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Production of Virus Free Seeds using Meristem Culture in Tomato Plant under Tropical Conditions

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Abstract

Protocol was established for production of virus free healthy seeds using meristem (0.3-0.5 mm in size) culture and field management under net house condition in tomato. The isolated meristem was found well established in MS liquid medium containing 0.1 mg l^{-1} of GA_3. For shoot and root development either from primary meristem or from nodal segment of meristem derived plants, semisolid MS medium having 0.5 mg l^{-1} of IBA was found most effective. The elimination of the studied viruses (ToMV, CMV, ToLCV) in meristem-derived plants was confirmed by DAS-ELISA test. For field management of the virus eradicated meristem-derived plants, use of net house was found very effective measures to check viral vector visit and eventually infection. The meristem-derived plants were vigor and high yielder than the native seed derived plants and produced healthy seeds. Due to stop vector visit, no viral symptoms were observed in both R_1 and R_2 plants cultivated in net house condition. Starting of viral infestation was observed in R_2 generation when they were planted in open house condition without control of vector visit. Therefore, for management of viral diseases, use of virus free meristem derived plantlets and their subsequent cultivation in soil under net house condition without using any vector killing insecticide can be recommended for producing healthy seeds in tomato. The developed protocol for environmentally healthy tomato seed production in Bangladesh may be used in the countries having similar tropical like environment conducive for viral vector visit.

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Introduction

Tomato (*Lycopersicon esculentum* Mill.) is specially honoured due to its high nutritive value, tests, versatile use as vegetable and preparation of various food items. Among the different factors responsible for low yield of tomato, viral diseases are one of them. Lukyanenko (1991) and Martelli and Quacquarelli (1982) reported that tomato is susceptible to more than 40 different viruses. In Bangladesh (considered as tropical like country) 16 different tomato viruses have been reported (Akanda et al. 1991; Akanda 1994). Among the recorded viruses tomato mosaic virus (ToMV), tomato leaf curl virus (ToLCV), and tomato yellow leaf curl virus (ToYLCV) causes 80%, 90% and 100% fruit yield losses, respectively (Lukyanenko 1991). Other tropical, subtropical and warm temperate regions have similar pictures (Green and Kallo 1994). These diseases are caused by geminiviruses that are transmitted by the whitefly *Bemisia tabaci* (Czosnck et al. 1988). Little resistance against those viruses was found among the existing cultivated tomato cultivars (Zakay et al. 1991). Therefore, there is limitation to solve these problems through conventional breeding procedures. As tomato is a systemic host for many plant viruses, some of which (e.g. Tomato mosaic virus) can be transmitted through seed tissues or carried on the seed coat to re-infect seedlings (George and Sherrington 1984). Tomato plants grown from fresh seeds collected in infected fields showed symptoms of the diseases and the presence of virus was confirmed by serological tests (Lourdes et al. 1986). So, proper virus elimination techniques are needed for successful disease free tomato production. Plant meristem culture is a unique

technique to free away various pathogens from plant including viruses, viroides, mycoplasma, bacteria and fungi (Morel and Martin 1952; Walkey 1978; Bhojwani and Razdan 1983; Pierik 1989). Tomato is therefore another example of where tissue culture might be used to propagate the virus free plants (George and Sherrington 1984). Therefore, present investigation was carried out to develop a suitable protocol for producing virus free tomato clones followed by healthy seeds production under net house management for tropical, subtropical and warm temperate environmental conditions where viral diseases are very frequent. For this purpose the experiment was conducted in two steps. In the first step, the experiment was done to examine the use of meristem culture for producing virus free clones, and in the second step, management of virus free meristem derived plants in net house condition for commercial production of healthy seeds.

