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Micropropagation of *Morus nigra* L. From Nodal Segments with Axillary Buds

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Abstract: Regeneration and production of multiple shoots were achieved from nodal segments with axillary buds of a field grown mature tree of *Morus nigra* L. it was observed that BAP (5.2mg/l) was found to be effective in inducing shoot regeneration from explants obtained from field grown trees. Multiple shoots were also achieved from *In vitro* raised shoots on MS medium supplemented with BAP (1.5mg/l). NAA (0.5mg/l) was found to be more effective than IAA and IBA for root induction. Complete plantlet development was achieved on MS medium supplemented with BAP (1.0mg/l) + NAA (2.0mg/l). The regenerated micro plantlets were successfully established in the soil under field conditions after a few days of indoor acclimatization.

Key words: *In vitro* response % Organogenesis % Rhizogenesis % *Morus nigra* L.

INTRODUCTION

Tissue culture techniques such as micropropagation provide a fast and dependable method for production of a large number of uniform plantlets in a short time and offer potential means not only for rapid mass multiplication of existing stocks but also for the conservation of important, elite and rare plants. It also provides novel approaches for the induction of genetic variability and production of plants which are resistant to biotic and abiotic stresses. Plant cell and tissue culture has contributed significantly to Crop improvement and has great potential for the future. Man's interest in mulberry cultivation originated with the growth of civilization and his fascination with quality fabric that led him search for silk. Mulberry leaves being the sole food for silkworm (*Bombyx mori*) bear great importance in sericulture as well as in forestry.

Nearly 70% of the silk produced by silkworm is directly derived from the proteins of the mulberry leaves [1]. Significant features of *in-vitro* propagation procedure are its enormous multiplicative capacity in a relatively short span of time; production of healthy and disease free plants; and its ability to generate propagules around the year [2]. Considerable work has been done in the last few years on *In vitro* propagation of *M. nigra* [3]. In the mulberry, studies have been conducted on the induction of division in pollen culture [4], the effect of sugars and alcohols [5] embryo differentiation [6] and production of haploid plants from anther culture [7].

Mulberry belongs to the Moraceae family and to the genus *Morus*. It is a perennial tree or shrub as an economically important plant used for sericulture. The economic importance of mulberry is primarily due to its leaf, which is being used for feeding the silk producing insect *Bombyx mori* L. it has been estimated that nearly 60% of the production cost of silkworm cocoon is incurred by mulberry leaf production [8]. Thus, mulberry varieties with higher leaf yield are important to sustain profitability in sericulture [9]. The black mulberry (*M. nigra*) is extensively cultivated in Turkey [10] for its fruits. With its Mediterranean climatic conditions, Isparta Province in the Lakes Region is notable for populations of black mulberry [11]. The fruits of mulberry has a tonic effect on kidney energy and thus, it is used as an antiphlogistic, a diuretic and an expectorant [12]. *M. nigra* fruit has good source of several phytonutrients and contain high amounts of total phenolics, total flavonoids and ascorbic acid [13-15]. Also, the fruit has pleasant taste with slightly acidic flavor and attractive dark red color. Tissue culture (micropropagation) is also used as a subculture and *In vitro* conservation for mulberry germplasm. It is believed that mulberry first originated in the foothills of Himalayas and later dispersed into Asia, Europe, Africa and America [16].

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Some of the economically important tree sps. of Kashmir Himalayas viz; *Morus nigra* L. *Ulmas wallichiana* planch, *Celtis austroails* L. *Betula utils* D. Don *and Corylus jacquemonti* Dene etc are also at the foot hold of being threatened and are at the verge of extinction. In the present study two of the above mentioned threatened tree species i.e., *Morus nigra* L. and *Betula utilis* D. Don were subjected to *In vitro* studies so to develop reproducible protocols for their mass multiplication. Mulberry is an important plantation crop grown primarily for foliage to rear the silkworm (*Bambyx mori* L). It grows in different climatic conditions ranging from tropical to temperate and is unique in expression of genetic characters under different environmental conditions. It is widely distributed in varied ecological and geographical zones from intensive cultivation in temperate, sub-tropical and tropical areas to natural occurrence in forests. This clearly indicates that mulberry has high degree of morphological and physiological adjustments to changes in the environment. There has been increasing interest in using tissue culture as a means of clonal propagation of woody plants. This is true in those plans whose cuttings are difficult to root, particularly when they are taken from the mature trees. Some reports have indicated that mulberry can be regenerated from axillary buds, meristems, winter buds, leaves and stem explants [17-21], but these explants are limited by season. There are two pathways for obtaining propagules through tissue culture. One is adventitious initiation of shoots or embryos from callus. The another is enhanced auxiliary branching by means of bud or meristem culture. The later system is useful in species where plant regeneration from cultured tissue is difficult to attain. Further, bud meristems are good material for producing multiple shoots in both gymnosperm and angiosperm plants. Pre-existing meristems easily developed into shoots and are likely to be genetically stable.

