Thidiazuron: A Potent Phytohormone for In Vitro Regeneration

22

Sujatha Govindaraj

Abstract

Thidiazuron ($C_9H_8N_4OS$) is one of the most effective substituted phenylureas that has been examined for cytokinin-like activity in plant tissue cultures. A wide range of physiological responses were ascertained in response to TDZ application in several plant species. Apart from its cytokinin-like activity, TDZ has been steered to modulate the endogenous auxin levels. However, it remains to be resolved whether it possesses an auxin activity or if it is concerned with auxin metabolism. It induces numerous morphogenic responses, starting from tissue proliferation to induction of shoot buds and somatic embryos. It has been shown to promote shoot regeneration expeditiously than that of other cytokinins, and organized centers of growth are attained at much lower concentrations. Other prospects embody modification in cell membrane, energy levels, nutrient absorption, transport, assimilation, etc. TDZ exhibits the distinctive property of mimicking both auxin and cytokinin effects on growth and differentiation of cultured explants, though structurally it is different from either auxins or purine-based cytokinins. The effectiveness of TDZ as an inductive chemical for ontogenesis is not restricted to tissue culture systems, and the regeneration is settled in vivo further. During this review, many recently revealed studies on characterization of TDZ-induced in vitro regeneration are bestowed and mentioned.

Keywords

Thidiazuron · In vitro morphogenesis · Callusing · Somatic embryos · Metabolism

S. Govindaraj (✉)
Department of Botany, Periyar E. V. R. College (Autonomous), Tiruchirappalli, Tamil Nadu, India

N. Ahmad, M. Faisal (eds.), *Thidiazuron: From Urea Derivative to Plant Growth Regulator*, https://doi.org/10.1007/978-981-10-8004-3_22

22.1 Introduction

Plant tissue culture technique is the culture of plant cells, tissues, or organs under specific in vitro conditions to create a great number of true-to-type plants in short time using assorted starting plant material through phases of explant selection and preparation, culture establishment, regeneration, and acclimatization of the plantlets to ex vitro conditions (George 2008). The technology is progressing in applications for clonal propagation of medicinal, horticultural, agronomic crops, and forest trees. Many factors influence in vitro response of plants including the selected explant to be cultured; physiological state of the explant, juvenile or mature state; genotype; the health status of the explant; and culture media (Lee 2004; Kane 2005). The chosen explant for in vitro studies needs physiological adjustment to the culture conditions so as to achieve enhanced clonal multiplication and for the cultivated plant to accomplish physiological stability, and recurrent subculture to fresh media is necessary as medium nutrients get exhausted over time (Lee 2004; Kozai and Xiao 2006). The capacity to regenerate the entire plant from cultured somatic cells, tissue, or organ has been known for several decades; however, the problem of how the cultures differentiate into a whole plant and various physiological and anatomical features of the regenerated plants and during transfer to field conditions is still being studied by many research groups (Skoog and Miller 1957; Pospisilova et al. 1999; Vogel 2005; Jariteh et al. 2015). Manipulation of the in vitro development of plants is of paramount and applied interest as it proffers a model to portray developmental stages at genomic and proteomic levels and also offers potential to rejuvenate plants for increased propagation (Lee 2004; Moyo et al. 2015). A profound understanding of the in vitro plant development, the morphophysiology, as well as stress physiology mechanism and potential for acclimatization to ex vitro environment are of significance in foreseeing and enhancing the survival rate of plantlets during the in vitro culture conditions and acclimatization stages (Pospisilova et al. 1999; Cassells and Curry 2001; Moyo et al. 2015).

The two primary morphogenic pathways leading to whole plant regeneration – which is a prerequisite for most plant breeding, genetic, and transgenic applications of in vitro biology – involve either somatic embryogenesis or shoot organogenesis followed by root organogenesis. Both developmental pathways can occur either directly without a callus intermediate stage, termed adventitious, or indirectly following an unorganized callus stage, termed de novo (Gamborg and Phillips 1995). Few plant species have been shown to regenerate by both organogenic and somatic embryogenic pathways, but many plant species can regenerate by one or the other of these pathways (Phillips 2004). 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 of the sixth 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). The capacity of cultured plant tissues and cells to undergo morphogenesis, resulting in the formation of discrete organs or whole plants, has provided opportunities for numerous applications of in vitro plant biology in studies of basic botany, biochemistry, propagation, breeding, and development of transgenic crops.

Thidiazuron (TDZ, 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea) with molecular formula $C_9H_8N_4OS$ and molecular mass 220.25 g/mol is a light yellow crystalline chemical that is sparingly soluble in water, but highly soluble in ethanol and at varying levels in other organic solvents such as acetone, benzene, DMSO, etc. (Table 22.1, Fig. 22.1). Ethanol is the preferred solvent in using TDZ for in vitro studies. TDZ was manufactured by the German Schering Corporation for defoliation of cotton (*Gossypium hirsutum*) (Arndt et al. 1976). The defoliating property of

Table 22.1 Physical and chemical properties of thidiazuron

Character	Description
Color	Light yellow crystals
Odor	Odorless
Trade name	DROPP
IUPAC name	1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea
Molecular formula	$C_9H_8N_4OS$
Molecular weight	220.25 g/mol
Melting point	210.5–212.5 °C
Vapor pressure	2.30 × 10–11 mmHg at 25 °C
pH	6.50 at 20 °C
Dissociation constant	pKa = 8.86
Storage	Dry conditions
Purity	≥98%
CAS number	51707-55-2

Source: Pub Chem; URL: https://pubchem.ncbi.nlm.nih.gov

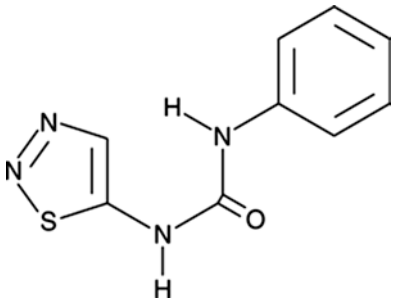

Fig. 22.1 Structure of thidiazuron

TDZ is restricted to a few species belonging to the Malvaceae family (Grossmann 1991; Zubkova et al. 1991). Thidiazuron induces abscission of cotton leaves, characteristically without the breakdown of chlorophyll or any alteration in the leaf water potential (Grossmann 1991).This chemical has now emerged as a highly efficacious bioregulant of morphogenesis in tissue culture of a diverse array of plant species, and the culture responses range from induction of callusing to the formation of somatic embryos. There are two functional groups in TDZ molecule, viz., phenyl and thiadiazol groups, and replacement of any of these groups with other ring structures results in the reduction in activity. A wide range of physiological responses were observed in response to TDZ application in different plant species. Examples of the diversity of physiological effects mediated by TDZ include enhanced seed germination in lettuce (Baskakov et al. 1981), substitution of chilling requirement for seed germination in *Pyrus* sp. (Lin et al. 1994), accelerated bud break in apple (Wang et al. 1986), stimulation of sprouting in potato (Ji and Wang 1988), cotyledon growth in pumpkin (Burkhanova et al. 1984), formation of branched trichomes and stomata on floral organs (Venglat and Sawhney 1994), and increased cluster and berry weight in grapes (Reynolds et al. 1992). TDZ has revealed both auxin- and cytokinin-like effects, though, chemically, it is totally different from frequently used auxins and cytokinins. A number of physiological and biochemical responses in cells are likely to be influenced by TDZ, but these may or may not be directly related to the induction of morphogenesis. 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 the modifications in cell membrane, energy levels, nutrient absorption, transport, assimilation, etc. (Guo et al. 2011). The precise mechanism of action of TDZ is explained by two hypotheses: It is possible that TDZ directly promotes growth due to its own biological activity in a fashion similar to that of N^6-substituted cytokinins, or it may induce the synthesis and (or) accumulation of endogenous cytokinins (Mok and Mok 1985). The latter notion is based on the effects of the high ability of TDZ in inducing cytokinin-dependent shoot regeneration and modulation of endogenous levels of cytokinins.

In vitro propagation of plants is widely used, quickly obtaining a large number of identical plants, with phytochemical and sanitary quality (Sivanesan et al. 2010). For the success of an in vitro culture, protocols specific to each species are necessary, using different culture media, salt concentrations, and plant growth regulators. Thidiazuron (TDZ) is widely used in tissue culture and promotes cell division and elongation (Murthy et al. 1998). It operates in the regeneration and proliferation of meristems and, in combination with other regulators, can be used for the formation and maintenance of callus (Kokotkiewicz et al. 2012). It was proved that TDZ, unlike traditional phytohormones, individually fulfilled the requirements of various regenerative responses of many different plant species. The morpho-regulatory potential of TDZ has led to its application in plant tissue culture for the development of feasible morphogenetic systems. High intrinsic activity (economic factor) coupled with stability against heat (ease of use) and enzymes renders TDZ a choice chemical for establishing regenerable tissue culture systems (Mok and Mok 1985).

