Research Article

Production of Leaf Curl Virus - Free Chilli by Meristem Tip Culture

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ABSTRACT

Chilli plants infected with leaf curl virus (geminivirus) are characterized by vein clearing, upward curling, deformation of leaves stunting of plants and abscission of flower buds. The virus was identified as chilli leaf curl virus (CLCV) on the basis of mechanical inoculation on *Nicotiana tabacum* and *Chenopodium quinoa* and by PCR. MS medium amended with BAP (3.0mg/l) and IAA (3.0mg/l) was used for shoot proliferation. A maximum of 34.26±0.06 number of shoots was obtained. Elongated shoots were rooted on 1/2 MS supplemented with IBA (2.0 mg/l) and 2% activated charcoal. Regenerated plants gave negative results for CLCV by PCR with specific primers. Virus free chilli plants (80%) were obtained from optimum size (0.5 mm) of meristem tips. Virus indexing by PCR was found to be a reliable method for confirmation of CLC virus free nature of the regenerated plantlets. This is the first report of geminivirus-free chilli plants production through meristem tip culture.

Keywords: Chilli leaf curl virus, Geminivirus, *In vitro* production, PCR, Virus free chilli.

INTRODUCTION

Chilli (*Capsicum annuum* L.) an important spice, is grown widely in the tropics and semi tropics throughout the world but the productivity is very low. One of the main reasons for the low productivity is the susceptibility to various pest and diseases including viral diseases which not only reduce yield but also the quality.[1]

Several viruses have been reported to affect chilli such as *Cucumber mosaic virus* (CMV), *Pepper vein banding virus*, *Pepper vein mottle virus*, *Potato virus y* (PVY), *Tobacco etch virus* and *Tobacco mosaic virus* (TMV).

In India, *Tomato Leaf Curl New Delhi Virus* (ToLCNDV) has recently been shown to be associated with chilli leaf curl disease. Several other *begomovirus* species associated with chilli leaf curl such as *Cotton leaf curl multan virus*, *Chilli leaf curl multan virus* and *Pepper yellow leaf curl Indonesian virus* were reported from Pakistan and Indonesia.[1-3] The PCR technique has been used by many workers for detection of virus in virus infected plants.[4-7]

In 2007, during a survey of chilli varieties grown in fields of Jodhpur and Jaipur (Rajasthan, India) plants were found infected with leaf curl disease. The virus induced leaf curl symptoms are characterized by vein clearing, upward curling, deformation of leaves stunting of plants and abscission of flower buds. The whole plant assumes a bushy appearance with stunted growth. Fewer flowers and fruits develop due to disease. The plants were tested for the presence of leaf curl gemini virus using PCR and were found to be positive.[8]

Apical meristem culture has been successfully used to produce virus free plants and in clonal micro propagation in a wide variety of plants However, knowledge of *in vitro* growth and developmental responses of *C. annuum* L., is limited.[9] The main advantage of using meristem tip culture in commercial micropropagation system is to attempt for recovering virus -free plants.[10-14] Meristem tip culture has been successful for Tobacco mosaic virus and or with Tomato spotted wilt virus or with both viruses in *C. annuum* but no attempts have been made to eradicate LCV from chilli.[15]

In the present paper, we report the detection of leaf curl disease in *Capsicum annuum* by PCR and *in vitro* production of LCV free chilli plants by meristem tip culture. This method will help to minimize virus infection and hence produce quality CLCV free chilli plants. This is the first report of leaf curl virus free plant production from meristem tip of mature chilli plants.

MATERIALS AND METHODS

Collection and maintenance of viral culture

Capsicum annuum var. Pusa jwala showing characteristic symptoms on leaves were collected from the field study. The virus was maintained on healthy chilli var. Pusa jwala by white fly *Bemisia tabaci* inoculation at 3 to 4 weeks interval.

The experimental plants used in this investigation were raised from seeds in pots filled with loamy clay soil, sand and farm yard (1:1:1) manure. From these plants young leaves and meristem tips were chosen for further detection and to produce virus-free plants.

