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Thidiazuron induced high frequency shoot bud formation and plant regeneration from cotyledonary node explants of *Capsicum annuum* L.

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An efficient protocol for rapid *in vitro* propagation of *Capsicum annuum* L. cv. Pusa jwala through multiple shoot bud formation from cotyledonary node explants of 15-day-old aseptic seedlings has been developed. Multiple shoot bud formation and highest rate of multiplication was standardized on Murashige and Skoog (MS) medium supplemented with 1.5 μ*M* thidiazuron (TDZ) and 0.5 μ*M* indole 3-acetic acid (IAA). The regenerated shoot buds when subcultured on hormone free MS medium gave the highest rate of shoot proliferation and shoot length by the end of third subculture. Individual elongated shoots were rooted best on MS media containing 1.0 μ*M* α-naphthalene acetic acid (NAA). *Ex vitro* rooting was also achieved when the basal cut ends of the *in vitro* regenerated shoots were dipped in 200 μ*M* indole-3-butyric acid (IBA) for half an hour followed by transplantation in plastic pots containing sterile garden soil. Plantlets went through a hardening phase in a controlled plant growth chamber, prior to field transfer. Of all the micropropagated plants, about 95% survived following transfer to soil. Growth performance of 2-month-old *in vitro* raised plants was compared with *in vivo* seedlings of the same age on the basis of selected morphological and physiological parameters. Carotenoids and relative water content were significantly higher in *in vitro* regenerated plants as compared to *in vivo* control plants of same age.

Keywords: *Capsicum annuum*, cotyledonary node explants, plant regeneration, *ex vitro* rooting, relative water content
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Introduction

Capsicum annuum L. commonly termed as "Hot pepper" forms an important economical crop of the family Solanaceae. It is utilized both in green and dried form in food. 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 diarrhoea. Chilli does not have natural ability for vegetative propagation. Therefore, *in vitro* propagation method may be used for its clonal multiplication. In order to facilitate development of plant biotechnology based varietal improvement for this species, considerable efforts have been devoted in developing and optimizing efficient *in vitro* regenerated protocols. The dose of cytokinin or cytokinin-auxin ratio is known to be critical in shoot organogenesis in pepper[1-3]. Therefore, the response of cotyledonary node explants of pepper using various concentrations of TDZ either alone or in combination with IAA was compared in the present experiment.

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In *C. annuum* cultivars, attaining elongation of *in vitro* formed shoot buds in itself poses a considerable challenge because the indistinct buds or shoot-like structures either resist elongation or produce rosettes of distorted leaves which generally do not produce normal shoots. This was considered a major obstacle for *Capsicum* plant transformation and regeneration[4,5].

TDZ (N phenyl 1,2,3 thidiazol 5-ylurea) has been used extensively in tissue culture studies. It exhibits strong cytokinin like activity and promotes the proliferation of axillary shoots[6] as well as stimulates adventitious organ regeneration[7,8] and induces somatic embryogenesis[9]. Although TDZ was successfully applied to induce organogenesis and genetic transformation in chilli using different explants[10], micropropagation, using TDZ from cotyledonary nodes of aseptic seedlings, has not yet been reported for any cultivar of *Capsicum*.

The most important and critical steps in field transfer of *in vitro* propagated plants is their transition during hardening from *in vitro* to *ex vitro* environment and subsequent field performance. The conditions, as low levels of light, aseptic conditions on a medium containing ample sugar, heterotrophic

mode of nutrition and high relative humidity result in the formation of plantlets of abnormal morphology, anatomy and physiology[11-15]. In the course of biological hardening, the tissue culture raised plants gradually overcome these inadequacies and adapt to *ex vitro* conditions.

Ex vitro rooting was attempted as means to decrease the micropropagation cost and also the time from laboratory to field. Data on growth and physiological parameters are likely to provide valuable information regarding the suitability of tissue culture raised plants for field plantations.

In this communication, the authors report a high frequency regeneration system for the first time from cotyledonary node explants of *C. annuum* cv. Pusa Jwala using TDZ alone or in combination with IAA and successful establishment of micropropagated plants to field conditions and evaluation of their performance on the basis of some morphological and physiological parameters in comparison to those of *in vivo* plants of the same age.

