Thidiazuron induced multiple shoot induction and plant regeneration from cotyledonary explants of mulberry

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Abstract

A rapid micropropagation protocol through induced multiple shoots from the cotyledonary explant of mulberry (*Morus alba* L) is described. The highest number of shoots (20.3) was obtained when explants from 14-d-old embryos were cultured on Murashige and Skoog (MS) medium supplemented with 7 μM thidiazuron for 45 d. Of the three cultivars used, cv. S-36 was the best followed by cv. K-2 and S-1. The shoots were transferred to MS medium supplemented with 5 μM 6-benzylaminopurine for elongation. The elongated shoots were rooted on half strength MS medium containing 1 - 7 μM indole 3-butyric acid or 1-naphthalene acetic acid. The rooted plants were transplanted to soil with 90 % success. The emerged shoot primordia probably initiated from the pre-existing meristems since the shoot bud show definite vascular connection to the major vascular tissue.

Additional key words: acclimatization, growth regulators, *Morus alba.*

Introduction

Mulberry (*Morus* spp.) is an invaluable tree for the sericulture industry as it is the only source of food for mori silk worms. The earliest report on mulberry micropropagation came from a Japanese scientist Ohyama (1970) who obtained the first complete plantlets of mulberry from axillary buds. Since then a number of reports on mulberry tissue culture using different explants were reported (for review see Thomas 2002). Regeneration of this tree species has been reported from nodal segments (Bapat *et al.* 1987, Sharma and Thrope 1990, Yadav *et al.* 1990, Hossain *et al.* 1992, Pattnaik *et al.* 1996, Pattnaik and Chand 1997), shoot tips (Ohyama and Oka 1975, Ivanicka 1987, Hayashi and Oka 1995), leaves (Machii 1992, Thinh and Katagiri 1994, Kathiravan *et al.* 1997, Sahoo *et al.* 1997), and callus (Oka and Ohyama 1986, Tewari *et al.* 1989).

Recently, thidiazuron (TDZ) has been extensively used for woody plant regeneration. TDZ is found to be as or more efficient than 6-benzylaminopurine (BAP) for shoot proliferation (Van Nieuwkirk *et al.* 1987) and adventitious shoot regeneration from leaves, cotyledons and hypocotyls of several woody species (Fasolo *et al.* 1989, Bretague *et al.* 1994). The present study was undertaken to establish an efficient and reproducible protocol for regeneration from the cotyledons of mulberry by using TDZ and BAP.

Materials and methods

Three cultivars of mulberry (*Morus alba* L.) S-36, S-1, and K-2 were used. The fruits 17 - 20 d after pollination (DAP) containing the seeds were surface sterilized with 70 % ethanol for 2 min, 0.1 % mercuric chloride for 13 min and washed three times with sterilized distilled water. The seeds were isolated from the fruit by squeezing the fruit with the help of a needle and forceps. The embryos were dissected out from the seeds and placed on Murashige and Skoog (1962; MS) medium solidified with 8 g dm^{-3} agar (*Qualigens*, Mumbai, India).

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Abbreviations: BAP - 6-benzylaminopurine; IBA - indole-3-butyric acid; NAA - 1-naphthalene acetic acid; TDZ - thidiazuron (N-phenyl N'1,2,3-thidiazol-5-yl urea).
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The growth regulators were added to the medium and the pH was adjusted to 5.8 before autoclaving at 1.5 kg cm^{-2} and 121 °C for 15 min. All cultures were maintained at 25 ± 2 °C under a 16-h photoperiod, (two *Philips TL* 40 W fluorescent tubes - irradiance of 22 µmol m^{-2}s^{-1}). At least twenty-four cultures were raised for each treatment and all experiments were repeated three times. Analysis of variance and Duncan's multiple range test was used for comparison among treatment means.

Cotyledonary explants were cultured 7, 14, and 21 d after embryo culture. Individual cotyledons were excised from seedlings about 1 mm below the cotyledonary node and cultured on MS medium supplemented with 2 - 9 µM TDZ and BAP. Shoots originated from cotyledons were multiplied on MS medium supplemented with 5 µM BAP. The multiplied shoots were transferred to half strength MS medium supplied with indole-3-butyric acid (IBA) or 1-naphthalene acetic acid (NAA) (1 - 7µM) for root induction. Individual plants were gently washed in distilled water to remove agar from their roots and were then transferred to garden soil in polythene bags and placed in a glasshouse under high humidity. After 2 months the plants were shifted out of the glasshouse and placed in shade under natural conditions. The plants were eventually transferred to pots.

