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Rapid Multiplication by Nodal Segment and Shoot tips through *in vitro* Micropropagation of Six Mulberry (*Morus alba*) Genotypes

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Key words: *In vitro* micropropagation, Regeneration, Nodal segment, Shoot tip, *Morus alba*

Abstract

A study was conducted to standardize *in vitro* propagation techniques of mulberry (*Morus alba*) using nodal segment and shoot tip culture. A high frequency of shooting (80%) from nodal segment and shoot tips (78%) were recorded on MS medium supplemented with various concentration and combination of BAP, Kn and two amino acids (L-asparagine and L-glutamine). BAP (2.0 mg/l) + NAA (0.2 mg/l) in addition with L-asparagine (1.0 mg/l) and L-glutamine (25 mg/l) with MS medium proven to be the best combination for multiple shoot formation in experimental mulberry genotypes. This medium facilitated the elongation of shoots and sprouting of axillary buds of *in vitro* grown shoots. Best performance was recorded in case of five genotypes *viz.* China white, BSRM19, BSRM63, BSRM34, and BSRM45 for calli induction and plant regeneration. 100% rooting was found when the medium was fortified half strength of MS + IBA (1.0 mg/l). The plantlets with well-formed root systems were gradually acclimatized in shade house using soil: coco peat (1:1) and finally plants were transferred to the field for further development.

Introduction

Mulberry belongs to the genus of *Morus* and its family is Moraceae. It is globally distributed and grown indifferent agro-climatic conditions in the world (Yuan et al. 2015, Rahman and Islam 2020). Mulberry is considered as an amazing plant of the planet due to its multiple uses for example feeding material for silkworms and domesticated animals (Shukla et al. 2019), edible berries (Ercisli and Orhan 2007), source of pharmaceutically important compounds (Raman et al. 2016, Rahman and Islam 2021), role in green synthesis of nanoparticles (Rohela et al. 2019) and for our environmental protection (Liu and Willison 2013). In Bangladesh, Sericulture Development Board and

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NGOs have been highly engaged in sericulture for three decades. Mulberry is a cross pollinated crop with heterozygous nature. Therefore, propagation through seeds does not conserve stable genetic makeup and does not assure a uniform quality of plants (Zaman et al. 1997). However, *in vitro* micropropagation techniques provide a fast and reliable way for production of an enormous number of uniform plantlets within shortening of time (Anis et al. 2003).

The propagation of mulberry by *in vitro* culture has been reported by many workers in different species of *Morus* in Japan (Oka and Ohyama 1974 1981), India (Yadav et al. 1990, Jain et al. 1990, Sharma and Thorpe 1990, Rao and Bapat 1993, Patnaik and Chand 1997, Chitra and Padmaja 1999) and in Bangladesh (Hossain et al. 1992). There are limited reports on the multiplication of mulberry germplasm of Bangladesh using *in vitro* techniques. To overcome the problems and to standardize a rapid multiplication from nodal segments and shoot tips were used under these studies and optimized suitable multiplication techniques of mulberry plants.

Materials and Methods

Six genotypes of mulberry (BSRM19, BSRM34, BSRM45, BSRM54, BSRM63 and China white) were considered as the main source of plant materials and shoots from 60 days old plants were used for this study. On the day of inoculation, healthy, disease free and actively growing shoots (4-5 cm) having tips and nodes were collected from the field grown mature plants cultivated at Mulberry Germplasm Bank of Bangladesh Sericulture Research Institute, Rajshahi. Only the explants of China white mulberry genotype were collected from *in vitro* grown seedlings and in this case explants were not surface sterilized. As basal medium MS was used in addition with different concentration and combination of plant growth regulators such as BAP, NAA, and Kn were used either individually or in combinations. Here 3% (w/v) sucrose and 0.6% (w/v) agar were used for shoot induction. Growth adjuvants, such as casein acid hydrolysate (100 mg/l) were also added to the medium. The pH of the medium was adjusted and maintained at 5.7 ± 0.1 by using NaOH or HCl through pH meter prior to autoclaving at 121°C for 15 min.

