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REVIEW

Thidiazuron-induced abnormalities in plant tissue cultures

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Abstract
Thidiazuron (TDZ) is a proven effective and potent synthetic plant growth regulator for organogenic, regeneration, and developmental pathways, including axillary and adventitious shoot proliferation, somatic embryogenesis, and in vitro flowering. TDZ has facilitated the establishment of in vitro cultures for several plant species, especially woody and recalcitrant plants, which has enabled their genetic transformation and improvement. Despite the effectiveness and advantages of using TDZ, several drawbacks are associated with its application in plant tissue culture. This review addresses the morphological, physiological, and cytogenetic abnormalities associated with the use of TDZ in vitro, and provides a summary of these abnormalities in several plant species.

Keywords Albinism · DNA polymorphism · In vitro culture · Micropropagation · Somaclonal variations · Physiological disorders

Introduction

Thidiazuron (TDZ; 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea, molecular weight 220.25, molecular formula $C_9H_8N_4OS$) is a synthetic plant growth regulator (PGR) that was originally registered as a cotton defoliant by Schering AG (Berlin, Germany) (Arndt et al. 1976). TDZ possesses cytokinin-like activity and does not contain the purine ring seen in other adenine-type cytokinins such as benzylaminopurine (BA), kinetin (Kin), and zeatin. The exploitation of TDZ in many aspects of plant cell, tissue, and organ culture studies, such as callus induction, somatic embryogenesis, and shoot organogenesis and proliferation has proved that TDZ is a potent regulator of these morphogenic processes. Lu (1993) reviewed the use of TDZ for adventitious shoot regeneration, axillary shoot induction, and somatic embryogenesis in many plant species, and indicated that TDZ induces as many or more adventitious shoots than adenine-type cytokinins for most of the species in which it had been tested. In some plant species, such as *Dianthus caryophyllus* (Lu et al. 1991; Nugent et al. 1991) and *Rosa* sp. (Lu 1993), TDZ was even more effective than BA, Kin, and zeatin at inducing shoot regeneration. The potency of TDZ has been demonstrated for the in vitro propagation of many recalcitrant, woody, and legume species (reviewed by Huetteman and Preece 1993; Lakshmanan and Taji 2000). A review by Schulze (2007) highlighted the role of TDZ in improving cereal tissue culture, minimising the recalcitrant nature of the Poaceae, and extending the application of transformation protocols to elite genotypes and more readily available explants. The morphoregulatory role of TDZ and the characterisation of TDZ-induced morphogenic effects was reviewed by Murthy et al. (1998), who indicated that TDZ stimulates endogenous plant-growth-regulating compounds in

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excised and intact tissues, and acts by modulating endogenous PGRs, either directly or as a result of induced stress. Guo et al. (2011) reviewed TDZ as a multi-dimensional PGR, and summarised the biochemical and biophysical responses of plant cells to TDZ and related mechanisms. TDZ was effective for flower induction in vitro in many plant species including *Bambusa edulis* (Lin and Chang 1998), *Dendrobium nobile* (Wang et al. 2009), and *Rauvolfia tetraphylla* (Faisal et al. 2005). Moreover, TDZ can significantly enhance transformation frequencies by improving the vigour of transgenic shoots (Joersbo et al. 1999). Recently, several authors contributed to a book edited by Ahmad and Faisal (2018) on TDZ and its applications to several aspects of plant tissue culture including morphogenesis, somatic embryogenesis, and micropropagation of herbaceous and woody plant species, highlighting the use of TDZ for the tissue culture of medicinal plants (Ahmad and Shahzad 2018; Deepa et al. 2018) and its potential use as an elicitor for the production of secondary metabolites (Unal 2018).

Unlike other cytokinins, TDZ is resistant to endogenous cytokinin oxidase, which makes it fairly stable in tissue culture (Mok et al. 1982). The metabolism of TDZ is extremely slow while that of zeatin is completely metabolised by plant tissues within hours of its application (Mok and Mok 1985). TDZ suppresses the activity of cytokinin oxidase (Horgan et al. 1988; Hare et al. 1994), which can result in the accumulation of purine cytokinins in plant tissues. When *Hordeum vulgare* leaves were treated with 10^{-8} to 10^{-5} M TDZ for 24 h, transcription was substantially accelerated in an in vitro system containing chromatin and RNA polymerase I from TDZ-treated leaves (Karavaiko et al. 2004). Nisler et al. (2016) reported two new TDZ derivatives (1-[1,2,3]thiadiazol-5-yl-3-(3-trifluoromethoxy-phenyl)urea (3FMTDZ) and 1-[2-(2-hydroxyethyl) phenyl]-3-(1,2,3-thiadiazol-5-yl)urea) as being very potent inhibitors of cytokinin oxidase/dehydrogenase, an enzyme that catalyzes the degradation of cytokinins. A possible modulation of endogenous gibberellin by TDZ has also been proposed (Hutchinson et al. 1997a). Treatment with TDZ enhanced stress-related genes (Zhang et al. 2006) leading to the accumulation of ethylene (Yip and Yang 1986; Hutchinson et al. 1997b; Murthy 1997). The accumulated ethylene in turn inhibits auxin transport (Radhakrishnan et al. 2009). The accumulation of stress signaling molecules such as abscisic acid and proline was also associated with TDZ treatment (Murch et al. 1999; Jones et al. 2007). Moreover, TDZ purportedly modulates the influx/efflux of calcium, which is an important signaling molecule that initiates a cascade of metabolic events (White and Broadley 2003; Jones et al. 2007). Several developmental patterns in plant tissue culture were attributed to the regulatory role of TDZ in the biosynthesis and accumulation of endogenous hormones.

Despite numerous studies characterising TDZ as a potent PGR across a wide range of plant species, disturbance of normal plant development is associated with its application. Early reports by Huetteman and Preece (1993) and Lu (1993) highlighted that the formation of fasciated and compact shoots, hyperhydricity and difficulty in rooting were the main undesirable effects of prolonged exposure to TDZ while several other negative effects were subsequently reported in literature. The focus of this review is to summarise and discuss the abnormalities associated with the use and application of TDZ, with a focus on different strategies used to avoid or overcome the occurrence of such abnormalities in in vitro cultures, with details described in Table 1.

Morphological, anatomical abnormalities and loss of morphogenic ability

TDZ (> 2.0 µM) induced undesirable changes in plant morphology, such as abnormal leaf morphology, fasciated shoots, and swollen shoot bases in many plant species (Table 1), including *Spathiphyllum cannifolium* (Fig. 1a, b; 2.27–8.08 µM TDZ; Dewir et al. 2006a), *Cordyline fruticosa* (Fig. 1c, d; 13.6–18.2 µM TDZ; Dewir et al. 2015), the *Aglaonema* hybrid 'Valentine' (9.1 µM TDZ; El-Mahrouk et al. 2016), *Prunus armeniaca* (20 µM TDZ; Goffreda et al. 1995), and *Musa* spp. (> 2.0 µM TDZ; Shirani et al. 2009). The differentiation of tetrafoliate single leaves and stalk-like structures without a shoot apex were reported in *Arachis hypogaea* in response to 4.5 µM TDZ (Akasaka et al. 2000). Histological observations revealed that the malformation most often obtained in *A. hypogaea* was a shoot-like structure that lacked a shoot apical meristem and had disorganised vascular bundles (Akasaka et al. 2000). Abnormal bulblets with small bulb scales and swollen basal plates from bulb scales of the *Lilium* oriental hybrid 'Casablanca' formed in media containing 4.5 µM TDZ (Han et al. 2005). Some of these abnormalities may appear during the first culture, or after continuous cultures. Continuous culture in TDZ-containing media induced abnormal bud primordia in *D. caryophyllus* (Ahmad et al. 2006) and *A. hypogaea* (Akasaka et al. 2000) which failed to develop into plantlets. Additionally, a TDZ-induced shoot culture of *Aloe polyphylla* developed swollen buds that did not develop into shoots (Ivanova and van Staden 2008).

Loss of morphogenic ability was also reported during somatic embryogenesis. Embryogenic masses of *A. hypogaea* were less responsive towards differentiation at high TDZ concentrations (13.62–45.41 µM) and several of these structures dedifferentiated and became necrotic (Joshi et al. 2008). TDZ-induced somatic embryos of *Oncidium*

Table 1 Studies on TDZ-induced abnormalities in plant tissue culture

Family	Plant species and/or cultivar[Y]	Explant used	Culture medium, TDZ concentrations and additives	Culture conditions	Abnormalities and remarks*	References
Apocynaceae	*Rauvolfia tetraphylla*[2] Cultivar NR	In vitro nodal segments (0.5–1 cm)	MS + 0.5–10 µM TDZ, pH 5.7, 3% sucrose, 0.25% phytagel (SIM, SMM)	16-h PP, 50 µmol m^{-2} s^{-1}, 25 ± 2 °C	5 µM TDZ for 4 weeks produced the highest shoot multiplication (18.5 shoots/explant). However, cultures grown continuously for 6 weeks on TDZ-containing media at ≥ 5 µM formed fasciated and distorted shoots	Faisal et al. (2005)
Araceae	*Spathiphyllum cannifolium*[2] Cultivar NR	In vitro axillary shoots (2.5 cm)	MS + 0–8.08 µM TDZ, pH 5.8, 3% sucrose, 0.2% gelrite (SMM)	16-h PP, 35 µmol m^{-2} s^{-1}, 25 °C	Shoots treated with TDZ (all tested concentrations) produced swollen shoots with narrow leaves and fewest axillary shoots (2.4 shoots/explant) compared to other cytokinins	Dewir et al. (2006a)
	Philodendron cannifolium[3.4] Cultivar NR	In vitro shoot tips (3 cm)	MS + 0–4.5 µM TDZ, pH 5.8, 3% sucrose, 0.8% gelrite (SMM)	16-h PP, 50 µmol m^{-2} s^{-1}, 25 ± 2 °C	Abnormal, swollen and hard callus formed from basal parts of shoots cultured on MS medium with TDZ for all concentrations tested. TDZ was less effective than BA for shoot proliferation. TDZ-proliferated shoots also showed necrosis and lacked chlorophyll	Han and Park (2008)
	Aglaonema[2] 'Valentine'	In vitro axillary shoots (2.5–3 cm)	MS + 0–9.1 µM TDZ, pH 5.7, 3% sucrose, 0.2% gelrite (SMM)	16-h PP, 35 µmol m^{-2} s^{-1}, 25 ± 2 °C	TDZ at 6.8 µM was superior to BA or Kin and produced the most axillary shoots (2.3 shoots/explant). Shoots cultured at 9.1 µM TDZ had a swollen shoot base and abnormal growth	El-Mahrouk et al. (2016)
Asphodelaceae	*Aloe polyphylla*[3.1] Cultivar NR	In vitro axillary shoots (3–4 cm)	MS + 5 and 15 µM TDZ, pH 5.8, 3% sucrose, 0.8% agar (SMM)	Continuous PP, 35 ± 2 µmol m^{-2} s^{-1}, 25 ± 2 °C	TDZ (5 and 15 µM) resulted in swollen base of initial explants and formation of buds, which did not develop into shoots	Ivanova and van Staden (2008)
	Aloe polyphylla [3.3] Cultivar NR	In vitro axillary shoots (3–4 cm)	MS + 1.25 or 2.5 µM TDZ, pH 5.8, 3% sucrose, 0.8% agar (SMM)	Continuous PP, 35 ± 2 µmol m^{-2} s^{-1}, 25 ± 2 °C	TDZ (2.5 µM) resulted in very low shoot regeneration and high hyperhydricity (79%). Explants grown on media with TDZ often displayed necrosis of the meristematic area, formed sponge-like callus at their base and produced many buds whose growth was inhibited, not developing into shoots	Ivanova and van Staden (2011)

Table 1 (continued)

