Tropical Agricultural Research and Extension 1 (1):28-33, 1998

Improvement of shoot proliferation in the micropropagation of mulberry (*Morus alba* L.)

A. Zaman[1] R. Islam[2], M. Islam[1] & O. I. Joarder[3]

[1] Plant Biotechnology Division, Bangladesh Sericulture Research & Training Institute, Rajshahi-6100, Bangladesh.

[2] Department of Botany, University of Rajshahi, Rajshahi-6205, Bangladesh.

[3] Department of Genetics & Breeding, University of Rajshahi-6205, Bangladesh.

Accepted 09 February 1998

ABSTRACT

Leaves of mulberry tree (*Morus alba* L.) are the only source of food for the silkworms (*Bombyx mori* L.). As the vegetative propagation of mulberry is strictly restricted to a single season (September-October) in Bangladesh, adequate production of leaves for silkworm rearing is interrupted every year. Efforts were made to search for an efficient protocol for year round mulberry propagation through *in vitro* techniques by single node culture. Among the media tested, Murashige and Skoog (MS) medium was found to be best followed by Woody Plant (WP) medium. Highest percentage (93.2%) of shoot proliferation was achieved in MS+5.0 µM benzyladenine (BA). Synergistic action of BA and kinetin (5.0 µM each) produced good number (15.3) of microshoots per culture. Addition of tyrosine (100 mg l^{-1}) coupled with synergistic action of cytokinins increased the number of microshoots (40.7%) remarkably. Best rooting was achieved in low concentration (1.0 µM) of indolebutyric acid (IBA) alone. *In vitro* derived plantlets were acclimatized conveniently and 85-90% survival of plants was observed without any abnormalities in soil.

Key words: acclimatization, mulberry, multiple shoot, single node culture.

INTRODUCTION

Mulberry (*Morus alba* L.) is a multipurpose, heterozygous, woody dicotyledonous plant. It is not only the food source of silkworms (*Bombyx mori* L.) but also used extensively in afforestation programmes and for medicinal purposes. In Bangladesh, Central Sericulture Board and NGOs have been engaged in sericulture for three decades. As mulberry is highly heterozygous (Das 1981) in nature, propagation through seed is not beneficial because the offsprings lack the exact qualities of parents.

In Bangladesh, mulberry is propagated largely through stem cuttings. Seasonal restriction is a significant drawback of this method of propagation. Optimum season for propagation through stem cuttings is early winter (September-October). In other seasons, the cuttings do not sprout properly and the plants remain stunted (Zaman *et al.* 1992a). For this reason, stem cutting propagation is inadequate to generate sufficient plant material to meet silkworm rearing targets every year (Zaman *et al.* 1996).

Considerable research work has been conducted in the last decade on *in vitro* cloning of mulberry in Japan (Oka and Ohyama 1986), India (Yadav *et al.* 1990), and Bangladesh (Zaman *et al.* 1996,1997a). However, information on a reliable protocol for year round commercial micropropagation of mulberry is still inadequate and yet to be established successfully.

Considering the shortcomings of stem cutting method of mulberry propagation, the present research work was undertaken to establish a reliable protocol for the micropropagation of mulberry through *in vitro* techniques.

MATERIALS AND METHODS

Plant material: Ten-year-old plants of *Morus alba* cv BM-1, one of the leading commercial cultivars of Bangladesh grown in the fields of Bangladesh Sericulture Research & Training Institute, Rajshahi were used as the source of plant materials for obtaining nodal segments in this study.

Abbreviations: MS, Murashige and Skoog medium; LS, Linsmeir and Skoog medium; WP, Woody Plant medium; BA, benzyladenine; Kin, Kinetin; IAA, Indole acetic acid.

Preparation of explants: Healthy branches were collected and nodal segments were carefully divided into one node explants. These explants were washed for 1 h under running tap water, and surface sterilized with 0.1% $HgCl_2$ (w/v) for 10 minutes followed by washing thrice in sterile distilled water.

