

The media used in the proposed study was Murashige Skoog media (MS medium), containing high concentration of organic salts. For preparing the media first we prepared a series of concentrated macronutrients, micronutrients, Fe-EDTA, vitamins and growth regulators. All stock solutions were stored in refrigerator at 4^0C. For preparation of the 1000ml of MS medium, the required volume of each stock solution was calculated and obtained into 1000ml beaker on a magnetic stirrer. Required amount of growth regulators were added (Table.1) followed by 30g sucrose and 0.1g myo-inositol, and then stirred until fully dissolved. The volume was adjusted to about 1000 ml with distilled water and the pH was adjusted to 5.8 by using 1N KCl or 1N NaOH. Finally, 8g/l phytagel added to the medium and stirred. Measured quantity of this medium was poured in suitable containers (conical flask, culture tubes and jars) and the medium was autoclaved at 15 lbp for 20 min. In this experiment we used three different compositions of media as treatments T_1 (T_1A, T_1B and T_1C); T_2 (T_2A, T_2B and T_2C) for explant establishment, shoot initiation and shoot proliferation and T_3, T_4 for root induction from the *in vitro* regenerated shoots (Table.1).

iii) Selection of plant material and explant collection

The explants used in the present study were axillary buds and tender shoot tips of *Persea bombycina* King collected from the experimental field no-2, Department of Sericulture, Assam Agricultural University, Jorhat. The collected material was free from any visible signs of diseases and pests. The material was excised with a scissor and kept in a flask containing ascorbic acid (0.5%) from 10-12-year-old plant and brought to the tissue culture lab. The explants were generally collected in the morning or in the evening time.

iv) Sterilization of explants

The explants were washed in running tap water for 30min followed by a drop of Tween-20 (5% v/v) added in the water washed with distilled water. After that treated with ascorbic acid solution (0.5%) in orbital shaker for 2 hours and washed 4-5 times with sterile double distilled water before taking them to sterile airflow chamber. The explants were surface sterilized under laminar hood with 0.1% $HgCl_2$ solution for 2-4 min followed by washing with sterile distilled water for 4-5 times. After blot-drying, nodal segments with one viable bud (1-2cm) and apical shoot with last two leaves (1-2cm) tip were inoculated onto the MS medium.

v) Cultural conditions

After inoculation, the cultures were transferred to the culture room and maintained at approximately 25 ± 2^0C under 16hr light/8hr dark photoperiod under white fluorescent tubes (30-35 mol m-2s-1). Observations of the cultures were done on a daily basis.

vi) Culture establishment

The explants were established under *in vitro* condition in modified MS media supplemented with different combinations and concentration of growth hormones (T_1A to T_1C). Healthy cultures without any contamination were transferred to fresh medium in every 15-20 days interval. Cultures were observed periodically and recorded the data.

vii) Shoot multiplication

Established shoots were transferred onto different modified MS media supplemented with different combination and concentration of growth hormones for shoot proliferation (T_2A to T_2C). The cultures were observed periodically and recorded the data.

viii) Rooting

In vitro regenerated shoots (2-3cm long) were cultured onto different modified MS media supplemented with different combination and concentration of growth hormones for root induction (T_3 and T_4). The cultures were observed periodically and recorded the data.

ix) Statistical analysis

CRD (Completely Randomized Design) was employed for the statistical analysis. The collected data were subject to statistical analysis by Fisher's method of analysis of variance. Significance of variance among the data was analyzed by calculating the "F" value and comparing it with the tabulated value of "F" at 5% level of probability.

The treatment means were compared among themselves by calculating critical difference (CD) as followed:

CD at 5% = S.Ed x "t" 5% (at error d.f.)

The standard error of differences (S.Ed) was calculated by using the following formula:

$$S.Ed = \sqrt{2Ems \div r}$$

Where,

S.Ed = Standard error of difference

"t" 5% = t for error d.f. at 5% level of probability.

The significance and non-significance of the treatments at 5% level of probability were calculated out by multiplying the S.Ed. With appropriate tabulated value for error degrees of freedom.

