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Review

Thidiazuron: A multi-dimensional plant growth regulator

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Thidiazuron (TDZ) has gained a considerable attention during past decades due to its efficient role in plant cell and tissue culture. Wide array of physiological responses were observed in response to TDZ-application in different plant species. TDZ has shown both auxin and cytokinin like effects, although, chemically, it is totally different from commonly used auxins and cytokinins. A number of biological (physiological and biochemical) events in cells are induced or enhanced by TDZ, but the mode of action of TDZ is yet unknown. However, varieties of underlying mechanisms were revealed by reports showing how morphogenic events were induced by application of TDZ. Other reports showed that TDZ may modify endogenous plant growth regulators, either directly or indirectly and produce reactions in cell/tissue, necessary for its division/regeneration. Other possibilities include modification in cell membrane, energy levels, nutrient absorption, transport and assimilation, etc. In this review, recent advancements in TDZ application in plant sciences are discussed.

Key words: Thidiazuron, plant growth regulators, somatic embryogenesis, regeneration, cell cultures, metabolism.

INTRODUCTION

Since plant cells can be maintained for extended periods in the apparent absence of all known plant hormones, it seems safe to conclude that no hormone is essential just to maintain the viability of plant cells (Davies, 1995). However, the auxins and cytokinins are very important for proper growth and maintenance of culture. Indole-3-acetic acid (IAA) is a major naturally occurring auxin, which is widely reported in plant tissue culture and morphogenesis. In addition to the natural auxin, a whole host of synthetic auxins are known. The most widely used are α-naphthalene acetic acid (NAA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D). The natural cytokinins are a series of adenine molecules modified by the addition of 5-carbon side chains off the 6 position. About 50 years ago, Skoog and Miller (1957) described the controlled organ regeneration in plants; however, developmental biologists were surprised by the unbelievable capacity of plant tissues to regenerate the whole plants (concept of totipotency).

During the last few decades, several compounds have been synthesized to induce regeneration potential in plant cell (Murthy et al., 1998). TDZ was synthesized by German Schering Corporation for defoliation of cotton (*Gossypium hirsutum*) (Arndt et al., 1976). Originally, TDZ was classified as a type of cytokinin that induces many responses that were similar to the responses induced by natural cytokinins. It was proved that TDZ,

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Abbreviations: ABA, Abscisic acid; **ATP,** adenosine triphosphate; **AVG,** amino ethoxyvinylglycine; **BA,** 6-benzyl adenine; **CAT,** catalase; **CBP,** cytokinin binding protein; **GA,** Gibberellic acid; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **iP,** isopentenyl adenine; **µM,** micro molar; **mM,** mili molar; **MS,** Murashige and Skoog; **NAA,** α-naphthalene acetic acid; **nM,** nano molar; **PCIB,** p-chlorophenoxyisobutyric acid; **POD,** peroxidase; **TDZ,** thidiazuron; **TFP,** trifluoperazine; **TIBA,** 2,3,5-triiodobenzoic acid; **ZR,** zeatin riboside; **2, 4-D,** 2,4-dichlorophenoxyacetic acid.

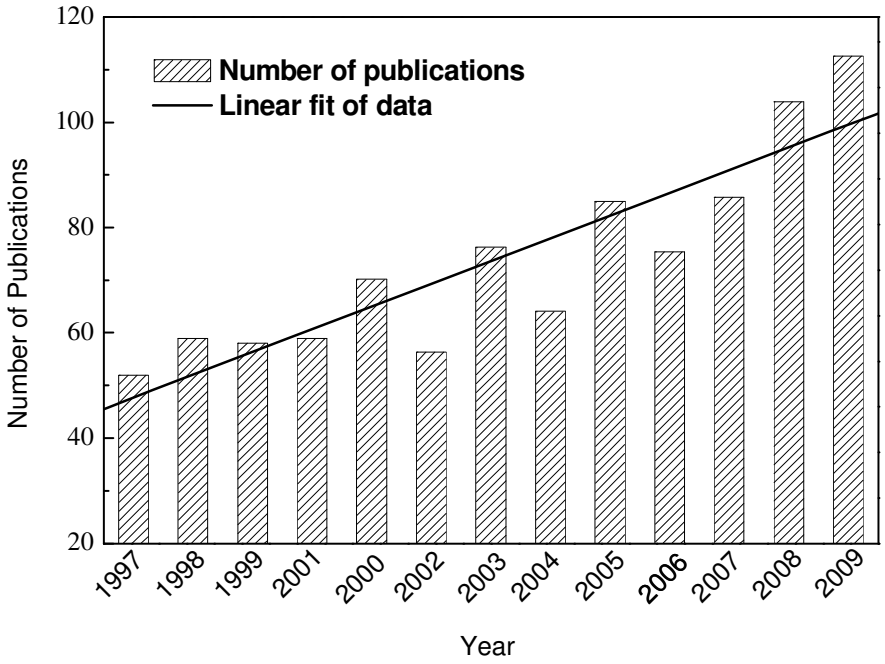

Figure 1. The number of paper published on the use of TDZ in regeneration of plants during last 10 years (from 1997 to 2009; data were collected from ISI web of science).

unlike traditional phytohormones, individual fulfilled the requirements of various regenerative responses of many different plant species. Recently, the morpho-regulatory potential of TDZ has led to its application in plant tissue culture for the development of feasible morphogenetic systems (Figure 1). TDZ emerged as an effective bio-regulant in cell and tissue cultures in wide array of plant species (Li et al., 2000; Hosseini-Nasr and Rashid, 2000; Svetla et al., 2003; Matand and Prakash, 2007).

TDZ application revealed great deal about Skoog and Miller (1957) postulate about morphogenesis. However, a short exposure to TDZ effectively induces a range of different morphogenetic responses. Pretreatment with TDZ can predispose a tissue to accept other inductive stimuli. Alternately, exposure to TDZ can commit a tissue to regenerative route that is expressed even after the inductive stimulus is removed. Therefore, experiments with TDZ both prove and disprove the hypothesized role of auxin and cytokinin in plant regeneration (Figure 2). If TDZ is in fact a cytokinin, then the activity of the compound in the absence of auxin would be in direct contrast to Skoog and Miller (1957) observations. However, the potential for a TDZ induced accumulation or increase in activity of endogenous auxin may account for the observations. Moreover, TDZ trigger a basic survival mechanism in plant tissues that includes asexual reproduction for species survival.

