In Vitro Morphogenesis of Woody Plants Using Thidiazuron

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Abstract

Thidiazuron (TDZ) has been in use for in vitro shoot regeneration, in particular, recalcitrant woody perennials. Owing to its superiority over natural cytokinins in plant regeneration, TDZ has been the plant growth regulator (PGR) of choice for mature tissues. In majority of the tree species, TDZ has induced regeneration via axillary shoot proliferation, adventitious shoot organogenesis and somatic embryogenesis. Interestingly, TDZ has evoked different regeneration routes from the same explant at different concentrations. In addition, various other factors like pretreatment, explant type, maturity, orientation, TDZ concentration, combination with other PGRs and organic additives interact synergistically to promote shoot regeneration. Despite being potent PGR, supra-optimal level of TDZ produces shoot abnormalities like vitrification/hyperhydricity (stunted shoots) or fasciation (fused shoots). In shoot organogenesis and somatic embryogenesis, prolonged exposure to TDZ resulted in callus necrosis or reversal of shoot buds or somatic embryos to callus. Therefore, this review paper is intended to bring out the effectiveness of TDZ in woody plant tissue culture. The authors also emphasize on various interacting factors to minimize the negative consequences of TDZ treatment.

Keywords

Thidiazuron · Woody plants · Shoot regeneration · Hyperhydricity · Fasciation

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Abbreviations

BA	Benzyladenine
GA_3	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kin	Kinetin
MS	Murashige and Skoog
NAA	α-naphthalene acetic acid
PGR	Plant growth regulator
SE	Somatic embryo
SSE	Secondary somatic embryo
TDZ	Thidiazuron

10.1 Introduction

Discovery and the application of plant growth regulators (PGRs) have revolutionized the field of plant tissue culture. Shoot regeneration of plants under in vitro conditions depends on the specific balance of PGRs (natural and synthetic) such as auxins and cytokinins. Cytokinins are N^6-substituted adenines that regulate cell division and cell differentiation in plants. Based on the chemical structure of the side chain, cytokinins fall into two categories: isoprenoid and aromatic. Benzyladenine (BA) or kinetin (6-furfurylaminopurine, Kin) are the most frequently used aromatic natural cytokinins (Kieber and Schaller 2014) in tissue culture systems but with limited success in woody plant regeneration (van Staden et al. 2008). On the other hand, synthetic analogues of natural cytokinins have evoked striking regeneration potential in recalcitrant woody tissues (Ďurkovič and Mišalová 2008). Thidiazuron (TDZ) is a synthetic phenyl urea (N-phenyl-N′-1,2,3-thiadiazol-5-yl-urea) with growth-promoting activity like cytokinins in plant cell cultures (Mok et al. 1982). Manufactured primarily for its use as a defoliant for cotton (Arndt et al. 1976), TDZ has induced a variety of morphogenetic responses in plant cells in vitro till date (Huetteman and Preece 1993; Zhang et al. 2016). TDZ was highly effective than other cytokinins at very minimal concentrations because of its stability to withstand degradation by cytokinin oxidases (Mok et al. 1987).

Propagation of woody plants by in vitro techniques is a challenging task, as majority of the tree species are recalcitrant. TDZ supplementation in the culture media significantly reduced the limitations encountered with the regeneration of recalcitrant tissues. Explants from mature trees that are non-regenerative using other cytokinins underwent rapid proliferation in the presence of TDZ (Huetteman and Preece 1993). Thus TDZ has played a significant role in ex situ conservation of trees by facilitating regeneration via micropropagation, shoot organogenesis and somatic embryogenesis (Murthy et al. 1998; Cuenca et al. 2000; Bunn et al. 2005). The morphogenetic route in TDZ media is greatly dependent on the factors like

pretreatment, explant type, maturity, orientation, TDZ concentration, combination with other PGRs and organic additives. This paper comprehends the successful application of TDZ in woody plant tissue culture with critical reviews on various parameters to be considered under different culture conditions when the media is amended with TDZ.

