Research Article

TDZ-induced direct shoot organogenesis and somatic embryogenesis on cotyledonary node explants of lentil (*Lens culinaris* Medik.)

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ABSTRACT

An efficient and simple procedure for inducing high frequency direct shoot organogenesis and somatic embryogenesis in lentil from cotyledonary node explants (without both the cotyledons) in response to TDZ alone is reported. TDZ at concentration lower than 2.0 µM induced shoot organogenesis whereas at higher concentration (2.5-15 µM) it caused a shift in regeneration from shoot organogenesis to somatic embryogenesis. The cotyledonary node and seedling cultures developed only shoots even at high concentrations of BAP and TDZ, respectively. TDZ at 0.5 and 5.0 µM was found to be optimal for inducing an average of 4-5 shoots per cotyledonary node in 93 % of the cultures and 55 somatic embryos in 68 % of the cultures, respectively. The somatic embryos were germinated when transferred to lower TDZ concentration (0.5-1.0 µM). The shoots were rooted on MS basal medium containing 2.5 µM IBA. The plantlets were obtained within 8 weeks from initiation of culture and were morphologically similar to seed-raised plants. The possible role of stress in thidiazuron induced somatic embryogenesis is discussed. **[Physiol. Mol. Biol. Plants 2008; 14(4) : 347-353]** *E-mail : jaiwalpawan@rediffmail.com*

Key words : *Thidiazuron, Lens culinaris, Somatic embryogenesis, Organogenesis*

Abbreviations : *2-iP: 2-isopentanyl adenine, TDZ: Thidiazuron, AdS: Adenine sulphate, KIN: Kinetin, BAP: 6-benzyl aminopurine, IBA: Indole-3-butyric acid*

INTRODUCTION

Lentil (*Lens culinaris* Medik.) is the third important cold-season food legume (after pea and chickpea) grown all over the world in 3.8 million hectare area (FAOSTAT, 2007) for its high nutritional value (20-36 % protein), easy cooking quality and easy digestibility (Muehlbauer *et al*., 2006). Almost all land races of lentil are poor in yield due to their prominent susceptibility to abiotic (drought, heat and salinity) and biotic stresses, such as Ascochyta blight caused by *Ascochyta lentis*, Anthracnose caused by C*olletotrichum truncatum*, Botrytis grey mold caused by *Botrytis fabae* and *B. cinerea*, Stemphylium blight caused by *Stemphylium botryosum*, lentil rust caused by *Uromyces fabae* and two species of *Orabanche* for which no resistance is available (Muehlbauer *et al*., 2006). An alternative approach for the improvement of this crop is to complement traditional breeding with molecular techniques to regenerate plants from single cells and organized tissues and to transfer desirable genes from other sources. Until recently, gene transfer in lentil has been difficult and challenging because of its recalcitrant nature to *in vitro* regeneration (Gulati and Mc Hughen, 2003). Regeneration and transformation procedures of lentil are not well developed compared to the success achieved in other grain legumes (Sarkar *et al*., 2003). Only a few reports have been published on *in vitro* plant regeneration through organogenesis and somatic embryogenesis. Direct regeneration of shoot without intervening of callus phase has been achieved from intact seedling (Malik and Saxena, 1992), shoot tip (Bajaj and Dhanju, 1979; Polanco and Ruiz, 1988, 1997; Singh and Raghuvanshi, 1989), cotyledonary node (Warkentin and Mc Hughen, 1993; Polanco and Ruiz, 1997; Gulati and Mc Hughen, 2001) cultures. Indirect organogenesis from calli, derived from shoot meristems and epicotyls (William and Mc Hughen, 1986) and leaves (Polanco and Ruiz, 1988, 1997) has also been achieved. There is only one report on plant regeneration by the process of somatic embryogenesis from embryonal axes-derived calli (Saxena and King, 1987). However, previous studies generally involved extensive manipulation of culture

