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Effect of genotype, explant type and growth regulators on organogenesis in *Morus alba*

B.S. Bhau* & A.K. Wakhlu
*Plant Tissue Culture Laboratory, Department of Botany Jammu University, Jammu 180 006 Jammu & Kashmir, India (*requests for offprints; E-mail: bsbhau@teri.res.in)*

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Abstract

Plantlets of the mulberry (*Morus alba* L. vars. Chinese White, and Kokuso-27) were produced from callus cultures. For callus induction, leaf, internodal segments, and petiole explants of Chinese White, Kokuso-27 and Ichinose varieties were grown on MS basal medium fortified with 2,4-D and 6-benzylaminopurine (BA). Callogenesis was dependent on the nature of explant used, the genotype and growth regulators supplemented in the medium. Leaves were the best explant type used for callus induction. Best callogenesis was obtained on MS medium containing a combination of 1 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BA (95-100%). Calluses formed shoots on MS medium supplemented with 1 mg l^{-1} BA. Supplementation with 0.1 mg l^{-1} 2,3,5-triiodobenzoic acid (TIBA) in this medium enhanced shooting response. Presence of TIBA in the medium also improved the long-term organogenic potential of the callus. Regenerated shoots produced roots on Murashige & Skoog (MS) medium containing either 0.5 mg l^{-1} indole-3-butyric acid (IBA) or α-naphthaleneacetic acid (NAA). Seventy percent of the rooted plants were established in the field where they are performing well.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid; BA – 6-benzylaminopurine; IAA – indole-3-acetic acid; NAA – α-napthaleneacetic acid; IBA – indole-3-butyric acid; MS – Murashige & Skoog; TIBA – 2,3,5-triiodobenzoic acid

Introduction

Mulberry (*Morus*) is an invaluable tree for the sericulture industry, as it is the only source of food for silkworm (Wakhlu and Bhau, 2000). *Morus alba* L. vars. Chinese White, Kokuso-27 and Ichinose are promising genotypes for sericulture industry in temperate regions of the world. In India, these varieties are cultivated for commercial purposes in Jammu and Kashmir, West Bengal, Karnataka and Tamil Nadu (Dandin and Sengupta, 1988; Rajan et al., 1992). Methods of conventional vegetative propagation through grafting is not economically viable for these varieties because it involves skilled manpower, expensive nursery facilities and a minimum time period of 4–5 years to obtain plants ready for harvest (Bhau, 1999). Propagation of plants through cuttings is also not viable for these varieties due to their poor rooting ability. An attempt to induce rooting in stem cuttings of these varieties by auxin application has not yielded encouraging results (Fotadar et al., 1990). Tissue culture propagation can be a viable alternative for rapid multiplication of the mulberries. The successful regeneration of plants *in vitro* has been achieved in several mulberry species by axillary shoot proliferation and organogenesis from callus cultures (see review Wakhlu and Bhau, 2000). These studies have revealed that regeneration ability in *Morus* spp. is greatly dependent on the growth regulator combinations, explant type and the mulberry genotype (Jain and Datta, 1992; Kathiravan et al., 1995; Sahoo et al., 1997; Vijayan et. al., 1998). These factors have been investigated separately without examining their interaction and the regeneration potential of these protocols was very low and inconsistent (Jain and

Datta, 1992; Kathiravan et al., 1995; Sahoo et al., 1997; Vijayan et al., 1998). Furthermore, a major problem with these studies was that immature tissues have been employed as explants during the standardisation of regeneration systems. This paper reports the studies on the role and interaction of genotype, explant type obtained from mature plants and growth regulators in callus mediated organogenesis of mulberry and evaluates the long-term regeneration potential of the callus.

Materials and methods

The plant material of *Morus alba* L. (var. Chinese White, Kokuso-27 and Ichinose) was collected from 10-year-old trees, maintained in the Mulberry Germplasm of Sericulture Division, Regional Horticulture Research Station, Sher-e-Kashmir University of Agriculture Science and Technology, Jammu, India, and Central Sericulture Research and Training Institute, Jammu, India. For each variety, explants were collected from a single tree between February to October 1993 to 1997.

