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Regeneration study in *Solanum lycopersicum*

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ABSTRACT

In vitro culture is one of the key tools of plant biotechnology that exploits the totipotent nature of plant cells, a concept proposed by Haberlandt and unequivocally demonstrated for the first time by Steward *et al.* Tomato (*Solanum lycopersicum* Mill.) is a high-yielding crop and has become most important plant in present. It is a valuable species for studying plant biology because it allows studying the integration of the tools and concepts of genetics, molecular biology and genetic engineering for studying and manipulating all of these processes.Many studies have been conducted on tomato to establish an efficient system of shoot multiplication and regeneration with growth regulators.

This vegetable is one of the most investigated crops both at genetic and genomic level not only because of its economic importance but also it is one of the best characterized plant systems. Biotechnological approaches are extensively employed for introducing foreign genes into high-yielding genotypes. However, the success of genetic manipulation of a plant depends on the regeneration frequencies of the explants.

This study presents tissue-culture based regeneration protocol for tomato (*Solanum lycopersicum* Mill.). It has been attempted through the use of

tomato hypocotyl as explants. Efficient and reproducible regeneration protocol was evaluated .

For tomato regeneration, a wide variety of plant growth regulators have been used with varying concentrations. Many cytokinin and auxin combinations could induce shoot proliferation in tomato. MS Regeneration media with BAP 0.5mg/ litre and kinetin 0.5mg/litre supplementation was found best for maximum number of shoot formation and reported that hypocotyl is the best explant source for callus formation and regeneration.

Successful regeneration of tomato will be greatly aided by genotype-specific determination of crucial parameters on improving *in vitro* regeneration by hypocotyl, followed by growth into a whole plant regenerated. As though tomato is recognized as a highly valuable and nutritious food. It is the second most popular vegetable.

Keywords: *Solanum lycopersicum*, Regeneration, Callus, Benzyl amino purine

1. INTRODUCTION

Botanically tomato belongs to family Solanaceae and named scientifically as *Solanum lycopersicum* L. It is recognized as a highly valuable and nutritious food. It is the second most popular vegetable crop next to potato in the world (Bhatia *et al.*, 2004; Foolad, 2004). This family also includes chilli, peppers, potato, eggplant etc. Like all known species of the genus Solanum, tomato is a diploid, it has 2n=24 chromosomes, and a genome size of 950 Mbp, which is composed of 77% heterochromatin and 23% euchromatin (Peterson *et al.*, 1996). Tomato plants are vines typically growing six feet or more above ground if supported. Most tomato plants have compound leaves, the leaves are 10-25 centimeter (4-10 inc) long odd pinnate, with 5-9 leaflets on petioles, each leaflet up to 8 centimeters (3 inc) long, with a serrated margin, both the stem and leaves are densely glandular–hairy. Their flowers appear on the apical meristem and self fertilizing. The flowers are 1-2 centimeters (0.4-0.8 Inc) across, yellow, with five petioled lobes on the corolla; they are borne in cymes of 3 to 12 together. Tomato fruit is classified as berry. The fruit is edible, bright colored, soft and succulent. Fruit size is generally 1-2 inches diameters in wild plants and commonly much larger in cultivated form (Islam, 2007).The tomato crop is very versatile and is grown either for fresh market or processing. Tomato fruits are eaten fresh and used in processing more productions like pastes and ketchup. Tomato is rich in vitamins A and C and fiber,and is also cholesterol free (Block *et al.*, 1992; Gerster,1997; Rao and Agarwal,2000).Tomatoes contain large amounts of vitamin C, providing 40 percent of the daily value (DV).They also contain 15 percent DV of vitamin A, 8 percent DV of potassium, and 7 percent of the recommended dietary allowance (RDA) of iron for women and 10 percent RDA for men.

