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Efficient TDZ and IAA-assisted Plant Regeneration from Cotyledon and Leaf Explants of *Capsicum annuum* L. – One-Step Protocol for Shoot Bud Differentiation and Elongation

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ABSTRACT

The present study was undertaken to investigate the possibility for developing a one-step regeneration protocol in *Capsicum annuum* L. For this, the effect of three different cytokinins on *in vitro* regeneration from cotyledon and leaf explants was studied in pepper cv. 'G4'. The cytokinins used were 6-benzylaminopurine (BAP), thidiazuron (TDZ), and kinetin (Kn), individually or in combination with indole acetic acid (IAA). Although organogenesis could be obtained on medium supplemented with BAP or TDZ, the regenerated shoots failed to elongate on the same medium and showed an albino phenotype. TDZ stimulated shoot regeneration and this effect was significantly enhanced when combined with IAA. TDZ (9.0 μM) in combination with IAA (2.8 μM) proved to be optimal for induction of maximum number of shoots from cotyledon and leaf explants within 4 weeks on the same medium. The regenerated shoots elongated 3-4 cm on the same medium within 4 weeks from the beginning of the culture. Microshoots were rooted (after 3-4 weeks of culture on rooting medium) on MS medium fortified with IAA (5.7 μM) followed by transfer to the greenhouse with a subsequent 70% acclimatization. We conclude that TDZ in combination with IAA induces significant regeneration as well as shoot elongation from cotyledon and leaf explants in pepper cv. 'G4'. For the first time our findings describe a single-step protocol for *in vitro* shoot regeneration and elongation in pepper.

Keywords: auxin, cytokinin, microshoots, rooting

INTRODUCTION

The use of transformation techniques such as biolistic or *Agrobacterium* mediated transformation requires, as a prerequisite, a protocol for plant regeneration. Thus the establishment of an efficient *in vitro* plant regeneration system becomes a primary need for plant genetic engineering to obtain useful traits. Chili pepper (*Capsicum annum* L.) is a world-wide important horticultural crop that is not easily amenable to tissue culture and transformation mainly due to its recalcitrant nature to *in vitro* regeneration. This has limited the applications of new genetic manipulation techniques to pepper improvement.

Plant regeneration from hypocotyl, cotyledon, leaf, and shoot tip explants has been reported in some pepper genotypes (Gunay and Rao 1978; Phillips and Hubstenberger 1985; Agrawal *et al*. 1989; Valera-Montero and Ochoa-Alejo 1992; Ebida and Hu 1993; Christopher and Rajam 1996; Rao *et al*. 1997; Golegaonkar and Kantharajah 2006). The above findings reported BAP as a potent cytokinin for *in vitro* regeneration in pepper. However, the response to culture conditions differed markedly depending on the pepper cultivar, explant type, and on the culture medium used for *in vitro* organogenesis. Only one isolated report described the role of TDZ in adventitious shoot induction and plant regeneration in pepper (Venkataiah *et al*. 2003).

A major concern limiting efficient *in vitro* pepper organogenesis from either cotyledon and/or leaf explants is that the regenerated shoots fail to elongate on the same medium and require an additional hormone supplement for shoot elongation. This makes it a two-step culture procedure for plant regeneration in pepper. Our earlier studies in pepper showed formation of shoots from leaf explants that did not elongate on the same medium and these were described as albino shoots (Rao *et al*. 1997). Several methods were tested to circumvent the problem for shoot elongation in pepper. In one strategy rooted hypocotyls were cultured upside down on shoot induction medium with three combination of BA and IAA supplemented with $AgNO_3$ (Valera-Montero and Ochoa-Alejo 1992) whereas Hyde and Phillips (1996) adopted four stages of pepper plant regeneration that included GA_3 and $AgNO_3$ as additional hormones for bud enlargement and shoot elongation. Franck-Duchenne *et al*. (1998) used 24-epibrassinolide for shoot elongation whereas Husain *et al*. (1999) used phenyl acetic acid in combination with BA in MS medium to stimulate bud induction and shoot elongation. Golegaonkar and Kantharajah (2006) reported that shoot elongation was observed when adventitious shoot buds were cultured on MS medium containing BA and GA_3. Joshi and Kothari (2006) increased the concentration of Cu 30 times its normal level in MS to improve shoot elongation from the cultured cotyledons of *C. annum*. These findings, to a certain extent, could resolve the problem associated with *in vitro* shoot elongation but did not define a common media formulation for shoot bud differentiation and elongation. As a result such protocols deserve time as well as delicate handling for transferring the shoot buds onto fresh culture medium in order to obtain elongated shoots for root induction and plantlet regeneration.