10.2 Micropropagation

Woody perennials are predominantly exploited for their timber products. Forest trees find their utilization in agroforestry (Kuzovkina and Volk 2009), phytoremediation (Perttu and Kowalik 1997; Vervaeke et al. 2003) and production of biofuels (Mashkina et al. 2010). Continued over-exploitation and indiscriminate logging of trees for the welfare of humankind had resulted in the shrinking of forest resources (Newton et al. 1999). Around 9000 tree species worldwide are threatened with extinction (Oldfield et al. 1998). In vitro propagation has emerged as a boon for the ex situ germplasm conservation of economically important tree species (Vinoth and Ravindhran 2013). It circumvents the need to exploit natural resources, thus preserving the tree populations in their natural habitats. Micropropagation is a useful means of mass propagating plantlets from young or mature tree tissues with a lower risk of genetic instability, than those obtained by other regeneration pathways (Rao and Lee 1986). Establishment of plantlets via micropropagation involves three phases, namely, axillary shoot proliferation, shoot elongation and rooting of in vitro shoots. TDZ plays a promising role in the entire regeneration processes. Protocols employing TDZ for the axillary shoot proliferation of various tree species until the 1990s have been described in Huetteman and Preece (1993). Table 10.1 reports the use of TDZ in micropropagation of woody plants from the late 1990s till date.

Table 10.1 Successful application of TDZ in micropropagation of woody plants

Species	Explant	Effective TDZ concentration (μM)	References
Dendrocalamus strictus	Node	2.27	Singh et al. (2001)
Leucaena leucocephala	Immature zygotic embryo	0.26	Pal et al. (2012)
	Cotyledonary node	0.23	Shaik et al. (2009)
Pinus massoniana	Node	4.0	Wang and Yao (2017)
Salix tetrasperma	Node	2.5	Khan and Anis (2012)
Sterculia urens	Node, cotyledonary node	0.90	Devi et al. (2011)
Vitex trifolia	Node	5.0	Ahmed and Anis (2012)

10.2.1 Axillary Shoot Proliferation

In micropropagation, TDZ revives the meristematic activity of lateral buds suppressed by apical dominance and promotes multiple shoot formation (Table 10.1). TDZ concentration and exposure are pivotal in modulating axillary shoot proliferation in woody plants (Fig. 10.1). In majority of the woody species, shoot proliferation showed an increasing trend with the increase in TDZ concentration (Singh et al. 2001; Faisal et al. 2005; Ahmad and Anis 2007). But the optimum concentration varied depending on the species as indicated in Table 10.1. In *Salix tetrasperma*, commonly called as 'Indian Willow', nodal explants cultured on 2.5 μM TDZ produced 90% regeneration response with 4.53 shoots per explant (Khan and Anis 2012). In *Leucaena leucocephala*, a fast-growing tree legume, the optimum concentration of TDZ varied based on the type of explants used (immature zygotic embryo (1.5 μM) and cotyledonary node (0.23 μM)) (Shaik et al. 2009; Pal et al. 2012). Unlike other cytokinins which are active at higher concentrations, TDZ is highly effective in axillary shoot proliferation at very low concentrations ranging from 0.1 nM to 10 μM. The physiological role of TDZ in shoot proliferation is attributed to the accumulation of endogenous cytokinins, conversion of cytokinin nucleotides to potent biologically active nucleosides and enhanced translocation of auxins to axillary meristems (Capelle et al. 1983; Thomas and Katterman 1986; Murch and Saxena 2001).

TDZ promotes shoot proliferation in different explants which are otherwise unresponsive to other PGRs. For example, in bamboo, nodal explants collected from the base to the shoot apex of in vitro germinated seedlings produced multiple shoot buds in TDZ liquid media. On the contrary, in TDZ-free media, only basal node showed slight response, while nodes closer to the apex failed to regenerate (Singh et al. 2001). In *Sterculia urens*, cotyledonary node from 15-day-old seedlings and nodal explants from 1-month-old seedlings were comparatively regenerative. The difference was observed only in the mean number of regenerated shoots per se with TDZ × explant age interaction, where the juvenile tissues produced maximum number of shoots (Devi et al. 2011). It gave a clear understanding that the effect of TDZ in promoting regeneration competence is well pronounced irrespective of the physiological gradients along the stem and the maturity of explants.

Prolonged culture under in vitro conditions will lead to epigenetic variation, thereby affecting the genetic integrity of micropropagated plantlets. TDZ favours rapid shoot proliferation over other adenine-type cytokinins by reducing the culture cycle. For instance, in *S. urens*, TDZ produced 19 shoots per cotyledonary node in 2 harvests within 45 days when compared to 16 shoots in 3 harvests (63 days) using BAP (Devi et al. 2011). This study signified the high cytokinin activity of TDZ as reported by Huetteman and Preece (1993).

