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Clonal Propagation of White Mulberry (*Morus alba* L.) Using *in vitro* Technique

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Abstract: The present study was undertaken with a view to develop a reproducible protocol for ***in vitro*** propagation of white mulberry (***Morus alba*** L.). For this purpose, surface sterilization of the explants from field grown mature plants was done with 0.1% $HgCl_2$ with a treatment duration of 5 minutes for shoot tip and that with 6 minutes for nodal segments. Between the two explants used, nodal explant exhibit comparatively better response to axillary shoot proliferation. Analysis of the results obtained from the proliferation experiment proved that effectiveness of cytokinin BA was better than that of Kn with respect to axillary shoot formation. And that MS medium supplemented with 1.0 mg l^{-1} BA was found to be best where 100% of the explants proliferated with the axillary shoots having average length of 5.46±0.02 cm and a multiplication rate of 7 fold per 4-week. On the other hand, percentage of root induction and number of roots per shoot were largely affected by the concentration of MS medium and type of the auxins used. The highest percentage (100%) of root regeneration was obtained in half MS medium supplemented with 0.5% mg l^{-1} IBA.

Key words: Clonal propagation, white mulberry, ***in vitro*** technique

Introduction

Man's interest in mulberry cultivation originated with the growth of civilization and his fascination with quality fabric that led him to search for silk. The mulberry cultivation has a great antiquity dating back to 2800 B.C. Mulberry is small to medium-sized shrub or tree, up to 15 m tall, wide spreading and round-topped. Mulberry leaves being the sole food for silkworm (***Bombyx mori***) bear great economic importance in sericulture as well as in forestry (Shajahan ***et al.***, 1996). Fukuda ***et al.*** (1959) reported that nearly 70% of the silk produced by a silkworm is directly derived from the proteins of the mulberry leaves (Purohit ***et al.***, 1996). Mulberry fruits are an ingredient of a particularly seductive drink known as mulberry wine and mulberry leaves are often eaten as a vegetable and are useful as a cattle feed.

Propagation of mulberry through ***in vitro*** culture has been established in Japan (Oka and Ohyama, 1974; 1981), India (Yadav ***et al.***, 1990) and Bangladesh (Hossain ***et al.***, 1990). However, Jain ***et al.*** (1990) reported that over all response in tissue culture of mulberry was very much genotype specific. Therefore, the present investigation was done to standardize the surface sterilization procedure for explants of the field grown plants and to develop a reproducible protocol for micropropagation of a commercial variety of mulberry that largely feed the Sericulture Industry of Bangladesh.

Materials and Methods

In the day of inoculation healthy, disease-free and actively growing shoots (4-5 cm) having tips and nodes portions were collected from field grown mature plants. For surface sterilization, they were immersed in 0.1% $HgCl_2$ for different durations of time. To remove any trace of the sterilant the materials were washed with autoclaved distilled water at least 4-5 changes of water. The surface sterilized explants were cultured on MS medium supplemented with BA and Kn in different concentrations either singly or in various combinations for regenerating the axillary shoots. For rooting, micro-shoots grown under ***in vitro*** condition were treated for root formation to establish themselves in the field. Auxin free medium and media containing two types of auxins (IBA and NAA) were used at different concentrations to regenerate roots under ***in vitro*** condition. Data from different treatments were recorded after 4 weeks of culture initiation. The pH of the medium was adjusted to 5.7-5.8 with the help of 0.1N NaOH or 0.1N HCl, whichever needed. All inoculations and aseptic manipulations were carried out in a laminar airflow cabinet. The temperature of the growth room was maintained at 26±1°C with a light intensity of 50-70 μE. m^{-2}. s^{-1}.

Results and Discussion

Overgrowth of surface contaminants inhibits the resumption of growth by the excised tissue (explants) under ***in vitro*** culture. Therefore, surface sterilization of the explants collected from the mature plants was found to be necessary before initiation of ***in vitro*** culture. Cent percent shoot tip explants were found alive and contamination free when they were treated for 5 minutes with 0.1% $HgCl_2$ after 10 days of inoculation. Similarly, the nodal segments produced highest frequency of contamination free cultures with elegant survivability (100%) when they were treated with 0.1% $HgCl_2$ for 6 minutes. It was found that explants were killed with the increasing duration of treatment with 0.1% $HgCl_2$ solution (Table 1). Although the $HgCl_2$ is considered as a potent surface sterilizing agent but its residual inhibitory effect is also greater than the other sterilizing agents commonly used in plant tissue culture (Bhojawani and Razdan, 1983; Torres, 1988; Razdan, 1993). As surface of young shoots of white mulbary is hairy and waxy use of a sterilant like $HgCl_2$ was essential. There are also many other reports on using $HgCl_2$ for surface sterilization of explants from ***ex vitro*** grown plants (Bennett and McComb, 1982; Roy ***et al.***, 1987).