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conditions and obtained unsatisfactory low frequencies of whole plant regeneration (Gulati and Mc Hughen, 2003). Moreover, there are no reports on the direct somatic embryogenesis and subsequent plant regeneration in *Lens* cultivars. Thidiazuron, a substituted phenylurea induced shoot organogenesis in several plant species and somatic embryogenesis in geranium, peanut and neem (Murthy *et al.*, 1998). Both shoot organogenesis and somatic embryogenesis have been induced simultaneously in white ash (Bates *et al.*, 1992), chickpea (Murthy *et al*., 1996) and African violet (Mithila *et al*., 2003). However, in the present study, TDZ at low concentration induced shoot organogenesis while at higher concentration it caused a shift in regeneration from induction of shoots to majority of somatic embryos. A simple procedure for induction of high frequency shoot organogenesis and somatic embryogenesis from the cotyledonary node explants in a single step of culturing them on a TDZ supplemented medium has been achieved in a recalcitrant grain legume, lentil.

MATERIALS AND METHODS

Plant material and preparation of explants

Lentil cultivars, L - 4076 and 4619 were obtained from the Pulse Research Laboratory, Division of Genetics, IARI, New Delhi. Seeds were surface sterilized by immersion in 70 % ethanol for one min and in aqueous mercuric chloride (0.1 %, w/v) for 5 min followed by rinsing four to five times with sterilized distilled water. The seeds were germinated aseptically on Murashige and Skoog's basal medium containing 3 % sucrose and 0.7 % agar in culture tubes (25 mm x 150 mm) at 25 ± 2 °C under light conditions. After 4 days, shoot tips, epicotyls and cotyledonary nodes without cotyledons were excised from *in vitro* – raised seedlings.

Induction of shoot organogenesis and somatic embryogenesis

In preliminary experiments, different explants, such as shoot tip, epicotyl and cotyledonary node were cultured on MS basal medium supplemented with BAP, KIN, 2-iP, AdS and TDZ. A higher regeneration potential was observed for cotyledonary node explants from cv. L - 4076, when cultured on BAP or TDZ. This explant and cultivar were used in subsequent experiments. The cotyledonary node explants (5 mm) without both the cotyledons were cultured on medium containing MS salts and vitamins, 30 g l^{-1} sucrose, 8 g l^{-1} agar-agar (Himedia, Mumbai, India) and BAP or TDZ (0-15 µM) . The pH of the media was adjusted to 5.8 prior to addition of agar and autoclaved at 121 °C for 20 min.

In a separate experiment, seeds were cultured on MS basal medium containing TDZ (0-15 µM). All the cultures were incubated at 25 ± 2 °C under a 16 h light photoperiod regime (80 µE m^{-2} s^{-1}). Each treatment consisted of 24 explants or seeds and all the treatments were repeated at least twice. Visual observations of the cultures were made periodically and the data was subjected to analysis of variance and significant treatment differences selected by Newman-Keul's multiple range test (Brunning and Kintz, 1977).

Plantlet formation and transplantation

Somatic embryos (4 weeks old) developed at cotyledonary node explants cultured on MS basal medium containing TDZ (2.5-15 µM) were germinated either on MS basal medium or MS basal medium containing TDZ (0.5-1.0 µM). The multiple shoots developed on cotyledonary node explants on MS basal medium supplemented with TDZ (0.5-1.0 µM) were excised and transferred to MS basal medium containing IBA (2.5 µM) for rooting. The rooted shoots were planted in pots containing soil, sand and manure (1:1:1) ratio and covered with polythene bags to maintain high humidity for first few days. Subsequently, the humidity was reduced gradually by making holes in the polythene bags to harden the plants.

Histology

For histological studies, somatic embryos at different developmental stages developed on cotyledonary nodes after 3-4 weeks of culture on MS medium containing TDZ (2.5 µM), were fixed in formalin, acetic acid and alcohol (90:5:5), dehydrated serially with ethanol and n-butanol and embedded in paraffin wax (melting point 58 °C). Longitudinal sections (10 µM thick) were cut on a rotary microtome. Paraffin ribbons were mounted on glass slide and passed through a series of deparaffinizing solutions and were stained with safranin and finally mounted on DPX.