TDZ has been successfully used for the propagation of several plants where the increase in the number and length of shoots is observed when there is an increase in concentrations of the regulator (Ahmed and Anis 2012; Grabkowska et al. 2014). Further advantage observed in the use of TDZ on proliferation of plants is the maintenance of genetic stability, important for obtaining plants *true-to-type* and germplasm conservation (Faisal et al. 2014). In this review, several recently published studies on characterization of TDZ-induced in vitro regeneration are presented and discussed.

22.2 TDZ-Mediated Organogenesis

22.2.1 *Stevia rebaudiana*

Stevia rebaudiana Bertoni, a member of Asteraceae family which is indigenous to certain regions of South America-Brazil and Paraguay, is one of the important antidiabetic medicinal herbs. The compounds in its leaves, stevioside and rebaudioside, taste about 300 times sweeter than sucrose. It is used as sweetening agent and has enormous commercial importance. Its other medicinal uses include regulating blood sugar, preventing hypertension and tooth decay, and treatment of skin disorders. *Stevia* also has healing effect on blemishes, wound cuts, and scratches, besides being helpful in weight and blood pressure management. Conventional propagation in this plant is restricted due to the poor seed viability coupled with very low germination rate. Role of vegetative propagation method is also limited as specific habitat conditions are mandatory to grow the plants in addition to low acclimatization rate in soil. A suitable alternative method to prepare sufficient amount of plants within short time duration is the use of in vitro cultures. An efficient high frequency plant regeneration protocol through direct organogenesis was successfully developed for *Stevia rebaudiana* Bertoni (Lata et al. 2013). Nodal segments containing axillary buds were used as an explant and inoculated on MS (Murashige and Skoog 1962) medium containing 3% (w/v) sucrose, 0.8% (w/v) agar supplemented with various concentrations of benzyladenine (BA), kinetin (KN), and thidiazuron (TDZ) ranging from 1.0 to 9.0 μM. Maximum multiple shoots (96%) were obtained in MS medium supplemented with 1.0 μM TDZ with an average of 60 shoots per culture, having an average shoot length of 6.0 cm, whereas BA (9.0 μM) produced a maximum of 32 shoots with an average shoot length of 2 cm, and KN (9.0 μM) produced a maximum of 22 shoots with an average shoot length of 2 cm (Fig. 22.2). It is evident that TDZ at very low concentrations was able to produce the maximum number of shoots than BA and KN in *Stevia rebaudiana*. Several reports have stated that TDZ results in better shoot regeneration than any other cytokinins (Lata et al. 2013). According to Capelle et al. (1983), TDZ directly promotes growth due to its own biological activities similar to that of an N-substituted cytokinin, or it may induce the synthesis and accumulation of an endogenous cytokinin.

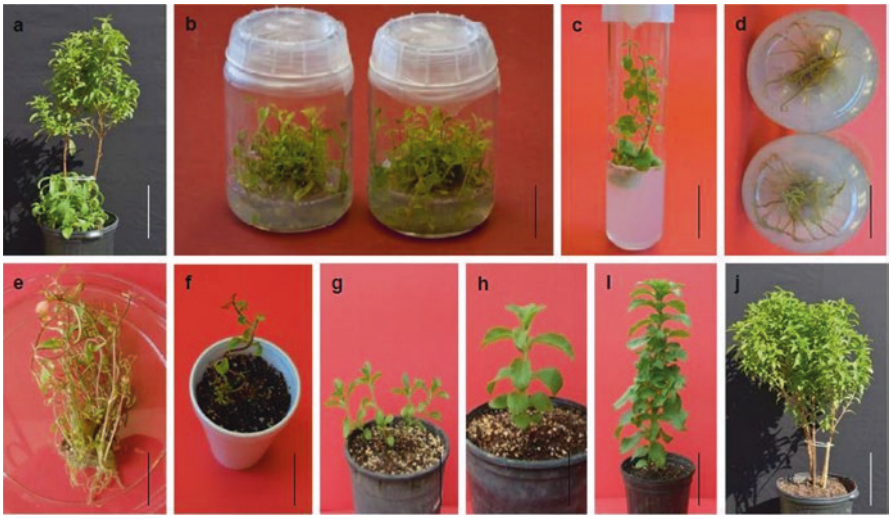

Fig. 22.2 Micropropagation of *Stevia rebaudiana* using nodal segment. (**a**) Mother plant, (**b**–**c**) in vitro shoot multiplication, (**d**–**e**) rooting, (**f**) fully rooted plant under acclimatization, (**g**–**i**) hardened tissue culture-raised plant, and (**j**) mature tissue culture-raised plants (*Source*: Lata et al. 2013; Article DOI – https://doi.org/10.4236/ajps.2013.41016)

22.2.2 *Withania somnifera*

Withania somnifera (L.) Dunal, Ashwagandha (Solanaceae), is cosmopolitan throughout the drier regions of India up to an altitude of 2000 m in range. It is useful in treating iatrogenic malnutrition in kids. In Ayurveda, the roots are prescribed for feminine disorders, bronchitis, arthritis, rheumatism, inflammation, central nervous system disorders, skin diseases, etc. The shortage of correct cultivation practices, the loss of habitats, and the illegal, indiscriminate collection of this plant from its natural habit create a heavy threat to its existence in the wild. Moreover, propagation through seeds is troublesome attributable to low germination percentage. Therefore, a protocol was standardized for in vitro regeneration of *Withania somnifera* on TDZ-supplemented medium (Fathima and Anis 2011). The nodal explants of *Withania somnifera* placed on MS basal medium lacking TDZ failed to show any morphogenetic response and did not produce shoots even after 6 weeks of incubation. On the other hand, MS basal medium supplemented with varied concentrations of TDZ (0.0–10.0 μM) showed swelling of explants, followed by differentiation of shoot bud primordia with different regeneration frequencies. Of the numerous concentrations of TDZ tested, 0.5 μM was found to be foremost effective in inducing highest percentage regeneration (98%) with the maximum number of shoots (23.8) and shoot length (4.83 cm) after 4 weeks of culture (Fathima and Anis 2011). TDZ has been used to induce shoot regeneration in several plants including *Psoralea corylifolia* L. (Faisal and Anis 2006) and *Cyamopsis tetragonoloba* L. (Ahmad and

Anis 2007). This behavior is believed to be due to the efficiency of TDZ to enhance the biosynthesis and accumulation of endogenous adenine-type cytokinins (Huettman and Preece 1993), thus creating TDZ as an effective cytokinin for the stimulation of shoot buds. In distinction, higher concentration of TDZ (1.0 μM and above) suppressed shoot formation during the same week culture period.

22.2.3 *Pluchea lanceolata*

Pluchea lanceolata is a perennial herb belonging to the family Asteraceae grown in warm climatic regions of India and known as Rasana. This plant is prized for its anti-arthritic and anti-inflammatory activity. A protocol was standardized for micropropagation of *P. lanceolata* by using nodal explants (Kher et al. 2014). The ability of the nodal explants for the bud break varied depending on the plant growth regulators and their concentration. Nodal explants were inoculated onto MS (Murashige and Skoog 1962) medium supplemented with 6-benzylaminopurine (BAP), kinetin (Kin), thidiazuron (TDZ), and 2iP (2-isopentenyladenine) at varied concentrations (0.0, 0.5, 1.0, 1.5, and 2.0 mg dm-3). Shoots developed with all the concentrations of cytokinins investigated and bud break occurred after 7–8 days of culture. Nodal explants cultured on MS medium augmented with 0.5 mg dm^{-3} thidiazuron (TDZ) exhibited the highest multiplication rate (9.7 shoots/explant). It was observed that in the cultures where shoot number was higher, the shoot length remained shorter.

22.2.4 *Cannabis sativa*

Hemp (*Cannabis sativa* L.) belongs to the Cannabaceae family. It is an annual herb that has been cultivated for the value of its fiber and more recently for paper manufacturing, oil extraction, and medicinal or drug preparation. Aseptic shoot tips were introduced to MS medium supplemented with different types of cytokinin for auxiliary bud induction. The effect of different concentrations of BA, KN, and TDZ on shooting response in the shoot tips of hemp was investigated. Among the three cytokinins tested, TDZ (0.2 mg l^{-1}) was found to provide the best bud induction, inducing an average of 3.22 buds with the thickest stem (Wang et al. 2009). Furthermore, the type of cytokinin in the medium also affected plantlet morphology, with the plantlets grown in TDZ-containing medium being more compact and vigorous. The suitability of TDZ for in vitro auxiliary shoot propagation has been well established in many woody plant tissue culture (Carl and John 1993) and also determinated in many herbage plants (Donna and John 2004).