Young greenish and healthy twigs were excised, trimmed and cut into pieces each containing 2-3 nodes and a shoot apex. These segmented twigs placed in running tap water for about 1 h followed by submerged in distilled water containing 100 ml distilled water + 1 ml Tween 80 and 1 ml Savlon (ACI Limited) for 15-20 min. The fully washed explants were placed in laminar air flow excised, separated (in node and shoot tip) and dipped in 70% ethanol for only about 1-2 sec, 1000 mg/l carbendazim-50% for 15 min, 7% sodium hypochlorite (NaOCl) for 5 min and then rinsed with sterile distilled water properly. Then the explants were treated with 0.1% $HgCl_2$ solution (w/v) for 9-10 min and washed with sterile distilled water for 6-7 times. The surface sterilized shoot tip and nodal explants were inoculated on MS medium with various concentrations and combinations of BAP (0, 1.0, 1.5, 2.0, 2.5, 3.0 mg/1), either individually or in combination

of NAA (0.1, 0.2, 0.3, 0.4 mg/1) and BAP (1.0, 1.5, 2.0, 2.5 mg/l) along with Kn (0.5 mg/l) to observed their effects for induction of shoots. Various concentration and combinations of phytohormones like BAP (0.5 to 2.0 mg/l), NAA (0.2 mg/l), and as amino acids L-glutamine (0.5 and 1 mg/1) and L-asparagine (12.5 and 25 mg/1) were added in medium for shoot multiplication. The shoots that were regenerated *in vitro* were consistently transferred to new culture media every 21-28 days in order to facilitate their further growth.

In vitro developed of shoots (3-4 cm long) were inoculated on half and full strength of MS medium fortified with five different concentrations and combination of IBA (0.1, 0.2, 0.3, 0.5 and 1.0 mg/1) and NAA (0.1, 0.2, 0.5, 1.0 and 2.0 mg/1) for root induction. The observation on number and length (cm) of roots were recorded. The culture vessels with inoculated explants were maintained in the culture room at 25 ± 2°C under a 16 h photoperiod at light intensity of 3000 lux. Well rooted micro-shoots were taken out from the culture vessels and washed thoroughly to remove the medium adhered to the surface of the root. These plants were then transferred to pot filled with garden soil, organic compost and sand or saw dust in a ratio of 2:1:1. For primary adaptation culture vessels were allowed to remain for a week outside the culture room.

The transfer of plantlets was covered with polybags to maintain high humidity. The polybags were finally removed after the second week and after one month, the plantlets were exposed to sunlight periodically, for proper acclimatization. After 2-3 months the plants were transferred to the field directly. For each treatment three replications containing five explants for in each culture vessel were considered. Data were recorded on the basis of different parameters subjected to analysis of variance (ANOVA) and the mean values of treatments were compared by least significant difference (LSD) test were calculated at the confidence level of $p < 0.05$ (Gomez and Gomez 1976) by SPSS (version 16.0) for Windows 7.

Results and Discussion

Various concentrations of BAP and Kn alone and the combinations of BAP and NAA as well as BAP with Kn were applied to observe their effects on the proper proliferation of six mulberry genotypes. Shoots were proliferated from both the explants within 7-16 days of culture in all the genotypes except BSRM54. The surface sterilization treatment applied in this experiment yielded approximately 85% of axenic cultures. MS medium fortified with 2.0 mg/1 BAP produced the highest percentage of shoots proliferation from nodal segments of China white (85%) and BSRM19 (80%) genotypes respectively (Table 1). The second highest shoots proliferation (80%) was recorded from China white and BSRM19 (75%) when the nodal segments were cultured on MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l NAA. In this combination of BAP and NAA shoot tip explants of China white genotype produced 78% proliferation response. The morphogenic responses was found to be better when BAP was applied singly as

compared to BAP + NAA and BAP + Kn supplemented medium (Table 1 and Fig. 1A-B). For shoot multiplication, three experiments were conducted, and in this case BAP and NAA with amino acids were used either single or in combinations (Table 2).