These findings raised many tantalizing questions in plant morphogenesis, most obviously, how could one single compound induce such different results in different species and yet induce the same response in such a wide range of species. The seeming contradiction provided the foundation for more than a decade of work to understand plant morphogenesis in TDZ regulated systems. The focus of this review is to summarize the biochemical and biophysical responses of plant cell to TDZ and to discern the mechanism(s) involved in the induction of morphogenic potential in plants.

METAMORPHOSIS OF TDZ

Exploitation of TDZ in plant cell culture systems in early 80's for induction of adventitious shoot regeneration produced a considerable interest in understanding the plant morphogenesis and different physiological parameters. The complex nature of the biochemical and morphological responses that have been reported for plant tissues exposed to TDZ has provided some indication of the cascade of physiological reactions within the plant tissues, but there have been relatively few investigations that have utilized radio-labeled TDZ for characterization of the fate of the TDZ molecule (Murthy et al., 1998). Mok and Mok (1985) found that TDZ did not metabolize in callus tissue of *Phaseolus lunatus* within the first 48 h of culture, following which the primary metabolites of TDZ were glucoside residue. In study with *Pelargonium x hortorum*, there was a limited oxidation of

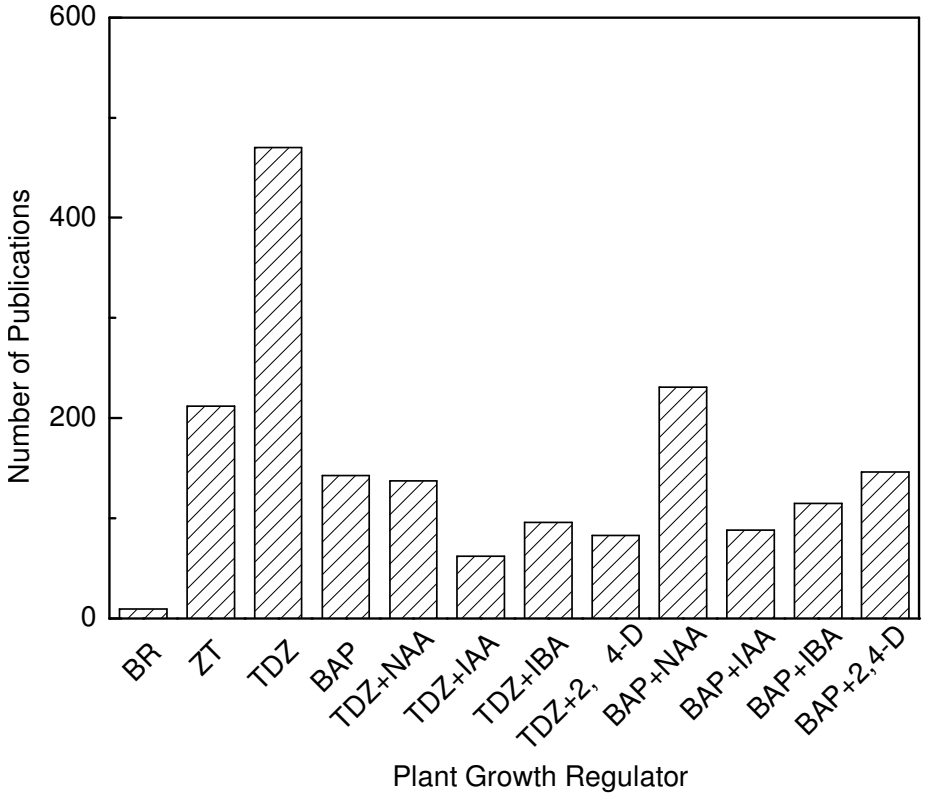

Figure 2. Comparison among number of papers on TDZ, BAP, ZT, 2,4-D, NAA, IAA, IBA and brassinoides, in regeneration of plant cell (from 1997 to 2009; data were collected from ISI web of science). TDZ: Thidiazuron; BAP 6-benzylaminopurine; ZT: zeatin; 2, 4-D: 2, 4-dichlorophenoxyacetic acid; NAA: napthalene acetic acid; IAA: indoleacetic acid; IBA: indolebutyric acid; BR: brassinosteroids.

the TDZ molecule within the etiolated hypocotyl explants as evidenced by the limited evolution of $14CO_2$ (Murch and Saxena, 2001). Few reports have shown that TDZ-molecule remains largely intact within the plant tissues (Murch and Saxena, 2001). However, the recovery of free, radiolabel TDZ molecules declined during the incubation period and radiolabel was detected in both the ethanol-soluble and ethanol-insoluble fractions. These data suggest that the TDZ molecule may exist in several forms within the plant tissue. Free TDZ molecules would be recovered in the ethanol soluble fractions, while TDZ molecules that had become associated with a protein or cell wall components which were sequestered would have been recovered in the ethanol insoluble fraction (Atkins and Canvin, 1971). These data may open many possibilities for the mode of TDZ action within the plant tissues including the induction of a metabolite stress response and the modulation of protein mediated responses. In spite of intensive efforts over the last decade, the precise determination of the mode of action of TDZ in the induction of plant regeneration has remained elusive.

TDZ LIAISED RESPONSES

TDZ is widely applied in plant *in vitro* or *in vivo* that influences a number of parameters in plants. A miscellaneous range of responses with a high grade of efficacy is induced via TDZ application. It was firstly used as a defoliant for cotton. Premature activation of the abscission zone affects water potential or chlorophyll content in some members of Malvaceae family (Grossmann, 1991). Although, TDZ protected chlorophyll degradation in detached leaves of geranium (Murthy et al., 1998; Visser et al., 1995). To encourage the growth of radish cotyledons and persuade adventitious shoots on tobacco leaf, TDZ was observed effective (Thomas and Katterman, 1986). Numerous plant species were induced to viable regeneration via TDZ application (Malik and Saxena, 1992; Çöçü et al., 2004; Faisal et al., 2005).

Some examples of the diversity of physiological effects mediated by TDZ include efficient seed germination, expedited bud break, induction and stimulation of sprouting, cotyledonary growth and development, formation of trichomes and stomata appearance on floral

parts and cluster and berry weight of grapes (Babiker et al., 1992; Baskakov et al., 1981; Lin et al., 1994). More recently, the morpho-regulatory potential of the chemical has led to its application in plant cell, tissue and organ culture for the betterment of regeneration protocols. It was also reported that TDZ-induced better response than BA (6-benzyl adenine) in shoot regeneration in peanut (Victor et al., 1999; Gairi and Rashid, 2004). Since 2000, a considerable increase in the number of reports involving TDZ in the induction of regeneration responses has been recorded. Although, TDZ had been reported for the production of economically important secondary metabolites in some plant species (Nabila et al., 2003).

