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Full Length Research Paper

Detection of pepper leaf curl virus through PCR amplification and expression of its coat protein in *Escherichia coli* for antiserum production

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Pepper leaf curl virus (PepLCV) is the most destructive pathogen of pepper and causes substantial economic losses of chilli production worldwide. Curling and puckering of leaves and stunted growth of the plants are typical symptoms of the viral infection. For a reliable detection of PepLCV, coat protein specific primer pairs were designed and after polymerase chain reaction amplification (PCR), -1 kb amplification product specific to coat protein gene was amplified. Cloning of this amplification product in an expression vector resulted in high-level expression of a 28 kD protein. Coat protein gene product is important for vector specificity and is also responsible for viral capsid formation and whitefly-mediated transmission. The purified protein can be used for production of coat protein specific antisera leading to development of an efficient detection system for this very important viral pathogen of pepper.

Key words: PepLCV, geminivirus, virus detection, polymerase chain reaction amplification (PCR), immunoblot.

INTRODUCTION

Pepper (*Capsicum annuum* L.) is one of the most diverse vegetable species and is considered to be a high value crop. Among the five domesticated species of the genus *Capsicum*, *C. annuum* is the most widely cultivated all over the world for its pungent (Chilli syn. hot pepper) and non-pungent (sweet pepper) fruits (Bosland and Votava, 2000). In India, chilli is an important commercial crop cultivated for vegetable, spice and industrial (oleoresin and capsaicin extraction) purposes (Kumar and Rai, 2005).

Chilli is susceptible to various pathogens including viruses, which cause heavy production losses (Villalon, 1975, 1981). Natural occurrence of several viruses, including, pepper leaf curl virus, pepper veinal mottle virus and pepper vein bending virus has been reported by different workers (Osaki and Inouuye, 1981; Martelli and Quacqarelli, 1983; Khan et al., 2006).

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Among all, the pepper leaf curl virus (PepLCV) is the most destructive virus in terms of incidence and yield loss. The typical symptom of PepLCV includes curling and puckering of leaves and stunted growth of the plants. Epidemics of PepLCV can result in 100% yield loss resulting in severe economic consequences (Senanayake et al., 2006). In India, PepLCV has been an emerging threat in chilli for a long time and several workers have reported their occurrence (Mishra et al., 1963; Dhanraj and Seth, 1968; Raj et al., 2005). The host range and virus-vector relationship of chilli leaf curl disease was reported as early as 1960 (Mishra et al., 1963; Dhanraj and Seth, 1968). PepLCV has also been reported from other countries, like, USA (Stenger et al., 1990), Nigeria (Alegbejo, 1990) and Pakistan, Bangladesh and Indonesia (Fauquet and Stanley, 2003).

The genus *Begomovirus* contains geminiviruses that infect dicotyledonous plants and are transmitted by white-fly (*Bemisia tabaci*). *Begomovirus* has been reported worldwide and they affect a number of economically important food and fiber crops such as cassava, tomatoes, cucurbits, pepper, beans and cotton (Varma

Figure 1. A PepLCV infected chili plant showing typical curling symptom.

and Malathi, 2003). Generally, *Begomovirus* indigenous to the western hemisphere has bipartite genome with components referred to as DNA-A and DNA-B. DNA-A encodes genes responsible for viral replication, regulation of gene expression, suppression of gene silencing and particle encapsidation. DNA-B encodes proteins involved in virus movement, host range determination and symptom development (Lazarowitz, 1992). But in the case of chilli, the PepLCV has monopertite genome that contains DNA-A and β-segment (Chattopadhyay et al., 2008).

