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Effect of plant growth regulators on *in vitro* organogenesis in cultivated tomato (*Solanum lycopersicum* L.).

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ABSTRACT:

An efficient and reproducible protocol for organogenesis from cotyledon explants in cultivated tomato (*Solanum lycopersicum* L.) cv S-22 is reported. The cotyledon explants of 10-12 days old excised from *in vitro* grown seedlings were cultured on MS medium supplemented with 0.5-5.0 mg/L BAP as a sole growth regulator and also in combination with 0.1 mg/L IAA (Indole-3-acetic acid). Highest percentage of response for callus induction was recorded in cotyledon explants at 3.0 mg/L BAP where as multiple adventitious shoots were formed at 0.1 mg/L IAA + 2.5-5.0 mg/L BAP containing medium. Shoots obtained were transferred on to MS medium augmented with 0.2-1.0 mg/L GA_3 + 3.5 mg/L BAP for shoot elongation. The medium supplemented with 0.6 mg/L GA_3 in combination with 3.5 mg/L BAP showed the maximum percentage of enhancement of shoot elongation. For *in vitro* rooting, elongated micro-shoots were transferred onto MS medium supplemented with 0.5mg/L Indole-3-acetic acid (IAA) / Indole butyric acid (IBA) / Napthalene acetic acid (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 and transferred to the field. These plants were found to be normal and similar to the donar plant. Thus, an efficient and reproducible protocol has been developed in cultivated tomato cv S-22 which is genotype dependent. This protocol can be used for *Agrobacterium tumefaciens* mediated genetic transformation in tomato cv S-22 .

Keywords:
Solanum lycopersicum, cotyledon explants, organogenesis, *in vitro* rooting.

Abbreviations:
IAA-Indole-3-acetic acid ; IBA-Indole-3-butyric acid; NAA-ά-naphthalene acetic acid; GA_3 -Gibberelic acid.

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INTRODUCTION:

Tomato (*Solanum lycopersicum* L.) is considered to be the second important vegetable crop next to potato (Bhatia *et al*., 2004). It is also considered as a model species for introduction of agronomically important genes (Wing *et al.,* 1994). Developing a good *in vitro* regeneration protocol 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). It 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 (Mc Cormick *et al*.,1986). The literature shows that the regeneration protocols have been published in cultivated tomato using different explants viz., cotyledons, hypocotyl and leaf , different plant growth regulators like BAP, Kinetin, TDZ and Zeatin alone and also in combination with various concentrations of auxins (Zelcer *et al.,* 1984; Park and Son 1988; Hamza and Chupeau1993; Ye Li and GL Zhou 1994; Plastira and Perdikaris 1997; Geetha *et al.,* 1998; Chen *et al* 1999; Gubis *et al.,* 2003). A good regeneration protocol is essential for enhanced percentage of the transformants. The success in tomato regeneration response has been found to be genotype dependent, and also depends upon the orientation of explants and plant growth regulators used in the culture medium (Praveen and Rama Swamy 2011). However there is no report on multiple shoots induction and regeneration from cotyledon explants in Tomato cv S-22 except on somatic embryogenesis (Godishala *et al.,* 2011). Hence in this communication we report on a simple and reproducible regeneration protocol for plantlet establishment in cultivated tomato cv S-22.

MATERIALS AND METHODS

Seeds of cultivated tomato cv S-22 were obtained from M/S Max Agri-Genetics Pvt Ltd, Hyderabad, India. The seeds were washed under running tap water for10 mins and soaked in sterile distilled water for 2 hours. These seeds were surface-sterilized with 0.1 % (w/v) $HgCl_2$ for 2 – 3 minutes followed by 3-4 rinses in sterile distilled water. Surface sterilized seeds were germinated on MS medium (Murashige and Skoog 1962) supplemented with 100 mg/l myoinositol and 30 g/l sucrose. 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 sterilized at 121^{o}C under 15 psi in an autoclave for 15-20 minutes. Cotyledon explants (1.0 cm^2) were excised from 10-12 day old *in vitro* grown seedlings and inoculated them on MS medium supplemented with varying concentrations of BAP and also in combination with IAA. The micro-shoots were excised and cultured for elongation on ½ strength MS, MSO and MS medium supplemented with 3.5 mg/L BAP in combination with 0.2-1.0 mg/L GA_3.For *in vitro* rooting the micro-shoots were transferred on to ½ strength MS, MSO and MS medium supplemented with 0.5mgL^{-1} IAA/IBA/NAA. All the cultures were incubated at 25^{o}C ± 1 under 16 h photoperiod with light intensity of 50µmol m^{-2} s^{-1}.

