Research Article

Improved method of *in vitro* regeneration in *Leucaena leucocephala* – a leguminous pulpwood tree species

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ABSTRACT

Leucaena leucocephala is a fast growing multipurpose legume tree used for forage, leaf manure, paper and pulp. Lignin in *Leucaena* pulp adversely influences the quality of paper produced. Developing transgenic *Leucaena* with altered lignin by genetic engineering demands an optimized regeneration system. The present study deals with optimization of regeneration system for *L. leucocephala* cv. K636. Multiple shoot induction from the cotyledonary nodes of *L. leucocephala* was studied in response to cytokinins, thidiazuron (TDZ) and N^6-benzyladenine (BA) supplemented in half strength MS (½-MS) medium and also their effect on *in vitro* rooting of the regenerated shoots. Multiple shoots were induced from cotyledonary nodes at varied frequencies depending on the type and concentration of cytokinin used in the medium. TDZ was found to induce more number of shoots per explant than BA, with a maximum of 7 shoots at an optimum concentration of 0.23 μM. Further increase in TDZ concentration resulted in reduced shoot length and fasciation of the shoots. Liquid pulse treatment of the explants with TDZ did not improve the shoot production further but improved the subsequent rooting of the shoots that regenerated. Regenerated shoots successfully rooted on ½-MS medium supplemented with 0.54 μM α-naphthaleneacetic acid (NAA). Rooted shoots of *Leucaena* were transferred to coco-peat and hardened plantlets showed ≥ 90 % establishment in the green house. **[Physiol. Mol. Biol. Plants 2009; 15(4) : 311-318]** *E-mail : bm.khan@ncl.res.in*

Key words : *Cotyledonary nodes, Multiple shoot induction, Pulse treatment, TDZ*

INTRODUCTION

Leucaena leucocephala is a fast growing, multipurpose, nitrogen fixing tree legume widely distributed throughout the tropics and subtropics. It is a native to Mexico (De Candolle, 1967) and is adapted to wide range of agro-climatic conditions of the world. It is used as a forage crop and its wood as a raw material for pulp and paper industry (Lopez *et al*., 2008). Global annual production of paper has increased more than three fold in the past forty years, amounting to a total production of 354 million tons in 2004 (FAOSTAT, 2005-06). Due to growing demand for pulp by paper industry and scarcity of cellulosic fibrous raw material in India, there is a need for high cellulosic high pulp yielding plants like *Leucaena,* which can grow in wide range of climatic and soil conditions (Malik *et al.,* 2004). *Leucaena* is a potential alternative to conventional sources of pulp (Owofadeju and Onilude, 2005). *Leucaena* is relatively rich in lignin, which is an undesirable component in pulp, that adversely influences the quality of paper produced (Thomas, 1970; Dean and Eriksson, 1992; Casler *et al.,* 2002). Lignin removal from the wood during processing by mechanical and chemical means is very expensive and cumbersome besides releasing many toxic compounds in the environment (Rastogi and Dwivedi, 2006). Also due to its intimate association with the cell wall polysaccharides, cellulose and hemicellulose, interferes with the digestibility of these carbohydrates by animals (Albrecht *et al.,* 1987). Identification and multiplication of elite trees of *Leucaena* with low lignin content will be a potential boost to paper and pulp industry. Also, there is a need for developing transgenics with altered or reduced lignin for its in-expensive and eco-friendly removal from the pulp (Rastogi and Dwivedi, 2006).

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Regeneration of complete plants through tissue culture has made it possible to introduce foreign genes in to plant cells and recover transgenic plants. Cotyledonary nodes are more responsive to multiple shoot induction compared to other nodes of the seedlings (Hussain *et al.,* 2007). Regeneration of shoots from meristem explants after *Agrobacterium* infection is a simple and relatively efficient method for transformation in a number of leguminous species like, *Vigna mungo* (Saini *et al.,* 2003), *Lotus japonicus* (Oger *et al.,* 1996), *Acacia mangium* (Xie and Hong, 2002), *Glycine max* (Olhoft *et al.,* 2003; Paz *et al.,* 2004) and *L. leucocephala* (Rastogi and Dwivedi, 2003; 2006). However, genetic transformation of *Leucaena* through *Agrobacterium*- or particle bombardment-mediated methods requires a simple, reproducible and efficient regeneration system.

