Thidiazuron: Modulator of Morphogenesis In Vitro

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Elham Tavakouli Dinani, Mukund R. Shukla, Christina E. Turi, J. A. Sullivan, and Praveen K. Saxena

Abstract

Thidiazuron (TDZ) is a substituted phenylurea first synthesized in 1967 by the Schering Corporation in Germany. Compared to other plant growth regulators, TDZ is a powerful and potent synthetic growth regulator, leading to a wide array of in vitro and in vivo applications in plants including prevention of leaf yellowing, enhanced photosynthetic activity, breaking of bud dormancy, fruit ripening, as well as proliferation of adventitious shoots, callus production, and induction of somatic embryogenesis. Despite the diversity of effects attributed to TDZ, its application and mode of action for induction of in vitro morphogenesis in plants are not well understood. Thus, this review aims to summarize current understandings for TDZ during in vitro morphogenesis in order to better understand the potential applications of TDZ for induction of in vitro morphogenesis and organogenesis.

Keywords

Thidiazuron · TDZ · Morphogenesis · Auxin · Cytokinin · Plant growth regulation

1.1 Introduction

Thidiazuron (TDZ) is a substituted phenylurea first synthesized in 1967 by the Schering Corporation in Germany, originally being used as a cotton defoliant and eventually becoming registered in the USA in 1982 (Arndt et al. 1976; Pavlista and

E. T. Dinani · M. R. Shukla · C. E. Turi · J. A. Sullivan · P. K. Saxena (✉)
Department of Plant Agriculture, Gosling Research Institute for Plant Preservation, University of Guelph, Guelph, Canada
e-mail: psaxena@uoguelph.ca

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Fig. 1.1 Summary of the physiological effect of TDZ on apple plant organs which include the stem, root, leaf, flower, and fruit

Gall 2011). Compared to other plant growth regulators (PGRs), TDZ is a powerful and potent synthetic growth regulator exhibiting both auxin- and cytokinin (CK)-like effects in plants, leading to a wide array of in vitro and in vivo applications including prevention of leaf yellowing, enhanced photosynthetic activity, breaking of bud dormancy, fruit ripening, as well as proliferation of adventitious shoots, callus production, and induction of somatic embryogenesis (Fig. 1.1). Despite this unique and dual effect, TDZ's action is often overgeneralized and referred to as a cytokinin. It is therefore important to note that although TDZ can mimic the effects of auxins and CKs, structurally it differs from both of these PGR groups, possessing both phenyl and thiadiazole functional groups, with both groups required for biological activity (Mok et al. 1987).

Compared with other PGRs, TDZ can be used for regeneration at much lower concentrations (10–1000 times lower) making it a valuable commercial agrochemical (Fig. 1.1; Guo et al. 2011). For instance, TDZ's ability to inhibit leaf yellowing, delay leaf senescence, maintain chlorophyll (Chl) concentrations, inhibit carotenoid degradation, inhibit abscisic acid (ABA) biosynthesis, and decrease ethylene sensitivity in cut flowers (Uthairatanakij et al. 2007; Ferrante et al. 2004) has led to its application in the horticultural industry for the purpose of increasing the longevity of cut flowers such alstroemeria (*Alstroemeria aurea* Graham), lilies (*Lilium* spp.), tulips (*Tulipa* spp.), and chrysanthemum (*Chrysanthemum* spp.) (Ferrante et al. 2002a, b; Sankhla et al. 2003). In addition to the above, TDZ's ability to increase fruit size without affecting seed number, through the promotion of cell division in the cortex layer of fruits (Stern et al. 2003), has led to its application for improving fruit size in a number of crops including pear (*Pyrus communis* L.), grape (*Vitis vinifera* L.), persimmon (*Diospyros virginiana* L.), cucumber (*Cucumis sativus* L.)

and kiwifruit (*Actinidia deliciosa* (A. Chev.) C.F. Liang and A.R. Ferguson) (Amarante et al. 2003; Stern et al. 2003). In stone fruits and cut flowers, TDZ has also been used to stimulate bud growth and opening and to accelerate bud breaking (Erez et al. 2006; Wang et al. 1986).

Despite the diversity of effects attributed to TDZ, its application and mode of action for induction of in vitro morphogenesis in plants is not well understood. This notion largely stems from TDZ's ability to display both CK- and auxin-like activities individually or simultaneously during in vitro regeneration. To complicate matters further, TDZ's ability to induce a defensive response in plant tissues can also initiate the up- or downregulation of other PGRs (i.e., ABA, ethylene, melatonin, serotonin) and secondary metabolites (i.e., polyamines) while also modulating the influx/efflux of specific cations (i.e., calcium) across biological membranes (Murch et al. 1997; Murch and Saxena 1997; Murthy et al. 1995; Proctor et al. 1996). In order to better understand potential applications of TDZ for induction of in vitro morphogenesis and organogenesis, the current review aims to summarize the current uses of this multipurpose synthetic PGR in plant tissue culture processes.

1.2 Application of TDZ During Plant Morphogenesis

Although shoot production and plant development reportedly vary in response to TDZ concentration, plant material, and species (Liu et al. 1998), generally, TDZ is more biologically active than BAP, kinetin, or zeatin (Capelle et al. 1983). For example, Lu (1993) observed that TDZ is more effective at lower concentrations compared to classical CKs during shoot regeneration of woody species. In addition to the above, TDZ's ability to exhibit its effects in explants well after the initial treatment (subsequently transferred to media without TDZ) indicates that some explants only require limited exposure (Matand and Prakash 2007). Short exposure time and low concentrations of TDZ have, in fact, been found to be highly effective in stimulation of shoot regeneration across diverse species (Mihaljević and Vršek 2009). TDZ's unique property of high efficacy at low doses and/or short exposure times may be explained by TDZ's ability to resist enzymatic degradation in vivo (Murthy et al. 1998; Kumar and Reddy 2012) which in turn enables TDZ levels to remain stable over time (Dey et al. 2012). For example, in bean callus incubated with radiolabeled TDZ for 33 days, TDZ was found to remain largely intact, with only a small fraction being glycosylated (Mok and Mok 1985). Tracer studies by Benezet and Knowles (1982) have also observed limited degradation (oxidation) of the TDZ molecule within etiolated hypocotyls by 13 species of microorganisms, as evidenced by limited evolution of $^{14}CO_2$, which is one of the principle degradation products of TDZ. This indicates TDZ molecules were not undergoing significant degradation and likely remained within plant tissues over the duration of the experiment, up to a 28-day incubation period. Furthermore, through the use of 14C-TDZ and fractionation experiments, Murch and Saxena (2001) noted that TDZ may in fact exist in several forms, i.e., TDZ-free molecules, sequestered TDZ molecules, and conjugated forms associated with proteins or cell wall components within plant tissues.

1.3 Shoot Bud Induction

In plants, the induction of shoot buds is dependent upon a balance between auxin and CK levels, whereby an increased presence of auxin and CKs can inhibit or initiate bud formation, respectively (Wang et al. 1986). TDZ appears to promote shoot bud initiation by stimulating cell division and multiplication in the apical meristem while also reprogramming cells to the appropriate developmental stage for initiation of shoot differentiation (Dey et al. 2012; Vu et al. 2006). As in other processes affected by TDZ, diverse factors may affect the ability of TDZ to induce shoot bud initiation and growth including: concentration of TDZ, type and source of explant, age or phase of growth, cultivar, presence of other PGRs, particularly auxin, in the medium, balance of endogenous growth regulators, and presence of light (Sanikhani et al. 2006; Visser et al. 1992; Table 1.1).