Materials and Methods

Shoot tips from 30-35 days old of the studied three viruses viz. ToMV, CMV (Cucumber Mosaic Virus) and ToLCV infected seed derived field grown native tomato plants were used as explants for initiation of meristem culture during the months of November to March (tomato cultivation time in Bangladesh). At first the excised shoot tips were sterilized in 70% ethanol and tween 20 followed by 3-5 times washing with sterilized distilled water. Then the explants were treated in 0.1% $HgCl_2$ (Mercuric chloride) solution with gently shaking for 3 min followed by 3-5 times washing with sterilized distilled water. All these operations were done inside the running laminar airflow. From sterilized shoot tips, immature leaves and leaf primordia were snapped off and then meristems (0.3-0.5 mm) were isolated using binocular stereoscopic microscope. After that the isolated meristem was quickly transferred on the filter paper bridge in test tubes containing liquid hormone free MS (MS_0), and MS (Murashige and Skoog 1962) medium supplemented with various concentrations of BA (6-benzyladenine) and GA_3 (gibberellic acid) singly for primary establishment of isolated meristem (Figure A and B). In this level, data were recorded on quick responses (days) and on percentage of meristem shows growth response for the establishment of primary meristem culture of the studied virus infected tomato cultivars (Table 1) After 3-4 weeks of inoculation the developed meristems were subcultured on semisolid MS_0 (control) and MS with GA_3, Kin (2-furfurylamino purine) and IBA (indole-3-butyric acid) singly for shoot and root induction (Figure C and D). Two concentration (1.0 and 0.5 mg L^{-1}) was used for each growth regulators. Data were recorded on number of root development explant^{-1}, shoot length and root formation (Table 2). The developed clones were checked for the elimination of three kind of viruses viz. ToMV, CMV, ToLCV using DAS- ELISA (double antibody sandwich enzyme linked immunosorbent assay) test (Clark and Adams 1977; Konate et al. 1995). Polyclonal antisera of ToMV, CMV and ToLCV were used in DAS- ELISA for serodiagnosis. DAS- ELISA was performed according to supplier's (Agia-UK, Identikit 2002) manual. Data were recorded on percentage of sample explants not reacted against the described four antisera (Table 3). The nodal segments of the virus eradicated plant stocks were cut and placed on semisolid MS_0, MS with 0.5 mg L^{-1} GA_3, and MS with 0.5 mg L^{-1} IBA media for clonal multiplication (Figure E). All the cultures were incubated in growth chamber, at 24±1℃ for 16 h photoperiod under cool white fluorescent lamps (Phillips Bangladesh Ltd.) and the light intensity was maintained at 28-34 mol $m^{-2}s^{-1}$. When the plantlets attained in 5-8 cm height with few leaves and well-developed root systems, were taken out from the controlled environment of growth chamber, and were successfully acclimatized (Figure F). After acclimatization, the mericlones (R_1 virus free plants) were transplanted in the two field conditions (open field, and net house, as shown in Figure G) during November 2000 to March 2001. The seeds collected from R_1 plants grown in both net and open field conditions were used for evaluation of R_2 generation. Like R_1, the R_2 plants were also evaluated in two field conditions during November 2001 to March 2002. Here it is mentioned that in every steps of evaluation control plants (native seed derived seedlings) were also planted as check, and no insecticides was used for controlling visit of virus spreading vectors under open field condition. As experimental design "split - plot design" was used, and was replicated three times. Here field condition

Table 1. Effect of different concentrations and combinations of growth regulators in MS liquid medium for primary establishment of apical meristem isolated form 30-35 days old field grown virus infected plants. Mean data of growth (%) and days to response of meristems were recorded by increasing their size and gradual change from translucent to light green in colour. Data were recorded after 21 days of inoculation. In each treatment 20 explants were inoculated.

Treatments	Morphogenic response of isolated meristem	Cultivars	
		Legend	Epoch
MS_0 (control)	Days to response	7-15	9-15
	Growth response (%)	30	25
GA_3 (0.1mg/L)	Days to response	6-11	8-12
	Growth response (%)	90	80
BA (0.1mg/L)	Days to response	9-15	12-15
	Growth response (%)	70	70

Table 2. Effect of different concentrations of cytokinin and auxin in MS medium on shoot and root development from developed primary meristem and nodal segments of meristems derived plantlets. The data were recorded after 4-6 weeks of culture initiation. Data on root number and shoot length are presented as $\overline{X} + SE$.