In *L. leucocephala,* shoot proliferation from the lateral buds from mature trees (Goyal *et al.,* 1985), seedling node segments (Dhawan and Bhojwani, 1985; Puthur *et al.,* 1998) and cotyledons (Saafi and Borthakur, 2002) was reported. In all the previous studies involving *in vitro* shoot regeneration from *L. leucocephala*, BA was the choice as the plant growth regulator. In addition, the number of shoots regenerated on BA was either low (3-4 shoots per explant) or the time taken for the plantlet formation was rather long (ranging from 31 to 172 days). Though TDZ was shown to induce multiple shoots in many tree species (Huetteman and Preece, 1993), no study was undertaken to assess the *in vitro* response of *L. leucocephala* to TDZ.

The present study compares the multiple shoot induction from cotyledonary nodes of *L. leucocephala* cv. K636 in response to TDZ and BA and also effect of these cytokinins on *in vitro* rooting of the regenerated shoots. The cultivar K636 was a selection from Hawaii (Bray *et al.,* 1988). The cultivar produces erect boles suitable for timber production and is highly tolerant to cold and psyllid infestation. This *in vitro* regeneration method from the cotyledonary nodes with high frequency can be used for mass propagation of the species and can find potential application in *Agrobacterium* as well as particle bombardment-mediated transformation.

MATERIALS AND METHODS

Explant preparation

Seeds of *L. leucocephala* cv. K636 were collected from a single source plant at National Chemical Laboratory, Pune, India. The seeds were sun-dried for 1 week and healthy seeds were treated with concentrated sulfuric acid for 7 min followed by 4-5 times washing with sterile distilled water. The seeds were then treated with 0.1 % (w/v) Mercuric Chloride for 10 min followed by 3-4 washes with sterile distilled water under laminar flow. The seeds were incubated in sterile distilled water in 300 mL culture bottle in dark at 25 ^{o}C on a rotary shaker at 90 rpm. After 24 h, imbibed seeds showing radicle emergence were shifted to culture bottles containing 50 mL of ½-MS (half strength of major and minor salts and full strength of vitamins of MS) (Murashige and Skoog, 1962) semi-solid medium supplemented with 2 % (w/v) sucrose and 1.5 % (w/v) glucose as carbon sources and the medium was gelled with 0.7 % (w/v) agar type I (HiMedia, Mumbai, India). Cotyledonary node explants were prepared from 1 week old *in vitro* seedlings by cutting out the radicle and epicotyls with sterile blade retaining cotyledonary node and cotyledons (Fig. 1A).

Multiple shoot induction from the cotyledonary nodes on continuous culture

Cotyledonary nodes with attached cotyledons were cultured in 300 mL screw-capped culture bottles containing ½-MS medium supplemented with thidiazuron (TDZ) (0.05 to 2.27 µM) or BA (N^6-benzyladenine) (2.22 – 13.32 µM). As TDZ at lower concentrations is more effective (Mundhara and Rashid, 2006), therefore direct comparison of TDZ with BA at the same concentration was not possible. ½-MS basal medium devoid of growth regulators served as control. Sucrose (2 %, w/v) and glucose (1.5 %, w/v) were added as carbon sources. Observations on the percentage of explants produced 2 or more shoots, average number of shoots per explant and the mean shoot length were recorded on 30th day after incubation.

