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Development of micropropagation protocol for *Morus nigra* L. (black mulberry) through axillary buds

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Abstract
A micropropagation protocol for *Morus nigra* L. (black mulberry) was developed using axillary buds as explants. Axenic cultures were established using sequential application of various antimicrobial agents *viz.* carbendazim-50%, cefotaxime, kanamycin, streptocycline and mercuric chloride ($HgCl_2$). Among all the treatments tested, highest shoot induction response was recorded on Murashige and Skoog (MS) medium supplemented with 2 mgl^{-1} 6-benzylaminopurine (BAP) and 0.2 mgl^{-1} 1-naphthylacetic acid (NAA). During shoot multiplication stage, shoots were best multiplied on MS medium supplemented with 0.2 mgl^{-1} BAP which produced maximum number of shoots (6.75 ± 0.48) and leaves (29.3 ± 0.85). *In vitro* developed shoots were successfully rooted on MS medium supplemented with 0.5 mgl^{-1} Indole-3-butyric acid (IBA) which produced highest number (16.25 ± 0.63) and length (7.30 ± 0.16 cm) of roots. The plantlets with well formed root systems were gradually acclimatized in greenhouse using soil:cocopeat (1:1) and later shifted to polyhouse.

Keywords: Axillary bud, Black mulberry, Micropropagation.

1. Introduction
The origin of mulberry is believed to be in China, Japan and in the Himalayan foothills (Sanchez, 2000). Mulberry belongs to the genus *'Morus'* and family *'Moraceae'* (Zaki *et al*., 2011) [20]. Among the various species belongs to this genus, *Morus alba* L. (white mulberry) and *Morus nigra* L. (black mulberry) are of great importance. *Morus alba* L. (white mulberry) is an invaluable species of immense economic importance in silk industry for its foliage, which constitutes the chief food for the silkworm, *Bombyx mori* L (Kavyashree, 2007) [10] whereas, *Morus nigra* L. (black mulberry) is cultivated for its tasteful fruits and potential use in pharmaceutical as well as cosmetic industries (Chiancone *et al*., 2007) [4]. The fruits of black mulberry has a tonic effect on kidney energy and thus, it is used as an antiphlogistic, a diuretic and an expectorant (Koyuncu, 2004) [13]. Practically, mulberry can grow on any type of land except on very steep lands. In India, mulberry is commercially cultivated in Andhra Pradesh, Karnataka, Manipur and West Bengal where as small scale plantations are also seen in Assam, Jammu-Kashmir, Kerala, Madhya Pradesh, Tamil Nadu and Uttar Pradesh (Datta, 2002) [5]. Nowadays, its cultivation has been greatly increased and it is mainly being used as an ornamental plant in gardens. Despite its huge demand, perennial nature of the plant coupled with prolonged juvenile period slows down the process of mulberry improvement (Kavyashree *et al*., 2001) [11]. 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 plants (Zaman *et al*., 1997) [21]. 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
Preparation of culture medium
MS (Murashige and Skoog, 1962) [14] media was prepared and dispensed into 80 × 150 mm glass

bottles (Borosil, India) using a Masterflex digi-static auto dispenser. The media bottles were sterilized by autoclaving at 121˚C at 15 psi pressure for 15 min. The pH of the medium was adjusted to 5.7 ± 0.1 prior to autoclaving.

Plant Material and Culture Conditions

Juvenile shoots were collected from two to three years old black mulberry plant during April, 2015. Explants were trimmed and made two to three centimeter (cm) long in size. The explants were then thoroughly washed in running tap water followed by washing in Tween-20 detergent for 10 minutes. The explants were then treated with 70% ethanol for 30 seconds, 1000 mgl^{-1} carbendazim-50% for 15 minutes, 500 mgl^{-1} cefotaxime and kanamycin each for 10 minutes, 500 mgl^{-1} streptocycline for 12 minutes and 0.1% $HgCl_2$ for 3 minutes for surface sterilization. The sterilized explants were given a fresh cut under laminar air flow and inoculated on MS medium.

