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Research Note

Zeatin induced direct multiple shoots development and plant regeneration from cotyledon explants of cultivated tomato (*Solanum lycopersicum* L.)

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Abstract:

An efficient protocol for multiple shoots induction and plant regeneration from cotyledon explants of cultivated tomato (*Solanum lycopersicum* L.) cv S-22 is reported. The cotyledon explants were cultured on MS medium supplemented with 0.2-2.0 mg/L Zeatin as a sole growth regulator and also in combination with 0.2/0.5 mg/L IAA. Maximum number of multiple shoots formation was found at 0.2 mg/L IAA with 1.2 mg/L Zeatin. Shoots were transferred to MS medium supplemented with 0.2-1.0 mg/L GA_3 and 1.0 mg/L Zeatin for elongation. The medium supplemented with 0.6 mg/L GA_3 in combination with 1.0 mg/L Zeatin showed the maximum percentage of enhancement of shoot elongation. For *In vitro* rooting, elongated microshoots were excised and transferred onto MS medium supplemented with 0.5mg / L IAA, IBA and NAA. Profuse rhizogenesis was observed at 0.5 mg/L IAA compared to NAA / IBA. The regenerated plants were acclimatized in the culture room and maintained in the green house. These plants were found to be normal and similar to the donor plant. Thus, an efficient and reproducible direct regeneration protocol has been developed in cultivated tomato cv S-22 using cotyledon explants which is genotype dependent. This protocol can be used for *Agrobacterium tumefaciens* mediated genetic transformation in tomato cv S-22.

Keywords: Acclimatization; Cotyledon explants; Plant regeneration; *Solanum lycopersicum*; Zeatin.
Abbreviations: IAA-Indole-3-acetic acid; IBA-Indole-3-butyric acid; NAA-α-naphthalene acetic acid; PGR- Plant growth regulator(s); GA_3 -gibberelic acid.

Introduction

Tomato (*Solanum lycopersicum* L.) is considered to be an important vegetable crop and a model species for introduction of agronomically important genes (Wing et al., 1994). *In vitro* regeneration of cultivated tomato has been a subject of research because of the commercial value of the crop and its amenability for further improvement via genetic manipulation (Evans, 1989). Tomato is one of the most studied higher plants because of its importance as a crop species, and of several advantages for genetic, molecular and physiological studies (McCormick et al., 1986). Various reports of multiple shoots induction in cultivated tomato using different explants viz cotyledons, hypocotyl and leaf with different plant growth regulators like BAP, Kinetin, TDZ and Zeatin alone and also in combinations with different concentrations of IAA was reported (Zelcer et al., 1984; Park and Son, 1988; Hamza and Chupeau, 1993; Ye Li and Zhou,1994; Plastira and Perdikaris 1997; Geetha et al., 1998; Chen et al., 1999; Gubis et al., 2003; Shivakumar et al., 2007; Devi et al., 2008; Shadin Ishag et al., 2009). A good regeneration protocol is essential which is limiting the efficiency of the percentage of the transformants. The success in tomato regeneration response has been found to depend largely on genotype, explant and plant growth regulators used in the culture medium (Praveen and Rama Swamy, 2011) Therefore, the development of an efficient regeneration protocol is essential for *Agrobacterium* mediated genetic transformation in tomato cv S-22.However there is no report on multiple shoots induction from cotyledon explants using Zeatin except on somatic embryogenesis (Godishala et al., 2011), hence in this communication we developed a reproducible regeneration protocol for induction of multiple shoots and plantlet establishment in cultivated tomato cv S-22.

