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ZEATIN INDUCED DIRECT *IN VITRO* SHOOT REGENERATION IN TOMATO (*SOLANUM LYCOPERSICUM* L.)

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ABSTRACT
The present investigation was undertaken to develop a highly efficient direct in vitro regeneration system, as an essential pre-requisite for the genetic transformation in tomato. Comparative studies on in vitro regeneration from cotyledon, hypocotyls and leaf explants of tomato revealed considerable variability in response to plant growth regulators. Direct shoot initiation was observed in medium supplemented with zeatin, while indirect shoot initiation with intermediate callusing phase, was observed in medium supplemented with benzylaminopurine (BAP) as cytokinin source. The MS medium supplemented with 2.0 mg/L zeatin + 0.2 mg/L indole acetic acid (IAA) was found to be the best regeneration medium with regards to regeneration efficiency, days to shoot initiation and number of shoots per explant. Among explants used cotyledons showed highest shoot regeneration efficiency (91.11%) followed by hypocotyls (87.77%) and leaf explant (85.55%). Earliest shoot initiation was observed in cotyledon explants (8.2 days), while highest numbers of shoots per explant (7.2) were obtained with hypocotyl explants. In vitro rooting initiation was observed within 14 days on MS medium devoid of any growth regulator. The in vitro rooted plantlets were successfully established in polycarbonated polyhouse with 86% survival rate.

INTRODUCTION

Tomato (*Solanum lycopersicum* L. formerly known as *Lycopersicon esculentum* Mill) (2n = 2x = 24) is commercially important vegetable crop throughout the world. It is grown under a wide range of climates (temperate or tropical) in the open field or under protected cultivation. Tomatoes are very valuable for human health since they are low in fat and calories, free of cholesterol and rich in vitamins A and C, b-carotene, lycopene and potassium, as well as octadecadienoic acid (Kim *et al.,* 2011).

There is a great potential for genetic manipulation in tomato to enhance productivity through increasing pest and disease resistance, environmental stress tolerance and to study gene function and regulation. Establishment of an efficient *in vitro* regeneration protocol is an essential prerequisite for harnessing the advantage of cell and tissue culture for genetic improvement. Till date several methods for *in vitro* regeneration of tomato have been described and cotyledons are the choice explant as they are quickly established and possess a high morphogenetic potential (Kaur and Bansal, 2010). However limitations with tomato regeneration system includes, morphogenetic response is highly growth regulator-dependent (Bhatia *et al.*, 2004), genotype specific response (Park *et al.,* 2003) and in most cases regeneration of shoots has been obtained through callus (Singh *et al.,* 2010; Kaur and Bansal, 2010).

Since tomato regeneration is genotype and procedure dependent, standardization of regeneration protocol for desired genotype is very important for genetic transformation. Therefore the aim of the present study was to simplify tomato regeneration procedure by simplifying medium conditions, avoiding callusing phase, eliminating frequent medium changes and systematically applying these conditions to achieve high regeneration efficiency.

MATERIALS AND METHODS

The seeds of Tomato (*Solanum lycopersicum* L.) variety "Dhanshree" were obtained from All India Coordinated Tomato Improvement Project, Mahatma Phule Krishi Vidyapeeth, Rahuri. These seeds were surface sterilized with 70% (v/v) ethanol for 1 min followed by 4% (w/v) sodium hypochlorite (NaClO) or 0.1% (w/v) mercuric chloride (**$HgCl_2$**) solution for 5/10 min. They were further rinsed five times with sterile distilled water and then inoculated on a Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar; and incubated at 25°C temperature with 16h light period and 8h dark period. Cotyledons and hypocotyls explants were obtained from 7-14 days old seedlings and leaves from 4-week old seedlings. Cotyledons, hypocotyls and leaves were aseptically excised from the *in vitro* grown seedling cultured on MS medium fortified with different levels of BAP, zeatin, IAA and naphthalene acetic acid (NAA). Those shoots greater than 2cm in length were excised and cultured on MS medium without any growth regulator or with either of 1mg/L of IAA, NAA and indole butyric acid (IBA). Plantlets with well developed roots were transferred to plastic pots containing coco peat: perlite (2:1). Those were initially covered with plastic bags for 5–7 days, and kept

at polycarbonated polyhouse. Plants were irrigated with half MS solution for 14 days and finally transferred to pot containing soil: cow dung (4:1) and irrigated with water at regular intervals. All treatments of regeneration experiments had three replicates and with 40 explants in each replication.

