8

Preconditioning of Nodal Explants in Thidiazuron-Supplemented Liquid Media Improves Shoot Multiplication in *Pterocarpus marsupium* (Roxb.)

Anees Ahmad, Naseem Ahmad, and Mohammad Anis

Abstract

Pterocarpus marsupium Roxb. (Fabaceae), commonly known as "Bijasal" or "Indian Kino," is a potential herbal drug-yielding tree since ancient times. In the present study, an efficient micropropagation system was developed for propagation of this valuable forest tree by pretreating the nodal explant in thidiazuron (TDZ) in half-strength Murashige and Skoog (MS) liquid medium before their inoculation onto the full-strength MS semisolid medium containing meta-Topolin (mT), a new aromatic cytokinin, at different doses either singly or in combination with auxins. Among the treatments tried, nodal explants treated with TDZ (10.0 μM) for 8 days followed by transfer to MS medium containing mT (5.0 μM) + NAA (1.0 μM) were found to be most effective combination in inducing maximum shoot number (11.16 ± 0.16) with an average shoot length (4.9 ± 0.13 cm) and maximum regeneration frequency (85%). A proliferating shoot culture was established by subculturing the original nodal explants on shoot multiplication medium supplemented with meta-Topolin after each harvest of newly formed shoots. For root induction, basal end of isolated shootlets (≥ 4.0 cm) was treated with high dose of indole-3-butyric acid (150.0 μM IBA) solutions for 1 week followed by their transfer to half-strength MS supplemented with low doses of IBA (1.5 μM) in the medium which produced an average of (6.32 ± 0.26) roots with mean root length (4.05 ± 0.27 cm) in 80% shootlets, after 4 weeks of culture transfer. The regenerated plantlets with proper root system were successfully acclimatized to field condition with 75% survival rate.

Keywords

Axillary bud · Meta-Topolin · Morphogenesis · Plant growth regulators · Pretreatment · Regeneration

A. Ahmad · N. Ahmad · M. Anis (✉)
Plant Biotechnology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, UP, India
e-mail: manis.bt@amu.ac.in

N. Ahmad, M. Faisal (eds.), *Thidiazuron: From Urea Derivative to Plant Growth Regulator*, https://doi.org/10.1007/978-981-10-8004-3_8

Abbreviations

BA	6-Benzyladenine
CKs	Cytokinins
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog medium
mT	Meta-Topolin
NAA	α-naphthalene acetic acid
NS	Nodal segment
PGRs	Plant growth regulators
TDZ	Thidiazuron

8.1 Introduction

World Health Organization (WHO) has assessed that approximately 80% of the people of developing countries depend on herbal drugs, and about 25% drugs obtained are from plants resources (Akshay et al. 2014). However, several countries including India are facing acute problems in maintaining the population of medicinal plants because of anthropogenic activities, unrestrained exploitation, pathogen attacks, plant diseases, low seed viability, or more recently climate change and uncontrolled use for making furniture goods, etc. These activities have resulted in the sharp decline in the population of many plant species. *Pterocarpus marsupium* Roxb. (Fabaceae) is a lofty, highly medicinal valued, and multipurpose forest tree of India. An aqueous extract of heartwood contains 5,7,2–4 tetrahydroxy 6–6 isoflavone-glucoside which are potent antioxidant and used to cure cardiovascular diseases, vasodilation, and inhibition of platelet aggregation (Mohire et al. 2007). Ethanolic extract of stem bark contains lupeol, tetradecanoic acid, and octadecadienoic acid which are very reputed components known to have cancer preventive and antitumor properties (Maruthupandian and Mohan 2011). One of the biggest problems with this plant is its low percentage of seed germination because of hard fruit coat and poor seed viability in natural environmental conditions. Micropropagation can offer great advantages over traditional method of plant propagation, and through this approach, a single explant can give rise to many plantlets within few months. Plant regeneration through in vitro techniques is potentially utilized in several areas like roadside tree plantation, forestry, and plant-derived pharmaceutical industries.