Multiple shoot induction by liquid pulse treatment

In another experiment, cotyledonary nodes were given liquid pulse treatment of TDZ in ½-MS for induction of multiple shoots and to improve their subsequent rooting. Cotyledonary nodes were dipped in ½-MS liquid medium supplemented with TDZ (0.45 – 2.27 µM) for a period of 8, 16 or 24 h. Higher concentrations of TDZ were selected as its lower levels (<0.45 µM) were found ineffective for pulse treatment. Hormone free ½-MS was used as control. After pulse treatment, explants were blotted on sterile filter paper and transferred to hormone free ½-MS medium containing sucrose 2 % (w/v) and glucose 1.5 % (w/v) for regeneration of multiple shoots. A stock solution of TDZ (1 mM) was

prepared by dissolving 22 mg TDZ (Sigma) in few drops of 1N KOH and made volume to 100 ml with sterile distilled water. Observations on the average number of shoots per explant and the mean shoot length were recorded on 30th day after incubation.

In vitro rooting

In preliminary studies on rooting, regenerated shoots measuring above 2 cm in length were excised from the mother explants (cotyledonary nodes) and transferred to MS medium at its half or full strength and supplemented with indole-3-aceticacid (IAA), indole-3-butyricacid (IBA) or α-naphthaleneacetic acid (NAA) at varied concentrations. Among all the treatments, rooting in terms of percent rooting, earliness in rooting and number of roots per shoots was superior in ½-MS medium supplemented with NAA (0.54 µM) (data not shown). In all further experiments, this pre-optimized medium was used for rooting of shoots. The percentage of rooting and the number of roots formed per shoot was recorded on 20th day after incubation.

Growth regulators, BA and NAA were added to the media before autoclaving and TDZ was filter sterilized with 0.22 µm filters and added to autoclaved medium. Media were sterilized by autoclaving at 121°C temperature and 15 psi pressure for 20 min after adjusting the pH to 5.8. All the cultures were incubated in a culture room maintained at 25±2 °C temperature and 16 h light period provided by cool white fluorescent lamps (Philips Ltd., India) at a light intensity of 24.4 µmol $m^{-2}s^{-1}$.

The *in vitro* shoots with well developed root system were washed with running tap water to remove agar sticking to them and transferred to plastic cups (250 mL) containing sterile coco-peat (Bio-Agro Ferticons, Pune, India), covered with poly ethylene bags and shifted to growth chamber maintained at 25±2 °C under continuous light at an intensity of 24.4 µmol $m^{-2}s^{-1}$ for hardening. After 2 weeks, poly ethylene covers were cut at two top corners and after another two weeks the covers were removed completely. The hardened plantlets then were transferred to round pots (20 cm Diameter x 20 cm Ht.) containing sterile sand: soil (1:1) mixture and shifted to green house for acclimatization.

Statistical analysis

Each treatment contained a minimum of 25 replicates and each experiment was repeated for a minimum of three times. All the data were analyzed using ANOVA and Critical Difference (CD) was calculated at 1 % level using tukey's test (Snedecor and Cochran, 1967).

RESULTS

About 90 % of the seeds of *L. leucocephala* cv. K636 showed imbibition and sprouting within 24 h. When sprouted seeds with radicle emergence were transferred to 300 mL culture bottles containing ½-MS medium showed vigorous growth and could attain an average height of about 5 cm in a week. For multiple shoot