The first step towards minimizing the effects of PepLCV includes the detection of virus. Virus indexing is currently performed by enzyme-linked immunosorbent assay. But, identification of whitefly-transmitted geminiviruses by traditional methods has been difficult and a reliable detection of PepLCV remains to be a challenge. Serology alone is not suitable, since high viral titre is difficult to prepare (Brown et al., 2001). Consequently, other approaches such as polymerase chain reaction amplification (PCR) amplification of specific genomic regions and DNA sequencing offer attractive opportunities for detection, identification and characterization of begomoviruses. In addition, Geminiviral genome is more suitable for PCR amplification because it replicates via a double-stranded circular DNA intermediate (replicate form), which can serve as template for DNA amplification. In recent past, PCR-based rapid detection methods have been developed for several viruses (Khan et al., 2006; Chattopadhyay et al., 2008). However, a method integrating the serological-based assay as well as PCR-based tools offers the most efficient system for rapid detection of virus (El-gaied et al., 2008). This study describes PCR amplification and cloning of a coat protein gene of PepLCV and its expression in a strain of *Escherichia coli* (DH5α) for anti-serum production.

MATERIALS AND METHODS

Isolation of PepLCV DNA and primer design

Leaves were collected from severely infected chilli plants variety Kashi Anmol growing at research farm of Indian Institute of Vegetable Research (IIVR), Varanasi, showing typical PepLCV symptoms (Figure 1). The viral DNA was isolated following CTAB method (Dellaporta et al., 1983) with minor modifications. Replicative form of viral genome was isolated by alkaline lysis of plant DNA as per the procedure of Birnhoim and Dolly (1979).

Using the software Primer 3 (www.justbio.com), a pair of primers specific to coat protein region were designed from the PepLCV genome sequence (GenBank accession no. EF1902179; Chattopadhyay et al., 2008). The forward primer 5'-AGAATTATGT CCAAGCGACCA-3' corresponds to nucleotide (nt) position 295 to 316 bp and the reverse primer 5'-AAGCGTTGGGGATACACAAA-3' corresponds to nt 1282 to 1262 bp. This primer pair was used to amplify and clone the PepLCV coat protein gene.

PCR amplification of coat protein gene

PCR amplification was carried out according to Kumar et al. (2006) with minor modifications. Amplification was carried out in 25 µl reaction volume containing 50 ng genomic DNA, 2.5 µl PCR buffer

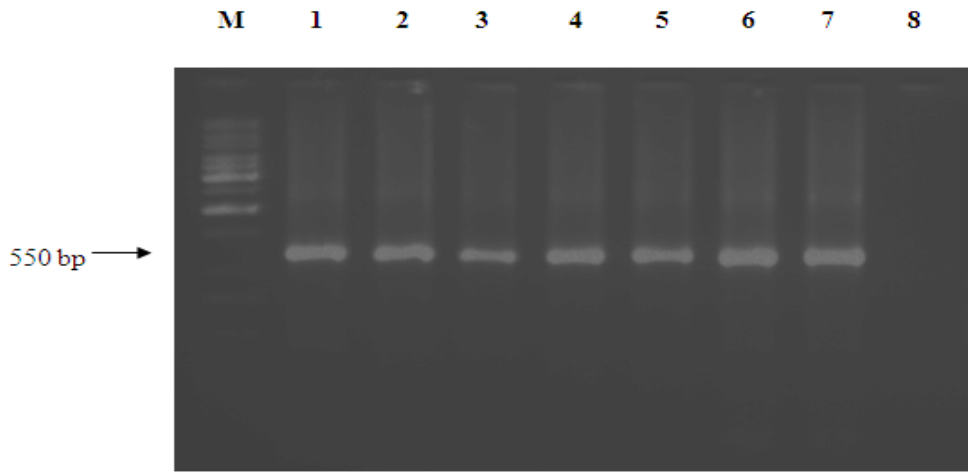

Figure 2. A 550 bp DNA specific to coat protein gene region amplified using universal degenerate primers. Lane M, 1 kb gene ruler marker; lanes 1 to 7, PepLCV affected samples; lane 8, healthy plant sample.

(MBI Fermentas, Hanover, USA), 200 µM dNTPs (Bangalore Genei, Bangalore, India), 1.5 U Taq DNA polymerase (MBI Fermentas) and 0.4 µM primer using a thermal cycler (PTC 1000, MJ Research, USA). The first amplification cycle consisted of 94°C for 4 min, at Tm 5°C for 1 min and at 72°C for 1 min. This was followed by 39 cycles with 1 min at 94°C, 1 min at 55°C and 1 min and 72°C; the final extension was allowed for 10 min at 72°C. The amplified DNA fragments were resolved through electrophoresis in 1.5% agarose gel prepared in TBE buffer (54.0 g Tris-base, 27.5 g boric acid, 0.5 M EDTA (pH 8.0) in 1000 ml) and visualized in a gel documentation system (AlfaImager 2200, Alfa Innotech Corporation, California). The 1 kb DNA ladder (MBI Fermentas) was used as molecular size marker. The amplification was repeated 2 to 3 times to ensure the reproducibility and consistency of the amplification product.

