Standardization of Protocol for *In Vitro* Micropropagation of *Morus alba* L., An Important Economical and Medicinal Plant

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Abstract

An efficient regeneration protocol for *Morus alba* L. was developed using nodal explants on MS medium augmented with different concentrations and combinations of plant growth regulators. The highest frequency (80.0%) of bud breaks and shoot induction was observed on medium supplemented with 1.0 mg/l BAP along with 0.5 mg/l NAA with the formation of 5.1 ± 0.1 number of shoots 30 days. MS medium fortified with the same combination of GR (1.0 mg/l BAP and NAA (0.5 mg/L) added with 10% Coconut water showed the highest percentage (80.0%) shoot multiplication, directly from the explants, without any callus formation. Elongated shoots were rooted best on MS medium with 20 mg/l AC producing maximum number of roots with average length 7.0 ± 0.6 cm within 20 days. The plantlets were gradually acclimatized and successfully transferred to field condition with 90% survival rate after rooting. The standardized protocol reported in this study may help in large scale propagation of this plant species which is currently exploited from the nature.

1 Introduction

Phytochemicals extracted from different parts of plant is used for the treatment of various diseases, for supplementing nutrition in foods and cosmetic industries has a great potential in this century. Most valuable phytochemicals are products of secondary metabolism and possess sufficient chemical or structural complexity, so that artificial synthesis is difficult. Plant tissue culture techniques now play an important role in the micropropagation and quantitative improvement of the medicinally important plant. Though the conventional breeding techniques have considerably increased the productivity of modern crops, the application of biotechnology could speed up further crop improvement. It overcomes the barriers in conventional vegetative propagation and fulfils the demand for large scale cultivation in a short period by rapid multiplication¹.

Morus alba is an important economical and medicinal plant, commercially grown for the usage of leaves as a food of insect's silkworm (*Bombyx mori* L.) in the commercial sericulture technologies². The species are also grown as fruit crop in the

countries like Turkey and Greece for the production of mulberries fruits which were used for various local food recipes³. Other than sericulture the plant own as fruit has huge economical and many unique and special importance. Several species like *Morus nigra* (black), *Morus rubra* (red), and *Morus alba* (white mulberries) are widely spread in India, Iran and Pakistan. *Morus alba* commonly called as mulberry belongs to family Moraceae is a medium to small tree mostly found in the tropical to temperate zones and are distributed in every range of environmental conditions⁴. The genus of this plant consist of near about sixteen members, and 150 species out of which *Morus alba* is more dominant^{5, 6}.

Despite its huge demand, perennial nature of the plant coupled with prolonged juvenile period slows down the process of mulberry improvement⁷. Further, mulberry is a cross pollinated crop and hence heterozygosity prevails.

Therefore, propagation through seeds does not conserve stable genetic makeup and does not assure a uniform quality of plants. Propagation through stem cuttings is restricted to only certain

months of the year and very small number of plants is generated due to poor rooting frequencies.

Additionally, the saplings obtained through cuttings show inferior vigor when compared with micro propagated plants⁸. In vitro establishment of nodal segments collected from mature trees has been reported in different species of mulberry⁹⁻¹¹ but to the best of our knowledge, very less significant prior information in the contemporary literature is available on in vitro micropropagation or plant regeneration in this economically important tree species of *M. alba*.

Thus, traditional propagation methods through seeds and vegetative method is not sufficient and reliable hence, plant production through tissue culture method is required. To overcome these problems and to produce large number of plants of same genetic makeup, a simple and efficient micropropagation protocol is presented in this investigation.

2 Materials and Methods

Nodal segments, for axillary and apical meristems of 5-10 centimeter length of *Morus alba* were excised from the mother plants were selected as explants for inoculation.

The explants were washed under running tap water for 30 minutes and then washed thoroughly in sterile double distilled water (DDW). These explants were then kept in 1.0% Bavistin (BVN) (Carbendazim Powder BASF India Limited) a broad spectrum fungicide for 10 minutes followed by 5.0% (v/v) Teepol, a liquid detergent for 5 minutes by continuous shaking method. Inside the laminar flow cabinet, the explants were surface sterilized with mercuric chloride solution at the time duration of 5 minute. The sterilant was then washed off by rinsing with five to six changes of sterile double distilled water.