In the present study, an attempt has been made to improve regeneration efficiency by giving a pretreatment with thidiazuron (TDZ) followed by their transfer to a secondary medium enriched with various dose of meta-Topolin (mT). The mT, a hydroxylated 6-benzyladenine derivative, is a new phytohormone identified in different plants and extracted from poplar leaves (Strnad et al. 1997). It promotes shoot elongation and multiplication in many plant species (Kubalakova and Strnad 1992; Werbrouck et al. 1996). It has the same mode of action to that of other cytokinins

such as benzyladenine, kinetin, etc. with small difference in molecular structure and has found to be effective in micropropagation system (Strnad et al. 1997). The intention of our study was to examine the role of TDZ in combination with mT on shoot induction and multiplication of axillary buds from nodal explant for developing a reliable in vitro regeneration protocol for procurement of true-to-type and healthy plantlets with maximum survival rate in natural condition.

8.2 Materials and Methods

8.2.1 Establishment of Aseptic Seedlings

Mature winged fruits of *Pterocarpus marsupium* were collected from forest area of Kharsia, District Raigarh (Chhattisgarh). The healthy seeds were excised from the fruits with the help of eastman stripper cutter and washed under running tap water for 15 min, treated with 1% Bavistin, again washed under running tap water for about 15 min, treated with a laboratory detergent (Labolene, Qualigens, India) 5% (*v/v*) for 10 min followed by 5–6 washes with sterilized distilled water. The treated seeds were imbibed in distilled water for 24 h to remove leachates and surface sterilized with 0.1% (*w/v*) $HgCl_2$ for 4 min under aseptic condition following repeated washes with sterile distilled water in order to remove traces of sterilant and placed on ½ MS (Murashige and Skoog 1962) medium for seed germination. Nodal segment (1.0–1.5 cm) was excised from 4-week-old aseptic seedlings and transferred on sterile culture medium containing various growth regulators (Fig. 8.1a, b).

8.3 Media and Culture Condition

The nutrient medium used in all the experiments consisted of MS salts and vitamins with 3% (w/v) sucrose (Qualigens, India) as a sole carbon source for plant growth in vitro and gelled with 0.75% (w/v) bacteriological grade agar (Qualigens, India) for shoot regeneration. The pH of media was adjusted to 5.8 with 1 N NaOH or HCl prior to autoclaving at 121 °C for 18 min. All culture tubes were incubated at 24 ± 2 °C under 16/8 h (light/dark) cycle with a photosynthetic photon flux density (PPFD) of 50 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (Philips, Kolkata, India) with 55 ± 5% relative humidity, regularly checked by thermohygrometer (Testo, India Pvt. Ltd.).

8.4 Experimental Design

The experiments were conducted in two sets; in the first set, 4-week-old aseptic nodal segments were treated in half-strength MS liquid medium containing various doses of TDZ (0.5, 2.5, 5.0, 10.0, or 20.0 μM; Fig. 8.1c) for different durations (4, 8, 12, or 16 days; Table 8.1). The exposure of TDZ was conduct in 50 ml liquid

Fig. 8.1 In vitro regeneration of *Pterocarpus marsupium* (Roxb.). (**a**) A 4-week-old aseptic seedling. (**b**) NS explant obtained from 4-week-old aseptic seedlings. (**c**) NS explant treatment in TDZ containing ½ MS liquid medium. (**d**) Shoots induction from NS explant on MS + mT (5.0 μM), after 4 weeks of culture. (**e**) Shoot induction from TDZ-pretreated NS on MS + mT (5.0 μM), after 4 weeks of culture. (**f**) Shoot multiplication from TDZ-pretreated NS on MS + mT (5.0 μM), after 8 weeks of culture. **g**. Pretreatment of isolated shootlet for root induction on ½ MS (liquid) + IBA (150.0 μM), employing a filter paper bridge. (**h**) An in vitro rooted shootlet on ½ MS (semisolid) + IBA (1.5 μM), after 2 weeks of culture transfer. (**i**) A 4-week-old well-rooted plantlet. (**j**) An acclimatized plantlet (2 months old) in Soilrite

media on a rotatory shaker at 120 rpm for different time periods in optimum culture condition. MS medium without TDZ supplementation served as control. In order to determine the optimal exposure concentration and duration, each culture flask was carefully observed with regard to bud breaking. Following the initial pretreatment, the explants pretreated with optimized exposure dose and duration with TDZ were transferred to a secondary medium. In the second set, TDZ-pretreated nodal explants were inoculated on full-strength MS semisolid medium supplemented with various concentrations of mT (0.5, 2.5, 5.0, 7.5, or 10.0 μM; Table 8.2) singly or in

Table 8.1 Effect of various doses of TDZ on axillary shoot bud breaking from 4-weeks old aseptic nodal segments on half-strength MS liquid medium at different durations

