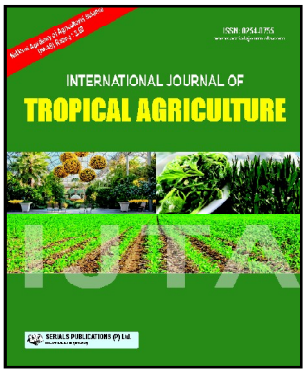

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Studies on Distribution of *Apple chlorotic leaf spot virus* in different parts of apple tree through RT-PCR

Santosh Watpade[1], Baswaraj R. [2], K. K. Pramanick[1], A. K. Shukla,[1] Usha Sharma[3] and Anil Handa[3]

[1] IARI Regional Station, Shimla 171004, [2] CPRI Shimla, [3]Dr. Dr YSPUHF Nauni, Solan
E-mail: santoshpathology@gmail.com

Abstract: Apple Chlorotic Leaf Spot Virus (ACLSV; family *Flexiviridae* genus *Trichovirus*) is one of the economically important latent virus infecting apples (*Malus*× *domestica* Borkh.). Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) procedures were used to amplify coat protein gene of ACLSV. Different plant parts including leaf, petals, sepal, anther, pollen, bark, bud and fruit were tested for the presence of ACLSV. Except pollen all other parts showed presence of ACLSV. Use of NAD5 gene in mitochondrial mRNA of the apple as an internal control, reduced the risk of false negative results that may occur with routine RT-PCR assays.

Key words: ACLSV, Apple, RT-PCR, *Malus,* Distribution

INTRODUCTION

Apple is commercially the most important temperate fruit and is fourth among the most widely produced fruits in the world after banana, orange and grape. China is the largest apple producing country in the world. India produces 2497680 MT of apple from 313040 Ha area with productivity of 7.97 MT/Ha (Anonymous 2017). The relatively low productivity of apple in India is due to several biotic and abiotic factors. Biotic factors comparises of various fungal, bacterial and viral diseases. Among various viral diseases Apple chlorotic leaf spot virus (ACLSV) is economically important virus of apple. ACLSV was first reported in *Malus* spp. from the US by Mink and Shay in 1959 (Burnt *et al.* 1996). ACLSV is one of the important latent viruses infecting apple its infection rates ranges up to 80–100% in many commercial apple cultivars with yield losses of the order of 30–40% (Nemchinov *et al.* 1995; Wu *et al.*

1998; Cembali *et al.* 2003). In Himachal Pradesh (India) ACLSV came across as a major virus on apple with disease incidence of 85-90% (Rana *et al* 2010).

ACLSV a type member of the genus *Trichovirus* (Martelli *et al.*, 1994) *Flexiviridae* family (Adams *et al.,* 2004), is a common virus of most fruit trees of the *Rosaceae* family, which include apple, pear, quince, sweet and sour cherry, peach, plum and apricot (Lister, 1970; Nemeth, 1986). Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) is ideal tool for virus diagnosis because it is reliable, rapid and sensitive technique. Plant parts used for RNA isolation plays crucial role in the virus indexing program. Hence this study was planned to study distribution of ACLSV in different parts of apple tree.

MATERIAL AND METHODS

Samples for RNA isolation

The samples for study were obtained from ACLSV infected tree planted at ICAR-Indian Agricultural Research Institute, Regional Station, Shimla (HP). Different plant part includes leaf, flower petals, sepal, anther, pollen, bark, bud and fruit were selected for study. Samples collected and placed in plastic sample bags labelled with the location and brought to the laboratory at 4°C. All the lab based techniques were carried out in Division of Plant Protection, Central Potato Research Institute (CPRI) Shimla, HP (India).

Isolation of total RNA and c-DNA synthesis

Total RNA was isolated from apple samples by using Spectrum™ Plant Total RNA kit (Sigma-Aldrich, USA) as per manufactures instructions. Total RNA isolated was quantified and used for c-DNA synthesis by using Revert Aid™ of Fermentas Life Sciences as per manufactures instructions.

Virus specific primers for RT-PCR and internal control

Earlier designed primers by Watpade *et al.*, 2012 for amplifying CP gene of ACLSV were selected for RT-PCR amplification. To minimize the risk of obtaining false negative results primers for internal control by Menzel *et al.* 2002 were used.

RT-PCR Conditions

RT-PCR was carried out in thin walled 1.25ml tubes and volume was made up to 20 ml with sterile nano pure water in a GeneAmp PCR 9700 system (applied biosystems, USA). The reaction mixture of 20 µl containing 0.8 units of Red Taq DNA polymerase (Genei, Banglore, India) 1.6µl of 2 mM dNTP mix (Fermentas), 2.5 µl of 10x taq DNA Polymerase buffer (Genei, Banglore, India), 0.5 µl of each downstream and upstream primers and 2 µl of cDNA. Amplification was carried out by following PCR conditions with selected primer pair along with an internal, water and healthy control. Denaturation was performed at 94°C/30 sec. annealing temperature of 57^{0}C/1 min. followed by extension at 72°C /1 min. for 40 cycles along with a final elongation step at 72°C for 10 minutes. About 10 ml of the reaction mixture from each tube was loaded onto 1 per cent agarose gel alongside 1 kb DNA ladder as molecular weight marker. Electrophoresis was done at 80 V, The buffer used was 1x TAE at 8.0 pH. The DNA bands in the gel were visualized on a UV-transilluminator.