Shoot Induction

MS medium supplemented with various concentrations and combinations of BAP (0, 2, 4 mgl^{-1}), TIBA (0, 1, 2 mgl^{-1}) and NAA (0, 0.1, 0.2, 0.4 mgl^{-1}) procured from Sigma Aldrich, Germany were used for shoot induction. The media were supplemented with 3% sucrose (Merck, USA) and 0.8% Agar Agar Type I (HIMEDIA chemicals, India). The observations on number of days to bud break, number and length of sprouts and number of leaves were recorded.

Shoot Multiplication

Two different experiments were carried out for multiple shoot induction. One to check the effect of various combinations of BAP (2 mgl^{-1}), TIBA (2 mgl^{-1}), NAA (0.2 mgl^{-1}), Glutamine (0.5 and 1 mgl^{-1}) and Aspargine (12.5 and 25 mgl^{-1}) on multiplication and other to check the sole effect of two different cytokinins at their variable concentration on multiplication. The media were supplemented with 3% sucrose and 0.8% Agar Agar Type I. The observations on number and length of shoots (cm) and number of leaves were recorded.

Root Induction

In vitro developed shoot clumps (cluster of two to three shoots), measuring around 3-4 cm long, were inoculated on MS medium supplemented with five different concentrations of IBA (0.1, 0.2, 0.3, 0.4 and 0.5 mgl^{-1}) for root induction. The observation on number and length (cm) of primary as well as secondary roots were recorded.

Hardening

The *in vitro* rooted plantlets were carefully removed from the culture bottles and washed thoroughly with distilled water to remove traces of the medium. Plantlets were treated with 200 mgl^{-1} carbendazim-50%, 200 mgl^{-1} rhidomil and 200 mgl^{-1} blitox-50% each for 2 minutes, followed by rinsing in distilled water. The plantlets were then transferred to 2" X 2" cm plastic bags containing soil: cocopeat (1:1) for acclimatization under greenhouse conditions. All the plantlets were fertigated with one-fourth MS daily for one month. Primary hardened plantlets were then transferred to polyhouse and maintained in the soil bags until transplantation to the field. The fungal and bacterial contaminants were reduced by an aerial spray of an antifungal (carbendazim-50%) and an antibacterial (streptocycline).

Statistical Analysis

The data generated from the experiments conducted for various stages *viz.* initiation, multiplication and rooting were statistically analyzed using completely randomized design (CRD). The data were subjected to one way analysis of variance (ANOVA) in the excel sheet followed by Duncan multiple range test (DMRT) using DSAASTAT software (Onofri and Pannacci, 2014) [16].

3. Results

Shoot induction

New sprouts emerged from axillary buds within 6 – 10 days of culture initiation. The surface sterilization treatment yielded approximately 85% of axenic cultures. All the sterilization treatments were reported to 100% bud break. Explants inoculated on MS medium supplemented with 2 mgl^{-1} BAP and 0.2 mgl^{-1} NAA produced maximum number of sprouts (3.75 ± 0.48) and leaves (8.50 ± 0.25) as well. The highest average shoot length (4.15 ± 0.64 cm) was also recorded within the same medium. Any increase or decrease in concentration of NAA, when used in combination with BAP, from 0.2 mgl^{-1} resulted in decrease in number of shoots. The second highest response was recorded in case of MS + 2 mgl^{-1} BAP + 2 mgl^{-1} TIBA, where in number of shoots, number of leaves and length of shoots was 2.75 ± 0.48, 7.00 ± 0.00 and 3.22 ± 0.14 cm, respectively. The morphogenic response was better on BAP + NAA as compared to BAP + TIBA. The detailed results are presented in table 1 and figure 1a-c.

Shoot Multiplication

Among two different experiments conducted for shoot multiplication, experiment involving individual treatments of BAP and Kn proved superior over amino acid based experiment. None of the amino acids based treatments could yield significant results. The number of shoots ranged from 1.00 ± 0.41 to 4.25 ± 0.25 while number of leaves ranged from 6.00 ± 0.58 to 21.00 ± 0.71. In cytokinin (BAP and Kn) based treatments BAP proved better as compared to Kn. MS supplemented with 0.2 mgl^{-1} BAP produced maximum response in terms of number of shoots (6.75 ± 0.48), number of leaves (29.3 ± 0.85) and length of shoots (9.98 ± 0.05 cm). Any increase in concentration of BAP from 0.2 mgl^{-1} showed marked decline in number shoots. The detailed results are presented in table 3 and figure 1d.