Hormone (μM)	Days			
TDZ	4	8	12	16
0.0	–	–	×	×
0.5	–	–	–	×
2.5	–	+	+	–
5.0	+	+	++	+
10.0	++	+++	+	–
20.0	+	–	×	×

Where, '–' no response; '+' responsive; '++' moderately responsive; '+++' best responsive; '×' explant dead

Table 8.2 Effect of various concentration of mT on multiple shoot induction from pretreated nodal segment on MS medium, after 4 weeks of incubation

Cytokinins (μM)				
TDZ- Pretreated	mT	% Response	Mean shoots/explant	Mean shoot length (cm)
–	0.00	00	0.00 ± 0.00^{f}	0.00 ± 0.00^{e}
–	0.5	10	2.24 ± 0.21^{e}	1.83 ± 0.22^{cd}
–	2.5	30	3.81 ± 0.28^{cd}	2.54 ± 0.20^{b}
–	5.0	55	5.65 ± 0.24^{b}	2.92 ± 0.04^{b}
–	7.5	25	3.37 ± 0.25^{d}	2.44 ± 0.35^{bc}
–	10.0	15	1.68 ± 0.21^{e}	1.56 ± 0.16^{d}
10.0	0.5	25	3.44 ± 0.17^{d}	1.61 ± 0.23^{d}
10.0	2.5	50	5.42 ± 0.10^{b}	2.76 ± 0.06^{b}
10.0	5.0	70	7.53 ± 0.21^{a}	3.53 ± 0.03^{a}
10.0	7.5	35	4.38 ± 0.35^{c}	2.37 ± 0.31^{bc}
10.0	10.0	20	3.70 ± 0.24^{cd}	1.49 ± 0.18^{d}

Values represented means ± SE. Means followed by the same letter within column are not significantly different ($P = 0.05$) using Duncan's multiple range test

combination with IAA or NAA at various concentrations (0.5, 1.0, or 1.5 μM; Table 8.3) for further proliferation and elongation of microshoots. Cultures were subcultured onto the same fresh media after every 3 weeks. Data on percentage of shoot regeneration, number of shoots per explant, and average shoot length were recorded after 4 weeks of subculture.

8.5 In Vitro Rooting

Healthy, well-elongated in vitro raised microshoots of about 4–5 cm in length with three to five fully expanded leaves were isolated from 8-week-old cultures, and rooting was achieved following two-step procedure previously established by Anis et al. (2005) in *P. marsupium* with few modifications. In the first step, the basal end

Table 8.3 Combined effect of various concentrations of IAA or NAA with mT (5.0 μM) for shoot proliferation from pretreated nodal segment on MS medium, after 8 weeks

Plant growth regulators (μM)			% Response	Mean shoots/explant	Mean shoot length (cm)
TDZ- Pretreated	IAA	NAA			
–	0.50		55	6.04 ± 0.21^{f}	3.63 ± 0.14^{de}
–	1.00		60	6.43 ± 0.23^{f}	3.76 ± 0.10^{de}
–	1.50		50	5.85 ± 0.21^{f}	3.55 ± 0.17^{e}
–		0.50	65	7.54 ± 0.16^{e}	3.79 ± 0.08^{de}
–		1.00	70	8.65 ± 0.14^{c}	3.92 ± 0.12^{cd}
–		1.50	60	6.24 ± 0.15^{f}	3.65 ± 0.09^{de}
10.0	0.50		70	7.95 ± 0.04^{de}	3.71 ± 0.11^{de}
10.0	1.00		80	8.80 ± 0.07^{bc}	3.84 ± 0.12^{cde}
10.0	1.50		65	7.74 ± 0.12^{e}	3.58 ± 0.10^{de}
10.0		0.50	80	9.26 ± 0.41^{b}	4.35 ± 0.04^{b}
10.0		1.00	85	11.16 ± 0.16^{a}	4.96 ± 0.13^{a}
10.0		1.50	75	8.34 ± 0.10^{cd}	4.15 ± 0.12^{bc}

Values represented means ± SE. Means followed by the same letter with in column are not significantly different ($P = 0.05$) using Duncan's multiple test

Table 8.4 Effect of higher dose (150.0 μM) of IBA pretreated microshoots followed by transfer to various low doses of IBA in half-strength MS semisolid medium for roots induction, after 4 weeks of transfer

