Short Communication

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TDZ - Mediated Differentiation in Commercially Valuable Indian Mulberry, *Morus indica* Cultivars K2 and DD

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Abstract

The effect of various growth regulators, especially of Thidiazuron (TDZ), on seed germination and subsequent differentiation capabilities of hypocotyl, cotyledon, leaf, internode, root and petiole explants was studied in the Indian mulberry, *Morus indica* cultivars K2 and DD. Of the various hormones tried, thidiazuron at a concentration of 5 μM, gave the highest percentage of adventitious bud formation in both the hypocotyl and cotyledon explants obtained from the seedlings germinated on 0.5 μM TDZ. The young tissue was more responsive and showed up to 50% regeneration from hypocotyl and 70% from cotyledon explants in *M. indica* cv. K2 when cultured for 10 days on TDZ containing medium. The leaf explants produced adventitious buds after 30 days of culture on 2.5 μM TDZ. The 7 - day old leaf explants taken from *in vitro* cultured axillary buds were more efficient in terms of regeneration percentage than the leaf explants derived from the seedlings or from the plants growing in the experimental field. The regenerated shoots were sub - cultured on to Shoot Elongation Medium (SEM: MS + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} GA_3 + 2 mg l^{-1} $AgNO_3$) for further growth. Healthy and vigorously growing roots were readily obtained on 1 mg l^{-1} NAA + 0.1 % activated charcoal (RIM: Root Induction Medium) within three weeks of culture at a rooting percentage of 70 - 76%. Successfully acclimatized plantlets (95 - 100%), transferred to the experimental plots, are now well established in the field.

Mulberry (*Morus* sp.) is an important plant of the sericulture industry as the foliage constitutes the chief feed for the silkworm, *Bombyx mori*. Some species of mulberry are also valued for good quality timber and edible fruits. Since efficient regeneration is a prerequisite for genetic manipulation and transformation studies, regeneration in mulberry has been attempted from the axillary buds, hypocotyl, cotyledon, leaf and stem explants (Oka and Ohyama, 1981, 1986; Kim *et al.*, 1985; Mhatre *et al.*, 1985; Ohyama and Oka, 1987; Narayan *et al.*, 1989; Saito and Katagiri, 1989; Chattopadhyay *et al.*, 1990; Jain and Datta, 1992; Saito, 1992; Machi, 1992) using different combinations of cytokinins and auxins. BAP has been reported to be more effective in terms of regeneration percentage than kinetin or zeatin (Oka and Ohyama, 1986; Mhatre *et al.*, 1985; Patel *et al.*, 1983).

Thidiazuron (TDZ), originally developed by A.G. Schering for utilization as a cotton defoliant (Arndt *et al.*, 1976), is known to mimic the effects of both cytokinins and auxins on growth and differentiation of cultured explants. TDZ has been employed for the induction of callus, shoot regeneration, somatic embryogenesis in cultured explants and on intact seedlings, protoplast culture and *in vivo* formation of outgrowths from roots and at the crown, in many herbs, shrubs and trees (see Huetteman and Preece, 1993; Lu, 1993; Murthy *et al.*, 1998). In mulberry, our earlier work has shown TDZ to be stimulatory for bud induction (Tewari *et al.*, 1999; Kapur *et al.*, 2000). Although micropropagation as a means of regeneration is quite popular in mulberry, varietal variations are immense. In this paper we report regeneration by TDZ application in two commercially valuable, relatively recalcitrant, Indian mulberry cultivars, K2 and DD, from the hypocotyl, cotyledon and leaf explants.

Seeds of *Morus indica*, i.e. K2 and DD, were collected from six- to seven-year old, apparently healthy looking, field-grown plants and stored in a desiccator at room temperature. Seeds were surface sterilized with 0.1% mercuric chloride (Qualigens, India) for 8 min, rinsed 5 - 6 times and imbibed in sterile distilled water for 24h prior to culture. Seeds

were sown on MS basal medium (Murashige and Skoog, 1962) or a hormone supplemented medium. For seed germination, cultures were kept in diffused light. The germination percentage was calculated after 15 days of inoculation. Hypocotyl and cotyledon explants were excised from 10-day old, *in vitro* raised seedlings. The leaf explants were obtained from three different sources, (a) field grown plants, (b) *in vitro* grown 30-day old seedlings, and (c) *in vitro* maintained axillary bud cultures.