Cloning of the amplification product and sequence analysis

The PCR amplification product of approximately 1 kb was excised form the agarose gel and the DNA was eluted using a gel extraction kit (Stratagene, USA). The DNA product thus, obtained was again amplified using the coat protein specific forward and reverse primers. The viral gene was ligated into pGEMT vector and ligation mix was used for transformation of *E. coli* (DH5-α) competent cells as per the method of Mendel and Higa (1970). The transformants were identified on a Luria agar plate containing X-gal/IPTG and ampicillin (100 µl/ml). Plasmid was isolated from the transformed cells as per the procedure of Birnboim and Dolly (1979) and the insert showing amplification of coat protein gene was sequenced (3130 Genetic Analyzer, Applied Biosystems, California). The viral ORF AV1 was identified from the partial sequence using NCBI Blast search (Altschul et al., 1997) and DNA analysis software MEGA (Ver. 4.1 beta, Kumar et al., 2008). Accession number was obtained for the CP nucleotide sequence and the sequence was deposited in GenBank.

Protein production and immunoblotting

Three milliliter of overnight grown cultures of *E. coli* (Degold) cells with cloned DNA into prokaryotic expression vector pET161/GW/D-TOPO® was inoculated to fresh 3 ml Luria Bertini broth containing 1.0 µg/ml ampicillin, the culture was incubated at 37°C with vigorous shaking (220 rpm) and allowed to grow for 2 h. When the cells reached to the $O.D._{600}$ 0.5, isopropyle-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 2 mM and incubation was continued at 37°C for additional 6 h.

Cells were harvested by centrifugation at 5000 rpm for 10 min, resuspended in 100 ml of 1x SDS sample loading buffer (50 ml Tris-Cl pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The resuspended cells were boiled for 4 min at 100°C and centrifuged at 13000 rpm for 2 min. 15 µl each sample (bacterial protein) was separated through 12% SDS-PAGE gel according to the standard procedure (Sambrook et al., 1989). Lysate from untransformed Degold cells was used as control and protein molecular weight marker (Biorad, USA) was used for molecular weight determination. The gel was stained through gentle rocking in Coomassie brilliant blue R-250 (1%) and visualized for protein bands on a white light transilluminator.

For further analysis by immunoblotting, PAGE-separated proteins were electrophoretically transferred onto nitrocellulose paper (Pall Gelmen Sciences, USA), following the procedure of Towbin et al. (1979) and immunoblotted as described by Harlow and Lane (1988) and probed with rabbit antiserum. The substrate buffer was added to the membrane, agitated until color was observed and the reaction was finally stopped by adding water.

RESULTS AND DISCUSSION

The leaf samples were collected from the plants showing typical curling symptom (Figure 1). Generally, the presence of virus in diseased plant samples could be checked by amplifying viral DNA using degenerate primers (Rojas et al., 1993; Wyatt and Brown 1996). In the present study, the infected leaf DNA samples produced a typical 550 bp amplification product (Figure 2), confirming it to be a geminivirus. However, the degenerate primers flanking the CP region are not of practical use except the checking the presence of geminivirus, since this region is not highly conserved within the genus (Brown et al., 2001). Therefore, pair of