Sterilized axillary and apical meristem were inoculated on Murashige and Skoog's (MS) media supplemented with different concentrations of 0.5-1.0 mg/l BAP (6-benzylaminopurine) and 0.5-1.0 mg/l KN (kinetin) either alone or with different combinations of 0.5 mg/l NAA (α -naphthalene acetic acid)¹². The cultures were incubated in a culture room at 25 $\pm$ 2°C under 16 hours photoperiod provided by cool white fluorescent tubes.

To standardize the media for shoot multiplication protocol, initiated and regenerated shoots from different explants from nodal segments were separated, isolated and transferred on fresh medium for rapid multiple shoot formation.

Experiments were carried out to check the effect of different concentration of higher cytokinin like 0.5- 2.0 mg/l BAP (6 benzylaminopurine) and 0.5-1.0 mg/l KN (kinetin) either alone or with combination of auxins 0.5 mg/l of NAA (Naphthalene acetic acid) an additive coconut water. Subsequent sub culture on fresh medium maximizes the multiplication of shoot. The growth response of cultures were studied at weekly interval. The parameters were taken as the average number of shoots initiated

and multiplied, and the length of regenerated and multiplied shoots were recorded.

After continuous shoot multiplication the shoots were separated and transferred from multiplication cytokinins containing medium to the root induction medium.

A low salt medium supplemented with auxin was found satisfactory for rooting of shoots in large number of plant species. After multiplication phase the regenerated multiplied shoots were separated and inoculated for root induction, in the medium containing different concentration of auxins like IAA, NAA, IBA and Activated charcoal (AC) the growth parameter was observed as percentage of root initiate, length in centimeters and number of roots formed.

For the hardening of *in vitro* raised plants of the well developed rooted shoots /plantlets were taken out washes thoroughly with running tap water to remove all traces of medium attached to the roots. Finally the plants were planted in pots containing mixture of soil sand and farmyard in the ratio of (1:2:1) for acclimatization and were maintained in green house.

2.1 Data analyses

Complete randomized design was used for all the experiments. The data were analyzed by ANOVA (Duncan's multiple range test) using SPSS release 12.0. The data were transformed where necessary by using various formulae.

3 Results and Discussions

3.1 Axillary shoot initiation

Axillary shoots were observed emerging from nodal explants of mature tree on MS medium supplemented with BAP, KN and NAA (Table 1). Generally, the rate of bud break was increased by increasing the BAP level in combination with either concentration of auxin after different periods of time. The frequency of bud break was maximum in MS medium supplemented with BA (1.0 mg/l) and NAA (0.5 mg/l) after 30 days of culture with little browning of the medium and longest (5.1 $\pm$ 0.1) shoot production. It was observed that shoot growth was not affected by the development of these calluses (Fig 1A & B).

3.2 In vitro shoot multiplication

The axillary shoots were excised and cut into further nodal segments for multiple shoot induction (Table 2). For this purpose, BAP alone or in combination with NAA and Coconut water were tested. Generally, the rate of shoot induction was low on individual cytokinins. Such shoot induction response was significantly improved by adding NAA with either cytokinin. BAP was superior over Kinetin to induce such responses when NAA was used along with Additive CW in MS medium. Multiple shoots produced by BAP were vigorous and green as compared to without CW supplemented media (Fig 1 C & D). The rate of

multiple shoot induction from nodal explants was highest (80%) in MS medium supplemented with BAP (1.0 mg/l) and NAA (0.5mg/l) with 10 %. CW having 10 ± 0.6 mean number of shoot

and 5.2 ± 0.7 cm shoot length after twenty days of culture. Multiple shoots induced on different treatments was also different.