In general, low concentrations (≥2.5 μM) of TDZ enhance axillary bud formation on cultured shoot tip meristems, while moderate concentrations of TDZ (5–10 μM) can result in somatic embryo formation. At higher concentrations, morphological abnormalities like hyperhydricity have been reported (Lu 1993; Mithila et al. 2003). Not surprisingly, TDZ is typically applied at low concentrations to a wide range of explant types in order to induce bud growth (Murashige 1974; Jiang et al. 2008); however, the concentration required varies with explant type. For instance, direct shoot bud formation occurred only on cotyledonary nodes when TDZ was applied at rates of 0.9–5.4 μM during in vitro regeneration of soybean (*Glycine max* (L.) Merr.) seeds. On the other hand, 10 μM TDZ was optimal for induction of shoot buds in leaf explants of apple (*Malus domestica* Borkh.) (Fasolo et al. 1989), while low concentrations of TDZ (0.02–0.56 μM) induced bud/shoot regeneration in excised roots (*Albizia julibrissin* Durazz.) (Sankhla et al. 1996). TDZ (10 μM) has also been found to induce bud formation and regeneration in thin cell layer (TCL) system from the common bean *Phaseolus vulgaris* L., where pretreatment significantly increased bud regeneration. Optimal bud induction and further development of the formed buds were observed in 2-week cultures of TCLs on 10 μM TDZ later reduced to 1 μM TDZ (Cruz De Carvalho et al. 2000). The length of time the explants are exposed to TDZ can also impact the ability of TDZ to induce bud formation. In *Curculigo orchioides* Gaertn., pretreatment with 15 μM TDZ for 24 h significantly stimulated adventitious shoot regeneration from leaves, while in *Tecomella undulata* (Sm.) Seem., exposure to a concentration of 0.7 μM for a duration of 1–3 weeks was most efficient for shoot regeneration (Varshney and Anis 2012). Interestingly, duration and level of exposure of explants to light during TDZ treatment can also influence shoot organogenesis. For example, de novo shoot bud formation in strawberry (*Fragaria* x *ananassa* Duch) was achieved using leaf disks cultured in the dark and on MS medium containing 9.08 μM TDZ (Husaini and Abdin 2007). Although it is not yet fully understood how light affects TDZ action, it is believed that TDZ's ability to induce shoot bud production in the dark is triggered by calcium stress, which in turn affects the production of ethylene (Mundhara and Rashid

Table 1.1 Summary of TDZ used during induction of shoot buds

Plant response	Optimal TDZ concentration	Explant used	Species	Family	Exposure time	Other PGRs used	Exposure to light/dark	References
Adventitious bud regeneration	0.01, 0.03, 0.1, 0.3, 1.0, 3.0 μM	Normal and hairy root cultures	*Centaurium erythraea* Rafn	Gentianaceae	30 days	Kinetin, BA, 6-γ,γ-dimethylallylaminopurine (2iP), N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU), 6-[4-hydroxy-3-methyl-but-2-enylamino] purine (ZEA)	16-h photoperiod	Subotić et al. (2009)
Shoot differentiation	0.1, 1, 10 μM	Leaf explants	*Rhododendron* spp.	Ericaceae	2 weeks	IBA, 2iP	16-h photoperiod	Preece and Imel (1991)
Plantlet regeneration	50 μM	Cell suspensions derived from leaf callus	*Actinidia chinensis* Planch	Actinidiaceae	1 month	Zeatin, NAA, BA, 2,4-D	Dark/16-h photoperiod	Suezawa et al. (1988)
Plant regeneration	1–50 μM	Protoplast-derived pea callus	*Pisum sativum* L.	Fabaceae	16 weeks	–	16-h photoperiod	Böhmer et al. (1995)
Adventitious bud formation	0.01–0.1 mg dm-3	Cotyledonary nodes	*Medicago sativa* L.	Fabaceae	28 days	NAA	14-h photoperiod	Li et al. (2009)
Bud induction	22.7 μM	Cotyledon explants	*Capsicum annuum* L.	Solanaceae	2–4 weeks	BA, IAA	16-h photoperiod	Hyde and Phillips (1996)
Shoot regeneration	5 μM	Root explant	*Hypericum perforatum* L.	Hypericaceae	28 days	–	16-h photoperiod	Zobayed and Saxena (2003)
Bud regeneration	1, 2.5, and 5 μM	Leaves	*Pyrus communis* L.	Rosaceae	20 days	BA, IBA	Dark	Leblay et al. (1991)

(continued)

Table 1.1 (continued)

Plant response	Optimal TDZ concentration	Explant used	Species	Family	Exposure time	Other PGRs used	Exposure to light/dark	References
Bud/shoot regeneration	0.02, 0.11, 0.56, 2.8, 14.1 µM	Root segment	*Populus alba* L.	Salicaceae	7 weeks	–	Dark/16-h photoperiod	Tsvetkov et al. (2007)
Shoot bud development	0.01–1 mg/l	Seed-derived rhizomes	*Cymbidium sinense* (Jacks.) Willd	Orchidaceae	4 months	NAA	16-h photoperiod	Chang and Chang (2000)
Shoot bud differentiation	0.90–22.72 µM	Leaf explants	*Jatropha curcas* L.	Euphorbiaceae	6 weeks	BAP, IBA	16-h photoperiod	Khurana-Kaul et al. (2010)
Shoot bud formation	0.1, 1.0 µM	Root segments, entire roots	*Albizia julibrissin* Durazz.	Fabaceae	30 days	6-BAP, TDZ	Light/dark	Hosseini-Nasr and Rashid (2002)
Shoot bud formation	0.05–0.2 mg/l	Flower clusters	*Vitis vinifera* L.	Vitaceae	3–6 weeks	2,4-D	16-h photoperiod	Oláh et al. (2003)
Shoot bud formation	4.54, 9.08, 13.12, 18.16 µM	Seedling	*Tamarindus indica* L.	Fabaceae	6 weeks	–	Dark/light	Mehta et al. (2004)
Shoot bud formation	0, 0.45, 2.27, 4.54 µM	Roots of 15-day-old seedlings	*Solanum melongena* L.	Solanaceae	28 days	BA, NAA	16-h photoperiod	Franklin et al. (2004)
Shoot bud induction	0.05, 0.1, 0.2, 0.4 µM	Leaves and cotyledons	*Solanum melongena* L.	Solanaceae	30 days	–	16-h photoperiod	Magioli et al. (1998)
Shoot bud induction	0.5–1 µM	Nodal and internodal segment	*Ephedra gerardiana* Wall. ex Stapf	Ephedraceae	10–15 days	–	Light	Sharma et al. (2013)
Shoot bud induction	0.01–0.1.0 mg/l	Axillary buds	*Prunus dulcis* (Mill.) D.A. Webb	Rosaceae	30 days	BAP, IAA, IBA, NAA	16-h photoperiod	Choudhary et al. (2015)

Shoot bud differentiation	0.1, 1, 10 µM	Leaf explants	*Rhododendron* spp.	Ericaceae	2 weeks	IBA, 2iP	16-h photoperiod	Preece and Imel (1991)
Plantlet regeneration	50 µM	Cell suspensions derived from leaf callus	*Actinidia chinensis* Planch	Actinidiaceae	1 month	Zeatin, NAA, BA, 2,4-D	Dark/16-h photoperiod	Suezawa et al. (1988)
Shoot bud regeneration	0.04, 0.45, 4.54, 13.62 µM	1-month-old root seedling	*Melia azedarach* L.	Meliaceae	3 weeks	BA, kinetin, adenine sulfate (AD)	14-h photoperiod or darkness	Vila et al. (2005)
Shoot regeneration	0.5–0.1 mg/l	Nodal explants	*Medicago scutellata* (L.) Mill., *M. rigidula* (L.) All.	Fabaceae	4 weeks	NAA, BAP	16-h photoperiod	Band et al. (2011)

2002). Given the above, future research is greatly needed to explore the interaction between light and TDZ as it will open new avenues for discovery in terms of its mechanism of action.