Fully expanded leaves (1.5–2.5 cm), petioles (1 cm) and internodal segments (1 cm) were excised from actively growing branches. They were washed under running tap water, followed by immersion in 0.5% (w/v) Bavistin solution for 30 min, and rinsed 3–4 times with sterile distilled water. Explants were surface-sterilised with 70% (v/v) ethanol for 30 sec, 15 min in sodium hypochlorite (4.6% available chlorine) solution, containing 2–3 drops of Tween-80 and were rinsed 4–5 times with sterilised distilled water.

The MS basal media (Murashige and Skoog, 1962) was supplemented with different concentrations and combinations of growth regulators, 3% (w/v) sucrose and 0.8% (w/v) agar (Ranbaxy India Ltd). The pH of the media was adjusted to 5.8 before autoclaving at 121 °C for 15 min. All cultures were incubated at a temperature of 28±2 °C under a 16-h photoperiod with a light intensity of 30μmol m^{-2}s^{-1} provided by cool white fluorescent tubes (40 W, Bajaj India Ltd).

After 8 weeks of culture, calluses were transferred onto the regeneration media with or without growth regulators. The effect of different concentrations of cytokinins (0.1–5 mg l^{-1} BA and kinetin) and auxins (0.1–2.0 mg l^{-1} NAA, IBA and IAA) were tested on shoot morphogenesis. The effect of TIBA (0.1–2.0 mg l^{-1}) on plant regeneration and long term culture was studied. Data were taken as the percentage of calluses forming shoots and the number of shoots formed per callus.

Nodal explants (1–1.5 cm) from *in vitro* formed shoots were excised with an axillary bud and cultured for rooting. Different auxins (IAA, IBA, NAA) were used for rooting. The rooted shoots were transferred to plastic cups containing a mixture of vermiculite and sand (1:1). The plantlets were covered with polythene bag in order to maintain high humidity and were placed in a shaded place. Plantlets were watered every 2 days with Knop's solution for a period of 4 weeks, and then transplanted to polythene bags containing garden soil, sand and farmyard manure (1:1:1) and kept in the shade for 2 weeks. The hardened plants were transferred to the field in July 1996 in the experimental beds of Department of Botany, Jammu University and in the fields of Central Sericulture Research and Training Institute, Jammu, India.

All treatments for each variety, explant type and growth regulator type consisted of 20 calluses. Each experiment was repeated at least 3 times. The results were recorded at a regular interval of 4 week of culture and analysed by analysis of variance using randomised block design method. Data taken in percentage was subjected to arcsin transformation for proportions before analysis and converted back to percentages for presentation in tables (Snedecor and Cochran, 1968). Means were compared using Duncan's new multiple range test (Duncan, 1955).

Results and discussion

Callus initiation occurred from the cut surface of the explant within one week on auxin supplemented media. 2,4-D was the most effective auxin for callus induction among the 4 auxins tested. Addition of BA (0.5 mg l^{-1}) in the medium containing 2,4-D enhanced the callus induction response. The maximum explants forming callus on this medium was 100% in Chinese White and Kokuso-27 and 95% in Ichinose (Table 1). High concentrations of BA ($\geq$ 1 mg l^{-1}) inhibited callus formation from different explants (data not shown). Pre-soaking of internodal explants in cytokinin has been demonstrated to be essential for callus initiation in *Morus bombycis* (Jain and Datta, 1992). In contrast, our study indicates that the pretreatment of explants with BA is not a prerequisite for callus initiation as earlier reported in *M. alba* (Kathiravan et al., 1995). Jain and Datta (1992) used explants from *in vitro* raised shoots that were produced

on an auxin-rich medium. It is possible that auxin level has increased in the explants during long term culture on an auxin-rich medium and pre-treatment of these explants with cytokinin compensates differences in endogenous cytokinin/auxin ratio. Leaf explants showed best callus forming ability, whereas petiole explants showed least callus initiation in the varieties investigated (data not presented). Variation in callus forming ability of different explant types has been reported in *M. alba* (Kathiravan et al., 1995). Significant genotypic differences in callus initiation response were observed among the three varieties investigated. Maximum callus initiation response was recorded from the explants of Kokuso-27, followed by Chinese White and Ichinose. Genotypic differences with respect to callus initiation have been previously observed in *Eucalyptus grandis* × *E. urophylla* (Barrueto Cid et al., 1999).