RESULT AND DISCUSSION

RNA isolation and cDNA synthesis

Total RNA isolated from different plant part i.e. leaf, petals, sepal, anther, pollen, bark, bud and fruit of apple tree were quantified by using Thermo scientific Nanodrop 2000 Spectrophotometer. Concentration of RNA was varied for different plant parts. Concentration of RNA was found highest in the petals of apple flower followed by young leaves. Proper grinding of sample is necessary for good quality of RNA. Plant samples such as petals, young leaves are easy to grind hence concentration of RNA is good in these samples. Isolation of RNA from

apple fruit is very difficult due to polysaccharides and polyphenolic compounds (Asif *et al.*, 2006).

RT-PCR Conditions

By following RT-PCR conditions standardized by Watpade *et al.*, 2012 and Watpade *et al.*, 2013 amplification of 432 bp was obtained. The designed primer pair for the internal control amplified consistently a fragment of the expected size i.e. 181 bp, irrespective of whether the extract came from infected or healthy plants. Amplification was not observed with respect to water control and healthy control (**Fig 1**). In all the reactions of RT-PCR a primer set amplifying apple mitochondrial NADH dehydrogenase subunit 5 (nad 5) gene was used as an internal control. Non DNase treated RNA extracts are often DNA contaminated (Nassuth *et al.*, 2000), making the use of other internal control primers as RT-PCR control difficult for such extracts, because they do not distinguish between RNA and DNA. DNase treatment of the extracts will eliminate these problems, but each additional extraction step is time consuming, will raise costs and increases the risk of contaminations. In order to avoid this, a primer pair designed by Menzel *et al.* (2002) was used which allows the specific amplification of mRNA even in the presence of genomic DNA.

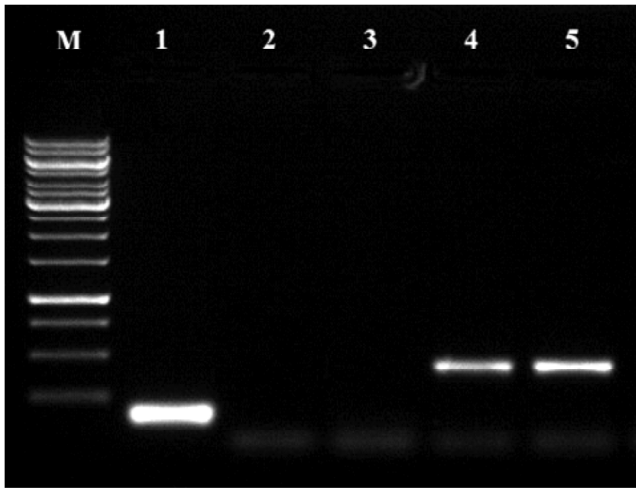

Figure 1: Amplification of coat protein gene of ACLSV along with internal control

Lane M: 1 kb DNA ladder, Lane 1: Internal control, Lane 2: water control, Lane 3: Healthy control, Lane 4 & 5: primer set 1F 1R with ACLSV positive sample

Detection of ACLSV in different plant part

RT-PCR conditions standardized for ACLSV and internal control were able to amplify desire amplicons from all plant part except pollen of apple flower. Petals were ideal part to isolate RNA because of highest RNA yield. Even RNA isolated from bark and fruit of virus positive plant showed desired amplification (**Fig 2**). In absence of leaves in the month of December-March bark of apple branches can be used for virus indexing. Even fruits collected from mother plants can be used for indexing for presence of ACLSV. Although virus is present in anthers but absence of virus in pollen clearly rules out pollen transmission possibility in apple. In similar study Kumar *et al.*, 2016 found that TYLCV had highest concentration in tomato twigs where as flower and fruit had low concentration.

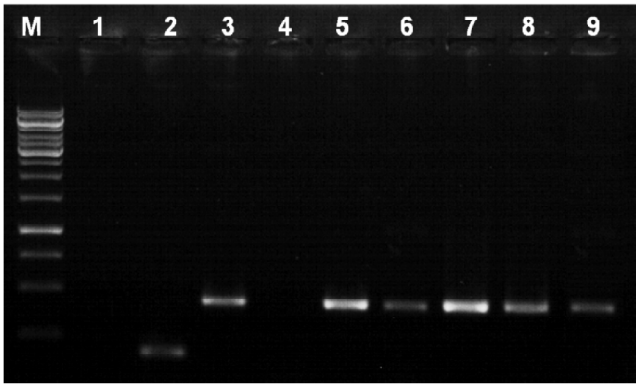

Figure 2: Detection of ACLSV in different plant part of apple

Lane 1: Marker, Lane 2: water control, Lane 3: Internal Control, Lane 4: anther, Lane 5: Pollen, Lane 6: Petal, Lane 7: sepal, Lane 8: Leaf, Lane 9: Bark

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