Note

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In Vitro Response of Commercially Valuable Cultivars of *Morus* Species to Thidiazuron and Activated Charcoal

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Abstract

Towards developing a rapid and reliable *in vitro* regeneration system for the propagation of mulberry, culture response of *Morus multicaulis* cv. Goshoerami and *M. indica* cvs. K2, RFS175 and S1, was examined. The presence of a cytokinin was essential for bud break. Thidiazuron (TDZ) at a concentration of 0.1 mg l^{-1} was found to be more effective than BAP for bud break and shoot proliferation in *M. indica* cvs. RFS175 and k2, whereas 0.5 mg l^{-1} TDZ was better than BAP in *M. indica* cv. S1. Although TDZ enhanced the efficiency of shoot multiplication in *M. indica*, 2.5 mg l^{-1} BAP was found to be superior in the case of *M. multicaulis*. TDZ not only significantly reduced the days required for bud break but also increased the percentage of bud breaks and the number of shoots per explant in *M. indica*. *In vitro* rooting of *Morus* shoots improved significantly by adding activated charcoal to culture media. A significant increase in the percentage of rooting and a decrease in the days required for rooting were observed by using 0.05% activated charcoal in *M. multicaulis* cv. Goshoerami and *M. indica* cv. S1, and 0.1% activated charcoal in *M. indica* cvs. K2 and RFS175. *In vitro*-raised plantlets were successfully acclimatized and transferred to the field.

Mulberry (*Morus* species) is an important tree in the sericulture industry. Propagation of mulberry through seeds is undesirable owing to its cross-pollination and enormous heterozygosity (Das, 1983). Season-specific response in rooting of cuttings and low survival of rooted cuttings of mulberry limit the application of conventional modes of vegetative propagation for large scale multiplication of selected strains and cultivars. To overcome these problems, micropropagation protocols have been developed for *Morus* species (Mhatre *et al.*, 1985; Sharma and Thorpe,1990; Chattopadhyay *et al.*, 1990; Tewari, *et al.*,1995). However, most of these protocols are largely genotype-specific and may not be applicable to valuable genotypes (Bhojwani, 1992). The present study was thus carried out to compare *in vitro* response of commercially valuable *Morus* species. Attempts to enhance the efficiency of the previously developed micropropagation protocols from field-grown plant material were also made.

Three commercially valuable cultivars of *Morus indica*, K2, RFS175 and S1, and one of *M. multicaulis*, Goshoerami, were used for the present investigation. Six- to seven-year-old, apparently healthy looking, field-grown plants of *M. indica*, and an old, mature tree of *M. multicaulis*, were chosen as explant sources. For each cultivar, the explants were collected from a single plant throughout the year. Shoot pieces, 1-2 cm long, containing a single axillary bud were excised and washed with the liquid detergent Teepol (Rickett & Colman, India) and then disinfected with 0.1% mercuric chloride (Qualigens, India) for 8 min. The surface-disinfected explants were rinsed 5-6 times with sterile distilled water prior to culture. MS medium (Murashige and Skoog, 1962) containing 3% sucrose (w/v), was supplemented with either 0.1-2.5 mg l^{-1} BAP or 0.1-2.5 mg l^{-1} TDZ. The pH of the medium was adjusted to 5.8 after adding 0.8% (w/v) agar (Hi media, Bombay, India). Culture tubes containing media were autoclaved at 104 Kpa and 121 ℃ for 15 min. The surface disinfected seeds were placed horizontally in the culture tubes. Cultures were maintained in the culture room at 25 ± 1 ℃ under a daily photoperiodic regime of 16h light and 8h darkness. Four cool white fluorescent tubes (Phillips, TL40 W/54) provided the light intensity of 65 μmol $m^{-2}s^{-1}$.

