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Thidiazuron Induced High Frequency Adventitious Shoot Formation and Plant Regeneration in *Capsicum annuum* L.

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Abstract

An efficient procedure was developed for adventitious shoot bud induction and plantlet regeneration from various explants of the ten genotypes of Pepper (*Capsicum annuum* L.) using Thidiazuron (TDZ). Among various treatments at 1.0-3.0 mg/L TDZ induced maximum number of adventitious shoots depending upon the explant type and genotype compared to other treatments. Among the explants tested, leaf induced maximum number of adventitious shoots than the cotyledons. TDZ-mediated organogenesis was possible in 10 pepper cultivars, the extent of the response being genotype-dependent. Of the ten genotypes tested, *C. annuum* cvs CA960, G_4 and X-235 were produced maximum number of adventitious shoots and Sel_1 was the least, and all other genotypes gave moderate response. Elongation of multiple shoots was observed on medium supplemented with BA (0.05 mg/L) in combination of IAA (0.05 mg/L). Differences in ability for *in vitro* shoot regeneration and elongation depend upon the variety and explant type. The elongated shoots were successfully rooted on MS medium containing at 1.0 mg/L IAA. Plantlets regenerated from different explants of ten genotypes were found to be diploid (2n=24) and were devoid of any chromosomal aberrations. Regenerated plants were successfully established in soil where 85-90% of them developed into morphologically normal and fertile plants.

Key words: organogenesis, cotyledon, leaf, genotype

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Introduction

Pepper (*Capsicum annuum* L.) is an economically important crop plant and two main consumption types of pepper spice and vegetable, are prevalent throughout the world. Sixty percent of this crop is produced in Asia and India is the leading producer in area and production (Berke and Shieh 2000). In order to facilitate development of plant biotechnology based on variety improvement for this species, considerable effort has been devoted in developing and optimizing efficient *in vitro* regeneration protocols. Plant regeneration via organogenesis from diverse explants, using different concentrations and combinations of auxins and cytokinins has been described in pepper (Fari and Andrasfalvy 1994; Steinitz et al. 1999; Ochoa-Alejo and Ramirez-Malagon 2001). However, plant regeneration in pepper is severely limited due to the formation of ill-defined buds or shoot-like structures either resisting elongation or producing rosettes of distorted leaves which generally do not produce normal shoots (Franck-Duchenne et al. 1998; Steinitz et al. 1999; Ochoa-Alejo and Ramirez-Malagon 2001). Additionally, the intervarietal differences in regeneration from various explants are highly pronounced. Therefore, cultivar and tissue specific media have been devised to optimize regeneration from specific cultivars (Christopher and Rajam 1996; Ramirez-Malagon and Ochoa-Alejo 1996; Venkataiah and Subhash 2001). Thus, the strong influence of the pepper variety/genotype makes it necessary to optimize regeneration protocols for specific cultivars/genotypes. Thidiazuron (TDZ) has a high efficiency on stimulating cytokinin-dependant shoot regeneration from a wide variety of plants (Huetteman and Preece 1993; Murthy et al. 1998). TDZ was successfully applied to induce organogenesis and somatic embryogenesis from different explants, such as wounded seedlings, intact seedlings, immature embryos, embryonic hypocotyl, embryonic cotyle-

dons, cotyledons, leaf, cotyledonary nodes and shoot-tip of pepper (Szasz et al. 1995; Hyde and Phillips 1996; Binzel et al. 1996; Ramirez-Malagon and Ochoa-Alejo 1996; Manoharan et al. 1998; Dabauza and Pena 2001; Kaparikas and Alderson 2002).

This study has been undertaken to demonstrate the relative importance of genotype and explant, and their interactions, as well as plant growth regulators for in vitro plant regeneration from hypocotyl, cotyledon and leaf explants of ten commercially grown Indian cultivars of pepper using Thidiazuron (TDZ). Further more, attempts have been made to identify a particular genotype and explant which produces a large number of shoots and complete plantlets. Such a morphogenetic system would be an attractive starting material for our investigations on genetic transformation studies in pepper.