Materials and Methods

Establishment of Aseptic Seedlings

Seeds of *C. annuum* cv. Pusa jwala were presoaked in water overnight and washed in running tap water for 30 min to remove any adherent particles. Thoroughly washed seeds were then immersed in 5% (v/v) Teepol for 20 min and rinsed 3 times with sterile distilled water. This was followed by the surface sterilization with 0.1% (w/v) $HgCl_2$ under the sterile conditions for 5 min and rinsed 4 times with sterile distilled water to remove all traces of sterilant. The sterilized seeds were then inoculated onto Murashige and Skoog basal medium[16] for germination. Cotyledonary nodes excised from 15-day-old aseptic seedlings were used as explants.

Culture Media and Conditions

MS medium containing 3% (w/v) sucrose and 0.8% (w/v) agar or 0.25% (w/v) gelrite (Sigma Aldrich Ltd.) were used in all the experiments. Plant growth regulators and their combinations were added to the medium as specified below. The *p*H of the medium was adjusted to 5.8 by 1N NaOH or 1N HCl prior to autoclaving at 121ºC at 1.06 kg cm^{-2} for 20 min. All the cultures were maintained in the culture room at 24 ± 2ºC under 16 h photoperiod with 50 µmol m^{-2} s^{-1} illumination from cool white fluorescent tubes with 60 -65% relative humidity.

Shoot Bud Induction

For shoot bud induction, explants were placed on MS medium supplemented with different concentrations of TDZ (0.1, 0.5, 0.8, 1.0, 1.5, 2.0, 3.0, 5.0, 8.0 and 10.0 µ*M*) either singly or in combination with IAA (0.5 µ*M*). The induced shoot buds were differentiated into multiple shoots in the same medium. The frequency of explant producing shoot buds and number of shoot buds per explant were scored after 4 weeks of culture.

Elongation of Multiple Shoots

Explants with multiple shoots were transferred to MS medium without plant growth regulator for elongation. These shoots were repeatedly subcultured in the same medium after every 2 weeks. Twenty replicates were employed for each treatment and the entire treatment was repeated thrice. The percentage of elongation, number of shoots elongated and shoot lengths were recorded at each subculturing.

Rooting and Acclimatization

In vitro root induction was carried out on MS medium supplemented with NAA and IBA at different concentrations (0.5, 1.0, 1.5 and 2.0 µ*M*). For *ex vitro* rooting, the basal end of the healthy shoots (≥3.0 cm in length) were dipped in IBA (50, 100, 150, 200 and 250 µ*M*) for half an hour and then planted in small plastic pots containing sterile garden soil and covered with transparent polythene bags to ensure high humidity. The bags were opened after 2 weeks in order to acclimatize plants to field conditions. After one month, plants were transferred to earthen pots filled with 3:1 mixture of soil and organic manure and maintained in green house under normal day length conditions. The number of roots per shoot and root length were recorded after 3 weeks of transplantation.

Plant Growth

Total shoot and root length, shoot, root fresh and dry mass and number of leaves per plant were determined after 2 month of transplanting of *in vitro* propagated plants and compared with control plants of same age.

Photosynthetic Pigments

Leaf chlorophyll and carotenoids were extracted with 80% acetone and estimated spectrophotometrically[17,18].

Relative Water Contents

Relative water content (RWC) of micropropagated plants were determined after 2 month of transplanting and compared with control plants of same age by using the formula[19]

$$RWC\ (\%) = (FM-DM)\times 100/(SM-DM)$$

where FM = Fresh mass, DM = Dry mass and SM = Saturated mass.

Statistical Analysis

All the experiments were repeated three times with 20 replicates per treatment. The effect of different treatments was quantified and the data was subjected to statistical analysis using one way analysis of variance (ANOVA) and means were compared using the Duncan's multiple range test at 5% level of significance.

Results and Discussion

The morphogenetic responses of cotyledonary node explants to TDZ alone or in combination with IAA are summarized in Figs 1 & 2. Explants cultured onto a growth regulator free MS medium failed to produce shoots even after four weeks. When MS medium was supplemented with different concentrations of TDZ (0.1-10.0 μ*M*), multiple shoots emerged from cotyledonary node explants after 15 d of culture. TDZ in combination with IAA at different concentrations induced more shoots per explant compared to TDZ alone in the present investigation. The explants cultured on a medium containing TDZ (1.0 μ*M*) produced a maximum of 11.16±0.73 shoot buds per explants with 72.67% regeneration. Optimum shoot differentiation was observed in a media containing TDZ (1.5 μ*M*) and IAA (0.5 μ*M*) after 4 weeks of culture. In this medium, the highest regeneration