22.2.5 *Gossypium hirsutum*

Most cotton *Gossypium hirsutum* (Malvaceae) genotypes of commercial interest present problems of in vitro regeneration. Aiming at improving regeneration rate,

meristems and caulinar apices (region with about 5 mm length, immediately below the meristematic region) of IAC 22 and COKER 312 cultivars were extracted from plants with two or three primordia leaves and grown in Murashige and Skoog (MS) medium containing thidiazuron (TDZ), with concentrations ranging between 0.02 and 5.0 μM, in 3- to 7-day periods, with naphthalene acetic acid (NAA) and gibberellic acid (GA_3). The number of shoots from meristems was higher in 0.02 μM TDZ (5.80 shoots/explant) concentrations. In the case of caulinar apices, best results were obtained with 0.5 and 1.0 μM of TDZ (4.08 shoots/explant) (Caramori et al. 2001).

22.2.6 *Curculigo latifolia*

The monocotyledonous plant, *Curculigo latifolia*, commonly known as lemba, is a perennial herb belonging to the Hypoxidaceae family and was thought to be natively from Malaysia. The plant is known for its sweet proteins, namely, curculin and neoculin, that have been proven to be 500–9000 times sweeter than sucrose by weight. Curculin which is a good low-calorie sweetener is absorbed by the human body and has a great potential for low-calorie sweetener-based industries. Besides the industrial and economic importance of *C. latifolia*, it is also considered as a valuable medicinal plant in having anticancer properties and antidiabetic properties and inhibiting hepatitis B virus. A procedure was developed for in vitro propagation of *Curculigo latifolia* through shoot tip culture (Babaei et al. 2014). Direct regeneration and indirect scalp induction of *Curculigo latifolia* were obtained from shoot tips grown on MS medium supplemented with different concentrations and combinations of thidiazuron and indole-3-butyric acid. Maximum response for direct regeneration in terms of percentage of explants producing shoot, shoot number (7.52 shoots/explant), and shoot length (2.71 cm) was obtained on MS medium supplemented with combination of thidiazuron (0.5 mg l^{-1}) and indole-3-butyric acid (0.25 mg l^{-1}) after both 10 and 14 weeks of cultures. Indole-3-butyric acid in combination with thidiazuron exhibited a synergistic effect on shoot regeneration. The shoot tips were able to induce maximum scalp from basal end of explants on the medium with 2 mg l^{-1} thidiazuron. Cultures showed that shoot number, shoot length, and scalp size increased significantly after 14 weeks of culture.

22.2.7 *Guizotia abyssinica*

Guizotia abyssinica Cass. belonging to family Asteraceae is an herbaceous crop with a lot of industrial as well as medicinal importance. The plant oil is good absorbent of fragrance of flowers used as base oil by perfume industry. The plant is used by the various tribal communities of India in the treatment of rheumatism, arthritis, microbial infections applied to treat burns, used for birth control, and treatment of syphilis. A simple, efficient, and reproducible regeneration protocol for in vitro propagation of *G. abyssinica* was established (Baghel and Bansal 2015). Different

explants, viz., apical and axillary buds, leaf, and internode, were selected for in vitro regeneration study to observe the effect of different concentrations of TDZ. Among all the four explants used, apical bud proved best in terms of shoot regeneration and multiplication. The variations in the regeneration potential of explants are attributable to the differences in the physiological and genetic makeup of cells. The best multiple shoot regeneration (4.44 ± 0.1) was observed on TDZ (0.45 μM)-supplemented medium. The reason for the better efficacy of apical bud is probably the presence of meristematic shoot bud at the already grown shoot tip. But in case of nodal explants, new shoot buds from the nodes are needed to be induced through purely hormonal control, or in other words, new shoot buds develop after inoculation in the absence of apical dominance. The role of TDZ in inducing regeneration is attributed to the ability of TDZ in enhancing the synthesis of adenine-type cytokinins. These results corroborate the fact that TDZ is an effective plant growth regulator for induction of shoot bud regeneration. The possible reason for the higher activity of individual TDZ treatment might be its high stability due to its resistance to cytokinin oxidase. TDZ-induced regeneration is linked to accumulation and transport of certain endogenous signals such as auxins or the related compounds like melatonin and serotonin (Jones et al. 2007).

22.2.8 *Artemisia vulgaris*

Artemisia vulgaris L. (mugwort) belongs to the family Asteraceae and is a tall aromatic perennial herb that grows in the hilly district of India in areas up to 2400 m in elevation. In traditional medicine, this plant is widely used for the treatment of diabetes, and extracts of the whole plant are used for epilepsy and in combination for psychoneurosis, depression, irritability, insomnia, and anxiety stress. Mugwort is commonly used in traditional European medicine as a choleretic and for amenorrhea and dysmenorrhea. The essential oil of the plant was reported to exhibit 90% mosquito repellency against *Aedes aegypti*, a mosquito that transmits dengue and yellow fever. An in vitro propagation system for *Artemisia vulgaris* has been developed (Sujatha and Ranjitha Kumari 2007). Hypocotyl segments (8–12 mm) excised from 10-day-old in vitro-grown seedlings were inoculated vertically on MS medium containing 3% (w/v) sucrose, 0.7% (w/v) agar supplemented with different concentrations of BA (6-benzyladenine) (0.44–13.32 μM), and TDZ (*N*-phenyl-*N′*-(1,2,3-thiadiazol-yl) urea) (0.23–11.35 μM) individually for multiple shoot induction (Fig. 22.3). The incubation of hypocotyl explants on MS media supplemented with BA (4.44 μM) or TDZ (4.54 μM) resulted in organogenic frequencies of 98.6% and 99.7%. The best organogenic response, including adventitious shoot number and elongation, was obtained when hypocotyl segments were cultured onto MS medium supplemented with 4.54 μM TDZ. Up to 28 shoots were formed per explant for an optimal duration of exposure of 48 days, and the maximum shoot length recorded was 9.8 cm. The media containing high concentrations of TDZ (>4.54 μM) decreased shooting frequency and shoot elongation.

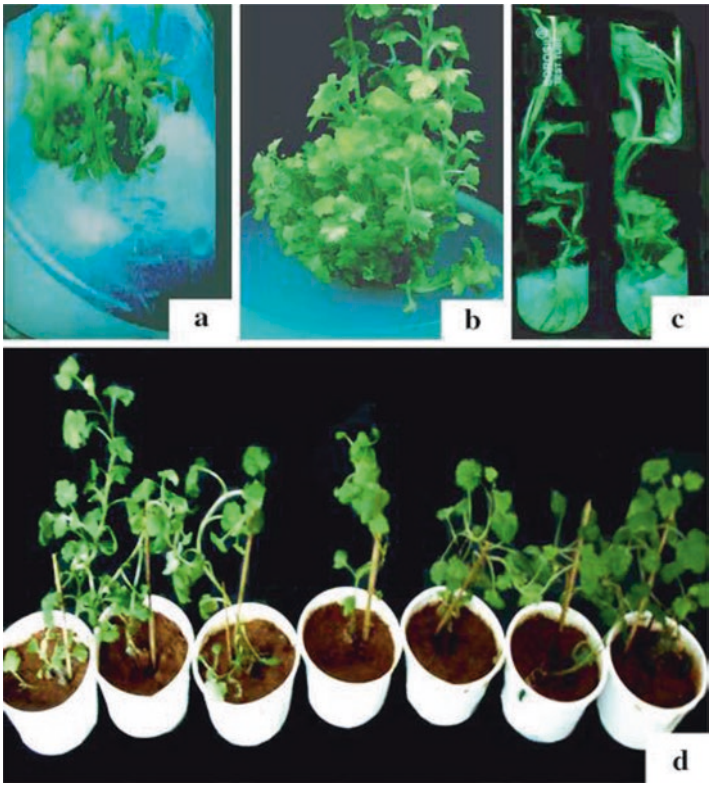

Fig. 22.3 Shoot multiplication in *Artemisia vulgaris* L. (**a**) Shoot initiation from hypocotyl segments, (**b**) shoot multiplication, (**c**) rooting of shoots, (**d**) hardened plants in plastic cups (*Source*: Sujatha and Ranjitha Kumari 2007; Article DOI https://doi.org/10.1007/s11816-007-0028-1)

22.2.9 *Capsicum annuum*

Capsicum annuum termed as "hot pepper" forms an important economical crop of the family Solanaceae. It acts on the circulation and the digestion and is used to treat a wide range of complaints from arthritis and chilblains to colic and diarrhea. An efficient protocol for rapid in vitro propagation of *Capsicum annuum* cv. Pusa Jwala through multiple shoot bud formation from cotyledonary node explants of 15-day-old aseptic seedlings has been developed (Siddique and Anis 2006). The morphogenetic responses of cotyledonary node explant to TDZ alone or in combination with IAA were studied. Explants cultured onto a growth regulator-free MS medium failed to produce shoots even after 4 weeks of culture. When MS medium was supplemented with different concentrations of TDZ (0.1–10.0 μM), multiple shoots emerged from cotyledonary node explants after 15 days of culture. TDZ in combination with IAA at different concentrations induced more shoots per explant

compared to TDZ alone in the investigation. The explants cultured on a medium containing TDZ (1.0 μM) produced a maximum of 11.6 shoot buds per explant with 72.67% regeneration. Optimum shoot differentiation was observed in a media containing TDZ (1.5 μM) and IAA (0.5 μM) after 4 weeks of culture. In this medium, the highest regeneration frequency (82.33%) and highest number of shoot buds per explant (19.0 ± 0.67) were achieved. At higher levels of TDZ, the number of shoot buds and regeneration frequency was reduced considerably, which may possibly be due to excessive callus growth while its specific concentration supports the maximum shoot bud induction.