2. Plant Regeneration Systems

In vitro culture is one of the key tools of plant biotechnology that exploits the totipotent nature of plant cells, a concept proposed by Haberlandt and unequivocally demonstrated for the first time by Steward *et al.* The tissue

culture regeneration system of tomato is influenced by genotype, type of explants, hormones, and other factors (Rai *et al.* 2012, 2013). Many studies demonstrate that variety characteristics are the key factors of tomato regeneration, so it is important to choose the appropriate acceptor. Selecting the appropriate varieties, explants and the additives of medium can increase the frequency of transformation of tomato. Although tomato plant regeneration has been achieved mainly through organogenesis, the somatic embryogenesis also has been explored.

3. Alternative way of regeneration:

Tomato has high degree of self pollination and non availability of suitable germplasm. Moreover, it is a per-dominantly inbreeding species and its genetic variation tends to decrease. So, these problems hamper to improve tomato characters through conventional breeding program. Besides, this method takes long time, extending over seven to eight years involving crossing and selection of desirable traits. *In vitro* regeneration technique helps to provide unique possibilities for overcoming the barriers of incompatibility between remote species and it facilitates rapid introduction of new varieties (Parveen, 2011). For raising transgenic crops with useful traits efficient *in vitro* plant regeneration protocol is necessary. As far as tomato is concerned, a good deal of tissue culture work. However, standard regeneration protocol with farmer popular tomato varieties of Bangladesh has not been explored extensively. Very few labs of India are working with establishment of reproducible regeneration protocol of locally grown tomatoes. (Chowdhury, 2008; Das, 2011; Ferdous, 2012 and Sarker, 2013).

Tomato is quite amenable and responsive to *in vitro* regeneration (Fari *et al.*, 1992). *In vitro* technique help to overcome the barrier of self incompatibility facilitates rapid introduction of new traits (Taji *et al.*, 2002) and development of disease free plant (Moghaleb *et al.*, 1999). For *in vitro* regeneration researchers have used various types of explants sources viz, cotyledon (Schutze and Wieczorrek, 1987), hypocotyls (Plastira and Perdikaris, 1997;

Gunay and Rao 1980), pedicel/peduncle (Compton and Veilleux, 1991), leaf (Duzyaman *et al.*, 1994), stem sections and inflorescence (Applewhite *et al.,* 1994).In tomato, adventitious shoot regeneration can be achieved either directly (Dwivedi *et al.*,1990) or indirectly through an intermediate callus phase (Behki and Lesley, 1980; Geetha *et al.*, 1998). However, both callus and shoots may be produced together (Bhatia, 2004).

Fari and her colleges reported a simple and efficient organogenetic mechanism of shoot regeneration via seedling decapitation method for tomato (Fari *et al.*, 1991).

In order to develop efficient and reliable procedure for regeneration, from different tomato varieties, various types of explants were cultured in MS media supplemented with various hormones in different concentrations and combinations.

4. *MATERIALS and METHODS*

The present investigations were carried out at Tissue culture and regeneration Lab, Institute of applied medicine and research, Ghaziabad. The materials in the conduct of experiment and methods employed are detailed in this chapter:

4.1. Materials

4.1.1. Glasswares and Plasticwares

All the glasswares used were of Scott Duran brand.

All plasticwares and disposable petri plates were of 'Tarson' brand. Micropipette tips were of 'Tarson' brand.

4.1.2. Sterilization

All glasswares were sterilized in an oven at 240^0C for 2 hours. Micropipette tips arranged in tip boxes and petriplates were packed in

autoclavable stainless steel containers (Himedia). Beakers and bottles containing double distilled water were properly covered with aluminium foil. Then they were autoclaved at 15 lbs (1.06 Kg cm^{-2}) pressure at approximately 121^0C for 15 minutes.

4.1.3. Equipments

Laminar airflow of Kirlosker brand, weigh machine of 'Avon weigh systems (AND)', autoclave, oven and microwave oven were used. 'Millipore' double distillation unit was used to get double distilled water. Shaker of 'Bio-rad' and Micropipettes used were of 'Tripette' brand. Membrane filter units of 'Nalgene' were used to filter sterilize antibiotic stocks and thermolabile hormones.

4.1.5. Chemicals

All chemicals, hormones, vitamins, salts used were of tissue culture and analytical grade from Himedia and Merck brand.