IBA concentrations (μM)	% Response	Mean roots/microshoot	Mean root length (cm)
Control	00	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}
0.0	30	2.36 ± 0.27^{d}	1.64 ± 0.19^{c}
0.5	45	3.83 ± 0.20^{c}	2.85 ± 0.38^{b}
1.0	60	4.44 ± 0.11^{b}	3.27 ± 0.14^{b}
1.5	80	6.32 ± 0.26^{a}	4.05 ± 0.27^{a}
2.0	55	3.57 ± 0.16^{c}	2.55 ± 0.25^{b}

Values represented means ± SE. Means followed by the same letter within column are not significantly different ($P = 0.05$) using Duncan's multiple test

of isolated microshoots was pretreated with high dose of IBA (50, 100, 150, or 200 μM; Fig. 8.1g) in half-strength MS liquid medium with 2% sucrose for 1 week using a filter paper bridge. Thereafter, these microshoots were transferred onto ½ MS semisolid medium supplemented with different low doses of IBA (0.0, 0.5, 1.0, 1.5, or 2.0 μM; Table 8.4) with 0.25% Phytagel and 2% sucrose. The data on percentage of rooting, mean root number, and root length were recorded, after 4 weeks of culture transfer.

8.6 Hardening and Acclimatization

Microshoots with well-developed roots were removed from the culture tube (Fig.8.1i), rinsed with running tap water to remove any adherent agar, and transferred to 10-cm-diameter thermocol cups containing sterile Soilrite™ (Keltech

Energies, Ltd., Bangalore, India). The cup was covered with transparent polythene bags as a safeguard for high humidity and irrigated with ½ MS (liquid) without organic salts and sucrose, after every 2 days. The polythene bags were opened, after a couple of week in order to harden the in vitro raised plantlets. These acclimatized plants were transferred to pots containing normal garden soil and kept at 25 ± 5 °C in incubation chamber for 64 days. Afterward, these potted plantlets were shifted to greenhouse.

8.7 Statistical Analysis

All experiments were based on a completely randomized block design (RBD) and repeated three times with ten replicates for each treatment. The data on various parameters were subjected to one-way analysis of variance (ANOVA) using SPSS version 16 (SPSS Inc., Chicago, USA). The significance of differences among means was carried out using Duncan's multiple range test at $P = 0.05$, and results were expressed as the mean ± SE of three repeated experiments.

8.8 Results

8.8.1 In Vitro Shoot Regeneration

The nodal explant did not show any response of bud break when cultured on a phytohormone-free medium and failed to induce any shoots even after 4 weeks of culture. Explants cultured on half-strength MS liquid medium with various doses of TDZ in a rotatory shaker for different days showed a discernible response on axillary shoot bud breaking. Of the five doses of TDZ tested, 10.0 μM for 8 days was found to be more effective than other treatments in respect to multiple shoot bud induction, and about 96% (data not shown) explants showed maximum response (Table 8.1). Further increase in the dose beyond the optimal level did not improve any bud breaking. When the TDZ-pretreated nodal explant was transferred to full-strength MS media supplemented with various concentrations of mT, a positive effect on shoot differentiation and elongation was recorded. Of the five treatments of mT used, 5.0 μM was found to be more effective where maximum number of shoots (7.53 ± 0.21) with mean shoot length (3.53 ± 0.03 cm) and maximum regeneration frequency (70%) were obtained, whereas in without pretreated explant, about 5.65 ± 0.24 shoots per explant with an average shoot length (2.92 ± 0.04 cm) and 55% regeneration frequency were recorded, after 4 weeks of culture transfer (Table 8.2; Fig. 8.1e, d). Basal callusing was removed during every subculturing as a precautious measure because it retarded shoot multiplication and elongation. Furthermore, we analyzed the combined effect of auxins (IAA or NAA) at various concentrations with the optimal concentration of mT (5.0 μM) for enhancing the shoot proliferation rate. Among all treatments tried, MS medium supplemented with mT (5.0 μM) + NAA (1.0 μM) showed highest number of shoots (11.16 ± 0.16)

with mean shoot length (4.96 ± 0.13 cm) per nodal explant and maximum regeneration frequency (85%), after 8 weeks of culture transfer (Table 8.3; Fig. 8.1f). Average number of shoots per explant with shoot length got increased with an increased concentration up to the optimal dose, whereas gradual decrease in shoot number with shoot length per explant was observed beyond the optimal dose (Table 8.3). The results further confirm that the ratio of auxin/cytokinin and their combination play an important role in plant tissue culture system.