1.4 Shoot Growth, Elongation, and Multiplication

TDZ's CK-like activity has also shown to be useful for the development of shoot buds and shoot proliferation/multiplication in plants (Table 1.2) (Mok et al. 1982; Thomas and Katterman 1986; Fiola et al. 1990; Malik and Saxena 1992; Huetteman and Preece 1993; Murch et al. 1997; Faisal et al. 2014; Singh and Dwivedi 2014; Parveen and Shahzad 2011; Jones et al. 2015). TDZ's CK-like activity is believed to be largely responsible for its ability to release lateral buds from dormancy or induce bud regeneration in vitro (Mok et al. 2005; Singh and Dwivedi 2014). Still it is important to note that TDZ likely modulates levels of other PGRs, including auxin, to achieve shoot bud regeneration by evoking regenerative responses, i.e., dedifferentiation and redifferentiation of tissue cells (Malik and Saxena 1992; Guo et al. 2011; Visser et al. 1992). For example, treatment of geranium hypocotyl explants with TDZ in combination with auxin increased shoot regeneration (Hutchinson et al. 1996). With respect to shoot proliferation, a wide spectrum of factors can influence TDZ's effects in vitro including: plant PGR perception and transduction, dedifferentiation and subsequent redifferentiation of cells, genotype, wounding of explants, donor plant condition (e.g. explant age), and duration of exposure to TDZ (Lazzeri and Dunwell 1984; Kumar and Reddy 2012; Magyar-Tábori et al. 2010; Sharifi et al. 2010). Furthermore, TDZ's ability to influence shoot proliferation has shown to be concentration and species specific. At low concentrations, between 1 and 10 μM TDZ can be used to enhance axillary shoot proliferation (Husain et al. 2007), while at much higher concentrations, shoot elongation can be either inhibited (Kumar and Reddy 2012) or stimulated to produce adventitious shoots (Feng et al. 2012; Guo et al. 2012). This trend has been observed for several spp. including "Gala" apples (*M. domestica*), where shoot production was found to decrease with increasing concentrations of TDZ (from 1 to 10 μM) (Liu et al. 1998), while TDZ concentrations greater than 22.7 μM inhibited shoot regeneration (Montecelli et al. 1999).

In addition to concentration, other factors can impact shoot organogenesis including the presence of other PGRs. For example, in vitro shoot multiplication of *Capsicum annuum* L. from cotyledonary node explants excised from seedlings was optimized on MS medium supplemented with 1.5 μM TDZ and 0.5 μM IAA. Compared to purine-type CKs, TDZ is superior at inducing shoot proliferation (Lu 1993) while also working synergistically with other PGRs to induce a response. The synergistic effect of TDZ with other CKs may be due to differences in uptake, recognition by the cells, or mechanisms of action of these different compounds (Huetteman and Preece 1993). For instance, the effect of TDZ on axillary meristem and shoot production was found to be 5–10 times greater compared to CKs (i.e., BA) in species such as soybean (*G. max*), peanut (*Arachis hypogaea* L.), and saffron

Table 1.2 Summary of TDZ used during shoot proliferation

Plant response	Optimal TDZ concentration	Explant used	Species	Family	Exposure time	Other PGRs used	Exposure to light/dark	References
Adventitious shoot regeneration	1, 2, 4, 8, 16 μM	Hypocotyl, cotyledon, and stem	*Hyoscyamus niger* L.	Solanaceae	3 weeks	BAP	16-h photoperiod	Uranbey (2005)
Adventitious shoot formation	2.0 mg/L	Leaves	*Malus domestica* Borkh.	Rosaceae	8 weeks	NAA, BAP, 2,4-D	16-h photoperiod	Bacha et al. (2009)
Adventitious shoot formation	10 μM	Leaves	*Malus domestica* Borkh.	Rosaceae	3 months	BA	Dark/light	Fasiola et al. (1989)
Adventitious shoot induction	22.7 or 27.3 μM	Leaf	*Paulownia tomentosa* Steud.	Paulowniaceae	2 weeks	IAA, BA	16-h photoperiod	Corredoira et al. (2008)
Adventitious shoot regeneration	0–20 mg/L	Root explants	*Gentiana triflora* Pall.	Gentianaceae	8 weeks	NAA	16-h photoperiod	Hosokawa et al. (1996)
Axillary shoot formation	2.2 μM	Seeds	*Malus domestica* Borkh.	Rosaceae	Not mentioned	BA	16-h photoperiod	Sharma et al. (2004)
Callus formation, shoot regeneration	0.05 μM	Leaf	*Populus tremula* L., *Populus alba* L.	Salicaceae	6 weeks	2,4-D	16-h photoperiod	Chupeau et al. (1993)
Direct organogenesis	0, 0.2, 0.5, 1, 2 mg/L	Shoot meristem	*Oryza sativa* L.	Poaceae	3–4 weeks	2,4-D, NAA, BA	16-h photoperiod	Chakrabarty et al. (2010)
Direct organogenesis	0.1 mg/L	Stem node	*Teucrium polium* L.	Lamiaceae	4 weeks	BAP, kinetin, NAA	16-h photoperiod	Rad et al. (2014)
Direct shoot regeneration	0.0, 0.005, 0.01, 0.02, 0.04, 0.08 mM	Excised root tips	*Populus alba* L., *P. tremula* L., *P. tremuloides* Michx.	Salicaceae	8 weeks	–	16-h photoperiod	Sherif and Khattab (2011)

(continued)

Table 1.2 (continued)

Plant response	Optimal TDZ concentration	Explant used	Species	Family	Exposure time	Other PGRs used	Exposure to light/dark	References
Direct shoot regeneration and callus-mediated protocorm-like body (PLB)	0.5–9.0 µM	Root tips	× *Doritaenopsis*	Orchidaceae	8 weeks	BA, zeatin	16-h photoperiod	Park et al. (2003)
Shoot formation	0.22 µM	Nodal explants	*Manihot esculenta* Crantz	Euphorbiaceae	6–8 weeks	BA, GA3	16-h photoperiod	Bhagwat et al. (1996)
Shoot formation	0.22 and 2.20 ppm	Root	*Panax quinquefolius* L.	Araliaceae	20 weeks	–	78% shade	Proctor et al. (1996)
Shoot formation	0.05 µM	Root segments from 15- to 20-day-old seedlings	*Albizia julibrissin* Durazz.	Fabaceae	15–30 days	IAA, IBA, NAA, BA, zeatin, 2iP	16-h photoperiod	Sankhla et al. (1996)
Shoot formation	0.1–30 µM TDZ	Root	*Pelargonium domesticum* L.H. Bailey	Geraniaceae	18 days	–	16-h photoperiod	Murch et al. (1997)
Shoot formation	1pM–100 nM	Node	*Camellia sinensis* (L.) Kuntze	Myrtaceae	2 months	BAP, NAA, IBA	16-h photoperiod	Mondal et al. (1998)
Shoot formation	0.046–4.6 µM	Cotyledonary nodes	*Dalbergia sissoo* DC.	Fabaceae	30 days	BA, kinetin, 2iP	16-h photoperiod	Pradhan et al. (1998)
Shoot formation	0, 5, 10, 15, 20 mM	Hypocotyls	*Hypericum perforatum* L.	Clusiaceae	3, 6, 9, and 12 days	BAP, NAA, IAA, and 2,4-D	16-h photoperiod	Murch et al. (2000)

Shoot formation	0.05, 0.1, 0.3 mg/L	Leaf, cotyledons, and stem	*Oenothera* spp.	Agavaceae	3 weeks	IBA, IAA, 2,4-D	16-h photoperiod	De Gyves et al. (2001)
Shoot formation	0.1 µM	Hypocotyl	*Linum usitatissimum* L.	Linaceae	5–7 days	NAA	Continues light	Jain and Rashid (2001)
Shoot formation	0.001–1 mg/L	Hypocotyl	*Cuminum cyminum* L.	Apiaceae	30 days	–	1S-h photoperiod	Gupta and Bhargava (2001)
Shoot formation	50–10,000 nM	Nodal shoot segments	*Wrightia arborea* (Dennst.) Mabb.	Apocynaceae	3 weeks	BAP, GA3, kinetin	16-h photoperiod	Purohit et al. (2004)
Shoot formation	0.5 and 5.0 µM	Petal, leave	*Dianthus* spp.	Caryophyllaceae	30 days	IAA, NAA	16-h photoperiod	Casanova et al. (2004)
Shoot formation	0.05, 0.1, 0.5, 1.0, 5.0 µM	Leaf	*Hydrangea quercifolia* W. Bartram	Hydrangeaceae	16 weeks	IBA	16-h photoperiod	Ledbetter and Preece (2004)
Shoot formation	4.5 µM	Cotyledon, hypocotyls	*Arnebia euchroma* (Royle)	Boraginaceae	12 days	–	16-h photoperiod	Jiang et al. (2005)
Shoot formation	2–10 µM	Leaf	*Tylophora indica* (Burm. f.) Merr.	Apocynaceae	45 days	2,4-D, BA, kinetin	16-h photoperiod	Thomas and Philip (2005)
Shoot formation	18.16–72.64 µM	Bud	*Curcuma longa* L.	Zingiberaceae	1 week	BA	16-h photoperiod	Prathanturarug et al. (2005)
Shoot formation	0, 0.4, 1, 2.5 µM	Shoot tip	*Musa* spp.	Musaceae	3 weeks	BAP, IAA	16-h photoperiod	Gubbuk and Pekmezci (2006)
Shoot formation	0.05, 0.10, 0.25, 0.50, 1.0 mg/L	Cotyledonary leaves	*Astragalus cicer* L.	Fabaceae	3–5 weeks	–	16-h photoperiod	Basalma et al. (2008)
Shoot formation	1.0–15 µM	Seedling	*Firmiana simplex* (L.) W. Wight	Malvaceae	8 weeks	BA, 2-iP, zeatin, kinetin, GA3	16-h photoperiod	Hussain et al. (2008)