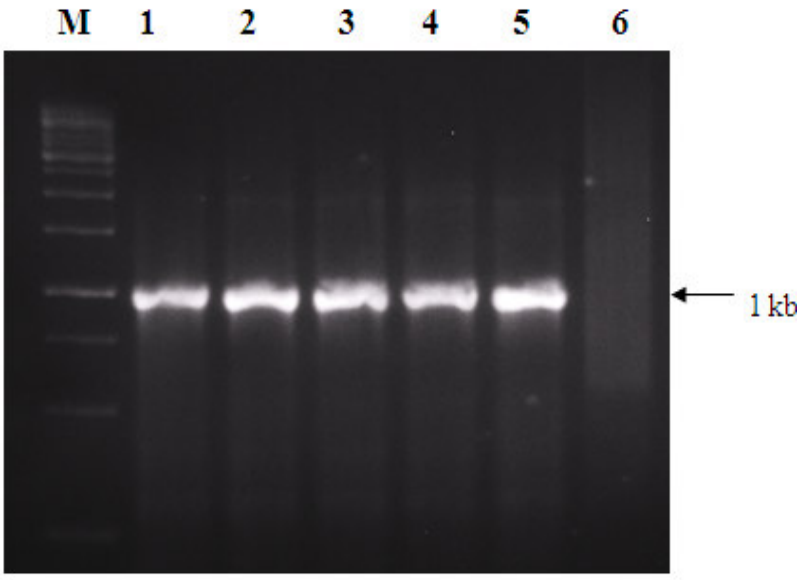

Figure 3. A 1kb DNA specific to coat protein gene region amplified using degenerate primers. Lane M, 1 kb gene ruler marker; lanes 1 to 5, PepLCV affected samples; lane 6, healthy plant sample.

specific primers was designed from the previously reported nucleotide sequence (Chattopadhyay et al., 2008). When the replicative form of viral DNA was subjected to PCR with this designed specific primers, it produced the expected ~1 kb amplification product (Figure 3) again confirming it to be a geminivirus CP clone. Earlier, Chattopadhyay et al. (2008) used degenerate primers for detection of PepLCV, but it can be used for preliminary detection of viruses. Use of a primer pair specific to coat protein gene or replicase gene region allows a more efficient detection of the virus particles. After cloning and partial sequencing of the amplified coat protein region as earlier stated, the viral ORF AV1 was identified. The identified CP-PepLCV consists of 792 nucleotide, which encodes 194 amino acid residues with a mass of 28 kD. The nucleotide sequence is accessible as accession number FJ968525 at GenBank data base (www.ncbi.nlm.nih.gov). The analysis of coat protein coding region sequence data and its alignment with other chilli leaf curl virus isolates revealed its 98% identity with pepper leaf curl virus previously reported from Narwan, Amritsar and Varanasi regions of India. It also showed 97% identity to papaya leaf curl virus from New Delhi, 96% identity to chilli leaf curl virus from Multan (Pakistan) and 95% identity to pepper leaf curl Bangladesh virus. This indicates that, the virus is an isolate of PepLCV. The cloned DNA could be of immense use, especially as DNA probe.

The coat protein gene region was also analyzed through expression studies using the vector pET161; a difference in the expression pattern of protein was recorded from the cells with and without the clone. In addition, a variation in the coat protein profile was also recorded, which may be attributed to the nature of cellular homogenate. The *E. coli* cells containing vector with the clone showed presence of a unique protein showing mobility with molecular weight 28 kD (Figure 4). The coat protein gene product is responsible for vector specificity of geminiviruses (Czousnek and Ghanim, 2002). In the immunoblot, the CP-antiserum gave a strong specific signal with the expressed CP protein of approximately 28 kD from the *E. coli* cells expressing coat protein gene as well as samples from the PepLCV infected plants (Figure 5).

Recent epidemic status shows that, geminivirus have become a major group of viruses causing huge economic yield loss in several vegetable crops. A PCR-based detection system that uses conserved region of coat protein gene was reported by Deng et al. (1994); however, not much emphasis has been given for the development of serology-based assay. The major bottleneck in PCR-based system is the requirement of purified viral DNA from plant samples, which is a tedious process. In addition, the presence of interfering secondary plant metabolites, low viral titer and degradation of viral DNA add difficulty in isolation of pure viral DNA. A PCR-based assay coupled with serological signal would help in overcoming some of these problems and could be used for routine, large-scale indexing of PepLCV. Cloning of viral coat protein gene using region specific primers and its expression in prokaryotic system is a step towards development of antisera and a detection system for Indian PepLCV isolates. The development of assay kit will help in better, quick and easy identification of PepLCV.