Cotyledonary explants with multiple shoots were fixed for 24 h in FAA (formalin + glacial acetic acid + 70 % ethanol in a 1:1:18 ratio by volume). Following dehydration in tertiary butanol the material was infiltrated and embedded in paraffin wax (Johansen 1940). Sections (8 µm thick) were cut on a rotary microtome and stained with safranin-Astrablau.

Results and discussion

The embryos cultured on MS medium supplemented with 5µM BAP produced well-developed cotyledons, hypocotyl and radicle 7 d after culture (Fig. 1*A*). The induction of multiple shoots varied with the age of the cotyledons as well as the concentration and type of growth regulators used (Table 1). A significantly greater number of shoots were formed from cotyledons of 14-d-old embryos cultured on MS medium containing TDZ and BAP compared to 7- and 21-d-old explants (Table 1). Explant age plays a major role to induce multiple shoots in a number of plants like eastern redwood, pea, lentil, and soybean (Mallick and Rashid 1989, Jackson and Hobbs 1990, Kim *et al.* 1990, Distabanjong and Geneve 1997).

TDZ at different concentrations induced more shoots per explant as compared to BAP at the same concentration and explant age. This showed that TDZ is more effective than BAP for multiple shoot induction in mulberry (Table 1). A maximum number of shoots per explant were observed on MS medium fortified with 7 µM TDZ. On this medium the 14-d-old explant gave an average number of 20.3 shoots per explant (Fig. 1*B*). TDZ induced multiple shoot formation and somatic embryogenesis is well known and reported in a number of plants including *Vicia faba*, *Psiadia arguta*, *Cicer arietinum* and *Cajanus cajan* (Kodja *et al.* 1998, Khalafalla and Hattori 1999, Mohan and Krishnamurthy 2002, Singh *et al.* 2002). The shoots were originated from the cut end of the cotyledons. The appearance of the shoot buds was observed two weeks after culture. The cotyledon size slightly enlarged before shoot formation. But the colour of the cotyledons remained green. No callusing was observed in any stages of culture. The emerging shoots were healthy and without any abnormality.

This procedure described here was found to be applicable to three mulberry genotypes (Table 1). The better response was observed in cultivar S-36 as compared to K-2 and S-1.

The shoot buds were emerged from the proximal end of the cotyledons as small nodular outgrowths without any sign of callusing. The histological analysis clearly showed that these buds were emerged from the epidermal region. The bud differentiation takes place early and shoot bud development was not synchronously induced. The formation of buds at different stages of development was observed on the proximal end of the cotyledons. The emergence of shoot primordia in cultured cotyledons seem to have initiated from the pre-existing meristems at the latter's proximal end since the shoot bud show definite vascular connection to the major vascular tissue (Fig. 1*C*).

The regenerated shoots (*ca.* 1 cm) from all the three cultivars were isolated and individual shoots were transferred to MS medium containing 5 µM BAP for 30 d for shoot elongation. 100 % of the cultures showed shoot elongation in all the three cultivars on this media. Root induction on the elongated shoots occurred when 3 cm long shoots were subcultured on half strength MS medium supplemented with IBA or NAA. IBA was comparatively better than NAA. 100 % of the cultured shoots rooted on half strength MS medium supplemented with 5 µM IBA in cultivar S-36. On this medium an average number of 7.6 roots per explant were observed (Table 2). The use of low salt MS medium for rooting of *in vitro* induced shoots has been reported in *Vicia faba* (Mohamed *et al.* 1992, Khalafalla and Hattori 1999). The rooted shoots were successfully transplanted to soil after acclimatization. The plants were eventually transferred to garden pots and 90 % of the transplanted plantlets survived (Fig. 1*D*).