Treatment		Morphogenic traits ($\overline{X} + SE$)	Morphogenic response			
			Primary meristem		Nodal segments	
			Cultivars		Cultivars	
			Legend	Epoch	Legend	Epoch
MS_0 (control)		number of roots	2.22 ± 0.61	1.88 ± 0.34	8.78 ± 0.13	6.75 ± 0.02
		shoot length	2.70 ± 0.8	2.49 ± 0.14	3.75 ± 0.11	2.98 ± 0.1
		root formation (%)	50	44	100	100
GA_3	0.5mg/L	number of roots	8.0 ± 0.18	7.87 ± 0.30	12.30 ± 0.67	10.10 ± 0.72
		shoot length	3.75 ± 0.31	2.75 ± 0.25	3.44 ± 0.21	2.11 ± 0.37
		root formation (%)	100	100	100	100
	1.0mg/L	number of roots	0	0	NC	NC
		shoot length	2.20 ± 0.31	1.80 ± 0.71		
		root formation (%)	0	0		
Kin	0.5mg/L	number of roots	0.80 ± 0.37	0	NC	NC
		shoot length (cm)	1.21 ± 0.17	0.65 ± 0.04		
		root formation (%)	10	0		
	1.0mg/L	number of roots	0	0	NC	NC
		shoot length (cm)	1.00 ± 0.90	0.80 ± 0.13		
		root formation (%)	0	0		
IBA	0.5mg/L	number of roots	14.2 ± 0.37	12.38 ± 0.19	15.13 ± 0.22	10.13 ± 0.38
		shoot length (cm)	6.12 ± 0.33	4.33 ± 0.52	6.73 ± 0.14	5.18 ± 0.56
		root formation (%)	100	100	100	100
	1.0mg/L	number of roots	12.30 ± 0.31	10.11 ± 0.31	NC	NC
		shoot length (cm)	3.44 ± 0.14	3.25 ± 0.37		
		root formation (%)	100	100		

NC = Not conducted

Table 3. Detection of virus elimination (%) from meristem derived plantlets by DAS- ELISA method. In virus negative plant no colour was detected.

Viruses	Cultivars	
	Legend	Epoch
ToMV	70-75	77-80
CMV	75-80	80-82
ToLCV	73-78	78-80

(open field and net house) was considered as main plot and cultivar was considered as subplot. Following data such as viral disease index, plant height (cm), number of leaves $plant^{-1}$, number of flower three $clusters^{-1}$, number of fruit $plant^{-1}$, total fruit weight $plant^{-1}$ (kg) were recorded from randomly selected field grown 10 plants. Except number of flowers three $clusters^{-1}$, data on other characters were collected at first harvest. Whereas, data on number of flowers were recorded at maximum flowering stage.

Table 4. Field evaluation of R_1 and R_2 plants.

Cultivars	Plant source	Plant height (cm) ($\overline{X} + SE$)		No. of leaves plant^{-1} ($\overline{X} + SE$)		No. of flower three cluster^{-1} ($\overline{X} + SE$)		Total no. of fruit plant^{-1} ($\overline{X} + SE$)		Total fruit weight (kg) plant^{-1} ($\overline{X} + SE$)		Viral disease index (%)					
												ToMV		ToLCV		CMV	
		R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2
Legend	meristem derived in net house	55.13 ± 0.13	55.01 ± 0.25	14.2 ± 0.3	14.23 ± 0.1	25.2 ± 0.21	25.1 ± 0.12	15.1 ± 0.12	16.11 ± 0.78	2.15 ± 0.6	2.69 ± 0.11	0	0	0	0	0	0
	meristem derived in open field	52.04 ± 0.01	48.32 ± 0.11	12.2 ± 0.3	10.23 ± 0.1	24.2 ± 0.21	21.1 ± 0.12	14.1 ± 0.12	12.11 ± 0.78	2.1 ± 0.65	1.89 ± 0.32	0	5.12 ± 0.31	0	3.12 ± 0.35	0	9.1 ± 0.32
	normal seed derived in open field (control)	50.98 ± 0.12	45.17 ± 0.66	10.8 ± 0.89	8.01 ± 0.11	20.1 ± 0.12	18 ± 0.23	10.1 ± 0.13	8.12 ± 0.30	1.5 ± 0.54	1.1 ± 0.32	15.12 ± 0.32	20.1 ± 0.39	12.11 ± 0.37	15.21 ± 0.98	25.2 ± 0.69	30.5 ± 0.98
Epoch	meristem derived in net house	65.34 ± 0.32	64.69 ± 0.11	10.1 ± 0.13	10.9 ± 0.12	30.3 ± 0.01	31.2 ± 0.11	23.1 ± 0.22	24.1 ± 0.36	3.18 ± 0.32	3.59 ± 0.32	0	0	0	0	0	0
	meristem derived in open field	64.13 ± 0.15	58.18 ± 0.88	11.3 ± 0.12	8.1 ± 0.12	27.3 ± 0.01	24.2 ± 0.11	21.1 ± 0.22	20.1 ± 0.36	3.11 ± 0.12	2.58 ± 0.30	0	4.85 ± 0.15	0	4.0 ± 0.69	0	7.85 ± 0.69
	normal seed derived in open field (control)	55.74 ± 0.32	50.11 ± 0.65	9.1 ± 0.23	7.5 ± 0.23	23.4 ± 0.12	19.1 ± 0.11	16.2 ± 0.30	10.6 ± 0.21	2.1 ± 0.41	1.89 ± 0.31	17.1 ± 0.21	21.7 ± 0.20	13.43 ± 0.34	14.22 ± 0.21	27.1 ± 0.13	32.1 ± 0.65

0 = No viral symptoms.