Shoots formed *in vitro* were isolated and sec-

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tioned into single-node pieces after removing the leaves. The nodal segments of *M. indica* cvs. K2 and RFS175 were cultured on MS medium with 0.1 mg l^{-1} TDZ whereas in the case of cv. S1, MS medium supplemented with 0.5 mg l^{-1} TDZ was used. The nodal segments of *M. multicaulis* cv. Goshoerami were cultured on MS medium supplemented with 2.5 mg l^{-1} BAP for further multiplication. Subcultures were made after 4 weeks in all cases. For rooting, *in vitro*-raised shoots with 3-5 leaves were excised and transferred to MS medium containing 2% sucrose, 0.8% agar, 1 mgl^{-1} α-naphthalene acetic acid (NAA) and 0.0-0.1% activated charcoal. Shoots with roots over 5 mm long were considered to be the rooted ones. Plantlets with well-developed roots were transferred to plastic pots (10 cm diameter) containing garden soil (soil:compost, 1:1) and maintained in the culture room for one month before transferring the plants to the field.

At the end of a 4-week culture period and a 4-week multiplication cycle, the percentage of bud break (emergence of shoots from the dormant meristem) and the number of shoots per explant were determined. Rooting experiments were evaluated by the percentage of rooting and the days required for rooting. Experiments were carried out in a completely randomized design with 3 replicates to determine the effect of various treatments. The data were subjected to Two Way Analysis Of Variance (ANOVA). The rooting and bud break data calculated in terms of percentage were subjected to arcsin transformation before performing ANOVA. The means of transformed values were retransformed for presentation in the tables. Least Significant Difference (LSD) at $p \leq 0.05$ among the means was estimated according to Snedecor and Cochran (1967).

Most of the recent reports on tissue culture of *Morus* species have used seedling-derived materials (Sharma & Thorpe, 1990). *In vitro* propagation using apical or axillary buds and nodal explants has also been reported for some mulberry species (Pattnaik *et al.*, 1996; Pattnaik and Chand, 1997). To date, however, there has been no report on the *in vitro* propagation of mulberry cultivars used in the present study.

Axillary buds of *M. indica* and *M. multicaulis* when placed on MS medium without growth regulators showed no symptoms of bud break even after 4-5 weeks, and the addition of a cytokinin was essential. Axillary buds of *M. indica* cultured on MS media supplemented with BAP developed into shoots following callus formation. This is in conformity with Patel *et al.* (1983). A strong monopodial growth habit was observed in *Morus* cultures when media containing low concentrations (0.1-0.5 mg l^{-1}) of BAP were used, and multiple shoots were induced by higher concentrations. However, these shoots failed to elongate at the later stage of culture in *M. indica* (**Table 1**).

The selected cultivars of *Morus* used in the present study responded differently to TDZ as well as activated charcoal. TDZ at a concentration of 0.1 mg l^{-1} significantly increased the percentage of bud break, number of shoots per explant, and decreased the days required for bud break in *M. indica* cvs. K2 and RFS175, whereas similar results were obtained by using 0.5 mg l^{-1} TDZ in *M. indica* cv. S1 (**Table 1**). These low concentrations of TDZ effectively inhibited callusing at the basal region of shoots in *M. indica* (**Fig. 1a**) and induced multiple shoots (**Fig. 1b**). Use of TDZ also gave rise to healthy looking, bright green shoots. TDZ, a substituted phenylurea, has been used for micropropagation of a wide range of woody species because it induces a much higher frequency of shoot regeneration than the commonly used cytokinins like BAP (Huetteman and Preece, 1993; Elobeidy and Korban, 1988).

Significantly higher bud break percentage, number of shoots per explant and a reduced time for bud break were observed in *M. multicaulis* cv. Goshoerami at 2.5 mg l^{-1} BAP than TDZ (**Table 1**). BAP was most effective for inducing bud break and multiple shoot formation in other species of *Morus* (Oka and Ohyama, 1986; Islam *et al.*, 1993; Pattnaik *et al.*, 1996). In contrast to *M. indica*, TDZ resulted in callus proliferation at the shoot base in *M. multicaulis*. This callus turned brown if left in the same media for a longer duration. Such interspecific as well as intraspecific variation in culture response of *Morus* has already been reported by earlier workers (Tewari *et al.*, 1995; Pattnaik *et al.*, 1996; Pattnaik and Chand, 1997). Using our protocol at least 7^6 shoots can be produced within 6 months from a single nodal explant.