RESULTS AND DISCUSSION

Tomato is considered as a model plant for plant genetic engineering to investigate various physiological, biochemical and molecular mechanisms (Goel *et al*., 2011). The absence of highly efficient regeneration procedures is a main obstacle in the application of gene transfer technology to economically important tomato cultivars (Velcheva *et al.,* 2005). The present study depicts development of a rapid and efficient plantlet regeneration system in tomato genotype "Dhanshree".

Sodium hypochlorite was found better for seed sterilization as synchronous and high seed germination percentage was observed (Table 1). While using mercuric chloride, there was decrease in seed germination and it was not synchronous. Wu *et al*. (2011) also found that sodium hypochlorite is better than mercuric chloride for tomato seed disinfection.

The type and the concentration of the phytohormones in the shooting medium were found to be crucial factor controlling the multiple shoot induction. Multiple shoot formation with varying numbers was observed in all hormonal treatments. There exists significant difference among the treatments for days required for callus initiation, days to shoot initiation, number of shoots per explant and percent response to shoot induction. Direct shoot initiation was observed from all three explants cultured on regeneration medium supplemented with zeatin, while indirect shoot initiation was observed on regeneration medium supplemented with BAP. Raj *et al*. (2005) and Velcheva *et al*. (2005) also observed direct shoot initiation in tomato on a zeatin supplemented medium whereas Singh *et al*. (2010) observed indirect shoot initiation on BAP supplemented medium.

Several studies have established that cotyledon was best explant for promoting shoot organogenesis in tomato (Velcheva *et al.,* 2005; Sharma *et al*., 2009, Bhushan and Gupta, 2010). Park *et al*. (2003) reported that cotyledons and hypocotyls were better to leaves for regeneration and the same result was noticed in the present study. Cotyledon explants were found better than hypocotyl and leaf explants in terms of days to callus initiation, days to shoot initiation and shoot regeneration efficiency. The maximum direct shoot regeneration efficiency was observed in cotyledon explants (91.11 %) followed by hypocotyl explants (87.77 %) and leaf explants (85.55 %) when cultured on MS medium with 2.0 mg/l zeatin + 0.2 mg/L IAA (Table 2, 3, 4). Early shoot initiation was observed in cotyledons (8.2 days) followed by hypocotyl (8.8 days) and leaf explants (10.4 days). The maximum number of shoots per explant was obtained from hypocotyl explants (7.20 with an average of 4.92) as compared to cotyledon

Table 1: Effects of disinfectant on seed germination and contamination percentage

Disinfectant	5 Min. seed germination%	10 Min. Contamination %	seed germination%	Contamination %
$HgCl_2$ (0.1%)	50	0	44	0
NaClO (**4%**)	92	15	88	0

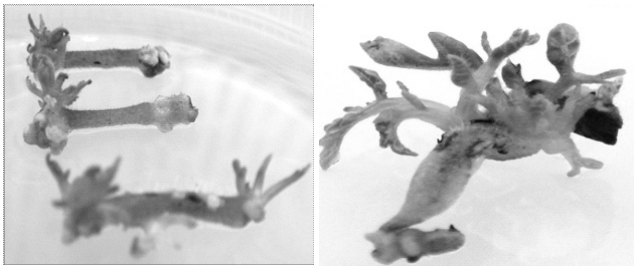

Figure 1: Direct multiple shoot induction after 8-10 days of incubation on MS + 2 mg/L zeatin + 0.2 mg/L IAA

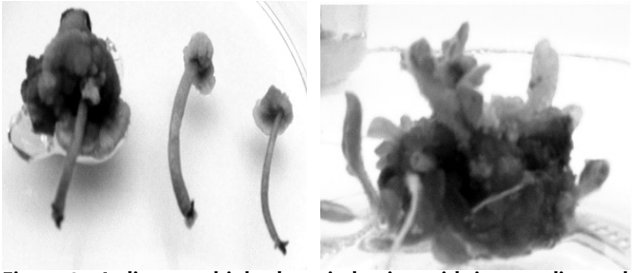

Figure 2: Indirect multiple shoot induction with intermediate callusing phase after incubation on MS + 2 mg/L BAP + 0.2 mg/L IAA

Table 2: Effect of medium composition on *in vitro* shoot regeneration from cotyledon explants