(continued)

Table 1.2 (continued)

Plant response	Optimal TDZ concentration	Explant used	Species	Family	Exposure time	Other PGRs used	Exposure to light/dark	References
Shoot formation	0.5, 0.75, 1, 1.25 mg/L	Seedling	*Hypericum triquetrifolium* Turra	Hypericaceae	8 weeks	IAA, BAP	Dark	Oluk and Orhan (2009)
Shoot formation	0.5–9.0 μM	Nodal	*Cannabis sativa* L.	Cannabaceae	28 days	–	16-h photoperiod	Lata et al. (2009)
Shoot formation	20, 40, 80 μM	Immature seed	*Epimedium alpinum* L.	Berberidaceae	7 days	CPPU, 2,4-D	Dark	Mihaljević and Vršek (2009)
Shoot formation	0.22–1.8 μM	Shoot tip	*Hoya wightii* Hook.f.	Apocynaceae	8 weeks	KN, BA, 2-iP, IBA, IAA, NAA	16-h photoperiod	Lakshmi et al. (2010)
Shoot formation	0, 0.5, 1.0, 3 mg/L	Immature embryos	*Sandersonia aurantiaca* Hook	Colchicaceae	4–6 weeks	NAA	16-h photoperiod	Deroles et al. (2010)
Shoot formation	1 mg/L	Male inflorescence	*Musa* sp.	Musaceae	4 weeks	BAP, kinetin, 2iP, zeatin	16-h photoperiod	Darvari et al. (2010)
Shoot formation	0.5, 1, 2, 3 μM	Hypocotyls and cotyledon	*Lycopersicon esculentum* Mill.	Solanaceae	2–8 weeks	BAP	16-h photoperiod	Osman et al. (2010)
Shoot formation	5–100 μM	Nodal segments	*Nyctanthes arbor-tristis* L.	Oleaceae	4, 8, 12, and 16 days	IBA	16-h photoperiod	Jahan et al. (2011)
Shoot formation	0.5, 1.0, 2.5, 5.0, 7.5, or 10.0 μM	Nodal explants	*Vitex trifolia* L.	Lamiaceae	4 weeks	BA, NAA	16-h photoperiod	Ahmed and Anis (2012)
Shoot formation	2.0 mg/L	Meristem-derived callus of leaf protoplasts	*Malus domestica* Borkh.	Rosaceae	2–3 months	2,4-D, BA, IAA, ABA	16-h photoperiod	Saito and Suzuki (1999)

Shoot formation and development	0.36 mM	Peach palm plants	*Bactris gasipaes* Kunth	Arecaceae	84 days	NAA, BAP	16-h photoperiod	Graner et al. (2013)
Shoot formation and somatic embryogenesis	0.01, 0.1, 1.0, 10.0 µM	Immature, mature nonstratified seed	*Fraxinus americana* L.	Oleaceae	4 weeks	BA, 2iP, 2,4-D	16-h photoperiod	Bates et al. (1992)
Shoot formation and somatic embryogenesis	0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, 10 µM	Leaf and petiole	*Saintpaulia ionantha* H.Wendl.	Gesneriaceae	3, 6, or 9 days	CPPU, 2,4-D, NAA, BA	16-h photoperiod	Mithila et al. (2003)
Shoot induction	0.1–10 µM	Cotyledonary nodes	*Pterocarpus marsupium* Roxb. i	Fabaceae	6 weeks	BA	16-h photoperiod	Husain et al. (2007)
Shoot organogenesis	0, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0 µmol/L	Leaf	*Artemisia judaica* L.	Asteraceae	20 days	–	Dark/light	Liu et al. (2003)
Shoot organogenesis	9.08, 13.62, 18.17, 22.7 µM	Leaf	*Morus* spp.	Moraceae	30 days	2,4-D, BAP, kinetin	16-h photoperiod	Chitra and Padmaja (2005)
Shoot organogenesis	0, 2.5, 5.0, 7.5, 10.0, 20.0 µM	Intact seedlings, etiolated hypocotyl, sterile stem	*Scutellaria baicalensis* Georgi	Lamiaceae	14–20 days	–	16-h photoperiod	Li et al. (2000)
Shoot organogenesis	0.5–9 mM	Nodal segment	*Kigelia africana* (Lam.) Benth.	Bignoniaceae	4 weeks	2,4-D, NAA	16-h photoperiod	Thomas and Puthur (2004)
Shoot organogenesis	0.45, 1.20, 2.30, 4.54, 22.70 µM	Leaf explants	*Rhododendron* spp.	Ericaceae	12 weeks	IAA	16-h photoperiod	Pavingerová (2009)
Shoot production	0.5–40 mg/L	Leaf, petiole, hypocotyl, internode, cotyledon, root	*Arachis hypogaea*	Legumes	15 days	BA	16-h photoperiod	Kanyand et al. (1994)

(continued)

Table 1.2 (continued)

Plant response	Optimal TDZ concentration	Explant used	Species	Family	Exposure time	Other PGRs used	Exposure to light/dark	References
Shoot regeneration	0.88–3.3 mg/L	Excised cotyledons and rooted hypocotyls	*Capsicum annuum* L.	Solanaceae	3 weeks	BAP, IAA	16-h photoperiod	Szász et al. (1995)
Shoot regeneration	1.0 or 2.0 mg/L	Cotyledons, leaves, cotyledonary nodes and shoot tip	*Capsicum annuum* L.	Solanaceae	30 days	–	16-h photoperiod	Dabauza and Pena (2001)
Shoot regeneration	0.1, 1, 5, and 10 μM	Untransformed and transformed roots	*Malus domestica* Borkh.	Rosaceae	–	NAA	16-h photoperiod	Pawlicki-Jullian et al. (2002)
Shoot regeneration	2–9 μM	Cotyledonary explants	*Morus alba* L.	Moraceae	7, 14, 21 days	BAP	16-h photoperiod	Thomas (2003)
Shoot regeneration	0.5, 1.0 μM	Root	*Fragaria* × *ananassa* (Duchesne ex Weston) Duchesne ex Rozier	Rosaceae	3–4 weeks	BA, 2,4-D, NAA	16-h photoperiod	Passey et al. (2003)
Shoot regeneration	0–12 μM	Cotyledon, hypocotyls	*Pinus strobus* L.	Pinaceae	6 weeks	2,4-D, NAA, IAA, BA, 2iP	16-h photoperiod	Tang and Newton (2005)
Shoot regeneration	0, 0.5, 2, 4 μM	Young expanding sepals	*Fragaria* × *ananassa* (Duchesne ex Weston) Duchesne ex Rozier	Rosaceae	4–5 weeks	Zeatin	Dark/light	Debnath (2005)