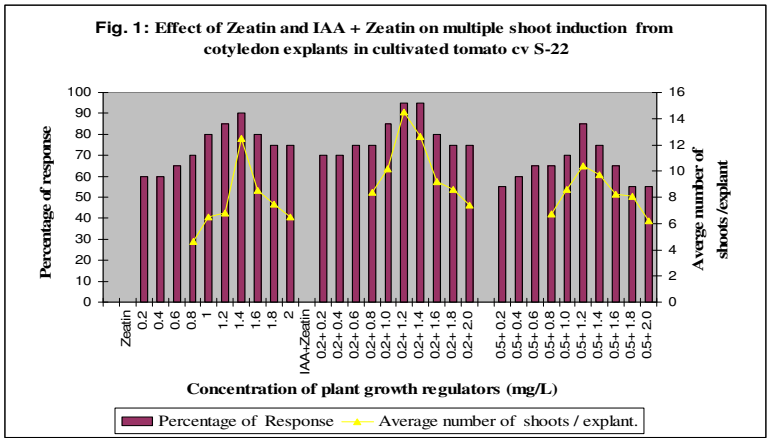
Results and Discussion

The cotyledon explants from 10-12 days old *in vitro* grown seedlings were cultured on MS medium supplemented with various concentrations of Zeatin and also in combination with IAA (0.2/0.5 mg/L) (Table 1). Shoot induction was initiated after 2nd week of inoculation and at the end of 4th week well developed multiple shoots were developed from the cut ends of the explants (Fig.2 a, b). Multiple shoots were induced directly from the explants in all the concentrations of Zeatin (0.2-2.0 mg/L) and also in combination with 0.2 / 0.5 mg/L IAA except at 0.2-0.6 mg/L Zeatin alone and also in combination with 0.2/0.5 mg/L IAA in which only callusing was observed. Maximum percentage of responding cultures with more number of multiple shoot formation was found at 1.4 mg/L Zeatin as a sole PGR, where as highest percentage of response and maximum frequency number of multiple shoots formation was found at 1.2 mg/L Zeatin in combin-

Table 1. Effect of Zeatin and IAA + Zeatin on multiple shoot induction in cultivated Tomato cv S-22

Concentration of plant growth regulators (mg/L)	Percentage of Response	Morphogenic Response	Average number of shoots/ explant (±SE)[a]	Average length (in cm) of shoots (±SE)[a]
Zeatin				
0.2	60	Callusing	---	---
0.4	60	Callusing	---	---
0.6	65	Callusing	---	---
0.8	70	Multiple Shoots	4.6±0.34	0.4±0.03
1.0	80	Multiple Shoots	6.5±0.30	0.6±0.06
1.2	85	Multiple Shoots	6.8±0.21	1.2±0.05
1.4	90	Multiple Shoots	12.5±0.40	0.8±0.07
1.6	80	Multiple Shoots	8.5±0.26	0.5±0.04
1.8	75	Multiple Shoots	7.5±0.35	0.4±0.08
2.0	75	Multiple Shoots	6.5±0.39	0.4±0.06
IAA + Zeatin				
0.2+0.2	70	Callusing	---	---
0.2+0.4	70	Callusing	---	---
0.2+0.6	75	Callusing	---	---
0.2+0.8	75	Multiple Shoots	8.4±0.31	0.5±0.09
0.2+1.0	85	Multiple Shoots	10.2±0.23	0.8±0.12
0.2+1.2	95	Multiple Shoots	14.5±0.26	1.4±0.08
0.2+1.4	95	Multiple Shoots	12.6±0.29	1.2±0.06
0.2+1.6	80	Multiple Shoots	9.2±0.40	0.8±0.05
0.2+1.8	75	Multiple Shoots	8.6±0.35	0.5±0.06
0.2+2.0	75	Multiple Shoots	7.4±0.39	0.4±0.06
0.5+0.2	55	Callusing	---	---
0.5+0.4	60	Callusing	---	---
0.5+0.6	65	Callusing	---	---
0.5+0.8	65	Callusing + Shoots	6.7±0.33	0.4±0.04
0.5+1.0	70	Callusing + Shoots	8.6±0.38	0.6±0.10
0.5+1.2	85	Callusing + Shoots	10.4±0.28	1.0±0.07
0.5+1.4	75	Callusing + Shoots	9.7±0.25	0.7±0.08
0.5+1.6	65	Callusing + Shoots	8.2±0.34	0.7±0.06
0.5+1.8	55	Callusing + Shoots	8.1±0.36	0.5±0.04
0.5+2.0	55	Callusing + Shoots	6.2±0..26	0.4±0.11