Family	Plant species and/or cultivar[Y]	Explant used	Culture medium, TDZ concentrations and additives	Culture conditions	Abnormalities and remarks*	References
Asteraceae	*Pluchea lanceolate*[3.3] Cultivar NR	Nodes from in vitro axillary shoots	MS + 2.3–11.4 µM TDZ, pH 5.8, 3% sucrose, 0.25% phytagel (SIM, SMM)	16-h PP, 35 µmol m^{-2} s^{-1}, 25 °C	Highest shoot multiplication (9.7 shoots/node) was obtained at 2.3 µM TDZ, but higher TDZ concentrations (> 2.3 µM) caused stunting and hyperhydricity of shoots	Kher et al. (2014)
Caryophyllaceae	*Dianthus caryophyllus*[3.3] 'Red Lena'	Axillary buds with small parts of the adjacent tissues from the stem	MS + 0.004–0.4 µM TDZ + 0.5 µM NAA, 0.8% Bacto agar (SIM)	16-h PP, 54 µmol m^{-2} s^{-1}, 25–26 °C	TDZ (0.04–0.4 µM) caused hyperhydricity, decreased pigment content, and explants developed many short stems with shortened internodes	Genkov et al. (1997)
Ericaceae	*Arbutus unedo*[3.3] Cultivar NR	In vitro axillary shoots	MS + 0–18.2 µM TDZ, pH 5.7, 3% sucrose, 0.8% agar–agar (SMM)	16-h PP, 25 µmol m^{-2} s^{-1}, 25 ± 2 °C	Best axillary shoot multiplication (3.9 shoots/explant) achieved on MS medium with 13.6 µM TDZ. Shoots cultured on medium with 18.2 µM TDZ showed symptoms of hyperhydricity	El-Mahrouk et al. (2010)
	Arbutus unedo [3.2] *A. andrachne* *A.* × *andrachnoides* Cultivar NR	Single-node stem explants of in vitro seedlings	MS or WPM + 0.9 or 9.1 µM TDZ, pH 5.6–5.7, 3% sucrose, 0.8% agar (SIM)	16-h PP, 37.5 µmol m^{-2} s^{-1}, 25 °C	TDZ (0.9 or 9.1 µM)-treated shoots had malformations and were very short, making them unsuitable for multiplication	Papafotiou et al. (2013)
	Vaccinium macrocarpon[3.2] 'Franklin' and 'Bergman'	Leaf explants from both in vitro shoots and greenhouse plants	Anderson's major salts (Anderson 1975) with MS minor salts and organics (3% sucrose) + 0.1–10 µM TDZ + 0 or 1 µM NAA, pH 4.5, 0.7% agar (SIM)	16-h PP, 35 ± 10 µmol m^{-2} s^{-1}, 25 °C	TDZ (10 µM TDZ)-regenerated shoots did not elongate until explants were transferred to PGR-free medium at which time only a minority of shoots elongated	Marcotrigiano et al. (1996)
	Vaccinium vitis-idaea[3.2] 'NL1' and 'EL1'	In vitro three-node stem sections	MMS + 0.1–5 µM TDZ, pH 5.0, 2.5% sucrose, 0.35% agar + 0.125% gelrite (SIM, SMM)	16-h PP, 30 µmol m^{-2} s^{-1}, 20 ± 2 °C	TDZ 1 µM produced the highest number of shoots (1.8/explant). A higher conc. (> 1 µM) inhibited shoot proliferation (1.4 shoots/explant). Shoot height, number of leaves and shoot vigour also declined with increasing TDZ concentration. Callus formation at basal ends at all TDZ concentrations	Debnath (2005)

Table 1 (continued)

Family	Plant species and/or cultivar[Y]	Explant used	Culture medium, TDZ concentrations and additives	Culture conditions	Abnormalities and remarks*	References
Fabaceae	*Albizia lebbeck*[4] Cultivar NR	Hypocotyls	MS + 0.5–7.5 µM TDZ, pH 5.8, 3% sucrose, 0.8% agar (SIM, SMM)	16-h PP, 50 µmol m^{-2} s^{-1}, 25 ± 2 °C	TDZ at 1 µM showed maximum number of shoots (12.6/explant) after 4 weeks of culture. However, TDZ-exposed hypocotyl explants subcultured onto the same fresh medium showed hyperhydricity and shoots did not elongate further. TDZ > 1.0 µM resulted in callus formation	Perveen and Anis (2015)
	Arachis hypogaea[2] 'Chico'	Leaf segments from in vitro plants	MS + 5.4 µM NAA + 0.5–45.4 µM TDZ, pH 5.8, 3% sucrose, 0.8% agar (SMM)	24-h PP, 60 µmol m^{-2} s^{-1}, 25 °C	TDZ at 4.5 µM resulted in the highest frequency of shoot regeneration (14.3%). However, regenerated shoots showed abnormal shoot morphologies, such as somatic embryo-like structures, fused shoots which elongated slightly, 'stalk'-like structures with expanded leaves, but lacking a shoot apex, and differentiation of a tetrafoliated single leaf with a 'stalk'	Akasaka et al. (2000)
	Arachis hypogaea[2] Cultivar NR	Embryogenic masses induced from mature zygotic embryo-derived immature leaflets	MS + 13.6 µM 2,4-D + (2.27–45.41 µM) TDZ, pH 5.8, 0.18 M sucrose, 0.6% agar (SEIM)	16-h PP, 35 ± 2 µmol m^{-2} s^{-1}, 25 ± 2 °C	Development of the embryos to form bipolar structures was restricted in the presence of TDZ in the majority of cultures. However, at high TDZ concentrations (13.62–45.41 µM), globular structures were less responsive to differentiation of shoot buds and several of these structures dedifferentiated and became necrotic	Joshi et al. (2008)
	Cassia alata[4] Cultivar NR	In vitro nodal segments from 20 days old seedlings	MS + 1–10 µM TDZ, pH 5.8, 3% sucrose, 0.8% agar (SMM)	16-h PP, 50 µmol m^{-2} s^{-1}, 25 ± 2 °C, RH 50–60%	The highest number of axillary shoots (17.9/explant) was achieved at 5 µM TDZ for 4 weeks followed by 8 weeks in TDZ-free medium. Shoots cultured on medium with TDZ at 5 µM for 6 weeks showed basal callusing and the development of thick and stunted shoots	Ahmed and Anis (2014)
	Cassia angustifolia[3.1] Cultivar NR	Shoot tips from ex vitro seedlings (< 2 cm)	MS + 0.5–10 µM TDZ, pH 5.8, 3% sucrose, 0.8% agar (SMM)	16-h PP, 50 µmol m^{-2} s^{-1}, 25 ± 2 °C	TDZ at 5 µM produced the highest axillary shoot multiplication (7 shoots) after 4 weeks of culture. However, cultures grown continuously on TDZ-containing medium formed fasciated and distorted shoots	Siddique et al. (2015)

Table 1 (continued)

Family	Plant species and/or cultivar[Y]	Explant used	Culture medium, TDZ concentrations and additives	Culture conditions	Abnormalities and remarks*	References
	Cicer arietinum[6] 'Kabuli'	Seeds	MS salts + B_5 vitamins + 1–100 µM TDZ, 5 or 10 mM L-proline pH 5.7, 3% sucrose, 0.3% gelrite (SGM, SMM)	Dark for 1 week then 16-h PP, 30–35 µmol m^{-2} s^{-1}, 25 °C	TDZ at 10 µM produced the highest adventitious shoots (23 shoots/explant) after 5 weeks of culture. Seedlings growth on 25 µM TDZ-supplemented medium were highly stunted, cotyledons became enlarged, and seedlings produced thicker and darker green leaves. Root growth was also inhibited and modified	Murthy et al. (1996)
	Glycine max 'Bragg'[2]	Cotyledons	MS + 5.0–7.5 µM TDZ, pH 6.15–6.25, 2.5% sucrose, 0.7% agar (SIM)	16-h PP, 40–50 µE, 24 °C	Adventitious shoots produced in the presence of TDZ (5.0–7.5 µM) appeared stunted with abnormal leaves	Mante et al. (1989)
	Leucaena leucocephala[4] 'K636'	Immature zygotic embryos	½ MS or MS + (0.03–1.5 µM) TDZ, pH 5.8, 3% sucrose, 0.65% phytagel (SIM)	16-h PP, 30 µmol m^{-2} s^{-1}, 25 ± 2 °C	The maximum number of shoots per explant (8.3) was achieved in medium with 0.26 µM TDZ. TDZ at 0.35–1.5 µM or higher concentrations resulted in abnormal stunted shoots, profuse callus, shorter internodes, and abnormal color of leaves with some degree of translucence	Pal et al. (2012)
	Pisum sativum[6] 'Finale' and 'Solara'	Protoplast-derived callus	MS salts + B_5 vitamins + 1.5–50 µM TDZ, pH 5.7, 2% sucrose, 3% mannitol, 0.05% MES, 0.7% agar (SIM)	16-h PP, light intensity NR, 22 ± 2 °C	TDZ at 10 µM induced maximum frequency of shoot formation (12% for Finale and 8% for Solara). However, the shoots were short and stunted and no rooting of these TDZ-induced shoots was achieved even after the elongation stage	Bohmer et al. (1995)
	Vigna subterranea[4] Cultivar NR; 7 landraces (Ci1–Ci6 and Ci8)	Shoot tips from in vitro seedlings	MS salts + B5 vitamins + 0–0.45 µM TDZ, pH 5.8, 3% sucrose, 0.7% agar–agar (SIM)	16-h PP, 100 µE m^{-2} s^{-1}, 25 ± 2 °C	TDZ at 0.45 µM was best for shoot multiplication with a mean of 11 shoots/explant, but callus formation at the basal end of the explants and reduced shoot length were observed	Silué et al. (2016)
Gramineae	*Oryza sativa*[5] Amaroo (*japonica*), Pelde (*japonica* × *indica*), Langi (*japonica* × *indica*), and Millin (*japonica*)	Callus initiated from excised zygotic embryos of mature dehusked seeds	MS + 0–9.1 µM TDZ + 1 mg l^{-1} NAA, 3% sucrose, 3% sorbitol and casamino acids (2 g/l), pH 5.8, 0.4% gelrite (SIM)	16-h PP, 55 µE m^{-2} s^{-1}, 28 ± 1 °C	Addition of TDZ (2.3–9.1 µM) to SIM resulted in the regeneration of some albino shoots in all varieties tested	Azria and Bhalla (2000)

Table 1 (continued)

Family	Plant species and/or cultivar[Y]	Explant used	Culture medium, TDZ concentrations and additives	Culture conditions	Abnormalities and remarks*	References
Hypericaceae	*Hypericum sampsonii*[4] *Hypericum perforatum* Cultivar NR	In vitro plantlets	MMS + (0.23–2.27 µM) TDZ, pH 5.7–5.8, 3% sucrose, 0.6% agar (SIM)	16-h PP, 72.35 ± 2.96 µmol m^{-2} s^{-1}, 25 ± 2 °C	Cultures elicited for only 6 days. In *H. perforatum*, hyperforin production was significantly decreased at 2.27 µM TDZ. Callus formation and malformed shoots were also observed. In *H. sampsonii*, TDZ (0.23–0.91 µM) induction decreased hypericin levels and resulted in malformed leaves and poor shoot growth	Liu et al. (2007)
	Hypericum hirsutum and *H. maculatum*[3.4] Cultivar NR	In vitro adventitious shoots	MS + 1.8 µM TDZ + 0.05 mg l^{-1} NAA, pH 5.7, 3% sucrose, 0.75% agar (SMM)	16-h PP, 32 µmol m^{-2} s^{-1}, 25 ± 2 °C	TDZ at 1.8 µM induced hyperhydricity, malformations and necrosis of regenerated shoots. TDZ induced an increase of hyperhydricity compared to other cytokinins (BA, Kin or 2iP)	Coste et al. (2011)
Juglandaceae	*Juglans nigra*[3.3] Cultivar NR	Nodal cuttings (0.5–1 cm with one to three buds each) and shoot tips (1–2 cm long) from in vitro plants	DKW + 0.01–0.1 µM TDZ, AdS (20 mg l^{-1}), 2% sucrose, pH 5.7–5.8, 0.2% gelrite (SMM)	16-h PP, 25–30 µmol m^{-2} s^{-1}, 23 ± 2 °C	High proliferation rates (75–87%) were obtained at 0.05–0.1 µM TDZ. The regenerated axillary shoots were typically hypertrophic (25%) with stem diameters of up to 5–7 mm compared to more typical diameters of 1.5–2 mm and were light green in color with epinastic leaves	Bosela and Michler (2008)
Lauraceae	*Cinnamomum camphora*[3.3] Cultivar NR	In vitro shoot tips (0.3–0.5 cm), nodal segments (2 cm)	MS + 0.00454–4.54 µM TDZ, pH 5.0, 3% sucrose, 0.2% gelrite (SIM, SMM)	16-h PP, 45 µmol m^{-2} s^{-1}, 25 ± 2 °C	BA stimulated callus development and shoot formation, whereas TDZ mainly promoted callus development and no lateral shoots emerged. TDZ at 0.454 and 4.54 µM TDZ caused hyperhydricity (50% and 100%, respectively)	Huang et al. (1998)
Laxmanniaceae	*Cordyline fruticosa*[2] Cultivar NR	In vitro shoots	MS + 0–18.2 µM TDZ, pH 5.8, 3% sucrose, 0.2% gelrite (SMM)	16-h PP, 55 µmol m^{-2} s^{-1}, 25 ± 2 °C	TDZ at 0.5 µM recorded the highest number of shoots (14 shoots/explant). Shoots cultured on medium with TDZ at 13.6 µM had thick broad leaves that were wrinkled, curled and brittle, all indicating symptoms of hyperhydricity	Dewir et al. (2015)