Callus was excised from the original explants, cut into pieces of roughly 1 cm^3 and subcultured on the fresh medium (MS + 1 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA). The callus turned green after 3 subcultures on optimal callus induction medium. Shoot buds appeared on the surface of the calluses obtained from leaf upon their transfer to MS media supplemented with growth regulators after 2 weeks of culture, whereas calluses obtained from internodal segment and petiole explants failed to differentiate shoot buds. A similar effect of explant type on shoot forming capacity of callus cultures has been reported for *M. alba* (Kathiravan et al., 1997). In contrast, calluses derived from internodal segments of *M. indica* was best for organogenesis (Sahoo et al., 1997). The shoot buds developed into 2–3 cm long shoots after 4 weeks of culture. Cytokinin type and concentration had a significant effect on shoot differentiation. BA was more effective than kinetin for shoot induction. Best shooting response in terms of percent of calluses forming shoots (80%) and number of shoots formed per callus (8.6) was obtained on a medium supplemented with 1 mg l^{-1} BA (Table 2). The superiority of BA has also been previously reported in *M. alba* (Kathiravan et al., 1995, 1997; Narayan et al., 1989), *M. indica* (Sahoo et al., 1997) and *M. bombycis* (Jain and Datta, 1992). High concentrations of BA decreased the shoot formation response. Addition of auxins inhibited the morphogenic response and increased callus proliferation (data not presented). The shoot forming ability of callus cultures of *M. alba* has also been reported to decrease with high concentrations of BA (1.5 mg l^{-1}) in the medium (Narayan et al., 1989; Kathiravan et al., 1995, 1997).

Table 1. Effect of growth regulators on callus induction from mature leaf explants obtained from three varieties of *Morus alba* after 4 weeks of culture

Growth regulators (mg l^{-1})	Ichinose	Kokuso-27	Chinese White
	% explants forming callus*	% explants forming callus*	% explants forming callus*
2,4-D			
0	0^{a}	0^{a}	0^{a}
0.1	16^{b}	10^{bc}	24^{c}
0.25	56^{f}	45^{f}	44^{ef}
0.5	84^{h}	50^{fg}	60^{h}
1.0	88^{h}	95^{i}	100^{i}
IBA			
0.1	12^{b}	0^{a}	16^{b}
0.25	28^{c}	20^{de}	36^{de}
0.5	56^{f}	45^{f}	40^{ef}
1.0	68^{g}	65^{h}	40^{ef}
IAA			
0.1	0^{a}	0^{a}	20^{bc}
0.25	28^{c}	10^{bc}	28^{cd}
0.5	32^{c}	20^{de}	44^{ef}
1.0	44^{de}	55^{g}	48^{fg}
NAA			
0.1	28^{c}	0^{a}	28^{cd}
0.25	36^{c}	0^{a}	36^{de}
0.5	48^{e}	15^{cd}	57^{gh}
1.0	60^{fg}	25^{e}	64^{h}
2,4-D + BA			
1.0 + 0.1	88^{h}	80^{h}	100^{i}
1.0 + 0.25	90^{hi}	95^{i}	100^{i}
1.0 + 0.5	95^{i}	100^{i}	100^{i}
1.0 + 1.0	85^{h}	60^{fg}	85^{h}

*Values within a column followed by the same letter are not significantly different from each other at a 5% level by Duncan's new multiple range test.

The effect of genotype was significant on shoot formation from callus cultures among the varieties. Shoots were formed from calluses of Kokuso-27 and Chinese White, whereas no shoot formation was observed from calluses of Ichinose. These differences in shoot regeneration from calluses in different mulberries seem to be genotypic as has also been earlier reported in *Liquidambar styraciflua* (Brand and Lineberger, 1992).