RESULTS AND DISCUSSION

Regeneration from cotyledonary node explants

Shoot organogenesis

In large-seeded grain legumes, regeneration from cotyledonary node with or without both the cotyledons is dependable and reproducible (Chaudhary *et al*., 2006). TDZ, a substituted phenylurea, is shown to be more

effective than all adenine type cytokinins in inducing high frequency shoot organogenesis as the most prevalent type of regeneration in legumes. (Amutha *et al.*, 2006). Therefore, the effect of different concentrations of TDZ and BAP was tested and compared on *in vitro* regeneration from cotyledonary node (without both the cotyledons) explants (Fig. 1a) of lentil (Table 1). The explants on MS basal medium containing BAP or TDZ swelled twice to their original size within 10-12 days. Subsequently, depending upon the type and concentration of cytokinin, the explants developed regenerants from the nodal region within 4 weeks of culture. BAP at all the concentrations (1.0-10.0 μM) tested and TDZ only at low concentrations (0.1-2.0 μM) induced shoot organogenesis (Fig. 1b). Although, the shoot regeneration frequency did not differ significantly among various BAP concentrations, the maximum frequency of shoot formation was 87 % on regeneration medium with 5.0 μM BAP and 93 % on that with 0.5 μM TDZ. Thus, the shoots were induced more efficiently at lower concentration of TDZ than that of BAP. TDZ was therefore concluded to be an effective cytokinin for lentil shoot organogenesis. These results support earlier reports on common bean (Malik and Saxena, 1992), peanut (Gill and Saxena, 1992) and soybean (Kaneda *et al.*, 1997).

Dose-response of TDZ on morpho-regulation

Cotyledonary node explants without cotyledons developed multiple shoots at lower concentration of TDZ (0.5-2.0 μM) and majority of the somatic embryos at higher concentrations (2.5-15 μM) from the nodal region within 4 weeks of culture. The TDZ dose-