The exogenous application of TDZ affects concentration of endogenous plant growth regulators in some members of dicots. TDZ affects pathways of purines and cytokinin metabolisms (Capelle et al., 1983; Mok et al., 1982; Laloue and Fox, 1989). TDZ stimulated sprouting in potato by inhibiting synthesis of abscisic acid (Li and Yang, 1998). However, TDZ is involved in modification of cell membranes, energy levels, nutrient uptake and nutrient assimilation. In depth study of the physiological responses of plant cells to TDZ will lead to an insight of the process of morphogenesis.

RANGE OF TDZ MEDIATED MORPHOGENESIS

Plant cells have the potential to reproduce intact plant via organogenesis or embryogenesis. Plant growth regulators play a role of backbone in process potentials, dedifferentiation and redifferentiation. These regenerative processes in cell and tissue cultures may be provoked by TDZ alone and in collaboration with other plant growth regulators. An array of complex physiological mechanisms like, functions of an intact molecule in both alone and in engaged system are involved in TDZ-treated somatic embryogenesis and also, TDZ-treated tissues maintain and enhance the accumulation and transport of auxin. All these results suggest that TDZ has a keen role in the induction of stimulation of plant growth regulator processes and physiological maintenance of plant tissues during culture process (Table 1). The ability of the explant tissue to survive the applied stresses of the culture process seems to be an integral part of the morphogenic phenomena and some studies provide indication of the factors involved in the regulation of plant regeneration (Murch and Saxena, 2001).

The undifferentiated mass of cells is called callus and the process of callus formation is called callogenesis, which is the primary step in the stimulation of shoot reproduction via indirect mode and adventitious organs regeneration, where auxin is the best choice. From woody explant, callus is achieved via TDZ at concentrations ≥0.1 µM (Huetteman and Preece, 1993). In different plant culture systems, TDZ induced callogenesis, in some cases higher cell proliferation rate is achieved at comparable levels to other growth substances. TDZ indicates high intrinsic activity when compared with other plant growth substances due to low absorbance in callus. Callus formation is induced via axillary shoot proliferation due to high fraction of TDZ. Few reports are available on type of callus induced by TDZ; however, compact-green nodular callus forms at low concentration (Murthy et al., 1998). In *Echinacea purpurea,* TDZ at concentration >0.1 µM induced significantly high growth rate (Jones et al., 2007). *Phaseola* explants, either from green house grown plants or *in vitro* germinated seeds were induced to morphogenic, green nodular callus via TDZ+IAA (Zambre et al., 2001).

It is reported that TDZ induces shoot regeneration in many plant species. TDZ fraction plays very important role in morphogenesis like, lower concentration induces axillary shoot proliferation, whereas higher fraction causes adventitious shoot development. Therefore, care must be given while using this as a potent cytokinin. It is also reported that higher dose of TDZ for longer time causes protracting effects in St John's Wort and Pelargonium (Murthy et al., 1998). Incorporation of IAA into medium containing TDZ resulted in enhanced shoot regeneration.

However, the intricacy of the *in vivo* signaling route, caused by TDZ during callogenesis and shooting, is still a mystery. Recently, TDZ alone or conjugated with other growth regulators, is used as somatic embryogenesis stimulant in tissue culture media (Nhut et al., 2006). It is also reported that in Neem and Pigeon pea, somatic embryogenesis was induced with TDZ only (Sreenivasu et al., 1998). Furthermore, TDZ and co-cultivation of microorganism gave supreme response in different plant species; however, TDZ alone was the second top choice. In Ringo Rose cultivar the embryogenic rate was increased by bacterial co-cultivation to such a range that this technology could be apt to the proliferation of this cultivar (Murthy et al., 1999).

The developmental pathway representing somatic embryogenesis in competent cells was stimulated by low fraction of TDZ (Jones et al., 2007). Sophisticated combinations of exogenous and endogenous plant growth substances regulate a process called somatic embryogenesis (Komamine et al., 1992; Skoog and Miller, 1957). *In vitro* alteration of auxin to cytokinin ratio induces somatic embryogenesis from somatic cells, whereas TDZ alone has the potential to induce somatic embryogenesis in several species (Murthy et al., 1998). It has been postulated that the concentration of endogenous phytohormones affect TDZ-induced morphogenesis. Murch et al. (1999) verified that TDZ-stimulated propagation causes accretion of abscisic acid, proline and particular ions (Tang and Newton, 2005a). Also, the TDZ mediated alteration in the cytokinin biosynthetic pathway might be responsible for the depletion of the endogenous 2iP pool and the elevated concentrations of the other purine metabolites (Victor et al., 1999; Zhang et al., 2005).

Table 1. Families of plants that respond to TDZ (data collected from 1991 to present).

Family	Explant	Response (s)	Reference
Aceraceae	Apical, axillary bud	Shoot formation	Linden and Riikonen (2006)
Actinidiaceae	Leaf , callus	Shoot formation	Seelye and Butcher (1991)
		Increase fruit size and yield	Famiani et al. (2002)
Agavaceae	Leaf, cotyledons and stem	Shoot formation	De Gyves et al. (2001)
Anacardiaceae	Plumule, hypocotyls and radicle sections	Shoot formation	Wilhelm (1999)
	Nucellar tissue	Somatic embryogenesis	Kidwai (2009)
Apocynaceae	Nodal	Shoot formation	Faisal et al. (2005)
	Epicotyls, hypocotyls, cotyledonary axis and shoot tips	Shoot formation	Kumar and Singh (2008)
Araceae	Shoot tip	Shoot formation	Du et al. (2006)
Araliaceae	Root	Shoot formation	Proctor et al. (1996)
Asclepiadaceae	Leaf	Shoot formation	Thomas and Philip (2005)
	Shoot tip	Shoot formation	Lakshmi et al. (2010)
Artemisia	Leaf	Shoot formation	Liu et al. (2003)
	Mesophyll protoplasts	Shoot formation	Pan et al. (2003)
	Hypocotyl	Shoot formation	Sujatha et al. (2007)
Begoniaceae	Petiole	Shoot formation	Nhut et al. (2005)
Bibnoniaceae	Nodal	Shoot formation	Thomas and Puthur (2004)
Boraginaceae	Cotyledon, hypocotyls	Shoot formation	Jiang et al. (2005)
Brassicaceae	Leaf and petiole	Shoot formation and somatic embryogenesis	Mithila et al. (2003)
		Branched trichomes formation	Venglat and Sawhney (1994)
	Immature embryos	Shoot formation	Cu et al. (2008)
Burseraceae		Seed germination	Rinaldi (2000)
Caprifoliaceae	Leaf	Shoot formation	Cambecedes et al. (1991)
Cannabaceae	Nodal	Shoot formation	Lata et al. (2009)
Caryophyllaceae	Petal, leave	Shoot formation	Casanova et al. (2004)
Clusiaceae	Hypocotyls	Shoot formation	Murch et al. (2000)
	Seedling	Shoot formation	Oluk and Orhan (2010)
Compositae	Leaf	Shoot formation and somatic embryogenesis	Jones et al. (2007)
	Cotyledonary leaves	Shoot formation	Basalma et al. (2008)