Table 2. Effect of cytokinins on shoot formation from calluses derived from leaf explant in *Morus alba* var. Kokuso-27 after 4 weeks of culture

Cytokinin	Concentration mg l^{-1}	Calluses forming shoots (Mean*)	Number of shoots per callus (Mean*)
BA	0.0	0^a	0^a
	0.5	20^b	1.4^b
	1.0	80^d	8.6^d
	1.5	50^c	3.8^c
	2.0	60^c	1.8^b
Kinetin	0.5	0^a	0^a
	1.0	0^a	0^a
	1.5	15^b	1.2^b
	2.0	15^b	1.0^b

*Values within a column followed by the same letter are not significantly different from each other at a 5% level by Duncan's new multiple range test.

Table 3. Organogenic response in *Morus alba* varieties on MS medium containing 1 mg l^{-1} BA and 0.1 mg l^{-1} TIBA after 4 weeks of culture

Variety	% calluses forming shoots (Mean)*	Number of shoots per callus (Mean)*
Chinese White	10^b	1.6^b
Kokuso-27	90^c	11.4^c
Ichinose	0^a	0^a

*Values within a column followed by the same letter are not significantly different from each other at a 5% level by Duncan's new multiple range test.

TIBA had a significant influence on shoot formation (Table 3). Incorporation of TIBA in media significantly enhanced the shoot formation response. Best results in terms of percent of calluses forming shoots (90%) and the number of shoots formed per callus (11.4) in Kokuso-27 was achieved on a medium supplemented with 0.1 mg l^{-1} TIBA. This observation is consistent with earlier report of Tetu et al., (1987) who observed improvement in morphogeneic response of callus cultures of *Beta vulgaris* in the presence of TIBA. It appears that TIBA inhibits transport of endogenous auxins in the callus cells and may confined the accumulation of auxin. It is also possible that high endogenous auxin level might be already present in the explant and TIBA counteracts the inhibitive effect of auxin on shoot regeneration as was earlier reported for *Beta vulgaris* (Detrez et al., 1989). Higher levels of TIBA ($\geq$ 0.25 mg l^{-1}) decreased shoot formation response. The addition of TIBA maintained the morphogenic response up to 10 subcultures, while media lacking TIBA lost morphogenic response after the 3rd subculture.

Table 4. Effect of auxins on root formation by shoots of *Morus alba* var. Kokuso-27 after 4 weeks of culture

Auxin	Concentration mg l^{-1}	% explants forming roots (Mean)*	Root length (cm) (Mean)*
None	0.0	0^a	0^a
IAA	0.5	20^b	1.3^a
	1.0	40^c	1.2^a
	2.0	60^d	1.3^a
IBA	0.5	80^{ef}	1.5^a
	1.0	70^{de}	1.2^a
	2.0	70^{de}	1.0^a
NAA	0.5	80^{ef}	1.3^a
	1.0	70^{de}	1.6^a
	2.0	70^{de}	1.2^a

*Values within a column followed by the same letter are not significantly different from each other at a 5% level by Duncan's new multiple range test.

Nodal explants (1–1.5 cm) from regenerated shoots were excised and transferred to MS medium enriched with different auxins (IAA, IBA, and NAA). IBA was most effective auxin (0.5 mg l^{-1}) in terms of percent of shoots forming roots (80%) and root length (1.5 cm) (Table 4). IBA has also been reported to be favourable auxin for root formation in *M. alba* (Kathiravan et al., 1997) and *M. bombycis* (Jain and Datta, 1992). Shoots were not able to develop roots on a medium that lacked auxins. Higher level of auxins encouraged callus formation from cut ends of the explants. Regenerated plants were hardened with a 65% (230 out of 355 plants) success rate. On their transfer to the potting mixture (sand and vermiculite 1:1) 70% (161 out of 230 plants) plants survived. Plants transferred directly to the soil were not able to survive.

Conclusions

A simple, reproducible and efficient *in vitro* protocol for regenerating plants of *M. alba* var. Kokuso-27 and Chinese White has been developed from leaf explants collected from mature trees. This procedure has four culture stages consisting of callus induction with an auxin, proliferation in presence of cytokinin, regeneration in presence of anti-auxin (TIBA) and finally a rooting stage with an auxin. Experiments are in

progress to evaluate the clonal fidelity and field performance of the regenerated plants in comparison to the mother plant.

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