Table 1. Effect of growth regulators on shoot initiation of *Morus alba*

MS+GR (mg/l)	%Bud break	Days of Cultures	%Callus formation	Mean No. of shoots induced $\pm$ SE	Mean shoot length (cm) $\pm$ SE
0.5 BAP	40.0	20	30	2.0 $\pm$ 0.3	3.0 $\pm$ 0.9
1.0 BAP	60.0	10	20	3.0 $\pm$ 0.21	5.0 $\pm$ 0.2
2.0 BAP	30.0	20	40	1.8 $\pm$ 0.8	1.4 $\pm$ 0.1
1.0 BAP + 0.5KN	50.0	20	50	2.0 $\pm$ 0.5	1.5 $\pm$ 0.4
2.0BAP + 0.5 KN	40.0	30	30	2.0 $\pm$ 0.1	1.2 $\pm$ 0.2
0.5 BAP+ 0.5 NAA	70.0	30	30	3.0 $\pm$ 0.4	4.4 $\pm$ 0.5
1.0 BAP+ 0.5 NAA	80.0	30	50	5.1 $\pm$ 0.1	4.2 $\pm$ 0.1
0.5 BAP + 1.0 NAA	60.0	30	40	2.5 $\pm$ 0.8	3.4 $\pm$ 0.1
1.0 BAP + 1.0NAA	70.0	30	20	2.0 $\pm$ 0.8	3.8 $\pm$ 0.2
2.0 BAP + 1.0 NAA	50.0	40	40	3.0 $\pm$ 0.9	3.0 $\pm$ 0.1

Table 2. Effect of growth regulators on shoot multiplication of *Morus alba*

MS+ GR (mg/l)	%Shoot multiplied	No. of culture days	Mean No. of shoots produced $\pm$ SE	Mean shoot length (cm) $\pm$ SE
MS + 1.0 BAP	50.0	20	5.0 $\pm$ 1.2	3.9 $\pm$ 0.02
MS + 2.0 BAP	30.0	20	3.0 $\pm$ 0.52	2.0 $\pm$ 0.3
MS + 1.0 BAP+0.5 NAA	70.0	20	8.0 $\pm$ 0.6	5.2 $\pm$ 0.01
MS + 2.0 BAP+0.5 NAA	60.0	30	3.0 $\pm$ 0.1	3.0 $\pm$ 0.5
MS +1.0 BAP+ 0.5 NAA+10% CW	80.0	20	10 $\pm$ 0.6	5.2 $\pm$ 0.7
MS + 2.0 BAP+0.5 NAA+10% CW	70.0	30	7.0 $\pm$ 0.6	4.4 $\pm$ 0.1
MS + 1.0 KN+0.5 NAA	30.0	20	7.0 $\pm$ 0.1	4.0 $\pm$ 0.8
MS + 1.0 KN+0.5 NAA+10% CW	60.0	20	8.0 $\pm$ 0.7	4.0 $\pm$ 0.2
MS + 2.0 KN+1.0 NAA	50.0	20	4.0 $\pm$ 0.2	4.5 $\pm$ 0.6
MS + 2.0 KN+0.5 NAA+10% CW	60.0	30	4.3 $\pm$ 0.1	3.8 $\pm$ 0.9

3.3 In vitro rhizogenesis

Well-developed in vitro shoots were used for rooting in MS full or half strength basal media or supplemented with 20% activated charcoal (Table 3). Rooting was observed after 20 days of culture and the data for further development of roots were recorded after 25 days. Significant difference was observed for root induction in MS half medium with the supplementation of AC and IBA.

However, rooting was better when activated charcoal was added in half strength MS medium. The rate of root induction was

highest (80.0%) in half-strength MS medium supplemented with 20% activated charcoal after 20 days of culture. The number of primary as well as secondary roots was also highest on this medium with mean root length (Fig 1 E).

3.4 Hardening and acclimatization

Plantlets (four-weekold) were transferred to plastic pots containing a mixture of soil:sand:peat moss (1:1:1) under glasshouse conditions (Fig 1 F). The plantlets were irrigated once a day with one-fourth strength of MS salts without vitamins,

myoinositol and sucrose for one month. The acclimatized plants were then shifted to the field conditions with 70-80% survival rate.