Table 1. Continue

Cucurbitaceae		Elevates plant resistance to adverse environments	Lukatkin et al. (2003)
	Cotyledon	Shoot formation, inhibition of root initiation	Leshem et al. (1994)
Dioscoreaceae	Bud	Shoot formation	Prathanturarug et al. (2005)
Echinacea	Leaf	Somatic embryos	Jones et al. (2007)
Elaeagnaceae	Leaf	Shoot formation	Karami and Piri (2009)
Epimedium	Immature seed	Shoot formation	Mihaljevic and Vrsek (2009)
Ericaceae	Leaf	Shoot formation	Pavingerova (2009)
	Hypocotyls	Shoot formation	Kim et al. (1997)
Euphorbiaceae	Nodal	Shoot formation	Bhagwat et al. (1996)
	Leaf	Shoot formation	Khurana-Kaul et al. (2010)
Fagacea	Cotyledonary node	Shoot formation	San-Jose et al. (2001)
	Immature embryo	Somatic embryogenesis	Tsvetkov (1999)
Geraniaceae		Inhibition of root initiation, delaying the onset of senescence	Mutui et al. (2005)
	Hypocotyl	Somatic embryogenesis	Hutchinson et al. (2000)
	Root	Shoot formation	Murch et al. (1997)
Gramineae	Mature embryo	Shoot formation	Ganeshan et al. (2006)
	Nodal and internodal	Somatic embryogenesis	Lin et al. (2004)
	Caryopsis	Shoot formation	Vikrant and Rashid (2002)
		Increase the yield of plants	Beckett and Vastaden (1992)
	Apical meristematic tissues	Shoot formation	Wamaitha et al. (2010)
Kalanchoe	Leaf	Polarized hypertrophy	Jaiswal and Sawhney (2008)
Labiatae	Stem, hypocotyl, cotyledonary petiole	Callus and shoot formation	Zhang et al. (2005)
Legaminosae	Nodal	Axillary shoot proliferation	Faisal and Anis (2006)
	Protoplast	Shoot formation	Bohmer et al. (1995)
	Otyledon node	Shoot formation	Aasim et al. (2010)
	Hypocotyl and epicotyl	Shoot formation	Mongomake et al. (2009), Basalma et al. (2008)
Liliaceae	Seedling, etiolated hypocotyl and stem segment	Shoot formation	Li et al. (2000)
	Leaf	Shoot formation	Faure et al. (1998)
	Immature embryos	Shoot formation	Deroles et al. (2010)
Linaceae	Hypocotyl	Shoot formation	Jain and Rashid (2001)
	Explants, apical node	callus induction	Rosu et al. (2010)
Icacinaceae	Seedling	Shoot formation	Thengane et al. (2001)

Table 1. Continue

Iridaceae	Korm	Globular embryo-like structures	Sharifi et al. (2010)
Malvaceae		Seed germination	Lamas and Athayde (1999)
Meliaceae	Hypocotyl, epicotyl, cotyledon, and leave	Somatic embryogenesis	Gairi and Rashid (2004)
Moraceae	Leaf	Shoot formation	Chitra and Padmaja (2005)
	Seeding	Shoot formation with growth of large leaves	Borkowska and Litwinczuk (1993)
Musaceae	Shoot tip	Shoot formation	Gubbuk and Pekmezci (2006)
	Male inflorescence	Shoot formation	Darvari et al. (2010)
Myrtaceae	Node	Shoot formation	Mondal et al. (1998)
Oleaceae	Immature, mature nonstratified seed	Shoot formation and Somatic embryogenesis	Bates et al. (1992)
Orchidaceae	Isolated shoots	Flower induction	Ferreira et al. (2006)
	Rhizome	Shoot formation	Chang and Chang (2000)
Pinaceae	Cotyledon, hypocotyls	Shoot formation	Tang and Newton (2005)
Restionaceae	Coleoptile	Somatic embryogenesis	Panaia et al. (2004)
Poaceae	Shoot meristem	Embryogenic calli formation	Chakrabarty et al. (2010)
	Mesocoptile	Direct organogenesis	Rey et al. (2010)
Rosaceae	Sepal	Shoot formation	Debnath (2005)
		Increases fruit size	Stern et al. (2003)
	Hypocotyl	Somatic embryogenesis	Hutchinson et al. (1997)
Quercus	Embryonic axes	Somatic embryogenesis	Martinez (2008)
Salicaceae	Leaf	Protoplasts culture, callus formation and shoot regeneration	Chupeau et al. (1993)
Sapindaceae	Cotyledonary node	Shoot formation	Husain et al. (2007)
Sterculiaceae	Seedlings	Shoot formation	Hussain (2008)
Saxifragaceae	Leaf	Shoot formation	Ledbetter and Preece (2004)
Scrophulariaceae		Seed germination	Logan and Stewart (1995)
	Leaf	Shoot formation	Corredoira et al. (2008)
Solanaceae	Stem and shoot tip	somatic embryogenesis	Khan et al. (2006)
	Hypocotyl, cotyledon and stem	Shoot formation	Uranbey (2005), Osman et al. (2010)
Umbelliferae	Hypocotyl	Shoot formation	Gupta and Bhargava (2001)

Table 1. Continue

Urticaceae	Cotyledon	Shoot formation	Wang et al. (2007)
Vitaceae	Root	Shoot formation	Olah et al. (2003)
	Cotyledon, hypocotyl and epicotyl	Shoot formation	Pelah et al. (2002)
Zingiberaceae	Rhizome sections	Shoot formation	Malabadi et al. (2004)