MATERIALS AND METHODS

During the present investigation, Murashige and Skoog's basal medium was used [22]. For the preparation of a given volume of MS medium, the desired volume of the stock solution from macrosalts, microsalts, vitamins and iron source were added to known quantity of double distilled water. To this solution 30 gm/l sucrose and 0.8gm/l agar was added. This solution was heated till agar got dissolved completely. It was then cooled and the pH of medium adjusted between 5.6-5.8 using 0.1 N NaOH or 0.1N Hcl. About 15 ml of this medium was dispensed in Boorsil culture tubes and about 25 ml in 100ml conical flasks which were plugged properly and tightly with sterilized non-absorbent cotton plugs. Finally, these culture vials and flasks containing nutrient medium were sterilized by autoclaving at 15 PSI pressure at 121°C for 15-20 minutes in an autocleave.

The explants used in the present study i.e., nodal segments with axillary buds were excised from the field grown authentic plants of *Morus nigra* L. These explants were firstly washed under a jet of running tap water in order to remove dust, dirt and other unwanted materials. They were then washed by a detergent solution (Labolenne) containing 2-3 drops of a wetting agent (tween 80). This was followed by washing with tap water to remove the detergent and finally washed 2-3 times with double distilled water under laminar air flow hood. Finally the explants were disinfected with 0.1% $HgCl_2$ solution for different time durations. The surface sterilized explants were then washed 5-6 times with autoclaved double distilled water so as to remove the last traces of the sterilant. The sterilized plant material was then put into pre-autoclaved petri-dishes, cut into suitable size and finally aseptically inoculated into the culture medium. Each treatment involved about 10-20 explants and each experiment were repeated twice.

The present study was undertaken to develop a reproducible protocol for *In vitro* propagation from nodal segments with axillary buds through shoot initiation and multiplication using MS-media supplemented with growth regulators, induction of root using various types and concentration from auxins, hardening of plants. Finally the plants transfer to field.

RESULTS

Shoot Regeneration: Nodal segments with 2-3 axillary buds obtained from Juvenile branches of *Morus nigra* L. when inoculated on MS basal medium showed no response. In order to induce axillary shoot proliferation these explants were inoculated on MS medium supplemented with various concentrations of cytokinins like BAP (0.2 and 0.5 mg/l) and Kn (5.0,5.1 and 5.2 mg/l) of which the most effective concentrations are given in (Table 1). On MS basal medium augmented with Kn (0.5mg/l) the shoot buds sprouted in 30% cultures after 10 days inoculation. The shoots obtained did not elongated on the same medium upto 4 weeks time. Later on, these explants were transferred to fresh MS basal medium of the same composition but still they did not showed any further growth upto a period of 4 weeks. Finally they turned yellowish in colour and afterwards necrosed.

Table 1: Effect of cytokinin (mg/l) on shoot regeneration from nodal segments of *Morus nigra* L.

Cytokinin (mg/l)	% age of culture forming usable shoots	Average No. of shoot/ explant	Average shoot length (cm)
MS basal Medium (Control)	No Response	0.0	0.0
Kn			
0.2	20	2.1	1.1
0.5	30	3.2	2.1
BAP			
5.0	90	7.5	5.4
5.1	60	2.5	3.0
5.2	70	2.1	1.1

Table 2: Effect of BAP (mg/l) on shoot multiplication from *In vitro* raised micro cuttings of *Morus nigra* L.

BAP (mg/l)	% age of culture forming usable shoots	Average no. of shoots / explant	Average length of shoots (cm)
5.5	30	2.0	2.2
4.5	30	3.0	2.3
3.5	40	4.1	2.5
2.5	70	7.1	2.4
0.5	80	8.0	2.3
1.5	90	10.2	2.8

Table 3: Effect of auxin (mg/l) on root induction from *In vitro* raised shoots of *Morus nigra* L.

