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EFFICIENT MICROPROPAGATION AND ROOTING OF KING WHITE MULBERRY (*MORUS MACROURA* Miq.) VAR. LAEVIGATA FROM NODAL EXPLANTS OF MATURE TREE

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Abstract

In the present study, nodal explants from mature trees were used for axillary shoot proliferation and rooting of *Morus macroura*. Nodal explants (1.5cm long) were cultured in MS medium supplemented with different levels of N^6-benzyladenine (BA: 2, 4, 8, 10 or 12µM) and 1-nephthaleneacetic acid (NAA: 2 or 3µM for each). The rate of bud break was 100% at 10µM BA in combination with either concentration of NAA (2 or 3µM) after 6 days of culture. Generally, the length of axillary shoots was significantly improved by increasing the level of BA with auxin. Most of the nodal explants (60.2%) developed surface calluses on the aerial portions of explants at comparatively lower BA levels (2-4µM). The elongating axillary shoots were cut to prepare further nodal explants for multiple shoot induction. MS medium supplemented with BA (8.8µM) and 2µM indole-3-butyric acid (IBA) was quite effective for 65% shoot induction with 4.7 mean number of shoots and 8.4mm shoot length, after a week. Rooting was highest (85.6%) in half strength MS medium supplemented with 4 µM IBA and activated charcoal (0.1%) after 25 days. The number of primary (2.8) as well as secondary roots (10) was also highest on this medium with a mean root length of 7cm. The rooted shoots were acclimatized and transferred to field conditions. We concluded that nodal explants from mature trees can be manipulated in vitro for clonal multiplication of king white mulberry.

Introduction

Morus macroura, commonly known as 'king white' is a medium-sized spreading tree with weeping habit. It is an important tree species in the Sericulture industry. The foliage of king white is used as a food of silkworms. It produces long catkins which when fully ripened become honey-sweet eaten as fresh but can also be sun-dried and eaten as a sweet. Mulberry wood is used in handicrafts, cabinet work and a major source for making field-hockey-sticks and tennis rackets (Sánchez, 2002). It is propagated through conventional methods such as grafting, air layering, cuttings and by seed sowing (Lu *et al*., 2002). Vegetative propagation of this plant is unsuccessful due to long time taken for adventitious shoot development and low rooting potential that might be due to several factors including physiological and environmental ones (Narayan *et al*., 1989).

In vitro clonal propagation through axillary bud proliferation is a reliable approach for the multiplication of trees mostly within a reasonable time frame irrespective of seasonal considerations (Shirin *et al*., 2005). This is an alternative method for clonal propagation of recalcitrant tree species that do not respond well by sexual or other vegetative means (Sharma *et al*., 2000). In vitro establishment of nodal segments collected from mature trees has been reported in different species of mulberry (Tewari *et al*., 1999; Thomas, 2002; Anis *et al*., 2003) but to the best of our knowledge, no significant prior information in the contemporary literature is available on in vitro micropropagation or plant regeneration in this economically important tree species of *M. macroura*. The present study was therefore conducted with an objective to establish an efficient micropropagation protocol for *M. macroura*.

Materials and methods

Plant material and culture conditions: One-month-old green shoots from eight-year-old king white mulberry tree were collected during April and surface sterilized. Nodal explants (2-3cm long) were prepared and thoroughly rinsed with running tap water for 10 minutes, 75% ethanol for 30 seconds and 15% commercial bleach (v/v) for 15 minutes and then rinsed with sterile distilled water 3 to 5 times. Dead tissues after surface-sterilization on both ends of the nodal explants were trimmed and 2cm long nodal segments were inoculated on the culture medium for bud break. MS (Murashige & Skoog, 1962) medium supplemented with BA (2, 4, 8, 10 or 12µM) in combination with NAA (2 or 3µM for each) was used for axillary shoot bud initiation. The data for bud break, shoot length, callus formation at the base as well as for surface calluses, medium browning and number of days to bud break were recorded. After 14 days, the elongating axillary shoots were further excised and nodal explants (0.5-1cm) were cultured on MS medium supplemented with BA or Kinetin (0.25, 0.5, 1, 2.2, 4.4 or 8.8µM) alone or in combination with IBA (1, 1.5 or 2µM for each) for multiple shoot induction. The data for shoot induction (%), number as well as length (mm) of shoots were recorded after seven days of culture incubation.