Activated charcoal was effective for rooting in both *M. indica* and *M. multicaulis*. Significant increase in the percentage of rooting and decrease in the days required for rooting (**Table 2**) was observed by adding activated charcoal. Addition of 0.05% activated charcoal was effective in *M. multicaulis* cv. Goshoerami and *M. indica* cv. S1, while 0.1% activated charcoal gave the similar response in *M. indica* cvs. RFS 175 and K2. Poor rooting response in the control may be attributed to a high level of endogenous cytokinin in the *in vitro*-generated shoots, which may be decreased by using activated charcoal (Steinitz and Yahel, 1982). Additionally, activated charcoal is also known to promote root growth by blocking the light reaching

Table 1. Effects of TDZ and BAP on *in vitro* responce of *Morus* species.

Growth Regulator	Conc. (mg l^{-1})	Bud break (%)	No. of shoots/ Explant	Days required for bud break
***M. indica* cv. K2**				
BAP	0.1	62.5	1.2	24.0
	0.5	75.0	2.8	20.0
	2.5	50.0	3.1	22.0
TDZ	0.1	87.5	7.2	12.0
	0.5	50.0	3.0	15.0
	2.5	50.0	3.2	16.0
LSD for $p \leq 0.05$		01.5	0.7	02.7
***M. indica* cv. RFS175**				
BAP	0.1	62.5	1.5	23.0
	0.5	75.0	3.0	20.0
	2.5	62.5	2.5	21.0
TDZ	0.1	100	8.0	11.0
	0.5	87.5	6.1	14.0
	2.5	62.5	5.0	17.0
LSD for $p \leq 0.05$		01.2	0.2	02.4
***M. indica* cv. S1**				
BAP	0.1	62.5	2.0	22.0
	0.5	75.0	3.2	19.0
	2.5	75.0	3.4	20.0
TDZ	0.1	87.5	6.0	14.0
	0.5	100	7.5	10.0
	2.5	75.0	5.4	15.0
LSD for $p \leq 0.05$		01.7	1.0	03.0
***M. multicaulis* cv. Goshoerami**				
BAP	0.1	62.5	2.0	20.0
	0.5	75.0	1.8	21.0
	2.5	87.5	7.0	13.0
TDZ	0.1	62.5	4.5	15.0
	0.5	50.0	5.1	17.0
	2.5	50.0	4.2	18.0
LSD for $p \leq 0.05$		02.7	0.6	02.9

roots through the medium (George, 1993). Use of activated charcoal also induced adventitious roots directly from the shoot base (**Fig. 1c**). In the control cultures, most of the roots developed via callus formation which has been reported to lead to a poor vascular connection between the shoot and root, and the subsequent survival of *M. alba* plantlets (Sharma and Thorpe, 1990). Shoot elongation was promoted on rooting media supplemented with activated charcoal in *M. indica* and *M. multicaulis*.

Emergence of new leaves was observed in the *in vitro* - generated plants maintained in the culture room, after about 3 weeks of transfer (**Fig. 1d**). A high percentage of survival (90%) was noted in all the cultivars. The regenerated plants did not show any variations in morphology or growth characteristics when compared with the respective donor plants after transferring to the field.

The protocol reported herein could thus be used for large scale propagation and for genetic manipulation of these commercially valuable cultivars of mulberry.