4.2 Methods

4.2.1. Tissue Culture Media

The media used for in vitro multiplication of tomato was MS (Murashige and Skoog 1962) based medium. Media was prepared in double distilled water using different constituents, the composition of which is given:

Table 3.1. ***Composition of modified MS basal medium.***

Compounds	**Concentration (mg L^{-1})**
Inorganic:	
Macronutrients	
NH4NO3	1650

KNO_3	1900
$CaCl_2$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Micronutrients	
KI	0.83
H_3BO_3	6.2
$MnSO_4.H_2O$	55.75
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	38.8
Vitamins	**Concentration (mg L^{-1})**
Inositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1

Amino acid	
Glycine	2.0
Carbon Source	
Sucrose	30’000
Solidifying agent	
Agar powder	6000

4.2.2. Preparation of Plant Tissue Culture Media:

1. Approximately 60% of the final required volume of double distilled water was measured and kept in a container twice the size of final volume.

2. Appropriate volume of the stock 1 to stock 4 was measured and mixed thoroughly.

3. Sucrose powder was added to the double distilled water and stirred until completely dissolved.

4. pH of the media was adjusted to 5.9.

5. Additional double distilled water was added to the medium solution to obtain the final required volume.

6. Required amount of agar was added and then the media was boiled in oven after cooling the media was packed in flasks and autoclaved.

Growth hormones were added in the modified basal media as per requirements. Sucrose was added at 3% concentration prior to the sterilization. pH of the media was adjusted 5.9 with the help of pH meter. If pH is high to 5.9 few microliter HCl was added, otherwise pH is low to 5.9

few microliter NaOH was added in the media. 0.6% agar was added. Then the media was dispended in conical flasks and boil in oven.

The flasks were capped and covered with double layer aluminum foil and autoclaved at 15 lbs (1.06 kg/m^2) pressure, 121^0C temperature for 30 min.

4.2.3. Source of explants

The starting materials of these experiments were fruits. The fruits of Tomato variety DVRT-1 were obtained from the herbal garden of Institute Of Applied Medicine And Research, Ghaziabad. Hypocotyl explants were excised from 3-9 days old *in vitro* germinated seedlings of DVRT-1.

4.2.4 Seed germination

4.2.4.1 Surface Sterilization of Seeds

Seeds were obtained directly from ripe fruits. Fruits taken from field were washed with running tape water. The fruits were dipped in ethyl alcohol and flamed, then the fruits were cut open and the seeds along with mucilaginous pulp were collected in a sterile beaker. Then 4 ml HCl was added and kept for 3 minute with vigorous shaking. The dissolved pulp was then decanted and again 2% HCl treatment was given for 3 minutes. After that pulp is decant and seeds so obtained were rinsed 2-3 times with sterile double distilled water followed by treatment of 6% Sodium hypochlorite solution for 3 minutes. Finally seeds were rinsed 3-4 times with sterile double distilled water and then were plated on half strength MS basal medium for germination. The seeds germinated in 4-5 days.

4.2.4.2 Seed Germination

After surface sterilization, seeds were blot dried in an autoclaved petriplate containing sterile tissue paper covered with lid. These seeds were inoculated into Magenta boxes for seed germination, containing

50 ml half strength Murashige and Skoog (1962) media. In each box 40-50 seeds were inoculated and all the boxes containing seeds were incubated initially in dark for 3 days and later transferred to 16:8 hour photoperiod at 25±2^{0}C. Up removal from incubation, the numbers of germinated and elongated seeds were counted. Contamination was determined by visual inspection for fungal and/ or bacterial growth.

4.2.4.3 Explant isolation

Hypocotyls explants were isolated from 8-15 days old seedlings. Prior to regeneration explants were isolated from aseptically grown seedlings and cultured on MS media supplemented with different concentrations and combination of BA and kinetin either alone or in combination. MS medium supplemented with 0.5 to 2mg/l BA and 0.5 to 2 mg/l kinetin was tested.