In vitro shoots (2-4cm long) were rooted on 6 combinations of MS full or half strength media with or without IBA (2 or 4µM) and/or 0.1% activated charcoal (MS basal, MS basal half-strength, MS half + 2 µM IBA, MS + 2 µM IBA, MS + 2 µM IBA + AC, MS half + 4 µM IBA + AC). The data for root induction (%), number of primary/secondary roots and mean root length (cm) were recorded after 25 days of culture on rooting medium. The rooted shoots were then transferred to pots (12 x 10 inch) containing soil: sand: peat moss (1:1:1v/v) for acclimatization under glasshouse conditions. During hardening process, the plants were watered on regular basis.

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.

Results

Axillary shoot initiation: Axillary shoots as well as inflorescence were observed emerging from nodal explants of mature tree on MS medium supplemented with BA and NAA (Table 1). Generally, the rate of bud break was increased by increasing the BA level in combination with either concentration of auxin after different periods of time. The frequency of bud break was 100% in MS medium supplemented with BA (10µM) and NAA (2 or 3µM) after 6 days of culture with little browning of the medium (Fig. 1a) and longest (16.3mm) shoot production. The established nodal explants showed various morphological characteristics, i.e., callus was observed from the base (20-65%) as well as from aerial portions of the explants referred to as surface callus (0-60.2%). The rate of callus formation at the basal cut ends of the explants was generally increased by increasing BA level whereas the development of surface calluses was reduced. Generally, shoot growth was not affected by the development of these calluses.

Table 1. Effect of BA and NAA on axillary bud break, shoot growth, medium browning and basal and surface callus formation from nodal explants of king white mulberry after different culture periods

Treatments (µM)		[2]Bud break (%)	Days to bud break	[3]Shoot length (mm)	Medium browning	[2]Basal cut end callus (%)	[2]Surface callus (%)[4]
BAP	NAA						
2	2	80.2 ± 5.24^{c}[1]	10 -12	5.2 ± 0.81^{def}	+	20.0 ± 2.14^{ef}	50.5 ± 3.33^{b}
2	3	85.5 ± 2.31^{c}	09 -11	8.1± 0.78^{cd}	+	36.6 ± 5.24^{de}	55.2 ± 3.33^{ab}
4	2	88.8 ± 5.24^{bc}	08 -10	10.0 ± 0.65^{bc}	+	25.5 ± 5.24^{ef}	60.2 ± 4.45^{a}
4	3	95.5 ± 4.24^{ab}	07 -10	14.3 ± 0.65^{ab}	++	36.6 ± 3.33^{de}	45.5 ± 4.55^{bc}
8	2	95.5 ± 5.24^{ab}	08 -10	15.2 ± 0.45^{a}	++	50.0 ± 5.22^{cd}	35.5 ± 3.33^{cd}
8	3	98.8 ± 3.25^{ab}	08-10	14.1 ± 0.45^{ab}	++	50.0 ± 2.22^{cd}	35.5 ± 4.44^{cd}
10	2	100 ± 1.25^{a}	06 -10	15.2 ± 0.81^{a}	+	60.6 ± 4.44^{ab}	25.1 ± 2.22^{ef}
10	3	100 ± 1.25^{a}	06 -11	16.3 ± 0.25^{a}	+	60.6 ± 3.11^{ab}	20.1 ± 5.24^{ef}
12	2	70.7 ± 2.25^{cd}	15 -17	5.1 ± 0.14d^{ef}	++	65.6 ± 3.11^{a}	0.0
12	3	75.7 ± 2.25^{cd}	15 -18	8.1 ± 0.14^{cd}	+++	65.4 ± 5.24^{a}	0.0

Each value is a mean of three replicates (10 explants per replicate) of three independent experiments.
[1]Mean values (±SE) followed by the same letters are not significantly different according to Duncan's multiple range test (DMRT) at $p<0.05$.
[2]The data were transformed using $2\sqrt{y}$ (y is the percent value for each treatment). Non-transformed values are presented.
[3]The data were transformed using $\sqrt{x+1}$ (x is the number and length of shoots). Non-transformed values are presented.
[4] Surface callus: white and fluffy callus on the outer surfaces of nodal explants above the culture medium.
Medium browning: '+'little browning, '++' moderate browning, '+++' high browning.