Table 1 (continued)

Family	Plant species and/or cultivar[Y]	Explant used	Culture medium, TDZ concentrations and additives	Culture conditions	Abnormalities and remarks*	References
Liliaceae	*Lilium* oriental hybrid[2] 'Casablanca'	In vitro bulb scales	MS + 0.5–22.7 µM TDZ pH 5.8, 9% sucrose (SIM)	16-h PP, 50 µmol m^{-2} s^{-1}, 25 ± 2 °C	TDZ at 4.5 µM produced the highest number of shoots/bulb scale (3.8) compare to control without TDZ (2.8) after 8 weeks in culture.TDZ at ≥ 4.5 µM caused abnormally swollen basal plates, a swollen basal part of bulb scales, and formed adventitious shoots with small foliage and leafy bulb scales	Han et al. (2005)
Musaceae	*Musa* spp.[2] 'Berangan Intan', 'Berangan' (AAA), 'Rastali', 'Nangka' (AAB) and 'Baka Baling' (ABB)	Shoot apices (0.5–0.7 cm) from ex vitro plants	MS + 0–7.5 µM TDZ, pH 5.7, 3% sucrose, 2.8% gelrite (SMM)	16-h PP, light intensity NR, 26 ± 2 °C	TDZ at 2 µM was optimal for shoot proliferation. TDZ concentration > 2.0 µM resulted in a high number of abnormal shoots. Higher TDZ concentrations (5–7.5 µM) enhanced exudation of phenolic compounds. Shoot length was reduced as TDZ concentration increased	Shirani et al. (2009)
Orchidaceae	*Cymbidium giganteum*[5]	Pseudostem segments with nodes from the base of in vitro-raised seedlings	Semi-solid or dual phase consisting of ½ MS + 0.909–20 µM TDZ, 2 g l^{-1} tryptone, pH 5.4, 3% sucrose, 1.5 g l^{-1} AC, 0.8% agar (PLB-IM)	16-h PP, light intensity NR, 25 ± 1 °C	Healthy shoots were differentiated at a low TDZ concentration (0.909 µM). Differentiated shoots at 20 µM TDZ were fasciated and hyperhydric. Assessment of molecular variation using 18 RAPD primers detected 5.81% change in regenerated plantlets at 0.909 µM TDZ	Roy et al. (2012)
	Phalaenopsis bellina[5]	Leaves from ex vitro plants	½ MS + 100 mg l^{-1} myo-inositol, 0.5 mg l^{-1} niacin, 0.5 mg l^{-1} pyroxidine HCL, 0.1 mg l^{-1} thiamine HCL, 2.0 mg l^{-1} glycine, 13.6 µM TDZ, 10% fresh ripen banana extract, pH 5.6, 2% sucrose, 0.3% gelrite (PLB-IM)	Culture conditions NR	TDZ-induced PLBs were subcultured for 6 months for proliferation which resulted in 17% dissimilarity with the mother plant using RAPD	Khoddamzadeh et al. (2010)

Table 1 (continued)

Family	Plant species and/or cultivar[Y]	Explant used	Culture medium, TDZ concentrations and additives	Culture conditions	Abnormalities and remarks*	References
	Oncidium orchid[2] 'Gower Ramsey' and 'Sweet Sugar'	Internodes of flower stalks (i.e., peduncle) (0.5 cm) from ex vitro plants	½ MS + 0–13.6 µM TDZ, pH 5.2, 2% sucrose, 0.22% gelrite (SEIM)	16-h PP, 28–36 µmol m^{-2} s^{-1}, 26 ± 1 °C	Supplementing 0.5–13.6 µM TDZ enhanced the percentages of embryo- and shoot bud-producing explants to 65 and 45%, respectively. However, embryos failed to develop into plantlets, and transformed to green abnormal structures after 1.5–2 months of culture on medium containing 1.4 µM TDZ and 2.7 µM NAA or PGR-free medium. These abnormal structures turned brown and eventually deteriorated on the same medium	Chen and Chang (2000)
Poaceae	*Bambusa edulis*[5] Cultivar NR	In vitro shoots (3–5 leaf primordia)	MS + 0.05–27.2 µM TDZ, pH 5.7, 3% sucrose, 0.2% gelrite (SMM)	16-h PP, 54 µmol m^{-2} s^{-1}, 26 °C	TDZ at 0.5 µM recorded the highest number of shoots (3.56/explant). Elongation was inhibited and considerable hyperhydricity occurred at a higher TDZ concentration (27.2 µM). TDZ-treated shoot cultures (30%) were albino, and albinism occurred by the second subculture	Lin and Chang (1998)
Ranunculaceae	*Cimicifuga racemosa*[3.1] Cultivar NR	Callus	MS + 2–18 µM TDZ, pH 5.7, 3% sucrose, 0.8% agar (SIM)	16-h PP, 52 µmol m^{-2} s^{-1}, 25 ± 2 °C	TDZ at 2 µM recorded the highest adventitious shoot frequency (85%) and number of shoots (14.8/explant), but a TDZ concentration > 4 µM suppressed shoot organogenesis from callus	Lata et al. (2002)
Rosaceae	*Cotoneaster wilsonii*[3.3] Cultivar NR	Nodal segments and shoot tips (0.5–1 cm) from ex vitro plants	MS + 0–9.1 µM TDZ, pH 5.7, 3% sucrose, 0.8% agar (SMM)	16-h PP, 30 µmol m^{-2} s^{-1}, 25 ± 1 °C	TDZ at 2.3 µM recorded the highest shoot induction from nodes (87.4%) and shoot tips (84.2%) as well as number of shoots from nodes (19.7) and shoot tips (11.3). TDZ (> 2.3 µM) had a negative effect on frequency of shoot induction, and number of shoots/explant. Callus formed at the base of explants and regenerated shoots were hyperhydric	Sivanesan et al. (2011)
	Prunus armeniaca[2] 'Zard' and 'NJA82'	Immature embryos and cotyledons	MS + 0–20 µM TDZ + 0–1 µM 2,4-D, 0.09 M sucrose, 0.7% gum agar (SIM)	40–50 µE m^{-2} s^{-1}, 27–28 °C	Induction of shoot primordia increased on media containing 5–20 µM TDZ and 1 µM 2,4-D. However, shoot morphology was abnormal, especially with the highest level of TDZ	Goffreda et al. (1995)

Table 1 (continued)

Family	Plant species and/or cultivar[Y]	Explant used	Culture medium, TDZ concentrations and additives	Culture conditions	Abnormalities and remarks*	References
	Rosa chinensis[2] 'Yueyuehong'	Embryogenic callus	SEP + 2.25–11.25 µM TDZ, pH 5.4, 3% sucrose, 0.4% agar (SEIM)	16-h PP, 27 µmol m^{-2} s^{-1}, 25 °C	Low concentrations of TDZ (2.25 µM) for 4 weeks increased the frequency of embryogenic callus induction. TDZ-induced somatic embryos at 11.25 µM for 12 weeks showed abnormal morphology, "wood ear"-like plantlets which had simple leaves rather than compound leaves	Chen et al. (2014)
Rutaceae	*Coleonema pulchellum*[3.4] Williams Cultivar NR	Shoot tips from ex vitro plants (≈2 cm)	MS + 4.2–22.7 µM TDZ and combination of 4.5 µM TDZ + 0.04% GM, 0.3% CH, MBZ, and HB, pH 5.8, 3% sucrose, 0.8% agar (SIM, SMM)	16-h PP, 40 µmol m^{-2} s^{-1}, 25 ± 2 °C	A high number of normal shoots (25/explant) were achieved with a low concentration of TDZ (4.5 µM) after 10 weeks of culture. Shoots cultured on medium with TDZ at 13.6 and 22.7 µM and a combination of 4.5 µM TDZ and 0.04% GM for 10 weeks showed shoot tip necrosis. Combinations of 0.3% CH, MBZ or HB with 4.5 µM TDZ produced hyperhydric shoots as well as green compact basal callus	Baskaran et al. (2014)
Salicaceae	*Salix tetrasperma*[3.1] Cultivar NR	Nodal segments from ex vitro sprouts	MS or WPM + 0–10 µM TDZ, pH 5.8, 3% sucrose, 0.8% agar (SIM, SMM)	16-h PP, 50 µmol m^{-2} s^{-1}, 25 ± 2 °C	Exposure to 2.5 µM TDZ for 4 weeks produced maximum axillary shoots (4.5/explant). Longer exposure time (> 4 weeks) resulted in fasciated or distorted shoots, conversion of shoots to callus or necrotic tissues	Khan and Anis (2012)
Scrophulariaceae	*Bacopa monniera*[3.1] Cultivar NR	Nodal cuttings (0.8–1 cm), internodes (1 cm) and leaves (0.6 cm) from in vitro plants	MS + 0–22.7 µM TDZ, pH 5.8, 3% sucrose, 0.8% agar–agar (SIM). MS + 2.2 µM TDZ, pH 5.8, 3% sucrose, 0.8% agar–agar (SMM)	16-h PP, 50 µmol m^{-2} s^{-1}, 25 ± 2 °C	TDZ at 6.8 µM was optimal for adventitious shoot induction in which 93 shoots/leaf explant were produced after 7 weeks of culture. TDZ > 6.8–22.7 µM reduced frequency of shoot bud induction and shoots had stunted growth in SIM. After three subcultures (each 7 weeks on SMM), the frequency and number of adventitious shoot buds declined from 85 to 50%	Tiwari et al. (2001)

Table 1 (continued)

Family	Plant species and/or cultivar[Y]	Explant used	Culture medium, TDZ concentrations and additives	Culture conditions	Abnormalities and remarks*	References
Verbenaceae	*Vitex trifolia*[3.1] Cultivar NR	Nodal segments (0.5–1 cm) from ex vitro plants	MS + 0–10 µM TDZ, pH 5.8, 3% sucrose, 0.8% agar–agar (SIM, SMM)	16-h PP, 50 µmol m^{-2} s^{-1}, 25 ± 2 °C	TDZ at 5.0 µM recorded the highest shoot regeneration frequency (90%) and maximum number (22.3) of shoots/explant. TDZ had negative consequences with prolonged exposure (> 7 days) resulting in distortion, hyperhydricity, and fasciation of induced shoot buds	Ahmed and Anis (2012)
Zingiberaceae	*Alpinia zerumbet*[3.2] Cultivar NR	In vitro plantlets	MS + 9.1 µM TDZ, pH 5.8, 3% sucrose, 0.78% agar (SMM)	16-h PP, 30 W m^{-2}, 25 ± 2 °C	TDZ-induced multiple shoots showed stunted growth with low fresh and dry weights	Victório et al. (2011)

AC activated charcoal, *AdS* adenine sulphate, *CARB* carbenicillin, *CH* casein hydrolysate, *CEF* cefotaxime, *DKW* Driver and Kuniyuki walnut medium (Driver and Kuniyuki 1984), *EDTA* (ethylenedinitrilo)-tetraacetic acid ferric sodium salt, *SEP* somatic embryo proliferation, *GM* glutamine, *MBZ* mebendazole, *MES* 2-(Nmorpholino) ethane acid, *MS* Murashige and Skoog (1962) medium, *NR* not reported in the study, *PEN* penicillin G, *PGR* plant growth regulator, *PLB* protocorm-like body, *PLB-IM* protocorm-like bodies induction medium, *PP* photoperiod, *PPFD* photosynthetic photon flux density, *RAPD* randomly amplified polymorphic DNA, *SEIM* somatic embryogenesis induction medium, *SGM* seed germination medium, *SIM* shoot induction medium, *SMM* shoot multiplication medium, *WPM* woody plant medium (McCown and Lloyd 1981)

*Even though the term "calli" may have been used in the original publication, the term "callus" has been standardized here based on the recommendation of Teixeira da Silva (2012), while any mentions of "vitrification" have been replaced with "hyperhydricity". Y, each plant species is assigned a number related to its TDZ-induced abnormality, corresponding to main text section numbers: 2 = morphological, anatomical abnormalities and loss of morphogenic ability; 3.1 = inhibition of shoot proliferation; 3.2 = inhibition of shoot growth and elongation; 3.3 = hyperhydricity; 3.4 = shoot tip, shoot and tissue necrosis; 4 = callus formation; 5 = cytogenetic variation; 6 = altered rooting or loss of rooting ability

sp. failed to develop into plantlets, and instead developed into green abnormal structures that eventually turned brown and deteriorated (Chen and Chang 2000). In *Rosa chinensis*, TDZ-induced somatic embryos showed abnormal morphology, and turned brown when TDZ was applied at 11.25 µM (Chen et al. 2014).