Table 1. Effects of different concentrations and combinations of BAP, NAA and Kn on *in vitro* shoot proliferation.

Plant growth regulators (mg/l)	Explants showed shoot proliferation (%)										Days taken for shoot proliferation
	Shoot tip					Nodal segment					
	BSRM 19	BSRM 34	BSRM 45	BSRM 63	China white	BSRM 19	BSRM 34	BSRM 45	BSRM 63	China white	
0 (Cont.)	20	15	15	20	20	30	20	18	30	30	8-15
BAP											
0.5	30	20	20	30	30	40	38	35	40	40	9-14
1.0	40	30	30	40	40	50	45	50	50	50	8-13
1.5	50	42	42	50	50	60	60	60	60	60	8-11
2.0	68	50	50	65	75	80	70	65	75	85	8-10
2.5	40	35	35	40	45	50	50	50	50	50	9-12
3.0	30	25	25	30	40	40	40	34	40	40	10-13
Kn											
0.5	15	15	15	15	20	30	30	30	30	40	10-16
1.0	20	20	20	20	25	40	40	40	40	45	9-15
1.5	30	30	30	30	30	50	50	50	50	50	7-14
2.0	40	40	40	40	40	60	60	60	60	65	8-12
2.5	32	32	32	32	30	40	40	40	40	40	7-14
3.0	20	20	20	20	20	30	30	30	30	20	8-15
BAP + NAA											
2.0 + 0.1	40	30	32	35	40	50	50	50	50	50	8-11
2.0 + 0.2	70	60	65	75	78	75	70	72	71	80	8-10
2.0 + 0.3	50	40	38	42	52	60	56	55	60	65	9-12
2.0 + 0.4	40	38	35	39	42	50	42	45	55	52	10-13
2.0 + 0.5	30	30	28	30	30	40	38	35	38	45	8-14
BAP + Kn											
0.1 + 0.5	20	18	20	25	28	30	28	30	35	36	10-16
0.2 + 0.5	30	32	30	34	35	40	38	40	42	45	9-15
0.5 + 0.5	40	35	38	40	45	50	48	45	50	56	8-14
1.0 + 0.5	50	45	45	50	55	60	50	52	58	60	7-12
1.5 + 0.5	32	35	38	40	42	50	45	50	50	55	8-14
2.0 + 0.5	30	28	30	32	35	40	35	38	40	45	9-15

All the concentrations and combinations showed multiple shoots formation with the addition of amino acids. The number of shoots ranged from 2.00 ± 0.20 to 6.50 ± 0.28.

Among the applied PGRs, maximum mulberry shoot tip and nodal segments were responded well on MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l NAA. To number (6.50 ± 0.28) and length of shoots (4.80 ± 0.12 cm) are presented in Table 2, Fig. 1 (C-D) and Fig. 2.

Fig. 1(A-G). *In vitro* proliferation of shoots from nodal segments and complete plant regeneration of *M. alba*. A) Shoot proliferation from nodal segments on MS with 2.0 mg/l BAP in BSRM19, B) Shoot proliferation from nodal segments on MS with 2.0 mg/l BAP in BSRM63, C) Shoot multiplication on MS with 2.0 mg/l BAP + 0.2 mg/l NAA in China white mulberry, D) Shoot multiplication from proliferated shoot on MS with 2.0 mg/l BAP+ 0.2 NAA in BSRM19, E) *In vitro* rooting on half MS with 1.0 mg/l IBA in BSRM 19, F) *In vitro* rooting on half MS with 1.0 mg/l IBA in China white, G) Plants were transferred to pots.

Table 2. Effects of different concentrations of BAP, NAA and Kn either single or in combination on MS medium for *in vitro* shoot multiplication.