22.2.10 *Rauvolfia serpentina*

Rauvolfia serpentina (L.) Benth. ex Kurz. (Apocynaceae) is an endangered medicinal plant recognized worldwide. Due to the presence of indole alkaloids and its usage as an antihypertensive drug, the demand for this plant has increased manifold in the global pharmaceutical industry. An efficient system for in vitro propagation of the endangered medicinal plant *Rauvolfia serpentina* has been developed (Alatar 2015). In vitro proliferation of shoots is usually promoted by incorporating growth regulators into the culture medium. TDZ is being selected for in vitro propagation of many plant species because of its tremendous ability to stimulate shoot proliferation. Compared to most of the other active compounds added to the media, extremely low concentrations of TDZ stimulate axillary shoot proliferation of many plant species. Proliferation of shoots was achieved from nodal segment explants, excised from field grown plants on Murashige and Skoog (MS) medium supplemented with thidiazuron (TDZ) (0.1–2.5 μ mol l^{-1}) although with low regeneration response and few number of shoots per explant. Greater number of shoots was achieved from nodal explants pretreated with higher concentrations of TDZ (5–100 μ mol l^{-1}) in liquid MS medium for different time periods (4, 8, 12, and 16 days), followed by their transfer on a growth regulator-free medium. The highest response in terms of percent regeneration (90%), average number of shoots/explant (23.17 ± 2.15), and maximum shoot length (5.3 ± 0.83 cm) was achieved by pretreating the nodal explants with 50 μ mol/L TDZ for 8 days. On increasing the concentration of TDZ, the number of shoots per explant was reduced. Similarly, at lower concentration, the percentages of regeneration as well as the number of shoots were drastically reduced.

22.2.11 *Kigelia pinnata*

Kigelia pinnata is a fast-growing, multipurpose tree used for ornamental and wayside planting belonging to the family Bignoniaceae. Various parts of the plant are employed for medicinal purposes by certain indigenous people. Traditional healers in India have used various parts of this plant to treat a wide range of skin ailments, from relatively mild complaints, such as fungal infections, boils, and psoriasis, to

the more serious diseases like leprosy, syphilis, and skin cancer. Other medicinal applications include the treatment of dysentery, ringworm, tapeworm, postpartum hemorrhaging, malaria, diabetes, pneumonia, and toothache. An antimalarial compound known as lapachol has been extracted from the root of *K. pinnata.* Another compound obtained from the wood, quinone, shows antimalarial activity against drug-resistant strains of *Plasmodium falciparum* superior to chloroquine and quinine. Conventionally, *K. pinnata* reproduces via viable seeds, but the low percentage of seed viability limits its natural propagation. Thus in vitro propagation of the plant was established (Thomas and Puthur 2004). The surface-sterilized nodal segments were cultured on MS (Murashige and Skoog 1962) medium supplemented with various concentrations (1–7 mM) of 2,4-D in a test for callus induction. Callus was subcultured onto fresh medium (MS + 3 mM 2,4-D) every 45 days. For multiple shoot induction, calli were transferred to MS medium supplemented with various concentrations (0.5–9 mM) of TDZ alone or in combination with NAA (0.5 and 1 mM). The optimum response in terms of percentage of explants producing shoots and the highest number of shoot buds per explant were recorded on MS medium supplemented with TDZ (3 mM) and NAA (0.5 mM). On this medium 100% cultures responded with an average 28 shoots per culture. The regenerated shoots attained a height of about 2 cm in about 45 days of callus culture.

22.2.12 *Solanum tuberosum*

Potato is one of the most important widely grown crops and is an integral part of diet in the entire world. It produces more protein (524 kg/ha) as compared to wheat (254 kg/ha). It also supplies at least 12 essential minerals including Vitamin C. Apical shoot explants of *Solanum tuberosum* L. cvs. Desiree and Cardinal were grown on MS (Murashige and Skoog 1962) medium containing three different concentrations of TDZ (10^{-8}, 10^{-9} or 10^{-10} M). The maximum number of shoots (2.66 and 2.96) was obtained on MS + TDZ (10^{-8}) in cvs. Cardinal and Desiree, respectively (Sajid and Aftab 2009). The highest number of roots (12.60 and 14.90) and nodes (7.90 and 7.20) was observed on MS medium in the two cultivars. The maximum fresh and dry weight of the plantlets (0.543 g and 0.0524 g) in cv. Cardinal was obtained on MS medium containing10^{-9} M TDZ. In Desiree, the highest fresh and dry weights (1.0560 and 0.0965 g, respectively) were observed on MS medium containing 10^{-10} M TDZ.

22.2.13 *Morus alba*

Morus sp. is an invaluable tree for the sericulture industry as it is the only source of food for mori silkworms. Three cultivars of mulberry S-36, S-1, and K-2 were selected for in vitro regeneration experiments (Thomas 2003). Cotyledonary explants were cultured 7, 14, and 21 days after embryo culture. Individual cotyledons were excised from seedlings about 1 mm below the cotyledonary node and

cultured on MS medium supplemented with 2–9 μM TDZ and BAP. The embryos cultured on MS medium supplemented with 5 μM BAP produced well-developed cotyledons, hypocotyl, and radicle 7 days after culture. The induction of multiple shoots varied with the age of the cotyledons as well as the concentration and type of growth regulator used. A significantly greater number of shoots were formed from cotyledons of 14-day-old embryos cultured on MS medium containing TDZ and BAP compared to 7- and 21-day-old explants. TDZ at different concentration was found to induce more shoots per explant as compared to BAP at the same concentration and explant age. A maximum number of shoots were produced on MS medium fortified with 7 μM TDZ. On this medium the 14-days-old explants produced an average number of 20.3 shoots per explant in S-36 cultivar. Whereas 7.3 shoots were produced in cultivar K-2 followed by 5.6 shoots in cultivar S-1. The shoots produced were isolated individually and elongated on MS medium augmented with 5 μM BAP.

22.2.14 *Salvia officinalis*

Salvia officinalis L., common sage (family, Lamiaceae), one of the important medicinal plant species, is cultivated in several countries mostly to obtain the dried leaves to be used as raw material in medicine and perfumery industries. Recent research has shown that sage essential oil can recover the memory and has shown promise in the treatment of Alzheimer's disease. It is also used in treating bronchial asthma, inflammatory affection, atherosclerosis, cataracts, ischemic heart disease, cancer, hepatotoxicity, and insufficient sperm mobility. Conventionally, *S. officinalis* is propagated through seeds; however, in nature, seeds germinate slowly and remain dormant for a long time. Alternatively, cutting can be used but low population size hampers the process. Therefore, in vitro methods for large-scale multiplication would be a viable option (Jafari et al. 2017). This research was conducted to develop an indirect organogenesis regeneration protocol for *Salvia officinalis* L. via callus which was obtained from leaf and internode explants. Among these explants internode explant gave best callus induction on MS medium supplemented with 0.5 mg/l 6-benzylaminopurine (BAP), 2.0 mg/l α-naphthalene acetic acid (NAA). The calli formed were subcultured on MS medium fortified with 0.5 mg/l thidiazuron (TDZ). A maximum of 70% shooting was observed, and a maximum of 2.5 shoots were produced on TDZ-augmented medium. The elongated shoots were transferred to MS/2 medium fortified with different concentrations of NAA and IBA for root induction.

22.2.15 *Lavandula angustifolia*

Lavandula angustifolia "Munstead" (English lavender) family Lamiaceae (Labiatae) is a hardy perennial shrub rich in aromatic essential oils and is valuable for its pharmaceutical, aromatic, and culinary properties. This genus is relatively rich in

phenolic constituents, with 19 flavones and 8 anthocyanins. *Lavandula angustifolia* is stated to be a carminative, spasmolytic, tonic, and antidepressant and is also used for treating nervous headache, neuralgia, rheumatism, depression, insomnia, windy colic, fainting, toothache, sprains, sinusitis, stress, and migraine. Aromatherapy involves massage using a much diluted essential oil or mixture of essential oil to the bath or a basin of hot water or using burners. The present investigation was carried out to study the in vitro shoot proliferation, root formation, and ex vitro acclimatization of English lavender (Munstead) (Hamza et al. 2011). Nodal explant showed a good response for producing the highest survival percentage and shoot and leaf numbers compared with the shoot tip one. Among the tested cytokinins, TDZ at 0.20 mg/L recorded the highest shoot number (30.55 shoots), followed by BAP at 0.80 mg/L (16.50 shoots). The weakest effects on shoot number were recorded for media supplemented with all KIN concentrations; however, it tabulated the tallest shoot length.