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 and maintained in the green house. Later they were shifted to field.

Data Analysis:

The data on multiple shoots formation was assessed after 6 weeks of culture. The following parameters were evaluated: percentage of response, average number of shoots per explant and mean length of shoots. The experiments were repeated at least twice and the data were analyzed statistically.

RESULTS AND DISCUSSION:

The cotyledon explants from 10-12 days old axenic grown seedlings were cultured on MS medium supplemented with various concentrations of BAP alone (**Table 1**). Callus induction was initiated after 2nd week of inoculation at the cut ends and gradually whole cotyledon turned into callus after 4 weeks of culture. Callus was developed on all the concentrations of BAP used from cotyledon explants of tomato cv S-22. Percentage of response was found to be increasing with increase in the concentration of the BAP and maximum percentage of response was recorded at 3.0 mg/L BAP for callus formation. Further increase in the hormonal concentration was showing a decline in the callus induction response. The nature of the callus was also found to be varied. Green nodular callus was developed at 2.5-3.5 mg/ L BAP (**Fig.1a**).

To know the morphogenetic event, the cotyledon explants were also cultured on MS

Table.1. Effect of various concentrations of BAP on morphogenesis from cotyledon explants in tomato cv S-22

Concentration of PGR (mg/L) BAP	% of Response	Morphogenic Response
0.5	40	Callusing
1.0	45	Callusing
1.5	45	Callusing
2.0	50	Nodular Callus
2.5	60	Green Nodular Callus
3.0	75	Green Nodular Callus
3.5	70	Green Nodular Callus
4.0	65	Brown Callus
4.5	50	Brown Callus
5.0	50	Brown Callus

medium supplemented with different concentration of BAP used in combination with 0.1 mg/L IAA (Table 2) .Multiple shoots were induced directly from the explants in all the concentrations of BAP in combination with 0.1 mg/L IAA (**Fig.1b**) except at 0.5-2.0 mg/L BAP. Maximum percentage of response in cultures with more number of multiple shoots formation was recorded at 3.0-3.5 mg/L BAP in combination with 0.1 mg/L IAA. But multiple shoots induction was found to be reduced when the concentration of BAP increased beyond to 3.5 mg/L BAP (**Table 2**).

Various 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 Chupeau1993, Van Roekel *et al.,* 1993, Ling *et al.,* 1998). Where as in the present investigation 10-12 day-old cotyledons were selected as the source of explants which were found to be superior and showed maximum percentage of shoot buds proliferation efficiency with more number of multiple shoots formation in tomato cv S -22.While.

Gunay and Rao (1980) have found more multiple shoots induction on MS medium augmented with 2.0 mg/L BAP + 0.2 mg/L IAA of tomato cv Rio Grande. According to our observation, BAP alone in the medium supported for the callus induction and addition of 0.1 mg/L IAA to the medium along with 2.5-5.0 mg/L BAP induced multiple adventitious shoots. Similar results were also obtained from the cotyledon explants on MS + BAP with IAA in tomato (Oktem *et al.,* 1999, Fariduddin *et al.,* 2004).

The regenerated micro-shoots when sub-cultured on ½ strength MS, MS basal media and also the same plant growth regulators combination and concentration did not support elongation. Elongation of micro-shoots was found on all the concentrations of GA_3+3.5 mg/L BAP used (**Table 3**). Maximum percentage of shoot elongation and also longer shoots were recorded at 0.6 mg/L GA_3 + 3.5 mg/L BAP (**Fig.1c**) without callus formation.