Materials and Methods

Plant materials

Seeds of *Capsicum annuum* L. cvs CA 960, G_4, NP46-A, PC1, Surya Mukhi Cluster (SMC), LCA-206, LCA-304, Selection 1(Sel_1), X-180, and X-235 were imbibed in sterile distilled water for 24 h, then surface sterilized with 0.1% $HgCl_2$ for 3 min., rinsed in several changes of sterile distilled water and germinated aseptically on MS basal medium (Murashige and Skoog 1962). The pH of the medium was adjusted to 5.8, solidified with 0.8% agar, dispensed into 250 mL Ehrelnmeyer flasks (Ca. 50 mL medium/flask) and autoclaved at 103.4 kPa for 15-20 min. Three-week-old axenic seedlings provided hypocotyl and cotyledon explants and 6-week old seedlings provided leaf explants.

Culture media and conditions

Shoot regeneration was tested on MS basal medium supplemented with various concentrations of Thidiazuron (TDZ) alone or in combination and Indole-3-acetic acid (IAA). The pH of the medium was adjusted to 5.8, solidified with 0.8% Difcobacto agar and autoclaved at 103.4 kPa for 15-20 min. A single explant was placed in each culture tube, and incubated at $25 \pm 1^{\circ}C$ with a 16 h photoperiod under fluorescent light (40-50 $\mu Mol\ m^2\ S^{-1}$).

Shoot bud induction

Effect of various concentrations of TDZ, alone or in combination IAA on shoot bud induction was evaluated. To investigate genotype and explant based adventitious shoot bud, the number of adventitious shoots per explant was also recorded after 6 weeks of culture. This experiment repeated thrice, using 30 explants in each treatment.

Elongation of adventitious shoots

Explants with adventitious multiple shoots and explants chopped into segments having shoot buds (5-10 buds/segment) were transferred to MS medium containing different concentration of BA, IAA, GA_3 and Kn., four different treatments viz., (i) BA (0.05 mg/L) + IAA (0.05 mg/L), (ii) BA (1.0 mg/L) + GA_3 (1.0 mg/L), (iii) Kn (1.0 mg/L) + GA_3 (1.0 mg/L), and (iv) GA_3 (1.0 mg/L) for elongation. Twenty replicates were employed for each treatment, and the entire experiment was repeated thrice. Shoots (>2 cm long) were counted and harvested after 2-4 weeks.

Rooting and acclimatization

Elongated shoots (>2 cm long) were excised from cotyledon and leaf explants of all the ten cultivars rooted and detailed below. Shoots collected in vitro were transferred to rooting medium containing 1.0 mg/L IAA (Christopher and Rajam 1996). The all experiments were repeated thrice. After 3 weeks of culture, plantlets recovered were washed with running tap water and agar sticking to the roots removed. Plantlets with fully expanded leaves and well-developed roots were first transferred to soil and vermiculite (50:50 v/v) mixture, covered with plastic bags. Pots were kept in the culture room for one week before being transferred to soil.

Chromosome number determination

Chromosome counts were carried out for regenerated plants. Root tips of about 100 plants randomly collected from ten cultivars were pretreated with 0.0002 M 8-hydroxyquinoline at 18-20°C for 3 h and then fixed in ethanol and glacial acetic acid (3:1). They were dipped in 1N HCl and 2% aceto-orcein (1:9) for about 2 h and then squashed with a drop of 45% acetic acid and chromosomes were counted in 2 to 5 well-spread cells of each regenerated plant.

Data analysis

All the experiments were repeated three times and the standard deviation and standard error were calculated. Data on adventitious bud regeneration and rooting were statistically analyzed using a completely randomized block design and means were evaluated at the $p<0.05$ level of significance using Duncan's new multiple range test.

