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BRIEF COMMUNICATION

Improved plant regeneration in *Capsicum annuum* L. from nodal segments

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Abstract

Multiple shoots were induced by culturing nodal explants excised from 1-month-old aseptic seedlings of red pepper (*Capsicum annuum* L. cv. Pusa Jwala) on Murashige and Skoog (MS) medium supplemented with (0.1 - 10 μM) thidiazuron (TDZ). The rate of multiple shoot induction per explant was maximum (14.4 ± 0.06) on MS medium supplemented with 1.0 μM TDZ. Regenerated shoots were elongated well on growth regulator free MS medium. Adventitious roots were induced two weeks after transfer of elongated shoots to MS medium supplemented with auxins (IAA, IBA or NAA) in different concentrations. Optimum root formation frequency was obtained in medium containing 1.0 μM IBA. *Ex-vitro* rooting was also achieved by pulse treatment with 300 μM IBA for 10 min. Rooted shoots were transplanted in plastic pots containing garden soil (with 90 % survival rate), where they grew well and attained maturity. Regenerated plants were phenotypically and cytologically normal.

Additional key words: axillary shoot, nodal segment, red pepper, thidiazuron.

The genus *Capsicum*, a member of family *Solanaceae* is an important vegetable and spice crop of India and many other regions around the world. Several attempts have been made on plant regeneration from different explants, shoot tip (Christopher and Rajam 1994), rooted hypocotyls (Valera-Montero and Alejo 1992), leaf, stem, hypocotyls, cotyledon, root, shoot tip and embryo (Agrawal *et al.* 1989) and induced somatic embryogenesis (Kintzios *et al.* 2000) but many of these investigations did not report satisfactory result in terms of enhanced number of shoots because plant regeneration in chilli 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).

Thidiazuron a substituted phenyl urea (N-phenyl-1,2,3-thidiazol-5-ylurea, TDZ) is a potent plant growth regulator, which exhibit cytokinin like activity in various culture systems including both organogenesis and somatic embryogenesis from different explants in several plant species such as in pigeon pea (Dolendro *et al.* 2003), *Sesbania drummondii* (Cheepala *et al.* 2004), *Calendula officinalis* (Çöçü *et al.* 2004) and *Vanda coerulea* (Malabadi *et al.* 2004). As far as we know, very few reports exist on successful application of TDZ for rapid and efficient propagation of pepper (Manoharan *et al.* 1998, Venkataiah *et al.* 2003) but the results did not include satisfactory figure of shoot production making it difficult to estimate the efficiency of the method used. Therefore, present study has been undertaken to demonstrate the relative efficiency of TDZ with those of earlier reports for *in vitro* plant regeneration from nodal explants and to find the optimal concentration of TDZ exposure for maximum shoot production and establishment of complete plantlets.

Cultures were started from aseptic germination of red pepper (*Capsicum annuum* L. cv. Pusa Jwala) seeds, which were initially soaked over night and then washed with running tap water for 30 min to remove adherent particles. Thoroughly washed seeds were then immersed in 5 % (v/v) *Teepol* for 10 min and then rinsed 3 times with sterile double distilled water. This was followed by the surface sterilization with 0.1 % (m/v) $HgCl_2$ under the sterile conditions for 5 min. These were then rinsed 5 times in sterile double distilled water to remove all traces of $HgCl_2$. The sterilized seeds were then placed on to the basal Murashige and Skoog (1962; MS) medium for germination. Nodal segments derived from one month old aseptic seedlings were used as explant.

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Abbreviations: IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; BM - basal medium; NAA - α-naphthalene acetic acid; MS - Murashige and Skoog; PGR - plant growth regulator; TDZ - thidiazuron.

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Jam bottles with the basal MS medium (BM) containing 3 % (m/v) sucrose and 0.8 % (m/v) agar or 0.25 % (m/v) *Gelrite* was adjusted to pH 5.8, were used as germination vessels. All media were autoclaved at 121 °C (1.4 kg cm^{-2}) for 15 min. BM was supplemented with different concentrations of TDZ for shoot bud induction. All cultures were incubated in growth room at temperature of 25 ± 2 °C, 16-h photoperiod with an irradiance of 50 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps.

Nodal segments (1.0 - 1.5 cm long) were excised and inoculated on MS medium supplemented with various concentrations of TDZ (0.1, 0.3, 0.5, 0.8, 1.0, 2.5, 5.0, 7.5, 10.0 µM) to evaluate its effect on shoot bud induction. Multiple shoot buds from nodal segments were induced in clumps after two weeks of culture which on subsequent subculturing differentiated into multiple shoots. These were then transferred to MS medium devoid of TDZ for elongation. The induced shoots were subcultured every 2 - 3 weeks onto the fresh medium. The percentage of explants on which multiple shoot developed, the number of shoots per explant and the shoot height were recorded after 4 and 8 weeks of culture.