Plant growth regulators (mg/l)	Amino acids (mg/l)		Number of multiple shoots per culture of shoot per Genotypes					Length of shoot (cm) per culture (average)
	L-glutamine	L-asparagine	BSRM 19	BSRM 34	BSRM 45	BSRM 63	China white	
0 (Control)	0	0	3.10 ± 0.15^{cde}	3.00 ± 0.09^{c}	2.50 ± 0.20^{de}	3.15 ± 0.10^{cde}	3.50 ± 0.08^{cd}	3.95 ± 0.15^{bce}
BAP								
0.5	0.5	12.5	3.80 ± 0.20^{cd}	3.50 ± 0.16^{bc}	3.00 ± 0.23^{c}	3.88 ± 0.16^{bc}	3.90 ± 0.20^{cd}	4.15 ± 0.14^{bc}
1.0	0.5	12.5	4.25 ± 0.25^{bc}	4.10 ± 0.22^{b}	$3.50 \pm 0.11b^{c}$	4.00 ± 0.23^{bc}	4.50 ± 0.29^{c}	4.26 ± 0.15^{bc}
1.5	1.0	25	5.00 ± 0.24^{b}	4.50 ± 0.23^{ab}	4.50 ± 0.24^{ab}	5.10 ± 0.33^{ab}	5.50 ± 0.35^{b}	4.50 ± 0.23^{ab}
2.0	1.0	25	6.00 ± 0.30^{a}	5.00 ± 0.25^{a}	4.90 ± 0.30^{a}	5.60 ± 0.20^{a}	6.00 ± 0.28^{ab}	4.70 ± 0.23^{a}
2.5	1.0	25	4.50 ± 0.20^{bc}	4.15 ± 0.30^{b}	4.00 ± 0.40^{b}	4.10 ± 0.22^{bc}	4.55 ± 0.24^{c}	4.11 ± 0.23^{bc}
3.0	1.0	25	3.20 ± 0.22^{cde}	3.00 ± 0.35^{c}	3.12 ± 0.28^{c}	3.11 ± 0.20^{cde}	3.29 ± 0.27^{de}	3.90 ± 0.11^{bc}
BAP + NAA								
2.0 + 0.1	1.0	25	5.00 ± 0.20^{b}	4.50 ± 0.12^{ab}	4.45 ± 0.14^{ab}	5.00 ± 0.26^{ab}	5.00 ± 0.09^{bc}	4.26 ± 0.15^{bc}
2.0 + 0.2	1.0	25	6.10 ± 0.30^{a}	5.00 ± 0.20^{a}	5.10 ± 0.30^{a}	5.60 ± 0.41^{a}	6.50 ± 0.28^{a}	4.80 ± 0.12^{a}
2.0 + 0.3	1.0	25	4.20 ± 0.25^{bc}	4.15 ± 0.23^{b}	4.00 ± 0.25^{b}	4.10 ± 0.10^{bc}	4.50 ± 0.34^{c}	4.60 ± 0.05^{ab}
2.0 + 0.4	1.0	25	3.00 ± 0.15^{cde}	3.00 ± 0.24^{c}	3.12 ± 0.11^{c}	3.11 ± 0.25^{cde}	3.20 ± 0.18^{de}	4.15 ± 0.08^{bc}
2.0 + 0.5	1.0	25	2.90 ± 0.11^{ef}	2.10 ± 0.35^{cd}	$3.50 \pm 0.14b^{c}$	3.00 ± 0.12^{cde}	3.00 ± 0.26^{de}	3.99 ± 0.20^{bce}
BAP + Kn								
0.1 + 0.5	1.0	25	2.80 ± 0.21^{ef}	2.00 ± 0.20^{cd}	2.15 ± 0.24^{d}	2.90 ± 0.07^{cde}	3.00 ± 0.20^{de}	2.50 ± 0.28^{def}
0.2 + 0.5	1.0	25	3.25 ± 0.20^{cde}	3.00 ± 0.30^{c}	3.15 ± 0.30^{c}	3.50 ± 0.19^{c}	3.50 ± 0.34^{cd}	2.98 ± 0.15^{de}
0.5 + 0.5	1.0	25	3.50 ± 0.16^{cd}	3.10 ± 0.24^{c}	$3.25 \pm 0.40b^{c}$	3.80 ± 0.28^{bc}	3.60 ± 0.06^{cd}	3.12 ± 0.06^{cd}
1.0 + 0.5	1.0	25	4.10 ± 0.24^{bce}	4.00 ± 0.38^{b}	4.25 ± 0.35^{ab}	4.50 ± 0.30^{b}	4.80 ± 0.09^{bc}	3.80 ± 0.12^{bce}
1.5 + 0.5	1.0	25	3.50 ± 0.30^{cd}	3.15 ± 0.19^{c}	3.58 ± 0.24^{bc}	3.50 ± 0.35^{cd}	3.60 ± 0.20^{cd}	3.15 ± 0.11^{cd}
2.0 + 0.5	1.0	25	2.20 ± 0.20^{ef}	2.00 ± 0.09^{cd}	2.25 ± 0.17^{de}	2.24 ± 0.39^{de}	2.50 ± 0.18^{def}	2.55 ± 0.14^{def}