***In vitro* shoot multiplication:** The axillary shoots obtained on MS medium supplemented with 10µM BA and 2 or 3µM NAA were excised after 14 days and cut into further nodal segments for multiple shoot induction (Table 2). For this purpose, BA or Kinetin alone or in combination with IBA were tested. Generally, the rate of shoot induction was low on individual cytokinins. Such shoot induction response was significantly improved by adding IBA with either cytokinin. BA was superior over Kinetin to induce such responses when IBA was used along with cytokinins in MS medium. Multiple shoots produced by BA were vigorous and green as compared to Kinetin-supplemented media (Fig. 1b). The rate of multiple shoot induction from nodal explants was highest (65%) in MS medium supplemented with BA (8.8µM) and IBA (2µM) with 4.7 mean number of shoot and 8.4mm shoot length after seven days of culture. Multiple shoots induced on different treatments was also different. However, the lower levels (0.25 or 0.5µM) of individual cytokinins did not favor shoot induction under the same experimental conditions.

***In vitro* rhizogenesis:** Well-developed in vitro shoots were used for rooting in MS full or half strength basal media or supplemented with IBA (2 or 4µM) along with 0.1% activated charcoal (Table 3). Rooting was observed after 15 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 medium alone or supplemented with 2 or 4µM IBA. However, rooting was better when activated charcoal was added in full or half strength MS medium supplemented with IBA. The rate of root induction was highest (85.6%) in half-strength MS medium supplemented with IBA (4µM) and 0.1% activated charcoal after 25 days of culture (Fig. 1c). The number of primary (2.8) as well as secondary roots (10) was also highest on this medium with mean root length of 7cm. The development of secondary roots was significantly improved by the use of IBA. Secondary roots generally developed one centimeter further down the root-shoot transition zone.

Hardening and acclimatization: Plantlets (four-week-old) were transferred to plastic pots containing a mixture of soil:sand:peat moss (1:1:2) under glasshouse conditions (Fig. 1d). The plantlets were irrigated once a day with one-fourth strength of MS salts without vitamins, myoinositol and sucrose for 1 month. The acclimatized plants were then shifted to the field conditions with 65% survival rate.