Culture media and experimental details: MS (Murashige and Skoog 1962), LS (Linsmeir and Skoog 1975) and Woody Plant (WP) medium (Mc Cown and Lloyd 1981) with 0.7% phytagar (Sigma), 3% sucrose (BDH) were used as basal media. These media were enriched with benzyladenine (BA) and kinetin (kin) separately and in combinations. The pH of the medium was adjusted to 5.7+ 0.1 using 0.1 N HCl or NaOH before autoclaving at 121°C and 1.1 kg cm^{-2} pressure for 20 minutes. To accelarate the shoot proliferation, 100 mg l^{-1} tyrosine was added in every subculture. The explants (experimental unit) were placed singly into 150x25 mm culture tubes, each containing 20 ml medium. For multiple shoot proliferation, clumps of shoots developed in the culture tubes were transferred to 250 ml Erlenmeyer flask containing 100 ml of medium to increase the rate of shoot proliferation. All cultures were incubated at 26+ 2°C and 70% relative humidity under 16 h photoperiod provided by cool fluorescent light where photosynthetic photon flux density (PPFD) was 40-60 $\mu Em^{-2}S^{-1}$.

Induction of rooting: Healthy microshoots (< 3cm) from initial culture stage were harvested, trimmed and planted separately in 150x25 mm culture tubes containing half MS + 0.5 μ M IBA + 2% sucrose + 5 g l^{-1} agar as this medium has showed better rooting in the previuous experiments (Zaman *et al.* 1992a). Rooting cultures were incubated under the same growth room conditions as in shoot proliferation.

Acclimatization phase: After proper rooting, the cotton plugs of the test tubes were kept ajar and placed in a growth chamber for a week, which made the roots harden. Then the tubes were transferred to the laboratory and were maintained at room temperature for another week which enhanced acclimatization process. Next the plantlets were transferred to polybags (12.5x17.5 cm) containing a mixture of non-sterile garden soil, sand and cowdung (1:1:0.5) and maintained for another 6-8 weeks. Plantlets were exposed to sunlight daily for 2-3 h during this period. From polybags the plantlets were transferred to field. Transplants were irrigated periodically.

Data collection and statistical analysis: The cultures were examined after 6-8 weeks and the morphological changes were recorded on the basis of visual observations. The effects of different treatments were quantified as the percentage of cultures regenerated, average number of shoots proliferated per culture and shoot length. There were 20 replications per treatment and the experiment was conducted twice. Analysis of Variance followed by New Duncan's Multiple Range Test (DMRT) and paired 't' test were used to determine significance of difference between mean values.

RESULTS

The objective of the present investigation was mainly confined to *in vitro* shoot multiplication, their rooting followed by successful acclimatization. High concentration of BA (5 μM) and combination of BA and kinetin were used as they exhibited performances in the previous experiments (Zaman *et al.* 1996, 1997a). In addition, effect of tyrosine was also evaluated as this specific amino acid enhanced better *in vitro* performance of other genotypes of mulberry (Islam *et al.* 1993). In the present investigation, with the change of mulberry genotype, the rate of shoot proliferation was changed considerably which was not exhibited in the previous experiments (Zaman *et al.* 1992a) with other genotypes of mulberry.

Shoot proliferation: Shoot proliferation from cultured nodal explants started following one week of lag period. In the second week of culture, 40-50% of the cultured explants exhibited bud breaking and within six weeks, about 83-93% explants produced proliferating shoot cultures except in LS medium. The results obtained from initial cultures are presented in Table 1. Maximum percentage of shoot proliferation (93.2%) was observed in MS medium supplemented with 5.0 μM BA. In MS medium supplemented with two cytokinins (BA 5.0 μM + Kinetin 5.0 μM), percentage of shoot proliferation was reduced slightly (87.3%) but the difference was statistically non-significant. In WP medium percentage of shoot proliferation was 83-86% but it was drastically reduced to 47-50% when cultured in LS medium.