For rooting, the effect of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA) at three concentrations (0.5, 1.0, 1.5 µM) were compared. The well rooted shoots were washed thoroughly under running tap water and transplanted in 10 cm diameter polycups filled with unautoclaved garden soil and irrigated with tap water regularly. Each plantlet was kept in growth room covered with transparent polythene bags to maintain high humidity and also to prevent desiccation with the same temperature, photoperiod and irradiance as stated above. For the reduction of humidity, holes were made in polythene bags after few days of transplantation to harden the plants. The hardened plants after 2 weeks were transferred to greenhouse where they attained maturity.

For cytological analysis, flower buds of appropriate size were collected randomly from 5 - 10 micropropagated plants and fixed in Carnoy's fluid (3 part ethyl alcohol + 1 part glacial acetic acid) for 24 h. The buds were finally stored in 70 % alcohol for analysis. Meiosis was studied from acetocarmine squashes of pollen mother

Fig. 1. *In vitro* plant regeneration of *Capsicum annuum* L. cv. Pusa Jwala. *A* - Multiple shoots from nodal explant on MS + TDZ (1.0 µM). *B* - Elongation of shoots on growth regulator free MS medium. *C* - Rooting of *in vitro* regenerated shoot on MS + IBA (1.0 µM). *D* - Regenerated plant in the soil.

cells and number of chromosomes were determined (Anis 1998).

Each treatment consisted of 20 replicates in three repeated experiments. Data were analyzed statistically using *SPSS Ver. 10* (*SPSS Inc.*, Chicago, USA) and significant differences among the means were assessed by Tukey's test at 5 % probability level.

Morphogenesis occurs in all the treatments except growth regulator free MS medium. Multiple shoots appeared from the nodal segment explants after 4 weeks (Fig. 1*A*), and the proliferated axillary shoots elongated by 2 - 3 cm within two weeks of subculture (Fig. 1*B*). Different concentrations of TDZ influenced the frequency of shoot bud formation and the number of shoot buds. The highest number of shoots (14.40 ± 0.06), longest shoot (6.02 ± 0.14 cm) and the greatest percentage (93.33 %) of shoot formation was observed in a treatment with 1.0 µM (TDZ) after eight weeks of culture (Table 1). The differential effect of various concentrations of TDZ on the stimulation of multiple shoot buds and plant regeneration in crop plants including pepper has been reported (Hyde and Phillips 1996, Manoharan *et al*. 1998). Hyde and Phillips (1996) studied the effect of different cytokinins (TDZ, BA or zeatin) and silver nitrate ($AgNO_3$) on shoot bud induction and plant regeneration from two chilly pepper cultivars, using cotyledon explants from 14 to 16 d old seedlings. They reported that shoots induced in a medium containing TDZ and $AgNO_3$ were usually numerous but with thin stem which did not elongate further. Manoharan *et al*. (1998) obtained a high percentage of shoot regeneration frequency from cotyledon explants of pepper on MS medium augmented with 0.5 mg dm^{-3} (TDZ). This differential response can be ascribed to the different explant and genotypes used in experiment. It appears that 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.

Table 1. Effect of thidiazuron on multiple shoot induction from nodal explants of *Capsicum annuum* L. in MS medium, eight weeks after culture. Means ± SE of 20 replicates per treatment in three repeated experiments. Means followed by the same letters are not significantly different by the Tukey's test at 5 % probability level.

TDZ [µM]	Response [%]	Number of shoots [expl.$^{-1}$]	Shoot length [cm] after 4 weeks	Shoot length [cm] after 8 weeks
0.10	70.0	2.80 ± 0.66^{cd}	2.62 ± 0.17^{de}	3.06 ± 0.11^{d}
0.30	76.6	2.60 ± 0.81^{cd}	2.96 ± 0.11^{de}	3.40 ± 0.19^{cd}
0.50	76.6	4.00 ± 0.54^{cd}	3.12 ± 0.19^{de}	3.38 ± 0.26^{cd}
0.80	80.0	8.86 ± 0.50^{b}	2.52 ± 0.17^{e}	3.20 ± 0.22^{d}
1.00	93.3	14.40 ± 0.06^{a}	4.78 ± 0.13^{a}	6.02 ± 0.14^{a}
2.50	70.0	5.40 ± 0.67^{c}	3.58 ± 0.16^{bc}	4.28 ± 0.23^{bc}
5.00	66.6	4.60 ± 0.50^{c}	4.22 ± 0.21^{b}	4.54 ± 0.14^{b}
7.50	66.6	4.00 ± 0.89^{cd}	3.26 ± 0.20^{cde}	3.46 ± 0.21^{cd}
10.00	56.6	1.20 ± 0.37^{d}	3.46 ± 0.29^{bcd}	3.80 ± 0.32^{cd}

Table 2. Effect of auxins on root induction from *in vitro* raised microshoots of *Capsicum annuum* L. in MS medium, after 2 weeks of culture. Means ± SE of 20 replicates per treatment in three repeated experiments. Means followed by the same letters are not significantly different by the Tukey's test at 5 % probability level.