Explants were cultured on MS medium containing 3% sucrose (w/v) and were supplemented with hormones. The pH of the medium was adjusted to 5.8 and autoclaved at 104 Kpa at 121 ℃ for 15 min. Cultures were maintained at 25 ± 1 ℃ under a daily photoperiodic regime of 16h light and 8h darkness. Four cool white fluorescent tubes (Philips, TL40 W/54) provided a light intensity of 65 μmol $m^{-2}s^{-1}$.

Explants displaying initiation of shoots were transferred to Shoot Elongation Medium (SEM: MS + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} GA_3 + 2 mg l^{-1} $AgNO_3$) for further growth of shoots. Multiple shoots were then excised individually and subcultured on the Root Induction Medium (RIM: 1 mg l^{-1} NAA + 0.1 % activated charcoal). Shoots with 1cm long roots were scored as rooted plantlet. Plantlets with well-developed roots were transferred to earthen pots containing autoclaved soil : Soilrite (1:1), and maintained in the culture room for about a month before transferring to field.

Seeds of *M. indica* cvs. K2 and DD were germinated on MS medium and differentiation capabilities of various explants was investigated. Various combinations BAP, kinetin, GA_3, NAA, IBA, IAA, 2,4-D and TDZ were tested and it was found that TDZ was most suitable for initiating multiple shoots.

When the amount of TDZ in the medium was varied from 1 to 10 μM, 9-10 shoots were induced per explant from the hypocotyl and cotyledon explants on 5 μM TDZ (**Fig. 1**). Shoots were formed at the cut ends of the hypocotyl (**Fig. 2A**) and the base of the cotyledon (**Fig. 2B**). At higher concentrations of TDZ (10 μM), many explants developed white papillae on their entire surface but did not form shoots. Leaf explants derived from *in vitro* grown axillary buds showed a maximum of 50% (K2) and 66% (DD) regeneration on 2.5 μM TDZ (**Fig. 1**). Regeneration could not be obtained from the root, petiole and internode explants on any of the media tested.

Explants were cultured on 5 μM TDZ for a period ranging from 5- to 45-days and thereafter, subcultured on SEM. Observations were made after 20-days of culture on SEM. The regeneration frequency of hypocotyl and cotyledon explants increased with an increase in duration of culture up to 10-days (**Fig. 3**). Thereafter, many shoot primordia were formed which did not develop into shoots. The leaf explants responded differently and there was no regeneration till 15 days of culture; shoot buds were

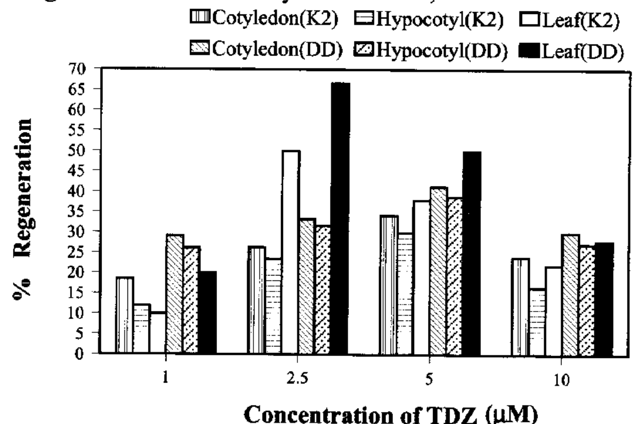

Fig. 1 Regeneration of shoots from cotyledon, hypocotyl and leaf explants of *Mours indica* cvs. K2 and DD on different concentrations of TDZ.