4.3 Effect of BA and Kinetin on shoot and root regeneration

Explants were cultured on MS medium supplemented with two cytokinins BA at (0.5, 1, 2 mg/l) and Kinetin at (0.5, 1, 2mg/l).

All cultured plates were incubated under 16 hr day and 8 hr night photoperiod conditions for 3 weeks, and temperature is adjusted to 25^{0}c ±2^{0}c. After one month each plate was tested for the shoot regeneration and root development.

5. RESULTS

5.1. *In vitro* Seed germination

Current study was conducted in three phases. The work started with a regeneration assessment study, followed by regeneration using hypocotyl explant and ended at establishment of a reproducible plant regeneration protocol.

For regeneration of tomato seeds were isolated from fruits. The fruits were collected from the plant of *Solanum lycopersicum* L. DVRT-1 from the herbal garden of Institute of Applied Medicine and Research, Ghaziabad.

Before germination the fruits and seeds were surface sterilized as per the procedure described earlier. The sterilized seeds of these cultivars were allowed to germinate in magenta boxes containing half strength Murashige and Skoog medium. The cultures were kept undisturbed for 2-9 days.

10 different seed lots were germinated on different dates. It has been observed that seed germination percentage range from 75.2% to 98.8%. The highest germination was found in Lot-4, followed by Lot no.-1and Lot no.-3.

In majority of the seed lots the germination percentage was recorded mostly above 80%.

Among 770 inoculated seeds 749 seeds were germinated. This accounts 97.2 % of average seed germination.

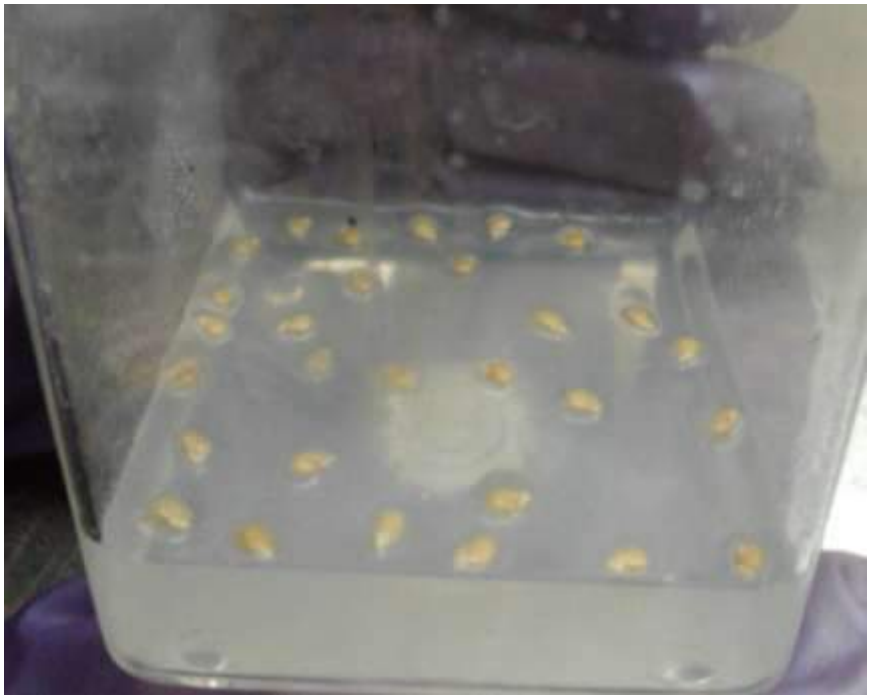

Fig.4.1.1. Day first seed inoculation .

Fig.4.1.2 Second day of seed inoculation .

Fig.4.1.3seed germination after 3 days of inoculation.

Fig.4.1.4 seedling after 4 days of inoculation

Fig4.1.5 seedling after 6 days of inoculation.

Fig.4.1.6 seedilng after 8 days of germination

Table 4.1. Germination percentage of seeds in different lots.