For *in vitro* rooting the elongated shoots were cultured on ½ strength MS, MSO and MS medium supplemented with 0.5 mg/L IBA / IAA/ NAA (**Table 4**). Rooting was absent on ½ strength MS and MSO media and callus was formed without rhizogenesis at the basal region of the shoots. Root formation was initiated within 2 weeks of incubation in all the auxins used.100 % rooting was observed on MS medium supplemented with 0.5 mg/L IAA with profuse rhizogenesis (**Fig.1d**). *In vitro* rooting was reported without PGRS in tomato cv UC 105 (Mensuali-Sodi *et al*., 1995). However, the current study could find that cultivation of the micro-shoots on MS medium substituted with

Table.2. Effect of various concentrations of BAP + 0.1 mg/L IAA on organogenesis from cotyledon explants of tomato cv S-22

Concentration of PGRS (mg/L) IAA + BAP	% of Response	Morphogenic Response	Average number of shoots/ explant (±SE)[a]	Average length (in cm) of shoots (±SE)[a]
0.1+0.5	45	White friable Callus	---	---
0.1+1.0	45	White friable Callus	---	---
0.1+1.5	50	White friable Callus	---	---
0.1+2.0	55	White friable Callus	---	---
0.1+2.5	65	Multiple Shoots	3.4±0.30	0.8±0.22
0.1+3.0	80	Multiple Shoots	3.8±0.12	1.2±0.32
0.1+3.5	80	Multiple Shoots	4.0±0.20	1.4±0.36
0.1+4.0	75	Multiple Shoots	3.6±0.36	1.6±0.42
0.1+4.5	65	Multiple Shoots	3.5±0.30	1.0±0.33
0.1+5.0	60	Multiple Shoots	3.3±0.26	0.6±0.40

[a] **Mean ± Standard Error**

different auxins resulted an effective rooting than culturing on an auxin-free medium.

Table.3. Effect of GA_3 + 3.5 mg/L BAP on elongation of the micro-shoots in tomato cv S-22.

Medium + Growth regulators (mg/L)	% of Response	Average length of shoots (in cm) (±SE)[a]
MS+0.2 GA_3 + BAP	55	2.6±0.30
MS+0.4 GA_3 + BAP	70	3.0±0.12
MS+0.6 GA_3 + BAP	95	3.8±0.26
MS+0.8 GA_3 + BAP	80	3.6±0.41
MS+1.0 GA_3 + BAP	66	2.9±0.36

[a] **Mean ± Standard Error**

The *in vitro* regenerated plants were taken for hardening by removing the residues of agar followed by washing with sterile distilled water under aseptic conditions. Later these were shifted to plastic pots containing sterile vermiculite: soil (1:1) covered with polythene bags for four weeks (**Fig.1e**) followed by shifting to earthenware pots containing garden soil and maintained in the green house (**Fig.1f**). Later these were transferred to research field and maintained under shady place. The survival rate was found to be 70 % and the plants were similar to donor plants. Thus, the results presented here describe an efficient protocol for multiple shoots induction and plantlet establishment 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 agronomically useful transgenic plants.

Table 4: Effect of IBA, IAA and NAA on *in vtiro* rooting of cultivated tomato cv S-22

	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
	Rooting*	40	16±0.21

[a] **Mean ± Standard Error**
***With Callusing**

CONCLUSION.

From the above study, it is concluded that the plantlet development was established through direct organogenesis form cotyledon explants in cultivated tomato cv S 22 on MS medium supplemented with BAP(2.5-5.0 mg/L) + 0.1 mg/L IAA. This protocol is simple and reproducible, which can be used for *Agrobacterium tumefaciens* mediated genetic transformation in cultivated tomato cv S-22.