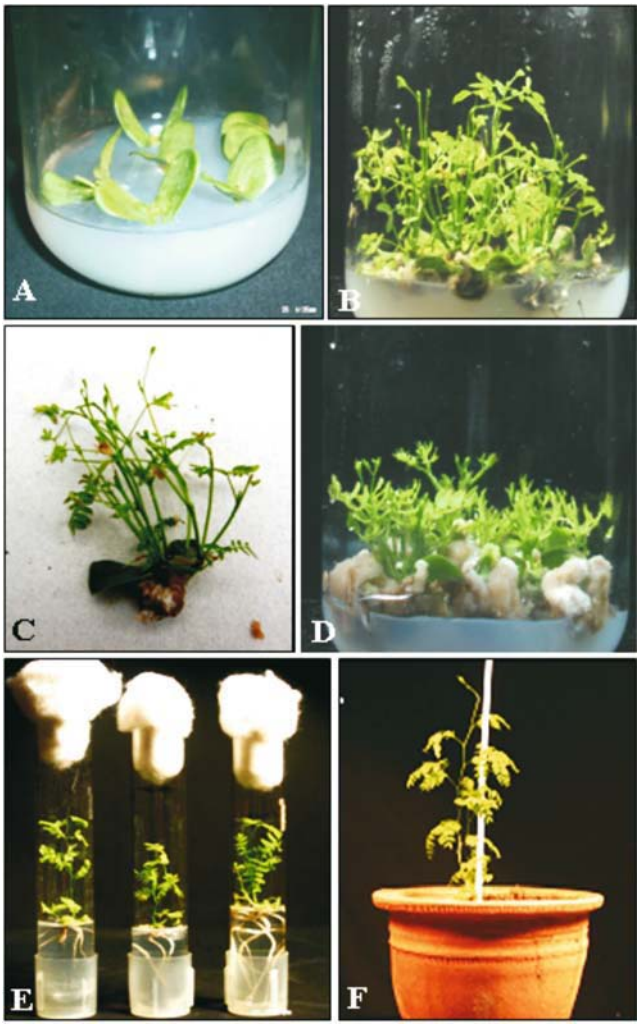

Fig. 1. *In vitro* shoot regeneration in *L. leucocephala*. A: Cotyledonary nodes, B-C: Multiple shoot induction in cotyledonary nodes on ½-MS + TDZ (0.23 µM), D: Shortened and fasciated shoots of *Leucaena* on ½-MS + TDZ (0.45 µM), E: Rooted shoots of *Leucaena* on ½-MS + NAA (0.54 µM) and F: Hardened *in vitro* propagated plant of *L. leucocephala* in Sand: soil mixture.

induction, cotyledonary nodes with the two cotyledons, excised from one week old axenic seedlings, were used as explants.

Multiple shoot induction from cotyledonary nodes on continuous culture

More than 95 % of the inoculated explants (cotyledonary nodes) in each treatment showed either single or multiple shoot regeneration irrespective of the growth regulator used. The data on percentage of explants showing shoot regeneration either single or multiples were found to be non-significant. Axillary bud swelling was observed after 4 days of incubation in the control as well as in cytokinin treatments. Explants on the hormone free ½-MS medium, as a consequence of axillary shoot bud activation, gave rise to only 1 or 2 shoots per explant in 30 days culture period. While in treatments supplemented with cytokinin (either TDZ or BA), multiple shoots induced in the axils of the inoculated explants (Fig. 1B,C). Number of explants produced 2 or more shoots increased with increase in the concentration of cytokinin in the medium (Table 1). Though all the inoculated explants on ½-MS + TDZ (1.36 μM) produced multiple shoots, the over all number of regenerated shoots was lesser compared to ½-MS + TDZ (0.14 or 0.23 μM). The number of shoots regenerated per explant also depended on type and concentration of the cytokinin used. In general, number of regenerated shoots increased with increase in the concentration of cytokinin in the medium up to an optimum level. Number of shoots increased from 1.55 in control to 6.98 (maximum) in the medium supplemented with TDZ (0.23 μM) (Table 1). With further increase in TDZ concentration in the medium, the number of shoots regenerated per explant decreased. But at the highest concentration of TDZ (2.27 μM), shoot number increased slightly due to the formation of adventitious shoots which showed poor elongation. These shoots further showed necrosis on sub-culture. Also, at higher concentrations of TDZ (0.45 – 2.27 μM), profuse callus formation was observed at the base of the explants. The response of number of shoots showed similar trend in BA treatments as that of TDZ though the shoot number was less than that of TDZ. Among BA treatments, BA at 8.89 μM effected higher number of shoots (4.93) per explant.