The present study demonstrated that BAP produced a better response than Kn for shoot multiplications compared with cytokinin-based treatments (Table 2 and Fig. 2). From the findings it was concluded that explants grown on BAP + NAA could yield better response as compared to that on BAP + Kn. Anis et al. (2003), Akram and Aftab (2012), and Desai et al. (2018) also used BA + NAA for shoot induction as well as multiplication from axillary buds explants of mulberry. Contradictorily, Kavyashree (2007) found 94% of the shooting response using 2.0 mg/1 BAP + 1.0 mg/1 TIBA in S54 variety of mulberry.

The obvious reason for moderate response in BAP + TIBA here may be due to inhibitory impacts of TIBA on polar transport of auxins in the cells (Venkatesh et al. 2009). Anis et al. (2003) found identical results on producing maximum number of shoots of mulberry on MS fortified with 2.0 mg/1 BAP + 0.2 mg/1 NAA + 25 mg/1 L-asparagine + 1.0 mg/1 L-glutamine. The elongated multiple shoots (2-3 cm) were cut off and transferred to rooting media (Table 3). Among the different concentrations of media half strength of MS + 1.0 mg/1 IBA showed the highest number of roots (10.1 ± 0.45).

Table 3. Effects of different concentration of IBA and NAA (auxin) in addition with half and full strength of MS medium for root formation in five mulberry genotypes.

Auxin supplement (mg/l)	Days taken for root formation	Percentage of root formation					No. of roots per shoot (average)	Average length of the roots (cm)
		Genotypes						
		BSRM 19	BSRM 34	BSRM 45	BSRM 63	China white		
Control	-	-	-	-	-	-	-	-
½MS + IBA								
0.1	14	60	55	55	60	65	2.1 ± 0.20^{g}	1.50 ± 0.09^{de}
0.2	13	70	65	65	70	75	4.2 ± 0.34^{e}	2.28 ± 0.10^{cd}
0.5	11	80	75	75	80	80	6.5 ± 0.40^{c}	3.20 ± 0.15^{bc}
1.0	10	100	80	80	100	100	10.1 ± 0.45^{a}	4.10 ± 0.20^{a}
2.0	13	65	60	60	65	65	6.4 ± 0.57^{c}	3.10 ± 0.08^{bc}
½MS + NAA								
0.1	14	40	35	35	40	42	1.9 ± 0.30^{g}	1.25 ± 0.13^{def}
0.2	14	50	45	45	50	55	3.1 ± 0.42^{f}	1.50 ± 0.21^{de}
0.5	13	65	60	60	65	68	4.5 ± 0.50^{e}	2.10 ± 0.08^{cd}
1.0	12	70	65	65	70	75	6.2 ± 0.45^{c}	2.50 ± 0.09^{c}
2.0	13	60	50	50	60	60	4.4 ± 0.55^{e}	2.15 ± 0.10^{cd}
MS + IBA								
0.1	14	30	25	25	30	35	2.0 ± 0.10^{g}	1.30 ± 0.05^{def}
0.2	15	40	30	30	40	45	3.2 ± 0.24^{f}	2.20 ± 0.10^{cd}
0.5	13	50	40	40	50	50	5.4 ± 0.30^{d}	3.10 ± 0.12^{bc}
1.0	12	75	65	65	75	80	7.2 ± 0.40^{b}	3.50 ± 0.18^{b}
2.0	14	60	50	50	60	65	6.4 ± 0.56^{c}	2.10 ± 0.06^{cd}

Values represent mean ± SE of ten replicates per treatment in three repeated experiments.