Protoplast technology has been used as a significant tool to produce new genotypes through parasexual hybridization, genetic engineering, etc. It also provides opportunity to improve biologically active constituents in elite medicinal plant species. It is beneficial to understand the biosynthesis of secondary metabolites related to particular medicinal contents from combination of medicinal plants with altered metabolites and could contribute efficiently in rapid and steady production of unique secondary metabolites (Murch and Saxena, 2001). Protoplast technology is exploited to isolate plant-derived secondary metabolites, where protoplast is isolated, cell division is induced and plant is regenerated. Few reports endorse the combination of auxin and cytokinin in medium promoted/stimulated protoplast division and development. So far, there is no report on combination of TDZ with different auxins like NAA or 2, 4 D at altered concentrations to stimulate cell wall synthesis around protoplast to initiate cell division and to regenerate plant from protoplast-derived callus (Hassanein and Dorion, 2006; Umate et al., 2005; Chupeau et al., 1993). It is reported that TDZ is the best choice than other phytohormones for protoplast proliferation (Murthy et al., 1998; Xiao et al., 2007).

Rooting is difficult and key step in plant micropropagation system. TDZ treatment causes rooting in the adult-derived tissues of *Bambusa vulgaris* (Ramanayake et al., 2006). Murch and Saxena (2001) reported the results of TDZ on the transport of root promoting auxin in many species. In woody plants, a parallel action may confine to rapid rooting with the TDZ pretreatment (Preece et al., 1991a). However, in most species, TDZ does not apt to promote adventitious rooting once shoots are removed. Mundhara and Rashid (2006) reported root inhibition in *Linum usitatissimum* cultured on TDZ fortified medium. Axillary shoots of bamboo could not develop roots on the medium containing TDZ and IBA. It is concluded from different reports that pretreatment with TDZ inhibited root formation, however, incorporation of TDZ in rooting medium showed positive response (Ramanayake et al., 2006). In some cases, retarded development of TDZ-stimulated propagules may obtain from application of supera-optimal values of TDZ in the media or the presence of compound for longer time in cultured tissues (Mundhara and Rashid, 2006). However, rooting reticence and several small plantlets regeneration resulted due to high values of suppression of cytokinin cessation and/or the consistent biosynthesis of purine cytokinins (John and Frank, 1986). A recent study confirmed an association among TDZ treatment which outcomes in ethylene-action and root elongation and shoot regeneration inhibition are correlated. Their research results also indicated that inhibitors of roots elongation by TDZ were related with the decrease in concentration of calcium in plant tissue (Mundhara and Rashid, 2006).

MECHANISM FOR TDZ ACTION

Involvement of phytohormones

Extensive progress has been made in many disciplines of plant organogenesis once the phytohormones were innovated and hormonal regulation was postulated (Skoog and Miller, 1957). TDZ is a powerful regulator of *in vitro* plant regeneration and subsequent growth (Murthy et al., 1998). Recent studies showed that TDZ is frequently associated with the metabolism of plant growth regulators. Actually, TDZ was categorized as cytokinin due to induction of natural cytokinins like responses. Later, an increase in endogenous auxin, ethylene and ABA is recorded in response to TDZ-treatment (Murthy et al., 1998; Murthy et al., 1995; Yip and Yang, 1986).

It is reported that TDZ's action in development is much closed to cytokinin metabolism in plant cell. Casanoval et al. (2004) determined the results of TDZ on endogenous plant growth regulators in organogenesis as low TDZ levels (0.0 to 0.005 µmol/l) induce ZR, while high concentrations (0.5 µmol/l) are associated with isopentenyl adenine (ip) that results in rapid cell division and the stimulation of shoot organogenesis I, that is, petals and maximum carnation cultivar leaves (*Dianthus* spp.). It is elucidated that phenylurea compounds can modify pre-existing cytokinins which results in the transformation of tissues independent of cytokinins. Many reports showed that TDZ replaces purine based cytokinins in *in vitro* and this is confirmed via purine metabolism inhibitors like, diaminopurine (DAP) halted TDZ stimulated somatic embryogenesis in geranium and peanut (Murthy et al., 1998).

Purines biosynthesis and storage is enhanced by TDZ. This may possibly occur via different strategies like, over synthesis, low catabolism rate or providing active

cytokinin molecules stored in in-active forms (Capelle et al., 1983; Jones et al., 2007; Ferreira et al., 2006; Zhang et al., 2005; Casanova et al., 2004; Murch et al., 2001; Victor et al., 1999; Murthy et al., 1998). Besides, Laloue and Fox (1989) reported that phenylurea can prevent the breakdown of purines by inhibiting cytokinin oxidase.

The structure of TDZ is totally different from naturally occurring purine-based cytokinins. However, certain findings have shown that normally, pure purine based cytokinin have not induced few morphogenic cascades like somatic embryogenesis (Victor et al., 1999). The mechanism in morphogenesis induced by zeatin riboside (ZR) and TDZ was studied by Anna et al. (2006). When the phytohormone supply is preceded by 3-day pre-culture, an alteration in response appears which is in favor of TDZ induced callus, while ZR activity is strongly inhibited. Therefore, it is assured that TDZ possess additional properties which are apparent via altered mechanisms. The two clusters of growth regulators, purine based cytokinin and phenylurea induce biotic response recommended a common location in the presence of competitive inhibitors (Hutchinson et al., 1996; Victor et al., 1999). Even though TDZ have proved to be the best option for adventitious shoot organogenesis from carnation petals (Nakano et al., 1994), but still, it is a mystery that whether phenylurea derivatives effect endogenous cytokinin metabolism directly or indirectly. .

Moreover, ethylene has dual effect on somatic embryogenesis that is, stimulative and inhibitory. Exposure of mung bean hypocotyls to TDZ responded in ethylene production and at the same time storage of auxin and Ca^{2+} occurred (Yip and Yang, 1986). A rise up in ethylene level was investigated during the somatic embryogenesis of geranium via TDZ in the upper space of culture vessel (Hutchinson et al., 1997). Embryogenic response in geranium hypocotyls was enhanced by incorporating ethylene inhibitor AVG (amino ethoxy-vinylglycine), which is a sign for the proof that ethylene is a harmful by-product of TDZ induced metabolic process (Hutchinson et al., 1997). However, the embryogenic response is decreased to the same extent as resulted in TDZ stimulated culture by the usage of exogenous ethylene or an ethylene precursor that is, 1-amino-cyclopropane-1-carboxylic acid. It was proposed that TDZ stimulate auxin-like response as auxin application enhanced ethylene production. So, it is likely to perceive that TDZ treatment may not cause leaf abscission directly, but as a result of TDZ mediated auxin response.