8.8.2 In Vitro Root Induction

In vitro rooting in the regenerated microhoots was a challenging step as none of the isolated microhoots rooted on full MS or its different reducing strengths (1/2 MS, 1/3 MS, 1/4 MS) with or without various concentrations of auxins (IAA, IBA, or NAA, data not shown) added to the medium. A two-step procedure for better induction of root was successfully used. In the first step, the basal end of isolated microshoots was treated with high dose of IBA (150.0 μM; Fig. 8.1g) for 1 week on a filter paper bridge followed by their transfer to the next stage. In the second step, the treated microhoots were transferred on a semisolid medium containing low dose of IBA (1.5 μM) where highest frequency (80%) of root formation and highest number of roots (6.32 ± 0.26) with an average shoot length (4.05 ± 0.27 cm) per microshoot were recorded, after 4 weeks of culture transfer. The roots were healthy and showed numerous secondary root hairs (Fig.8.1i), which helped in establishing plantlets in soils. Therefore, IBA (150.0 μM) pretreated microshoots followed by transfer to lower dose of IBA (1.5 μM) was found to be best as compared to other concentrations used (Table 8.4).

8.8.3 Acclimatization of In Vitro Raised Plantlets

In vitro raised plantlets with fully expanded and healthy leaves were removed from culture tube and transferred to thermocol cups containing sterile Soilrite™ for 4 weeks (Fig. 8.1j). Acclimatization and hardening procedure of in vitro regenerated plantlets was the same as described in materials and methods. The 75% of regenerated plantlets got survived during acclimatization and successfully transferred to pots containing normal garden soil. The plantlets grew well and did not show any morphological variation in growth parameters.

8.9 Discussion

A successful and efficient protocol for axillary shoot multiplication from nodal segment of *P. marsupium* has been established. The nodal segments are a good source of axillary shoot production in tissue culture system, and even a single explant can give rise to multiple copies of true-to-type plantlets within few months. The nodal

explants were first given a high dose of TDZ followed by their transfer to MS medium supplemented with meta-Topolin, a new cytokinin for multiple shoot induction.

Thidiazuron (TDZ—N-phenyl- N′-1,2,3-thiadiazol-5-ylurea) is a synthetic phenylurea having potent cytokinin-like activity and widely used as a phytohormone in in vitro regeneration protocols. It is more effective as compared to other cytokinins for inducing multiple de novo shoots from different explants of several plant species such as *Paulownia* species (Chalupa 1988), many woody trees (Huetteman and Preece 1993), *Pterocarpus marsupium* (Husain et al. 2007), *Vitex negundo* (Ahmad and Anis 2007), *Embelia ribes* (Dhavala and Rathore 2010), and *Dendrocalamus strictus* (Singh et al. 2013). According to Murthy et al. (1995), TDZ modifies the endogenous level of phytohormones especially cytokinins and auxins in plant tissue culture system and affects the metabolism of cytokinins biosynthetic pathways which is responsible for the degradation of endogenous level of elevated concentrations of purine metabolites (Zhang et al. 2005). There are many reports available on TDZ-supplemented media which showed positive response on multiple shoot bud break but failed to shoot elongation in many plants including in *Rhododendron* (Preece and Imel 1991), *Adhatoda beddomei* (Sudha and Seeni 1994), *Dalbergia sissoo* (Pradhan et al. 1998), *Rauvolfia tetraphylla* (Faisal et al. 2005), *Capsicum annuum* (Ahmad et al. 2006), and *Psoralea corylifolia* (Faisal and Anis 2006). But in some cases the use of these highly active cytokinin (Ck) in plant regeneration protocol was limited (Huetteman and Preece 1993). The antagonistic effect of this Ck may be due to the presence of phenyl group which on over exposure beyond the optimal level can lead to many drawbacks such as bunching, fasciation, hyperhydricity, poor microshoot quality, and loss of rooting capacity (Singh and Syamal 2001; Lu 1993).