[a] Mean ± Standard Error



ation with 0.2 mg/L IAA (Fig.1).But multiple shoots induction was found to be reduced when 0.5 mg/L IAA was added to all the concentrations of Zeatin used in cv S-22 in comparison to 0.2 mg/L IAA + Zeatin combinations(Fig.1). Previous studies demonstrated that 8-10 days–old cotyledons of tomato were superior to other source of explants, including hypocotyls, stems and leaves for promoting shoot organogenesis of tomato (Hamza and Chupeau, 1993; Van Roekel et al., 1993; Ling et al., 1998). In our experiments 10-12 day-old cotyledons showed maximum percentage of adventitious shoot buds proliferation efficiency in tomato c v S-22. Gubis et al. (2004) have also reported that the medium fortified with 1.0 mg/L Zeatin with 0.1 mg/L IAA was the most effective (100 %) in induction of adventitious shoots (5.89±0.35) from cotyledon explants in tomato cultivar Premium. According to our observation, addition of 0.2 mg/L IAA to the medium supplemented with Zeatin enhanced the number of adventitious shoots (14.5 ±0.26) proliferation in cultivar S-22. Our results are also in conformity with the results of Nogueira et al. (2001) in tomato cultivar Santa

Table 2. Effect of IBA, IAA and NAA on *in vtiro* rooting of cultivated tomato cv S-22

Medium + Growth regulators (mg/L)	Type of Response	% of Rooting	Average number of roots/shoot (±SE)[a]
½ MSO	Callusing	---	
MSO	Callusing	---	
MS + IBA (0.5)	Rooting	70	20±0.16
MS + IAA (0.5)	Rooting	100	26±0.08
MS + NAA (0.5)	Rooting*	40	16±0.21

[a] Mean ± Standard Error, *With Callusing

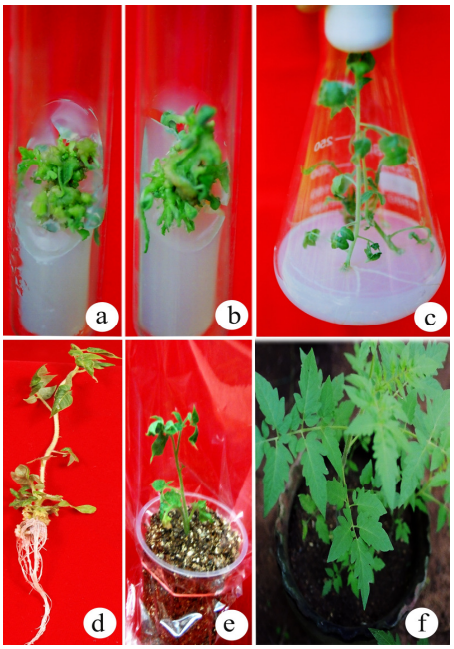
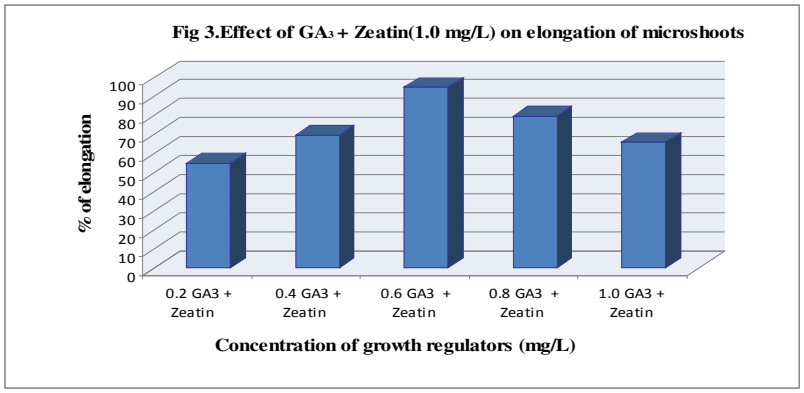


Fig 2. Multiple shoots induction and plantlet regeneration from cotyledon explants in tomato cv S-22. a & b : Explants showing multiple shoot induction on MS + 0.2 mg/L IAA + 1.2 mg/L Zeatin after 3 and 4 weeks of culture respectively. c: Shoot elongation on MS + 0.6 mg/L GA_3 + 1.0 mg/L Zeatin. d: Profuse rooting on MS+ 0.5mg/L IAA. e: Acclimatization in culture room. f. The regenerated plant growing in the research field .