Except in LS medium, mean shoot length was from 3-4 cm in both MS and WP. However, in LS the shoot length was less than 3 cm. A higher number of shoots per culture was obtained when BA and kinetin were used together than when either of the cytokinins was used separately (Table 1).Thus the two cytokinins had a synergistic effect on the number of shoots per explant. However, this synergistic action was not observed in LS medium. In MS medium BA alone induced 7.6 shoots per explant, whereas combination of BA and kinetin

Table 1. Effect of different basal media supplemented with cytokinins on *in vitro* shoot proliferation of *Morus alba* cv. BM-1 after 6 weeks of culture.

Basal salts	Cytokinins, µM	% cultures regenerated	no. of shoots culture^{-1}	shoot length culture^{-1}, cm
MS	BA 5.0	93.2a*	7.6b	4.3a
	BA 5.0+Kin 5.0	87.3ab	12.9a	3.9ab
LS	BA 5.0	50.2c	4.1cd	2.7c
	BA 5.0+Kin 5.0	47.4d	3.7d	2.9bc
WP	BA 5.0	83.1b	4.3c	3.2bc
	BA 5.0+Kin 5.0	85.7ab	8.2b	3.5abc

*Values within the column followed by the same letters are not significant according to Duncan's Multiple Range Test at 5% probability.

produced 12.9 shoots. This observation allowed to choose MS in the second phase of experiment.

In the second phase, healthy single nodes and very short shoots having 2 nodes or less were aseptically harvested from the combined applications of BA and kinetin (MS + 5.0 µM BA + 5.0 µM kinetin) and subcultured onto the same media of its origin but with the addition of tyrosine (100 mg l^{-1}). The synergistic effect of cytokinins coupled with the action of tyrosine led to a higher root proliferation rate. Consequently, the number of shoots per culture increased significantly compared to the tyrosine free media (Table 2). After 8 weeks of subculture, the number of shoots per culture recorded 40.7 (Table 2, Fig. 1A) which was the highest that has ever been observed previously in *in vitro* culture of other genotypes of *Morus alba* L.

***In vitro* rooting and transfer to *ex vitro* environment:** In the present and previous experiments, it was observed that, unlike shoot proliferation, synergistic action of two auxins was not mandatory for successful *in vitro* rooting. Low concentrations (0.5-1.0 µM) of either IBA or NAA alone could easily induce sufficient roots from *in vitro* grown microshoots (Zaman *et al.* 1992 b, 1993, 1994, 1997 b). In the present investigation, NAA was discarded as it induced considerable amount of callus at the basal portion of microcuttings which in turn interrupted *in vitro* rooting in this genotype (Fig. 1 B). This phenomenon was not so common in other genotypes (Zaman *et al.* 1994). Nevertheless, microshoots rooted well in IBA (1.0 µM) enriched media irrespective of their origin (MS, LS or WP) and more than 90% success, with 15-20 roots per microshoot achieved after 6 weeks of culture. Due

Table2. Effect of tyrosine on *in vitro* shoot proliferation and average shoot length (each value is the mean per culture).

Treatments	No. of shoots	Length of shoots, cm
MS+BA 5.0µM+Kin 5.0µM	15.3	3.3
MS+BA 5.0µM+Kin 5.0µM+ 100 mg l^{-1} tyrosin	40.7	3.5
"Paired t" test	***	ns

*** Significant at the 0.1% level, ns non-significant.

to heavy aggregation of microshoots into clumps in conical flasks, the number and size of leaves were reduced (Fig. 1A). This was overcome when individual microshoots were harvested and transferred onto the rooting media. After 3-4 weeks of incubation many lateral roots were induced from the basal portion of microshoots (Fig.1C). Acclimatization of the plantlets was conveniently achieved when they were taken to the polybags during active growth, with no more than 4-5 internodes and short (2-3 cm) roots. Within 7-8 weeks of transfer to polybags, the *in vitro* derived plantlets showed satisfactory growth (Fig. ID).