Auxins (mg/l)	% of cultures forming useful roots	Average no. of roots/ explant	No. of days taken for rhizogenesis
IAA			
0.5	40	3.1	12
1.0	50	3.5	10
IBA			
2.0	20	3.0	18
3.0	20	3.1	18
NAA			
0.5	60	5.3	16
1.7	60	5.2	16
1.0	70	3.6	8-10
0.5	80	7.5	5-6

BAP as a source of cytokinin was found to be more effective than Kn, as these explants responded to its varied concentrations. On MS basal medium augmented with BAP (5.1-5.2 mg/l) explants sprouted and produced shoot buds in 60-70% cultures after 1 week of inoculation. The shoot buds elongated into multiple shoots in the same media upto 2 weeks of inoculation. These shoots were then transferred to a fresh medium of the same composition and were maintained for a period for 4 weeks time. When inoculated on MS basal medium fortified with BAP (5.0mg/l) these explants showed the maximum response as initiation of shoot buds was achieved in 90% cultures from 6th day of inoculation. These buds developed into multiple shoot after 2 weeks of inoculation.

Shoot Multiplication: The shoots regenerated during 1st passage of clonal propagation were cut into nodal segments of 2-3cm and subcultured on the best of the induction medium i.e., MS+BAP in order to increase the number of shoots. It was observed that the explants responded better to the reduced concentrations of BAP as compared to the 1st stage of shoot regeneration (Table 2). Explants inoculated on MS medium augmented with BAP (1.5mg/l) induced the best response as initiation of multiple shoot buds was achieved in 90% cultures within 4-5 days of inoculation. The microshoots produced lobed as well as unlobed leaves which were similar to those produced by the mother plant in nature. The shoots started growing and after 15th day of inoculation the cultures turned light green in color and got established as shoots (Fig. 1, 2).

Induction of Rooting: For the production of complete plantlets the *In vitro* raised shoots were transferred on MS basal medium adjuvanted with various concentrations of auxins including IAA, IBA and NAA (Table 3). In MS basal medium augmented with IAA (0.5mg/l) the cultures showed root initiation after 12th day of inoculation from basal cut portion of shoot explants in 40% cultures and

Fig. 1:

Fig. 2:

Fig. 3:

Fig. 4:

within 4 weeks roots developed in each responsive culture. The micro shoots when transferred onto MS basal medium fortified with IAA (1.0mg/l) exhibited root initiation in 50% cultures after 10 days of inoculation. The sprouted roots increased in size in the same medium upto 4 weeks of inoculation. MS basal medium supplemented with IBA (2.0-3.0mg/l) exhibited root initiation only in 20% cultures after 18th day of inoculation.

When MS basal medium was supplemented with different concentrations of NAA, the explants started sprouting within 6 days of inoculation and microcuttings showed different rooting response at different concentrations. At a concentration of NAA (0.5-1.7mg/l) microcuttings sprouted only in 60% cultures and produced (5.3) number of roots within 2 weeks of culture. The maximum numbers of roots (7.5) were formed in 80% microcuttings, when subcultured on MS medium supplemented with NAA (0.5 mg/l), within 5 to 6 days of inoculation (Fig. 3). Further, roots developed in this medium were comparatively stout and also produced lateral roots within 2 weeks of inoculation.

Effect of Auxin-Cytokinin Combinations: In order to develop a single step protocol for production of clonal plants, the nodal segments were also subcultured on MS medium supplemented with various auxin-cytokinin

Table 4: Effect of auxin- cytokinin combination on nodal segments of *Morus nigra* L.

PGR's (mg/l)	% of cultures regenerated	Average no. of shoots/explants	Average no. of roots/explants
Kn+IBA			
1.0+0.5	20	2.1	Creamish friable callus
2.0+1.0	30	3.0	-
BAP+IBA			
1.0+0.5	30	3.5	2.1
2.0+1.0	40	4.6	3.2
BAP+NAA			
0.5+1.0	70	9.1	6.2
1.0+2.0	80	10.2	8.1

Table 5: Effect of Planting out media on survival of micro propagated plantlets of *Morus nigra L.* during hardening, irrigated with $^{1}/2$ MS solution + tap water

Planting out media	Irrigating solution	Survival percentage	Plant height
Vermiculture	½ MS salt solution +tap water	50%	20 cm
Sand	½ MS salt solution +tap water	30%	25 cm
Soil	½ MS salt solution +tap water	30%	28 cm
Sand + vermiculture (1:1)	½ MS salt solution +tap water	80%	34 cm
Soil + vermiculture	½ MS salt solution +tap water	60%	32 cm
Sand + soil (1:1)	½ MS salt solution +tap water	40%	30 cm

Fig. 5:

Fig. 6:

combinations. Of the different combinations used, nodal segments responded very well in the combinations mentioned in (Table 4). MS basal medium fortified with Kn (1.0mg/l) + IBA (0.5mg/l) induced formation of friable callus at the cut end of nodal segments along with shoot formation within 10-12 days of inoculation in 30% cultures. When subcultured in the same media composition, the shoots elongated which were later maintained in the same medium upto a period of 4 weeks. MS basal medium augmented with Kn (2.0mg/l) + IBA (1.0mg/l) induced only shoot differentiation in 20% cultures within 14-16 days of inoculation however, on MS basal medium supplemented with BAP (1.0-2.0mg/l) + IBA (0.5-1.0mg/l) the explants exhibited shoot as well as root initiation after 10-15 days of inoculation in 30-40% cultures. These plantlets were finally maintained on the same medium composition upto a time period of 4 weeks. Complete plantlet development was achieved when explants were cultured on MS basal medium fortified with BAP (0.5-1.0mg/l)+NAA (1.0-2.0mg/l). On this medium the explants exhibited best morphogenetic potential as in 70-80% cultures shoot as well as root differentiation started after 6-8 days of inoculation. In the next 4 weeks these cultures showed enhanced growth along with the production of roots (Fig. 4).

Fig. 7:

Hardening and Transplantation: During the present work the *In vitro* raised rooted plantlets were carefully removed from the cultural vials and were washed thoroughly with running tap water in order to remove agar adhering to their roots. They were treated with (0.5%) Baveston solution (Fungicide) for 10 minutes and transferred to small polythene bags or pots containing of either vermiculite or mixtures of sand and soil (1:1) or vermiculite and soil (1:1). The plantlets were covered with polythene bags so as to attain maximum humidity around the plantlets and were grown in shaded places under natural day lights with an average temperature of 25°C (Fig. 5, 6).

These plantlets were watered every alternate day with tap water and ½ strength MS salt solution. Each treatment was repeated at least once. The bags covering the plantlets were cut open from the top after 2 weeks so as to bring the humidity to the level of ambient atmosphere. Finally the covers were removed after 2 weeks time. After 8 days these platelets were transferred to polythene bags or pots containing garden soil, Sand and farm yard manure (1:1:1). The survival percentage of micropropagated plantlets of *Morus nigra* L was influenced by different planting out mixtures. Maximum survival percentage of rooted plantlets of *Morus nigra* L. in soil was obtained after hardening in sand and vermiculite (1:1) mixture (Table 5). plantlets survived with 80% frequency. These plants showed normal growth and attained an average height of 34 cm after 2 months of transfer and finally showed vigorous growth in the field (Fig. 7).

DISCUSSION

The regeneration potential from the nodal segments of *Morus nigra* L as observed in present work was attention creating. On MS medium as control, explants showed no response for a couple of weeks. MS medium supplemented with BAP (5.0 mg/l), influenced shoot proliferation from the nodal explants of mature trees (Fig. 1). Similar results have been achieved by in case of various mulberry species from nodal segments on MS medium supplemented with different concentrations of BAP and NAA.

These *In vitro* raised shoots when subcultured in reduced concentration of BAP (1.5 mg/l), sprouted and multiple shoot proliferation was observed in 4-5 days of inoculation. The leaves produced by *In vitro* raised shoots were similar to those of mother plant (Fig. 2).

Same results were obtained by Hussain *et al.* [19] from nodal explants of *Morus laevigata*. The production of shoots from nodal segments was also established by Anis *et al.* [23]. But these workers achieved shoot multiplication by culturing explants on MS medium fortified with BAP (2.5 mg/l) along with Glutamin (1 mg/l). This medium facilitated the elongation of shoots and sprouting of nodal segments from *In vitro* grown micro cuttings.

NAA (0.1-0.5 mg/l) was found to be the optimum concentration for the production of roots in the *In vitro* raised shoots (Fig. 3). Same results have been reported by Ohyama and Oka [24], while studying effect of NAA on rooting from *Morus alba*, Anis, *et al.* [23] also achieved 80% rooting in case of shoots cultured on MS medium supplemented with NAA (1.0 mg/l).