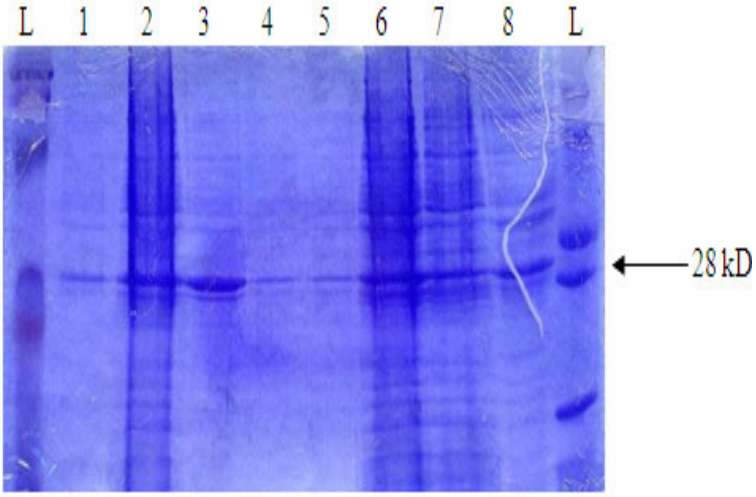

Figure 4. Detection of PepLCV coat protein: Lane L, Molecular protein markers; lane: 1, 2, 3, 6, 7 and 8, coat protein induced samples; lane 4 and 5, uninduced samples.

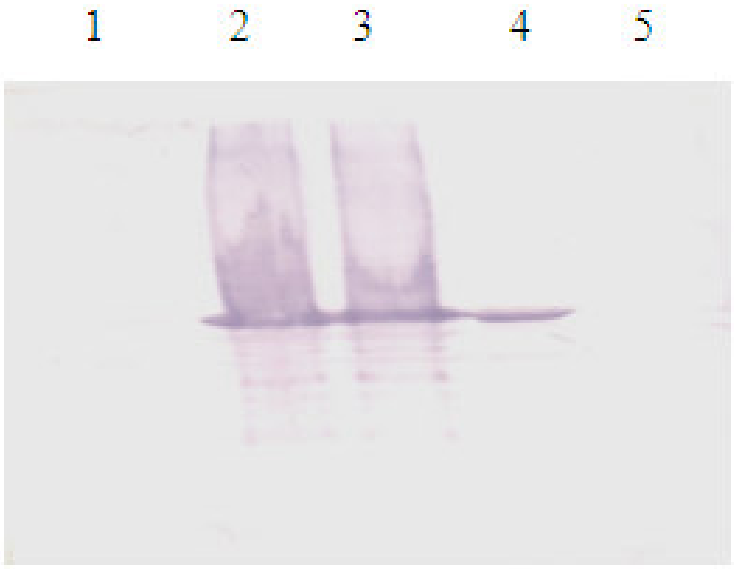

Figure 5. Immunoblot analysis of protein synthesized in *E. coli* cells harboring the T161 plasmid. Lanes 2 to 4, Coat protein induced samples; lane 1 and 5, control uninduced healthy plant sample.

Thus, the data generated could be useful for detection of PepLCV at molecular level leading to virus-free chilli breeding programme.

REFERENCES

Alegbejo MD (1990). Screening of pepper cultivars for resistance to pepper leaf curl virus. Capsicum Eggplant Newslett. 18: 69-72.

Altschul SF. Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acid. Res. 25: 3389-3402.

Birnboim HC, Dolly J (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Res. 7: 1513-1523.

Bosland PW, Votava EJ (2000). Pepper: Vegetables and Spice Capsicum. CABI Publishing, CAB International, Wallingford, U.K.

Brown JK, Idris AM, Torres-Jerez I, Banks GK, Wyatt SD (2001). The core region of the coat protein gene is highly useful for establishing the provisional identification and classification of begomoviruses. Arch. Virol. 146: 1581-1598.