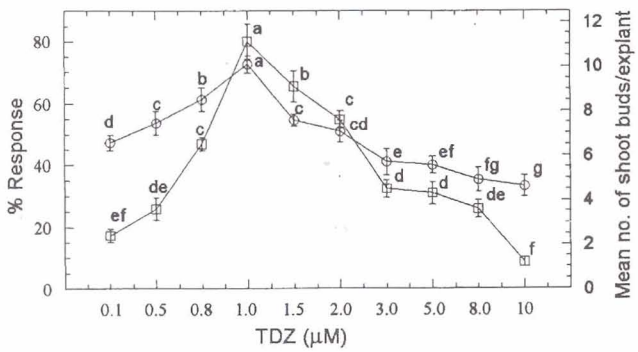

Fig. 1 — Effect of different concentrations of TDZ alone on regeneration frequency and mean number of shoot buds/explant after 4 weeks of culture. % Response (○), Mean number of shoot buds per explant (□).

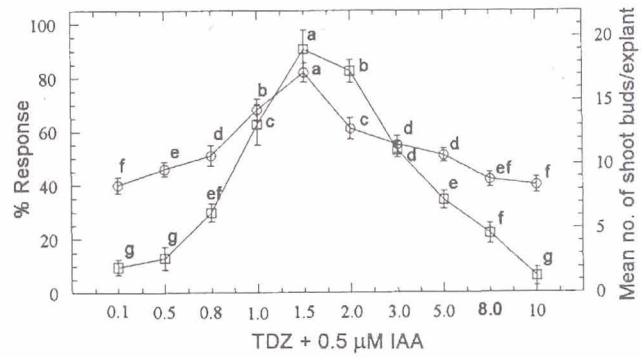

Fig. 2 — Effect of different concentrations of TDZ in combination with IAA (0.5 μ*M*) on regeneration frequency and mean number of shoot buds/explant after 4 weeks of culture. % Response (○), Mean number of shoot buds per explant (□).

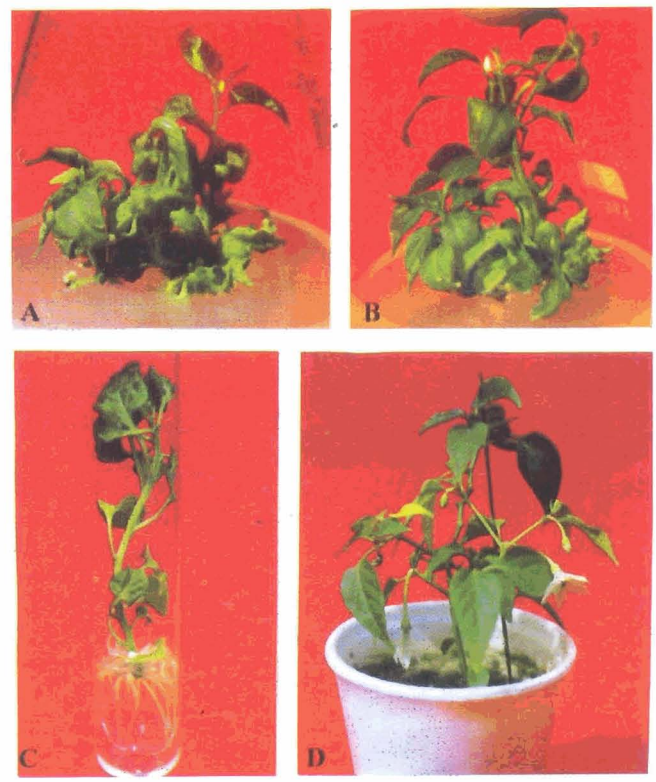

Fig. 3 — A-D Multiple shoot bud induction and plant regeneration from cotyledonary node explants of *Capsicum annuum* L.: A, Multiple shoot bud induction on MS medium + TDZ (1.5 μ*M*) + IAA (0.5 μ*M*) after 4 weeks of culture; B, Multiplication and elongation of shoots on hormone free MS medium; C, Rooting of *in vitro* regenerated shoots on MS + NAA (1.0 μ*M*); & D, Plant acclimatized to greenhouse condition.

frequency (82.33%) and highest number of shoot buds per explant (19.0±0.67) was achieved (Figs 2 & 3A).

At higher levels of TDZ (5.0, 8.0 and 10.0 µ*M*), 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[3,10].

The regenerated shoot buds were transferred onto the same medium after 2 weeks of induction. In most of the cases, the shoot buds failed to elongate into plantlets, instead leaf like structure were formed in clusters[5,10]. Elongation of shoot buds into long shoots has been a consistent problem in pepper. This problem was overcome by continuous subculturing of regenerated shoot buds to hormone free MS medium after every 2 weeks.