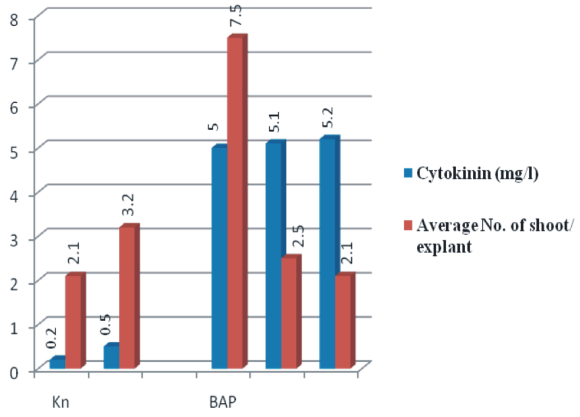

Fig. 1: Effect of cytokinin (mg/l) on shoot regeneration from nodal segments of *Morus nigra* L.

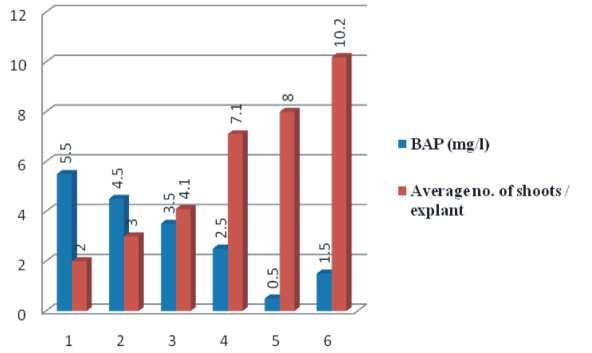

Fig. 2: Effect of BAP (mg/l) on shoot multiplication from *In vitro* raised micro cuttings of *Morus nigra* L.

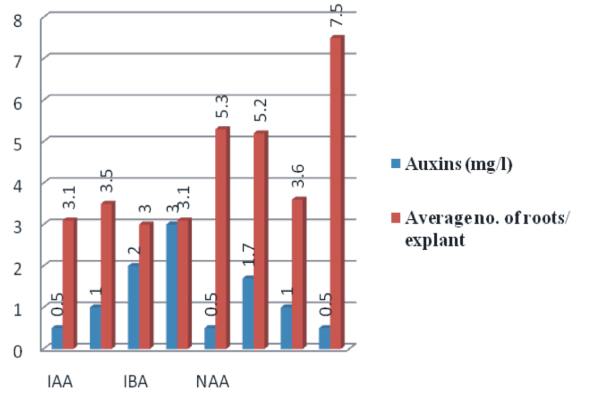

Fig. 3: Effect of auxin (mg/l) on root induction from *in vitro* raised shoots of *Morus nigra* L.

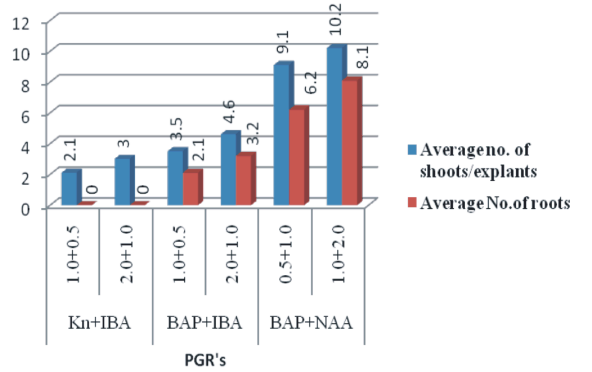

Fig. 4: Effect of PGR's on nodal segments of *Morus nigra* L.

Complete plantlet development was achieved when explants were cultured on MS basal medium fortified with BAP (0.5-1.0mg/l)+NAA (1.0-2.0mg/l) on this medium combination the explants exhibited best morphogenetic potential in 70-80% cultures shoot differentiation started after 6-8 days of inoculation. In the next 4 weeks the micro plantlets in the same medium showed enhanced shoot multiplication along with the production of roots (Fig. 4). Regeneration of whole plantlets from isolated shoots tips of mulberry trees were also achieved Ohyama and Oka [24].

The nodal segments of *M. nigra* exhibited different morphogenetic growth response in MS basal medium supplemented with various growth regulators viz; auxins and cytokinins either separately or in combination. The explants developed into shoots on MS+BAP (5.0 mg/l) and on reduced concentration of BAP (1.5mg/l) multiple shoot production occurred from *In vitro* raised micro cuttings of *M. nigra*. Induction of rooting from *In vitro* raised shoots was achieved on MS medium supplemented with NAA (0.5 mg/l).

The most inductive auxin-cytokinine combination for the production of complete plantlets was BAP (0.5-1.0mg/l)+ NAA (1.0-2.0mg/l) at which explants exhibited best morphogenetic potential in 70-80% cultures.

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