The lack of media composition for shoot bud differentiation and elongation, and coupled with strong genotypic dependency made us initiate research to optimize the regeneration protocol for a specific pepper cultivar. For the first time ever the present investigation describes a single-step culture procedure for plant regeneration in pepper cv. 'G4', a popular genotype among farmers and which is cultivated on a wide scale in southern parts of India.

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Original Research Paper

Table 1 Effect of TDZ and TDZ + IAA on *in vitro* regeneration from cotyledon and leaf cultures of *Capsicum annum* L. 'G4' after 28 days.

Cotyledon explant			Leaf explant		
Phytohormone (µM)	**Response (%)**	**№ of shoots/explant[a]**	**Phytohormone (µM)**	**Response (%)**	**№ of shoots/explant[a]**
TDZ			**TDZ**		
4.5	62	2.1 ± 0.45 a	4.5	63	2.3 ± 0.62 a
9.0	91	5.4 ± 0.22 b	9.0	92	5.9 ± 0.19 b
13.6	90	4.3 ± 0.16 c	13.6	90	5.6 ± 0.39 c
18.0	82	3.8 ± 0.09 d	18.0	86	4.2 ± 0.21 d
TDZ+IAA			**TDZ+IAA**		
9.0 + 2.8	91	5.8 ± 0.63 e	9.0 + 2.8	96	5.8 ± 0.64 b
13.6 + 2.8	94	4.4 ± 0.38 c	13.6 + 2.8	92	5.6 ± 0.13 c
9.0 + 5.7	90	5.1 ± 0.33 b	9.0 + 5.7	94	5.3 ± 0.28 e
13.6 + 5.7	93	4.2 ± 1.18 c	13.6 + 5.7	93	5.3 ± 0.34 e

[a] Data (mean ± S.E) are the average of two experiments (n = 40)
In each column, means followed by the same letter were not significantly different ($p \leq 0.01$) according to Duncan's multiple range test.

MATERIALS AND METHODS

Seed germination

Seeds of *Capsicum annuum* L cv. 'G4' were obtained from Pocha seed Co., Maharastra, India. The seeds were soaked in distilled water for 24h, and surface sterilized using 0.1% $HgCl_2$ for 3 min, followed by four to five rinses with sterile distilled water. Seeds were germinated aseptically on Murashige and Skoog (MS) (1962) medium containing 2% sucrose and solidified with 0.8% agar (Himedia).

Regeneration and elongation medium

Cotyledon and leaf explants were excised from 15 and 25 day-old *in vitro* grown seedlings, respectively. Explants from cotyledons and leaf of 1 cm^2 were dissected and cultured separately in culture tubes in such a way that the abaxial surface was in close contact with the medium. When leaf explants were cultured, the leaf tips and basal portions including the petiole were discarded. MS basal medium containing 2% sucrose was fortified with plant growth regulators, thidiazuron (TDZ) (4.5, 9.0, 13.6 and 18.0 µM), 6-benzylaminopurine (BAP) (4.4, 8.9 and 13.3 µM), kinetin (Kn) (4.6, 9.2 and 13.9 µM) alone, or in combination with IAA (2.8 and 5.7 µM). MS medium without TDZ, BAP or Kn served as controls for shoot initiation and shoot elongation.

Rooting medium and greenhouse transfer

After approximately one month's growth on regeneration medium the elongated shoots 3-4 cm in length were transferred for rooting onto MS medium supplemented with 5.7 µM IAA. The effect of plant growth regulators on rooting was investigated 3-4 weeks after culture on rooting medium (MS medium without IAA served as the rooting control). Rooted plants (4-5 weeks old) were weaned away from the tubes, the roots cleaned for agar by washing with sterile water and the plantlets were transferred to pots (10 cm in length, 15 cm in diameter, 1.3-1.7 cm deep; one plant per pot) containing soil and compost (1:1) followed by transfer to greenhouse. A 65% relative humidity was maintained in the greenhouse (28°C maintained during the daytime, 16-18°C at night; 60 µmol $m^{-2}s^{-1}$ light intensity; plantlets were sprinkled with tap water everyday), and the pots were covered with polythene bags until the plantlets were acclimated under greenhouse.

All media pH was adjusted to 5.7 with 1M KOH prior to addition of 0.8% agar, and the media were autoclaved at a pressure of 1.05 kg cm^{-2} for 15 min at 121°C. All cultures were incubated at 25 ± 2°C under white fluorescent light (40-60 µmol $m^{-2}s^{-1}$; IS 2416 L 7434877, two bulbs model, Phillips, India) with a 16-h photoperiod. All data were statistically analyzed by ANOVA followed by Duncan's multiple range test for mean comparison. Data pertaining to shoot regeneration was obtained from 10 explants in each of two replicates for each treatment and the experiment was repeated twice.