TDZ, not like natural cytokinin, has the potential to fulfill auxin requirements of different regenerative responses of several plant species. Dedifferentiation and differentiation of cultured tissues is vital for regeneration where in auxin has critical role. More and more researches indicated that the action mechanism of TDZ can be closely linked to the biosynthesis and transportation of IAA. Jaiswal and Sawhney (2008) observed an organized hypertrophic growth at the vein ending on the proximal side of *K. pinnata* leaf disks induced by TDZ, which is considered an exclusive auxin-mediated response. Further experiment proved that the effect is completely reversed by auxin efflux inhibitor, 2,3,5-triiodobenzoic acid (TIBA). The translocation of auxin appears to be vital for the TDZ-stimulated morphogenesis since exposure. TDZ-induced calli of *Scutellaria baicalensis* responded in high fraction of IAA and it is possible that TDZ altered indirectly endogenous hormonal values, particularly IAA/BA ratio which is responsible for calli growth and bud formation (Zhang et al., 2005).

TDZ-induced somatic embryogenesis in geranium was significantly inhibited by both the auxin-action inhibitor p-chlorophenoxyisobutyric acid (PCIB), but did not reduce the concentration of endogenous auxin (Hutchinson et al., 1996). Hypocotyl sections that had been exposed to TDZ were found to accumulate more 14C-IAA from the culture medium and to translocate the auxin over a greater distance within the tissues, which indicated that TDZ-exposure enhances the accumulation and translocation of auxin within the tissues (Murch and Saxena, 2001). The accumulation of IAA was also reported in the regeneration of *E. purpurea* (Jones et al., 2007). Wagner et al. (2006) also investigated the first samples of *Dendrobium* (Second Love; taken at day 5) grown on TDZ-supplemented medium that a considerable improvement in endogenous IAA was associated with stimulation, while after 25 days of culturing flower development was associated with second increase in hormones concentration. It is postulated that auxin transport inhibitors like, TIBA hamper efflux of auxin from cells (Fujita and Syono, 1996). Jones et al. (2007) reported that the movement of the radio labeled auxin beyond the first 5 mm of the tissue was significantly inhibited by TIBA, which substantiated the earlier evidence for the inhibition of auxin transport by TIBA.

Other research fields in the relationship of endogenous hormones with TDZ include ABA, GA and melatonin. The modulation of endogenous ABA and GA by TDZ was also reported (Murthy et al., 1996; Murch et al., 1999). But unfortunately, no progress was made in this field later on. The hypothesis that gibberellins can be affected by TDZ based on the evidences of few researchers' findings like, TDZ mediates endogenous GA, TDZ-stimulated somatic embryogenesis in geraniums by GA-synthesis inhibitors (Hutchinson et al., 1975b; Murch and Saxena, 1998).

Involvement of enzymes

A variety of biochemical reactions are involved in the process of differentiation, which is a complex biological phenomenon. Numerous studies have been carried out with the objective of explaining the role of antioxidant enzymes in plant morphogenesis. TDZ treatment resulted in accumulation of phenols, catalase and peroxidase

(Wang et al., 1991a). Todor and Iordanka (1995) investigated that TDZ enhances POD activity. Tang and Newton (2005) determined TDZ influences on antioxidant enzymes performance during morphogenesis of Eastern white pine. They showed that during culture, at the beginning, POD activity decreased and increased at later stages of shoot bud formation and CAT activity linearly decreased throughout the complete culture duration. All these findings showed that TDZ-induced direct adventitious shooting in Eastern white pine resulted in decreased activities of POD and CAT. Peroxidase is a multifunctional enzyme. As said by Edreva (1988) and Mamaghani et al. (2010) that various active forms of peroxidase involved in growth regulation, development and organogenesis. TDZ promotes the activities of POD, which may be one reason for shoot regeneration or embryo formation. It is remarkable that cytokinin oxidase may be involved in TDZ induced physiological responses. It has been reported that TDZ hampers the action of cytokynin oxidase possibly via a composite, freely occurring uncompetitive mechanism (Hare et al., 1994). Apart from antioxidant enzymes, it was reported that TDZ application causes alterations in nitrate reductase, ATP, ribulose diphosphate carboxylase oxidase and pentose phosphate enzymes (Kulaeva et al., 1982; Chernyadev and Kozlovskikh, 1990; Mok et al., 1987). So, it can be determined that morphological changes appeared in TDZ-induced tissues and organs might be the consequence of alterations in enzyme kinetics. Wang et al. (1991b) reported that many of the TDZ-stimulated enzymes were associated with cell walls, membranes and membrane fluidity was modified.

Involvement of ions

TDZ treatment promoted accumulation of mineral ions which induced process of regeneration. Somatic embryogenesis was induced by incorporation of zinc, copper or sodium into the culture medium of carrots. Thus, it was inferred that application of TDZ enhanced accumulation of minerals or other metabolites and predisposes the explant to stress. To get rid of this physiological constrain, the plant tissues reformed their metabolic pathways, the alternate result of which is the synthesis and storage of different metabolites and favoring the production of regenerants. In this situation, regenerants in TDZ-treated plants to get rid of enforced hassle may be an adaptive reproductive tool (Murch et al., 1997). In recent years, calcium signal response to TDZ has been made a point of doing. Calcium, a ubiquitous second messenger, plays the role of a facilitator of stimulus-response pairing in the regulation of miscellaneous cellular activities (Allen and Schroeder, 2001; Yang and Poovaiah, 2003). A calcium ion prickle produces when the calcium ion level in plants is getting to its peak due to maximum calcium ion influx and swift to basal level via calcium ion efflux, various stimuli responsible are: light, gravity, physio-chemical and biological stresses and hormones.

Response of plant cells to different hormones is due to external calcium concentration or rise in the level of this cation in the tissue (Trewavas, 1999). Thus, TDZ was classified as plant growth regulator. The mechanism of TDZ in the response of plant cells, especially in the plant tissue and organ culture was conjectured in relation with the balance of calcium concentration in plant tissue. This is substantiated by the effectiveness of TDZ at concentration of externally supplied calcium which is insufficient for shoot formation.