The shoot buds isolated from TDZ (1.5 µ*M*) and IAA (0.5 µ*M*) combination were repeatedly subcultured on hormone free MS medium for elongation. The use of hormone free MS medium for shoot elongation has been reported in soybean[20] and *Vicia faba*[21].

The per cent elongation, number of shoots elongated and shoot heights were recorded at every subculture, which reached its maximum by third subculture (Fig. 3B). The number of shoots elongated and shoot height increased considerably from first to third subculture and then it became stable at fourth subculture (Fig. 4) and then declined in subsequent subcultures (data not shown). Similar effect of subculturing has also been reported in Cranberry[22]. Rooting experiment was carried out by transferring the microshoots onto the basal MS medium supplemented with different concentrations of NAA and IBA (0.5, 1.0, 1.5 and 2.0 µ*M*) for obtaining complete plantlets (Figs 5 & 6). The maximum frequency of root formation (90%) and root length (6.0 cm) was achieved on MS medium supplemented with NAA (1.0 µ*M*) after 3 weeks of culture (Fig. 3C).

For *ex vitro* rooting, the basal portion of the regenerated shoots (3-4 cm) was dipped in different concentrations of IBA (50, 100, 150, 200 and 250 µ*M*) for half an hour and then planted in plastic pots containing sterile garden soil. Best results were recorded when shoots were dipped in IBA (200 µ*M*) for half an hour (Fig. 7). The growth of *ex vitro* and *in vitro* rooted plantlets was almost similar. Given the reduced time for establishment, IBA dipped *ex vitro* rooting has been found to be more favourable method than *in vitro* rooting. Rooted plantlets were transferred to pots containing garden soil and organic manure (3:1) and kept in the greenhouse for

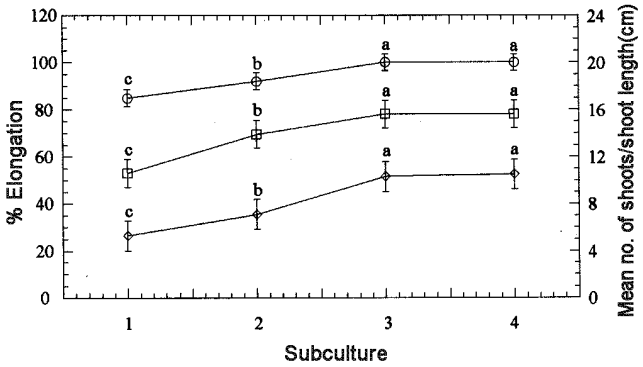

Fig. 4 — Evaluation of morphogenetic potential of shoot bud culture obtained from combination of TDZ (1.5 µ*M*) and IAA (0.5 µ*M*) after being tested for four subculture passages on growth regulator free MS medium. % elongation (○), Mean number of elongated shoots (□), Mean shoot length (◊).

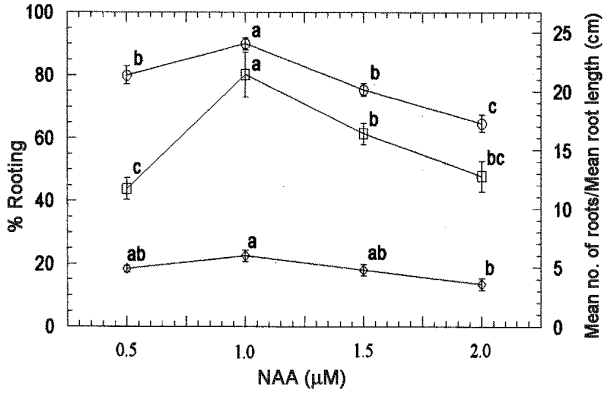

Fig. 5 — E ffect of different concentrations of NAA on root induction, mean number of roots and root length from *in vitro* raised shoots of *C. annuum* after 3 weeks of culture. % rooting (○), Mean number of roots/shoot (□), Root length (◊).