Virus detection and indexing of naturally infected and in vitro grown plants 2.2.1 Biological indexing

Mechanical inoculation was done using the leaf sap from naturally infected and *in vitro* grown chilli plants. Young leaves were homogenized in 0.02 M sodium phosphate buffer, pH.7.0 with mortar and pestle. The slurry thus

obtained was squeezed through double layered muslin cloth. *Nicotiana tabacum* (systemic host) *Chenopodium quinoa* (local lesion host) were inoculated with sap to test the presence of virus.

PCR Detection

The leaves of chilli infected with leaf curl disease used in present study were obtained from field samples from Jodhpur, Jaipur and New Delhi.

Total DNA was isolated from the infected and healthy leaves using DNA easy mini kit (Qaigen Inc. USA). About 50 mg of leaves were ground in liquid nitrogen and processed as described in Qaigen DNA manual. The total DNA was eluted with 100 ml autoclaved double distilled water.

The PCR was carried out to amplify the genome of the virus associated with chilli leaf curl disease. Thus, a pair of primers (AVF9 & AVR10) over the putative coat protein gene was effective in detecting the virus by polymerase chain reaction. The specific primers AVF9 and AVR10 designed at IARI, New Delhi were used in the present investigation for comparing the putative coat protein gene. Sequence available for present study and from the gene bank was very useful in diagnosis of 750bp fragments. These specific primers are valuable molecular tools in the diagnosis of chilli leaf curl virus. Two primers (AVF9, AVR10) of following sequences were used.

Name of the primer	Sequence	Expected product (Kb)	Used for amplification of
AVF_9	5´ATGTCG/CAAGCGAG /CCTAGCC/AGATAT3´	0.75	Coat protein gene
AVR_{10}	5´ TG/TAATTCGT/ATAC /ACGAG/TTC ATA3´		

The PCR reaction mixture of 50 mlconsisted of 1 mlof total DNA (about 200 ng), 2 mlof 10 mM dNTP 1 mleach of forward and reverse primers (about 1 ng/ml), 5 ml of 10X PCR reaction buffer, 0.5 mlof *Taq* DNA polymerase and autoclaved double distilled water to make up the volume. The thermo cyclic programme was started at 90°C for 5 minutes followed by 35 cycles of denaturation at 90°C for 1 minute annealing at 42°C for 1 minute and synthesis at 72°C for 2-3 minutes. The final extension was at 72° C for 10 minutes.

PCR product was separated by electrophoresis on a 1.0% agarose gel at 80 V. The gel was stained in ethidium bromide (1mg/ml) and visualized in transilluminator. An aliquot (500 ng) of 1kb DNA ladder (M.B.I. fermentas, USA) was used as a molecular weight marker. The PCR product was cut from the gel and isolated from gel elution kit (Promega inc. USA) in 30 mlof autoclaved double distilled water.

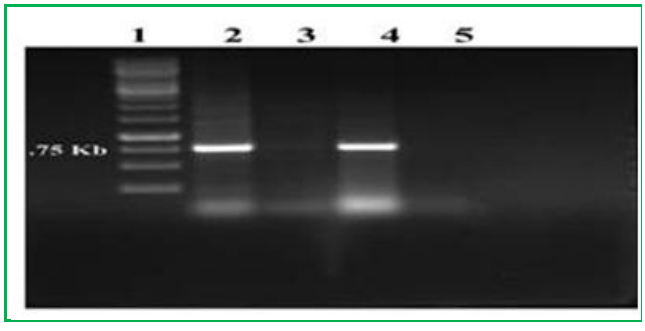
***In vitro* production of chilli (Capsicum annuum)**

Source of explants

Shoot tips (2-3mm long) with two leaf primordia were chosen from chilli var. Pusa jwala field grown infected plants.