SPECIAL APPREHENSIONS

Effects of concentration

Among other agents with cytokinin activity, comparatively low amount of TDZ promote shoot multiplication or somatic embryogenesis in several plants. Direct comparisons between TDZ and the amino purine cytokinins at equimolar concentrations complicated the statistical analyses. As various amino purine cytokinins have closed operative ranges of activity (1 to 10 µM), that is why experiments are in progress to compare fractional combinations of these cytokinin at parallel levels. TDZ with this capacity normally results in callogenesis and shoot growth inhibition. Mithila et al. (2003) reported that low concentration of TDZ induced shoot organogenesis of African violet explants, whereas at higher doses (5 to 10 µM) somatic embryos were formed. Studies on regeneration of *E. purpurea* L. reported that at high concentration of TDZ (up to 2.5 µM) on solid media, maximum number of regenerants were produced, while the plantlets obtained from low concentrations were healthy and rooted earlier (Jones et al., 2007). It is studied that in spite of the reality that all trials with TDZ generated equal number of regenerants; higher concentrations of TDZ hindered further growth and development of the regenerants (Shirani et al., 2010). Considerable maximum number of regenerants are generated with exposure of 0.1 to 1.0 µM TDZ when compared with 10.0 µM TDZ or MS zero medium. Thomas and Philip (2005) showed that shoot induction is achieved at range 1 nM to 10 µM TDZ. At maximum level of TDZ, shoots might be prone to maximum hydricity and fasciation (Huetteman and Preece, 1993). Zhang et al. (2005) suggested that such a harmful effect may be due to hindering consequences of TDZ on endogenous auxin (IAA) and particularly cytokinin (BA) concentration. That is why Huetteman and Preece (1993) proposed that initial experiments should be directed at concentrations of equal strength for both the amino purine cytokinin and TDZ. To begin *in vitro* regeneration of a novel species, it is recommended that 100 nM is keeping as reference and estimation of two preparations of measurements above and below that level.

The length of exposure to TDZ also affected shoot proliferation or somatic embryogenesis. Liu et al. (2003) reported that cultures grown on medium supplemented with 1 µM TDZ for 20 days produced a maximum number of shoots (16) per intact seedling in *Artemisia judaica* L. and there was no significant increase in the number of shoots formed when the duration of exposure to TDZ was increased. Tang and Newton (2005) reported that the 6 days in medium supplemented 3 to 6 µM are necessary for the morphogenic ability of Eastern white pine. Tapan et al. (1998) observed the callus of tea overgrowth and later necrosis when the responsive explants were continuously grown on the medium containing TDZ. The deleterious effect of the continued presence of TDZ on the growth and multiplication have been reported for chickpea (Murthy et al., 1996), *Pisum sativum* (Bohmer et al., 1995), *Anoectochilus formosanus* (Ket et al., 2004), *Rauvolfia tetraphylla* (Faisal et al., 2005) and *Psoralea corylifolia* (Faisal and Anis, 2006). Chitra and Padmaja (2005) reported that only few buds derived leaves of mulberry developed into shoots of 0.5 to 1.5 cm at the end of 30 days, and growth of the rest of the adventitious shoot buds remained arrested on medium containing 18.17 and 22.7 mM TDZ. Further experiments indicated that the optimal duration of exposure to TDZ was 8 to 10 days and then subsequent transfer to TDZ-free medium, produced better results. Cultured hypocotyl and cotyledonary explants of *Morus* indica for 10 days on medium supplemented with 5mM TDZ and obtained 50 and 70% regeneration, respectively. In certain cases like geranium, there is short incubation of TDZ-exposure which is tracked by morphogenesis period in the lack of any external growth substance (Hutchinson et al., 1996). In contrast, other species have longer incubation periods for regeneration induced by TDZ. TDZ is a urea based cytokinin and therefore, is non-degradable by cytokinin-oxidase enzymes in plant tissues. This quality causes TDZ to be persistent in tissues, hence, transforming them from cytokinin dependence to cytokinin autonomy. Makara et al. (2010) reported that TDZ had a carry-over effect that enabled shoots to continue proliferation on a hormone free medium as the culture cycles increased and that this effect was significantly ($P < 0.05$) higher than that of BAP. Various plants may need altered incubation periods for differentiation and morphogenesis. This is probably due to the capacity of TDZ in stimulating endogenous cytokinin biosynthesis or in altering cytokinin metabolism (Sankhla et al., 1994; Zhou et al., 1994). Therefore, it is suggested that double stage culture procedure consisting of culture of plant tissue on TDZ-medium for appropriate period (usually 10 to 20 days) followed by a secondary TDZ-free medium, is beneficial for shoot proliferation and/or somatic embryogenesis.

Involvement of plant growth regulators

Although, TDZ alone is highly effective in inducing plant cell redifferentiation such as White Sim petals or other cultivars and species (Frey and Janick, 1991, Ricci et al., 2001), in few cases, combination of TDZ with other plant growth regulators promoted shoot proliferation or embryogenesis. The early report suggested that TDZ + NAA treatments maximized shoot regeneration both in leaf and petal explants. An optimal level of auxin was found to be required for induction of regeneration and cell proliferation in TDZ-exposed geranium hypocotyls (Hutchinson et al., 1996). Combination levels of exo-genous 2, 4-dichlorophenoxyacetic acid (2,4-D) and thidiazuron (TDZ) changed granular and yellow callus of *Oncidium* into more friable or compact embryogenic callus and the most significant proliferation of this callus was observed after the third subculture (Jheng et al., 2006). Histological studies observed that cell proliferation is enhanced by TDZ with 2, 4-D rather than TDZ with IBA. Shoot organogenesis is induced by TDZ with IBA or IAA, while treatment of TDZ/2, 4-D for more than 6 days results in deterioration of shoot organgenesis (Yancheva et al., 2003). Nhut et al. (2006) obtained embryogenic cells from friable calli cultured in media containing 0.2 mg/l TDZ with 1.0 mg/l NAA. Similar results have been reported for *Nemesia strumosa* (Cui and Ezura, 2003; Tang and Newton, 2005). Auxins represent one of the most important classes of signaling molecules involved in the regulation of cell division, cell elongation and cell differentiation in higher plants (Bennett et al., 1998).