Treatment	Days to callus initiation	Days to shoot initiation	No. of shoots par explant	Efficiency of shoot regeneration (%)
MS + 1 mg/L BAP +0.1 mg/L NAA	8.1	16.9	2.7	59.14
MS +1 mg/L BAP+ 0.2 mg/L NAA	8.2	16.4	3	64.44
MS + 1 mg/L BAP + 0.1 mg/L IAA	7.5	14.7	3.7	74.44
MS + 1 mg/L BAP + 0.2 mg/L IAA	6.9	14.4	4.1	75.55
MS + 2 mg/L BAP + 0.2 mg/L IAA	6.6	13.9	4.5	76.70
MS + 1 mg/L zeatin + 0.1 mg/L IAA	No callus	10.8	5.4	81.11
MS +1 mg/L zeatin + 0.2 IAA	No callus	10.3	5.8	82.72
MS + 2 mg/L zeatin	No callus	11.6	3.5	68.89
MS + 2 mg/L zeatin + 0.1 mg/L IAA	No callus	8.6	6.4	88.89
MS + 2 mg/L zeatin + 0.2 mg/L IAA	No callus	8.2	6.6	91.11
Grand mean	7.46	12.58	4.57	76.30
Range	6.6-8.2	8.2-16.9	2.7-6.6	59.14-91.11
Standard Error	0.223	0.310	0.285	0.883
C.D. at 5 percent	0.6359	0.87	0.797	2.597

Table 3: Effect of medium composition on *in vitro* shoot regeneration from hypocotyl explants

Treatment	Days to callus initiation	Days to shoot initiation	No. of shoots par explant	Efficiency of shoot regeneration (%)
MS + 1 mg/L BAP +0.1 mg/L NAA	8.39	18.7	2.8	60
MS +1 mg/L BAP+ 0.2 mg/L NAA	8.3	18.4	3.4	61.11
MS + 1 mg/L BAP + 0.1 mg/L IAA	7.8	15.5	4.2	70
MS + 1 mg/L BAP + 0.2 mg/L IAA	7.8	15.4	4.7	73.33
MS + 2 mg/L BAP + 0.2 mg/L IAA	7.5	14.3	5.1	74.44
MS + 1 mg/L zeatin + 0.1 mg/L IAA	No callus	10.4	5.4	78.86
MS +1 mg/L zeatin + 0.2 mg/L IAA	No callus	10.3	5.7	80
MS + 2 mg/L zeatin	No callus	11.7	3.8	65.55
MS + 2 mg/L zeatin + 0.1 mg/L IAA	No callus	8.89	6.9	86.66
MS + 2 mg/L zeatin + 0.2 mg/L IAA	No callus	8.8	7.2	87.77
Grand mean	7.96	13.24	4.92	73.77
Range	7.5-8.39	8.8-18.7	2.8-7.2	60-87.77
Standard Error	0.232	0.314	0.297	0.997
C.D. at 5 percent	0.649	0.879	0.831	2.932

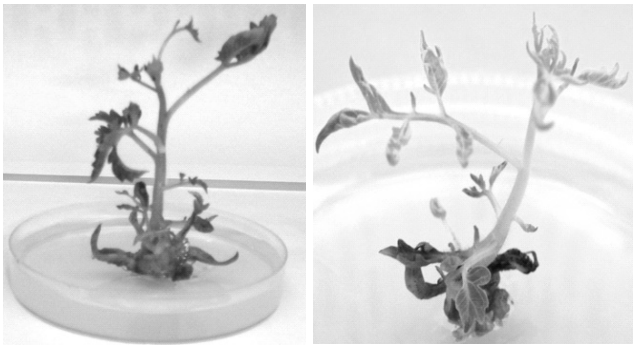

Figure 3: Elongation of *in vitro*-raised shoot

explants (6.60 with an average of 4.57) and leaf explants (6.60 with an average of 4.13). Singh *et al*. (2010) also reported maximum number of shoots per explant from the hypocotyls than cotyledon explants.

Maximum indirect shoot regeneration efficiency (76.70 %) was observed when cotyledon explants were cultured on MS medium with 2.0 mg/L BAP and 0.2 mg/L IAA. Earliest callus initiation in cotyledons (6.6 days) followed by hypocotyl (7.5 days) and leaf explants (7.9 days) was observed on same medium. Singh *et al*. (2010) also observed maximum indirect shoot regeneration on regeneration medium comprising MS medium in combination with 2.0 mg/L BAP.