Results and Discussion

Adventitious shoot bud induction

The regeneration regimes elucidated so far red pepper (*Capsicum* spp.) has largely been aided by species-specific determination of critical parameters such as explant source, genotypic response and effect of media constituents. The dose of cytokinin or cytokinin-auxin ratio is known to be critical in shoot organogenesis (Fari and Andrasflavy 1994; Steinitz, et al. 1999; Ochoa-Alejo and Ramirez-Malagon 2001; Venkataiah and Subhash 2001). Therefore, we compared the response of three (hypocotyl, cotyledon and leaf) explants of ten cultivars to various concentrations TDZ alone or in combination with IAA. In the presence of TDZ during the first week of the culture period, cotyledon, hypocotyl and leaf explants became swollen twice their original size. After an additional 2 to 3 weeks, multiple shoot primordia emerged from the explant cut surfaces. Adventitious shoot buds were emerged from both ends of hypocotyl (Figure 1a) and cut ends cotyledon (Figure 1b). Leaf explants produced shoot buds all over the surface (Figure 1c). The type of explant, genotype and the concentration of TDZ influenced the frequency of shoot bud formation and the number of shoot buds. Taking into account the mean number of adventitious shoots produced by explants from 10 chilli pepper genotypes grown on 8 different concentrations of TDZ, it is possible to separate the cultivars as highly responsive (X-235, CA960 and G_4), intermediate (NP46-A, PC1 and X-180) and low responsive (LCA-206, LCA-304, SMC, and Sel_1). The number of adventitious shoot buds per explant in red pepper cultivars ranged from 20 to 60 (as observed under stereomicroscope) depending upon the explant, genotype and medium, but no significant differences were found for genotypes medium interactions (Table 1, 2).

The shoot bud regeneration frequency decreased with increase in the concentration of TDZ, the optimal level for maximum frequency of shoot buds formation was not occurred on medium containing TDZ. On the medium the frequency of shoot bud formation decreased in high levels of TDZ concentration due to callus growth. Among the different explants used, leaf explants showed highest frequency and number of shoot bud formation (2-25 shoots /explant) followed by cotyledon. In the case of leaf explants, cytokinin TDZ exerted a profound influence on their proliferation capacity. The number of shoots per leaf explants ranged between 2 to 25 and was maximal on TDZ-supplemented media (Table 1, 2). The differential effect of various concentrations of TDZ on the stimulation adventitious shoot buds and plant regeneration in crop plants

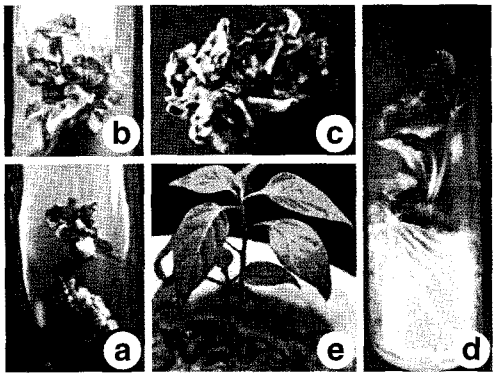

Figure 1. Plant regeneration from different explants of *Capsicum annuum* L. cv X-235 (a) Adventitious shoot buds from cut ends of hypocotyls explants after two weeks of incubation in regeneration (2.0 mg/L TDZ). (b) Adventitious shoot buds from cut surface of cotyledon explants after two weeks of incubation in regeneration (2.0 mg/L TDZ). (c) Adventitious shoot bud proliferation all over cut surface of leaf explants after two weeks of incubation in regeneration (2.0 mg/L TDZ). (d) Complete regenerated plantlet on rooting medium (1.0 mg/L IAA) after three weeks. (e) Potted regenerated plant of C. annuum L. cv X-235 after four weeks of transplantation.

Table 1. Effect of TDZ concentration of adventitious shoot bud formation from cotyledon explants of different genotypes of *Capsicum annuum* L.