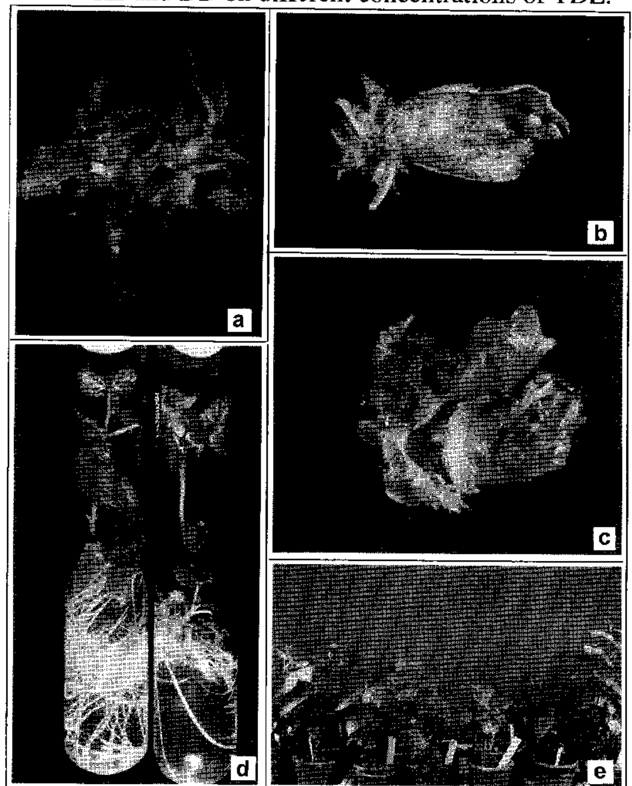

Fig. 2 A-E Differentiation of *M. indica* cvs. K2 and DD to TDZ. **A & B**, Shoot regeneration from hypocotyl and cotyledon explants, respectively, in *M. indica* cv. K2, on 5 μM TDZ; **C**, Shoot regeneration from the axillary bud derived leaves of *M. indica* cv. DD on 2.5 μM; **D**, Rooting of shoots in *M. indica* cv. DD on medium with (left) and without (right) 0.1% activated charcoal; **E**, Acclimatized plantlets of *M. indica* cv. K2 10 weeks after transfer to pots.

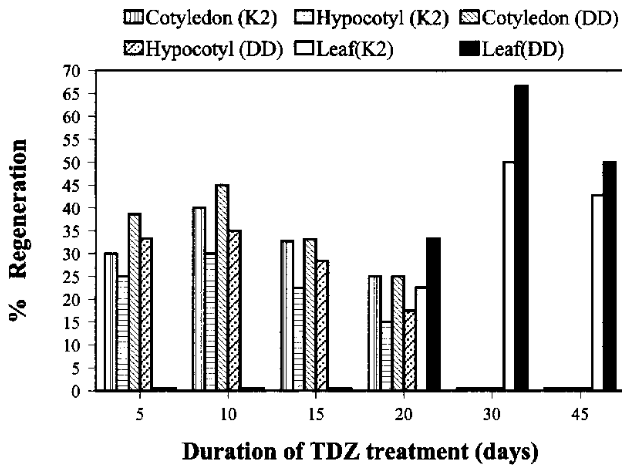

Fig. 3 Effect of TDZ treatment on regeneration frequency of cotyledon, hypocotyl and leaf explants of *M. indica* cvs. K2 and DD .

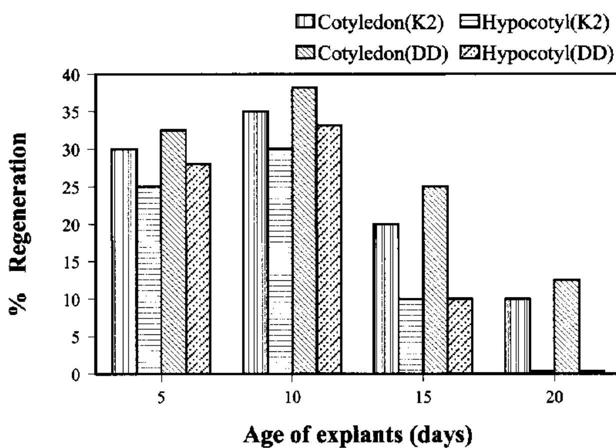

Fig. 4 Variation in regeneration response of cotyledon and hypocotyl explants of *M. indica* cvs. K2 and DD with the age of explant at the time of culture .

obtained after 21-days and the optimal response was obtained after 30-days of culture (**Fig. 4**). Upon continuous culture on TDZ for 45 days, callus developed at the cut margins of the leaf and at the base of the regenerating shoots (see Kapur *et al.*, 2000).

The 7-day old leaf explants taken from *in vitro* maintained axillary buds were more efficient in terms of regeneration percentage than the leaf explants derived from the *in vitro* raised seedlings, or from the field-grown plants (**Table 1**). In leaves derived from the seedlings, 7-8 adventitious buds developed from the base of the leaf, and at the base as well as along the veins from field-plants. However, from axillary bud derived leaves, the entire cut margin differentiated adventitious buds (15-20 buds/explant), thus indicating the high morphogenic potential of the later explant.

The regenerated shoots were sub-cultured on SEM for further growth. Multiple shoots were excised and sub-cultured individually for rooting

Table1. Regeneration response of leaves of *M. indica* cv. K2 and DD derived from different sources and cultured on 2.5 μM TDZ for 30 days.