Shoot regeneration	0.1, 0.5, 1.0, 2.5, 5.0 µM	Root explant from 30-day-old seedlings	*Senna alexandrina* Mill.	Fabaceae	6 weeks	BA, kinetin, NAA	16-h photoperiod	Parveen and Shahzad (2011)
Shoot regeneration	0.22, 0.90, 2.27, 4.54, 9.08 µM	Cotyledonary petiole	*Jatropha curcas* L.	Euphorbiaceae	6 weeks	Kinetin, BAP, NAA, IAA	16-h photoperiod	Kumar and Reddy (2012)
Shoot regeneration	15 mg/L	Young leaf	*Prunus persica* (L.) Batsch	Rosaceae	5 weeks	NAA, IBA	Dark/light	Soliman (2013)
Shoot regeneration	0, 2.5, 5, 7.5 µM	Nodal explants, leave	*Pyrus communis* L.	Rosaceae	4 weeks	NAA, BAP	Dark/16-h photoperiod	Yousefiara et al. (2014)
Shoot regeneration	0.45–22.7 µM	Apical and axillary buds, leaf, and internode	*Guizotia abyssinica* (L.f.) Cass	Asteraceae	3–4 months	–	16-h photoperiod	Baghel and Bansal (2014)
Shoot regeneration	4.54, 6.81, 9.08 µM	Leaf explant	*Prunus persica* (L.) Batsch	Rosaceae	4–6 weeks	NAA	Dark/16-h photoperiod	San et al. (2015)
Shoot regeneration	1.0 µM	Leaf disks	*Mentha × piperita* L.	Lamiaceae	6 weeks	NAA, BA	16-h photoperiod	Faure et al. (1998)
Shoot and root regeneration	1.0 mg/L	Aerial stem explants	*Zingiber officinale* Roscoe	Zingiberaceae	3–4 months	IBA, BAP, NAA	16-h photoperiod	Lincy and Sasikumar (2010)
Shoot induction	4.5, 9.1 µM	Mature embryos		Poaceae	4 weeks	BAP	16-h photoperiod	Ganeshan et al. (2006)
Shoot multiplication	0.5, 1, 2, 3, 4, 5 µM	Nodal	*Cullen corylifolium* (L.) Medik.	Fabaceae	4 weeks	–	16-h photoperiod	Faisal et al. (2006)
Shoot regeneration	1.0 µM	Leaf disks	*Mentha x piperita* L.	Lamiaceae	6 weeks	NAA, BA	16-h photoperiod	Faure et al. (1998)

(continued)

Table 1.2 (continued)

Plant response	Optimal TDZ concentration	Explant used	Species	Family	Exposure time	Other PGRs used	Exposure to light/dark	References
Shoot regeneration	0.45–45.41 μM	Rhizome sections	*Cheilocostus speciosus* (J.Koenig) C.D.Specht	Costaceae	6 weeks	–	16-h photoperiod	Malabadi et al. (2004)
Shoot regeneration	0.045, 0.23, 1.14, 2.27, 4.54 μM	Cotyledons excised	*Boehmeria nivea* (L.) Gaudich.	Urticaceae	4 days	2,4-D, IAA, IBA, NAA	16-h photoperiod	Wang et al. (2007)
Shoot regeneration	2 mg/L	Root, cotyledon, hypocotyl, and intact seedlings	*Brassica oleracea* L.	Brassicaceae	6 weeks	BA, zeatin	16-h photoperiod	Ćosić et al. (2015)

(*Crocus sativus* L.) (Victor et al. 1999; Radhakrishnan et al. 2009; Sharifi et al. 2010). Furthermore, TDZ alone or in combination with other auxins/CKs (e.g., BA) can induce shoot bud formation and multiplication especially after transfer of shoots to TDZ-free medium (Singh and Dwivedi 2014). Consequently, the transfer of explants from enriched TDZ medium to a secondary medium without growth regulators has been successfully applied in plant regeneration systems for a variety of species (Malik and Saxena 1992; Victor et al. 1999).

1.5 Somatic Embryogenesis

TDZ is a substitute for the auxin/CK requirement that is needed during somatic embryogenesis, thereby increasing the number of formed somatic embryos (Visser et al. 1992). Somatic embryogenesis changes somatic cells to embryonic cells in a physiological sequence that is tightly regulated by a delicate balance of PGRs (Murthy et al. 1998). Induction and development of somatic embryogenesis are associated with endogenous PGRs including auxins and CKs; not surprisingly, TDZ promotes somatic embryogenesis, alone or in combination with other PGRs, for a wide range of recalcitrant species (Durkovic and Misalova 2008; Nhut et al. 2006) as well as a variety of commercial crops including tobacco (*Nicotiana tabacum* L.), peanut (*A. hypogaea*), geranium (*Pelargonium* spp.), African violet (*Saintpaulia* spp.; Mithila et al. 2003; Shukla et al. 2013), and chickpea (*Cicer arietinum* L.) (Visser et al. 1992; Saxena et al. 1992; Gill and Saxena 1993; Murthy et al. 1995) (Table 1.3).

Different types of tissues can be selected for induction, for instance, TDZ has been described to induce somatic embryos on hypocotyl, epicotyl, cotyledonary node, cotyledon, and leaves of intact seedlings of *Azadirachta indica* A. Juss. (Gairi and Rashid 2004; Saxena et al. 1992; Iantcheva et al. 1999). In peanut, induction of direct somatic embryogenesis occurs by culturing mature intact seeds on a medium supplemented with 0.5–10 μM TDZ or N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU). Explants with no cotyledons, and thus no embryogenic potential, did not respond to increasing levels of TDZ. In contrast, retention of one or both cotyledons resulted in increased response to TDZ (Saxena et al. 1992; Murthy and Saxena 1994; Murthy et al. 1995). Exposure time can impact the effectiveness of TDZ. For example, application of TDZ on plant tissues alone or in combination with other PGRs for short periods of time at low concentration (10 μM) has been found to induce embryogenic responses (Hutchinson et al. 1997; Malik and Saxena 1992; Murthy et al. 1998), while exposure to TDZ for longer than 3–4 weeks (10 μM) led to a reduced induction of roots (Malik et al. 1993). This is interesting, as it reflects patterns also observed in induction of somatic embryogenesis by the synthetic auxin and pesticide, 2,4-dichlorophenoxyacetic acid (2,4-D). Similar to TDZ, short exposure to 2,4-D followed by explant transfer to growth regulator-free medium allows for first an accumulation of 2,4-D in tissues followed by a gradual decrease over time with somatic embryos developing with these falling concentrations (Zee 1981; Fujimura and Komamine 1980; Feher et al. 2002). The similarity between this

Table 1.3 Summary of TDZ used during somatic embryo formation

Plant response	Optimal TDZ concentration	Explant used	Species	Family	Exposure time	Other PGRs used	Exposure to light/dark	References
Somatic embryogenesis	10 µM	Hypocotyl explants	*Pelargonium* × *hortorum* Bailey	Geraniaceae	3 days	Gas	16-h photoperiod	Hutchinson et al. (1997)
Somatic embryogenesis	0. 045, 0.45, 4.54 µM	Immature embryo		Fagacea	2, 4, and 8 weeks	–	Dark	Tsvetkov (1999)
Somatic embryogenesis	0.01–5.0 µM	Embryonic axes	*Quercus robur* L.	Fagacea	4 weeks	BA, NAA	16-h photoperiod	Martínez et al. (2008)
Somatic embryogenesis	2.5, 5, 7.5 mg/L	Nucellar tissue	*Mangifera indica* L.	Anacardiaceae	1–2 months	BAP, 2iP, IAA	15-h photoperiod	Kidwai et al. (2009)
Somatic embryogenesis	5.0 or 10.0 µM	Leaf disk	*Vitis vinifera* L.	Vitaceae	40 days	2,4-D, BA, KIN, ZEA, 2iP	16-h photoperiod	Matsuta and Hirabayashi (1989)
Somatic embryogenesis	0.455 µM	Nodal and internodal tissues	*Bambusa odashimae* Hatus. ex D.Z.Li & Stapleton	Poaceae	3–4 weeks	Kinetin, 2,4-D	16-h photoperiod	Lin et al. (2004)
Somatic embryogenesis	0.1–2.5 µM	Stem segments and shoot tips	*Capsicum annuum* L.	Solanaceae	4 weeks	–	16-h photoperiod	Khan et al. (2006)
Somatic embryos	1 µM	Leaf	*Echinacea purpurea* (L.) Moench	Asteraceae	4 weeks	–	Dark	Jones et al. (2007)
Somatic embryos	1,5, 10, 20, 40 µM	Seedling	*Arachis hypogaea* L.	Fabaceae	4–6 weeks	–	16-h photoperiod	Saxena et al. (1992)

well-documented process and the pattern observed in TDZ treatment supports a strong auxin-like role for TDZ in this mechanism. Further, it is likely that the inherent stability of TDZ in living tissues is a strong contributing factor in establishing this function.