After passing a semi-acclimatization phase in ploybags, the plantlets were directly transferred to the field (Fig. IE) where they are growing normally, similar to the sister plants grown conventionally by stem cuttings.

DISCUSSION

In the present investigation, MS was found to be the best medium for micropropegation of mulberry followed by WP. LS medium was unsatisfactory. Jain *et al.* (1990) found MS and LS both as suitable media for *in vitro* cloning of mulberry.

The synergistic effect of cytokinins (BA+kinetin) increased the shoot number singnificantly. An equimolar concentration of BA and kinetin also showed best results for multiple shoot proliferation in other woody species (Nair *et al.* 1984; Brave and Mehta 1993; Amin and Jaiswal 1993) and ornamental plants (Williams *et al.* 1984).

Synergism of cytokinins and tyrosine surprisingly and significantly increased shoot number per culture which is also supported by other workers. Islam *et al.* (1993) observed improved shoot proliferation in mulberry (*Morus alba* cv S_1) by adding tyrosine (50 mg l^{-1}) in initial media. Positive effect of tyrosine was also observed by

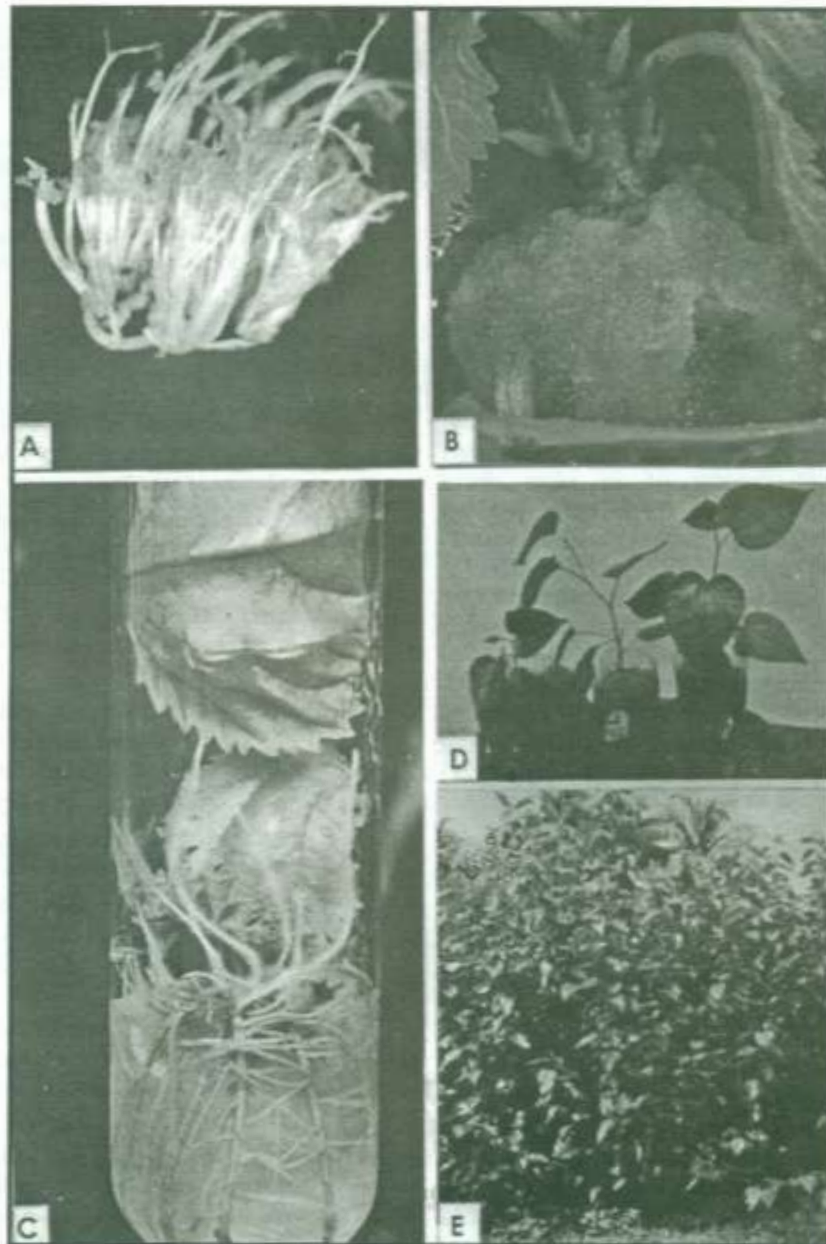

Fig. 1 (A-D) Micropropagation of *Morus alba* cv. BM-1

A. Multiple shoot proliferation with maximum number of shoots in MS + BA 5.0 μ M + Kin 5.0 μ M + 100 mg l^{-1} tyrosine after 8 weeks of culture.

B. Formation of massive callus at the basal portion of *in vitro* derived microcuttings in 1/2 MS + NAA 0.5μ M + IBA 0.5μM after 4 weeks of culture.

C. Root induction on *in vitro* derived microoshoots in 1 /2 MS + 0.5μ M IBA after 2 weeks of culture.

D. Rooted plantlets in polybags after 6 weeks of transfer.

E. Six months old field grown micropropagated mulberry plants.

Zaman *et al*. (1994) in mulberry (*Morus alba* cv C776) and by Hossain *et al*. (1990) in *Sesbania cannabina*. In these experiments, tyrosine was added in initial cultures. However, in the present investigation, it was observed that, when explants from axenic cultures were subcultured in tyrosine enriched media higher numbers of shoots were obtained. Hossain (1990) also reported that the presence of tyrosine (100 mg l^{-1})in the media sufficiently improved *in vitro* shoot regeneration potential in *Aegle marmelos* which confirms Our findings. Besides, there are other examples which