Axillary shoot proliferation was induced in shoot tip and nodal segments of two sources on MS media fortified with 0.2, 0.5, 1.0 and 2.0 mg l^{-1} BA for selecting the better explant type. Nodal and shoot tip segments were collected from mature field grown mulberry plants as well as from aseptically raised ***in vitro*** grown shoots/ plantlets. In case of the explants collected from field grown mature plants, the highest of 60% shoot tips showed proliferation on MS medium supplemented with 1.0 mg l^{-1} BA while 70% of the nodal segments produced shoot (proliferated) on the same media composition (Table 2). Similarly with the explants collected from the ***in vitro*** proliferated shoots, at the best cytokinin concentration, the highest 80% shoot tips showed proliferation on medium fortified with 1.0 mg l^{-1} BA while 100% of the nodal segments showed proliferation (Fig. A) on the same media formulation (Table 2). From the above observations it was

Table 1: Effects of $HgCl_2$ treatment period on surface sterilization of shoot tip and nodal segment collected from field grown mature plants

Treatment duration (min) with 0.1% $HgCl_2$	Number of explants	Rate of contamination (after days of treatment) 2	4	6	8	10	Percentage of contamination free explants after 10 days
For shoot tip							
1	10	-	4	6	10	10	0
2	10	-	-	5	8	10	0
3	10	-	-	6	8	8	20
4	10	-	-	-	4	5	50
5	10	-	-	-	-	-	100
6	10	-	-	-	-	-	100*
7	10	-	-	-	-	-	100**
8	10	-	-	-	-	-	100***
For nodal segment							
2	10	-	4	6	10	10	0
3	10	-	3	5	9	10	10
4	10	-	-	2	3	5	50
5	10	-	-	-	1	2	80
6	10	-	-	-	-	-	100
7	10	-	-	-	-	-	100*
8	10	-	-	-	-	-	100**
9	10	-	-	-	-	-	100***

"-"indicates no contamination

" * "indicates culture death due to tissue killing (* = 5-25%; ** = 26-50%; *** = 50-75%)

Table 2: Effects of different concentrations of BA on explants of field and *in vitro* grown for axillary shoot proliferation. There were 10 explants for each treatment and data ($\bar{x}$±SE) were collected after 4 weeks of culture

Growth Regulators (mg l^{-1})	% of explant showed proliferation		Length of shoots per culture (cm)	
Field grown mature plants	ST	NS	ST	NS
BA				
0.2	30	40	2.53±0.06	3.76±0.05
0.5	40	50	3.46±0.08	4.12±0.07
1.0	60	70	4.10±0.05	4.37±0.06
2.0	50	60	3.15±0.07	3.52±0.06
In vitro proliferated shoot				
0.2	50	70	3.72±0.08	4.56±0.05
0.5	70	90	4.76±0.06	5.26±0.05
1.0	80	100	5.0±0.07	5.54±0.05
2.0	60	80	4.14±0.05	4.88±0.06

ST= Shoot tip, NS= Nodal segment

found that nodal segments from the mature plants and ***in vitro*** grown shoots produced considerably higher number of axillary shoots than the shoot tip explants. Above all, between the two sources of the explants nodal segments from ***in vitro*** grown shoots were found to be best for rapid proliferation of axillary shoots. Xin and Zhang (1987) found better result from the nodal explants of ***in vitro*** plantlet regeneration.

Table 3: Effects of different concentrations of two cytokinins either separately or combinedly in MS medium on direct regeneration of shoot from explants of *Morus alba* L. There were 10 explants for each treatment and data (x̄±SE) were recorded after 4 weeks of culture