22.3 TDZ-Mediated Embryogenesis

22.3.1 *Tylophora indica*

Tylophora indica (Burm. f.) Merrill, previously called as *Tylophora asthematica*, a member of Asclepiadaceae, is an important indigenous medicinal plant found in restricted localities in the Indian subcontinent. The roots have a sweetish taste turning acrid, an aromatic odor, and a brittle fracture. They possess stimulant, emetic, cathartic, expectorant, stomachic, and diaphoretic properties and are used for the treatment of asthma, bronchitis, whooping cough, dysentery, diarrhea, and rheumatic gouty pains. Apparently due to non-availability of sufficient quality planting materials, commercial plantations of this important aromatic and medicinal species have not been widely attempted, and presently only the wild population is exploited for extraction purposes. Due to overexploitation and lack of organized cultivation, the wild populations have declined fast. An efficient procedure has been developed for inducing somatic embryogenesis from mature leaves of *Tylophora indica* was established (Chandrasekhar et al. 2006). Leaf bits were cultured facing the adaxial and abaxial side toward the medium. They were inoculated on MS medium containing 2,4-D (1.0, 1.5 and 2.5 μM), TDZ (0.25, 0.5, and 0.75 μM), or BA (0.5 and 1.0 μM) alone or in combinations. Leaf sections were initially cultured on Murashige and Skoog's (MS) medium supplemented with thidiazuron (TDZ) in addition with 2,4-dichlorophenoxy acetic acid (2,4-D); particularly 0.5 μM TDZ along with 1.5 μM 2,4-D was very effective in inducing somatic embryos (71.6%). Plants were regenerated from in vitro somatic embryos plated on semisolid medium devoid of growth regulators.

22.3.2 *Vitis vinifera*

Grapes (*Vitis vinifera*) are one of the most commonly consumed fruits in the world. Grapes can be used in numerous forms, viz., raisins, jam, jelly, and beverages like juice and wine, and also added for other culinary purposes. Being an inexpensive fruit, the demand for grapes is increasing day by day due to its immense potential toward improving the health of the humans. The biological activities of grapes has been studied widely which showed that it is rich in phenolic compounds with approximately two-thirds of grape polyphenols present in skin and seeds. These grapes owing to its phenolic compounds provide wholesome health benefits including cardioprotective, anti-inflammatory, anticarcinogenic, antimicrobial, and antioxidant properties. In vitro protocols for callus induction, somatic embryogenesis, and plant regeneration using leaf explants of three varieties of grapes (Thompson seedless, Sonaka, and Tas-e-Ganesh) were developed (Malabadi et al. 2010). Surface-sterilized leaf explants were cultured on Nitsch and Nitsch basal medium (Nitsch and Nitsch 1969) supplemented with a range of TDZ concentrations (0.45–11.35 μM) and 2,4-D at a concentration of 4.52 μM singly and in combination. The leaf explants responded well, and callus was induced after 2–4 weeks of culture on NN medium supplemented with 4.52 μM 2,4-D and 4.54 μM TDZ in all the three varieties. The highest percentage of somatic embryogenesis (Thompson seedless, 78%; Sonaka, 56.2%; and Tas-e-Ganesh, 48%) was observed. Somatic embryos recovered per gram fresh weight of embryogenic tissue were 55.0 in Thompson seedless, and the number of seedlings recovered per gram fresh weight of embryogenic tissue was 43.0. This study has opened possibilities for large-scale clonal propagation of grapes.

22.3.3 *Theobroma cacao*

Cacao trees (*Theobroma cacao*) are grown principally in rainforest areas in the tropical regions of the world. Cacao seeds are the sources of cacao powder and butter, which are important ingredients in chocolate and confectionary products. Cocoa butter is also used in a number of pharmaceutical and cosmetic products. Vegetative propagation of cacao is limited because of the low propagation rate, intensive labor, and associated costs. Simultaneously cacao has proven to be recalcitrant to in vitro shoot regeneration and organogenesis. Plant regeneration via somatic embryogenesis provides an alternative approach for clonal propagation of cacao since somatic embryos are produced through bipolar development of somatic cells; plants derived from somatic embryos are genetically identical to their parental donor cells and have the growth characteristics of seed-derived plants. A procedure for the regeneration of cacao plants from staminode explants via somatic embryogenesis was developed (Li et al. 1998). Rapidly growing calli were induced by culturing staminode explants on primary callus growth medium supplemented with 20 g l^{-1} sucrose, 9 μM 2,4-D, and various concentration of TDZ (22.7–454.4 nM). Calli were further subcultured, and somatic embryos were formed from embryogenic calli. A TDZ

concentration of 22.7 nM was found to be the optimal concentration for effective induction of somatic embryos from cacao. Two types of somatic embryos were identified on the basis of their visual appearance and growth behavior. Plants raised via somatic embryos showed morphological and growth characteristics similar to those of seed-derived plants.

22.3.4 *Myrica rubra*

Myrica rubra is a dioecious species and its progeny is highly heterozygous. Conventional vegetative propagation methods such as air layering and grafting are not rapid to meet the need of elite varieties. Somatic embryogenesis provides great promise of mass propagation and could be used as genetic engineering vehicle to develop non-chimeric transgenic plants. This present study accomplishes plant regeneration through direct somatic embryogenesis from cotyledon explants of *Myrica rubra*. Somatic embryogenesis was induced on woody plant medium (WB) (Sugawara et al. 1994) supplemented with thidiazuron (TDZ) alone or in combination with 2,4-D from mature cotyledon explants of *Myrica rubra* (Asghar et al. 2013). All concentrations of TDZ except 1.0 mgL^{-1} induced somatic embryos and adventitious shoots simultaneously within 2 months of culture. Addition of 2,4-D in the medium significantly improved induction of somatic embryos. Frequency of embryogenesis was only 3.34% with 7.00 embryos per explants when TDZ was fortified as a single growth regulator which was improved to 22.00% with the addition of 0.1 mgL^{-1} 2,4-D in the media. Repetitive embryogenesis was induced on optimized concentrations (0.5 mgL^{-1} BA and 0.05 mgL^{-1} TDZ) of two cytokinins in combination with various concentrations of 2,4-D. Continuous culture of the explants with cluster of embryos on the induction media did not induce repetitive embryogenesis. On repetitive embryogenesis induction media, most of the embryos induced were smaller in size than those of the primary embryos during their induction stage. TDZ in combination with IBA induced adventitious shoots on the surface of somatic embryo explants. TDZ (0.2 mgL^{-1}) plus IBA (1.0 mgL^{-1}) was the most effective combination with maximum number (8.5) of shoots per explant. Shoot elongation was achieved on the media supplemented with 0.5 mgL^{-1} BA concentration plus 0.1 mgL^{-1} NAA. The plants were rooted and successfully hardened.

22.3.5 *Murraya koenigii*

Murraya koenigii (L.) Spreng, popularly known as curry leaf plant, is a small aromatic tree belonging to the family Rutaceae that grows widely in Southeast Asia. Its leaves are slightly pungent, bitter, and acidulous in taste. Fresh and dried leaves are used extensively as a flavoring agent in many Indian culinary practices. The aromatic components of this tree are widely utilized in the medicinal field. A reproducible protocol for direct and indirect somatic embryogenesis was established (Paul et al. 2011). Embryogenic callus was obtained from 90% zygotic embryonic axis

(ZE) and 70% cotyledon (COT) explants in Murashige and Skoog (MS) basal medium supplemented with 8.88 μM 6-benzyladenine (BA) and 2.675 μM α-naphthalene acetic acid (NAA). Globular somatic embryos were induced and further matured from such embryogenic callus by subsequent culture on the same basal media containing thidiazuron (TDZ) (2.27–9.08 μM). The highest frequency of somatic embryos (14.58 ± 0.42) was recovered from ZE-derived callus after 6 weeks. The age and type of explant and concentration of TDZ played an important role in the development of somatic embryos. Explants excised from 60-day-old seed differentiated from 96.67% of ZE explants and 86.67% from COT explants when cultured on MS basal medium supplemented with 4.54 and 9.08 μM TDZ, respectively, after 4 weeks. The best result obtained for the average frequency of somatic embryos (11.28 ± 0.32) was from ZE explants, which was significantly higher than COT explants (7.34 ± 0.97). Most of the somatic embryos (above 95%), irrespective of their origin, germinated after 4 weeks in 1/2 MS basal media.