However, the twofold culture strategy was applied to address the problem of shoot elongation. Primarily, we have evaluated the effect of TDZ on nodal explant for axillary buds breaking. Half-strength MS liquid media supplemented with TDZ (10.0 μM) for 8 days were found to be more responsive than other doses tried. When pretreated nodal segments were transferred on a secondary medium containing mT, a positive change in enhancing shoot multiplication and elongation was observed. Some positive results of mT have been reported in several plant species (Werbrouck et al. 1996; Wojtania 2010; Aremu et al. 2012; Mala et al. 2013; Clapa et al. 2014). It has the same mode of action as BA and kinetin, but due to little difference in molecular structure, it was found to be effective on micropropagation in many plant species (Kubalakova and Strnad 1992; Werbrouck et al. 1996; Strnad et al. 1997). Among the different concentrations of mT tested, the efficient shoot elongation was recorded at 5.0 μM mT where maximum shoot numbers (7.53 ± 0.21) with an average shoot length (3.53 ± 0.03 cm) were recorded in pretreated explants, after 4 weeks of culture transfer. Similar type of culture strategy using shoot bud breaking (primary) and shoot elongation (secondary) medium was effectively applied in many plant species, such as *Malus alba* (Thomas 2003), *Acacia sinuata* (Vengadesan et al. 2002), and *Vitex negundo* (Ahmad and Anis 2007).

Furthermore, we have evaluated the combined effect of cytokinins with auxin for improving the microshoots quality or number for obtaining healthy plantlets of *P. marsupium.* TDZ-pretreated nodal explants when transferred to MS medium augmented with mT (5.0 μM) plus NAA (1.0 μM) in combination were found to be best where highest proliferation rate (11.16 ± 16 shoots) with an average shoot length (4.96 ± 0.13 cm) per explant was achieved, after 8 weeks of culture (Table 8.3; Fig. 8.1f). Our results are in agreement with earlier findings of several workers in many recalcitrant or woody species like *A. catechu* (Kaur et al. 1998), *Eucalyptus grandis* (Luis et al. 1999), *Lagerstroemia parviflora* (Tiwari et al. 2002), *Melia azedarach* (Husain and Anis 2009), *Balanites aegyptiaca* (Siddique and Anis 2009), and *Acacia ehrenbergiana* (Javed et al. 2013). This is an improved and cost-effective protocol than the earlier described by Husain et al. (2008) in *P. marsupium* where only 8.6 shoots per explant with 4.8 cm shoot length were obtained. By repeated subculturing onto the fresh medium, successful healthy shoot cultures were established.

In vitro rooting has been a difficult process in woody trees. Indole-3-butyric acid is known to have stimulatory effect on the multiple root formation due to its preferential uptake, transport, and stability as compared to other auxins and may play an important role in rooting gene activation (Ludwing-Muller 2000). Husain et al. (2008) reported the root induction in BA-regenerated microshoots where a maximum root (3.8) with average length (3.9 cm) was recorded. In several other reports, exogenous application of cytokinins like BA used in micropropagation has negative effect on rooting, resulting in poor acclimatization of many plant species (Werbrouck et al. 1995; Bairu et al. 2008; Amoo et al. 2011). These negative effects are due to the formation of biologically inactive and chemically stable metabolites such as N-glucosides or alanine conjugates (Werbrouck et al. 1995). BA-treated in vitro regenerated shootlets have the propensity of accumulation of BA metabolites as a toxic substance in a basal rooting zone, and these toxic metabolites interfere rooting and acclimatization of in vitro raised plantlets (Werbrouck et al. 1995; Valero-Aracama et al. 2010). But in our study, we have tried mT in place of other CKs for regeneration of multiple and healthy microshoots. The mT-treated regenerated shootlets gave highest positive response in respect to root number as well as root length with 75% of successful acclimatization. According to Werbrouck et al. (1996), mT produce relatively less stable and low toxic metabolites as compared to other CKs. Bairu et al. (2011) postulated that mT-derived shootlets produce more roots with respect to BA because of small differences in hydroxyl groups of meta-Topolin. Thus an improved regeneration protocol has been established using a new cytokinin, meta-Topolin.

8.10 Conclusion

The research finding emphasizes the role of TDZ and mT on in vitro regeneration of a woody legume for ensuring better transplant success due to proper continuity of conducting tissues of shoot and root in the absence of callusing. The thidiazuron-pretreated nodal explants, when transferred onto mT-supplemented medium,

showed positive results on in vitro regeneration such as shoot multiplication, shoot elongation, rooting and acclimatization, and improving physiological parameters. The rapid in vitro propagation using TDZ and mT will not only help in conservation but also low-cost multiplication of this tree species. This research work might be an initial step in the breakthrough of in vitro propagation revolution which is yet to firmly establish its roots in plant biotechnology field.