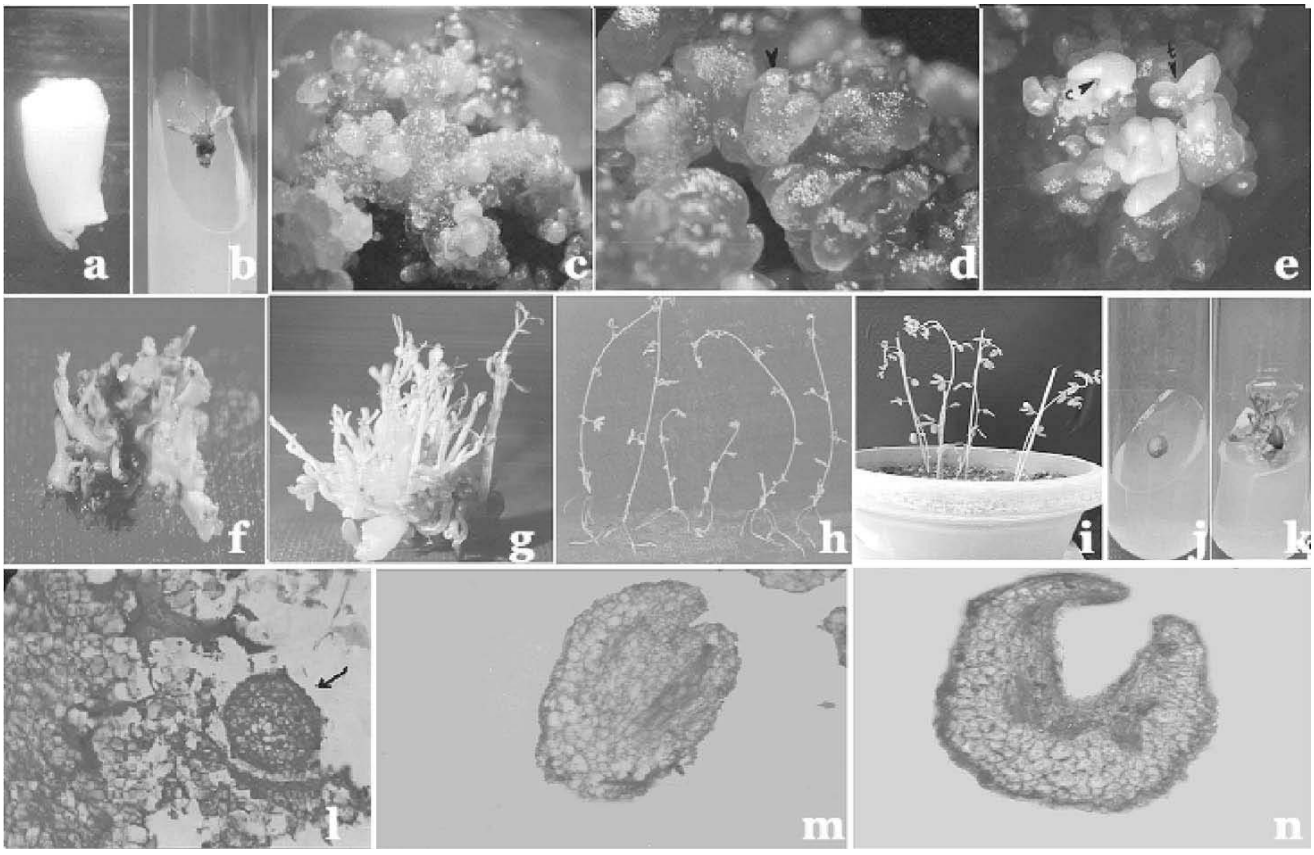

Fig. 1. *In vitro* organogenesis and induction of somatic embryogenesis in lentil. (a) Cotyledonary node without both the cotyledons at the time of culture, (b) Node with multiple shoots on TDZ (0.5 μM), (c-e) Explant showing globular, (c) heart shaped (d) and torpedo and cotyledonary (e) stages of somatic embryogenesis on medium containing TDZ (2.5 μM), (f-g) Embryos germinating on TDZ (1.0μM), (h) Shoots rooted on MS+IBA (2.5 μM), (i) Plantlets growing in pot containing soil (Photographed after 4 weeks of transplantation), (j-k) Mature seed at the time of culture (j) and development of multiple shoots from nodal region of seedling on MS+TDZ (5.0μM) (k) l-n. Longitudinal sections of different stages of somatic embryos: globular (l) heart shaped (m) and cotyledonary stage (n).

Table 1. Effect of different concentrations of TDZ and BAP on shoot regeneration from cotyledonary node explants (without both the cotyledons) of *Lens culinaris cv*. L-4076★.

Growth regulator	Concentration (μM)	% regenerating explants	Average number of shoots per explants	Average length of shoot (cm)
None	0	70	1.4±0.1[b]	5.8±0.2
TDZ	0.5	93	3.8±0.3[a]	3.2±0.1
	1.0	89	2.0±0.1[b]	1.8±0.0
	1.5	86	1.9±0.2[b]	1.7±0.1
	2.0	80	0.3±0.1[c]	0.5±0.0
	2.5	0	0	0
BAP	1.0	85	1.6±0.1[b]	4.1±0.3
	2.5	87	2.2±0.1[b]	3.9±0.2
	5.0	86	4.4±0.3[a]	2.7±0.1
	10.0	85	1.7±0.3[b]	1.9±0.3

★Culture medium = MS basal, Data based on 24 explants per treatment and each treatment was repeated twice. Data was taken after 4 weeks of culture. Mean followed by same letter are not significant according to Newman-Keul's multiple range test (P=0.5).

dependent organogenic and embryogenic competency of explants of lentil is a unique finding clearly reaffirming the efficacy of TDZ for eliciting both organogenesis as well as embryogenesis.