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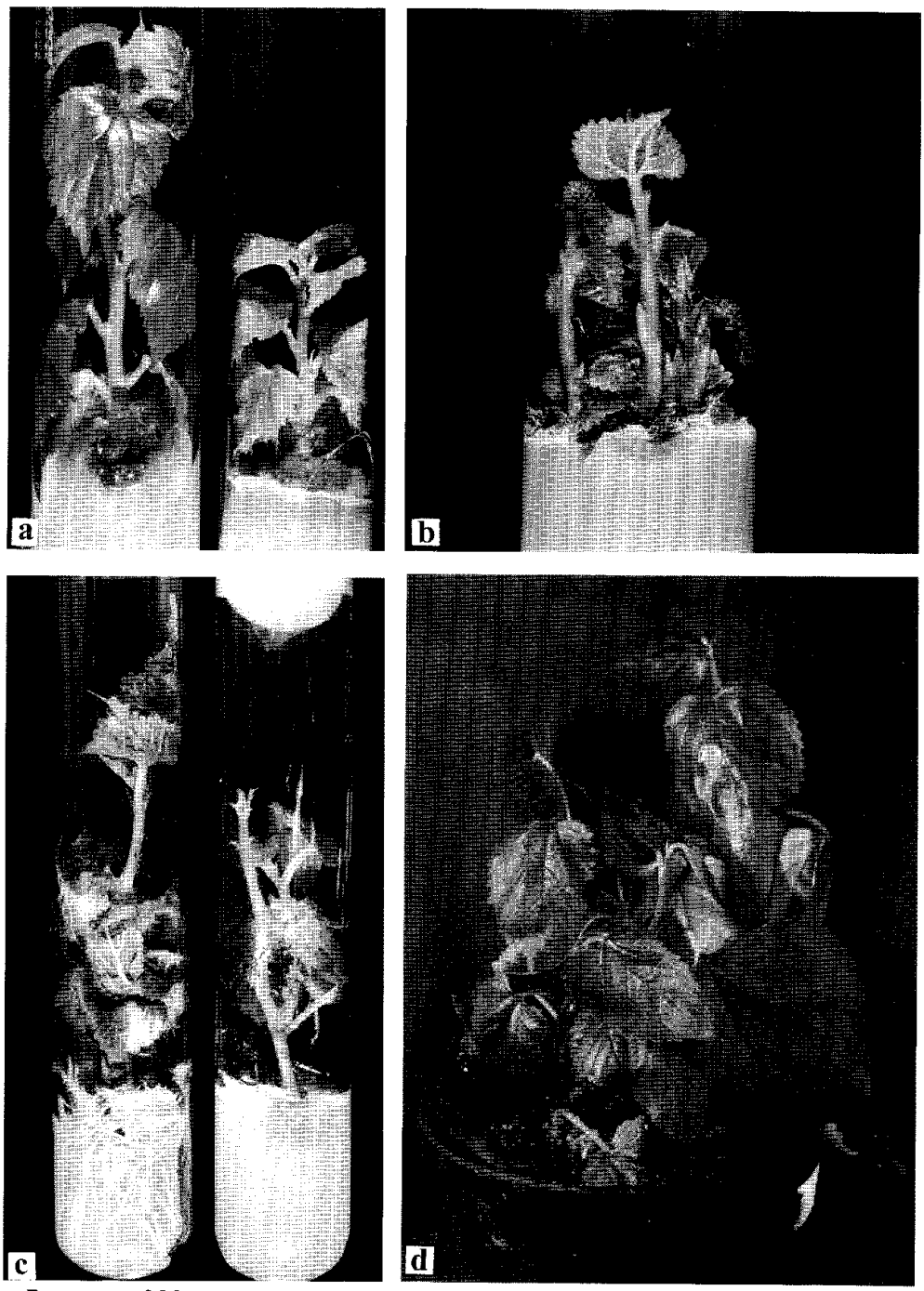

Fig. 1 a–d. Response of *Morus* spp. to TDZ and activated charcoal. **a,** Shoot induction in *M. indica* cv. RFS175 on MS media containing 0.1 mg l^{-1} TDZ (left) or BAP (right) ; **b,** multiple shoots of *M. indica* cv. K2 on MS medium containing 0.5 mg l^{-1} TDZ ; **c,** rooting of shoots in *M. multicaulis* cv. Goshoerami on media with (left) or without (right) 0.05% activated charcoal; and **d,** acclimatized plantlets of *M. indica* cv. S1 6 weeks after transfer to soil.
Bars represent 1cm in all photographs.

Table 2. Effect of activated charcoal on rooting response (%) and days required for rooting of *in vitro* raised shoots of *Morus species*.