Devi *et al*. (2008) also reported that with increase in hormonal concentration there was corresponding increase in the frequency of callus formation, percent plant regeneration and the number of shoots per explants. In the present study also gradual increase in regeneration efficiency, number of shoots per explant was observed with increasing concentrations of either BAP or zeatin (1.0 - 2.0 mg/L) in combination with IAA (0.1- 0.2 mg/L) in all the explant tested. Medium supplemented with zeatin in combination with IAA exhibited better results than medium supplemented BAP and IAA. However when zeatin was used alone in culture medium it resulted in less number of multiple shoot per explant, required more days for shoot initiation and also showed low shoot regeneration as compared to medium supplemented with combination of BAP and IAA. It has been reported earlier that zeatin in combination with IAA improved the efficiency of shoot regeneration in tomato (Park *et al.,* 2003; Van *et al.,* 2010).

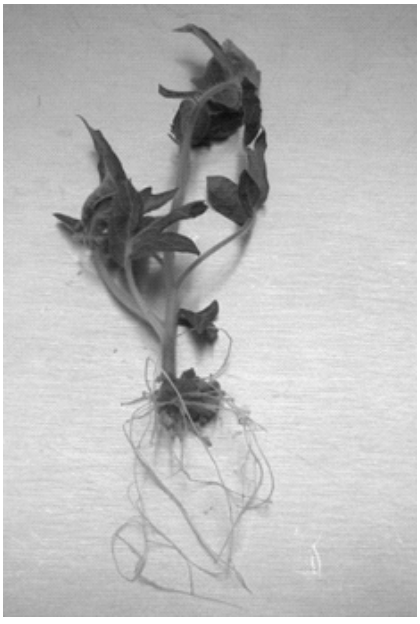

Figure 4: *In vitro* regenerated young plantlet with well developed roots

Figure 5: Well developed tomato plant in polycarbonated polyhouse

Table 4: Effect of medium composition on *in vitro* shoot regeneration from leaf explants

Treatment	Days to callus initiation	Days to shoot initiation	No. of shoots par explant	Efficiency of shoot regeneration (%)
MS + 1 mg/L BAP +0.1 mg/L NAA	8.89	20.9	2.4	57.77
MS +1 mg/L BAP+ 0.2 mg/L NAA	8.8	20.6	2.5	58.89
MS + 1 mg/L BAP + 0.1 mg/L IAA	8	16..1	3.3	67.77
MS + 1 mg/L BAP + 0.2 mg/L IAA	8.1	16.4	3.5	68.89
MS + 2 mg/L BAP + 0.2 mg/L IAA	7.9	14.7	4.1	73.33
MS + 1 mg/L zeatin + 0.1 mg/L IAA	No callus	12.5	4.8	77.77
MS +1 mg/L zeatin + 0.2 mg/L IAA	No callus	11.2	5.3	78.89
MS + 2 mg/L zeatin	No callus	13.3	2.7	63.33
MS + 2 mg/L zeatin + 0.1 mg/L IAA	No callus	10.6	6.1	84.44
MS + 2 mg/L zeatin + 0.2 mg/L IAA	No callus	10.4	6.6	85.55
Grand mean	8.34	14.67	4.13	71.66
Range	7.9-8.89	10.4-20.9	2.4-6.6	57.77-85.55
Standard Error	0.232	0.362	0.319	0.864
C.D. at 5 percent	0.651	1.013	0.892	2.54

Table 5: Effect of culture medium on the rooting of *in vitro* multiplied and elongated shoots

Treatment	Rooting (%)	Days to rooting
MS	94.66	14
MS +1 mg/L IBA	68.66	19.33
MS + 1 mg/L IAA	72.66	15.66
MS + 1 mg/L NAA	65.33	17.66
Grand mean	75.33	16.66
Range	65.33-94.66	14-19.33
Standard Error	2.74	0.64
C.D. at 5 percent	2.32	1.63

In the present investigation, full-strength MS medium without any growth regulator gave the best results in terms of rooting percentage (94.66%) and days to root initiation (14 days). MS medium supplemented with either of 1mg/L IBA, NAA or IAA showed lower rooting percentage (65.33-72.66%) and also required more days to rooting (15.66-19.33 days) (Table 5). Bhushan and Gupta, (2010), Velcheva *et al*. (2005) and Singh *et al*. (2010) also reported rooting in hormone free MS medium. All the rooted plantlets were hardened in polycarbonated polyhouse of which 86 % plants survived and exhibit normal morphological growth.

In summary, we report a highly improved, efficient and reproducible regeneration protocol for *in vitro* direct plant regeneration in tomato. Cotyledons were found to be more responsive to direct shoot regeneration as compared to hypocotyls. Direct plant regenera-tion which is desired for genetic transformation, was achieved due to zeatin. This optimized regeneration protocol can be efficiently used for *Agrobacterium* mediated genetic transformation in tomato.

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