Chattopadhyay B, Singh AK, Yadav T, Fauquat CM, Sarin NB, Chakraborty S (2008). Infectivity of the cloned components of a begomovirus: DNA beta complex causing chilli leaf curl disease in

India. Arch. Virol. 153: 533-539.
Czousnek H, Ghanim M (2002). The circulative pathway of begomoviruses in the whitefly vector *Bemisia tabaci* insights from studies with tomato yellow leaf curl virus. Ann. Appl. Biol. 140: 215-231.
Dellaporta SL, Wood J Hicks JB (1983). A plant DNA minipreparation version II. Plant Mol. Biol. Rep. 1: 19-21.
Deng D, McGrath PF, Robinson DJ, Harrison BD (1994). Detection and differentiation of whitefly transmitted geminivirus in plants and vector insects by the polymerase chain reaction with degenerate primers. Ann. Appl. Biol. 125: 327-336.
Dhanraj KS, Seth ML (1968). A strain of tobacco leaf curl virus causing enation chilli (*Capsicum annuum L.*). Indian J. Hort. 25: 70-71.
El-gaied LF, Salama MI, Salem AM, Amani F. Nour El-deen, and Naglaa A, Abdallah (2008). Molecular and serological studies on a plant virus affecting strawberry. Arab J. Biotechnol. 11: 303-314.
Fauquet CM, Stanley J (2003). Geminivirus classification and nomenclature: progress and problems. Appl. Biol. 142: 165-189.
Harlow E, Lane N (1988). Antibodies: A Laboratory Manual, New York: Cold Spring Harbor Laboratory.
Khan MS, Raj SK, Singh R (2006). First report of Tomato leaf curl New Delhi virus infecting chilli in India. Plant Pathol. 55: p. 289.
Kumar S, Rai M (2005). Chile in India. Chile Pepper Institute Newslett. xxii: 1-3.
Kumar S, Singh V, Rai S, Kumar S, Singh M, Rai M (2006). Tagging of male sterility and fertility restorer genes in chilli (*Capsicum annuum* L.). Sci. Hortic. 111: 197-202.
Kumar S, Nei M, Dudley J, Tamura K (2008). MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief. Bioinfo. 9(4): 299-306
Lazarowitz SG (1992). Geminivirus: genome structure and gene function. Crit. Rev. Plant Sci. 11: 327-349.
Martelli GP, Quacquarelli A (1983). The present status of tomato and pepper viruses. Acta Hort. 127: 39-64.
Mendel M, Higa A (1970). Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. 53: 159-162.
Mishra MD, Raychaudhari SP, Jha A (1963). Virus causing leaf curl of chilli (*Capsicum annuum L.*). Indian J. Microbiol. 3: 73-76.
Osaki T, Inouuye T (1981). Tobacco leaf curl virus CMV/AAI No.232, p. 4.
Raj SK, Khan MS, Pandey SK, Singh BP (2005). Association of a Begomovirus with leaf curl disease of chilli (*Capsicum annuum*). CABI Abstract.
Rojas MR, Gilbertson RL, Russell DR, Maxwell DP (1993). Use of degenerate primers in the polymerase chain reaction to detect whitefly-transmitted geminiviruses. Plant Dis. 77: 340-347.
Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
Senanayake DMJB, Mandal B, Lodha S, Varma A (2006). First report of chilli leaf curl virus affecting chilli in India. New Disease Reports 13. http:// www.bspp.org.uk/ndr/july2006/2006-35.asp]
Stenger DC, Duffus JE, Villalon B (1990). Biological and genomic properties of a gemini virus isolated from Pepper. J. Am. Phytopath. Soc. 80: 704-709.
Towbin H, Staehlin T, Gordon J (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Nat. Acad. Sci. 76: 4350-4354.
Varma A, Malathi VG (2003). Emerging geminivirus problems: A serious threat to crop production. Ann. Appl. Biol.142: 145-164.
Villalon B (1975). Virus disease of bell peppers in South Texas. Plant Dis. Rep. 59: 858-862.
Villalon B (1981). Breeding peppers to resist virus diseases. Plant Dis. 65: 557-561.
Wyatt SD, Brown JK (1996). Detection of subgroup III geminivirus isolates in leaf extracts by degenrate primers and polymerase chain reaction. Phytopathol. pp. 86-128.