22.3.6 *Phalaenopsis aphrodite*

Phalaenopsis aphrodite subspecies *formosana* is qualified to be a model plant in the recent orchid research which has been intensively studied in the past 10 years, including in vitro protocols, flowering and photosynthetic physiology, chloroplast genomic analysis, global analysis of transcriptome, and modified ABCDE model of flowering. In the global horticultural trade, *Phalaenopsis* (i.e., moth orchids) is one of the most popular plants in the production of pot plants and cut flowers. It is mainly due to their beautiful flowers, ease of cultivation in the artificial conditions, and a long vase life. An alternative in vitro protocol for embryo induction directly from intact living seedlings of *Phalaenopsis aphrodite* subspecies *formosana* was established (Feng and Chen 2014). Without the supplementation of plant growth regulators (PGRs), no embryos were obtained from all the seedlings when cultured on the solid medium. In contrast, embryos formed from the seedlings on the two-layer medium and the two-step culture system without the use of PGRs (Fig. 22.4). It was found that the age of the seedlings affected embryo induction. The 2-month-old seedlings typically had higher embryogenic responses when compared with the 4-month-old seedlings in the two-layer medium or two-step system. For the 2-month-old seedlings, 1 mg/L TDZ resulted in the highest number of embryos at the distal site of the shoot. However, on the leaves' surface, 0.5 mg/L TDZ induced the highest number of embryos. When the 2-month-old seedlings were cultured using the two-step method at 1 mg/L of TDZ, the highest embryogenic response was obtained, with an average of 44 embryos formed on each seedling. These adventitious embryos were able to convert into plantlets in a PGR-free 1/2 MS medium, and the plantlets had normal morphology and growth.

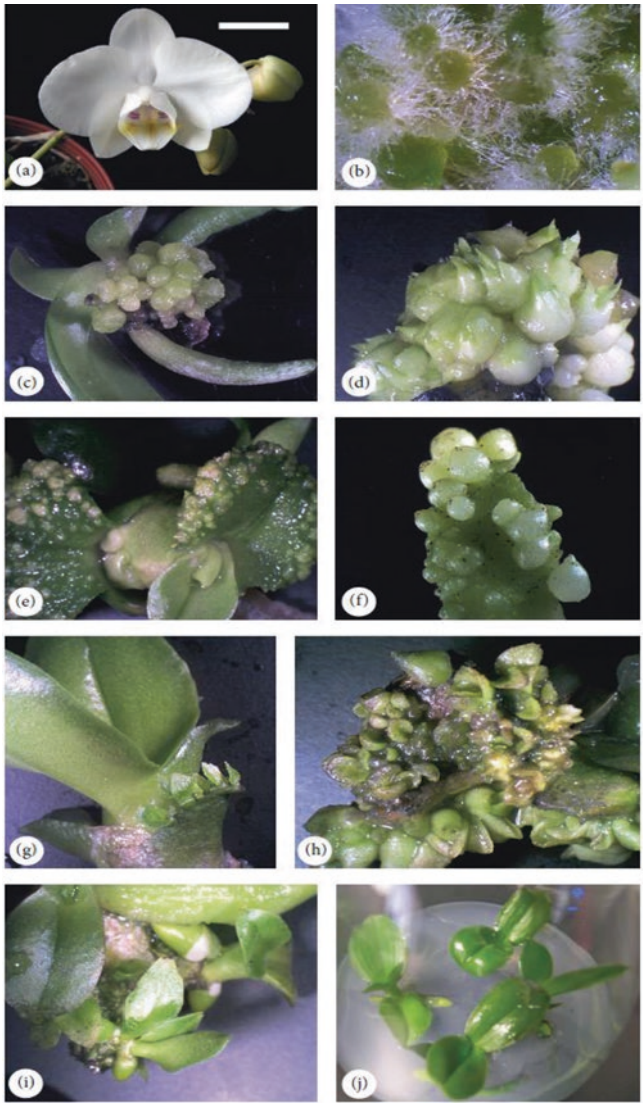

Fig. 22.4 *Phalaenopsis aphrodite* subsp. *formosana*, direct somatic embryogenesis from intact seedlings. (**a**) A flowering potted plant, (**b**) in vitro seed germination, (**c**) cluster of embryos, (**d**) embryos turned into protocorm-like bodies, (**e**) embryogenesis from the leaf surface, (**f**) foliar embryos formed, (**g**) foliar embryos of developing leaves, (**h**) numerous embryos initiated, (**i**) plantlet conversion, (**j**) rooted plantlets (*Source*: Feng and Chen 2014; Article DOI: https://doi.org/10.1155/2014/263642)

22.3.7 *Coffea arabica*

Coffea is an extremely important perennial agricultural crop in tropical areas with more than 6.5 million tons of green beans being produced every year on about 11 million hectares. The genus *Coffea* (Rubiaceae) consists of about 80 species in which only *Coffea arabica* (arabica) and *Coffea canephora* (robusta) are important for the production of *Coffea* beans. *C. arabica* contributes nearly 70% of the coffee consumed worldwide due to its superior quality, and *C. canephora* accounts for the rest 30%. Conventional breeding of coffee is difficult because of the long duration of cultivation before the seeds are set. Plant regeneration via various tissue culture methods could be very effective for propagation and improvement of coffee plants. Somatic embryogenesis is a highly useful method for the large-scale propagation of species of economic interest. Somatic embryos are widely considered to be of single cell origin; hence this is advantageous for transformation studies. The rapid direct and repetitive somatic embryogenesis in *Coffea arabica* and *C. canephora* genotypes was tested on Murashige and Skoog medium containing thidiazuron (TDZ) in concentrations of 2.27–11.35 μM (Giridhar et al. 2004). Segments taken from cotyledon leaf, first leaf, and stalk of regenerated plantlets produced clusters of somatic embryos directly from cut portions of explants on TDZ (9.08 μM)-containing medium within a period of 2 months. Subculturing of these embryo clusters produced more secondary embryos on reduced TDZ (0.045–0.91 μM)-containing medium, and these subsequently developed into plantlets (80–85%) on development medium followed by rooting on MS basal medium. This direct somatic embryogenesis from leaf and hypocotyl explants in *Coffea* sp. is a strong evidence of cell totipotency. The rapid somatic embryo induction protocol would be useful for the mass propagation, direct regeneration, and genetic transformation of selected elite lines.

22.3.8 *Vigna umbellata*

Rice bean, an under exploited tropical legume, is a native of Southeast Asia. In India, the crop is mainly found in the Western and Eastern Ghats and the NE Himalayas but is also grown in the sub-temperate Western Himalaya in the Uttaranchal and Himachal Pradesh hills. It is grown for its nutritious seeds and green pods as vegetable and also as a leguminous fodder crop in Kerala, Orissa, and West Bengal because of its higher fodder production potential. Rice bean has a comparatively higher content of proteins than other crops. Amino acid content is well suited for human digestion. But despite the great advantages, there are some biotic and abiotic constraints which affect its potential. Strategies to overcome these yield-limiting factors by conventional breeding have been slow due to the lack of desirable level of genetic variability in germplasm. An efficient in vitro regeneration protocol for Indian cultivar (RBL-50) of rice bean *Vigna umbellata* (Thunb.).

Fig. 22.5 Somatic embryogenesis and plant regeneration in rice bean. (**a**) Callus induction from cotyledonary node explant. (**b**) Formation of shoot buds on regenerative callus. (**c**–**d**) Induction of somatic embryos. (**e**–**i**) Various stages of somatic embryos: (**f**) globular, (**g**) heart, (**h**) torpedo, and (**i**) cotyledonary. (**j**) Germination of somatic embryos. (**k**) Abnormal germination of somatic embryos. (**l**) Potted plantlets raised from germination of somatic embryos (*Source*: Saini and Chopra 2012)

Ohwi and Ohashi via somatic embryogenesis has been developed (Saini and Chopra 2012). Highly proliferating (98%) calli cultures were initiated from the cotyledonary node containing both half cotyledons on semisolid MSB medium (MS salts and B5 vitamins) supplemented with 12.5 μM thidiazuron (TDZ) alone. Type and concentrations of growth regulators influenced the frequency of somatic embryogenesis. TDZ was found responsive for somatic embryogenesis than BAP, 2,4-D, and picloram, and the best result (18 somatic embryos per explant) was obtained with 12.5 μM TDZ in combination with 2.5 μM 6-benzylaminopurine (BAP). Sustained cell division resulted in the formation of cell aggregates, which progressed to the globular and heart-shaped somatic embryos and then, if they differentiated properly, to the torpedo shape and cotyledonary stages. The transfer of embryos onto MS basal medium enabled the embryos to achieve complete maturation and germination (Fig. 22.5). The percentage of germinating embryos increased significantly from 20 to 50 when 1.5 μM BAP and 2.0 μM gibberellic acid (GA_3) were supplemented to the MS basal media. In vitro-raised plantlets with well-developed roots were successfully hardened in a greenhouse and established in soil.