Physiological abnormalities in shoots and stems

Inhibition of shoot proliferation

Despite the high cytokinin-like activity of TDZ during shoot proliferation, the inhibition of this process may also occur due to its application. The negative effects of TDZ on shoot proliferation have been reported in many plant species, including *S. cannifolium* (Dewir et al. 2006a) and *Cotoneaster wilsonii* (Sivanesan et al. 2011). In *Bacopa monniera*, the frequency and number of adventitious shoot buds (initiated at 6.8 µM TDZ) declined from 85 to 50% and from 53.5 to 16.9, respectively, after three subcultures (7 weeks in each passage) in media containing 2.2 µM TDZ (Tiwari et al. 2001).

Explants in plant tissue experiments tend to be continuously exposed to TDZ for a long duration, similar to other cytokinins, until organogenesis. Thus, explants are subjected to an overdose, resulting in the inhibition of shoot proliferation and other abnormalities. The optimal duration of TDZ exposure was less than 7 days in *Phaseolus vulgaris* (Malik and Saxena 1992), *Pelargonium* × *hortorum* (Hutchinson and Saxena 1996), and *Manihot esculenta* (Bhagwat et al. 1996). Malik and Saxena (1992) reported that a TDZ pulse treatment (10 µM) of *P. vulgaris* seeds for just 1 day was sufficient to induce direct shoot organogenesis (ten shoots/seedling), and the number of shoots after 7 days (35 shoots/seedling) was comparable to numbers after a continuous 4-week treatment (30 shoots/seedling). However, the optimal TDZ concentration and duration of exposure are species-dependent due to genotypic differences. In *M. esculenta*, exposure for 6–8 days to 0.22 µM TDZ was optimal for shoot proliferation (12.4 and 11/nodal explant, respectively), while longer or shorter durations (4 and 10–26 days) reduced shoot proliferation to 6–8 shoots (Bhagwat et al. 1996).

To avoid TDZ-induced inhibition of shoot proliferation, the TDZ concentration, exposure duration, plant genotype, and subculture of TDZ-exposed cultures to a secondary medium should be considered. A secondary medium lacking TDZ was used to improve *Cassia angustifolia* shoot proliferation and elongation by exposing shoot tips to 0.5 µM TDZ for 4 weeks (Siddique et al. 2015), while a secondary medium for 12 weeks containing 1 µM BA and 0.5 µM NAA effectively enhanced shoot multiplication from 4.5 to 20.3

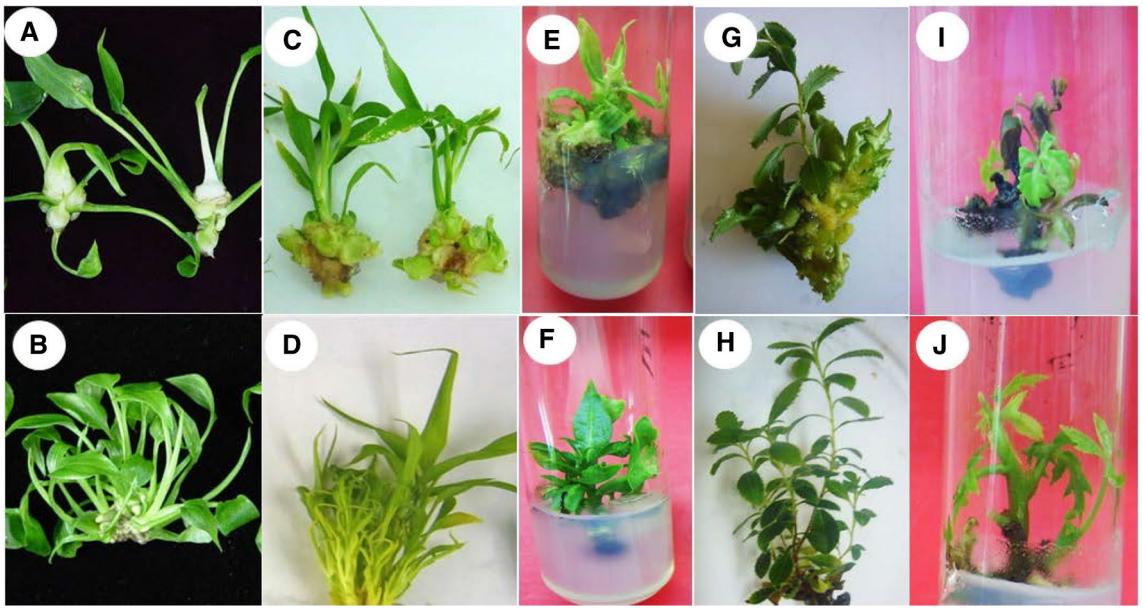

Fig. 1 Detrimental side effects of thidiazuron (TDZ) during shoot proliferation of several plants in vitro. *Spathiphyllum cannifolium* (**a** swollen shoot base and abnormal morphology at 2.27–8.08 µM TDZ; **b** normal proliferation at13.9 µM kinetin—in vitro shoots were cultured on MS medium solidified with 0.2% gelrite and incubated in a 16-h photoperiod (PP), 35 µmol m^{-2} s^{-1}, and 25 °C), *Cordyline fruticosa* (**c** stunted and swollen shoots at 13.6 µM TDZ; **d** normal proliferation at 0.5 µM TDZ—in vitro shoots were cultured on MS medium solidified with 0.2% gelrite and incubated at 16-h PP, 55 µmol m^{-2} s^{-1}, and 25 ± 2 °C), *Conocarpus erectus* (**e** callus formation at the shoot base with 2.3 µM TDZ; **f** normal proliferation with 8.9 µM benzyl adenine [BA]—in vitro shoots were cultured on MS medium solidified with 0.8% agar and incubated at 16-h PP, 35 µmol m^{-2} s^{-1}, and 25 °C), *Arbutus unedo* (**g** hyperhydric shoots at 18.2 µM TDZ; **h** normal shoots at ≤ 13.6 µM TDZ—in vitro shoots were cultured on MS medium solidified with 0.8% agar and incubated at 16-h PP, 25 µmol m^{-2} s^{-1}, and 25 ± 2 °C), and *Cissus rhombifolia* (I, shoot tip necrosis with 4.5 µM TDZ; J, normal shoot in response to 4.4 µM BA—in vitro shoots were cultured on MS medium solidified with 0.8% agar and incubated at 16-h PP, 25 µmol m^{-2} s^{-1}, and 25 ± 2 °C). Unpublished photographs (Y.H. Dewir)

shoots and shoot length from 1.4 to 4.7 cm 4-week-old cultures of *Salix tetrasperma* exposed to 2.5 µM TDZ (Khan and Anis 2012). The efficiency of a secondary medium lacking PGRs or containing 1.0 µM BA singly or in combination with 0.5 µM NAA on explants exposed for 7 days to TDZ (5 µM) was also evaluated for 4 and 8 weeks in *Vitex trifolia* (Ahmed and Anis 2012). BA (1.0 µM), when applied with NAA (0.5 µM), resulted in highest shoot number (22.3) and shoot length (5.2 cm) after 8 weeks of culture (Ahmed and Anis 2012).

Inhibition of shoot growth and elongation

Although TDZ has been shown to be superior to other cytokinins for shoot proliferation (reviewed by Huetteman and Preece 1993; Lu 1993; Murthy et al. 1998), problems with short and compact shoots have been reported in many plant species, including *Juglans nigra* (Van Sambeek et al. 1997), *Rollinia mucosa* (Figueiredo et al. 2001), and *Alpinia zerumbet* (Victório et al. 2011). Despite the high efficiency of TDZ at 2.5 µM, compared to zeatin, Kin, BA and 2-isopentenyladenine (2iP), in the regeneration of *Saccharum officinarum* embryogenic callus, it produced a high percentage (84%) of stunted shoots shorter than 1 cm (Chengalrayan and Gallo-Meagher 2001). Huetteman and Preece (1993) indicated that TDZ-inhibited shoot elongation may be due to its high cytokinin activity while the presence of a phenyl group in TDZ may be a possible cause of shoot bud fasciation. A study by Hutchinson et al. (1997a), which used gibberellin-synthesis inhibitors (triazoles and ancymidol) to improve TDZ-induced somatic embryogenesis in *Pelargonium* × *hortorum*, indicated the possible modulation of endogenous gibberellin by TDZ. TDZ-induced stunted growth may be related to gibberellin metabolism or proline biosynthesis, but this remains unclear. In general, stunted growth is not desired for micropropagation due to the difficulty in isolating individual shoots from multiple shoots, which increases the labour and time taken, and the cultivation of extra subcultures for their elongation and growth. On the other hand, TDZ-stunted shoots facilitates excision due to thick stems, for example, in *Dendranthema* (Teixeira da Silva 2003).

Previous studies showed different strategies to overcome the TDZ-induced inhibition of shoot elongation, such as sub-culturing onto PGR-free medium or medium containing another cytokinin, or the inclusion of other cytokinins with TDZ. Shoot elongation was favoured in the absence of PGRs

in *Lavandula stoechas* (Nobre 1996) while inhibited elongation in *Vaccinium vitis-idaea* was overcome by transferring cultures inhibited by TDZ (1 µM for 8 weeks) to media containing 1 µM zeatin for 4 weeks (Debnath 2005). A two-step regeneration strategy, using TDZ to induce bud/shoot formation, followed by the use of zeatin to promote shoot elongation, was also reported for northern highbush blueberry cultivars and *Vaccinium angustifolium* (Song and Sink 2004; Debnath 2009; Liu et al. 2010). In *Picea glauca*, adventitious shoots elongated more when TDZ was combined with BA or zeatin (Ellis et al. 1991). In *Vaccinium macrocarpon*, the inclusion of 5 µM 2iP in the TDZ (10 µM)-containing culture initiation medium for 15 days and transfer of explants to a secondary medium containing a low TDZ concentration (1 µM) and 10 µM 2iP for 7 days followed by transfer to PGR-free medium resulted in enhanced shoot elongation in 2 weeks while no elongation was observed on initiation medium (5 µM 2iP and 10 µM TDZ) even after 4 months (Qu et al. 2000). A secondary medium containing 25 µM 2iP alone was used to elongate TDZ (10 µM)-regenerated *Pisum sativum* adventitious shoots within 4–10 weeks while shoots remained stunted when secondary medium with auxins or free of PGRs was used (Bohmer et al. 1995).