There was a gradual decrease in the average shoot length of the regenerated shoots with the increase in the concentration of TDZ (Table 1). However, the decrease in average shoot length among BA levels was non-significant. Shoots regenerated on BA treatments were healthy and measured above 3 cm length on an average. Incorporation of higher concentrations of TDZ (0.45 to 2.27 μM) in the medium resulted in the regeneration of fasciated shoots, which failed to elongate. The fasciated shoots were short and thick, light green in colour with small leaflets which showed frequent fall (Fig. 1D).

Multiple shoot induction by liquid pulse treatment

The number of shoots produced per explant also improved with the addition of TDZ in liquid ½-MS used for pulse treatment. Though the response of multiple shoot induction gradually increased with increase in the concentration of the TDZ in liquid ½-MS, the average number of shoots per explant was less when compared with continuous culture of explants at similar concentrations of TDZ. While the average shoot length of the regenerated shoots decreased with the addition of TDZ in liquid ½-MS medium used for pulse treatment. The maximum response, 4.37 shoots per explant was observed when the explants were given a pulse treatment of ½-MS medium containing TDZ (2.27 μM) for 8 h (Table 2).

***In vitro* rooting**

The regenerated shoots of *L. leucocephala* from different BA and TDZ treatments, showed both shoot elongation as well as robust root induction on ½-MS medium supplemented with NAA (0.54 μM) (Fig. 1E). The regenerated shoots, from different BA and TDZ treatments showed varied degrees of rooting response. In general, percent rooting decreased in shoots regenerated on the media supplemented with cytokinins and the response was still lower with shoots proliferated on higher concentrations of cytokinins (Table 1). In case of shoots regenerated on media in continuous culture, TDZ in the medium strongly inhibited the subsequent rooting of the shoots. After 20 days of culture, shoots from half strength MS medium with BA (2.22 - 4.44 μM) showed a maximum of 86.7 % of rooting, which was on par with that of control (88.9 %). Percentage rooting was lower from the shoots regenerated on lower concentrations of TDZ *i.e.* 0.05 – 0.23 μM than in control. While shoots regenerated on higher concentrations of TDZ (0.45 – 2.27 μM) did not root at all. Shoots regenerated on TDZ (0.05 μM) produced a maximum of 3.25 roots per shoot (Table 1). Shoots regenerated from the cotyledonary nodes given pulse treatment with hormone free ½-MS showed a substantial increase in rooting response (97.5 %) and number of roots per shoot (3.79) (Table 2). Shoots rooted *in vitro* were transferred to pots containing sterile coco-peat for

Table 1. Effect of TDZ and BA on *in vitro* shoot regeneration from cotyledonary nodes of *L. leucocephala* cv. K636 and on subsequent rooting

TDZ (µM)	BA (µM) (µM)	% of explants produced 2 or more shoots	Number of shoots per explant	Av. shoot length (cm)	% of shoots rooted	Number of roots per shoot
0.00	-	45.0	1.55	3.99	88.9	1.33
0.05	-	66.7	3.55	3.13	76.6	3.25
0.14	-	82.7	6.18	2.33	63.3	2.2
0.23	-	93.3	6.98	2.10	43.3	1.45
0.45	-	93.3	5.13	1.43	0	0
1.36	-	100.0	4.67	1.12	0	0
2.27	-	90.7	5.80	0.96	0	0
-	2.22	48.0	2.33	4.15	86.7	1.60
-	4.44	48.0	3.87	3.45	86.7	1.20
-	6.67	60.0	4.13	2.98	60.0	1.06
-	8.89	73.3	4.93	3.09	60.0	0.80
-	11.11	80.0	4.07	3.05	53.3	0.60
-	13.32	80.0	4.27	3.21	20.0	0.33
SEm±	5.12	0.52	0.21	4.71	0.22	
CD (p=0.01)	9.20	1.03	0.86	8.51	0.87	
		**	**	**	**	**

Basal medium: ½-MS + sucrose 2 % (w/v) + glucose 1.5 % (w/v).
**Significant at 1 % level

Table 2. Effect of liquid pulse treatment of TDZ on *in vitro* shoot regeneration from cotyledonary nodes of *L. leucocephala* cv. K636 and on subsequent rooting