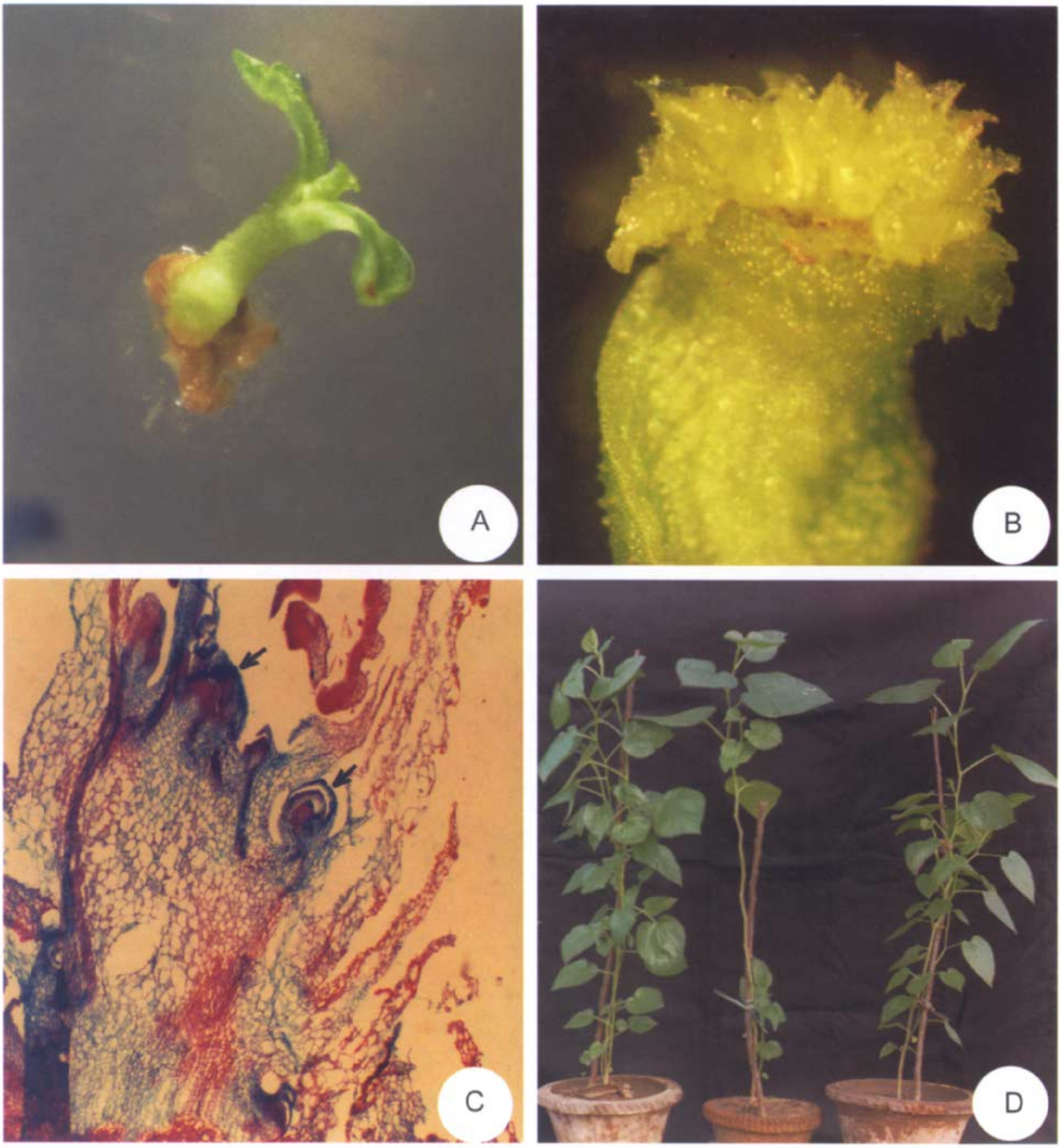

Fig. 1. Different stages of shoot regeneration from the cotyledonary explants of *Morus alba* L. (cv. S-36): *A* - a 7-d-old embryo showing the green cotyledon and hypocotyl on MS medium supplemented with 5 µM BAP; *B* - the emergence of several shoots from the proximal end of a 14-d-old cotyledon on MS medium containing 7 µM TDZ, 45 d after culture; *C* - longitudinal section through an explant from 14-d-old embryo, 45 d after culture on MS medium supplemented with TDZ (7 µM), showing the emergence of shoot buds (*arrow* marked), the shoot buds show definite vascular connection to the major vascular tissue; *D* - 90-d-old actively growing healthy plants after acclimatization.

Table 1. Effect of cotyledon age and various BAP and TDZ concentrations on shoot induction from the cotyledonary explants of three cultivars (S-36, K-2 and S-1) of mulberry. Culture period 45 d. Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P > 0.05$).