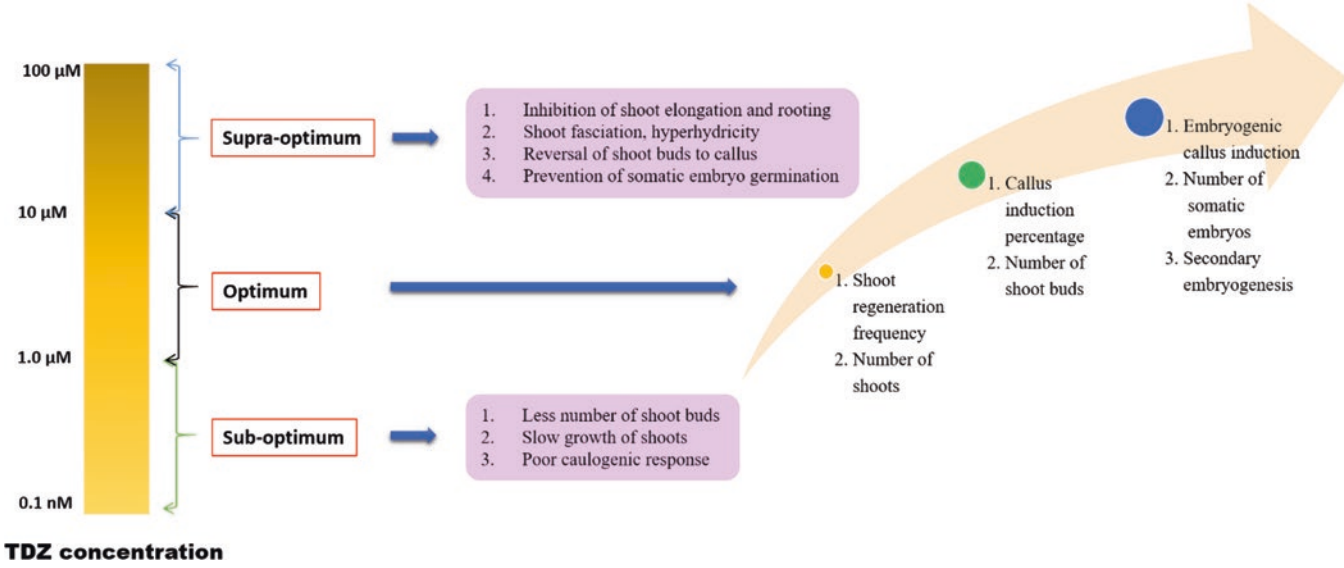

Fig. 10.1 Dose-dependent role of TDZ in shoot regeneration of woody plants. Within the optimum range, increase in TDZ had a positive correlation with different morphogenetic responses. At supra-optimal range, negative consequences were observed, while at suboptimal range, poor regeneration response is recorded. This schematic diagram does not take into account the genotype dependency

10.2.2 Shoot Elongation

Shoot elongation was found to have an inverse relation to TDZ concentration and exposure in solid as well as liquid medium (Fig. 10.1). Shoot buds initiated at concentrations greater than the optimum level (0.26 μM) of TDZ failed to elongate (Pal et al. 2012) or showed basal callusing, shoot fasciation and necrosis (Shaik et al. 2009), even after the removal of TDZ from the culture media in further subcultures. The mean number of elongated shoots was highly dependent on the dosage of TDZ in the shoot initiation medium (Singh et al. 2001; Shaik et al. 2009). Inhibition of shoot elongation by TDZ, in general, was found to be reversed upon transfer to PGR-free medium or to media supplemented with adenine-type cytokinins and auxins. In *Vitex trifolia*, a shrub by tree used by tribes and native medical practitioners, highest shoot regeneration frequency was achieved when explants exposed to TDZ for 7 days were repeatedly subcultured on MS media containing a combination of BA (1.0 μM) and NAA (0.5 μM). Prolonged exposure in TDZ media for more than 7 days resulted in fasciated or distorted shoots with occasional reversal of shoots into callus or necrotic tissues (Ahmed and Anis 2012). Similar results were observed in *S. tetrasperma,* primarily used for fuel wood and timber, when nodal explants were cultured in TDZ media for more than 4 weeks (Khan and Anis 2012). Shoot fasciation (fused shoots) is attributed to the high cytokinin activity and resistance/inhibition of cytokinin oxidases by TDZ (Huetteman and Preece 1993).