clearly indicate that, synergistic action of hormones coupled with amino acids certainly play a significant role in the improvement of *in vitro* shoot proliferation. Highest frequency of shoot formation in *Commiphora wightii* was achieved by culturing axillary buds in MS supplemented with 17.8 μM BA + 18.6 μM kinetin + 100 mg l^{-1} glutamine + 10 mg l^{-1} thiamine HCl (Brave and Mehta 1993). Furthermore, highest frequency of plant regeneration was observed when MS was enriched with BA in combination with IAA plus alanine in peanut and BA in combination with IAA plus aspartic acid in pigeon-pea (Eapen and George 1993).

Finally, when the synergistic effect of cytokinins and tyrosine in improving of *in vitro* shoot proliferation in mulberry is considered, present findings significantly differed from previous results (Zaman et al. 1992b, 1994, 1996, 1997a, 1997b; Islam et al. 1993). This improvement might be due to genotypic effect that we have often experienced before. Genotypic effects in mulberry on *in vitro* culture was also demonstrated by Jain *et al.* (1990). They reported that various genotypes of mulberry under similar *in vitro* conditions exhibited different morphogenic performances. Zaman *et al.* (1992a) also found genotypic differences in mulberry for regeneration capacity under *in vitro* conditions.

CONCLUSION

The foregoing research elucidates an optimistic protocol for rapid multiplication of axenic microshoots in mulberry (*Morus alba*. cv BM-1) by single node culture. The genotype BM-1 was found to be outstanding for *in vitro* cloning exceeding the previous *in vitro* performance of cv S1, the leading commercial genotype of mulberry in Bangladesh. Current results may possess high applied value in the micropropagation of mulberry (*Morus alba* L.) and thus can be utilized in fulfilling the demand of clonal planting material in large scale multiplication programmes.

REFERENCES

Amin MN and Jaiswal VS 1993 *In vitro* response of apical bud explants from mature trees of jackfruit (*Artocarpus heterophyllus*). Plant Cell Tissue Organ Cult. 33: 59-65.