Hyperhydricity

Hyperhydricity is a disorder of tissue-cultured plants in which leaves become translucent and stems become swollen, distorted, and brittle. The environment inside a tissue culture vessel affects the normal growth and physiology of plants, and ultimately gives rise to morphological and physiological malformation and malfunctions (Park et al. 2004; Chakrabarty et al. 2006; Dewir et al. 2006b, 2015). Cytokinins are among the various factors that cause hyperhydricity in vitro (reviewed by Vieira de Vasconcelos 2012; Dewir et al. 2014). Kadota and Niimi (2003) indicated that phenylurea derivatives (11 µM *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea (CPPU) or 44 µM TDZ) promoted nearly a tenfold increase in the number of hyperhydric shoots in *Pyrus pyrifolia* than adenine derivatives (44 µM BA or 0.44 µM Kin). Different cytokinins (BA, Kin, 2iP, and TDZ) induced hyperhydricity in *Hypericum hirsutum* and *H. maculatum*, and TDZ had the greatest negative effect at 1.8 µM. These anomalies could explain the reduced shoot biomass production and inhibition of pseudohypericin biosynthesis (Coste et al. 2011). Hypericin (R=CH_3) and pseudohypericin (R=CH_2OH) are dianthrone derivatives with antiviral and anticancer activities (Prince et al. 2000; Kirakosyan et al. 2008). In *Hypericum* species, Liu et al. (2007) reported that TDZ at 0.23 µM resulted in malformed leaves and poor shoot growth and decreased hypericin content while 2.27 µM increased the formation of clustered shoots and hypericin content in *H. perforatum*. There was a 25-fold increase in the number of dark glands between *H. sampsonii* and *H. perforatum*, but only a 4.21-fold increase in the production of hypericin, suggesting that there was no direct correlation between gland number and hypericin production. Although hypericin metabolism occurred in black glands and the number of black glands increased following TDZ treatment, the production of hypericin was dependent on the metabolic efficiency of black glands on leaves. Thus, TDZ concentration and its induced anomalies affect the accumulation of secondary metabolites in tissue culture.

TDZ-induced hyperhydricity of regenerated shoots was reported in several plant species (Table 1), including *Arbutus unedo* (18.2 µM TDZ; Fig. 1g, h; El-Mahrouk et al. 2010), *Pluchea lanceolata* (> 2.3 µM TDZ; Kher et al. 2014), *A. polyphylla* (2.5 µM TDZ; Ivanova and van Staden 2011), and *J. nigra* (0.05–0.1 µM TDZ; Bosela and Michler 2008). High concentrations of TDZ (6.8–9.1 µM) and repeated subcultures resulted in hyperhydric *C. wilsonii* shoots (Sivanesan et al. 2011). Among different cytokinins (2iP, TDZ and zeatin; 2.25–22.5 µM) tested for shoot multiplication of *Hymenocallis littoralis*, TDZ showed the lowest total chlorophyll content at all tested concentrations (Yew et al. 2010). Ultrastructural analysis of *Annona glabra* in vitro leaves from regenerated axillary shoots on WPM medium (McCown and Lloyd 1981) supplemented with cytokinins (4.5 µM TDZ, 4.4 µM BA, 4.6 µM Kin, or 4.6 µM zeatin) revealed that TDZ, compared to other cytokinins, resulted in a significant reduction of chlorophyll *a* content and formation of abnormally shaped chloroplasts rich in plastoglobules, which is associated with a marked disorganization of the chloroplast endomembrane system (Oliveira et al. 2008). In *D. caryophyllus*, TDZ at 0.04–0.4 µM induced hyperhydricity in regenerated axillary buds and decreased the stability of the photosynthetic membrane, and the autolytic destruction of photosynthetic pigments of isolated chloroplasts was very strong in TDZ-treated material but weak in material treated with 0.4 µM BA (Genkov et al. 1997). TDZ (0.93 µM) induced the expression of stress-related genes in *Medicago sativa* callus, including the trehalose-6-phosphate phosphatase (TPP), 1-aminocyclopropane-1-carboxylate synthase (ACS) and proline dehydrogenase genes (Zhang et al. 2006) while TDZ-induced expression of ACS resulted in enhanced ethylene accumulation. Several studies indicated increased ethylene in response to TDZ treatment (Yip and Yang 1986; Hutchinson et al. 1997b; Murthy 1997). In wilted *Triticum aestivum* leaves, 10 µM TDZ exerted stress-induced ethylene production equal to that exerted by 1 mM BA, indicating that TDZ is more active than BA by two orders of magnitude (Yip and Yang 1986). During somatic embryogenesis of *Pelargonium* × *hortorum*, hypocotyl explants treated with 10 µM TDZ resulted in elevated levels of ethylene within 6 h in the headspace of culture vessels (Hutchinson et al. 1997b). Ethylene has been proposed as a

negative by-product of the TDZ-mediated metabolic cascade (Hutchinson et al. 1997b), so it is likely that TDZ-induced hyperhydricity could be an indirect response to increased ethylene production.

Shoot tip, shoot and tissue necrosis

Shoot tip necrosis (STN), a common physiological deformity in plant tissue culture, is associated with the type and concentration of cytokinin in the culture medium (Bairu et al. 2009). In STN, the necrotic shoot tip initially becomes brown and eventually dies (Fig. 1i, j—unpublished results on *Cissus rhombifolia*; Lakshmi and Raghava 1993; Bairu et al. 2009). Baskaran et al. (2014) noted that although 13.6 µM TDZ produced a significantly higher number of shoots (37 per explant) of *Coleonema pulchellum* after 10 weeks of culture than BA (4.4–22.2 µM) or *meta*-topolin (4.2–20.7 µM), those shoots exhibited symptoms of STN. TDZ at 20 µM induced tissue necrosis in *Hypericum erectum* (Kim et al. 2006). In *H. hirsutum* and *H. maculatum*, the addition of 1.8 µM TDZ to liquid culture medium led to malformation and STN (Coste et al. 2011). Murch et al. (1999) noted the accumulation of abscisic acid, proline, and ions in TDZ-induced regeneration in peanut. Axillary shoots of *Cercis canadensis* proliferated using TDZ (0.2–22.7 µM) had leaves that were chlorotic and at a high concentration of TDZ (22.7 µM) had excessive necrosis and phenolic accumulation (Mackay et al. 1995). Prolonged exposure to TDZ may also result in the accumulation of phenolic compounds, as was reported during the induction of *Pelargonium*×*hortorum* somatic embryogenesis (Hutchinson and Saxena 1996). The exudation of phenolic compounds was enhanced by TDZ (Shirani et al. 2009). The accumulation of these stress-related substances might be responsible for TDZ-induced STN. However, several other factors including aeration, medium pH, nutrients and salt strength were reported to affect STN (reviewed by Bairu et al. 2009).

Callus formation

The use of TDZ may result in the formation of callus during axillary shoot proliferation, which is technically difficult and laborious to dissect from single shoot buds. Moreover, it could inhibit axillary shoot proliferation, as the regeneration pathway is directed toward callogenesis and indirect organogenesis. At 10 µM TDZ, axillary shoot proliferation of *Cassia alata* was coupled with callus formation and the number of shoots was reduced from 17.9 to 7.2 when nodal segments were exposed for 4 weeks to TDZ at 5 and 10 µM, respectively (Ahmed and Anis 2014). The formation of callus at basal cut ends of node explants on cytokinin-enriched medium is frequent in species with strong apical dominance (Preece et al. 1991). Marks and Simpson (1994) attributed the formation of basal callus to the action of accumulated auxins at the basal ends, stimulating cell proliferation. TDZ also exhibits auxin-like activity by modulating the biosynthesis and accumulation of endogenous auxins (Mok and Mok 1985; Murthy et al. 1995; Murch and Saxena 2001) thus, enhancing callus formation. In *B. monniera*, treatment with TDZ (> 6.8–22.7 µM for 7 weeks) produced excessive callus, and the shoot buds remained stunted (Tiwari et al. 2001). Callus type in *Chirita swinglei* could be altered depending on the use of TDZ at 1 µM or other cytokinins (Zhang et al. 2016). Callus formation has been reported as an undesirable side-effect of TDZ in a number of plants (Table 1), including *Conocarpus erectus* (2.3 µM TDZ for 6 weeks; Fig. 1e, f; Dewir et al. 2018), *Albizia lebbeck* (> 1 µM TDZ for 4 weeks; Perveen and Anis 2015), *Leucaena leucocephala* (0.35–1.5 µM TDZ for 3 weeks; Pal et al. 2012), *Vigna subterranea* (0.11–45 µM TDZ for 4 weeks; Silué et al. 2016), and Ericaceous species (Cao and Hammerschlag 2000; Qu et al. 2000; Debnath 2005; Guo et al. 2011).

Cytogenetic variation

In vitro plants are usually proliferated for several subculture cycles prior to rooting and acclimatisation and cytogenetic variations may occur in this long process. TDZ-induced somaclonal variations can be a valuable source for new genetic material. All off-type plants of *Tulipa gesneriana* 'Blue Parrot' micropropagated through long term cyclic subcultures (4 years) on TDZ (4.5–9.1 µM)-supplemented medium and grown in an insect-proof tunnel for four growing cycles, had a red–purple flower color instead of purple–violet flowers (Podwyszyńska 2005; Podwyszyńska et al. 2006). However, high genetic fidelity and true-to-type clones are critical for commercial micropropagation to maintain the essential characteristics of the mother plant. Several indicators, including morphological characteristics, chromosome numbers, isozyme profiles, and PCR-based molecular markers [randomly amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), and start codon targeted (SCoT) polymorphism] can be used to assess genetic fidelity (Gostimsky et al. 2005; Podwyszyńska et al. 2006), including of tissue-cultured plants (Kacar et al. 2006). Khoddamzadeh et al. (2010) reported DNA polymorphism with 17% dissimilarity among the TDZ (13.6 µM) regenerated plants to the mother plant *Phalaenopsis bellina* using RAPD. Less variability (5.95%) was detected within TDZ (6.8 µM)-propagated *D. nobile* using RAPD and SCoT (Bhattacharyya et al. 2014) and TDZ (0.909 µM)-propagated *Cymbidium giganteum* (5.81%) plants using RAPD (Roy et al. 2012). RAPD and ISSR analysis of micropropagated *T. gesneriana*

'Blue Parrot' through cyclic subcultures (4 years) on TDZ (4.5–9.1 µM) medium revealed 45 and 55% polymorphism, respectively, while no polymorphism was detected in progeny lines derived from 2-year-old cultures (Podwyszyńska et al. 2006). DNA polymorphism (20%) was also reported in protocorm like-bodies (PLBs) of *Phalaenopsis gigantea* after 20 weeks of culture on medium containing 4.5 µM TDZ and 65.5 µM chitosan while reducing the culture period to 16 weeks resulted in no variations (Samarfard et al. 2014). Therefore, limiting the number of subculture cycles could maintain clonal characteristics, but the amenability to a TDZ-induced mutagenic effect is species-dependent.

In plants, albinism is a phenomenon in which the partial or complete loss of chlorophyll may result from differences in genotype, environmental conditions, or genome-based modifications that reduce chlorophyll biosynthesis and eventually damage the photosynthetic apparatus, causing photo-bleaching (Kumari et al. 2009). Albinism has been reported to be a cytogenetic variation associated with TDZ treatment in vitro (Table 1). Albino plants are difficult to root in vitro, and do not survive ex vitro, therefore, albinism is clearly undesirable for plant mass propagation. Lin and Chang (1998) reported that 30% of TDZ-regenerated (4.5 µM for 3 weeks) *B. edulis* axillary shoots were albino, albinism occurred by the second subculture and all albino plants did not survive ex vitro conditions. Lin et al. (2007) induced flowers from adventitious albino shoots of *Dendrocalamus latiflorus* after an 8-month subculture on medium with 0.45 µM TDZ, and shoots were multiplied on medium containing 0.45 µM TDZ, but no seeds were produced. Thus, albinism is also considered as a biotechnological limitation to in vitro hybridization. Plant regeneration from mature zygotic embryo-derived callus in several rice varieties on medium containing 2.3–9.1 µM TDZ and 5.4 µM NAA resulted in the regeneration of some albino shoots in every variety tested compared to 8.9–17.8 µM BA with 5.4 µM NAA (Azria and Bhalla 2000). Albinism is affected by the genotype, physiological state of the donor plants, culture temperature, and medium composition including sucrose concentration, and PGRs (reviewed in Kumari et al. 2009).