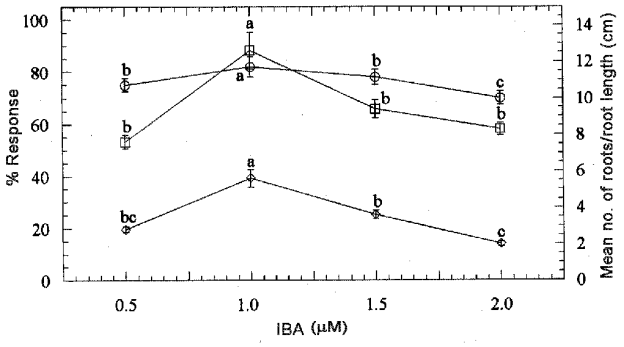

Fig. 6 — Effect of different concentrations of IBA on root induction, mean number of roots and root length from *in vitro* raised shoots of *C. annuum* after 3 weeks of culture. % rooting (○), Mean number of roots/shoot (□), Root length (◊).

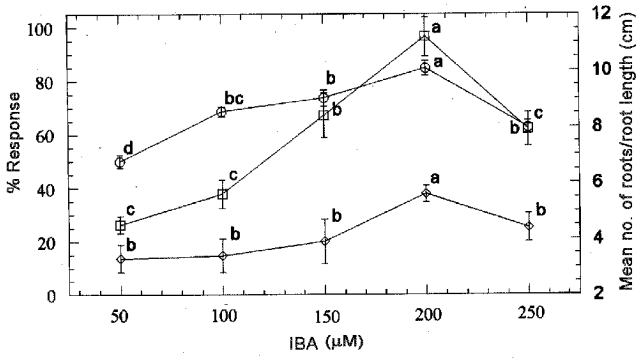

Fig. 7 — Efficiency of root induction from shoots of *C. annuum* dipped in IBA solution for half an hour before field transfer. The data were taken after three weeks of transplantation. % rooting (○), Mean number of roots/shoot (□), Root length (◊).

Table 1 — Comparison of some morphological features, chlorophyll and carotenoids contents of micropropagated plants and seedlings of *C. annuum*

Parameters	Micropropagated plants	Seedlings
Root length (cm)	6.48±0.27	7.27±0.46
Shoots length (cm)	14.34±0.78	17.89±0.81
Root fresh mass (g)	0.15±0.01	0.18±0.01
Shoot fresh mass (g)	1.49±0.08	1.86±0.06
Root dry mass (g)	0.03±0.01	0.07±0.01
Shoot dry mass (g)	0.38±0.01	0.56±0.02
Leaf dry mass g plant^{-1}	0.27±0.03	0.32±0.03
Leaf number/plant	15.16±0.72	17.0±0.57
Chlorophyll a mg g^{-1} fresh mass	4.01±0.13	5.05±0.18
Chlorophyll b mg g^{-1} fresh mass	1.48±0.14	1.98±0.22
Chlorophyll a + b mg g^{-1} fresh mass	5.51±0.10	7.03±0.03
Carotenoids mg g^{-1} fresh mass	0.22±0.01	0.17±0.01
Relative water content (%)	91.02±1.49	76.76±2.35

Data recorded in triplicate and five plants used for each case. Determinations made on 2-month-old plants.

acclimatization. After one month of transfer to soil, 95% of the plants survived. They flowered normally (Fig. 3D) and were able to set viable seeds.

Comparative data on some morphological features, chlorophyll content and relative water content of *in vitro* propagated plants and seedlings is summarized in Table 1. Slight reduction in the morphology of *in vitro* propagated plants in terms of shoot, root length and dry and fresh mass and leaf number were observed in comparison to control plants after considerable period of establishment in the green house. Chlorophyll a and b and total chlorophyll were found lower in regenerated plants while relative water content and carotenoids were higher in regenerated plants. Decrease or increase in chlorophyll content, carotenoid and relative water content depends on the environmental conditions. The regenerated plants appeared to have poorly developed chloroplast with low chlorophyll, protein and disorganized grana[23]. The plantlets dehydrated quickly and experienced water stress as soon as they were transferred from the tissue culture vessels into field conditions. Under low relative humidity, plantlets became somewhat wilted and because of this RWC is decreased. However, regenerants slowly recovered from water stress with the lapse of time and obtained higher relative water content than *in vivo* seedlings[24].

In conclusion, the present study describes for the first time, an effective regeneration and multiplication protocol for *in vitro* propagation of *C. annuum* using TDZ from cotyledonary node explants. The comparison of several physiological and morphological parameters in micropropagated plants and seedlings showed considerable similarities.

High multiplication efficiency, *ex vitro* rooting, easy establishment in the soil and normal growth performance of micropropagated plants as reported in this study, are features necessary for the adoption of *in vitro* propagation technology for large-scale multiplication and genetic transformation using biolistic and other gene transfer experiments.

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