Rooting

Among all the different concentrations tested of IBA, MS + 0.5 mgl^{-1} IBA produced maximum number (16.25 ± 0.63) of primary roots and MS + 0.2 mgl^{-1} IBA produced maximum number (10.75 ± 0.48) of secondary roots. The highest length of primary (7.30 ± 0.16 cm) and secondary (4.22 ± 0.13 cm) roots were recorded with MS + 0.5 mgl^{-1} IBA and MS + 0.1 mgl^{-1} IBA, respectively. The detailed results are presented in table 4 and figure 1e.

Hardening and acclimatization

Four week old plantlets transferred to greenhouse conditions were successfully primary hardened in poly bags using a mixture of soil: cocopeat (1:1) (Fig. 1 f-g). The primary hardened plantlets attained further growth in polyhoyse using soil as the hardening substrate. The acclimatized plants were later transplanted to the field and had a survival rate of 100%.

Table 1: Effect of BAP, NAA and TIBA on *in vitro* shoot induction

Sr. No.	Plant Growth Regulators (mgl^{-1})			Number of days to bud break			
	BAP	NAA	TIBA		No of sprouts	No of leaves	Length of sprouts (cm)
1	0	0	0	6	1.75 ± 0.25^{c}	6.00 ± 0.00^{bcd}	1.81 ± 0.26^{fg}
2	2	0	0	7	1.25 ± 0.25^{cde}	4.75 ± 0.25^{efgh}	2.07 ± 0.11^{defg}
3	4	0	0	6	1.00 ± 0.00^{de}	3.75 ± 0.25^{h}	1.50 ± 0.68^{g}
4	0	0	1	6	1.50 ± 0.29^{cd}	4.25 ± 0.25^{gh}	1.72 ± 0.12^{fg}
5	2	0	1	8	1.25 ± 0.25^{cde}	4.25 ± 0.25^{gh}	1.95 ± 0.06^{efg}
6	4	0	1	9	1.00 ± 0.00^{de}	5.50 ± 0.29^{cdef}	2.52 ± 0.19^{bcdef}
7	0	0	2	6	1.00 ± 0.00^{de}	4.25 ± 0.25^{gh}	1.90 ± 0.11^{fg}
8	2	0	2	10	2.75 ± 0.48^{b}	7.00 ± 0.00^{b}	3.22 ± 0.14^{b}
9	4	0	2	6	1.00 ± 0.00^{de}	5.25 ± 0.25^{defg}	2.90 ± 0.09^{bcd}
10	2	0.1	0	6	0.75 ± 0.25^{e}	5.00 ± 0.00^{defg}	2.55 ± 0.09^{bcdef}
11	4	0.1	0	9	1.00 ± 0.00^{de}	3.75 ± 0.25^{h}	2.17 ± 0.13^{cdefg}
12	0	0.2	0	6	1.00 ± 0.00^{de}	5.00 ± 0.00^{defg}	2.77 ± 0.18^{bcde}
13	2	0.2	0	6	3.75 ± 0.48^{a}	8.50 ± 0.25^{a}	4.15 ± 0.64^{a}
14	4	0.2	0	7	1.00 ± 0.00^{de}	5.75 ± 1.19^{cde}	3.00 ± 0.17^{bc}
15	0	0.4	0	8	1.00 ± 0.00^{de}	4.50 ± 0.29^{fgh}	2.27 ± 0.13^{cdefg}
16	2	0.4	0	6	1.00 ± 0.00^{de}	6.50 ± 0.29^{bc}	3.25 ± 0.10^{b}
17	4	0.4	0	7	1.00 ± 0.00^{de}	5.00 ± 0.00^{defg}	1.90 ± 0.09^{fg}