Activated Charcoal (%)	Percentage of Rooting*				Days required for Rooting**			
	0.0	0.05	0.1	Varietal Mean	0.0	0.05	0.1	Varietal Mean
***M. indica* cv.**								
S1	50.0	75.0	62.5	52.4	22.0	13.0	20.0	18.3
RFS175	37.5	75.0	100	62.6	25.0	21.0	14.0	20.0
K2	25.0	50.0	87.5	48.1	26.0	19.0	13.0	19.3
***M. multicaulis* cv.**								
Goshoerami	12.5	87.5	62.5	47.4	28.0	12.0	16.0	18.7
Treatment mean	33.4	58.6	66.0		25.3	16.3	15.8	

* LSD for $p \leq 0.05$, for treatment mean=13.3, for varietal mean=22.7

** LSD for $p \leq 0.05$, for treatment mean=3.8, for varietal mean=7.6

References

Bhojwani, S.S., 1992. Plant Tissue Culture and its relevance to mulberry breeding. Brainstorming meeting on Genetics and Biotechnology of Silk Worm and Mulberry,1-11. CSRTI, Mysore, India.

Chattopadhayay, S., Chattopadhayay, S., Dutta, S.K., 1990. Quick *in vitro* production of mulberry (*Morus alba*) plantlets for commercial purpose. Indian J. Exp. Biol., **28**: 522-525.

Das, B.C.,1983. In: National Seminar on Silk Research and Development, March 10-13,9. Bangalore, India.

Elobeidy, A., Korban, S.S., 1988. The effect of thidiazuron on shoot regeneration from apple leaf discs. HortSci., **23**: 755.

George, E.F., 1993. Plant propagation by tissue culture Part I, 470-471. Exegetics Ltd. England.

Huetteman, C.A., Preece, J.E., 1993. Thidiazuron: A potent cytokinin for woody plant tissue culture. Plant Cell Tiss. Org. Cult., **33**: 105-119.

Islam, R., Zaman, A., Zoarder, O.I., Barman, A.C., 1993. *In vitro* propagation as an aid for cloning of *Morus laevigata* Wall. Plant Cell Tiss. Org. Cult., **33**: 339-341.

Mhatre, M., Bapat, V.A., Rao, P.S., 1985. Regeneration of plants from the culture of leaves and axillary buds in mulberry (*Morus indica* L.). Plant Cell Rep., **4**: 78-80.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., **15**: 473-497.

Oka, S., Ohyama, K., 1986. Mulberry (*Morus alba* L.). In: Bajaj, Y.P.S. (Ed.) Biotechnology in Agriculture and Forestry Vol.I, Trees I, 384-392, Springer, Berlin-Heidelberg-New York-Tokyo.

Patel, G.K., Bapat, V.A., Rao, P.S., 1983. *In vitro* culture of organ explants of *Morus indica*: Plant regeneration and fruit formation in axillary bud culture. Z. Pflanzenphysiol., **111**: 465-468.

Pattnaik, S.K., Sahoo, Y., Chand, P.K., 1996. Micropropagation of a fruit tree, *Morus australis* Poir. Syn. *M. acidosa* Griff. Plant Cell Rep., **15**: 841-845.

Pattnaik, S.K., Chand, P.K., 1997. Rapid clonal propagation of three mulberries, *Morus cathayana* Hemsl., *M. ihou* Koiz. and *M. serrata* Roxb., through in vitro culture of apical shoot buds and nodal explants from mature trees. Plant Cell Rep., **16**: 503-508.

Sharma, K.K., Thorpe, T.A., 1990. *In vitro* propagation of mulberry (*Morus alba* L.) through nodal segments. Sci. Hortic., **42**: 307-320.

Snedecor, G.W., Cochran, W.G., 1967. Statistical methods. 6th edition, Oxford & IBH Publishing Co., New Delhi.

Steinitz, B., Yahel, H., 1982. *In vitro* propagation of *Narcissus tazetta*. HortSci., **17**: 333-334.

Tewari, P.K., Sarkar, A., Kumar, V., Chakraborti, S., 1995. Rapid *in vitro* multiplication of high yielding mulberry (*Morus* spp.) Genotypes V1 and S34. Indian J. Sericult., **34**: 133-136.