22.3.9 *Crocus sativus*

Saffron (*Crocus sativus* L.) is one of the most valuable industrial crops which is particularly important in exploration and income revenue. In vitro propagation of saffron either through somatic embryogenesis or cormogenesis is considered as an efficient alternative method for large-scale propagation of pathogen-free corms. In order to develop an efficient protocol for in vitro propagation of saffron, a factorial experiment was carried out based on completely randomized design to investigate the effects of various concentrations of TDZ (0, 0.1, 0.25, and 0.5 mg l^{-1}) on somatic embryogenesis induction from five different types of corm explants (terminal or axillary buds, upper or lower parts of the corm tissue, and terminal buds from pre-treated corms at 4 °C for 2 weeks) (Sheibani et al. 2007). The results revealed that TDZ concentration affected the induction of somatic embryogenesis significantly, while different types of corm explants showed no significant effect on this process. Among TDZ concentrations used, 0.5 mg l^{-1} was the most effective treatment for embryogenesis induction. Embryogenic calli proliferated well when subcultured into MS medium supplemented with 0.25 mg l^{-1} TDZ before transferring to hormone-free MS medium containing 6% sucrose for maturation. Matured embryos were transferred to half-strength MS medium without growth regulators for further development, from which microcorms were produced at the basal part after 3 months.

22.3.10 *Psoralea corylifolia*

Psoralea corylifolia L. (Fabaceae), commonly known as "babchi," is an endangered medicinal plant distributed in the tropical region of the world. The plant is used in indigenous system of medicine as a laxative, aphrodisiac, anthelmintic, diuretic, and diaphoretic in febrile conditions. It is specially recommended in the treatments of leucoderma, leprosy psoriasis, and inflammatory diseases. It is a seed-propagated species; however, the germination percentage is very low (5–7%). The low percentage of seed germination coupled with non-judicious wild collection for pharmaceuticals pose a serious threat to its existence in the nature. Tissue culture and in vitro plant regeneration system provide an alternative means for mass proliferation and ex situ conservation of endangered plant species. The development of efficient in vitro regeneration systems is needed to facilitate the application of recombinant DNA technology to the improvement of crop germplasm. In the present study, a simple, rapid, and effective system to regenerate *Psoralea corylifolia* plants via direct somatic embryogenesis from nodal segments has been established (Faisal et al. 2008). The embryogenic cells proliferated, formed somatic embryos, and were subsequently converted into normal plantlets under optimized culture conditions. The frequency of somatic embryogenesis was strongly influenced by the concentration of thidiazuron (TDZ) (0.0, 10.0, 11.0, 12.0, 13.0, 14.0 15.0, 16.0, 17.0, 18.0, 19.0, 20.0 μM) in the medium. The highest frequency (82%) of somatic embryogenesis was observed on Murashige and Skoog medium containing 16.0 μM TDZ. The

somatic embryos, when transferred to plant growth regulator-free MS basal medium, developed further to heart-shaped, torpedo, and cotyledonary stages within 2 weeks. Conversion of somatic embryos into plantlets was achieved by isolating somatic embryos with distinct cotyledons and transferring them onto half-strength MS medium containing 1.0 μM gibberellic acid (GA_3). Subsequently, the regenerated plantlets were successfully established in ex vitro condition with 90% survival.

22.3.11 *Pimpinella tirupatiensis*

Pimpinella tirupatiensis Balk and Subr., locally known as "adavikothimeera" (forest coriander), is a herbaceous medicinal plant, distributed on Tirumala Hills (1000 m above MSL) of Chittoor District, Andhra Pradesh. It is a narrow endemic species (Umbelliferae) of seasonal occurrence with underground tuberous root system. Dried roots of *P. tirupatiensis* are administered along with few other ingredients to cure colic and rheumatic ailments in cattle. The local Yanadi tribal community uses the tuberous roots of *P. tirupatiensis* to cure severe ulcers of the stomach, throat, and genital organs and also as aphrodisiac and as abortifacient agents. Fruits are used to cure asthma and are considered as an effective remedy for "flatulent colic." Conventional propagation methods through seed and root tubers for cultivation of *P. tirupatiensis* are beset with limited planting material and poor fruit setting. The availability of the seed is also very less due to its dispersal by wind, on attaining maturity. In the present investigation, a regeneration protocol through somatic embryogenesis is attempted to conserve this rare species of Umbelliferae for posterity (Prakash et al. 2001). Hypocotyl segments were excised from 4-week-old aseptic seedlings of *Pimpinella tirupatiensis* and were cultured on MS medium with TDZ (1 mg/l) and NAA (0.5 mg/l), which gave rise to friable, pink callus after 4 weeks of culture. Embryogenic callus on transfer to MS medium containing TDZ (1 mg/l) produced somatic embryos after 8 weeks having dark green shoots and white hairy roots. On MS + TDZ (1 mg/l) + BA (1 mg/l), somatic embryo formation was enhanced. Embryos isolated and germinated in the presence of MS + TDZ (1.0 mg/l) and GA3 (1.0 mg/l) showed normal flowering without any morphological variation on transplantation to soil.

22.4 Conclusion

TDZ is widely applied in plant in vitro or in vivo that influences a number of parameters in plants. It was firstly used as a defoliant for cotton. A miscellaneous range of responses with a high grade of efficacy is induced via TDZ application. Exploitation of TDZ in plant cell culture systems in the early 1980s 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. It was reported that TDZ induces shoot regeneration in many plant species. TDZ fraction plays a very important role in morphogenesis like lower concentration which induces axillary shoot proliferation, whereas higher fraction causes adventitious shoot development. 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. 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 transferred from amino purine cytokinin-cultured medium onto TDZ-fortified medium. TDZ is much effective in concentrations 10–1000 times less than the 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 twofold 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. 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.

References

Ahmad N, Anis M (2007) Rapid plant regeneration protocol for cluster bean (*Cyamopsis tetragonoloba* L. Taub.) J Hortic Sci Biotechnol 82:585–589

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

Alatar AA (2015) Thidiazuron induced efficient in vitro multiplication and ex vitro conservation of *Rauvolfia serpentina* – a potent antihypertensive drug producing plant. Biotechnol Biotechnol Equip 29(3):489–497

Arndt FR, Rusch R, Stillfried HV, Hanisch B, Martin WC (1976) A new cotton defoliant. Plant Physiol 57:S–99

Asghar S, Abbas SJ, Wahab F, Khan NH, Ahmad N, Chen L, He X, Qin Y (2013) Direct induction of somatic embryogenesis and plant regeneration from cotyledon explants of *Myrica rubra* Sieb. & Zucc. Afr J Agric Res 8(2):216–223

Babaei N, Abdullah NAP, Saleh G, Abdullah TL (2014) An efficient in vitro plantlet regeneration from shoot tip cultures of *Curculigo latifolia*, a medicinal plant. Sci World J 2014:1–9

Baghel S, Bansal YK (2015) Thidiazuron promotes *in vitro* plant regeneration and phytochemical screening of *Guizotia abyssinica* Cass. – a multipurpose oil crop. World J Pharm Pharm Sci 4(1):1193–1217

Baskakov YA, Shapovalov AA, Zhirmunskaya NM, Ovsyannikov TV (1981) Interrelationship of growth-regulating activity and phytotoxicity of synthetic cytokinins. Dokl Akad Nauk SSSR 267:1514–1517

Burkhanova EA, Fedina AB, Baskakov YA, Yu A, Kulaeva ON (1984) Comparative study of the action of 6-benzylaminopurine, thidiazuron, and cartolin on growth of intact pumpkin seedlings. Sov Plant Physiol 31:8–12

Capelle SC, Mok DWS, Kirchner SC, Mok MC (1983) Effects of thidiazuron on cytokinin autonomy and the metabolism of N6-(Y2-Isopentyl) [8-14c] adenosine in callus tissues of *Phaseolus lunatus* L. J Plant Physiol 73(3):796–802

Caramori LPC, Favaro S, Vieira LGE (2001) Thidiazuron as a promoter of multiple shoots in cotton explants (*Gossypium hirsutum* L.) Acta Sci 23(5):1195–1197

Carl AH, John EP (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tissue Organ Cult 33:105–119

Cassells A, Curry R (2001) Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: implications for micro-propagators and genetic engineers. Plant Cell Tissue Org Cult 64(2–3):145–157

Chandrasekhar T, Mohammad Hussain T, Rama Gopal G, Srinivasa Rao V (2006) Somatic embryogenesis of *Tylophora indica* (Burm.f.) Merril., an important medicinal plant. Int J Appl Sci Eng 4(1):33–40

Davies PJ (1995) The plant hormones: their nature, occurrence, and functions. In: Davies PJ (ed) Plant hormones: physiology, biochemistry and molecular biology. Kluwer, Boston, pp 3–38

Donna IL, John EP (2004) Thidiazuron stimulates adventitious shoot production from *Hydrangea quercifolia* Bartr., leaf explants. Sci Hortic 101:121–126

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

Faisal M, Shahzad A, Anis M (2008) Somatic embryogenesis and plant regeneration from nodal explants in *Psoralea corylifolia* L. Int J Plant Dev Biol 2(2):111–113

Faisal M, Alatar AA, Hegazy AK, Alharbi SA, El-Sheikh M, Okla MK (2014) Thidiazuron induced in vitro multiplication of *Mentha arvensis* and evaluation of genetic stability by flow cytometry and molecular markers. Ind Crop Prod 62:100–106