Plastid development requires compatibility between nuclear and chloroplast genomes which encode proteins essential for chloroplast development and function. Genetic studies in albino plants indicated that it is a recessive trait governed by many loci (Kumari et al. 2009). A large deletion of plastid DNA may be responsible for the regeneration of many albino rice plants (Harda et al. 1992). Recently, Zeng et al. (2017) observed that an albino rice plant had a mutation in the *OsABCI8* gene which is localized in the chloroplast and plays a major role in chloroplast development and biosynthesis of chlorophyll precursor. Albino plants accumulated significantly higher levels of iron and nickel than wild type plants. In vitro albino *Agave angustifolia* plantlets regenerated on MS medium supplemented with 0.11 µM 2,4-D and 22.2 µM BA showed low expression levels of genes involved in photosynthesis and carotenoid biosynthesis, suggesting a disruption of these enzymes and processes in albino plants (Us-Camas et al. 2017). In in vitro plant cells, TDZ promotes the passage and storage of endogenous plant signals and iron, and modifies endogenous PGRs as well as the activities of antioxidant enzymes such as catalases and peroxidases (reviewed by Murthy et al. 1998; Guo et al. 2011). However, the mutagenic effect of TDZ and associated molecular mechanisms remain unknown, calling for further investigations.

Altered rooting or loss of rooting ability

TDZ influences the morphogenesis and rooting efficiency of shoots in culture when used at concentrations above the threshold levels and/or for long durations (Dewir et al. 2016). The loss of rooting ability is due to carryover effects of TDZ when used at the shoot multiplication stage which is dependent on TDZ concentration. In *Solanum melongena*, adventitious shoots regenerated by 0.2 µM TDZ failed to develop roots in several rooting induction media while at 0.01 µM TDZ, only 6% rooting was obtained in ½ MS medium supplemented with 0.6 µM IAA (Magioli et al. 1998). Interestingly, rooting reached 70% when callus was maintained on PGR-free medium before shoot excision for 2 weeks after bud induction by TDZ (Magioli et al. 1998). In *L. leucocephala*, rooting was 0% for axillary shoots regenerated on 0.45–2.27 µM TDZ for 30 days and there was a gradual increase in rooting up to 76.6% by decreasing TDZ concentration to 0.05 µM (Shaik et al. 2009). Moreover, the authors indicated that a TDZ (0.45 µM) pulse for 24 h for shoot multiplication caused high rooting (82–87%) of the regenerated shoots. TDZ-related inhibitory effects on rooting were also reported for *Cicer arientinum* (Murthy et al. 1996), *Tamarindus indica* (Mehta et al. 2004), and *T. aestivum* (Li et al. 2003). Inhibited root initiation caused by an extended period of exposure to cytokinins could be induced by modified metabolic enzyme activity or an edited receptor site ultimately reducing the effectiveness of auxins (Javed et al. 2013). The period of exposure to TDZ can also impact the timing of root initiation, with longer rooting times observed as a side effect of incubation with high concentrations of TDZ. In cultures of *S. melongena* initiated from root explants on root induction medium supplemented with auxin and TDZ, only shoots formed, but not roots (Franklin et al. 2004). The carryover effects of TDZ on the loss of rooting ability in the Fabaceae family can be overcome or minimised through several techniques and alternative methods to

promote rooting (reviewed by Dewir et al. 2016), including subcultures on a PGR-free medium, using a reduced concentration of salt, adjusting the auxin type, concentration and timing of application, use of liquid rather than semi-solid medium, or exposing unrooted shoots to a high concentration of auxin as a pulse. Although grafting is a skill-based and tedious technique, it can be an alternative to in vitro rooting in difficult-to-root or recalcitrant species. Rooting was impossible for adventitious shoots of *P. sativum* regenerated with TDZ (1–50 µM) and so in vitro grafting was used resulting in a 100% grafting rate (Bohmer et al. 1995).

Species-specific effects of TDZ concentration

Some plant species are receptive to a wide range of TDZ concentrations and subculture cycles than others as confirmed by numerous successful protocols for tissue culture using TDZ, in which these plant species exhibited normal growth. In contrast, several other plants (Table 1) such as *Cassia alata* (Ahmed and Anis 2014), *Glycine max* (Mante et al. 1989), *Leucaena leucocephala* (Pal et al. 2012), *Juglans nigra* (Bosela and Michler 2008), and *Musa* sp. (Shirani et al. 2009), exhibited abnormal growth and showed sensitivity to very low TDZ concentrations (> 0.1–10 µM) while other species such as *Aglaonema* 'Valentine' (El-Mahrouk et al. 2016), *Bambusa edulis* (Lin and Chang 1998), and *Cordyline fruticosa* (Dewir et al. 2015) showed abnormalities at relatively high concentrations (> 13.6–27.2 µM). A slight increase in TDZ concentration reduced shoot proliferation in *Cimicifuga racemosa*, but increasing the TDZ concentration from 2 to 18 µM reduced the number of shoots from 14.8 to 3.5 per explant (Lata et al. 2002). Additionally, increasing TDZ concentration switched the regeneration pathway in *Saintpaulia ionantha*, where a low concentration (˂2.5 µM) induced shoot organogenesis while 5–10 µM induced somatic embryogenesis (Mithila et al. 2003). A similar switch from shoot organogenesis to somatic embryogenesis was observed in *Ochna integerrima* when TDZ concentration was increased from 5 to 10–15 µM (Ma et al. 2011) or in *Metabriggsia ovalifolia* when 2.5 µM TDZ was increased to 25 µM (Yao et al. 2016). Tremendous improvement in the tissue culture of woody and recalcitrant plant species has been achieved using TDZ. While a low concentration of TDZ in the range of nM to a few µM has shown the ability to induce axillary shoots, higher concentrations favor callus formation and proliferation of adventitious shoots (Huetteman and Preece 1993; Debnath 2018; Vinoth and Ravindhran 2018).

Conclusions

The efficiency of TDZ has been proved in tissue culture applications in several plant species, but it can lead to deformities if not used properly. To understand and avoid these deformities, we reviewed the detrimental side effects of TDZ in 40 plant species belonging to 23 families (Table 1), nine of which belong to the Fabaceae. These detrimental effects included morphological abnormality, loss of morphogenic ability, inhibition of shoot proliferation and shoot elongation, STN, hyperhydricity, cytogenetic variations such as albinism and DNA polymorphism, and altered rooting or loss of rooting ability, all of which hinder the in vitro propagation of plant species. These detrimental effects occur due to the inappropriate use of TDZ. TDZ is highly stable due to its slow metabolism and resistance to cytokinin oxidase. It enhances the synthesis of adenine-type cytokinins, modulates endogenous hormones, and exhibits both auxin and cytokinin-like activities. Moreover, it promotes stress genes and leads to the production of ethylene and stress signaling molecules. TDZ can thus be used solely to achieve different morphogenic and regeneration pathways. The appropriate and optimal concentration of TDZ is species-specific. However, the use of TDZ at low concentrations, pulse treatment and short periods of exposure are effective strategies to avoid TDZ-induced abnormalities. In *Bactris gasipaes*, a pulse treatment with 0.36 µM TDZ for 14 days minimized the negative effects caused by prolonged exposure to TDZ (Graner et al. 2013). Recently, Kumari et al. (2018) reported that overnight soaking of *C. arietinum* seeds in a 20 µM TDZ solution prior to culture in PGR-free medium reduced the detrimental effects of TDZ in proliferated axillary shoots. The inclusion of other cytokinins with TDZ in the primary medium and the use of a secondary medium lacking PGRs or containing adenine-derived cytokinins is an efficient two-step regeneration strategy to overcome growth deformities, enhance shoot elongation and minimize the carry-over effects of TDZ during the rooting stage. Other culture conditions including medium type and strength, gelling agent, ventilation and light intensity are important factors to maintain normal growth characteristics. Limiting the number of subculture cycles on TDZ-supplemented medium needs to be considered to maintain the clonal characteristics of regenerants. Further studies are required to improve our understanding of the physiological and metabolic mechanisms associated with using TDZ. Additionally, studies on the molecular mechanisms of TDZ-induced cytogenetic variations are necessary since genetic fidelity is a serious concern, particularly for commercial propagation. Finally, many plant research-based journals have historically placed greater emphasis on positive results than on negative results, so it is expected that

the negative impact of TDZ on plant morphogenesis in vitro may be much higher than that reported in this review, simply because negative results tend to be deemphasized (Teixeira da Silva 2015).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

Ahmad N, Faisal M (2018) Thidiazuron: From urea derivative to plant growth regulator. Springer, Singapore, p 489

Ahmad Z, Shahzad A (2018) Thidiazuron influenced morphogenesis in some medicinal plants. In: Ahmad N, Faisal M (eds) Thidiazuron: from urea derivative to plant growth regulator. Springer, Singapore, pp 231–246

Ahmad N, Srivastava R, Anis M (2006) Improvement in carnation shoot multiplication using thidiazuron in vitro. Propag Ornam Plants 6:109–113

Ahmed MR, Anis M (2012) Role of TDZ in the quick regeneration of multiple shoots from nodal explant of *Vitex trifolia* L.—an important medicinal plant. Appl Biochem Biotechnol 168:957–966

Ahmed MR, Anis M (2014) Changes in activity of antioxidant enzymes and photosynthetic machinery during acclimatization of micropropagated *Cassia alata* L. plantlets. In Vitro Cell Dev Biol Plant 50:601–609

Akasaka Y, Daimon H, Mii M (2000) Improved plant regeneration from cultured leaf segments in peanut (*Arachis hypogaea* L.) by limited exposure to thidiazuron. Plant Sci 156:169–175

Anderson WC (1975) Propagation of rhododendrons by tissue culture. Part 1. Development of a culture medium for multiplication of shoots. Proc Intl Plant Prop Soc 25:129–134

Arndt FR, Rusch R, Stillfried HV, Hanisch B, Martin WC (1976) SN 49537, a new defoliant [abstract]. Plant Physiol 57:99

Azria D, Bhalla PL (2000) Plant regeneration from mature embryo-derived callus of Australian rice (*Oryza sativa* L.) varieties. Aust J Agric Res 51:305–312

Bairu MW, Stirk WA, Staden JV (2009) Factors contributing to in vitro shoot-tip necrosis and their physiological interactions. Plant Cell Tiss Org Cult 98:239–248

Baskaran P, Moyo M, Van Staden J (2014) In vitro plant regeneration, phenolic compound production and pharmacological activities of *Coleonema pulchellum*. S Afr J Bot 90:74–79

Bhagwat B, Vieira LGE, Erickson LR (1996) Stimulation of in vitro shoot proliferation from nodal explants of cassava by thidiazuron, benzyladenine and gibberellic acid. Plant Cell Tiss Org Cult 46:1–7

Bhattacharyya P, Kumaria S, Diengdoh R, Tandon P (2014) Genetic stability and phytochemical analysis of the in vitro regenerated plants of *Dendrobium nobile* Lindl., an endangered medicinal orchid. Meta Gene 2:489–504

Bohmer P, Meyer B, Jacobson HJ (1995) Thidiazuron induced high frequency of shoot induction and plant regeneration in protoplast derived pea callus. Plant Cell Rep 15:26–29

Bosela MJ, Michler CH (2008) Media effects on black walnut (*Juglans nigra* L.) shoot culture growth in vitro: evaluation of multiple nutrient formulations and cytokinin types. In Vitro Cell Dev Biol Plant 44:316–329

Cao X, Hammerschlag FA (2000) Improved shoot organogenesis from leaf explants of highbush blueberry. HortScience 35:945–947

Chakrabarty D, Park SY, Ali MB, Shin KS, Paek KY (2006) Hyperhydricity in apple: ultrastructural and physiological aspects. Tree Physiol 26:377–388

Chen JT, Chang WC (2000) Plant regeneration via embryo and shoot bud formation from flower-stalk explants of *Oncidium* sweet sugar. Plant Cell Tiss Org Cult 62:95–100

Chen J, Wu L, Hu B, Yi X, Liu R, Deng Z, Xiong X (2014) The influence of plant growth regulators and light quality on somatic embryogenesis in china rose (*Rosa chinensis* Jacq.). J Plant Growth Regul 33:295–304

Chengalrayan K, Gallo-Meagher M (2001) Effect of various growth regulators on shoot regeneration of sugarcane. In Vitro Cell Dev Biol Plant 37:434–439

Coste A, Vlase L, Halmagyi A, Deliu C, Coldea G (2011) Effects of plant growth regulators and elicitors on production of secondary metabolites in shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum*. Plant Cell Tiss Org Cult 106:279–288

Debnath SC (2005) Micropropagation of lingonberry: influence of genotype, explant, orientation and overcoming TDZ-induced inhibition of shoot elongation using zeatin. HortScience 40:185–188