Growth regulators (mg l^{-1})	% of culture forming usable shoot	Days taken for shoot formation	Average length of shoot (cm)	Number of nodes per shoot
Control	40	9.6±0.51	3.54±0.05	3.6±0.50
BA				
0.1	70	7.0±0.31	4.32±0.05	3.8±0.37
0.2	80	6.8±0.37	4.48±0.06	4.4±0.24
0.5	90	6.0±0.31	5.14±0.05	6.2±0.37
1.0	100	5.4±0.51	5.46±0.02	9.2±0.58
1.5	80	5.8±0.59	5.02±0.05	5.4±0.40
2.0	60	7.4±0.51	4.68±0.06	4.8±0.58
Kn				
0.1	50	8.2±0.58	3.84±0.05	3.4±0.24
0.2	50	7.2±0.59	4.04±0.05	3.6±0.50
0.5	60	6.2±0.37	4.16±0.06	4.6±0.50
1.0	80	6.0±0.70	4.52±0.03	5.0±0.31
1.5	70	6.4±0.51	4.22±0.03	5.0±0.54
2.0	60	7.2±0.58	3.98±0.06	3.8±0.58
BA + Kn				
0.1 + 0.5	60	7.4±0.60	3.76±0.05	2.6±0.24
0.2 + 0.5	70	7.0±0.70	3.82±0.06	4.2±0.58
0.5 + 0.5	90	6.2±0.58	4.18±0.03	4.6±0.50
1.0 + 0.5	100	6.0±0.63	4.92±0.06	6.2±0.58
1.5 + 0.5	80	6.8±0.73	4.76±0.06	5.4±0.24
2.0 + 0.5	60	7.6±0.51	4.18±0.05	4.4±0.24

Table 4: Effect of different concentrations of auxins in ½ MS medium on *in vitro* root formation. There were 10 cuttings in each treatment and data (x̄±SE) were collected after 4 weeks of culture

Auxin supplement (mg l^{-1})	Percentage of root formation	Number of roots per rooted cutting	Average length of the roots (cm)	Days taken for root formation
Control	-	-	-	-
IBA				
0.1	80	3.8±0.37	1.68±0.03	13.0±0.31
0.2	80	5.0±0.31	2.46±0.05	10.0±0.44
0.5	100	10.0±0.54	4.20±0.07	9.2±0.58
1.0	90	8.8±0.37	3.18±0.06	10.4±0.50
1.5	70	7.8±0.37	3.54±0.05	10.6±0.51
2.0	-	-	-	-
NAA				
0.1	60	6.0±0.44	2.36±0.07	13.2±0.37
0.2	70	7.4±0.50	2.28±0.05	11.8±0.38
0.5	70	6.2±0.37	1.96±0.06	11.2±0.38
1.0	60	5.2±0.37	1.66±0.05	12.6±0.51
1.5	40	4.0±0.44	1.52±0.08	13.0±0.44
2.0	-	-	-	-