Source of Leaf Explants	% Regeneration	
	K2	DD
In vivo plants	25 ± 2.89	35 ± 1.65
In vitro seedlings	20 ± 0.00	30 ± 3.19
In vitro axillary buds	50 ± 3.06	66 ± 2.60

on RIM. Healthy and vigorously growing roots were readily obtained within three weeks of culture at a rooting percentage of 70-76% (**Fig. 2D**). To realize the full potential of this protocol for genetic transformation purposes, the *in vitro* regenerated plantlets were transferred to the earthen pots (**Fig. 2E**) and observed carefully for any morphological variability. Successfully acclimatized plantlets (95-100%) appeared phenotypically normal and are presently well established in the field.

In our initial studies on *M. indica* cvs. K2, RFS175, S1 and *M. multicaulis* cv. Goshoerami, the regeneration capabilities of the internode (stem segment), leaf and petiole explants from mature (7- to 8-year old) plants were investigated. MS (Murashige and Skoog, 1962) medium was found superior to B5 (Gamborg *et al.*, 1968) medium. Although extensive callusing was obtained from all the explants, shoot regeneration via dedifferentiation was sporadic. Only 10% internodes developed shoots (on 2 mg l^{-1} BAP + 1 mg l^{-1} NAA) while none of the leaves and petiole regenerated on any of the hormonal combinations tested (Bhatnagar, 1998). Regeneration from stem segments could not be obtained in the experiments performed by Patel *et al.*, (1983) and Mhatre *et al.*, (1985), although a regeneration frequency of 53% has been reported by Oka and Ohyama, (1986) from young leaves of *M. alba*, 76% in *M. alba* (Kim *et al.*, 1985), 20% in *M. alba* (Mhatre *et al.*, 1985), and up to 50% regeneration from the stem segments of *M. alba* cv. S1 by Narayan *et al.* (1989).

Thidiazuron (TDZ) has been shown to stimulate shoot organogenesis from several woody species (see Huetteman and Preece, 1993). Thus, employing the seedling-derived young explants of *M. indica* cvs. K2 and DD, the effect of various growth regulators, especially of TDZ, was investigated. TDZ at a concentration of 5 μM, induced highest percentage of adventitious bud formation from both the hypocotyl and cotyledon explants. Multiple shoots emerged at the base of the cotyledon and from the cut ends of the hypocotyl. At higher

concentrations white-papillae like structures were formed on many explants, and the overall percentage of bud formation was reduced. Young tissue was more responsive in terms of frequency of shoot formation. TDZ (5 μM) for ten days was sufficient for the induction of adventitious buds. Thereafter, sub-culture on TDZ free SEM, lead to the formation of multiple shoots. As prolonged culture on TDZ is not beneficial, use of primary and secondary medium has been reported e.g., in pear (Singha and Bhatia, 1988), apple (Fasola *et al.*, 1989), *Populus* (Russell and McCown, 1986) and *Rhododendron* (Preece and Imel, 1991).

As against the hypocotyl and cotyledon explants, the leaf explants showed optimal response at 2.5 μM of TDZ for 30 days. Although, at higher concentrations the number of adventitious buds increased, they failed to elongate as also reported by Pijut *et al.* (1991) in *Pinus strobus*. Of the various sources of leaf explants, the response from the leaves derived from axillary buds, cultured on BAP 1 mg l^{-1}, was almost double than those obtained from *in vivo* or field plants and also from *in vitro* seedlings. This may be attributed to the inductive effect of BAP present in the pre-culturing medium. No shoots could be induced in the BAP untreated explants (Oka and Ohyama, 1981; Kim *et al.*, 1985; Mhatre *et al.*, 1985; Narayan *et al.*, 1989). Similarly, Sugimura *et al.* (1999) have reported enhanced regeneration by pre-culturing on BAP, while Thinh and Katagiri, (1994) have reported stimulatory effect of seeds grown on TDZ in Mexican mulberry. Shoots were rooted on NAA and activated charcoal according to Tewari *et al.*(1999).

In conclusion, a highly efficient and rapid regeneration of shoots from the hypocotyl, cotyledon and leaf explants could be obtained on TDZ supplemented medium. The protocol described here can be used for large-scale multiplication of the recalcitrant varieties of mulberry. The highly regenerative callus obtained from the leaf and the multiple shoots regenerated from the hypocotyl, cotyledon and leaf explants are suitable material for genetic manipulation both via *Agrobacterium*-mediated co-cultivation and direct gene transfer by particle bombardment.

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