Growth regulator	Mean number of shoots per explant (Cotyledon)									
TDZ (in mg/L)	CA960	G4	NP46-A	LCA-206	LCA-304	PC1	SMC	S1	X-180	X-235
0.05	2.4 ± 0.56^{h}	2.8 ± 0.64^{gh}	2.1 ± 0.38^{fg}	-	-	-	-	2.1 ± 0.28^{cd}	2.2 ± 0.32^{de}	4.6 ± 0.62^{ef}
0.50	3.8 ± 0.48^{g}	3.2 ± 0.47^{fg}	2.6 ± 0.43^{ef}	2.1 ± 0.28^{cd}	2.3 ± 0.32^{cd}	2.2 ± 0.28^{de}	2.3 ± 0.38^{cd}	1.4 ± 0.52^{cd}	4.3 ± 0.48^{bcd}	6.4 ± 0.68^{de}
1.00	8.3 ± 0.86^{cd}	6.6 ± 0.48^{cd}	3.2 ± 0.48^{de}	2.2 ± 0.32^{cd}	2.6 ± 0.48^{cd}	2.3 ± 0.26^{cd}	2.9 ± 0.42^{abc}	1.9 ± 0.22^{cd}	4.6 ± 0.52^{bc}	8.8 ± 0.54^{c}
1.50	12.8 ± 1.34^{b}	8.4 ± 0.68^{b}	4.8 ± 0.56^{cd}	2.6 ± 0.42^{cd}	2.8 ± 0.38^{cd}	2.8 ± 0.28^{bcd}	3.2 ± 0.36^{abc}	2.5 ± 0.26^{bc}	6.8 ± 0.82^{a}	11.3 ± 1.26^{b}
2.00	16.4 ± 1.48^{a}	12.4 ± 1.16^{a}	5.2 ± 0.68^{b}	4.6 ± 0.57^{ab}	3.8 ± 0.52^{bc}	4.4 ± 0.62^{ab}	4.2 ± 0.49^{ab}	2.8 ± 0.59^{bc}	5.4 ± 0.66^{a}	18.6 ± 1.48^{a}
3.00	8.2 ± 0.78^{cd}	6.4 ± 0.73^{cd}	8.6 ± 0.78^{a}	5.4 ± 0.63^{ab}	4.8 ± 0.64^{ab}	3.2 ± 0.48^{bc}	3.8 ± 0.57^{ab}	3.6 ± 0.43^{ab}	4.8 ± 0.58^{bc}	6.8 ± 0.86^{de}
4.00	6.8 ± 0.54^{de}	5.6 ± 0.44^{de}	4.6 ± 0.54^{cd}	4.8 ± 0.38^{ab}	4.2 ± 0.58^{ab}	3.8 ± 0.42^{ab}	3.6 ± 0.48^{ab}	3.2 ± 0.48^{ab}	3.6 ± 0.52^{cd}	5.6 ± 0.68^{ef}
5.00	4.6 ± 0.38^{fg}	3.8 ± 0.46^{fg}	3.4 ± 0.43^{de}	2.6 ± 0.42^{cd}	3.8 ± 0.34^{bc}	3.2 ± 0.38^{bc}	3.4 ± 0.28^{abc}	2.8 ± 0.32^{bc}	2.8 ± 0.46^{de}	4.8 ± 0.76^{ef}

Each experiment had 20 replications and was repeated thrice. Mean within a column followed by the same letters are not significantly different at $P<0.05$ according to DNMRT. All the data were scored other 6 weeks (after elongated treatment).