Clara and its natural mutant 'Firme'. The regenerated micro-shoots sub-cultured on ½ strength MS and MS basal media did not support elongation. The micro-shoots cultured on 0.2-1.0 mg/L GA_3 with 1 mg/L Zeatin supported the elongation (Fig 3). Absolute percentage of shoot elongation was recorded at 0.6 mg/L GA_3 with 1.0 mg/L Zeatin (Fig.2c). For *in vitro* rooting the micro-shoots were cultured on ½ strength MS, MSO and MS medium supplemented with 0.5 mg/L IBA / IAA and NAA (Table 2). Rooting was absent on ½ MS and MSO and callus was formed without rhizogenesis at the basal region of micro-shoots. Root formation was initiated within 10-12 days of inoculation in all the auxins used. 100 % rooting was observed on MS medium supplemented with 0.5 mg/L IAA with profuse rhizogenesis (Fig. 2d). Mensuali-Sodi et al., 1995 have reported the *invitro* rooting without PGRS. However, the current study could find that cultivation of the micro-shoots on MS medium substituted with different auxins resulted an effective rooting than culturing on an auxin-free medium which supported the promotive effect of auxins on root initials (De Klerk et al., 1999). The

regenerated plants were taken out and washed with sterile distilled water under aseptic conditions to remove remains of agar medium. Later these plants were shifted to plastic pots containing sterile vermiculite: soil (1:1) covered with polythene bags in order to maintain 80% RH and kept in culture room for 3 weeks (Fig.2e). Later, they were shifted to earthenware pots containing garden soil and maintained in the research field (Fig.2f) under shady place and the survival rate was found to be 60 %. Thus, the results presented here describe an efficient and reproducible protocol for direct regeneration from cotyledon explants of tomato cv S-22. Since cotyledon is a favoured source of explant for transformation studies, the cotyledon based direct regeneration protocol is a pre-requisite for *Agrobacterium tumefaciens* mediated genetic transformation in the cultivar S-22 for producing transgenic plants.

Material and methods

Plant material

Seeds of tomato cv S-22 were obtained from M/S Max Agri-Genetics Pvt Ltd, Hyderabad, India. These seeds were washed under running tap water for 2 hrs and soaked in sterilized distilled water for 2 hours. The seeds were surface-sterilized with 0.1 % (w/v) $HgCl_2$ for 2 – 3 minutes followed by 3-4 rinses with sterile distilled water. These sterilized seeds were allowed to germinate on half-strength MS medium (Murashige and Skoog, 1962)(MS salts, 100 mg L^{-1} myoinositol, 2 mg L^{-1} thiamine-HCl, 0.5 mg L^{-1} pyridoxine-HCl, 0.5 mg L^{-1} nicotinic acid and 3 % (w/v) sucrose.

The cotyledon explants (1.0 cm^2) from 10- 12 days old *in vitro* grown seedlings were excised and cultured on MS medium supplemented with various concentrations of Zeatin and also in combination with IAA to induce shooting. The micro-shoots were excised and cultured for elongation on ½ strength MS, MSO and MS medium supplemented with 0.5 mg/L GA_3 and 1.0 mg/L Zeatin. After elongation, these micro-shoots were transferred on to MS medium supplemented with 0.5mgL^{-1} IAA/IBA/NAA for *in vitro* rooting.

Culture Media and Culture Conditions

The pH of the media was adjusted to 5.8 either with 0.1 N NaOH or 0.1 N HCl before adding 0.8% (w/v) agar-agar prior to autoclaving. The medium was autoclaved at 121^oC under 15 p.s.i in an autoclave for 15-20 minutes. All the cultures were maintained at 25^oC ± 2 under photoperiod of 16 hr illumination with light intensity of 50µmol m^{-2} s^{-1}.

Plantlet Establishment

In vitro rooted plants derived from cotyledon explants were washed with sterile distilled water and shifted to plastic pots containing sterilized vermiculite: garden soil (1:1). Each plastic pot was covered with polythene bag to maintain the RH (80-90 %) and kept in culture room for 4 weeks. Later these polythene bags were removed and the plants were shifted to earthenware pots containing garden soil. These regenerated plants are maintained in the green house.

Data Analysis

Regeneration of explants was assessed after 6 weeks. The following parameters were evaluated: percentage of response, average number of shoots per explant and mean length of shoots. Each experiment was consisting of 20 replicates and each experiment was repeated at least twice. The data were analyzed statistically.

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