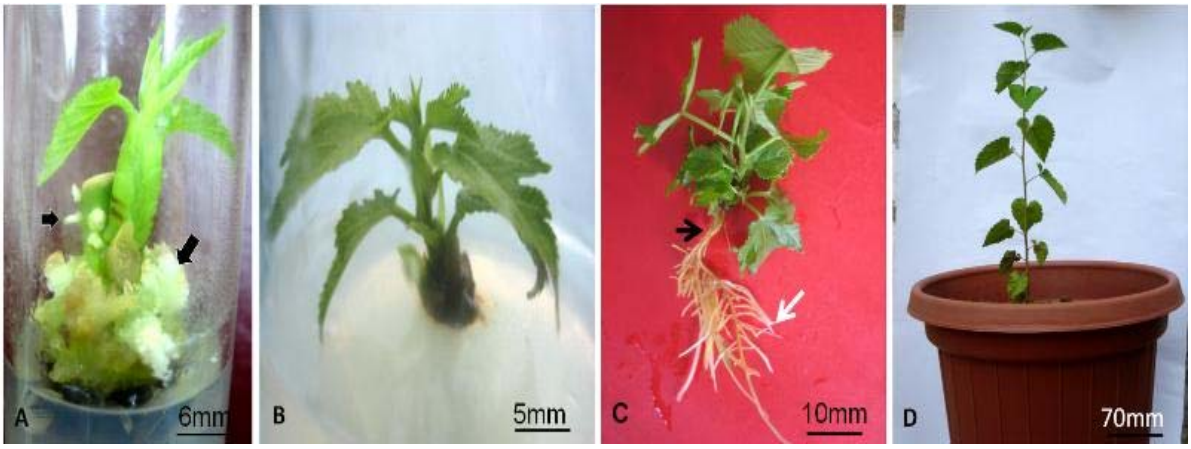

Fig. 1. Micropropagation via axillary shoot bud induction and acclimatization of king white mulberry. a) Axillary bud induction and development of surface callus (arrows) on the explants in MS medium supplemented with BA (10 µM) and NAA (3 µM), after 7 days of initial culture. b) Shoot proliferation in MS medium supplemented with BA (8.8 µM) and IBA (2 µM) after 14 days of culture incubation. c) A twenty-five-day-old rooted shoot with well developed primary (black arrow) and secondary roots (white arrow) in half strength MS medium + 4 µM IBA and 0.1 % activated charcoal. d) Further hardening and acclimatization of rooted shoot in pot under glasshouse conditions (30 day-old).

Table 2. Effect of BA or Kinetin separately or in combination with IBA for multiple axillary shoot induction from in vitro-derived nodal explants of king white mulberry shoots.

Treatments (µM)			Shoot induction (%)[2]	Number of shoots[3]	[3]Shoot length (mm)
BAP	Kinetin	IBA			
0.25			0.0	0.0	0.0
0.5			0.0	0.0	0.0
1			15.2±4.2^{def}	0.2±0.12^{ef}	5.1±1.2^{cd}
2.2			15.1±2.2^{def}	0.4±0.09^{def}	3.4±1.0^{de}
4.4			16.4±4.2^{def}	0.8±0.07^{de}	5.1±1.1^{cd}
8.8			20.3±2.2^{def}	1.0±0.70^{de}	7.3±0.8^{bc}
	0.25		0.0	0.0	0.0
	0.5		0.0	0.0	0.0
	1		10.4±5.5^{efg}	0.4±0.02^{def}	6.3±1.1^{bcd}
	2.2		12.5±5.5^{def}	0.6±0.01^{def}	4.3±2.2^{cde}
	4.4		15.2±2.2^{def}	0.4±0.01^{def}	5.4±1.5^{cd}
	8.8		20.2±4.5^{def}	0.9±0.05^{de}	5.1±1.5^{cd}
2.2		1	50.3±5.5^{c}	2.0±0.84^{abc}	9.2±1.5^{ab}
4.4		1.5	60.0±6.6^{ab}	3.5±0.57^{ab}	10.0±3.3^{a}
8.8		2	65.0±6.6^{a}	4.7±0.84^{a}	8.4±3.3^{abc}
	2.2	1	55.4±2.2^{c}	2.1±0.47^{abc}	7.2±2.2^{bc}
	4.4	1.5	45.5±3.3^{cd}	2.2±0.65^{abc}	5.4±1.0^{cd}
	8.8	2	42.4±5.5^{cd}	2.2±0.45^{abc}	8.7±1.0^{abc}

The data were recorded after 7 days of initial culture.
Each value was a mean of three replicates (10 explants per replicate) of three independent experiments.
[1]Mean values (±SE) followed by the same letters are not significantly different according to Duncan's multiple range test (DMRT) at $p<0.05$.
[2]The data were transformed using $2\sqrt{y}$ (y is the percent value for each treatment). Non-transformed values are presented.
[3]The data were transformed using $\sqrt{x+1}$ (x is the number and length of shoots). Non-transformed values are presented.

Table 3. Effect of various MS media formulations on rooting of in vitro shoots of king white mulberry.

Treatments (µM)	Root induction (%)[2]	Number of primary roots[3]	Number of secondary roots[3]	Mean root length (cm)[3]
MS basal	10.5±2.2^{d}	0.8±0.9^{bc}	5.2±1.2^{c}	3.1±1.2^{c}
MS basal half strength	10.4±3.2^{d}	1.0±0.4^{bc}	8.1±0.8^{b}	2.1±0.8^{d}
MS half+IBA (2 µM)	50.2±2.5^{bc}	1.5±0.6^{ab}	7.3±0.4^{bc}	5.4±0.7^{bc}
MS+IBA (4µM)	50.5±3.2^{bc}	1.8±0.2^{ab}	8.2±0.5^{b}	6.6±0.5^{b}
MS+IBA (2µM) + AC	80.8±4.5^{ab}	2.2±0.7^{a}	9.2±1.4^{ab}	6.6±0.6^{b}
MS half +IBA (4µM) + AC	85.6±4.5^{a}	2.8±0.7^{a}	10.0±2.1^{a}	7.0±0.4^{a}