Acknowledgment Anees Ahmad is thankful to the Department of Biotechnology, Govt. of India, New Delhi, for financial support as a Junior Research Fellow under the Major Research Project (Vide No. BT/PR2189/PBD/17/744/2011 Dated 19.12.2012). The award of UGC-BSR Faculty Fellowship (2017) to M. Anis is duly acknowledged.

References

Ahmad N, Anis M (2007) High frequency axillary shoot multiplication from mature tree of *Vitex negundo* L. Agrofor Syst 71:195–200

Ahmad N, Siddique I, Anis M (2006) Improved plant regeneration in *Capsicum annuum* L. from nodal segments. Biol Plant 50(4):701–704

Akshay KR, Sudharani N, Anjali KB, Deepak TM (2014) Biodiversity and strategies for conservation of rare, endangered and threatened medicinal plants. Research and Reviews: J Pharmacogn Phytochem 2:12–20

Amoo S, Finnie JF, Van Staden J (2011) The role of meta-topolins in alleviating micropropagation problems. Plant Growth Regul 63:197–206

Anis M, Husain MK, Shahzad A (2005) In vitro plantlet regeneration of *Pterocarpus marsupium* Roxb. an endangered leguminous tree. Curr Sci 88:861–863

Aremu AO, Bairu MW, Dolezal K, Finnie JF, Van Staden J (2012) Assessment of the role of meta-topolins on in vitro produced phenolics and acclimatization competence of micropropagated 'Williams' banana. Acta Physiol Plant 34:2265–2273

Bairu MW, Stirk WA, Dolezal K, Van Staden J (2008) The role of topolins in micropropagation and somaclonal variation of banana cultivars 'Williams' and 'Grand Naine' (Musa spp. AAA). Plant Cell Tissue Organ Cult 95:373–379

Bairu MW, Novak O, Dolezal K, Van Staden J (2011) Changes in endogenous cytokinins profiles in micropropagated *Harpagophytum procumbens* in relation to shoot-tip necrosis and cytokinins treatments. Plant Growth Regul 63:105–114

Chalupa V (1988) Large scale micropropagation of *Quercus robur* L. using adenine type cytokinins and thidiazuron to stimulate shoot proliferation. Biol Plant 30:414–421

Clapa D, Fira A, Simu M, Vasu LB, Buduroi D (2014) Improved *in vitro* propagation of *Paulownia elongata*, *P. fortunei* and its interspecific hybrid *P. elongata* x *P. fortunei*. Bull UASVM Hortic 71(1):6–14

Dhavala A, Rathore TS (2010) Micropropagation of *Embelia ribes* Burm f. through proliferation of adult plant axillary shoots. In Vitro Cell Dev Biol Plant 46:180–191

Faisal M, Anis M (2006) Thidiazuron induced high frequency axillary shoot multiplication in *Psoralea corylifolia*. Biol Plant 50:437–440

Faisal M, Ahmad N, Anis M (2005) Shoot multiplication in *Rauvolfia tetraphylla* L. using thidiazuron. Plant Cell Tissue Organ Cult 80:187–190

Huetteman CA, Preece JE (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tissue Organ Cult 33:105–119

Husain MK, Anis M (2009) Rapid in vitro multiplication of *Melia azedarach* L. (a multipurpose woody tree). Acta Physiol Plant 31:765–772

Husain MK, Anis M, Shahzad A (2007) In vitro propagation of Indian Kino (*Pterocarpus marsupium* Roxb.) using thidiazuron. In Vitro Cell Dev Biol Plant 43:59–64

Husain MK, Anis M, Shahzad A (2008) *In vitro* propagation of a multipurpose leguminous tree (*Pterocarpus marsupium* Roxb.) using nodal explants. Acta Physiol Plant 30:353–359

Javed SB, Anis M, Khan PR, Aref M (2013) In vitro regeneration and multiplication for mass propagation of *Acacia ehrenbergiana* Hayne: a potential reclaiment of denude arid lands. Agrofor Syst 87:621–629

Kaur K, Verma B, Kant U (1998) Plants obtained from the Khair tree (*Acacia catechu* wild) using mature nodal segment. Plant Cell Rep 17:427–429

Kubalakova M, Strnad M (1992) The effect of aromatic cytokinins (populins) on micropropagation and regeneration in vitro. Biol Plant 34:578–579

Lu CY (1993) The use of thidiazuron in tissue culture. In Vitro Cell Dev Biol Plant 29:92–96

Ludwing-Muller J (2000) Indole-3-Butyric acid in plant growth and development. Plant Growth Regul 32:219–230