"-"indicates no response

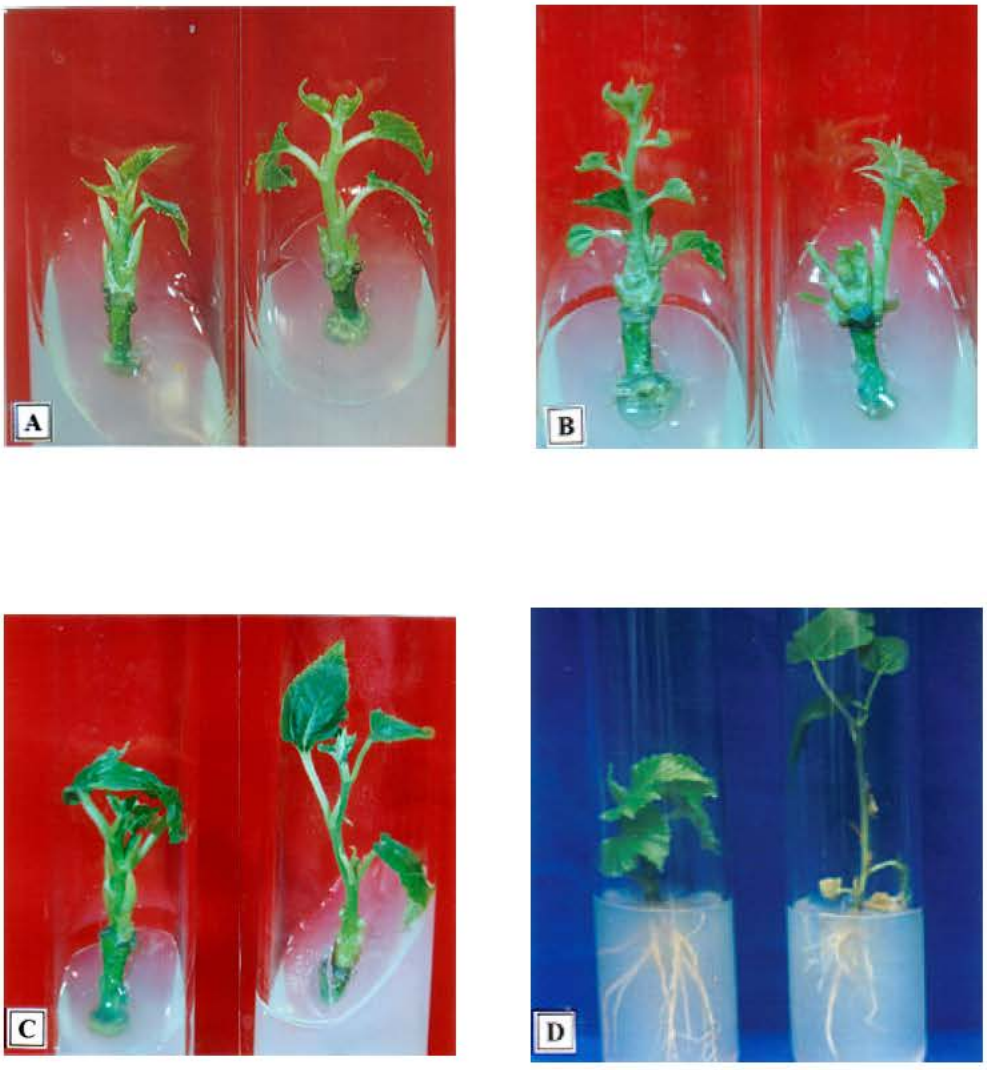

Fig. A-D: *In vitro* proliferation of shoots and development of complete plantlets in white mulberry Roxb

A: Single shoot formation from a nodal explant on MS + 1.0 mg l^{-1} BA after three weeks of culture

B: Multiple shoot proliferation from a nodal explant on same medium after six weeks of culture

C: Elongation of shoots from nodal explants on same medium after eight weeks of culture.

D: Root formation in the regenerated shoot on ½ MS + 0.5 mg l^{-1} IBA after six weeks of culture

Shoot tip and nodal segments collected from the field grown plants were cultured on MS medium supplemented with BA and Kn individually at concentrations of 0.1, 0.2, 0.5, 1.0, 1.5 and 2.0 mg l^{-1} for selecting optimum cytokinin concentration for the maximum proliferation of shoots. Between the two cytokinins, incase of Kn highest 80% of cultures formed usable shoots with average length of shoot was 4.52±0.03 cm when explants were cultured on MS medium fortified with 1.0 mg l^{-1} Kn. On the other hand 100% cultures showed usable shoot formation (Fig. B) with the average length of 5.46±0.02 cm (Fig. C) when they were cultured on MS medium supplemented with 1.0 mg l^{-1} BA (Table 3). In another experiment, explants from ***in vitro*** grown shoots were cultured on MS medium supplemented with different concentrations (0.1, 0.2, 0.5, 1.0, 1.5 and 2.0 mg l^{-1}) of BA along with only one concentration (0.5 mg l^{-1}) of Kn. Among theses different combinations, the highest percentage (100%) of shoot formation was found when explants were cultured on 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn. The highest average length (4.92±0.06 cm) of shoot and number of nodes (6.2±0.58) per shoot were also found on the same combination. Therefore, 1.0 mg l^{-1} BA + 0.5 mg l^{-1} Kn showed the best proliferation result than other combinations (Table 3). Analysis of the results obtained from the above experiments clarify that BA is relatively better for axillary shoot formation than Kn. Superiority of BA over other cytokinins in producing ***in vitro*** shoots has also been confirmed in other plants like ***Ocimum sanctum*** (Begum ***et al.***, 2000), ***Rosmarinus officinalis*** (Misra and Chatruvedi, 1984), ***Arachis hypogaea*** (Mhatre ***et al.***, 1985) and ***Atropa belladona*** (Benjamin ***et al.***, 1987).

Induction and development of roots at the base of the ***in vitro*** grown shoots is an essential step to establish tissue culture derived plantlets on the soil. For this purpose 3-4 cm long microcuttings were prepared from the ***in vitro*** proliferated usable shoots and cultured on ½ MS medium supplemented with 0.1 to 2.0 mg l^{-1} of either IBA/ NAA or without any auxin. In medium without auxin no rooting was found. Between two types of auxin, IBA was found to be comparatively more effective than NAA. Among various concentrations of IBA and NAA, the highest percentage (100%) of shoots that produced roots were found when they cultured on MS medium having 0.5 mg l^{-1} IBA (Fig. D). The highest growth and maximum length (4.2±0.07 cm) of the root were on the same medium (Table 4). In this experiment percentage of root induction and number of roots per shoot were greatly influenced by the concentration and type of auxin. Here between the two types of auxin used, IBA was found to be comparatively more effective than NAA and 0.5 mg l^{-1} IBA showed the best result. The findings are in agreement with those observed in other plants such as ***Datura metel*** (Muthukumar ***et al.***, 2000), ***Elaeocarpus robustus*** (Rahman ***et al.***, 2001) and ***Punica granatum*** (Khatun ***et al.***, 2001).

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