RESULTS AND DISCUSSION

In the present study three kinds of cytokinins (BAP, Kn and TDZ) were used either alone or in combination with IAA to investigate *in vitro* regeneration in pepper 'G4'.

Effect of TDZ

TDZ showed efficient shoot bud induction and regeneration from cotyledon and leaf explants. Medium containing TDZ induced formation of shoot buds predominantly at the cut surface of the cultured explants within 1-2 weeks of culture (**Fig. 1A**). Shoot buds were proliferated and developed into shoot primordia (**Fig. 1B**). Among the tested concentrations of TDZ (4.5, 9.0, 13.6 and 18.0 µM), lower (4.5 µM) and higher (18.0 µM) concentrations showed least regenerative response with fewer shoots produced per explant compared to 9.0 and 13.6 µM of TDZ (**Table 1**). TDZ at 9.0 µM proved to be the optimal concentration producing a maximum number of shoots from cotyledon (5.4 ± 0.22) and leaf (5.9 ± 0.19) explants. These regenerated shoots did not elongate on the same medium and showed an albino phenotype (**Figs. 1C, 1D**). TDZ at 13.6 µM was less significant in terms of the number of shoots/explant produced as compared to 9.0 µM TDZ. At 13.6 µM TDZ, the number of shoots produced from cotyledon (4.3 ± 0.16) and leaf (5.6 ± 0.39) explants were less than with 9.0 µM TDZ (**Table 1**). In the control treatment, no shoot development was observed.

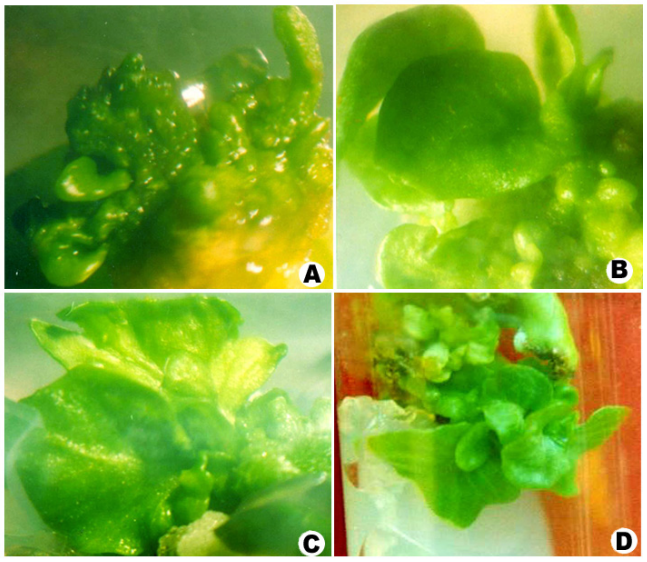

Fig. 1 Leaf explants cultured on MS medium containing 9.0 µM TDZ. (**A**) Induction of shoot buds (21 days); (**B**) shoot bud proliferation and shoot initiation (28 days); (**C, D**) regeneration of albino shoots (28 days).

Table 2 Effect of BAP and BAP + IAA on *in vitro* regeneration from cotyledon and leaf cultures of *Capsicum annum* L. 'G$_4$' after 28 days.

Cotyledon explant			Leaf explant		
Phythormone (µM)	**Response (%)**	**№ of shoots/explant[a]**	**Phythormone (µM)**	**Response (%)**	**№ of shoots/explant[a]**
BAP			**BAP**		
4.4	48	NR	4.4	42	NR
8.9	52	NR	8.9	48	NR
13.3	60	1.3 ± 0.10 a	13.3	53	NR
BAP+IAA			**BAP+IAA**		
4.4 + 2.8	68	2.9 ± 0.32 b	4.4 + 2.8	61	2.6 ± 0.47 a
8.9 + 2.8	74	3.2 ± 0.36 b	8.9 + 2.8	98	3.2 ± 0.15 b
13.3 + 2.8	80	5.0 ± 0.11 c	13.3 + 2.8	80	4.1 ± 0.18 c
4.4 + 5.7	78	1.9 ± 0.21 d	4.4 + 5.7	68	1.8 ± 0.39 d
8.9 + 5.7	81	2.4 ± 0.30 e	8.9 + 5.7	79	2.1 ± 0.14 e
13.3 + 5.7	88	4.2 ± 0.26 f	13.3 + 5.7	83	3.8 ± 0.30 c

[a] Data (mean ± S.E) are the average of two experiments (n = 40)
In each column, means followed by the same letter were not significantly different ($p \leq 0.01$) according to Duncan's multiple range test.
NR- No Response (no shoots were observed)

Effect of TDZ and IAA combination

In the present study the effect of TDZ and IAA combination on shoot bud proliferation and elongation was analyzed in cotyledon and leaf explants of pepper cv. 'G4'. For this, two concentrations each of TDZ (9.0 and 13.6 µM) and IAA (2.8 and 5.7 µM) were tested in combination (**Table 1**).