Debnath SC (2009) A two-step procedure for adventitious shoot regeneration on excised leaves of lowbush blueberry. In Vitro Cell Dev Biol Plant 45:122–128

Debnath SC (2018) Thidiazuron in micropropagation of small fruits. In: Ahmad N, Faisal M (eds) Thidiazuron: from urea derivative to plant growth regulator. Springer, Singapore, pp 139–158

Deepa AV, Anju M, Thomas TD (2018) The applications of TDZ in medicinal plant tissue culture. In: Ahmad N, Faisal M (eds) Thidiazuron: from urea derivative to plant growth regulator. Springer, Singapore, pp 297–316

Dewir YH, Chakrabarty D, Hahn EJ, Paek KY (2006a) A simple method for mass propagation of *Spathiphyllum cannifolium* using an airlift bioreactor. In Vitro Cell Dev Biol Plant 42:291–297

Dewir YH, Chakrabarty D, Ali MB, Hahn EJ, Paek KY (2006b) Lipid peroxidation and antioxidant enzyme activities of *Euphorbia millii* hyperhydric shoots. Environ Exp Bot 58:93–99

Dewir YH, Indoliya Y, Chakrabarty D, Paek KY (2014) Biochemical and physiological aspects of hyperhydricity in liquid culture system. In: Paek KY, Murthy HN, Zhong JJ (eds) Production of biomass and bioactive compounds using bioreactor technology. Springer, Berlin, pp 693–703

Dewir YH, El-Mahrouk ME, El-Banna AN (2015) In vitro propagation and preliminary results of *Agrobacterium*-mediated genetic transformation of *Cordyline fruticosa*. S Afr J Bot 98:45–51

Dewir YH, Murthy HN, Ammar MH, Alghamdi SS, Al-Suhaibani NA, Alsadon AA, Paek KY (2016) In vitro rooting of leguminous plants: difficulties, alternatives, and strategies for improvement. Hortic Environ Biotechnol 57:311–322

Dewir YH, Aldubai AA, El-Hendawy S, Alsadon AA, Seliem MK, Naidoo Y (2018) Micropropagation of buttonwood tree (*Conocarpus erectus*) through axillary shoot proliferation. HortScience 53:687–691

Driver JA, Kuniyuki AH (1984) In vitro propagation of Paradox walnut rootstock. HortScience 19:507–509

Ellis DD, Barczynska H, McCown BH, Nelson N (1991) Comparison of BA, zeatin, and thidiazuron for adventitious bud formation from *Picea glauca* embryos and epicotyl explants. Plant Cell Tiss Org Cult 27:281–287

El-Mahrouk ME, Dewir YH, Omar AMK (2010) In vitro propagation of adult strawberry tree (*Arbutus unedo* L.) through adventitious shoots and somatic embryogenesis. Propag Ornam Plants 10:93–98

El-Mahrouk ME, Dewir YH, Naidoo Y (2016) Micropropagation and genetic fidelity of the regenerants of *Aglaonema* 'valentine' using randomly amplified polymorphic DNA. HortScience 51:398–402

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

Figueiredo SFL, Albarello N, Viana VRC (2001) Micropropagation of *Rollinia mucosa* (Jacq.) Baill. In Vitro Cell Dev Biol Plant 37:471–475

Franklin G, Sheeba CJ, Sita GL (2004) Regeneration of eggplant (*Solanum melongena* L.) from root explants. In Vitro Cell Dev Biol Plant 40:188–191

Genkov T, Tsoneva P, Ivanova I (1997) Effect of cytokinins on photosynthetic pigments and chlorophyllase activity in in vitro cultures of axillary buds of *Dianthus caryophyllus* L. J Plant Growth Regul 16:169–172

Goffreda JC, Scopel AL, Fiola JA (1995) Indole butyric acid induces regeneration of phenotypically normal apricot (*Prunus armeniaca* L.) plants from immature embryos. Plant Growth Regul 17:41–46

Gostimsky SA, Kokaeva ZG, Konovalov FA (2005) Studying plant genome variation using molecular markers. Russ J Genet 41:378–388

Graner EM, Oberschelp GP, Brondani GE, Batagin-Piotto KD, de Almeida CV, de Almeida M (2013) TDZ pulsing evaluation on the in vitro morphogenesis of peach palm. Physiol Mol Biol Plants 19:283–288

Guo B, Abbasi BH, Zeb A, Xu LL, Wei YH (2011) Thidiazuron: a multi-dimensional plant growth regulator. Afr J Biotechnol 10:8984–9000

Han BH, Park BM (2008) In vitro micropropagation of *Philodendron cannifolium*. J Plant Biotechnol 35:203–208

Han BH, Yae BW, Yu HJ, Peak KY (2005) Improvement of in vitro micropropagation of *Lilium* oriental hybrid 'Casablanca' by the formation of shoots with abnormally swollen basal plates. Sci Hortic 103:351–359

Harda T, Ishikawa R, Niizeki M, Saito K (1992) Pollen derived rice calli that have large deletions in plastid DNA do not require protein synthesis in plastids for growth. Mol Gen Genet 233:145–150

Hare PD, Staden J, Van Staden J (1994) Inhibitory effect of TDZ on the activity of cytokinin oxidase isolated form soybean callus. Plant Cell Physiol 35:11221–11125

Horgan R, Burch LR, Palni LMS (1988) Cytokinin oxidase and the degradative metabolism of cytokinins, plant growth substances. In: Pharis RP, Rood SB (eds) Plant growth substances. Springer, Berlin, pp 282–290

Huang L-C, Huang B-L, Murashige T (1998) A micropropagation protocol for *Cinnamomum camphora*. In Vitro Cell Dev Biol Plant 34:141–146

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

Hutchinson MJ, Saxena PK (1996) Acetylsalicyclic acid enhances and synchronizes thidiazuron-induced somatic embryogenesis in geranium (*Pelargonium* × *hortorum* Bailey) tissue cultures. Plant Cell Rep 15:512–515

Hutchinson MJ, KrishnaRaj S, Saxena PK (1997a) Inhibitory effect of GA_3 on the development of thidiazuron-induced somatic embryogenesis in geranium (*Pelargonium* × *hortorum* Bailey) hypocotyl cultures. Plant Cell Rep 16:435–438

Hutchinson MJ, Murr DP, KrishnaRaj S, Saxena PK (1997b) Does ethylene play a role in thidiazuron-regulated somatic embryogenesis of geranium (*Pelargonium* × *hortorum*) hypocotyl cultures. In Vitro Cell Dev Biol Plant 33:136–141

Ivanova M, Van Staden J (2008) Effect of ammonium ions and cytokinins on hyperhydricity and multiplication rate of in vitro regenerated shoots of *Aloe polyphylla*. Plant Cell Tiss Org Cult 92:227–231

Ivanova M, Van Staden J (2011) Influence of gelling agent and cytokinins on the control of hyperhydricity in *Aloe polyphylla*. Plant Cell Tiss Org Cult 104:13–21

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

Joersbo M, Brunstedt J, Marcussen J, Okkels FT (1999) Transformation of the endospermous legume guar (*Cyamopsis tetragonoloba* L.) and analysis of transgene transmission. Mol Breed 5:521–529

Jones M, Yi Z, Murch SJ, Saxena PK (2007) Thidiazuron-induced regeneration of *Echinacea purpurea* L.: micropropagation in solid and liquid culture systems. Plant Cell Rep 26:13–19

Joshi M, Sujatha K, Hazra S (2008) Effect of TDZ and 2, 4-D on peanut somatic embryogenesis and in vitro bud development. Plant Cell Tiss Org Cult 94:85–90

Kacar YA, Byrne PF, Teixeira da Silva JA (2006) Molecular markers in plant tissue culture. In: Teixeira da Silva JA (ed) Floriculture, ornamental and plant biotechnology: advances and topical issues, vol II, 1st edn. Global Science Books, Ltd., Isleworth, pp 444–449

Kadota M, Niimi Y (2003) Effects of cytokinin types and their concentrations on shoot proliferation and hyperhydricity in in vitro pear cultivar shoots. Plant Cell Tiss Org Cult 72:261–265

Karavaiko NN, Selivankina SY, Kudryakova NV, Maslova GG, Burkhanova EA, Zubkova NK, Kulaeva ON (2004) Is a 67-kD cytokinin-binding protein from barley and *Arabidopsis thaliana* leaves involved in the leaf responses to phenylurea derivatives? (a review). Russ J Plant Physiol 51:790–797

Khan MI, Anis M (2012) Modulation of in vitro morphogenesis in nodal segments of *Salix tetrasperma* Roxb. through the use of TDZ, different media types and culture regimes. Agrofor Sys 86:95–103

Kher MM, Joshi D, Nekkala S, Nataraj M, Raykundaliya DP (2014) Micropropagation of *Pluchea lanceolata* (Oliver & Hiern.) using nodal explant. J Hort Res 22:35–39

Khoddamzadeh AA, Sinniah UR, Kadir MA, Kadzimin SB, Mahmood M, Sreeramanan S (2010) Detection of somaclonal variation by random amplified polymorphic DNA analysis during micropropagation of *Phalaenopsis bellina* (Rchb. f.) Christenson. Afr J Biotechnol 9:6632–6639

Kim OT, Bang KH, In DS, Kim TS, Seong NS, Cha SW, Ahn JC, Hwang B (2006) Micropropagation of *Hypericum erectum* Thunberg by using thidiazuron. Korean J Med Crop Sci 14:278–281

Kirakosyan A, Gibson D, Kaufman P (2008) The production of dianthrones and phloroglucinol derivatives in St. John's Wort. In: Ramawat K, Merillon J (eds) Bioactive molecules and medicinal plants. Springer, Berlin

Kumari M, Clarke HJ, Small I, Siddique KHM (2009) Albinism in plants: a major bottleneck in wide hybridization, androgenesis and doubled haploid culture. Crit Rev Plant Sci 28:393–409

Kumari P, Singh S, Yadav S, Tran LSP (2018) Pretreatment of seeds with thidiazuron delimits its negative effects on explants and promotes regeneration in chickpea (*Cicer arietinum* L.). Plant Cell Tiss Org Cult 133:103–114

Lakshmanan P, Taji A (2000) Somatic embryogenesis in leguminous plants. Plant Biol 2:136–148

Lakshmi SG, Raghava SBV (1993) Regeneration of plantlets from leaf disc cultures of rosewood: control of leaf abscission and shoot tip necrosis. Plant Sci 88:107–112

Lata H, Bedir E, Hosick A, Ganzera M, Khan I, Moraes RM (2002) In vitro plant regeneration from leaf-derived callus of *Cimicifuga racemosa*. Planta Med 68:912–915

Li W, Ding C-H, Hu Z, Lu W, Guo G-Q (2003) Relationship between tissue culture and agronomic traits of spring wheat. Plant Sci 164:1079–1985

Lin CS, Chang WC (1998) Micropropagation of Bambusa edulis through nodal explants of field-grown culms and flowering of regenerated plantlets. Plant Cell Rep 17:617–620

Lin SA, Liang CJ, Hsaio HW, Lin MJ, Chang WC (2007) In vitro flowering of green and albino *Dendrocalamus latiflorus*. New For 34:177–186

Liu XN, Zhang XQ, Sun JS (2007) Effects of cytokinins and elicitors on the production of hypericins and hyperforin metabolites in *Hypericum sampsonii* and *Hypericum perforatum*. Plant Growth Regul 53:207–214

Liu C, Callow P, Rowland LJ, Hancock JF, Song G-Q (2010) Adventitious shoot regeneration from leaf explants of southern highbush blueberry cultivars. Plant Cell Tiss Org Cult 103:137–144

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

Lu C, Nugent G, Wardley-Richardson T, Stephen F, Chandler SF, Young R, Dalling MJ (1991) *Agrobacterium-mediated* transformation of carnation (*Dianthus caryophyllus* L.). Nat Biotechnol 9:864–868

Ma G-H, Lü J-F, Teixeira da Silva JA, Zhang X-H, Zhao J-T (2011) Shoot organogenesis and somatic embryogenesis from leaf and shoot explants of *Ochna integerrima* (Lour). Plant Cell Tiss Org Cult 104:157–162