Age [d]	Growth regulator	Conc. [μM]	Explants forming shoots [%]			Number of shoots [explant^{-1}]		
			S-36	K-2	S-1	S-36	K-2	S-1
7	BAP	2.0	21b	12a	10a	2.2 ± 0.4a	1.8 ± 0.2a	1.1 ± 0.1a
		5.0	34c	19a	14a	3.2 ± 0.6b	2.0 ± 0.2a	1.4 ± 0.1a
		7.0	38d	22b	16a	4.1 ± 0.5c	2.1 ± 0.5a	1.8 ± 0.2a
		9.0	30c	21b	15a	3.8 ± 04b	2.0 ± 0.3a	1.5 ± 0.1a
	TDZ	2.0	18a	9a	6a	6.3 ± 0.2e	2.1 ± 0.1a	1.8 ± 0.3a
		5.0	37c	24b	18a	8.8 ± 0.7f	2.8 ± 0.3b	2.0 ± 0.2a
		7.0	33c	26b	21b	9.7 ± 0.5g	3.0 ± 0.3c	2.5 ± 0.3b
		9.0	29b	22b	20ab	8.4 ± 0.2f	2.7 ± 0.2b	2.2 ± 0.4a
14	BAP	2.0	88f	60c	55c	3.8 ± 0.3b	2.2 ± 0.2a	2.1 ± 0.1a
		5.0	91f	72c	61d	6.5 ± 0.4e	2.3 ± 0.1a	2.4 ± 0.3a
		7.0	93f	78d	65d	5.5 ± 0.4d	2.6 ± 0.4b	2.2 ± 0.3a
		9.0	90f	70c	64d	4.0 ± 0.2c	2.1 ± 0.2a	2.3 ± 0.2a
	TDZ	2.0	100g	89e	75e	10.8 ± 0.6h	2.8 ± 0.5b	2.7 ± 0.1b
		5.0	100g	93f	78e	15.8 ± 0.8j	3.1 ± 0.4c	3.5 ± 0.2c
		7.0	100g	97f	80f	20.3 ± 0.6k	7.3 ± 0.6e	5.6 ± 0.5d
		9.0	100g	91f	76e	11.2 ± 0.4i	4.1 ± 0.4d	5.1 ± 0.4d
21	BAP	2.0	83e	69d	65d	3.3 ± 0.2b	2.2 ± 0.2a	2.0 ± 0.2a
		5.0	87e	76d	72e	3.8 ± 0.5b	2.8 ± 0.3b	2.3 ± 0.3a
		7.0	90f	81e	71e	4.1 ± 0.7c	2.6 ± 0.3b	2.2 ± 0.3a
		9.0	90f	78d	68d	4.0 ± 0.3c	2.4 ± 0.2a	2.1 ± 0.4a
	TDZ	2.0	84e	81e	68d	5.2 ± 0.5d	2.1 ± 0.1a	2.5 ± 0.3b
		5.0	90f	88e	71e	9.8 ± 0.7g	2.8 ± 0.3b	2.7 ± 0.4b
		7.0	95f	91f	78e	10.5 ± 0.4h	2.7 ± 0.3b	2.8 ± 0.5c
		9.0	94f	90f	76e	8.0 ± 0.5f	2.5 ± 0.5b	2.6 ± 0.3b

Table 2. Effect of different auxins (IBA and NAA) on rooting of the shoots in three cultivars. Half strength MS medium. Culture period 30 d. Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P > 0.05$).

Growth regulator	Conc. [μM]	Explants forming roots [%]			Number of roots [explant^{-1}]		
		S-36	K-2	S-1	S-36	K-2	S-1
IBA	1	52b	48b	56b	2.5 ± 0.3a	2.3 ± 0.2b	2.0 ± 0.3b
	3	76d	75d	72d	3.2 ± 0.5b	2.8 ± 0.3b	2.6 ± 0.4b
	5	100g	98e	93f	7.6 ± 0.7e	4.3 ± 0.5d	3.8 ± 0.4c
	7	92f	94e	89e	6.4 ± 0.6d	4.0 ± 0.4d	4.1 ± 0.3d
NAA	1	43a	39a	48a	2.1 ± 0.2a	1.8 ± 0.2a	1.4 ± 0.2a
	3	68c	66c	67c	3.2 ± 0.3b	3.6 ± 0.6c	2.4 ± 0.3b
	5	87e	79d	89e	4.1 ± 0.4c	3.9 ± 0.7c	3.2 ± 0.4c
	7	78d	75d	78d	3.9 ± 0.4bc	3.6 ± 0.5c	3.0 ± 0.4c

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