TDZ in combination with IAA enhanced the number of shoots formed per cotyledon explant compared to the use of TDZ alone. The TDZ (9.0 µM) and IAA (2.8 µM) combination proved to be optimal for the production of a maximum number of shoots per cotyledonary explant (5.8 ± 0.63) (**Table 1**). Interestingly, the regenerated shoots elongated on the same medium. A higher concentration of TDZ (13.6 µM) in combination with IAA (2.8 µM) showed increased percentage of responding cultures (94%) but induced fewer shoots per cotyledon explant (4.4 ± 0.38). Further, increasing IAA concentration (5.7 µM) in combination with TDZ (9.0 and 13.6 µM) did not show a significant increase in the number of shoots/explant produced (**Table 1**).

When leaf segments were cultured, the addition of IAA in combination with TDZ did not show significant differences in terms of number of shoots/explant regenerated compared to the use of TDZ alone. Further, we observed an increase in the percentage of responding cultures (96%) when TDZ (9.0 µM) and IAA (2.8 µM) were used in combination compared to a 92% of response when TDZ alone was used at 9.0 µM (**Table 1**).

Venkataiah *et al.* (2003) reported regeneration from cotyledon and leaf explants of pepper on TDZ-containing medium. However regenerated shoots did not elongate on the same medium and a low concentration of BA and IAA (0.05 mg/L each) was used to elongate shoots. Thus the pepper regeneration protocol described by Venkataiah *et al.* (2003) is essentially a two-step protocol. In our study regeneration medium (TDZ 9.0 µM and IAA 2.8 µM) both shoot bud induction and shoot elongation could be achieved in a single step.

Effect of BAP and Kn

In the present study, we analyzed in parallel experiment the effect of BAP and Kn on pepper regeneration. Medium with 4.4 and 8.9 µM of BAP induced compact callus from both leaf and cotyledon explants. No shoots regenerated from either of the explants at these BAP concentrations. Shoot buds were formed directly on cotyledon explants only at a higher concentration of BAP (13.3 µM). Several of these shoot buds failed to regenerate into shoots. At 13.3 µM of BAP only a small number of albino shoots (1.3 ± 0.10) regenerated from cotyledon explants (**Table 2**). At a similar concentration, leaf explants showed no shoot bud induction. At all tested concentrations, the presence of Kn in the medium induced callus formation and did not support shoot bud development from leaf and cotyledon explants in pepper 'G4'. The presence of Kn in the medium induced callus formation from leaf and cotyledon explants without shoot regeneration (data not shown). No shoot regeneration was observed in the control treatment.

From the above results we conclude that TDZ is the most potent cytokinin to induce *in vitro* regeneration from cultured leaf and cotyledon explants in pepper 'G4'. Among the tested cytokinins, BAP at a higher concentration proved to be second best to induce shoot regeneration; no shoots regenerated from explants cultured on Kn-supplemented medium. Thus *in vitro* regeneration in this genotype was highly influenced by the type of cytokinin used in the medium.

Effect of BAP and IAA combination

Cotyledon and leaf explants showed a positive morphogenic response on medium containing BAP (4.4, 8.9 and 13.3 µM) in combination with IAA (2.8 and 5.7 µM). Interestingly, BAP at lower concentrations (4.4 and 8.9 µM) did not induce *in vitro* regeneration however, when combined with IAA (2.8 and 5.7 µM) shoot formation was initiated from both cotyledon and leaf explants (**Figs. 2A, 2B; Table 2**). This clearly implies the additive effect of IAA with BAP on cotyledon and leaf regeneration in pepper 'G4'. Further, a higher BAP concentration (13.3 µM) when combined with IAA (2.8 and 5.7 µM) produced more shoots/explant from cotyledon and leaf explants than when lower levels of BAP (4.4 and 8.9 µM) in combination with IAA were used. The