Somatic embryogenesis

The cotyledonary node explants without cotyledons on MS basal medium containing high concentration of TDZ (2.5-15 μM) developed a cluster of globular somatic embryos at the swelled nodal region within 20 days (Fig. 1c). There was no visible sign of callus formation during development of somatic embryos. After 24 days of culture, the embryos developed further to heart shaped (Fig. 1d), torpedo and cotyledonary stage (Fig. 1e). Development of somatic embryos is asynchronous. The frequency of embryogenesis and the number of embryos formed per explant increased with the increase in TDZ concentration up to 5 μM, thereafter, decreased with further increase in concentration. TDZ at 5 μM induced an average of 55 embryos per explant in 68 % of cultures (Table 2). Embryogenesis was not observed on the explants cultured on MS basal medium or MS basal medium containing TDZ lower than 2.5 μM (Table 1). The cluster of embryos induced by TDZ (2.5-10 μM) when transferred to MS basal medium without any hormone or MS basal medium containing low concentration of TDZ (0.5-1.0 μM) germinated into well-

Table 2. Morphogenetic response of cotyledonary node explants (without both the cotyledons) of lentil *cv*. L-4076 on MS basal medium supplemented with TDZ at concentrations higher than 2.5 μM★.

TDZ concentration (μM)	% explants forming embryos	No. of embryos per explant ± S.E
0	0	0
2.5	60	33.7±1.1[a]
5.0	68	55.4±4.5[b]
10.0	62	31.4±3.1[a]
15.0	50	25.0±2.0[c]

★Data taken after 4 weeks and based on 24 explants per treatment, Mean followed by same letter are not significantly different according to Newman-Keul's multiple range test (P=0.5)

Table 3. Effect of MS basal medium or MS basal medium containing lower concentrations of TDZ on germination of somatic embryos. The cluster of embryos (an average of 55.4 ± 4.5) induced on 5.0 μM TDZ were transferred to MS basal medium or MS basal medium containing lower concentrations of TDZ for germination of somatic embryos.

TDZ concentration (μM)	No of embryo cultures transferred	No of cultures showing germination of embryos	No of embryos germinated per culture
0.0	17	6 (35)★	2 (4)★★
0.5	21	13 (61)	9 (16)
1.0	23	19 (82)	14 (26)

★Values in parentheses are percentage of cultures showing germination of embryos.
★★Values in parentheses are percent embryos germination

formed shoots (Figs. 1f-g). Only 4 % of the embryos in 35% of the cultures germinated on the MS basal medium. The frequency and number of embryos germinated into shoots was higher in the presence of lower concentrations of TDZ (0.5-1.0 μM). A maximum of 26 % of embryos were able to form shoots in 82 % of the cultures on medium containing 1 μM TDZ (Table 3).

Regeneration from mature seeds

A unique system of TDZ-induced regeneration is the development of somatic embryos on intact seedlings of large-seeded legumes namely pea, peanut and chickpea (Murthy *et al*. 1998), pigeonpea (Singh *et al*. 2003) and other plants including neem (Gairi and Rashid, 2004). However, in present study, axenic seedling cultures of lentil established from mature dry seeds (Fig. 1j) on MS supplemented with TDZ (0.1-10.0 μM) developed multiple shoots from the nodal and the basal regions of primary shoots (Fig. 1k). There was no visible sign of somatic embryogenesis. TDZ at 2.0 μM induced a maximum of 6 shoots per explant in 86 % of the cultures (data not shown). Similar morphogenic response of lentil seedlings even at high concentration (1.0-50.0 μM) of TDZ was observed earlier (Malik and Saxena, 1992).

Rooting and transplantation

Sixty six percent of the shoots excised from cotyledonary node explants or seedlings or derived from embryos were rooted on MS basal medium supplemented with IBA (2.5 μM) (Fig. 1h). The resulting plantlets on transplantation in pots containing soil survived (Fig 1i) with 60 % success and reached to maturity. The *in vitro* developed plants were morphologically similar to the seed derived plants and produced viable seeds.