10.2.3 Rooting

Shoots regenerated from TDZ medium often failed to root in auxin-free basal medium. A negative correlation was commonly observed between TDZ concentration and mean rooting percentage (Fig. 10.1). In vitro shoots of *L. leucocephala* regenerated on lower concentrations of TDZ (0.05–0.23 μM) showed poor rooting response compared to control, while those from higher concentrations (0.45–2.27 μM) did not root at all (Shaik et al. 2009). This phenomenon was observed due to the residual effect of TDZ on the cells at the shoot base which retained their shoot organogenic capacity even after the removal of TDZ (Meyer and van Staden 1988). Rooting of such shoots required transfer to media containing different dosages of auxins like IBA, NAA or IAA. In a study by Khan and Anis (2012), in vitro regenerated shoots of willow species underwent rooting only in medium containing 0.5 μM IBA. Contrastingly, shoots induced from media supplemented with TDZ higher than 0.6 μM showed no root development in rooting medium containing NAA or IBA and Kin (Pal et al. 2012). Rooting of in vitro shoots is a vital phase which affects their subsequent acclimatization and survivability in field conditions. Though TDZ promotes rapid proliferation of axillary shoots, dosage and exposure time in the shoot induction medium are two critical parameters to be monitored to facilitate proper root development.

10.3 Adventitious Shoot Organogenesis

Adventitious shoot regeneration is a promising approach for genetic transformation in tree species. Standardized protocol for adventitious shoot bud initiation is a prerequisite to introduce desired traits into elite tree genotypes. Shoot organogenesis occurs through three vital developmental stages, namely, competence (dedifferentiation), determination (redifferentiation) and morphogenesis (Sugiyama 1999). Transition to different stages occurs by the balance of exogenously supplied PGRs. Among the PGRs, TDZ is highly influential in regenerating shoot buds from the explants of woody plants, in particular, recalcitrant genotypes (Table 10.2).

10.3.1 Callus Induction (Dedifferentiation)

Callus induction is the preliminary step to achieve shoot organogenesis from woody plants as explants from mature tissues fail to produce shoot buds de novo. TDZ is an effective PGR known for its greater ability to induce callus (Table 10.2). Various explants respond differently to TDZ concentration in the media. In a study on regeneration of *Eucalyptus grandis* × *E. urophylla*, 2.0 μM TDZ induced callus in 95–100% of the explants (hypocotyl, cotyledon, primary leaves and cotyledonary node) (Barrueto Cid et al. 1999). Despite the difference in the types of explant, callus was fast-growing, homogeneous and highly regenerative. The regeneration potential was however maintained only until 30 days of TDZ exposure, and further extension beyond this time frame resulted in callus necrosis. Shoot regeneration in beeches (*Fagus* sp.), commercially important trees for timber production, was achieved using internodal explants through callus culture (Cuenca et al. 2000). In this study, callus with higher organogenic capacity was induced from different genotypes of *F. orientalis* and *F. sylvatica*. TDZ was thus able to overcome the limitations offered by explant type and genotypes in producing organogenic callus.

10.3.2 Shoot Bud Initiation (Redifferentiation)

Induction of adventitious shoot buds occurs either from the explant (direct) or from the organogenic callus (indirect). Leaf, petiole and cotyledon were the most suitable explants for adventitious shoot organogenesis. In *Robinia pseudoacacia*, seeds and hypocotyl were used as explants, wherein seeds formed shoots via callusing from the root region, while hypocotyl exhibited de novo meristematic activity in TDZ medium (Hosseini-Nasr and Rashid 2004). Explants from juvenile tissues of in vitro grown seedlings were highly competent to shoot regeneration compared to the in vivo explants from mature trees (Mante et al. 1989; Liu and Pijut 2008; Kumar et al. 2010a, b; Aggarwal et al. 2015). Explant preparation and orientation in medium are vital factors to be considered in shoot organogenesis. Shoot initials were induced from cotyledon explants primarily in the proximal regions devoid of embryonal axis (Mante et al. 1989; Sujatha et al. 2008). Excision of proximal part from cotyledon