Results and discussion

To remove the phenolic exudates from explants 0.5% of ascorbic acid (an antioxidant) was used during collection of explants and further treatment. Various antioxidants and adsorbents for removal of phenolic exudates from explants of *Tectona grandis* was studied where they treated the explants with 0.1% (w/v) solution of various inorganic compounds and adsorbents viz. ascorbic acid, citric acid, glutamine, polyvinylpyrolidone, boric acid and activated charcoal for 18 hours prior to their surface sterilization with 0.1% (w/v) mercuric chloride solution. Among these boric acid and ascorbic acid was proved to be the most effective resulting in 50-60% establishment of nodal segments on culture media [14].

In the present investigation, preliminary tests of explant disinfection using 15% sodium hypochloride (NaOCl) for 5 min for surface sterilization was inefficient, with an average contamination above 40%. Mercuric chloride ($HgCl_2$) is stronger than sodium hypochloride (NaOCl), which is the likely reason for its effectiveness in combating fungi, bacteria

and endogenous species [15]. The mortality of the cultures may be higher due to damage caused by stronger disinfectants, as was the case with *Calophyllum apetalum* [16].

In the current study, we demonstrated the possibility of *Persea bombycina* King for mass propagation of *som* using axillary bud and shoot tip culture. For successful micropropagation axillary buds or shoot tip cultures are preferred as pre-existing Meristem easily develops into shoots while maintaining clonal fidelity [17]. The *in vitro* production of plants from axillary buds has been reported by various workers in different species of Morus [5, 9, 10, 18]. In the present investigation, slightly tender nodal segments of medium thickness (0.5cm-0.6cm) with emerging greenish axillary buds responded more favorably in terms of bud sprouting and shoot differentiation. The successful establishment percentage and proliferation rate varied from 36-58% and 45-75% respectively (Table.2). The frequency of sprouting was comparatively lower in treatment T_1/A (0.5mg/l kinetin and 2mg/l BAP) (Table.2).

Different combinations and concentrations of cytokinins, auxin and gibberrelic acid were used in MS medium for optimizing shoot initiation, multiple shoot proliferation and rooting (Table.1). Among various combinations best result in terms of shoot initiation and multiple shoot regeneration was observed on treatment T_2C (1.5mg/l kinetin, 4mg/l BAP, 0.5mg/l GA_3 and 100mg/l fenbendazole) (Table.2). There are not much significant differences among treatment T_2B and T_2C in case of multiple shoot proliferation (Table.2). But at later stages the cultures in T_2/B showed high fungal contamination and eventually the cultures died (Fig.H). By using fungicide @100mg/l in the medium (treatment T_2C) minimized the fungal attack and lead to further growth and development of cultures. In treatment T_2C a mean of 3 multiple shoots regenerated from nodal segments (Table.2 & Fig. D). Two experiments were conducted to evaluate bedazole fungicidal effects I neem *(Azadirachta indica A. Juss)*, where in the first experiment explants in Driver and Kuniyuki (DKW) culture medium was observed for 30 days containing different concentrations of bendazol (M_1 -50, M_2 - 100, M_3 -200, and M_4 - 400 mg /L) and In the second experiment, the explants were maintained for one week in medium supplemented with bendazol or BAP, and then they were transferred to bendazol/BAP free medium for three weeks. The variables analyzed included the formation of calli and roots, lateral bud development, shoot height, contamination and plant death. There was no significant difference in these variables (shoot, calli formation and shoot height) between treatments in both experiments [19].