including pepper has been reported (Szasz et al. 1995; Ramirez-Malagon and Ochoa-Alejo 1996; Hyde and Phillips 1996; Manoharan et al. 1998). Since the level of TDZ in medium was critical for shoot organogenesis efficiency, the response of pepper explants on various TDZ concentrations was compared (Table 1, 2). Szasz et al (1995) obtained shoot regeneration from cotyledon explants of two Italian and two Hungerian genotypes using TDZ, which were considered to be non-responsive to the usual methods. Ramirez-Malagon and Ochoa-Alejo (1996) evaluated 21 genotypes and the percentage of shoot bud regeneration was 27.2 on MS medium supplemented with 1.0 mg/L TDZ in wounded hypocotyls and decapitated seedling explants of pepper. Hyde & Phillips (1996) studied the effect of different cytokinins (TDZ, BA or Zeatin) and silver nitrate ($AgNO_3$) on bud induction and plant regeneration from two chili pepper cultivars, using cotyledon explants from 14 to 16 d old seedlings. The only treatment that consistently failed to produced plantlets in the both cultivars was that in which $AgNO_3$ was absent in both primary (bud induction medium) and second-stage medium (shoot elongation medium), regardless of the cytokinins. They reported that shoots induced in medium TDZ and $AgNO_3$ were usually numerous but very small, with a thin stem which did not elongate further or did not develop roots, though those that did not root survived transplanting better. However, they preferred BA to TDZ because of its earlier stimulation of shoot development. Manoharan et al (1998) obtained a high percentage of shoot regeneration and the frequency from cotyledon explants of pepper on MS medium augmented with 0.5 mg/L TDZ. This discrepancy can be ascribed to the different explants and genotypes used in experiments. Our investigations have also revealed the determining role of genotype and explant in the efficiency of adventitious shoot bud formation (Table 1, 2). Shoot bud formation depends primarily on cultivar used as the source of explants, and the culture medium is important for the expression of this capacity; Cultivars X-235, CA 960 and G_4 have consistently shown a high shoot forming capacity independent of the type of explant and culture medium employed. While, remaining genotypes gave intermediate response for adventitious shoot bud formation, some of these genotypes shows green nodular callus at cut ends of the explants, which does not show either proliferation or shoot bud formation on low concentrations of TDZ. Where as Sel1 cultivar has been found to be deficient in shoot formation in all tested media (Table 1, 2). Such genotypic differences for shoot regeneration have been reported in pepper (Szasz et al. 1995; Christopher and Rajam 1996; Ramirez-Malagon and Ochoa-Alejo 1996; Hyde and Phillips 1996; Dabuaza and Pena 2001; Venkataiah and Subhash 2001). The cut surface of the hypocotyls was swollen greatly and adventitious shoot buds were rarely found in all other cultivars. The hypocotyl explants of cultivar X-235 produced maximum number of shoot buds on medium containing TDZ at 2.0 mg/L (data not shown). Among the ten genotypes tested; Leaf explants consistently showed maximum number of regenerated adventitious shoots than cotyledon explants (Table 1, 2). Similar observations were also made in *Capsicum* is that leaf explants are more amenable to adventitious shoot formation than other explants (Agrawal et al. 1989; Christopher and Rajam 1996; Zhou et al. 1996; Dubauzia and Pena 2001; Venkataiah and Subhash 2001). The responsiveness of the ten pepper varieties investigated turned out to be very different. This was not surprising because the strong dependency on the regeneration ability in excised seedling parts had been known earlier in pepper (Fari and Andrasfalvy 1994; Szasz et al. 1995; Christopher and Rajam 1996; Ramirez-Malagon and Ochoa-Alejo 1996; Dabauza and Pena 2001; Venkataiah and Subhash 2001). It has been shown in our results that a fraction of the pepper genotypes not inducible adventitious shoot buds by conventional methods employing BA and IAA (Szasz et al. 1995; Venkataiah and Subhash 2001) as plant growth regulators could regenerate shoots in cotyledon and leaf explants after TDZ treatments.

Shoot elongation

Shoot buds obtained from different explants on TDZ containing media did not elongate and resulted in a rosette of shoots when continued to be cultured on same medium. In most instances shoot or shoot bud cluster were transferred to a shoot/stem elongation medium *in vitro* because shoot elongation has repeatedly been found as major obstacle in obtaining normal pepper plants (Steinitz et al. 1999). Cytokinins commonly stimulate shoot proliferation and inhibit their elongation. Therefore, inhibition of shoot elongation by TDZ may be consistent with its high cytokinin activity. The problem of shoot elongation can be overcome by transfer of shoot clusters to a secondary medium often lacking TDZ or with a different balance of plant growth regulators (Huetteman and Preece 1993). Normally one, occasionally two shoots were elongated in explants with adventitious shoot buds from the total of 10 to 60 shoot buds per explant (counted under stereo microscope). For the purpose of obtaining shoot elongation the explants were chopped into segments carrying small number of buds and short shoots and then transferred to medium containing different combinations of growth regulators. Segmentation prevented crowding of developing shoots and facilitated further bud elongation. After elongation treatment each segment produced one to four well-developed shoots from segment, depending upon explant and genotype and concentration of TDZ in adventitious shoot bud induction medium. A total of 2 to 20

complete plantlets per explant were recovered from cotyledon and leaf explants depending upon the genotype of pepper (Table 1, 2). Shoot elongation occurred only when the shoot buds were transferred to medium containing low levels of BA and IAA. However, shoot elongation may, in some cases took place after transplantation of small shoots *ex vitro* (Arroyo and Revilla 1991; Ebida and Hu 1993; Hyde and Phillips 1996).