IAA [µM]	IBA [µM]	NAA [µM]	Response [%]	Number of roots [shoot^{-1}]	Root length [cm]
0.5			60.0	6.20 ± 0.86^{c}	2.46 ± 0.24^{abc}
1.0			80.0	6.60 ± 0.81^{c}	2.38 ± 0.31^{bc}
1.5			66.6	6.60 ± 0.67^{c}	2.24 ± 0.33^{c}
	0.5		70.0	11.20 ± 0.58^{b}	3.40 ± 0.44^{abc}
	1.0		90.0	12.20 ± 0.86^{a}	3.74 ± 0.20^{a}
	1.5		60.0	11.00 ± 0.70^{ab}	3.64 ± 0.30^{ab}
		0.5	56.6	8.20 ± 0.37^{bc}	2.50 ± 0.17^{abc}
		1.0	70.3	8.40 ± 0.50^{bc}	2.90 ± 0.27^{abc}
		1.5	60.3	8.60 ± 0.50^{bc}	2.34 ± 0.20^{abc}

Shoot buds induced from nodal segment on TDZ containing media did not elongate and resulted in a rosette of shoots when continued to be cultured on the same medium. Cytokinins commonly stimulate shoot proliferation and inhibit their elongation, therefore; inhibition of shoot bud elongation by TDZ may be consistent with its high cytokinin activity. The problem of shoot elongation was overcome by transfer of shoot clusters to secondary medium lacking TDZ where the multiple shoots elongated further within four weeks of subculture.

Well developed shoots (> 6 cm) were excised and transferred to MS medium augmented with different auxins (IAA, IBA, NAA) in various concentrations of 0.5, 1.0, 1.5 µM each, for root initiation (Table 2). Regenerated shoots rooted within 2 weeks of culture but with different rooting frequency in each treatment. The maximum rooting (90 %) and vigorous growth of microshoots was observed at 1.0 µM IBA (Fig. 1*C*). Our observations are consistent with the earlier finding in which IBA was successfully employed for rooting in *Capsicum* (Agrawal *et al*. 1988). For *ex vitro* root induction, individual microshoots were excised and the basal portion of shoots were dipped in IBA (300 µM) for 10 min, washed with distilled water and transferred to pots containing garden soil (Fig. 1*D*). Approximately 90 % of the treated shoots formed roots after 2 weeks. Given the reduced time for establishment of micropropagated plants, IBA dipped *ex vitro* rooting is more favourable method than *in vitro* rooting. From rooting of the isolated shoots to obtaining healthy plants (15 cm high) in potted soil, it took about two and a half months. 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 were able to set fruits.

Cytological studies revealed that *in vitro* regenerated

plantlets were found to be normal diploid (2n=24) having 12 bivalents in pollen mother cells at diakinesis, and metaphase-I (Fig. 2) devoid of any chromosomal aberrations. Different concentrations of TDZ used in the experiment had no effect on chromosomal behavior of *in vitro* regenerated plantlets.

Our findings are significant in obtaining the maximum regeneration with minimum concentrations of growth hormone, a primary objective in any morphogenetic study, since the excess concentrations of growth hormone not only increase the cost of production, but also the chances of genetic variation.

In conclusion, we have established a promising protocol for an efficient regeneration from nodal explant of *Capsicum annuum* using TDZ. It employs uniform nodal segments, a large number of shoot meristems differentiated simultaneously, all the differentiated shoot meristems developed into shoots and many healthy plants can be grown *ex vitro* under field condition. The protocol could be useful for large scale production of individual genotypes and provides a practicable system towards genetic improvement of the crop.

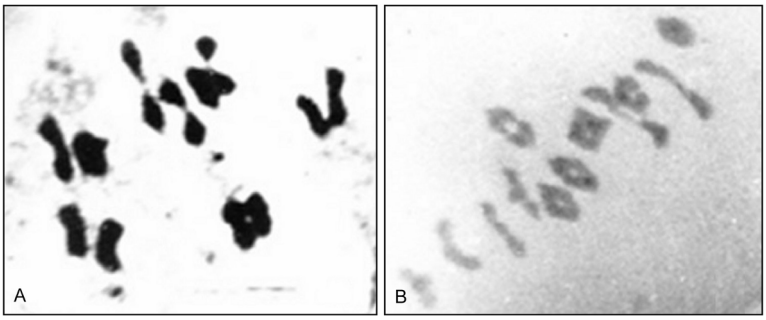

Fig. 2. Pollen mother cells of regenerated plant showing normal chromosome number (2n = 24): *A* - diakinesis;. *B* - metaphase I.

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