In the current study, treatment T_1A (0.5mg/l kinetin and 2mg/l BAP) showed slow growth of cultures in respect of shoot initiation as well as elongation after 4 weeks of culture (Table.2). In the same medium when BAP concentration was increased (Table.1) to 3mg/l and added GA_3 at the concentration of 0.5mg/l, the cultures showed better shoot initiation and increase in shoot length (Treatment T_1B) (Table.2). BAP exhibiting superiority over other sources of cytokinins for differentiations and growth of new shoots is well documented in other species, the possible reason could be that BAP is much closely related to natural cytokinins as far as the structures of latter is concerned [20]. A simple protocol was reported for shoot induction via micropropagation of *Morus indica* in different nutrient media where among all the MS formulation was found to be the best medium for shoot induction. Multiple shoots were also achieved from *in vitro* raised shoots in MS medium supplemented with BAP (1-1.5mg/l), NAA (0.25-0.5mg/l) and GA_3 (0.5mg/l). The highest shoot lengths (2.12±1.01) with maximum number of leaves were obtained in same MS medium [21].

In the current study, it was also observed that kinetin stimulates shoot initiation and proliferation but when concentration was increased it showed slight callusing at the base of the explants (Fig. F&G). For multiple shoot regeneration the frequency of sprouting was lower on kinetin supplemented medium (Kn+IAA) and slight callusing was also observed from the lower cut edge of the explant [22]. BAP was more effective than kinetin in inducing shoot induction from both, shoot tip and nodal explants in the three different mulberry species [9, 10].

IBA was the most effective auxin for inducing rooting from *in vitro* regenerated micro shoot. MS media supplemented with 10.0 µM IBA, screened out to be significantly excellent for induction and growth of adventitious roots, resulting in 72.2% rooting and 0.72 root number per explant at 30 days after inoculation in *Litsea glutinosa* L [21]. In the present study when *in vitro* grown shoots (2-3cm long) were cultured on modified MS medium fortified with IBA (0.5mg/l), $CuSO_4$ (0.05mg/l) and $ZnSO_4$ (17.2mg/l), it observed that most of the shoots developed calli at the base and failed to regenerate roots (Fig. F&G). Single healthy elongated shoots sub-cultured on rooting media (MS+IBA 0.5mg/l) with or without activated charcoal shows 100% initiations of roots [17]. About 80% rooting was obtained from shoots cultured on the MS supplemented with NAA (1mg/l) in case of mulberry [22]. Efforts are on using different combinations and concentration of auxin to induce rooting from the *in vitro* regenerated shoots.

Table 1: Composition of modified MS media for explant establishment and shoot initiation (T_1A, T_1B & T_1C), shoot multiplication (T_2A, T_2B & T_2C), Rooting from the *in vitro* regenerated shoots (T_3 &T_4)

Treatments	Plant Growth Regulators (mg/l)				Copper (Cu) (mg/l) CuSO4	Zinc (Zn) (mg/l) ZnSO4	Fenbendazole (mg/l)	Sucrose (gm/l)	Phytagel (gm/l)
	Kinetin	BAP	GA_3	IBA					
T_1A	0.5	2	-	-	-	-	-	30	8
T_1B	0.5	3	0.5	-	-	-	-	30	8
T_1C	0.5	3	0.5	-	-	-	100	30	8
T_2A	0.5	1.5	-	-	-	-	-	30	8
T_2B	1	4	0.5	-	-	-	-	30	8
T_2C	1.5	4	0.5	-	-	-	100	30	8
T_3	-	-	-	0.5	-	-	-	30	8
T_4	-	-	-	0.5	0.05	17.2	-	30	8

Table 2: Percentage of culture successfully established and initiation rate, shoot multiplication, shoot lengths (cm) and number of leaves developed per culture in different treatments after 2nd, 4th, 6th and 8th weeks of culture.