Results

Primary establishment of isolated meristem

Shoot tips from field-grown plants were surface sterilized with 0.1% $HgCl_2$ for aseptic isolation of meristem. Among the different time period used for sterilization, three (3) minutes treatment was found best for survive of the explants. Isolated meristems of two cultivars viz. Legend and Epoch were cultured on filter paper bridge containing test tubes in liquid medium MS_0 and MS medium (Figure A) supplemented with different concentrations of growth regulators and their response are presented in Table 1. Initial growth of the cultured meristem starts within 6-15 days by increasing the size and gradually change to light green in colour (Figure B). For primary establishment of the isolated meristem, MS medium supplemented with 0.1 mg $L^{-1}GA_3$ showed better response for both cultivars compared to MS_0 medium (without growth regulators). About 90% of the isolated meristems were survived and responded in respect of growth within 6-11 days of cultivar Legend. On the other hand the survival rate was 80% and responded within 8-12 days of cultivar Epoch.

Better growth response in terms of healthy shoot development of the primary established meristem was also observed in MS medium containing 0.1 mg $L^{-1}BA$ compared to MS_0 medium.

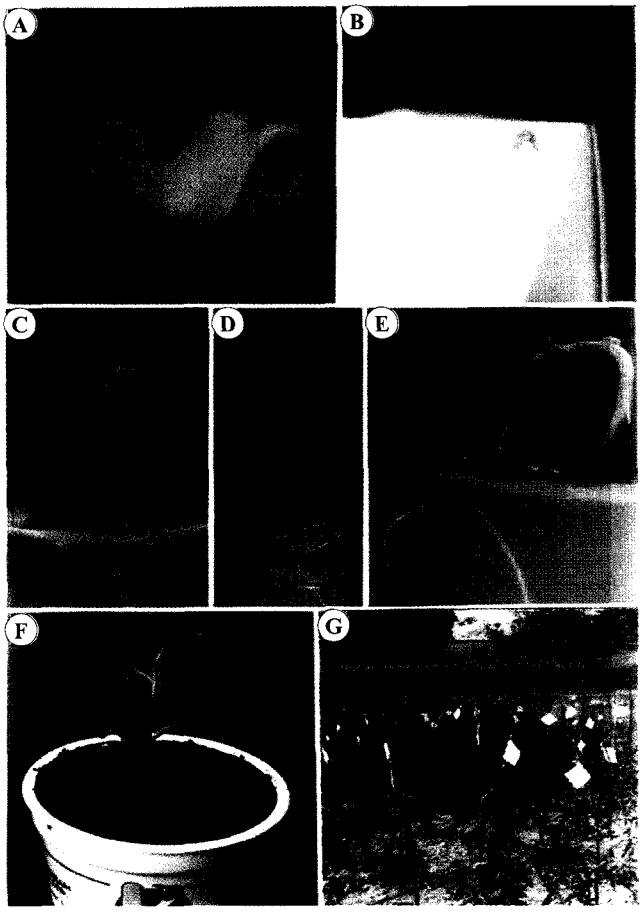

Figure 1. A, Isolated meristem (5 days old) on filter paper bridge in liquid medium. B, Developed (14 days old) isolated meristem in liquid medium. C, Primary developed meristem sub-cultured on semi solid medium. D, Rooting of meristem-derived plantlet. E, Nodal segment of virus free plantlet for clonal multiplication. F, Acclimatization of plantlet. G, Meristem derived plantlets in net house for field evaluation.

Shoot and root development from established primary meristem

The tiny shoots developed from meristem were carefully rescued and transferred on semisolid MS_0, and MS with GA_3, IBA and Kin singly at different concentrations for shoot and root development (Figure C), and the results are presented in Table 2. Among the three growth regulators (GA_3, IBA, kin), IBA (0.5 mg L^{-1}) was found most effective for proper shoot and root development. The results of other growth regulators show that GA_3 (0.5 mg L^{-1}) was found effective for root induction but the shoot length and root number was not induced enough, where as, GA_3 (1.0 mg L^{-1}) resulted short shoot development without root induction.

Confirmation of virus elimination in meristem derived plant sample by DAS-ELISA test

The result on DAS-ELISA test for checking virus elimination in meristem-derived plantlets are presented in Table 3. From this test it was found about 70-82% of the tested plantlets were free from the studied four viruses.