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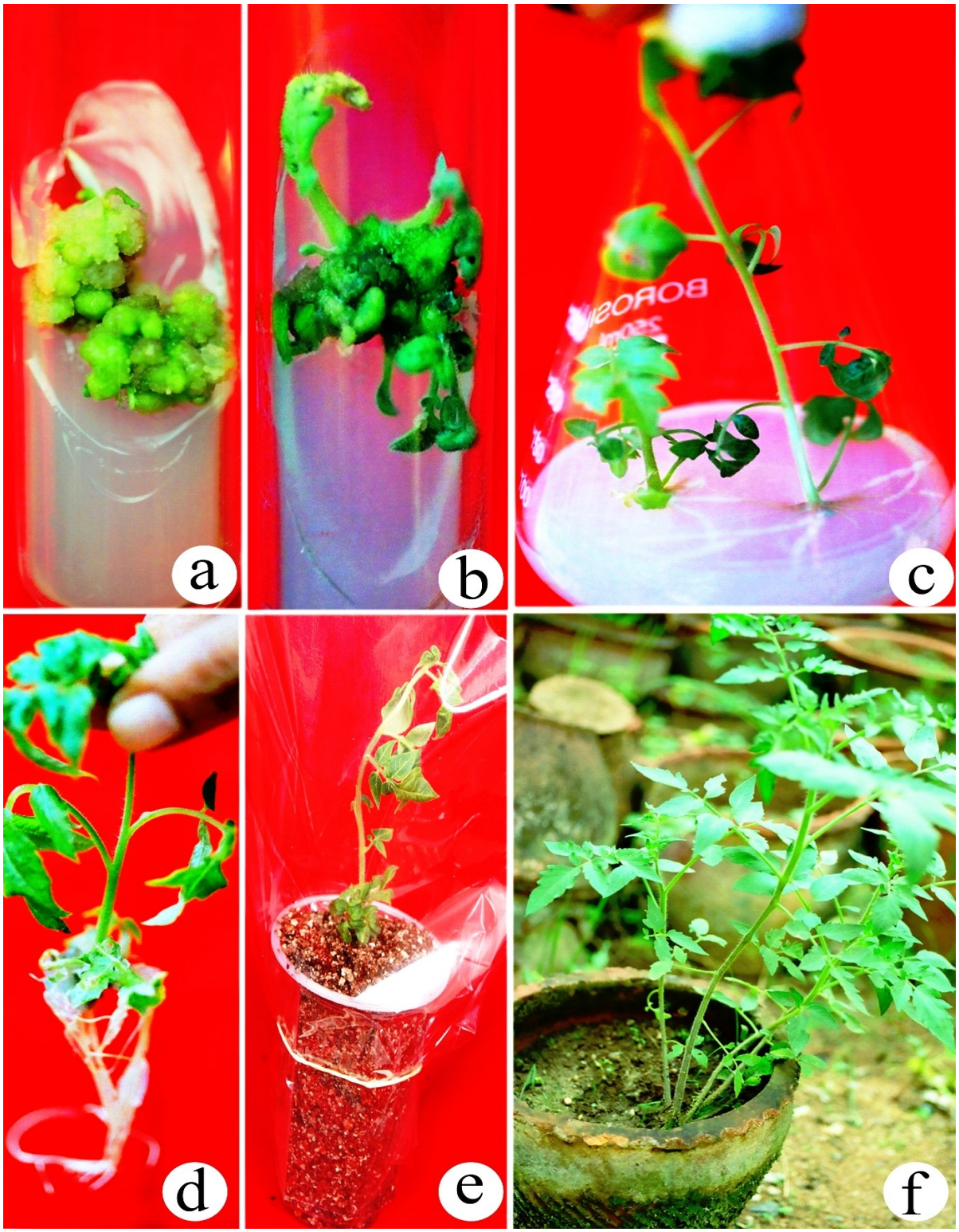

Fig 1: Callus induction and organogenesis from cotyledon explants in cultivated tomato cv S-22.
a: Callus induction on MS + 3.0 mg/L BAP (after 4 weeks of culture)
b: Multiple shoots induction from cotyledon explant on MS + 0.1 mg/L IAA + 3.5 mg/L BAP.
c: Shoot elongation on MS + 0.6 mg/L GA_3 + 3.5mg/L BAP.
d: Profuse rhizogenesis on MS+ 0.5mg/L IAA.
e: Acclimatization of regenerated plant.
f: Regenerated plant growing in the research field.