Remarkably, Mutasim and Kazumi (1999) developed a procedure for multiple shoot formation from cotyledonary node explants of faba bean (*Vicia faba*) cultured on MS medium containing benzyladenine (BA) and thidiazuron (TDZ). They found that explants obtained from 7-day-old seedlings cultured on MS medium containing BA in combination with TDZ (2 mg l^{-1} each) produced a higher number of shoots than with either cytokinin alone. A model was proposed by Nielsen et al. (1995) for cytokinin action in the plant cell. Both BA and TDZ can bind to a receptor, a cytokinin-binding protein (CBP) which has two binding sites. One site binds adenine-type cytokinins naturally, while the other is able to bind phenylurea-type cytokinins. The functions of combined BA and TDZ enhance the shoot morphogenesis possibly due to their active binding to both CBP sites. Similar results have been reported by Tegeder et al. (1995).

Amino acids

In the last few years, certain studies recommended that culture media, fortified with amino acids, expanded the shoot organogenesis or embryogenesis. To improve embryogenesis in eucalyptus, glutamine and casein have been employed (Pinto et al., 2002), while in *Miscanthus* and Cherries cultures, 1-proline is studied to boost up early stages of somatic embryogenesis (Holme et al., 1997; Cheong and Pooler, 2004). The accumulation of amino acids during somatic embryogenesis was also

reported for alfalfa, Anise (Andarwulan and Shetty, 1999), cowpea (Ramakrishnan et al., 2005) and for the shoot regeneration of strawberry (Qin et al., 2005). Moreover, Zhang et al. (2006) reported that the transcription levels persuaded by TDZ were observed to vary all over TDZ treatment. TDZ persuaded the transcription of stress-related genes, comprising proline dehydrogenase in both the treatments that is, early (day 2) and late stage (day 28), which are indicative of amino acid responses from the TDZ-treated callus. For other species, the proline or glutamine fortified culture media have reduced number of total embryos which were larger in size and greener.

Indole amines (melatonin)

Melatonin is a most attractive chemical discovered in plants recently (Murch et al., 1997). Elevated levels of melatonin and serotonin were detected in the TDZ treated tissues (Murch et al 2001; Jones et al., 2007), which indicated that melatonin played the role of hormone autonomously or in combination with auxin and peculiar precursors and metabolites. Biosynthesis of melatonin occurs in the *epiphysis* of mammals (Murch and Saxena, 2002; Chen et al., 2003; Kolar and Machackova, 2005). Comparatively, high concentrations of melatonin have been stated in certain medicinal plants (Murch et al., 1997b, 2000; Chen et al., 2003). Now, some researches on plant melatonin and serotonin focused on their roles in plant growth and development. It has been observed that melatonin and serotonin storage is allied with TDZ-stimulated shoot formation in St John's wort. TDZ-induced regeneration, triggered by TIBA, PCIB and the ion channel activators/blockers, resulted in considerable higher levels of melatonin and serotonin. Melatonin and serotonin absolute concentrations were higher in the inhibited tissues when compared with that induced by TDZ alone. The high antioxidant efficiency of melatonin is mediated through a direct binding or indirectly by improving the action of extra free radical scavenging antioxidants and related enzymes (Pandi-Perumal et al., 2006; Hardeland et al., 2006). During the regeneration, melatonin may encounter the oxidative stress caused by explanting and exposure to growth regulators like TDZ. High levels of melatonin and serotonin are the signs for their byproduct nature of regenerative stress by TDZ. Further research is required to comprehend relationship between TDZ and melatonin to open new horizons in field of micropropagation.

Antioxidant potential

In early time, it has been proven that the physiological effect of TDZ is the induction of a stress response. Murthy et al. (1998) reported that seeding of several species including legumes and pumpkin exhibited stunted growth, darkly colored leaves, swollen green cotyledons and reduced primary root growth with few secondary roots in the presence of TDZ. Along with apparent physical indications of constrain in TDZ-treated tissues, there was an associated storage of numerous mineral ions and other stress associated metabolites like, proline, abscisic acid and 4- amino butyrate stored in the TDZ-tested wheat roots during the first week of induction, suggesting that TDZ can provoke stress response. Proline accumulation to 6% of the dry weight was observed when seedlings of peanuts were cultured on TDZ fortified medium (Murch and Saxena, 1997). TDZ-treated plant tissues enhanced endogenous auxin metabolism and transport (Murthy et al., 1995; Hutchinson et al., 1996; Murch and Saxena, 2001). More recently, the auxin related compounds serotonin and melatonin were found to be important in TDZ-induced regeneration (Murch and Saxena, 2001). Indoleamine and stress associated agents are accumulated in TDZ-induced regeneration (Murch et al., 1997a, b; Murch 1999, 2003). Melatonin can balance the oxidative stress generated by explanting and exposure to TDZ during the culture process (Mundhara and Rashid, 2006). Plants utilize reactive oxygen species as second messengers in signal transduction cascades in diverse physiological functions including cell division, senescence and tropisms (Foyer and Noctor, 2005). Oxidative stresses have earlier been shown to stimulate cell division, differentiation and morphogenesis analogous to auxins. Therefore, plant's response to TDZ may be attributed to the change of oxidative stresses in plant cell, especially during the shoot regeneration or embryo formation.

CONCLUSION

Organogenesis, either *in vivo* or *ex vivo,* needs stable regulation of cell proliferation and organogenesis, which are assumed to confine to plant hormones, auxin and cytokinins. TDZ is believed to be the best synthetic cytokinin present for the regeneration of numerous plant species. TDZ improved greatly the *ex vivo* generation and multiplication of species recalcitrant to propagation. In several cases, growth of explants accelerated when transfer them from amino purine cytokinin cultured medium onto TDZ fortified medium. TDZ is much effective in concentrations 10 to 1000 times less than other phytohormones. It has been observed that an expanded range of concentrations to be effective *ex vivo,* dependent on the species, explant status and objective. In certain procedures, a two-fold culture system is conducted with pronounced success, where TDZ fortified initial medium induces shoot multiplication which is followed by secondary medium containing low level of TDZ or other phytohormones to enhance shoot organogenesis. This is amenable if callogenesis or shoot elongation is difficult. In order to get a wide variety of

responses *ex vivo*, combination of TDZ and other phytohormones (including other cytokinins) can be more effective than when used alone. TDZ-promoted organogenesis comprises a metabolic cataract including primary signaling event, storage, passage of endogenous plant signals and iron in plant cell, a system of secondary messengers and a simultaneous stress response which may or may not be established as organogenesis. However, in spite of the popularity of TDZ as a phytohormone and more than thirty years of research work, the exact biological role of TDZ is still a mystery.

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