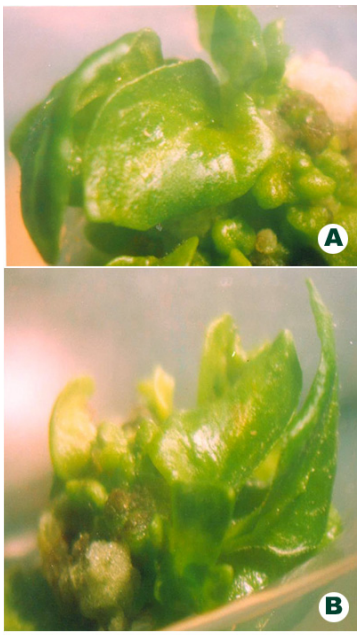

Fig. 2 Cotyledon explants on MS medium supplemented with BAP (13.3 µM) and IAA (2.8 µM). (**A**) Shoot bud proliferation (21 days); (**B**) shoot initiation (28 days).

Fig. 3 (**A**) Development of albino shoots on MS medium with 9.0 µM TDZ (21 days); (**B**) elongated shoot on MS + TDZ (9.0 µM) + IAA (2.8 µM) (28 days); (**C**) rooted microshoot on MS + 5.7 µM IAA.

Fig. 4 Regenerated plantlet under field conditions 10 days after the rooting period

Table 3 Effect of IAA on rooting of regenerated shoots of *Capsicum annum* 'G$_4$' after 28 days.

IAA (µM)	Culture response (%)	№ of roots/shoot (± S.E)[a]	Mean root length (±S.E)[a]
2.8	38	1.0 ± 0.1 a	2.0 ± 0.3 a
5.7	90	4.1 ± 0.1 b	3.2 ± 0.1 a
8.5	62	3.2 ± 0.3 b	1.2 ± 0.3 b
11.4	60	1.8 ± 0.1 c	1.3 ± 0.3 b

[a]Mean values with the same letter within a column are not significantly different at p≤0.05 according to Duncan's multiple range test.

shoots that regenerated from leaf or cotyledon explants in the presence of BAP and IAA were of albino type.

From the above data it can be concluded that the addition of IAA to TDZ or BAP enhanced shoot bud induction and the percentage of responding cultures from both explant types. However, the concentration of TDZ (9.0 µM) and IAA (2.8 µM) proved to be optimum for inducing the maximum number of shoots from cotyledon and leaf explants. Hence, TDZ and IAA can be used as a standard hormone combination to support regeneration from pepper 'G4'.

Shoot elongation

The major problem encountered during tissue culture studies in pepper is the formation of rosettes leaf-like structures that do not develop further into shoots (Franck-Duchenne *et al*. 1998; Steintz *et al*. 1999; Ochoa-Alejo and Ramirez-Malagon 2001; Joshi and Kothari 2006). In addition, the regenerated shoots do not elongate on the same medium and retain the albino phenotype as was also observed during present investigation when using TDZ (**Figs. 1C, 1D, 3A**). Interestingly, during our investigation shoot elongation occurred on medium supplemented with TDZ (9.0 µM) in combination with IAA (2.8 µM) (**Fig. 3B**). Fully elongated microshoots developed on the same medium, and thus transferring the stunted shoots for further elongation onto a fresh medium was avoided. Thus the described protocol is essentially a one-step protocol for plant regeneration in pepper 'G4'.

Rooting of microshoots

Prominent shoots (1.5-2.5 cm) were developed from cut ends of explants three weeks after culture on regeneration medium. Upon additional 7 days of culture on the same medium, fully elongated shoots of 3-4 cm length were excised and transferred onto rooting medium supplemented with 5.7 µM IAA (Shyam Prasad 2002). It was observed that IAA induced rooting from cut ends of microshoots within 3-4 weeks (28days) of culture on rooting medium (**Fig. 3C**). Maximum number of roots per rooted microshoot was 4.1 ± 0.1 that attained an average length of 3.2 ± 0.1 (**Table 3**). Rooted microshoots were successfully transferred to the greenhouse with a 70% survival rate (**Fig. 4**). Microshoots from all hormone treatments rooted to a similar extent and were acclimatized in the greenhouse with a 70% survival rate. A total of 84 plantlets were initially transferred for acclimatization, out of which 59 plantlets survived. No shoots regenerated from the control treatment.

In conclusion, our findings describe an efficient *in vitro* regeneration protocol from cotyledon and leaf explants of pepper 'G4'. The optimized regeneration method is based on a one-step protocol that does not need any additional culture steps to achieve shoot regeneration and elongation. Shoot bud initiation, proliferation, and shoot elongation occur on a common TDZ- and IAA-supplemented medium. Thus the regeneration protocol is simplified to a large extent and can be completed in a short duration. The present protocol facilitates genetic manipulation studies for chili pepper improvement.

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