4 Discussion

Clonal propagation via in vitro establishment of nodal explants is quite a reliable method for the maintenance of clonal fidelity, high

proliferation rate and easy manipulation¹³⁻²⁰. During elongation and development of axillary shoots, development was also observed. Similar results have been reported in several other species of *Morus*^{16, 17, 19} reported female inflorescence from nodal explants collected during May from 57-day old pruned shoots of *M. atropurpurea*.

Table 3. Effect of growth regulators on Root induction of *Morus alba*

MS+ GR (mg/l)	%age of Root formation	No. of days	Mean Root length (cm) $\pm$ SE
MS + 20 AC	60.0	20	4.0 $\pm$ 0.4
MS 1/2 +20 AC	80.0	20	7.0 $\pm$ 0.6
MS 1/2 + 0.5 IBA	60.0	20	5.3 $\pm$ 0.8
MS 1/2 + 1.0 IBA	50.0	20	3.5 $\pm$ 0.0
MS 1/2 + 2.0 IBA	70.0	20	6.5 $\pm$ 0.6
MS 1/2 + 0.5 NAA	50.0	20	3.8 $\pm$ 0.2
MS1/2 + 1.0 NAA	40.5	30	2.5 $\pm$ 0.7
MS + 2.0 NAA	60.0	30	5.0 $\pm$ 0.1
MS + 0.5 IAA	30.0	20	4.0 $\pm$ 0.2
MS + 1.0 IAA	30.0	30	2.0 $\pm$ 0.2

Fig. A, B: Shoot initiation in MS + 1.0 mg/l BAP + 0.5 mg/l NAA; C, D: Shoot multiplication in 1.0 mg/l + 0.5 mg/l NAA; E: Root induction MS 1/2+ 20 mg/l AC; F: Hardened plant of *Morus alba*

It has been observed in earlier studies that BA was quite effective for shoot development than other purine based cytokinins^{15, 18, 21, 22}. BA played significant role for in vitro bud break and shoot multiplication with vigorous shoot growth in *Morus* sp²¹. These

results agree with the present investigation. However, 10 or 8.8 μ M BA along with NAA or IBA at 2 μ M each were quite supportive concentrations for bud break and shoot multiplication during the present study (*Morus alba*) similar to *Morus latifolia*¹⁹.

Positive effect of BA for shoot growth has been documented in other tree species of tropical origin^{18,22}. Shirin *et al.*, (2005) reported synergistic effect of BA and NAA for in vitro shoot growth from mature nodal explants of teak. The interaction of BA (22.2µM) and IAA (0.57µM) produced large number of shoots in teak²³. The optimal endogenous and exogenous levels of cytokinins and auxins might control the balance for in vitro development and multiplication of axillary shoots²².

Rooting was good in half strength MS medium supplemented with IBA (4µM) along with activated charcoal (AC) as compared to AC-deficient medium. The use of AC has also been reported in *M. indica* and *M. multicaulis* for promising growth of rooting⁹. Addition of 0.1% AC was effective for root growth of *M. indica* cv. RFS 175 and K2 similar to the present study for root induction in *M. macroura*. Activated charcoal provides dark conditions to the rooting zone by blocking light reaching roots through the medium²⁴. Agarwal & Kanwar (2007) reported 72% rooting response in *M. alba* on MS basal medium supplemented with 0.005g/L AC. From these reports we may conclude that AC at various concentrations significantly affects the growth of in vitro root cultures of *Morus* sp. The potential use of AC has been well documented and reviewed in various plant species²⁵.

5 Conclusion

the present study demonstrates an efficient micropropagation system for axillary bud proliferation from nodal explants of mature mulberry tree for the first time in species of M.P. This is a rapid and reproducible method as compared to traditionally used propagation approaches. Acclimatized plants were morphologically uniform with fairly good growth potential under field conditions.

6 Conflict of interest

None

7 Author's contributions

VD, KWS and RKR performed the experimental work. SK drafted the manuscript, and all author approved the article for publication.

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