Table 10.2 Adventitious shoot organogenesis in woody plants using TDZ

Regeneration route	Species	Explant	Effective TDZ concentration (μM)	References
Direct	*Acacia crassicarpa*	Phyllode	2.27	Yang et al. (2006)
	Alstroemeria sp.	Leaf	10.0	Lin et al. (1997)
	Hagenia abyssinica	Leaf	<1.0	Feyissa et al. (2005)
	Nothapodytes foetida	Leaf	1.36	Thengane et al. (2001)
		Hypocotyl	2.27	
	Populus alba × *P. berolinensis*	Stem	0.45	Wang et al. (2008)
	Paulownia tomentosa	Leaf	22.7	Corredoira et al. (2008)
	Pongamia pinnata	Cotyledon	11.35	Sujatha et al. (2008)
	Populus tremula	Root	0.04	Vinocur et al. (2000)
	Prunus serotina	Leaf	4.4	Hammatt and Grant (1998)
	P. avium	Leaf	9.08	Liu and Pijut (2008)
	P. serotine		22.7	
	Ricinus communis	Hypocotyl	1.0	Ahn et al. (2007)
Indirect	*Acacia mangium*	Embryo axes, cotyledon, leaf, petiole and stem	4.55	Xie and Hong (2001)
	Eucalyptus grandis × *E. urophylla*	Hypocotyl, cotyledon, primary leaves and cotyledonary node	2.0	Barrueto Cid et al. (1999)
	Fagus sylvatica	Leaf	2.3	Vieitez and San José (1996)
	F. orientalis	Internode	4.5	Cuenca et al. (2000)
	F. sylvatica			
	Hagenia abyssinica	Leaf	>1.0	Feyissa et al. (2005)
	Jatropha curcas	Leaf	2.27	Deore and Johnson (2008)
	Pinus strobus	Zygotic embryo	6.0	Tang and Newton (2005)
	Prunus persica × *P. davidiana*	Leaf	9.08	Zhou et al. (2010)
	Robinia pseudoacacia	Seed	1.0	Hosseini-Nasr and Rashid (2004)
	Santalum album	Node	0.6	Singh et al. (2016)
	Ulmus americana	Leaf	22.5	George and Tripepi (1994)

explants failed to induce shoot buds even with the supplementation of TDZ. Culturing of cotyledon explants with abaxial surface in the medium was more effective and generated more buds than the adaxial side (Sujatha et al. 2008). Leaf explants responded contrastingly with higher shoot regeneration frequency when the adaxial side was in contact with the media (Kim et al. 2007). With petiole explants, horizontal positioning induced more shoot buds than vertical placement as more surface area was in contact with the medium (Kumar et al. 2010b). Leaf explants closer to the shoot apex formed adventitious buds not only on the petiolar end but also on the laminar end (Corredoira et al. 2008). Similarly, internodal explants displayed decrease in regeneration frequency and shoot bud number basipetally along the stem (Cuenca et al. 2000).

Culturing of explants in TDZ media under varied photoperiod conditions also affected the regeneration frequency. Pre-culturing of leaf explants under darkness for a short duration improved the regeneration percentage in *Ficus carica* (Kim et al. 2007). Genotype dependency was another notable factor that interplayed with TDZ in adventitious shoot regeneration. In trees like apple, cherry, beech and poplars, TDZ was prominently superior over other cytokinins in shoot bud induction across different species. However, the range of TDZ concentration varied greatly across different genotypes (Hammatt and Grant 1998; Cuenca et al. 2000; Magyar-Tábori et al. 2010; Aggarwal et al. 2015).

In majority of the tree species, amendment of TDZ in the shoot induction medium (liquid or solid) hastened the regeneration process (Sriskandarajah and Goodwin 1998). TDZ supplementation exhibited dose-dependent response with increase in the regeneration efficiency until a saturation point at 10 μM (Fig. 10.1). Cuenca et al. (2000) reported significant interaction between bud-forming capacity and TDZ concentration in beech, with optimal concentration being 4.5 μM. There are exceptions where the optimum TDZ concentration shifted to above 10 μM. In vitro regeneration frequency of *Paulownia tometosa* (empress tree) from leaf explants was maximum in the induction media supplemented with 22.7–27.3 μM TDZ (Corredoira et al. 2008) and that of *Ulmus americana* (American elm) at 22.5 μM TDZ (George and Tripepi 1994).