½-MS + TDZ (µM)	8 h		16h		24h		% of shoots rooted	No. of roots per shoot
	Av. No. of shoots	Av. shoot length (cm)	Av. No. of shoots	Av. Shoot length (cm)	Av. No. of shoots	Av. shoot length (cm)		
0.00	1.75	4.38	1.00	4.50	1.5	3.75	97.50	3.79
0.45	2.85	2.37	2.90	2.86	2.31	3.40	82.69	2.33
1.36	3.62	1.87	4.30	1.75	4.15	1.46	84.54	2.62
2.27	4.37	2.12	4.60	1.71	4.55	1.42	86.88	2.45

	Av. No. of shoots	Av. shoot length	% shoots rooted	No. of roots per shoot
SEm±	0.27	0.47	4.64	0.29
CD (p=0.01)	0.65	1.22	2.02	0.95
	**	**	**	*

Medium used for regeneration: ½-MS + sucrose 2 % (w/v) + glucose 1.5 % (w/v).
**Significant at 1 % level; *Significant at 5 % level.

hardening and above 90 % of plantlet survival was recorded under green house conditions (Fig. 1F). The regenerated plants did not show any visible phenotypic variation among themselves.

DISCUSSION

In the present study, single or multiple shoots were induced from decapitated cotyledonary nodes of *Leucaena* with attached cotyledons depending on the treatment. Removal of apical dominance might have triggered activation of accessory buds to give rise to more number of shoots. It was observed in our preliminary studies that removal of cotyledons or cutting of half of the cotyledons significantly reduced the number of multiple shoots regenerated (unpublished result). This suggests mobilization of nutrients from cotyledons to axils and aided in proliferation of axillary buds in the present study. This assumption was further confirmed by the report of Gulati and Jaiwal (1994) in which they reported more number of shoots from embryo axes with both the cotyledons attached compared to those without cotyledons with delayed regeneration. In the present study, mostly single shoots were induced from the explants cultured on ½-MS medium necessitating the requirement of cytokinin for multiple shoot induction as reported earlier (Hussain *et al.,* 2007). Cytokinins were reported to play a key role in DNA synthesis and cell division which might be the reason for induction of multiple shoots (Skoog and Miller, 1957).

Multiple shoots were induced from cotyledonary nodes by the use of BA and TDZ at varied frequencies depending on the type and concentrations of cytokinin (Mundhara and Rashid, 2006). Though BA (2.22 – 8.89 μM) has been commonly used for induction of multiple shoots and direct shoot organogenesis studies in legumes (Prakash *et al.,* 1994; Saafi and Borthakur, 2002), higher response of multiple shoot induction was observed in TDZ (0.14 - 2.27 μM) treatments in the present study. TDZ has been reported to induce bud break, axillary and adventitious shoots (Huetteman and Preece, 1993; Pradhan *et al.,* 1998). TDZ may act through modulation of the endogenous plant growth regulators, modification in cell membrane, energy levels, nutrient uptake, or nutrient assimilation (Murthy *et al.,* 1998). TDZ has been used for multiple shoot induction from cotyledonary nodes of *Populus* (Russell and McCown, 1986), *Quercus robur* L. (Chalupa, 1988), *Cercis Canadensis* (Yusnita *et al.,* 1990) and *Dalbergia sissoo* (Pradhan *et al.,* 1998). TDZ also has been used for shoot regeneration from immature cotyledon explants of *Macadamia* (Mulwa and Bhalla, 2006) and *Poplar* (Cseke *et al.,* 2007). Urtubia *et al.* (2008) reported improved shoot regeneration from hypocotyls slices of *Prunus,* when TDZ was combined with IBA as compared to TDZ alone in the regeneration medium.