1.6 Intact Seedling Development

TDZ enhances seed germination via improvement of shoot regeneration, with positive effects being reported in soybean (*G. max*), pea (*Pisum sativum* L.), common bean (*P. vulgaris*), chickpea (*C. arietinum*), and lentil (*Lens culinaris* Medik) (Radhakrishnan et al. 2009; Malik and Saxena 1992) (Table 1.4). In contrast, the intact seedling regeneration system is a unique morphogenetic system which involves the direct development of multiple shoots on the germinating seedling. For the first time, Malik (1993) reported a direct seed culture method for de novo differentiation of shoots from intact seedling without explanting. The number of shoots regenerated from intact seedling of *Lathyrus sativus* L., *L. cicera* L., and *L. ochrus* L. DC. was significantly higher than that observed with explants. These results indicated that excision of explant is not always necessary for induction of morphogenesis and also that the morphological integrity of intact seedlings plays a critical role in the induction of organogenesis/somatic embryogenesis (Malik 1993). TDZ induction of shoot production in the intact seedling system effectively depends on the applied concentration. For example, intact seedlings of silk tree (*A. julibrissin*) grown on MS medium containing 0.1–10 μM TDZ produced shoots indirectly through callus. Interestingly, at higher TDZ concentrations (2.5–10 μM), shoots were produced, but did not form callus (Mok et al. 1987). Sankhla et al. (1994) also reported high efficiency of TDZ in inducing shoot formation from roots of intact seedling of *A. julibrissin* at 0.1–1.0 μM TDZ. Regeneration of multiple shoots from intact seedlings of switch grass (*Panicum virgatum* L.) was induced on MS medium supplemented with 4.5 μM2, 4-dichlorophenoxyacetic acid (2,4-D), and 18.2 μM TDZ (Gupta and Conger 1998). An in vitro propagation system for *Artemisia judaica* L., a medicinal plant, induced shoot organogenesis by culturing intact seedlings on medium supplemented with 1 μM TDZ for 20 days (Liu et al. 2003). In a study with seeds of *Firmiana simplex* (L.) W. Wight, induction of shoot proliferation was assessed on MS medium supplemented with 5.0 μM TDZ + 1.5 M GA_3 + 0.1% ascorbic acid compared to various levels (1.0–15 M) of several different cytokinins (BA, 2-iP, zeatin, and kinetin). Shoots formed within 8 weeks of culture and the shoot-forming capacity of seeds were found to be influenced by the type and concentration of CKs, with TDZ showing up to 13% greater regeneration rates than other cytokinins tested (Hussain et al. 2008). Induction of shoot organogenesis for felty germander (*Teucrium polium* L.), an endangered medicinal plant, was obtained using intact seedlings cultivated in MS medium supplemented with 22.72 μM TDZ (Rad et al. 2014). Regeneration ability of kohlrabi (*Brassica oleracea var. gongylodes*) cultivars Vienna Purple (VP) and Vienna White (VW) has also been tested. Intact seedlings were cultivated on MS media

Table 1.4 Summary of TDZ used during seedling development

Plant response	Optimal TDZ concentration	Explant used	Species	Family	Exposure time	Other PGRs used	Exposure to light/dark	References
Somatic embryogenesis	0.1–1.0 μM	Hypocotyl, epicotyl, cotyledon, leaves	*Azadirachta indica* A. Juss.	Meliaceae	1 week	–	24-h light	Gairi and Rashid (2004)
Direct somatic embryogenesis	0, 0.91, 2.27, 4.54, 45.41 mM	Hypocotyls, cotyledons, petioles, nodal stem	*Medicago* spp.	Fabaceae	20 days	BAP	16-h photoperiod	Iantcheva et al. (1999)
Shoot formation	10 μM	Mature seeds and growing seedlings	*Phaseolus vulgaris* L.	Fabaceae	7 days to 4 weeks	BAP	Dark/light	Malik and Saxena (1992)
Somatic embryogenesis	0.5–10 μM	Seedling	*Arachis hypogaea* L.	Fabaceae	5 weeks	–	Dark/light	Murthy et al. (1995)
Direct embryogenesis	1, 5, 10, 20, 40 μM	Seedling	*Arachis hypogaea* L.	Fabaceae	6–9 weeks	–	16-h photoperiod	Saxena et al. (1992)
Somatic embryogenesis	0.05, 1, 5, 10, 20 μM	Seeds	*Cajanus cajan* (L.) Millsp.	Fabaceae	24 h to 14 days	–	16-h photoperiod	Singh et al. (2003)
Shoot formation	1–100 μM	Cotyledonary region of the seedlings	*Cicer arietinum* L.	Fabaceae	2–3 weeks	NAA, BAP	Dark/light	Murthy et al. (1996)
Somatic embryogenesis and regeneration	1, 5, 10, 15, 20, 25 μM	Seedling explants	*Arachis hypogaea* L.	Fabaceae	2 weeks	BAP	Dark/light	Gill and Saxena (1992)
Shoot formation	1 μM	Seedling	*Linum usitatissimum* L.	Linaceae	4 days	BAP	24-h light	Mundhara and Rashid (2006)
Bud induction	10, 1 μM	Seedlings	*Phaseolus vulgaris* L.	Fabaceae	2 weeks	BAP	Dark or light	de Carvalho et al. (2000)
Shoot formation	0.88, 1.1, 1.76, 2.2, 2.75, 3.3 mg/L	Seedling	*Capsicum annuum* L.	Solanaceae	1 week	BAP, IAA	16-h photoperiod	Szasz et al. (1995)

supplemented with BA, TDZ, and trans- or cis-zeatin. All tested CKs induced shoot regeneration with 47.5–60% shoot regeneration frequency from hypocotyl explants and intact seedlings (Ćosić et al. 2015).

1.7 Mechanisms of TDZ Activity

1.7.1 Cytokinin-Related Effects of TDZ

TDZ was first reported to have CK activity in 1982 by Mok et al. and later confirmed by Visser et al. (1992). TDZ exhibits a considerably higher degree of biological activity when compared with traditional CKs for inducing regeneration in plant species (Mok et al. 1987; Van Nieuwkerk et al. 1985; Escalettes and Dosba 1993), stimulating organogenesis and somatic embryogenesis, and retarding senescence or leaf yellowing in plants (Mehrotra et al. 2015). For example, callus tissue of *Phaseolus lunatus* L. which cannot grow without CKs is able to grow after exposure to TDZ (Murthy et al. 1998). Similarly, lower concentrations of TDZ are needed to initiate shoot differentiation and regeneration responses compared to levels required for CKs (Baker and Bhatia 1993). TDZ's CK-like activity is believed to stem from its ability to modulate pathways responsible for CK biosynthesis in plants (Mok et al. 1987) by acting on endogenous adenine-based CK metabolism (Capelle et al. 1983). To date it is unclear whether TDZ causes CK responses by interacting directly with CK receptors or indirectly either by stimulating the conversion of CK nucleotides to active ribonucleosides or by inducing the accumulation of endogenous adenine-based CKs.