Treatments	Culture successfully established %	Explants showed shoot initiation %	Shoot multiplication (nos) After 2nd, 4th, 6th & 8th week of culture				Shoot length (cm) After 2nd, 4th, 6th & 8th week of culture				Number of leaves After 2nd, 4th, 6th & 8th week of culture			
			2nd	4th	6th	8th	2nd	4th	6th	8th	2nd	4th	6th	8th
T_1A	36	45	-	-	-	-	-	-	-	-	-	-	-	-
T_1B	45	68	-	-	-	-	-	-	-	-	-	-	-	-
T_1C	58	75	-	-	-	-	-	-	-	-	-	-	-	-
T_2A	-	-	0.7	1	1.4	1.4	0.89	1.04	1.36	1.49	1.8	2.5	3.4	3.5
T_2B	-	-	1.6	2	2.9	3.1	1.57	1.98	2.23	2.43	3.6	4.5	5.2	8
T_2C	-	-	1.9	2.5	3.4	3.3	1.69	2.31	2.54	3.02	3.7	4.6	6.3	8.8
S.Ed (±)	-	-	0.23	0.34	0.3	0.34	0.17	0.2	0.21	0.16	0.38	0.47	0.49	0.8
C.D. at 5%	-	-	0.4	0.57	0.51	0.57	0.3	0.34	0.36	0.28	0.66	0.79	0.83	1.36

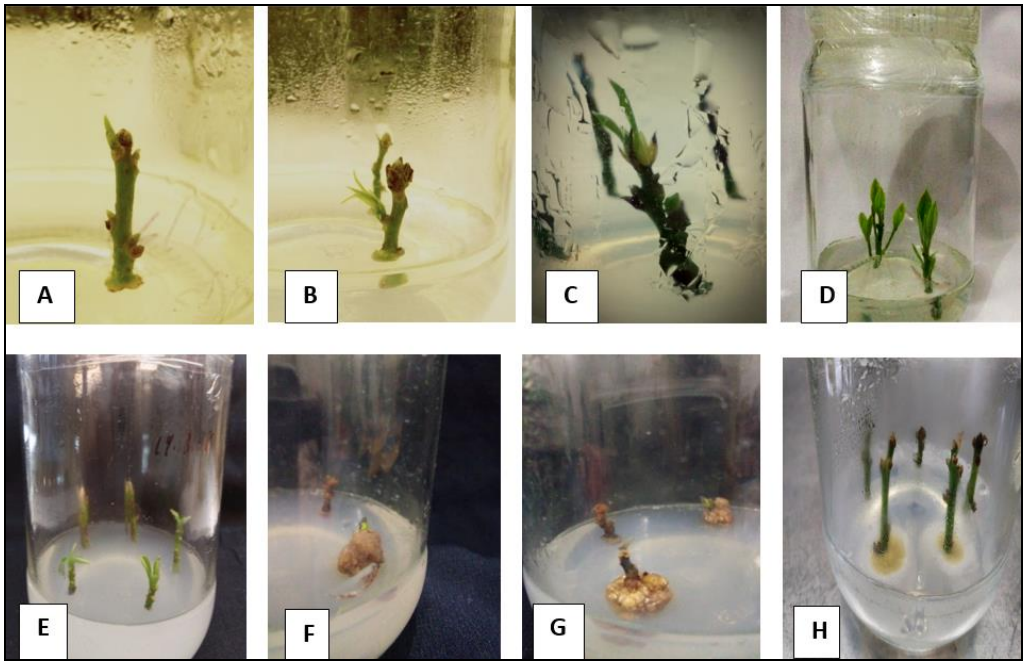

Fig: A & B. Bud enlargement and shoot initiation, C. Shoot developed after 8 weeks of culture in (T_1/C), D. Multiple shoots developed after 8 weeks of culture in T_2/C, E. Sub-culture of multiple shoots after 4 weeks of culture on to same composition medium, F & G. Individual shoot cultured on T_3 and T_4 medium for root induction. Most of the explants developed callus at the base of the shoots and failed to develop any roots, H. Fungal contamination observed after 2 weeks of culture.

Conclusion

There is scanty of literature available in micropropagation of *som* plant (*Persea bombycina* King) and no such commercial mass propagation method of *som* available. The ultimate aim of the investigation was to find out regeneration protocol for *in vitro* propagation of *som* to overcome problems faced by the conventional methods and the results obtained from the present study might have proved to do so till multiple shoot induction. As rooting was not successful efforts are on using different combinations and concentration of auxin to induce rooting from the *in vitro* regenerated shoots**.**

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