Establishment of aseptic cultures

Shoot tips were washed with 2% liquid detergent solution (Extran, E. Merck- a commercial grade detergent) for 5 minutes under running tap water and immersed in 1.0% Sodium hypochlorite solution for 2-3 minutes followed by three washings with sterile distilled water. Shoot tip meristems, 0.25–1.0 mm in size were excised aseptically under stereomicroscope and were kept in laminar flow clean bench. The excision was performed in a sterile glass petridish, lined with sterile moist filter paper to avoid desiccation of the small explant. MS-medium supplemented with various concentrations and combinations of auxins (1-5 mg/l) and cytokinins (1-5 mg/l) was prepared. The pH of the medium was adjusted to 5.8 and autoclaved at 15 psi for 20 minutes. These were planted with the cut-end slightly embedded in the medium. All the cultures were incubated at 26±2°C and exposed to 16 hr photoperiod illuminated by fluorescent light of about 1500-2500 lux light intensity. Relative humidity of 55±5% was maintained in the culture room. The cultures were regularly subcultured on fresh medium after 4 weeks. Multiple shoots obtained were later transferred to elongation medium supplemented with different growth hormones. Elongated and healthy shoots were transferred to ½ MS medium supplemented with IBA (2mg/l) with activated charcoal (0.2%) for rooting. Regenerated plantlets were then transferred to pots containing sterilized soil and vermiculite (3:1) in an insect proof net house. They were routinely checked for presence of virus.



1. Marker (1 kb); 2. Infected leaf (Jaipur); 3. Healthy leaf of Chilli; 4. Infected leaf Jodhpur; 5. Infected leaf (New Delhi).

Figure 1: PCR detection of chilli leaf curl virus by coat Protein gene amplification Position of sample

RESULTS AND DISCUSSION

Virus indexing

On mechanical inoculation from naturally leaf curl infected chilli and in vitro grown plants, *Nicotiana tabacum* produced leaf curl symptom on the leaves in 11-13 days and on *Chenopodium quinoa* chlorotic local

lesions appeared in 6-7 days.However the *in vitro* grown plants, did not produce local lesions on *C. quinoa*.

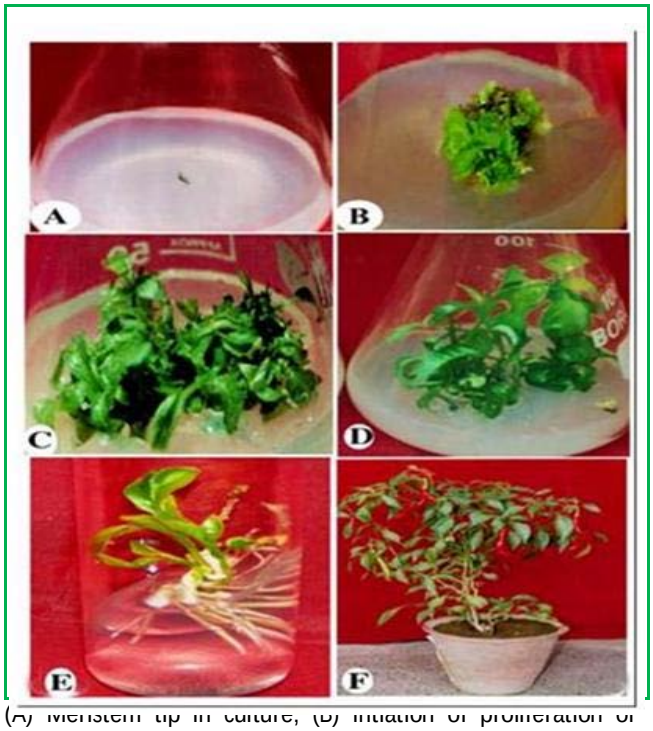
PCR was found to be a reliable method in comparision to biological indexing. Chilli leaf curl disease in Jaipur and Jodhpur is caused by geminivirus because these samples gave an amplification of 0.75Kb fragment but the sample collected from New Delhi fields did not show the band so it can be concluded that leaf curl disease of chilli in New Delhi is caused by some other virus. (Figure 1)

Meristem culture and shoot multiplication

Shoot tips of *Capsicum annuum* L. were isolated aseptically and cultured on MS-medium supplemented with cytokinins and auxins for initiating vegetative growth and inducing a maximum number of plantlets. Therefore, in the present study experiments were aimed at obtaining multiple shoot proliferation in *capsicum annuum* L. In the present study, MS-medium fortified with a combination of BAP (3 mg/l) and IAA (3 mg/l) produced maximum number of (34.26±0.06) multiple shoot (Table.1, Fig.2 A-D).