Parameters have been recorded after 8 weeks of transfer in shoot induction media. Data are in the form of mean ± SE
Means with the same letter along the column are not significantly different at p=0.05

Table 2: Effect of BAP, NAA and TIBA along with different concentrations of glutamine and asparagine on multiple shoot induction

Sr. No.	Plant Growth Regulators (mgl^{-1})			Amino acids (mgl^{-1})				
	BAP	NAA	TIBA	Glutamine	Asparagine	No of shoots	No of leaves	Length of shoots (cm)
1	0	0	0	0	0	3.00 ± 0.41^{bc}	21.00 ± 0.71^{a}	8.35 ± 0.30^{a}
2	2	0	2	0.5	25	2.00 ± 0.41^{cde}	6.00 ± 0.58^{f}	5.08 ± 0.70^{b}
3	2	0	2	1	25	1.25 ± 0.25^{de}	8.00 ± 0.41^{ef}	4.35 ± 0.24^{bcd}
4	2	0	2	0.5	12.5	3.00 ± 0.41^{bc}	10.75 ± 0.25^{cd}	3.60 ± 0.39^{d}
5	2	0	2	1	12.5	2.25 ± 0.25^{cd}	9.25 ± 0.63^{de}	2.45 ± 0.06^{e}
6	2	0.2	0	0.5	25	4.25 ± 0.25^{a}	14.50 ± 1.04^{b}	4.90 ± 0.20^{bc}
7	2	0.2	0	1	25	3.50 ± 0.29^{ab}	11.25 ± 1.49^{cd}	3.88 ± 0.11^{d}
8	2	0.2	0	0.5	12.5	2.25 ± 0.25^{cd}	13.25 ± 0.75^{bc}	3.95 ± 0.17^{cd}
9	2	0.2	0	1	12.5	1.00 ± 0.41^{e}	12.75 ± 1.03^{bc}	3.55 ± 0.06^{d}

Parameters have been recorded after 12 weeks of transfer in shoot multiplication media. Data are in the form of mean ± SE.
Means with the same letter along the column are not significantly different at p=0.05

Table 3: Effect of different concentrations of BAP and Kn on multiple shoot induction

Sr. No.	Plant Growth Regulators (mgl^{-1})				
	BAP	Kn	No of shoots	No of leaves	Length of shoots (cm)
1	0	0	3.00 ± 0.41^{e}	21.0 ± 0.71^{d}	8.35 ± 0.30^{b}
2	0.2	0	6.75 ± 0.48^{a}	29.3 ± 0.85^{a}	9.98 ± 0.05^{a}
3	0.4	0	4.25 ± 0.48^{bcde}	25.5 ± 1.04^{b}	8.73 ± 0.21^{b}
4	0.6	0	3.25 ± 0.25$^{c\ e}$	21.5 ± 0.65^{cd}	8.73 ± 0.11^{b}
5	0.8	0	5.25 ± 0.25^{b}	24.3 ± 1.25^{bc}	8.73 ± 0.64^{b}
6	1	0	4.50 ± 0.29^{bc}	21.8 ± 0.63^{cd}	7.85 ± 0.41^{bc}
7	0	0.2	4.50 ± 0.29^{bcd}	19.0 ± 0.91^{d}	5.23 ± 0.17^{e}
8	0	0.4	5.50 ± 0.29^{b}	11.5 ± 0.65^{f}	6.33 ± 0.20^{d}
9	0	0.6	3.25 ± 0.25^{ce}	14.0 ± 0.41^{ef}	7.05 ± 0.27^{cd}
10	0	0.8	4.50 ± 0.65^{bcd}	12.0 ± 1.29^{ef}	5.10 ± 0.16^{e}
11	0	1	5.50 ± 0.29^{b}	14.8 ± 0.85^{e}	6.18 ± 0.06^{d}

Parameters have been recorded after 12 weeks of transfer in shoot multiplication media. Data are in the form of mean ± SE.
Means with the same letter along the column are not significantly different at p=0.05