It has been proposed that TDZ promotes the conversion of CK ribonucleotides (inactive CKs) to active forms of CKs (i.e., ribonucleosides and free bases) by encouraging the synthesis of endogenous purine CKs while also inhibiting their degradation (Capelle et al. 1983; Lu 1993; Murthy et al. 1995; Mok and Mok 1985). On the other hand, TDZ has demonstrated binding affinity for CK receptors such as CRE1 as well as CRE1/AHK4, AHK2, and AHK3 (de Melo Ferreira et al. 2006; Susan 1996; Rolli et al. 2012). It is interesting to note that both purine- and urea-type CKs have demonstrated binding affinities for cytokinin-specific binding proteins (CSBPs). A stronger association has, however, been demonstrated for compounds containing phenylurea derivatives (Murthy et al. 1998); this could help to explain TDZ's ability to modulate plant morphogenesis at lower concentrations. In addition to the above, TDZ can also increase endogenous levels of CKs by reducing catabolism, increasing synthesis, and changing non-active CK molecules to active forms (Kefford et al. 1968; Murthy et al. 1995), possibly through inactivation of CK oxidase/dehydrogenase (CKX) (an enzyme responsible for CK inactivation through cleavage of the unsaturated N6 side chain of most isoprenoid CKs) (Nikolić et al. 2006). TDZ can also modify CK biosynthesis pathways by decreasing endogenous pools of the CK 2iP and by increasing the concentration of purine-based CKs (Zhang et al. 2005).

In general, reduced rooting capacity and inhibition of shoot elongation are attributable to the high CK activity of TDZ. Medium concentrations (approx. 10–20 μM) of TDZ may result in both axillary and adventitious shoot organogenesis, and high concentrations tend to stimulate callus formation. Concentrations of TDZ much smaller than most CKs often stimulate higher shoot proliferation. Combinations of TDZ with other CKs result in better shoot proliferation due to differences in uptake, recognition by the cells and receptors, or mechanisms of action of different compounds (Huetteman and Preece 1993). TDZ facilitates efficient multiplication of apical meristem cells and their reprogramming to appropriate developmental stages for shoot differentiation (Dey et al. 2012).

1.7.2 Auxin-Related Activity of TDZ

The auxin-like activity of TDZ was first assessed by Suttle (1984). Following this work, TDZ's ability to modulate auxin levels in plants was reported by Yip and Yang (1986) who found that TDZ stimulated auxin concentrations in mung bean (*Vigna radiata* (L.) R.Wilczek) hypocotyl tissue. Similarly, results by Visser et al. (1992) suggested that auxin(s) were involved during the induction and/or expression of TDZ-induced morphogenic differentiation.

To date TDZ's auxin-like activity is believed to act through the modulation of metabolism and transport for endogenous hormones including auxins, cytokinins, ethylene, abscisic acid, and gibberellins (Feng et al. 2012; Murch and Saxena 2001). While a significant amount of work has been performed to understand TDZ's cytokinin-like effects in plants, far less is understood in terms of its relationship to auxin. Currently, two concepts have been proposed: (1) TDZ directly promotes growth due to its own biological activity, and (2) TDZ may modulate the synthesis and accumulation of endogenous auxins or auxin-like bioregulators in synergism with CKs (Capelle et al. 1983; Mok and Mok 1985).

Auxins including natural (IAA) and synthetic auxins (e.g., naphthaleneacetic acid (NAA) and 2,4-D) are responsible for cell proliferation and development of callus (a mass of dedifferentiated cells), which are the first part of the morphogenetic process. They are also strongly associated with regeneration and somatic embryogenesis (Murthy et al. 1998). TDZ via auxin-like activity has been shown to induce callus formation on the graft and bud cutting of grape and leaf disks of cotton (Lin et al. 1988; Kartomysheva et al. 1983), increasing proliferation and growth rate of callus 30 times more than the common auxins. Tracer studies by Murch and Saxena (2001) noted that the translocation of auxin is essential for TDZ-induced morphogenesis through the observation that radiolabeled IAA accumulated in the hypocotyl of geraniums and was translocated over a great distance within the tissues. TDZ may also mimic an auxin response by modifying endogenous auxin metabolism, for example, TDZ had a stimulating effect on auxin synthesis when peanut seedlings were treated with TDZ, causing an increase in cytosolic auxin followed by induction of somatic embryogenesis (Murthy et al. 1995).

The relationship between TDZ and auxin metabolism has also been confirmed through inhibitor studies. Suppression of TDZ-induced regeneration by inhibitors of auxin action and transport has been employed in several studies to better understand the relationship between auxin and TDZ across several different regeneration studies (Hutchinson et al. 1996; Murch and Saxena 2001; Murch et al. 2002). For example, application of 2-(ρ-chlorophenoxy)-2-methylpropionic acid (PCIB, an auxin biosynthesis inhibitor) in peanut and geranium demonstrated an increasing effect of TDZ during somatic embryogenesis (Murthy et al. 1998). Although use of 2,3,5-triiodobenzoic acid (TIBA, an inhibitor of polar auxin transport) in samples treated with TDZ did not change auxin levels, a decrease in the rate of somatic embryogenesis was observed (Hutchinson et al. 1996). Reduced rate of embryogenesis in TDZ-exposed tissues treated with TIBA and PCIB suggests TDZ may modulate auxin metabolism during developmental processes such as embryogenesis (Hutchinson et al. 1996). Furthermore, in TDZ-exposed leaf tissue of *Echinacea purpurea* L., inclusion of TIBA and PCIB decreased TDZ-induced morphogenesis (shoot organogenesis and somatic embryogenesis) but increased concentrations of auxin and endogenous indoleamines (i.e., melatonin and serotonin) (Jones et al. 2007). The above examples indicate that TDZ-induced regeneration is correlated with a metabolic cascade, i.e., accumulation and transport of endogenous signals auxin and melatonin, and the activation of a stress response.

Endogenous and exogenous auxin levels are closely associated with somatic embryogenesis in plants, and TDZ plays a crucial role in modulating the interaction among different hormones. It is important to note that TDZ's ability to induce somatic embryogenesis is not solely dependent upon its auxin-like properties, as CKs have also been implicated. For example, embryogenesis was repressed in TDZ-treated geranium tissues by applying diaminopurine (DAP, an inhibitor of a purine-based CK) (Hutchinson and Saxena 1996). Unlike purine-based CKs, TDZ alone can induce somatic embryogenesis (Murthy et al. 1998), which in turn highlights the ability of TDZ to act as both an auxin and cytokinin. In addition to somatic embryogenesis, TDZ's auxin-like activity has also been shown to be beneficial during callus formation by increasing proliferation and growth rate of callus (Lin et al. 1988). Synthetic auxins such as NAA and 2,4-D are responsible for stimulation, multiplication, and differentiation of cells into somatic embryos and callus development (Murthy et al. 1998). The regulatory role of TDZ appears to be partially mediated through inactivation of genes responsible for auxin and CK biosynthesis, which in turn causes changes in developmental patterns in plants (Malik 1993).

In general, TDZ inhibits root meristem activity effectively by acting as an auxin antagonist (Rolli et al. 2012). Auxin-like activity of TDZ is also strongly associated with regeneration, somatic embryogenesis, organogenesis, and development of adventitious shoots in many plant species (Huetteman and Preece 1993; Lu 1993; Feng et al. 2012; Guo et al. 2012). A low concentration of TDZ induces organogenesis of axillary buds on cultured shoot tip meristem by reducing apical dominance (Lu 1993). However, it is important to note that auxin-like properties of TDZ are dependent on a multitude of factors including the basal medium used, type of cultivar, source of the explant, developmental stage of explant, and age of the donor

plant (Radhakrishnan et al. 2009). TDZ seems to act via reprogramming the fate of cells, developmental pathway, and interaction between endogenous hormones (Malik 1993).

1.7.3 Calcium Signaling

TDZ is believed to modulate plant morphogenesis through its ability to influence inter- and intracellular calcium (Ca^{2+}) concentrations and signaling cascades (Trewaves 1999). Plant cells and tissues react to different hormones due to changes in concentration of external Ca^{2+} (Guo et al. 2011), and the balance of cytosolic Ca^{2+} may relate to the TDZ induction. Ca^{2+} is an important secondary messenger and signaling molecule in plants, facilitating different morphological responses in plant cells and tissues through modulation of PGR levels (Guo et al. 2011; Allen and Schroeder 2001). In response to TDZ, Ca^{2+} channels will open, leading to changes in plant cytosolic Ca^{2+} levels; intermittent signals are then sent across the cell initiating a cascade of metabolic events (White and Broadley 2003). Several studies have confirmed the above noted theory. Hosseini-Nasr and Rashid (2002) reported that addition of Ca^{2+} uptake inhibitors (lanthanum, calmodulin, trifluoperazine (TFP), chlorpromazine (CPZ)) to culture medium supplemented with TDZ led to decreased levels of shoot production, while Jones et al. (2007) applied a Ca^{2+} channel activator, (S)-Bay K8644, in TDZ-treated explants of *E. purpurea* and noted changes in cell polarity, increased auxin concentration, callus induction, and regeneration. Murch et al. (2003) found that treatment with the calcium channel antagonist (S)-Bay K8644 increased influx of Ca^{2+}, leading to a change in the pattern of somatic embryogenesis. Increases in cytosolic Ca^{2+} for a long period, however, can also lead to apoptosis and cell death (White and Broadley 2003).