The highest length of roots (4.10 ± 0.20 cm) was recorded using half strength of MS in supplemented with 1.0 mg/1 IBA. The second highest number of rooting (7.2 ± 0.40) was recorded when shoots were cultured on full strength of MS medium with 1.0 mg/l IBA. From this finding it is clear that the half strength of MS showed best performance on rooting formation of the studied mulberry genotypes (Fig. 1E-F and Fig. 3). Anuradha and Pullaiah (1992) reported that NAA was a more effective rooting agent for *M. alba*. Chitra and Padmaja (1999) did not get any response with NAA as a rooting agent and reported 2,4-D to be more effective.

Well rooted plantlets were transferred to pots which were mixture with garden soil, organic compost and sand or saw dust in a ratio of 2:1:1 (Fig. 1G). The primary hardened plantlets attained further growth in polyhouse using soil as the hardening substrate. The acclimatized plants were later transplanted to the field and had a survival rate of 80% (data not shown). Acclimatized plants appeared to be morphologically similar with mother plant grown under field conditions.

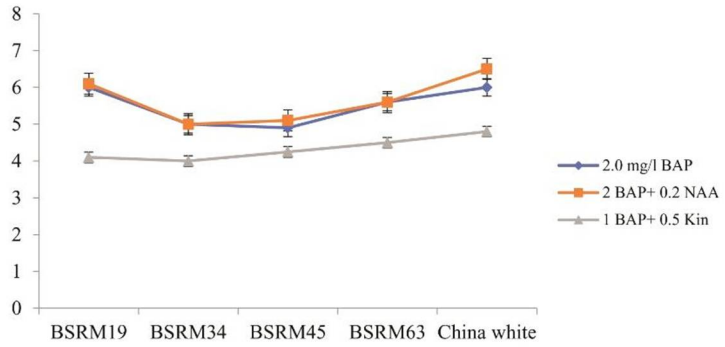

Fig. 2. Graphical presentation of multiple shoot induction on MS medium supplemented with different concentration of PGRs either singly or in combination.

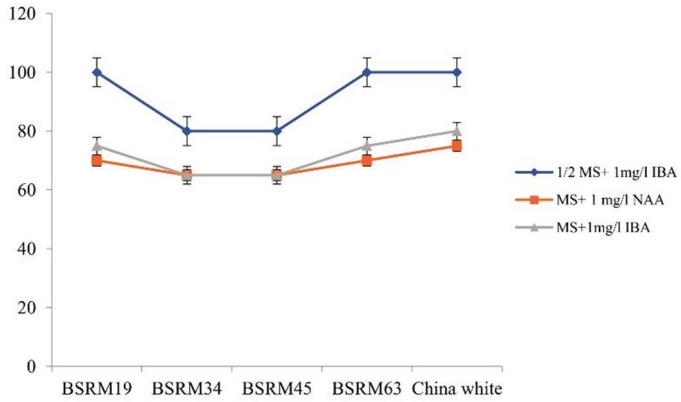

Fig. 3. Graphical presentation of root induction percentage on half and full strength of MS which supplemented with different concentration of IBA and NAA.

Similar type of observations was reported in *M. indica* var. C176 and C776 (Bhatnagar et al. 2002) and other mulberry varieties (Raghunath et al. 2009, Raghunath and Ravindran 2011). Based on the findings of this study, it can be stated that the nodal segment is a suitable explant for achieving an efficient micropropagation in mulberry. The regeneration process will offer significant benefits for large-scale production of mulberry in Bangladesh.

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