Table 4: Effect of various concentrations of IBA on root induction

Sr. No.	Plant Growth Regulator (mgl^{-1})	No of primary roots	Length of primary roots (cm)	No of secondary roots	length of secondary roots
	IBA				
1	0	7.30 ± 1.00^{b}	1.98 ± 0.27^{f}	16.75 ± 0.63^{b}	1.95 ± 0.06^{c}
2	0.5	16.25 ± 0.63^{a}	7.30 ± 0.16^{a}	10.75 ± 0.48^{c}	1.10 ± 0.20^{d}
3	0.4	1.50 ± 0.29^{c}	6.15 ± 0.08^{b}	7.50 ± 0.29^{d}	0.70 ± 0.6^{e}
4	0.3	7.00 ± 0.41^{b}	3.10 ± 0.05^{e}	4.50 ± 0.29^{e}	0.15 ± 0.03^{f}
5	0.2	3.50 ± 0.50^{c}	5.29 ± 0.15^{c}	21.50 ± 0.29^{a}	2.42 ± 0.17^{b}
6	0.1	8.00 ± 0.70^{b}	4.53 ± 0.17^{d}	16.75 ± 0.25^{b}	4.22 ± 0.13^{a}

Parameters have been recorded after 4 weeks of transfer in root induction media. Data are in the form of mean ± SE. Means with the same letter along the column are not significantly different at p=0.05

Fig 1: (a) Explant preparation **(b)** Culture initiation **(c)** Shoot induction **(d)** Shoot multiplication **(e)** Root induction **(f)** *In vitro* regenerated plant **(g)** Fully hardened plant

4. Discussion

In the present investigation, nodal explants were collected in the April month when the climate remains dry in Anand, Gujarat. Hence, large number of the explants was converted to axenic cultures. These results are in accordance with the findings of Akram and Aftab (2012) [1] where they initiated the cultures in April and obtained more than 90% of the axenic cultures. Use of 70% ethanol, antibiotics and 0.1% $HgCl_2$ has no adverse effect on shoot induction and proliferation. These findings were well supported by the work of Niratker *et al*. (2015) [15] and Kavyashree (2007) [10] where they obtained 80% and 94% of the axenic cultures, respectively. To promote shoot induction and development, present study used BAP in combinations with NAA and TIBA. From the results it was concluded that explants grown on BAP + NAA could yield better response as compared to that on BAP + TIBA. Anis *et al*. (2003) [2] and Akram and Aftab (2012) [1] also used BA + NAA for shoot induction as well as multiplication. Contradictorily, Kavyashree (2007) [10] reported 94% of the shooting response using 2 mgl^{-1} BAP + 1 mgl^{-1}TIBA in S_{54} variety of mulberry. The obvious reason for moderate response in BAP + TIBA here may be due to inhibitory effects of TIBA on polar transport of auxins in the cells (Venkatesh *et al*., 2009) [18]. During shoot multiplication phase, treatment supplemented with 0.2 mgl^{-1} BAP produced significant results over Kn and amino acid based treatments. Yadav *et al*. (1990) [19] also obtained similar findings and suggested the use of BAP over kinetin. The moderate response generated on Kn based treatments might be due to its different uptake rate in different genomes (Blakesly, 1991) [3]. Contrarily, Anis *et al*. (2003) [2] obtained maximum number of shoots on MS supplemented with 2 mgl^{-1} BAP + 0.2 mgl^{-1} NAA + 25 mgl^{-1} aspargine + 1 mgl^{-1} glutamine. Instead of checking the effect of various auxins and their combinations (NAA, IBA and IAA), present investigation used different concentrations of IBA for *in vitro* root induction. One obvious reason for choosing IBA in the present investigation was due to its beneficiary effect on lateral root induction and overall root growth. Kim *et al*. (1985) [12], Ivanicka (1987) [8], Jain *et al*. (1990) [9], Islam *et al*. (1993 and 1994) [6-7] also favored the use of IBA for root induction in mulberry shoots. The *in vitro* regenerated plantlets were successfully acclimatized in primary and secondary hardening units. Acclimatized plants seemed morphologically similar with mother plant grown under field conditions. In conclusion, present study demonstrates an effective micropropagation technology for black mulberry from nodal explants.

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