TDZ pretreatment promoted autonomous competence in cells, that is, to regenerate in the absence of PGRs. An interesting observation was recorded in *Hagenia abyssinica*, where TDZ concentrations less than 1.0 μM induced direct shoot buds while above 1.0 μM produced callus. The calli exhibited 96–100% regeneration when TDZ was removed or supplemented at lower concentration (0.1 μM) (Feyissa et al. 2005). In another study, TDZ-pretreated calli derived from cotyledons, hypocotyls or cotyledonary nodes were able to regenerate shoot buds in PGR-free medium (Barrueto Cid et al. 1999). Therefore, it is clearly evident that TDZ could act as a sole PGR that positively influenced the regeneration ability without requiring the need for combination with other auxins/cytokinins in the induction medium. Even the report discussed below describing the combination of auxins/cytokinins with TDZ for shoot bud induction indicated the prominent role of TDZ in the synergistic interaction. In a study by Deore and Johnson (2008), TDZ (2.27 μM) + BA (2.22 μM) + IBA (0.49 μM) induced maximum adventitious shoot buds (53.5%) in

leaf disc explants. Elimination of TDZ from this combination drastically reduced the shoot bud induction and resulted in higher callus induction, thereby displaying its necessity for the promotion of organogenic competence.

Conclusively, in spite of TDZ being potent cytokinin to initiate shoot organogenesis in recalcitrant tissues, explant type, explant age, preprocessing of explants, physiological gradients in parent tissue, orientation in the medium, photoperiod and genotype exhibited synergistic interactions to promote regeneration competence in cells.

10.3.3 Shoot Morphogenesis and Rooting

Amendment of TDZ greater than 10 μM in the shoot induction medium suppressed the elongation of shoots, thereby reducing the number of harvestable shoots. In *Fagus* sp., TDZ greater than 8.9 μM produced bud clusters on callus, which were dense and compact mass of minute buds. At still higher concentration, shoot buds appeared as green nodular organogenic patches which failed to elongate into shoots (Cuenca et al. 2000). This was attributed to the compaction of shoot buds due to spatial constraint. The shoot clusters also required longer time to develop into individual shoots (Liu and Pijut 2008; Kumar et al. 2010b). In some cases, shoots were hyperhydric and fasciated (Pawlicki and Welander 1994; Caboni et al. 1996; Dobránszki et al. 2002).

Combination of auxins, adenine-type cytokinins or organic additives was found to be necessary to promote elongation of shoot buds that were suppressed by TDZ. Regeneration of macadamia trees (*Macadamia tetraphylla*) from cotyledon explants underwent three developmental stages including callus formation, shoot bud initiation and shoot regeneration. Supplementation of coconut milk (2%) + TDZ (15 μM) improved both callus and shoot bud induction. However, the conversion of buds into individual shoots required the elimination of TDZ and coconut milk from the shoot initiation medium and subsequent transfer to shoot development medium containing BA alone or combined with GA_3 (Mulwa and Bhalla 2006). Likewise, in Himalayan poplar, shoot buds initiated from MS medium supplemented with 0.02 μM TDZ + 79.7 mg/L adenine were transferred to elongation medium containing BAP and GA_3 (Aggarwal et al. 2015). Barrueto Cid et al. (1999) reported shoot regeneration and elongation from TDZ-derived callus only upon transfer to medium containing BAP, NAA and GA_3. Shoot elongation in *Jatropha curcas* was best achieved in medium containing 2.25 μM BA and 8.5 μM IAA (Kumar et al. 2010b). In all the above reports, the shoot buds did not elongate simultaneously, and the elongated shoots have to be excised continuously to reduce the growth suppression of young buds. As described in Sect. 10.2.3, rooting of in vitro shoots produced from TDZ media was prominent in auxin-based medium.

10.4 Somatic Embryogenesis

Somatic embryogenesis is the most preferred regenerative pathway for mass propagation of recalcitrant woody plants. As somatic embryos (SE) originate by bipolar development of somatic cells, SE-derived plantlets are genetically identical to the seed-grown plantlets. Somatic embryogenesis occurs through three stages comprising of embryogenic callus induction, SE maturation and conversion of SE into plantlets. In addition, SEs undergo cyclic embryogenesis resulting in the formation of secondary somatic embryos (SSE) originating directly from primary SEs. Auxins, in general, are potent inducers of somatic cells transition to embryogenic cells in vitro. TDZ, though being a cytokinin, mimics auxins by stimulating direct and indirect somatic embryogenesis in several woody plants (Table 10.3).