AC: Activated charcoal
IBA: Indol-3-butyric acid
The data were recorded after 25 days of initial culture.
Each value was a mean of 15 explants from three independent experiments.
[1]Mean values (±SE) followed by the same letters are not significantly different according to Duncan's multiple range test (DMRT) at $p<0.05$.
[2]The data were transformed using $2\sqrt{y}$ (y is the percent value for each treatment). Non-transformed values are presented.
[3]The data were transformed using $\sqrt{x+1}$. Non-transformed values are presented.

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 manipulation (Hossain *et al*., 1992; Katase, 1993; Pattnaik *et al*., 1996; Pattnaik & Cland, 1997; Chitra & Padmaja, (1999); Lu, 2002; Akram & Aftab, 2009; Memon *et al*., 2010). During the present investigation, nodal explants were collected during April from one-month-old shoots of eight-year-old tree. During elongation and development of axillary shoots, axillary inflorescence development was also observed. Similar results have been reported in several other species of *Morus* (Lin *et al*., 1996; Pattnaik *et al*., 1996; Chitra & Padmaja, (1999); Lu, 2002). Lin *et al*., (1996) reported female inflorescence from nodal explants collected during May from 57-day-old pruned shoots of *M. atropurpurea*. The development of inflorescence has also been reported from the explants collected during November through February in *M. australis* with high frequency bud break and maximum number of shoots (Pattnaik *et al*., 1996). These reports indicate that in vitro development of inflorescence from mature axillary buds appear to be a common reproductive feature of *Morus* species by exogenous application of growth factors.

It has been observed in earlier studies that BA was quite effective for shoot development than other purine-based cytokinins (Yadav *et al*., 1990; Pattnaik & Cland, 1997; Shirin *et al*., 2005; Akram & Aftab, 2009). BA played significant role for in vitro bud break and shoot multiplication with vigorous shoot growth in *Morus* sp. (Yadav *et al*., 1990). These results agree with the present investigation. The concentration of BA also affected shoot growth. BA higher than 4.4µM suppressed shoot growth in different *Morus* species (Hossain *et al*.,1992; Pattnaik & Cland, 1997). However, 10 or 8.8µM BA along with NAA or IBA at 2µM each were quite supportive concentrations for bud break and shoot multiplication during the present study (*Morus macroura*) similar to *Morus latifolia* (Lu, 2002). Positive effect of BA for shoot growth has been documented in other tree species of tropical origin (Shirin *et al*., 2005; Akram & Aftab, 2009). 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 teak (Tiwari *et al*., 2002). Akram & Aftab (2009) also reported the interactive effect of BA (8.8µM) and IBA (2µM) for multiple shoot induction from nodal explants of teak. The optimal endogenous and exogenous levels of cytokinins and auxins might control the balance for in vitro development and multiplication of axillary shoots (Shirin *et al*., 2005).

The development of callus is a common phenomenon during shoot induction in *Morus* species (Sharma & Thorpe, 1990; Pattnaik *et al*., 1996). Callus growth at the shoot base makes rooting difficult by interfering with the connection between shoot and root growth (Williams & Taji, 1989; Jain *et al*., 1990; Quraishi *et al*., 1996). Auxin concentrations (Kim *et al*., 1985) and nodal explants maturity (Sharma & Thorpe, 1990) were the determining factors for callus development at the cut basal ends in different *Morus* species. Kim *et al*., (1985) reported considerable amount of callus on NAA (0.5-2mg/l) containing medium in *M. alba*. In the present study, the frequency of callus formation was low when plant growth regulators were individually used in the medium (data not presented). More calluses from the base as well as from aerial portions of nodal explants were observed on higher levels of BA and IBA thus supporting Lu, (2002) who suggested cooperative role of auxin and cytokinins in callus formation of *M. latifolia* (Lu, 2002).

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 rooting (Tewari *et al*., 1999). 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 medium (George, 1993). 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 species (Thomas, 2008).

In conclusion, the present study demonstrates an efficient micropropagation system for axillary bud proliferation from nodal explants of mature king white mulberry tree for the first time. 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.

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