Histology

Histological sections of regenerating explants confirmed the presence of somatic embryos and clearly revealed their direct development without an intervening callus phase. Well-organized globular shaped embryo (Fig. 1l) developed further through characteristic heart-shaped (with notch) (Fig. 1m) to form welldeveloped mature cotyledonary somatic embryo that had a shoot primordia with a pair of cotyledons and no distinct root primordial (Fig. 1n). These embryos had no vascular connection with parental tissue and thereafter, can be easily detached.

In the present study, TDZ induced high frequency direct shoot organogenesis at low concentration (0.5-2.0 μM) and direct somatic embryogenesis at high concentration (2.5- 15 μM) on the same tissue, i.e. the cotyledonary node explants of an important but recalcitrant large-seeded grain legume, lentil. This is the first report on direct somatic embryogenesis in lentil. The concentration dependent redirection of development from shoot organogenesis to somatic embryogenesis provides a model system to investigate the role of specific signals in the induction of these developmental processes. The process of *in vitro* shoot organogenesis in various types of explants is well-documented in lentil and the protocols clearly indicate that a combination of auxin and cytokinin is essential for shoot formation (Polanco *et al*., 1988; Sarker *et al*., 2003). The most interesting aspect of the present study is the TDZ induced direct regeneration of somatic embryos from well-differentiated cotyledonary node explant, a response usually mediated by higher doses of auxins or by auxin-cytokinin ratio in legumes (Lakshmanan and Taji, 2000). These results corroborated the findings of the earlier

reports that TDZ can be substituted for auxins or auxin-cytokinin required to induce somatic embryogenesis. This is probably due to the involvement of TDZ in the modulation of endogenous growth regulators especially auxins and cytokinins (Murthy *et al*., 1995). The redirection of development from shoot organogenesis to somatic embryogenesis at higher TDZ (2.5-15 μM) doses may have occurred due to an optimum phytohormone balance within the tissue or as a result of increased stress imposed by high concentrations of TDZ. TDZ induced somatic embryogenesis on seedlings of neem has been shown to be a stress related response (Gairi and Rashid, 2004). One week old seedlings of several species including legumes (Murthy *et al*., 1995) on TDZ medium exhibited stress symptoms like stunted growth, darkly coloured leaves and reduced root growth. In addition to the obvious physical manifestations of stress in TDZ treated tissues, there was a concomitant accumulation of several minerals ions, indoleamines, stress related metabolites including proline, abscisic acid (ABA) and γ-aminobutyrate and detoxification of free radicals generated on exposure to TDZ (Murch *et al*., 1999, Jones *et al*., 2007). Addition of high concentration of ABA (Nishiwaki *et al*., 2000), NaCl (Kiyosue *et al*., 1989) and heavy metal ions, i.e., Cd^{2+}, zinc and copper (Kiyosue *et al*., 1990a,b) to culture media induced somatic embryogenesis in the absence of external growth regulator stimuli in carrot tissue culture. Several stresses (osmotic, heavy metal ion, and dehydration stress) also induced somatic embryogenesis in *Arabidopsis thaliana* (Ikeda-Iwai *et al*., 2003). In case of stress-induced embryogenesis, embryos were found to differentiate only from the epidermis and without appreciable amount of calli (Kamada *et al*., 1993) as in the present study. Moreover, TDZ-induced somatic embryos revealed repression of well-developed root meristem, a sign of stress. In the lentil somatic embryogenesis, TDZ functions as a stress chemical. To overcome the stress, the plant tissues modified their metabolic processes, resulting in the accumulation of various metabolites and culminating in the formation of regenerants. However, characterization of biochemical and physiological responses of plant tissue to TDZ will clarify the mechanisms responsible for morphogenesis.

In conclusion, the cotyledonary nodes derived from the mature dry seeds of lentil can be induced to form direct shoots and somatic embryos in high frequency and density in almost a single step on a simple medium containing only TDZ. This system is rapid with initiation of tissue culture to transplanting of regenerants to soil completed in 8 weeks. Since the regenerant developed directly without an intervening callus phase, the somaclonal variation can be avoided. The regeneration system developed in this study can be employed in lentil genetic transformation via *Agrobacterium* where no success has yet been achieved and also in propagation of this species.

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