10.4.1 Embryogenic Callus Induction

Embryogenic callus induction is the primary approach to generate SEs from woody tissues which are otherwise recalcitrant to de novo somatic embryogenesis. Embryogenic calli are characterized by nodular structures called 'pro-embryogenic masses' (Jiménez and Bangerth 2001). Supplementation of TDZ had a positive influence on indirect somatic embryogenesis from staminode explants of 19 genotypes in *Theobroma cacao* (Li et al. 1998). Previously published reports on *T. cacao* expressed poor embryogenic response using other PGRs. In the study by Li et al. (1998), development of SEs required three different regeneration media: primary

Table 10.3 In vitro regeneration of woody plants through somatic embryogenesis using TDZ

Regeneration route	Species	Explant	Effective TDZ concentration (μM)	References
Direct	*Melia azedarach*	Immature zygotic embryo	13.62	Vila et al. (2003)
	Santalum yasi × *S. album*	Node	4.55	Zhang et al. (2016)
Indirect	*Eucalyptus microtheca*	Green twigs	0.45	Mamaghani et al. (2009)
	Murraya koenigii	Zygotic embryo, cotyledon	4.54	Paul et al. (2011)
	Paulownia elongata	Leaf, internode	0.45	Ipekci and Gozukirmizi (2004)
	Theobroma cacao	Staminode	0.02	Li et al. (1998)
Secondary embryogenesis	*Cinnamomum camphora*	Immature zygotic embryo	0.90	Shi et al. (2010)
	M. koenigii	Zygotic embryo, cotyledon	9.08	Paul et al. (2011)
	Prunus avium × *P. pseudocerasus*	Transgenic roots	0.45	Pesce and Rugini (2004)

callus induction, secondary callus growth and somatic embryo development. TDZ (22.7–454.5 nM) was amended only in the primary callus induction medium for a short exposure of about 2 weeks. Inclusion of TDZ in the initial stages displayed a positive correlation between embryogenic callus induction and conversion of pro-embryogenic masses into SEs compared to TDZ-free medium. The highest number of SEs (46 per responsive staminode) was obtained in medium containing minimal concentration (22.7 nM) of TDZ. This dosage of TDZ was 20–600-folds less, when compared to other studies in Table 10.3, thus providing a clear evidence of its high growth-promoting activity even at negligible concentrations. Deviation above this optimum concentration (22.7 nM) resulted in necrotic tissues while below produced poor callogenic response (Li et al. 1998). In *Paulownia elongata*, higher concentration of TDZ (18.16 μM) favoured embryogenic callus induction from leaf and internode explants of micropropagated plantlets (Ipekci and Gozukirmizi 2004). Nevertheless, the dosage of TDZ required to induce embryogenic competence varied according to the tree species.

10.4.2 Somatic Embryo Maturation

Maturation of SEs occurs through four developmental stages (globular, heart-shaped, torpedo and cotyledonary stage) commencing from small rounded structure and terminating into bipolar structure bearing shoot apical meristem embedded between a pair of cotyledons, hypocotyl and a root axis. Differentiation of SEs is characterized by accumulation of lipid-rich globular bodies (Bandyopadhyay and Hamill 2000). Usually, SEs of various medicinal plants undergo maturity when transferred to media containing cytokinins like BA and Kin, while TDZ resulted in reversion to callus (Baskaran and van Staden 2012). In woody plants, TDZ had higher embryogenic activity than that of adenine-type derivatives.

Development of globular, heart-shaped and torpedo stage SEs in *P. elongata* was achieved on medium containing a combination of 0.45 μM TDZ and 4.64 μM Kin (Ipekci and Gozukirmizi 2004). Inclusion of TDZ modulated the embryogenic pathway as direct or indirect in *Murraya koenigii*, commonly referred as curry plant (Paul et al. 2011). Culturing of cotyledon and zygotic embryo explants in TDZ medium produced direct SEs, while embryogenic calli obtained from medium containing 4.44 μM BA and 2.675 μM NAA formed mature SEs on exposure to TDZ. Concentration of TDZ above or below the optimum level (4.54 μM) could not trigger the sequential development of SEs from globular to cotyledonary stages (Paul et al. 2011). Likewise, in *Eucalyptus microtheca*, inclusion of TDZ facilitated SE formation in 81% of embryogenic calli obtained from 18.56 μM Kin + 2.68 μM NAA (Mamaghani et al. 2009). In total, 244 plantlets were regenerated from SEs with the supplementation of 0.45 μM TDZ.