Transfer of shoot buds to media containing GA_3 (1.0 mg/L) and Kn (1.0 mg/L) or BA (1.0 mg/L) leads to excessive callus growth and did not support shoot bud elongation (data not shown). Elongation of shoot buds was observed on medium containing GA_3 in pepper (Szasz et al. 1995). Silver nitrate ($AgNO_3$) an ethylene inhibitor in plant tissue culture systems, found to be an essential compound in the induction and elongation of shoots in pepper (Hyde and Phillips 1996). Manoharan et al. (1998) reported that GA_3 in combination with Kn was not favourable for shoot elongation. Hussain et al. (1999) reported the successful shoot elongation when the shoot buds were subcultured on elongation medium supplemented with either IAA or PAA in combination with lower concentrations of BA. The best elongation medium was the medium supplemented with BA at 2.0 mg/L + PAA at 1.0 mg/L. Franck-Duchenne et al. (1998) have observed that 24-eip-brassinolide (0.1 μM) at lower concentration would stimulate the shoot bud elongation in sweet pepper. Dubauza and Pena (2001) reported adventitious bud shoot formation and shoot elongation was achieved by culturing cotyledons and leaves on medium supplemented with TDZ alone or in combination with GA_3. In over case, adventitious shoot buds were induced from cotyledon, hypocotyl and leaf explants on medium containing TDZ, and subsequent shoot elongation was occurred on medium containing low levels of BA and IAA. IAA in combination with BA (0.05 mg/L each) treatment elicited high frequency of shoot elongation. Our results are consistent with those in previous reports in which low levels of BA and IAA were used successfully to elongate the induced adventitious shoot buds (Phillips and Hubstenberger 1985; Agrawal et al. 1989; Christopher and Rajam 1996; Manoharan et al. 1998; Venkataiah and Subhash 2001; Venkataiah et al. 2001).

Rooting and acclimatization

Well developed shoots (>2 cm) from cotyledon and leaf explants of all cultivars were excised and transferred to MS medium augmented with IAA at 1.0 mg/L for root initiation. Hypocotyl explants of all genotypes, regenerated fewer shoots failed to elongate and produced rosettes. Therefore, shoots from hypocotyl explants were not used for the rooting experiments. Identical observations were also made in hypocotyl explants of several pepper cultivars (Szasz et al. 1995; Christopher and Rajam 1996; Ramirez-Malagon and Ochoa-Alejo 1996; Venkataiah and Subhash 2001). Rooting occurred within two weeks of culture on MS medium supplemented with 1.0 mg/L IAA (Figure 1d). The initiation of rooting depends upon the genotype. IAA was found to be most suitable auxin for root formation as well as shoot elongation. Our observations also support the earlier findings, in which IAA was successfully employed for shoot elongation and rooting in *Capsicum* spp. (Gunay and Rao 1978; Phillips and Hubstenberger 1985; Szasz et al. 1995; Christopher and Rajam 1996; Manoharan et al. 1998; Venkataiah and Subhash 2001; Venkataiah et al. 2001).

Plantlets regenerated from various explants (cotyledon or leaf) were found to be normal diploid (2n=24) and devoid of any chromosomal aberrations. The establishment of *in vitro* grown plants in the soil (soil: vermiculite 50:50 v/v) was easily achieved (Figure 1e). Percentage of the plantlets surviving after transfer to soil in the field was 85-90%. The regenerated plants did not show any detectable variation in morphology or growth characteristics as compared to the respective donor plants and they flowered normally and able to set viable seeds.

In conclusion, we have established a promising system for direct regeneration in cotyledon and leaf explants of recalcitrant hot pepper genotypes, that efficient shoot organogenesis of red pepper (*C. annuum* L.) induced by TDZ over the traditional use of BA in combination with IAA (Szasz et al. 1995; Venkataiah and Subhash 2001). Genetic improvement of pepper cultivars for disease and insect pest resistance, among other characters, can be efficiently achieved through genetic engineering. To date, difficulties in regeneration (shoot elongation from induced adventitious shoot buds) and non-reproducibility of results in tissue cultures of pepper have restricted work on genetic transformation. Cotyledon and leaf explants are found to be most compatible tissue for *Agrobacterium*-mediated genetic transformation in *Capsicum* spp. (Steinitz et al. 1999; Ochoa-Alejo and Ramirez-Malagon 2001; Venkataiah et al. 2001).

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