Fathima N, Anis M (2011) Thidiazuron induced high frequency axillary shoot multiplication in *Withania somnifera* L. Dunal J Med Plants Res 5(30):6681–6687

Feng JH, Chen JT (2014) A novel in vitro protocol for inducing direct somatic embryogenesis in *Phalaenopsis aphrodite* without taking explants. Sci World J 2014:1–7

Gamborg OL, Phillips GC (1995) Plant cell, tissue and organ culture–fundamental methods. Springer – Verlag, Berlin

George EF (2008) Plant tissue culture procedure – background. In: George EF, Hall MA, De Kler KGJ (eds) Plant propagation by tissue culture, 3rd edn. Springer, Dordrecht, pp 1–28

Giridhar P, Kumar V, Indu EP, Ravishankar GA, Chandrasekar A (2004) Thidiazuron induced somatic embryogenesis in *Coffea Arabica* L. and *Coffea canephora* P ex Fr. Acta Bot Croat 63(1):25–33

Grabkowska R, Sitarek P, Wysokińska H (2014) Influence of thidiazuron (TDZ) pretreatment of shoot tips on shoot multiplication and ex vitro acclimatization of *Harpagophytum procumbens*. Acta Physiol Plant 36:1661–1672

Grossmann K (1991) Induction of leaf abscission in cotton is a common effect of urea and adenine-type cytokinins. Plant Physiol 95:234–237

Guo B, Abbasi BH, Zeb A, Xu LL, Wei YH (2011) Thidiazuron: a multi-dimensional plant growth regulator. Afr J Biotechnol 10(45):8984–9000

Hamza AM, Omaima M, Kafie AE, Kasem MM (2011) Direct micropropagation of English Lavender (*Lavandula angustifolia* Munstead) plant. J Plant Prod 2(1):81–96

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

Jafari S, Daneshvar MH, Salmi MS, Abadi LJ (2017) Indirect organogenesis and plant regeneration in common sage (*Salvia officinalis* L.): an important medicinal plant of Iran. Mod Appl Sci 11(5):22–29

Jariteh M, Ebrahimzadeh H, Niknam V, Mirmasoumi M, Vahdati K (2015) Developmental changes in protein, proline and some antioxidant enzymes activities in somatic and zygotic embryos of Persian walnut (*Juglans regia* L.) Plant Cell Tissue Org Cult 122:101–115

Ji ZL, Wang SY (1988) Reduction of abscisic acid content and induction of sprouting in potato, *Solanum tuberosum* L., by thidiazuron. J Plant Growth Regul 7:37–44

Jones MPA, Yi ZJ, 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
Kane ME (2005) Shoot culture procedures. In: Trigiano RN, Gray DJ (eds) Plant development and biotechnology. CRC Press, Washington, DC
Kher MM, Joshi D, Nekkala S, Nataraj M, Raykundaliya DP (2014) Micropropagation of *Pluchea lanceolata* (Oliver & Hiern.) using nodal explant. J Hortic Res 22(1):35–39
Kokotkiewicz A, Luczkiewicz M, Hering A, Ochocka R, Gorynski K, Bucinski A, Sowinski P (2012) Micropropagation of *Cyclopia genistoides*, an endemic South African plant of economic importance. Zurich Nat Sci 67:65–76
Kozai T, Xiao Y (2006) A commercialized photoautotrophic micropropagation system. In: Gupta SD, Ibaraki Y (eds) Plant tissue culture engineering. Springer, Dordrecht, pp 355–371
Lata H, Chandra S, Wang YH, Raman V, Khan IA (2013) TDZ-induced high frequency plant regeneration through direct shoot organogenesis in *Stevia rebaudiana* Bertoni: an important medicinal plant and a natural sweetener. Am J Plant Sci 4:117–128
Lee JM (2004) Plant cell culture and its applications. In: Goodman RM (ed) Encyclopedia of plant and crop sciences. Marcel Dekker, New York, pp 931–933
Li Z, Traore A, Maximova S, Guiltinan MJ (1998) Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using thidiazuron. In: In Vitro Cell Dev Biol Plant, vol 34, pp 293–299
Lin CH, Lee LY, Tseng MJ (1994) The effect of stratification and thidiazuron treatment on germination and protein synthesis of *Pyrus serotina* Rehd. cv. Niauli. Ann Bot 73:515–523
Malabadi RB, Vijayakumar S, Nataraj K, Mulgund GS (2010) Induction of somatic embryogenesis and plant regeneration in grapes (*Vitis vinifera* L.) Bot Res Int 3(2):48–55
Mok MC, Mok DWS (1985) The metabolism of [^{14}C]-thidiazuron in callus cultures of *Phaseolus lunatus*. Physiol Plant 65:427–432
Moyo M, Aremu AO, Vanstaden J (2015) Insights into the multifaceted application of microscopic techniques in plant tissue culture systems. Planta 242(4):773–790
Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
Murthy BNS, Murch SJ, andSaxena PK (1998) Thidiazuron: a potent regulator of in vitro plant morphogenesis. In: In Vitro Cell Dev Plant, vol 34, pp 267–275
Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163:85–87
Paul S, Dam A, Bhattacharya A, Bandyopadhyay TK (2011) An efficient regeneration system via direct and indirect somatic embryogenesis for the medicinal tree *Murraya koenigii*. Plant Cell Tissue Organ Cult 105:271–283
Phillips GC (2004) In vitro morphogenesis in plants – recent advances (invited review). In: In Vitro Cell Dev Biol Plant, vol 40, pp 342–345
Pospisilova J, Ticha I, Kadlecek S et al (1999) Acclimatization of micropropagated plants in ex vitro conditions. Biol Plant 42:481–497
Prakash E, Khan SV, Meru E, Rao KR (2001) Somatic embryogenesis in *Pimpinella tirupatiensis* Bal. and Subr., an endangered medicinal plant of Tirumala hills. Curr Sci 81(9):1239–1242
Reynolds AG, Wardle DA, Zurowski C, Looney NE (1992) Phenylureas CPPU and thidiazuron affect yield components, fruit composition, and storage potential of four seedless grape selections. J Am Soc Hortic Sci 1(17):85–89
Saini R, Chopra AR (2012) In vitro plant regeneration via somatic embryogenesis in rice bean *Vigna umbellata* (Thunb.) Ohwi and Ohashi: an underutilized and recalcitrant grain legume. J Environ Res Dev 6(3):452–457
Sajid ZA, Aftab F (2009) Effect of thidiazuron (TDZ) on in vitro micropropagation of *Solanum tuberosum* L. cvs. Desiree and Cardinal. Pak J Bot 41(4):1811–1815
Sheibani M, Azghandi AV, Hemati SH (2007) Induction of somatic embryogenesis in Saffron using Thidiazuron (TDZ). Pak J Biol Sci 10(20):3564–3570
Siddique I, Anis M (2006) Thidiazuron induced high frequency shoot bud formation and plant regeneration from cotyledonary node explants of *Capsicum annuum* L. Indian J Biotechnol 5:303–308

Sivanesan I, Hwang SJ, Jeong BR (2010) Influence of plant growth regulators on axillary shoot multiplication and iron source on growth of *Scrophularia takesimensis* Nakai-a rare endemic medicinal plant. Afr J Biotechnol 7:4484–4490

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plants tissue cultured in vitro. Symp Soc Exp Biol 11:118–131

Sugawara F, Yamamoto N, Tanaka O (1994) Plant regeneration in in vitro culture of leaf, stem and petiole segments of *Actinidia polygama* Miq. Plant Tissue Cult Lett 11:14–18

Sujatha G, Ranjitha Kumari BD (2007) High-frequency shoot multiplication in *Artemisia vulgaris* L. using thidiazuron. Plant Biotechnol Rep 1:149–154

Thomas TD (2003) Thidiazuron induced multiple shoot induction and plant regeneration from cotyledonary explants of mulberry. Biol Plant 46(4):529–533

Thomas TD, Puthur JT (2004) Thidiazuron induced high frequency shoot organogenesis in callus from *Kigelia pinnata* L. Bot Bull Acad Sin 45:307–313

Venglat SP, Sawhney VK (1994) Ectopic formation of trichomes and stomata in floral organs of *Arabidopsis thaliana* induced by thidiazuron. Can J Bot 72:671–677

Vogel G (2005) Deriving 'controversy-free' ES cells is controversial. Science 310:416–417

Wang SY, Steffens GL, Faust M (1986) Breaking bud dormancy in apple with a plant bioregulator, thidiazuron. Phytochemistry 25:311–317

Wang R, He LS, Xia B, Tong JF, Li N, Peng F (2009) A micropropagation system for cloning of hemp (*Cannabis sativa* L.) by shoot tip culture. Pak J Bot 41(2):603–608

Zubkova NF, Bukashkina ZV, Markina LG, Belova NA (1991) Defoliating effect of fusicoccin. Agrokhimiya 12:86–92