10.4.3 Somatic Embryo Germination

In several woody plant species' embryogenic systems, the limiting step is the conversion of SEs into plantlets. The reduction in plant recovery from SE is due to poor embryo quality, lack of maturation and desiccation tolerance (Etienne et al. 2013). Germination of SEs thus depends on the cellular organization of meristem regions and the sizes of vacuoles in embryo cells (Nickle and Yeung 1993; Taylor and Vasil 1996). Prolonged culture and exposure of SEs to higher concentration of TDZ prevented their precocious germination. Conversion of SEs into plantlets thus required transfer of cotyledonary stage SEs either to basal medium or to medium containing other PGRs. In *Murraya koenigii*, direct SEs that originated in TDZ medium failed to regenerate into plantlets in the same medium, while germination was achieved in medium containing NAA and Kin (Paul et al. 2011). Similarly, direct SEs from zygotic embryo of *Melia azedarach* germinated into well-developed plantlets in quarter-strength MS basal medium containing activated charcoal (Vila et al. 2003).

10.4.4 Secondary Somatic Embryogenesis

Cyclic secondary somatic embryogenesis maintains the embryogenic competence of cells for longer time period. TDZ facilitates production of secondary somatic embryos (SSE) by interplaying with various factors like carbon source, photoperiod conditions, etc. (Fig. 10.1). In transgenic cherry rootstock 'Colt' (*Prunus avium* ×*P. pseudocerasus*), interaction of TDZ with maltose produced higher number of SSEs. On the contrary, combination with sucrose dedifferentiated the embryogenic masses into non-morphogenic callus. Dark incubation of embryogenic calli in TDZ medium was superior to light conditions in promoting secondary somatic embryogenesis (Pesce and Rugini 2004). In cherry and apple, higher TDZ concentration resulted in reversal of SSE to callus, thereby indicating the modifications in the endogenous auxin/cytokinin ratio (Daigny et al. 1996; Pesce and Rugini 2004). Higher concentration of TDZ (9.08 μM) induced secondary embryogenesis in *M. koenigii* but produced abnormal embryos with fused margin, fused polycotyledons and a single cotyledon (Paul et al. 2011). Lower concentration of TDZ (0.90 μM) formed opaque white SSEs in camphor tree (*Cinnamomum camphora*), an indicative character of embryo maturation (Shi et al. 2010). Recurrent embryogenesis was observed when primary SE was cultured back in TDZ medium (Vila et al. 2003; Shi et al. 2010).

10.5 TDZ-Induced Stress

TDZ, being a cytokinin analogue, has severe negative consequences on in vitro morphogenesis (Fig. 10.1). Though it has maximum cytokinin activity, prolonged exposure of explants resulted in the production of fasciated or vitrified shoots and sometimes leading to reversal of shoots into callus or necrotic tissues (Poudyal et al. 2008). On the contrary, suboptimal exposure results in the dormancy or slow growth

of shoot buds. In somatic embryogenesis, prolonged subculture and higher concentration of TDZ reduced the frequency of embryogenesis and resulted in reversal of embryogenic masses into non-morphogenic callus (Pesce and Rugini 2004). This may be attributed to the changes in the levels of the endogenous auxin/cytokinin and elevated levels of ethylene induced by TDZ (Lu 1993).

TDZ pretreatment also suppressed the rooting of regenerated shoots (Khan and Anis 2012). A report indicated the negative consequence of TDZ on seed germination which resulted in rapid expansion of cotyledons, stunted shoots and impairment of root growth (Murthy and Saxena 1998). Higher concentration of TDZ produced an abnormality termed 'burr knots' in leaf explants of pear. Burr knots are brown, radicle-like structures that cannot differentiate into roots or shoots (Poudyal et al. 2008).

10.6 Conclusion and Recommendations

Plant tissue culturists have exploited TDZ to the maximum potential to induce a wide variety of morphogenetic responses in recalcitrant woody plants. Because of its greater stability and high cytokinin activity at very low concentrations, TDZ stands alone as a potent PGR influencing regeneration in recalcitrant tissues compared to its natural counterparts. However, the use of TDZ in plant cell cultures has its own limitations. TDZ produces toxic effects at higher concentration and prolonged exposure, thereby resulting in the growth of abnormal shoots. TDZ also failed to overcome the differential explant response and genotype dependency, a similar phenomenon exhibited by other PGRs. Henceforth, it is recommended to carefully consider all the interplaying factors to negate the shoot abnormalities in using TDZ. Further investigations are also needed to elucidate the molecular mechanism behind in vitro shoot regeneration by TDZ. This could provide a better understanding for experimental design to achieve regeneration via suitable regeneration pathway.

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Conflict of Interest The authors declare that the review paper was written in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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