Explants inoculated in all the BA treatments produced multiple shoots directly from axils without intervening callus formation as observed by Saafi and Borthakur (2002). Slight to moderate amount of callus formation was observed at the bases of the inoculated explants at higher concentration of BA (11.11 - 13.32 μM) as observed earlier (Dhawan and Bhojwani, 1985). While, profuse amount of callusing was noticed at the bases of the explants inoculated in higher concentrations of TDZ (0.45 – 2.27 μM) in continuous culture. Multiple shoots were induced as clusters of stunted and fasciated shoots, failed to elonagate and differed markedly from normal shoots. They appear as several shoots fused together similar to the phenomenon observed in silver maple (Preece *et al.,* 1991) and *Dalbergia* (Pradhan *et al.,* 1998). Fasciation of the shoots might be due to the high cytokinin activity and stability of TDZ due to its resistance to cytokinin oxidase (Heutteman and Preece, 1993). TDZ is also reported to inhibit various cytokinin oxidases (Armstrong, 1994). In a study on *Salix nigra*, Lyyra *et al.* (2006) reported that buds developed from un-expanded inflorescence explants on Woody Plant Medium supplemented with TDZ (0.5 μM) did not elongate, while buds developed on BA (2.25 μM) could elongate in to shoots. In *Nothapodites foetida*, multiple shoots induced on TDZ (0.23 μM) needed to be transferred to reduced level of TDZ (0.05 μM) to achieve elongation (Sateeshkumar and Seeni, 2000). In the present study, a short period exposure of explants to TDZ for 8 h was found to be enough to elicit multiple shoots. Further, unlike in case of continuous culture of explants in higher concentrations of TDZ, shoots regenerated by pulse treatment were found to be healthy with no fasciation.

The *in vitro* formed shoots of *Leucaena* were successfully rooted on a NAA (0.54 μM) containing medium and this lower concentration of NAA resulted in satisfactory rooting of regenerated shoots as reported earlier (Naik *et al.,* 2000). Rooting was higher in case of shoots regenerated on ½-MS medium devoid of growth regulators and the rooting response decreased on use of BA or TDZ for multiple shoot induction. As the concentration of BA or TDZ in the regeneration medium increased, the rooting response of the shoots decreased. Shoots regenerated on higher concentrations

of TDZ (>0.45 µM) did not root at all. These results are in conformity with earlier findings by Naik *et al.* (2000). In few other studies, poor root initiation was also observed from shoots of *Leucaena* regenerated on higher concentrations of BA (Dhawan and Bhojwani, 1985; Saafi and Borthakur, 2002). In the present study, shoots regenerated in BA and in lower concentrations of TDZ (<0.14 µM) showed both shoot elongation and the rooting without any callus formation at the shoot-root junction. Higher rooting response in shoots regenerated from explants given pulse treatment might be due leaching of phenolic compounds and rooting inhibitors from the shoots.

Present study demonstrates successful use of cotyledonary nodes for induction and proliferation of multiple shoots. Cotyledonary nodes failed to give multiple shoots in hormone free ½-MS medium and the addition of cytokinins to the medium was found essential for multiple shoots induction. *L. leucocephala* cotyledonary nodes carry a high potential for rapid multiple shoot regeneration on medium containing lower concentrations of TDZ (0.05 or 0.23 µM). Also, a 8 h exposure of the cotyledonary nodes to 2.27 µM TDZ supplemented in ½-MS medium was adequate for the induction of multiple shoots and for their efficient rooting. As multiple shoots originated from the mass of closely placed shoot initials of axillary meristems, this system could be efficiently used for particle bombardment mediated transformation (Prakash *et al.,* 1994). This efficient and high frequency *in vitro* regeneration system is highly reproducible and can be used for mass propagation and genetic transformation of *L. leucocephala*.

ACKNOWLEDGEMENTS

Financial support in the form of Junior Research Fellowship to N.M. Shaik, M. Arha, S.K. Gupta, P.S. Kulkarni, O.U. Abhilash, T. Rajeshri, Kannan Chinathambi and A. Nookaraju by the Council of Scientific and Industrial Research (CSIR), New Delhi; by University Grants Commission (UGC), New Delhi, India to S. Srivastava and A.K. Yadav is gratefully acknowledged.

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