Lot no.	No. of seeds inoculated	No. of seeds Germinated	Percentage of seed Germination
Lot 1	120	115	95.8%
Lot 2	108	92	85.1%
Lot 3	142	130	91.3%
Lot 4	89	88	98.8%
Lot 5	105	92	87.6%
Lot 6	125	94	75.2%
Lot 7	94	85	90.4%
Lot 8	68	53	77.9%
Total	**770**	**749**	**97.2%**

5.2 Explant regeneration

For regeneration, hypocotyls explants were isolated from the seedling of *Solanum lycopersicum* L DVRT-1 from the herbal garden of Institute of Applied Medicine and Research,Ghaziabad.

10 experiments were conducted with hypocotyl explants. Seedling age for regeneration was kept variable. It has been observed that, the hypocotyl explants isolated from 11 days old seedlings give best regeneration frequency. The explants were then inoculated in the MS media having different concentrations of BAP and kinetin. As far as age of explants is concerned, which is related to the meristematic

activity of explants, young explants were more amenable to regenerate than older explants . The hypocotyl explants which were inoculated at a young age showed more regeneration then the explants of an older age.

After one months, regeneration of hypocotly explants were observed. MS Regeneration media supplemented with 0.5mg/ litre BAP and 0.5mg/litre kinetin was found best for maximum number of shoot formation and reported that hypocotyl is the best explant source for callus formation and regeneration. Out of 801 hypocotyl explants only 660 explants showed regeneration.

The surviving explants were further transfer in same MS medium for shoot elongation and root induction. On that medium putatively regenerated plantlets were grown.

Fig.4.2.1 seedling after 10 days of germination.

Fig.4.2.3 Hypocotyl explants after 5 days of inoculation

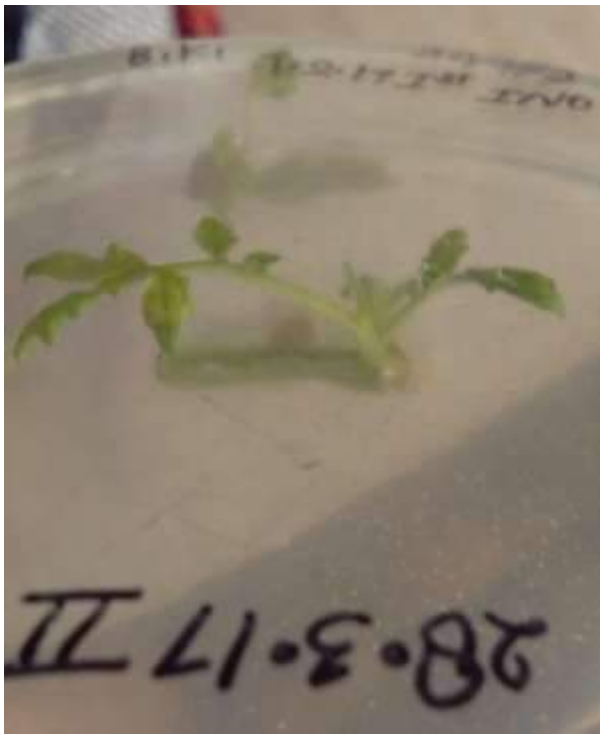

Fig.4.2.4 Regeneration recorded in hypocotyl explant

Fig.4.2.5 regeneration and callus formation

Fig.4.2.6 Putative plant regeneration seen in hypocotyl explant

Table4.2.1- Effect of seedling age on regeneration.

S.no	No. of explants	Age of explants [Seedlings] (Days)	No.of explants grown on regeneration medium after 15 Days	No. of explants grown on regeneration medium after 1 months	Regeneration frequency (%)
1.	78	8	32	50	64%
2.	81	9	35	65	80%

3.	76	10	42	70	92%
4.	82	11	55	80	97%
5.	87	11	40	86	98%
6.	78	12	34	66	84%
7.	79	12	47	64	81%
8.	85	13	39	74	87%
9.	80	14	34	65	81%
10.	75	15	35	66	88%
TOTAL	**801**			**686**	**85.64%**

Table 4.2.2. Effect of hormone concentration on regeneration of hypocotyl explant.

s.no	Explants type	MS medium supplemented with hormone concentration	No.of explants inoculated	No.of explants showing regeneration	% of regeneration