Table 1: Effect of Plant Growth Regulators (PGR) on shoot formation from shoot tip explant of *Capsicum annuum* L on MS-medium

PGR's mg/l			No. of shoots buds per explant
IAA	BAP	Kn	Mean ±SE
0.5	-	-	21.58± 0.29
1.0	-	-	25.81 ± 0.37
2.0	-	-	31.33 ± 0.15
3.0	-	-	32.22 ± 0.18
4.0	-	-	30.20 ± 0.18
5.0	-	-	25.01± 0.22
-	0.5	-	4.82 ± 0.33
-	1.0	-	5.22 ± 0.11
-	2.0	-	8.15± 0.21
-	3.0	-	**21.11 ± 0.10**
-	4.0	-	15.22 ± 0.45
-	5.0	-	6.80± 0.54
0.5	0.5	-	9.0 ± 0.336
1.0	1.0	-	5.4 ± 0.401
2.0	1.5	-	23.4 ± 0.220
3.0	2.0	-	21.0 ± 0.427
4.5	2.5	-	16.4 ± 0.336
3.0	3.0	-	**34.26 ± 0.06**
	0.5	0.5	**Nil**
	1.0	1.0	3.10 ± 0.72
	1.5	1.5	3.22 ± 0.88
	2.0	2.0	4.16 ± 0.63
	2.5	2.5	5.38 ± 0.45
	3.0	3.0	4.35 ± 0.60



(A) Meristem tip in culture, (B) Initiation of proliferation of shoots; (C) Prolifiration of several shoots; (D) Elongated virus free (tested) shoots; (E) Rooting of virus free (tested) shoot in the medium; (F) The chilli leaf curl virus-free plant with green and red fruits.

Figure 2: Production of leaf curl virus free plants of chilli using meristem tips.

Effect of meristem size on its establishment and virus elimination

Small size meristems (0.2-0.3mm) taken in this study transformed into callus and were hence not tested for the presence or absence of virus. The 0.5 mm long meristem produced only shoots and 80% plants regenerated from these meristems tested negative for leaf curl virus as indexed by PCR. As the size of meristem increased, the percentage of obtaining the virus free plants decreased and with 0.7-1.0 mm long meristem, no virus –free plants were obtained. (Table 2)

Table 2: The effect of size of meristem tip in the production of leaf curl virus free chilli plants

Size of meristem (mm)	Tissue differentiation	No of shoots grown in vitro	Virus indexing by PCR No of virus free plants	% virus free plants
0.2-0.3	Callus	-	-	-
0.5	Shoots	34	27	80
0.7	Shoots	34	0	0
1.0	Shoots	34	0	0

Rooting, hardening and acclimatization

Proliferated shoots after 8 weeks were transferred to MS-medium fortified with IBA (2.0 mg/l) and activated charcoal (0.2%).On this medium, rooting percentage (85%), number of roots (37-38) and root length (10.6 ±

0.185) were maximum (Table 3). Roots were thick with white root hairs and healthy in nature (Figure 2, E) Plantlets with 6-7 leaves and well developed root system were removed and transferred to pots containing soil rite. These pots were kept in growth chamber for 15 days at 26±2^{o}C and 1000-2000 lux light intensity for acclimatization. (Figure 2, F) In order to maintain high humidity the pots were covered with inverted glass beaker. After six months when new leaves emerged from these plantlets, they were taken outside the growth chamber and kept in shady place under normal temperature and light. 70 % survival rate in *C. annuum* was observed.