Luis PBC, Adraine CMGM, Silvica BRCC, Anna Christina MB (1999) Plant regeneration from seedling explants of *Eucalyptus grandis* x *E. urophylla*. Plant Cell Tissue Organ Cult 56:17–23

Mala J, Machova P, Cvrckova H, Karady M, Novak O, Mikulık J, Dostal J, Strnad M, Dolezal K (2013) The role of cytokinins during micropropagation of wych elm. Biol Plant 57:174–178

Maruthupandian A, Mohan VR (2011) GC-MS analysis of some bioactive constituents of *Pterocarpus marsupium* Roxb. Int J Chem Tech Res 3(3):1652–1657

Mohire NC, Salunkhe VR, Bhise SB, Yadav AV (2007) Cardiotonic activity of aqueous extract of heartwood of *Pterocarpus marsupium*. Indian J Exp Biol 45:532–537

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

Murthy BNS, Murch SJ, Saxena PK (1995) Thidiazuron- induced somatic embryogenesis in intact seedlings of peanut (*Arachis hypogaea*): endogenous growth regulator levels and significance of cotyledon. Physiol Plant 94:268–276

Pradhan C, Kar S, Pattnaik S, Chand PK (1998) Propagation of *Dalbergia sissoo* Roxb. through in vitro shoot proliferation from cotyledonary nodes. Plant Cell Rep 18:122–126

Preece JE, Imel MR (1991) Plant regeneration from leaf explant of *Rhododendron* P J M hybrids. Sci Hortic 48:159–170

Siddique I, Anis M (2009) Direct plant regeneration from nodal explant of *Balanites aegyptiaca* L. (Del.)- valuable medicinal tree. New For 37:53–62

Singh SK, Syamal MM (2001) A short pre-culture soak in Thidiazuron or Forchlorfenuron improves axillary shoot proliferation in rose micropropagation. Sci Hortic 91:169–177

Singh M, Jaiswal VS, Jaiswal U (2013) Thidiazuron-induced anatomical changes and direct shoot morphogenesis in *Dendrocalamus strictus* Nees. Scro Res Ann Rep 1:44–47

Strnad M, Hanus J, Vanek T, Kaminek M, Ballantine JA, Fussell B, Hanke DE (1997) Meta-Topolin, a highly active aromatic cytokinin from poplar leaves (*Populus x canadensis* Moench, cv Robusta). Phytochemistry 45:213–218

Sudha GC, Seeni S (1994) In vitro multiplication and field establishment of *Adhatoda beddomei* CB Clark, rare medicinal plant. Plant Cell Rep 13:203–207

Thomas TD (2003) Thidiazuron induced multiple shoot induction and plant regeneration from cotyledonary explants of mulberry. Biol Plant 46:529–533

Tiwari SK, Kashyap MK, Ujjaini MM, Agrawal AP (2002) In vitro propagation of *Lagerstroemia parviflora* Roxb. from adult tree. Indian J Exp Biol 40:212–215

Valero-Aracama C, Kane M, Wilson S, Philman N (2010) Substitution of benzyladenine with meta-topolin during shoot multiplication increases acclimatization of difficult- and easy– to acclimatize sea oats (*Uniola paniculata* L.) genotypes. Plant Growth Regul 60:43–49

Vengadesan G, Ganapathi A, Anand PR, Anbazhagan RV (2002) In vitro propagation of *Acacia sinuata* (Lour.) Merr. via cotyledonary node. Agrofor Syst 55:9–15

Werbrouck SPO, Van der Jeugt B, Dewitte W, Prinsen E, Van Onckelen HA, Debergh PC (1995) The metabolism of benzyladenine in *Spathiphyllum floribundum* 'Schott Petite' in relation to acclimatisation problems. Plant Cell Rep 14:662–665

Werbrouck SPO, Strnad M, Van Onckelen HA, Debergh PC (1996) Meta-topolin, an alternative to benzyladenine in tissue culture. Physiol Plant 98:291–297

Wojtania A (2010) Effect of meta-topolin on in vitro propagation of *Pelargonium* x *hortorum* and *Pelargonium* x *hederaefolium* cultivars. Acta Soc Bot Pol 79:101–106

Zhang CG, Li W, Mao YF, Zhao DL, Dong W, Guo GQ (2005) Endogenous hormonal levels in *Scutellaria baicalensis* calli induced by thidiazuron. Russ J Plant Physiol 52(3):345–351