1.7.4 Relationship to Other PGRs and Stress Signaling Molecules

Plants interpret TDZ as stress, and it has been suggested that TDZ's ability to initiate stress in plants helps to induce morphogenesis through modulation of PGRs as well as other metabolites and ions. For instance, proline is considered a marker of stress as it enables plants to produce more $NADP^+$/NADPH. Proline levels have been found to increase in tissues which have been treated with TDZ and which show a capacity to switch from shoot formation to somatic embryogenesis (Hare and Cress 1997). In addition to proline, accumulation of mineral ions in TDZ-treated tissues may also act as a trigger factor for induction of somatic embryogenesis and regeneration in carrot (*Daucus carota* subsp. *sativus* (Hoffm.) Arcang) (Guo et al. 2011). Stress-related metabolites 4-aminobutyrate, ABA, proline, and mineral ions increased in the TDZ-treated root tissues of geranium (*Pelargonium domesticum* L.H. Bailey) (Murch et al. 1997, Murch and Saxena 1997).

TDZ treatment has also been found to significantly improve accumulation of endogenous hormones (IAA, zeatin, GA3, and ABA) during shoot organogenesis. For instance, in leaf explants of *E. purpurea*, the levels for auxin, melatonin, and serotonin were found to increase after exposure to TDZ during regeneration. Furthermore, TDZ exposure stimulates ethylene production concurrent to accumulations of ABA, auxin, proline, and Ca^{2+} (Jones et al. 2007). Inhibition of rooting and hypocotyl elongation, swelling at the base of hypocotyl, and tightening of cotyledons toward the apex induced by TDZ are characteristics of an ethylene action (Mundhara and Rashid 2006); not surprisingly, TDZ is more effective than CK for inducing "stress ethylene" production in plants (Yip and Yang 1986). Some negative effects of TDZ on growth parameters like rooting can be related to the stimulatory effect of TDZ on endogenous ethylene production (Pourebad et al. 2015). An increase in ethylene production following TDZ treatment results in an inhibition of auxin transport in many dicots (Radhakrishnan et al. 2009), which in turn further highlights the complex relationship between TDZ's auxin-like activity in terms of downstream effects with other PGRs.

1.7.5 Morphological Abnormalities Resulting from TDZ Use

Genetic evaluation of TDZ-induced explants using flow cytometry, inter-simple sequence repeat (ISSR), molecular markers, and directed amplification of minisatellite-region DNA (DAMD) has shown uniformity and stability in genome size and consistent ploidy level (Faisal et al. 2014). Still unfavorable side effects involving TDZ have been reported including hyperhydricity, dwarfing, uncontrolled callusing, abnormal shoot growth, and difficulty in rooting of shoots. The above side effects are manageable by transferring samples to TDZ-free medium and altering concentration and exposure time (Huetteman and Preece 1993; Mok et al. 2005; Singh and Dwivedi 2014; Magyar-Tábori et al. 2010). In addition to the concentration, undesired side effects associated with the use of TDZ will increase over time as a result of overexposure to TDZ (Manjula et al. 2014; Zhihui et al. 2009; Franklin et al. 2004).

Observed abnormalities demonstrated by cultured tissues exposed to TDZ are likely to be specific to plant organ and species; still certain trends have been observed including: enlarged dark-green cotyledons and leaves (Lu 1993; Murch et al. 1999), short, compact shoots and shoot buds, inhibited shoot elongation, deformation and hyperhydricity of seedlings (Franklin et al. 2004; Hosokawa et al. 1996; Varshney and Anis 2012; Zaytseva et al. 2016; Zhihui et al. 2009; Hare and Van Staden 1994; Dobránszki and da Silva 2010; Lu 1993), inhibited rooting, stunted and thickened root systems (Lu 1993; Murch et al. 1999; Proctor et al. 1996; Dobránszki and da Silva 2010), and necrosis and browning of tissue in seedlings (Zhihui et al. 2009). Morphological effects caused through exposure to TDZ can also be species specific, for instance, morphological abnormalities have been observed in *C. annuum* and *Malus* spp. along with positive effects including increased bud production. TDZ promotes abnormal regenerated shoots from roots of *Bixa orellana* L. (da Cruz et al.

2014). Seedlings developed in the presence of TDZ exhibited reduced root, epicotyl, and hypocotyl elongation. Sankhla et al. (1994) found that *A. julibrissin* roots developed under the influence of TDZ were very thick and short and the development of secondary roots was inhibited. On the other hand, no abnormalities (i.e., including fasciated shoots, hyperhydricity, and inhibited shoot elongation) were reported for other species including white pine and *Dendrocalamus strictus* (Roxb.) Nees (Mihaljevic and Vrsek 2009; Huetteman and Preece 1993; Tang and Newton 2005; Singh and Dwivedi 2014).

Abnormalities caused by TDZ can be overcome. For instance, vitrification can be reduced by using unsealed petri dishes during shoot bud initiation, vented caps for jars during shoot elongation, and a higher concentration of gelling agent. Also, transferring regenerated shoots induced by TDZ to a second medium containing different CKs BA, 2iP, or IBA but lacking TDZ, can lead to regenerated shoots with normal growth and development (Lu 1993; Husain et al. 2007). Another solution to reduce the frequency of shoot fasciation is subculturing induced shoots to medium without TDZ which results in elongated shoots and normal leaves (Huetteman and Preece 1993; Varshney and Anis 2012). Furthermore, the type and combination of other CKs with TDZ significantly influence the occurrence of morphologically abnormal plants (Manjula et al. 2014). Generally, most morphological abnormalities associated with applying TDZ can be overcome by reducing TDZ concentrations and exposure time (Lu 1993). Additionally, TDZ-induced abnormal variations may be overcome by testing various concentrations and times of TDZ application in balance with other phytohormones. Application of TDZ in root-based regeneration systems may also be useful as roots are considered to be genetically more stable in regeneration responses. Regardless of these shortcomings of TDZ in inducing regeneration, it still remains a very useful tool to achieve the designed goals in a range of short-term and long-term micropropagation projects.

1.8 Conclusion

Although TDZ was discovered half a century ago, many questions still remain with respect to its mode of action and function during morphogenesis and organogenesis, which in turn provides interesting opportunities for researchers to explore. For instance, it is still largely unclear as to how plants metabolize TDZ upon exposure and how the mode of action of TDZ may contribute toward its ability to induce morphogenesis in plants even after being removed from growth media. Similarly, the observation that a relationship between TDZ and photoperiod exists suggests that additional mechanisms of action may exist for TDZ, potentially via downstream interactions with phytochrome. While a wealth of attention has been given to TDZ's auxin- and CK-like properties, there is growing information in the literature to suggest that its mode of action is far more complicated than once initially thought, with PGRs and regulatory signals likely playing a greater role than once imagined. The diversity of mechanisms with which TDZ is thought to act is reflected across the wide spectrum of morphological responses that has been observed for TDZ in

plants. For example, specific responses including bud development, shoot proliferation, somatic embryogenesis, and seedling development are known to vary significantly across species, explant, concentration, exposure time, and photoperiod, as well as in the presence or absence of other PGRs. As greater efforts are put forth to understand TDZ's multifaceted role in vivo, new ways for utilizing this intriguing PGR will undoubtedly be realized as researchers will be better equipped to predict plant growth and developmental responses when inducing morphogenesis in vitro.

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