Table 3: Effect of auxin (IBA) along with 2.0% activated charcoal on root induction from shoots of *Capsicum annuum after* 4 weeks of culture. [Values are mean ± SE from 5 replicates in each treatment]

IBA (mg/l)	% of cutting rooted	No. of roots per shoots	Average length of the root (cm)
1.0	60	26.8 ± 0.179	8.2 ± 0.395
2.0	85	37.6 ± 0.220	10.2 ± 0.185
3.0	65	29.4± 0.179	6.8 ± 0.283
4.0	40	20.6 ± 0.180	5.0 ± 0.317
5.0	25	18.6± 0.447	4.6 ± 0.350

Ansari *et al.* (2006) used the same primer sequence in detection of geminivirus.[16] The primers have been used by many workers for detection of virus in virus infected plants. [6,7] Cotton leaf curl disease has been detected using virus nucleic acid based on hybridization test in infected host and seed.[17] Shih *et al.*. (2003) carried out molecular characterization of an isolate of Begomovirus in Pakistan and found that isolate was not related to the two tomato isolates of Begomovirus from Pakistan.[2]

The nutrient medium generally used to initiate cultures is composed of basal salts (major and minor elements) and organic supplements. Besides the vitamins, growth regulators also play a vital role in growth of meristem. As meristems are incapable of synthesizing the growth hormones other than auxins,external supply through medium becomes absolutely essential.[18]

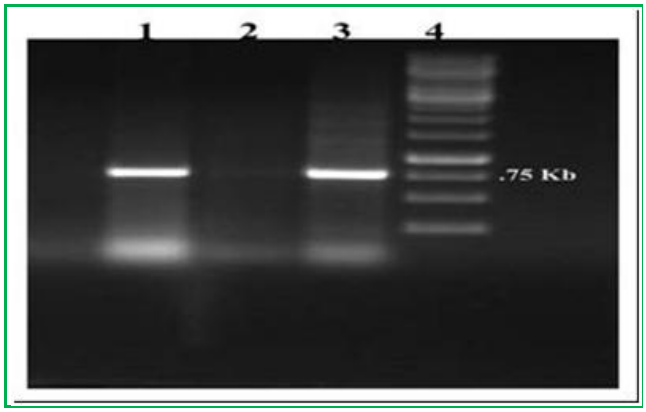
In contrast to above mentioned results some researchers observed that the combination of BAP and IAA on MS-medium favoured multiple shoot bud induction in *Capsicum annuum* (Sobhakumari and Lalithakumari, 2003) and in *Salvia nemorosa* L. by Skaia and Nska (2004).[19,20] Similar effect of ½ MS media combination with IBA was observed by Rao *et al.* (2006) in the same plant species.[21] In most species IBA has also been found to be potent root auxin for in vitro grown shoots and potentiality of activated charcoal on rooting was assessed by several workers in many plant species.[21-24]

In consonance to this, Kanyand *et al.* (1994) reported successful transfer of in vitro regenerated plantlets from flask to field condition in *Arachis hypogaea*. Virus elimination depends on various factors such as meristem size, the virus concerned, physiological condition of mother plants and meristem position on it. The larger the size of the meristem cultured, the greater is the number of regenerated plants, while the number of virus-free plantlets obtainable is inversely proportional to the size of the cultured tips.[25]

In our experiments, meristem tips of size 0.5 mm were found to be optimum for eliminating leaf curl virus from chilli. Meristem of 0.7-1.0 mm size carried the virus particle with it and all the shoots produced from these were found to be virus infected.

There are various explanations for virus elimination during in vitro culture e.g. action of growth regulators particularly cytokinin phenol-amines loss of enzymes necessary for viral replication and viral RNA degradation due to cell injury during explants excision.[26,27]

Regenerated plants were longer, fruits were healthy, green and elongated. Seeds obtained from *in vitro* plants were more in number than that of in vivo plants. Plants regenerated from shoot tip meristem (0.5 mm) did not show the 0.75 kb band hence they were gemini virus (LCV) free. This was observed morphologically and confirmed by PCR. (Figure 3) Meristem–tip culture to produce leaf curl virus free plants of chilli is an efficient technique and the indexing by PCR is time saving and a reliable method for certification of chilli leaf curl free tissue culture raised plants. This proposal can be used for the production of CLCV-free plants of chilli.



1. Sample from leaf of chilli leaf curl virus infected plants growing in pots; 2. Sample from leaf of chilli plants raised in vitro; 3. Standard DNA of Gemini virus; 4. Marker (1 Kb)

Figure 3: Detection of virus free plant by PCR Position of Samples

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