1	Hypocotyl	BAP 0.5mg/l	78	56	71%
2	Hypocotyl	Kin 0.5 mg/l	81	62	76%
3	Hypocotyl	BAP 1 mg/l	76	58	76%
4	Hypocotyl	Kin 1 mg/l	82	68	82%
5	Hypocotyl	BAP 2mg/l	87	70	80%
6	Hypocotyl	Kin 2 mg/l	78	66	84%
7	Hypocotyl	BAP 0.5mg/l+Kin 0.5 mg/l	79	78	98%
8	Hypocotyl	BAP 1mg/l+Kin 1 mg/l	85	73	85%
9	Hypocotyl	BAP 2mg/l+Kin 2 mg/l	80	67	83%
10	Hypocotyl	BAP 3mg/l+Kin 3mg/l	75	62	82%
			801	**660**	**82%**

6. Discussion

In the present investigations, efforts were made to regenerate DVRT-1 cultivars of tomato by in-vitro regeneration method. The regeneration studies were carried to regenerate the tomato cultivar DVRT-1. The explants like hypocotyls were isolated from 10 to 11 days old *in vitro* germinated seedling; and were cultured on MS media carrying different concentrations and combinations of Kinetin and BAP. For tomato regeneration, a wide variety of plant growth regulators were used with varying concentrations.

Many cytokinin and auxin combinations were induced for shoot proliferation in tomato. MS Regeneration media supplemented with BAP 0.5mg/ litre and kinetin 0.5mg/litre was found best for maximum number of shoot formation and reported that hypocotyl is the best explant source for callus formation and regeneration.

Putative plants were regenerated from the hypocotyl explants of tomato. In this study, shoot regeneration from hypocotyl was observed to be 96-98% . Varieties and explant sources showed significant difference in results from different media combinations. Hypocotyl derived regeneration varied significantly among the treatments used. Another factor studied was seedling age taken for regeneration. Explants excised from 10 to 11 days old seedlings give best regeneration frequency.

This observation has been found to be similar with the regeneration potential 90%). Earlier, Efficient plantlet regeneration in tomato has been reported from the hypocotyls Asakura N, Misoo S, Kamijima O & Sawano M (1995). Regeneration of tomato has been attempted through the use of explants viz. cotyledon Rai *et al.* (2012). Gubis *et al.* (2004) studied the effect of different growth regulators and plant regeneration of tomato explants, where tomato regeneration response has been found to depend largely on genotype, explants, and plant growth regulator used in culture medium.

No visual phenotypic abnormalities were recorded in any of the plants. The present regeneration protocols are considered to be more

suitable for regeneration of tomato. The explants selected for regeneration are programmed basically for direct shoot organogenesis. While seed embryo explants selected for regeneration, are programmed for direct plant regeneration with minimal tissue culture steps. The progeny developed from explants will be true to the type and free from virus.

7. Conclusion

The development of efficient regeneration protocol is a pre-requisite for incorporation of agronomically important gene(s). Regeneration of tomato hypocotyl by McCormic *et al.* (1986,) has opened a new era in tomato transgenic technology. It shows that a whole plant could be regenerated from a single cell. It has created an exciting scenario in the field of genetic manipulation and crop improvement. The growing realization of the potentialities of plant cell and tissue culture for plant breeding has itself provided a substantial impetus for research related to generation of variability and selection of variants.

In this study, regeneration by shoot organogenesis has been investigated. For efficient regeneration, the preparation of explants plays an important role. The regeneration response found to be highly dependable on genotype, explant and plant growth regulators used in the culture medium (Bhatia *et al.,* 2004; Ishag and Osman, 2009; Chaudhry *et al.,* 2010; Praveen, 2010). Inspired from these findings, present study was conducted to verify regeneration efficiency of the chosen varieties through previously optimized tissue culture protocol.

In the present studies, hypocotyl explants were excised from *in vitro* geminated seedlings. The best results were found in the MS medium having the concentration BAP+Kin (0.5 mg/l each).

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