Mackay WA, Tipton JL, Thompson GA (1995) Micropropagation of Mexican redbud, *Cercis canadensis* var. *mexicana*. Plant Cell Tiss Org Cult 43:295–299

Magioli C, Rocha APM, De Oliveira DE, Mansur E (1998) Efficient shoot organogenesis of eggplant (*Solanum melongena* L.) induced by thidiazuron. Plant Cell Rep 17:661–663

Malik KA, Saxena PK (1992)) Regeneration in *Phaseolus vulgaris* L.: high frequency induction of direct shoot formation in intact seedlings by N^6-benzyladenine and thidiazuron. Planta 186:384–389

Mante S, Scorza R, Cordts J (1989) A simple, rapid protocol for adventitious shoot development from mature cotyledons of *Glycine max* cv Bragg. In Vitro Cell Dev Biol 25:385–388

Marcotrigiano M, McGlew SP, Hackett G, Chawla B (1996) Shoot regeneration from tissue- cultured leaves of the American cranberry (*Vaccinium macrocarpon*). Plant Cell Tiss Org Cult 44:195–199

Marks TR, Simpson SE (1994) Factors affecting shoot development in apically dominant *Acer* cultivars in vitro. J Hortic Sci 69:543–551

McCown BH, Lloyd G (1981) Woody plant medium (WPM)—a revised mineral formulation for micro-culture of woody plant species. HortScience 16:453

Mehta UJ, Barreto SM, Hazra S (2004) Effect of thidiazuron in germinating tamarind seedlings. In Vitro Cell Dev Biol Plant 40:279–283

Mithila J, Hall JC, Victor JMR, Saxena PK (2003) Thidiazuron induces shoot organogenesis at low concentrations and somatic embryogenesis at high concentrations on leaf and petiole explants of African violet (*Saintpaulia ionantha* Wendl.). Plant Cell Rep 21:408–414

Mok MC, Mok DWS (1985) The metabolism of [^{14}C]-thidiazuron in callus tissues of *Phaseolus lunatus*. Physiol Plant 65:427–432

Mok MC, Mok DWS, Armstrong DJ (1982) Cytokinin activity of *N*-phenyl-*N*-1, 2, 3-thidiiazol-5ylurea (TDZ). Phytochemistry 21:1509–1511

Murch S, Saxena PK (2001) Molecular fate of thidiazuron and its effects on auxin transport in hypocotyls tissues of *Pelargonium* × *hortorum* Bailey. Plant Growth Regul 35:269–275

Murch SJ, Victor JMR, Krishnaraj S, Saxena PK (1999) The role of proline in thidiazuron-induced somatic embryogenesis of peanut. In Vitro Cell Dev Biol Plant 35:102–105

Murthy BNS (1997) Morpho-physiological role of thidiazuron in plants. PhD thesis, University of Guelph, Canada

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

Murthy BNS, Victor J, Singh RPS, Fletcher RA, Saxena PK (1996) In vitro regeneration of chickpea (*Cicer arietinum* L.): stimulation of direct organogenesis and somatic embryogenesis by thidiazuron. Plant Growth Regul 19:233–240

Murthy BNS, Murch SJ, Saxena PK (1998) Thidiazuron: a potent regulator of in vitro plant morphogenesis. In Vitro Cell Dev Biol Plant 34:267–275

Nisler J, Kopečný D, Končitíková R, Zatloukal M, Bazgier V, Berka K, Zalabák D, Briozzo P, Strnad M, Spíchal L (2016) Novel thidiazuron-derived inhibitors of cytokinin oxidase/dehydrogenase. Plant Mol Biol 92:235–248

Nobre J (1996) In vitro cloning and micropropagation of *Lavandula stoechas* from field-grown plants. Plant Cell Tiss Org Cult 46:151–155

Nugent G, Wardley-Richardson T, Lu C (1991) Plant regeneration from stem and petal of carnation (*Dianthus caryophyllus* L.). Plant Cell Rep 10:477–480

Oliveira LM, Paiva R, Santanaa JRF, Alves E, Nogueira RC, Pereira FD (2008) Effect of cytokinins on in vitro development of autotrophism and acclimatization of *Annona glabra* L. In Vitro Cell Dev Biol Plant 44:128–135

Pal A, Negi VS, Borthakur D (2012) Efficient in vitro regeneration of *Leucaena leucocephala* using immature zygotic embryos as explants. Agrofor Syst 84:131–140

Papafotiou M, Bertsouklis KF, Trigka M (2013) Micropropagation of *Arbutus unedo, A. andrachne*, and their natural hybrid, *A.* × *andrachnoides* from seedling explants. J Hortic Sci Biotechnol 88:768–775

Park SW, Jeon JH, Kim HS, Park YM, Aswath C, Joung H (2004) Effect of sealed and vented gaseous microenvironments on the hyperhydricity of potato shoots in vitro. Sci Hortic 99:199–205

Perveen S, Anis M (2015) Physiological and biochemical parameters influencing ex vitro establishment of the in vitro regenerants of *Albizia lebbeck* (L.) Benth.: an important soil reclaiming plantation tree. Agrofor Syst 89:721–733

Podwyszyńska M, Niedoba K, Korbin M, Marasek A (2006) Somaclonal variation in micropropagated tulips determined by phenotype and DNA markers. Acta Hortic 714:211–220

Podwyszyńska M (2005) Somaclonal variation in micropropagated tulips based on phenotype observation. J Fruit Ornam Plant Res 13:109–122

Preece JE, Hutterman CA, Ashby WC, Roth PL (1991) Micro and cutting propagation of silver mapple. I. Results with adult and juvenile propagules. J Am Soc Hortic Sci 116:142–148

Prince AM, Pascual D, Meruelo D, Liebes L, Mazur Y, Dubovi E, Mandel M, Lavie G (2000) Strategies for evaluation of enveloped virus inactivation in red cell concentrates using hypericin. Photochem Photobiol 71:188–195

Qu L, Polashock J, Vorsa N (2000) A highly efficient in vitro cranberry regeneration system using leaf explants. HortScience 35:948–952

Radhakrishnan R, Ramachandran A, Kumari BR (2009) Rooting and shooting: dual function of thidiazuron in in vitro regeneration of soybean (*Glycine max* L.). Acta Physiol Plant 31:1213–1217

Roy AR, Sajeev S, Pattanayak A, Deka BC (2012) TDZ induced micropropagation in *Cymbidium giganteum* Wall. Ex Lindl. and assessment of genetic variation in the regenerated plants. Plant Growth Regul 68:435–445

Samarfard S, Kadir MA, Kadzimin SB, Saud HM, Ravanfar SA, Danaee M (2014) In vitro propagation and detection of somaclonal variation in *Phalaenopsis gigantea* as affected by chitosan and thidiazuron combinations. HortScience 49:82–88

Schulze J (2007) Improvements in cereal tissue culture by thidiazuron: a review. Fruit Veg Cereal Sci Biotech 1:64–79

Shaik NM, Arha M, Nookaraju A, Gupta SK, Shrivastava S, Yadav AK, Kulkarni PS, Abhilash OU, Vishwakarma RK, Singh S, Tatkare R, Chinnathambi K, Rawal SK, Khan BM (2009) Improved method of in vitro regeneration in *Leucaena leucocephala*—a leguminous pulpwood tree species. Physiol Mol Biol Plants 15:311–318

Shirani S, Mahdavi F, Maziah M (2009) Morphological abnormality among regenerated shoots of banana and plantain (*Musa* spp.) after in vitro multiplication with TDZ and BAP from excised shoot-tips. Afr J Biotechnol 8:5755–5761

Siddique I, Bukhari NAW, Perveen K, Siddiqui I (2015) Influence of plant growth regulators on in vitro shoot multiplication and plantlet formation in *Cassia angustifolia* Vahl. Braz Arch Biol Technol 58:686–691

Silué N, Koné T, Soumahoro AB, Koné M (2016) In vitro shoot tip multiplication of bambara groundnut [*Vigna subterranea* (L.) Verdc.]. Plant Cell Tiss Org Cult 127:603–611

Sivanesan I, Song JY, Hwang SJ, Jeong BR (2011) Micropropagation of *Cotoneaster wilsonii* Nakai—a rare endemic ornamental plant. Plant Cell Tiss Org Cult 105:55–63

Song G-Q, Sink KC (2004) *Agrobacterium tumefaciens*-mediated transformation of blueberry (*Vaccinium corymbosum* L.). Plant Cell Rep 23:475–484

Teixeira da Silva JA (2003) Chrysanthemum: advances in tissue culture, postharvest technology, genetics and transgenic biotechnology. Biotechnol Adv 21:715–766

Teixeira da Silva JA (2012) Callus, calluses or calli: multiple plurals? Asian Australas J Plant Sci Biotechnol 6:125–126

Teixeira da Silva JA (2015) Negative results: negative perceptions limit their potential for increasing reproducibility. J Negat Results BioMed 14:12

Tiwari V, Tiwari KN, Singh BD (2001) Comparative studies of cytokinins on in vitro propagation of *Bacopa monniera*. Plant Cell Tiss Org Cult 66:9–16

Unal BT (2018) Thidiazuron as an elicitor in the production of secondary metabolite. In: Ahmad N, Faisal M (eds) Thidiazuron: from urea derivative to plant growth regulator. Springer, Singapore, pp 463–470

Us-Camas R, Castillo-Castro E, Aguilar-Espinosa M, Limones-Briones V, Rivera-Madrid R, Robert-Díaz ML, de la Peña C (2017) Assessment of molecular and epigenetic changes in the albinism of *Agave angustifolia* Haw. Plant Sci 263:156–167

Van Sambeek JW, Lambus LJ, Kahn SB, Preece JE (1997) In vitro establishment of tissues from adult black walnut. In: Van Sambeek JW (ed), Knowledge for the future of black walnut. Proceedings of the fifth black walnut symposium, 28–31 July 1996, USDA Forest Service, North Central Forest Experiment Station, pp 78–92

Victorio CP, Arruda RDCDO, Riehl CAS, Lage CLS (2011) Leaf volatiles and secretory cells of *Alpinia zerumbet* (pers.) Burtt et Smith (Zingiberaceae). Nat Prod Res 25:939–948

Vieira de Vasconcelos AG, Tomas LF, Camara TR, Willadino L (2012) Hyperhydricity: a metabolic disorder. Cienc Rural 42:837–844

Vinoth A, Ravindhran R (2018) In vitro morphogenesis of woody plants using thidiazuron. In: Ahmad N, Faisal M (eds) Thidiazuron: from urea derivative to plant growth regulator. Springer, Singapore, pp 211–230

Wang ZH, Wang L, Ye QS (2009) High frequency early flowering from in vitro seedlings of. *Dendrobium nobile*. Sci Hortic 122:328–331

White P, Broadley M (2003) Calcium in plants. Ann Bot 92:487–511

Yao O-Y, Chen Y-L, Lü J-F, Teixeira da Silva JA, Zhang X-H, Ma G-H (2016) Somatic embryogenesis and enhanced shoot organogenesis in *Metabriggsia ovalifolia* WT Wang. Sci Rep 6:24662

Yew CK, Balakrishnan B, Sundasekaran J, Subramaniam S (2010) The effect of cytokinins on in vitro shoot length and multiplication of *Hymenocallis littoralis*. J Med Plants Res 4:2641–2646

Yip WK, Yang SF (1986) Effect of thidiazuron, a cytokinin-active urea derivative, in cytokinin-dependent ethylene production system. Plant Physiol 80:515–519

Zeng X, Tang R, Guo H, Ke S, Teng B, Hung YH, Xu Z, Xie XM, Hsieh TF, Zhang XQ (2017) A naturally occurring conditional albino mutant in rice caused by defects in the plastid-localized OsABCI8 transporter. Plant Mol Biol 94:137–148

Zhang CR, Huang XL, Wu JY, Feng BH, Chen YF (2006) Identification of thidiazuron-induced ESTs expressed differentially during callus differentiation of alfalfa (*Medicago sativa*). Physiol Plant 128:732–739

Zhang Y-Y, Cheng Q-W, Niu M-Y, Liang H-Z, Yan H-F, Zhang X-H, Teixeira da Silva JA, Ma G-H (2016) Plant regeneration via direct and callus-mediated organogenesis from leaf explants of *Chirita swinglei* (Merr.) WT Wang. In Vitro Cell Dev Biol Plant 52:521–529