Brave DM and Mehta AR 1993 Clonal propagation of mature elite trees of *Commiphora wightii.* Plant Cell Tissue Organ Cult. 35:237-244.

Eapen S and George L 1993 Plant regeneration from leaf disc of peanut and pigeonpea: Influence of BA, IAA and aminoacids. Plant Cell Tissue Organ Cult. 35:223-227.

Das DC 1981 Mulberry Taxonomy, Cytogenetics and Breeding. National Seminar on Silk Research and Development. March 10-13, Bangalore, India.

Hossain M 1990 *In vitro* propagation of *Aegle marmelos*. Ph.D thesis. Univ. of Rajshahi, Bangladesh.

Hossain M and Ahmad G 1990 Rapid clonal propagation of *Sesbania cannabina* through *in vitro* shoot proliferation from shoot tip explants. Bangladesh J. Bot. 19 (2): 183-187.

Islam R , Zaman A and Joarder OI 1993 *In vitro* propagation of mulberry (*Morus alba* L.): Effect of cytokinin types and tyrosine. Bull. Seric. Res. 4:6-9.

Jain AK, Dandin SB and Sengupta K 1990 *In vitro* propagation through axillary bud culture in different mulberry genotypes. Plant Cell Rep. 8:737-740.

Linsmaier EM and Skoog F 1975 Organic growth factor requirements of tobacco tissue culture. Physiol. Plant. 18:100-127.

Mc Cown BH and Lloyd G 1981 Woody Plant Medium (WPM)- a minaral nutrient formulation for micro culture of woody plant species. Hort. Sci. 16:89.

Murashige T and Skoog F 1962 A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473-497.

Nair S, Gupta PK, Shirgurkar MV and Mascarenhas AF 1984 *In vitro* organogenesis from leaf explants of *Annona squamosa* L. Plant Cell Tissue Organ Cult. 3: 29-40.

Oka S and Ohyama K 1986 "Mulberry (*Morus alba* L.)". In: Bajaj YPS (ed.), Biotechnology in Agriculture and Forestry Vol. I, Trees I , Springer-Verlag, Berlin, pp.384-392.

Williams RR, Taji AM and Bolton JA 1984 *In vitro* propagation of *Dampriera* and *Prostenthera rotandifolia*. Plant Cell Tissue Organ Cult. 3:273-281.

Yadav V, Madan L and Jaiswal VS 1990 Micropropagation of *Morus nigra* from shoot tip and nodal explants of mature trees. Scientia Hort. 44:61-67.

Zaman A, Islam R and Joarder OI 1992a Clonal propagation through *in vitro* shoot proliferation of nodal explants of seven mulberry genotypes. Plant Tissue Cult. 2(2):71-74.

Zaman A, Islam R and Joarder OI 1992b Micropropagation of *Morus laevigata* Wall. from mature trees. Plant Cell Rep. 11:522-524.

Zaman A, Islam R and Joarder OI 1993 *In vitro* propagation as an aid for cloning of *Morus laevigata* Wall. Plant Cell Tissue Organ Cult. 33: 339-341.

Zaman A, Islam R and Joarder OI 1994 Effects of amino acids on *in vitro* cloning of mulberry microshoots: Observations , analysis and consequences. Bull. Seric. Res. 5:1-4.

Zaman A, Islam R and Joarder OI 1996 Micropropagation of mulberry, a food plant of silkworms (*Bombyx mori* L.). Plant Tissue Culture and Biotech. 2(3):156.

Zaman A, Islam R and Joarder OI 1997a Micropropagation of *Morus alba* L. cv S1 from shoot apices of mature trees. J. Col. Sci. 10(1): 7-14.

Zaman A, Islam R and Joarder OI 1997b Field performance and biochemical evaluation of micropropagated mulberry plants. Plant Cell Tissue Organ Cult. 51: 61-67.