Clonal multiplication of virus free plantlets

Based on the earlier results (Table 2) the nodal segments of the virus free plantlet were inoculated in MS_0 and MS medium containing either 0.5 mg L^{-1} GA_3 or 0.5 mg L^{-1} IBA for clonal multiplication, and the results are also presented in Table 2. Here root development (100%) was noticed in MS medium with or without using of plant hormone (Figure D). However, distinct variation was observed between them in number of root development and shoot elongation. Use of phytohormone was found effective for increasing length of shoot and root number. Similar to primary meristem culture use of IBA (0.5 mg L^{-1}) was also found good for clonal multiplication of virus free plantlets.

Field trial of R_1 and R_2 plants for checking disease incidence

The analysis of variance of the studied five yield contributing characters reveals that the items field condition and cultivar were significant (data not shown). These significant results show the differences of performance of the cultivars under two planting conditions. The performance of the cultivars was better in net house condition than the open house condition (Table 4). The data further shows that the performance of meristem derived plants in both R_1 and R_2 generations did not change much under net house condition. On the other hand, in open field condition the performance was decreased in R_2 generation than the R_1 generation. In all cases the performance of native seed derived plants was lower than the meristem derived plants. No viral disease infection was occurred in meristem derived plants in both the R_1 and R_2 generation cultivated in net house condition, whereas in open field condition, the disease infection was observed in R_2 generation. The disease index (%) of the native seed derived plants was very high as expected.

Discussion

The results of this study provide that the use of meristem for plant regeneration and their subsequent maintenance under net house condition was found very effective method for viral disease eradication management in tomato seed. In addition to this no apparent somaclonal variation resulted from the tissue culture process. The entire protocol was in two steps: production of virus free clones, and management of the virus free plants in field. The first step of protocol was, production of high frequent virus free tomato clone using meristem culture, and their subsequent acclimatization in the soil. Meristem with 0.3 - 0.5mm length was found effective size for virus elimination using shoot tips collected from infected plants (data not shown). According to Stone (1963) only shoot tips between 0.2 and 0.5 mm most frequently produce virus free carnation plants. Use of shoot tips for meristem culture for disease elimination was also reported in different crops (Morel and Martin 1952; Walkey 1978, Bhojwani and Razdan 1983, Pierik 1989; Senula et al. 2000; Balukiewicz and Kryczynski 2001; Fajardo et al. 2002; Zhang et al. 2002; Nagib et al. 2003). The result further shows that use of GA_3 (0.1 mg L^{-1}) in MS liquid medium helped for quick establishment of primary meristem culture. This indicates the role of growth regulators for effective establishment of meristem culture. Use of liquid culture method for tissue culture has also been reported (Goodwin 1966; White et al. 1934; Nagib et al. 2003). Effective role of GA_3 for establishing meristem culture was also reported by others (Foxe et al. 1986; Polevaya et al. 1988; Ahmad et al. 2000; Vasanthi et al. 2001; Nagib et al. 2003). The results on shoot and root development from primary established meristems using GA_3, IBA and Kin showed, use of growth regulators was also needed for rapid shoot and root induction and their further proliferation. Use of auxin including IBA for shoot and root development from isolated meristems was reported in several plants (Langhe and Bruijne 1993; Morris et al. 1997; Radhakrishnan et al. 1999; Vasanthi et al. 2001; Nagib et al. 2003). The DAS-ELISA results indicate that virus can successfully be eradicated from the infected plants by meristem culture. Now currently this test is being used for virus detection in various crops all over the world (Morris et al. 1997; Allam et al. 2000; Fajardo et al. 2002; Nagib et al. 2003). Distinct variation was observed in *in vitro* response for the studied two cultivars. Others also observed differential *in vitro* response due to different genotypes (Zelcer et al. 1984; Gorbatenko 1986). The second step of protocol includes field management of meristem derived virus free plants against contamination of viral vectors. The data on viral disease index (%) show that the use of net house for cultivation of meristem-derived plants was very effective to protect them from viral disease vector attack and eventually from disease infestation. Net house use shows advantage of using no insecticides for killing virus spreading vectors. Therefore, for commercial production of environmentally friendly healthy tomato seeds, the described protocol in this paper can be followed.

In conclusion this can be recommended that proper attention should be given in field management of meristem derived plants for producing disease free healthy seeds under net house condition. The developed protocol in Bangladesh condition can be followed for other countries having similar tropical like environments.

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