

Short Communications

Nucleic acid probe based technique for detection of cotton leaf curl virus in India

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Cotton leaf curl disease, caused by a geminivirus, has been detected using viral nucleic acid based hybridization tests in infected hosts and weeds. Cotton leaf curl was transmitted by whitefly, *Bemisia tabaci* to *Gossypium hirsutum* varieties, which showed typical symptoms of the disease. Hybridization with [α -³²P] dCTP radiolabelled CLCuV-DNA-A probe, detection of viral nucleic acids in different cotton cultivars viz., HS-6, H-1098, F-846, H-777, H-182, LH-1556 and RST-9 grown under glasshouse conditions, samples collected from Hisar, Sirsa and Dabwali and six weed hosts was carried out. Nucleic acid based tests could therefore be useful in screening of cotton germplasm in cotton breeding programme to detect minor infections.

Keywords: diagnostics, geminivirus, cotton, plant virus disease

Cotton (*Gossypium hirsutum*) is an important cash crop that plays a dominant role in India's agrarian and industrial economy. *G. hirsutum* is most widely grown and contributes to about 80% of the total cotton production in Asia. Whitefly-transmitted geminiviruses pose serious constraint to the production of cotton grown in subtropical and temperate regions of the world¹. The geminiviruses, cotton mosaic virus (CotMV), cotton leaf crumple virus (CLCrV) and cotton leaf curl virus (CLCuV) are known to cause infection on cotton under natural conditions²⁻⁷. All these viruses are transmitted through the whitefly, *Bemisia tabaci*¹.

Cotton leaf curl virus, a new member of Begomovirus genus of the family *Geminiviridae*, is a recently recognized pathogen of the leaf curl disease of cotton in Haryana⁸. During the past few years, 100% incidence of leaf curl disease of cotton was reported in India and Pakistan^{9, 10}. Being present in low concentration in infected tissues coupled with their extremely fragile nature and susceptibility to some buffers besides poor contrast in electron dense

stains, geminiviruses are very difficult to isolate and purify. Consequently introduction of antibodies against these viruses for immunodiagnostic purpose is a difficult process. A preferred choice for detection of viruses in these cases is the use of DNA probes. The objective of this study was to use CLCuV-DNA-A as a hybridization probe in molecular tests and detection of presence of CLCuV in leaf samples of *G. hirsutum* and weed hosts.

G. hirsutum plants showing typical leaf curl, vein thickening and enation type of symptoms were collected from C C S HAU, Hisar and the virus was maintained in susceptible variety HS-6 by whiteflies transmission in insect proof cages. Healthy whiteflies were maintained on healthy cotton and then given an acquisition period of 24 hrs on CLCuV infected cotton plants in cages and also sometimes in muslin cloth bags under the controlled environment at 35°C with supplementary lighting to give a 16 hrs photoperiod. Plants were inoculated by whiteflies at primary leaf stage. After inoculation, whiteflies were killed by spraying with Imidachloprid.

Plant DNA from CLCuV infected tissues was extracted^{11, 12}. Infected plant tissues were ground to fine powder using liquid nitrogen with a prechilled pestle and mortar in 2.5 vol of extraction buffer (100mM Tris HCl, pH 8.0; 100mM NaCl; 10mM EDTA; 1% SDS). While grinding, 2% water-insoluble PVP (polyvinyl pyrrolidone) was added. The supernatant of this extract was treated with equal vol of phenol : chloroform : isoamyl alcohol (25:24:1) and DNA was precipitated by adding 0.8 vol of isopropanol and 1/10th of 3M NaOAc, (pH 4.8). DNA pellet was then washed with 70% ethanol, air-dried and dissolved in sterile double distilled water. DNA used for different dot-blot and southern hybridization assays was derived from the same set of infected tissues.

Cloned DNA-A derived from CLCuV was radiolabelled with [α -³²P] dCTP. Cloned fragment of DNA A (~2.7 kb) was obtained by digestion with Eco RI and electrophoresed in 1% low melting point agarose gel. The desired DNA fragment was eluted from the gel¹³. The desired DNA was eluted from the gel and radiolabelled by random primer extension¹⁴.

Before dotting, Hybond nylon membranes (Amersham International, UK) were marked for grid,

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soaked in 10×SSC for 10-15 min. Excess SSC was allowed to be drained off and kept on filter paper. After spotting the known quantities of DNA (1µg, 0.1µg, 0.01µg), membranes were allowed to air dry and baked at 80°C for 2hrs. The [α - ^{32} P] dCTP labelled DNA probes were used for hybridization for 16 hrs and the detection was carried out by exposing to X-ray film at -70°C for overnight with intensifying screen.

Nucleic acid based technique appears to be more sensitive for detecting the presence of viruses even in very small quantities of test samples. Labelled nucleic acid probes have been used in the detection of several plant viruses^{15,16}. Cloned DNA can be prepared in large amounts and provide absolute purity and specificity of the probe to the tests in question.

The disease is transmitted through whiteflies from infected cotton to healthy cotton varieties (HS-6, H-1098, F-846, H-777, H-182, LH-1556 and RST-9), which showed typical symptoms of leaf curl disease. Total DNA was extracted from 200 mg each from healthy and virus infected cotton cultivars. DNA-A of all whitefly-transmitted geminiviruses shares a high degree of sequence homology in the coat protein gene¹⁷. Therefore, DNA-A of CLCuV could be used as a specific probe to detect the presence of whitefly-transmitted geminiviruses in glasshouse, field and its alternate hosts exhibiting symptoms of geminivirus infection.

A strong hybridization signal was obtained with DNA-A probe in CLCuV infected tissues (Fig. 1). This probe did not give any signals with DNA from healthy cotton tissue used as a control (Lane 8). Using the same probe the detection limit of CLCuV was determined. For this purpose, a blot was prepared with DNA obtained from infected cotton leaves (10^{-1} to 10^{-2} dilution). Leaves from various varieties of cotton showing leaf curl, vein-thickening symptoms along with some apparently healthy looking cotton plants were collected from the farmers fields of Hisar, Sirsa and Dabwali. Total DNA isolated from these samples was subjected to dot-blot hybridization analysis. Intense hybridization was obtained (Fig. 2). The probe could detect the presence of geminivirus in DNA obtained from up to 10^{-1} dilution and therefore is very sensitive.

Certain weeds might play an important role in the epidemiology of geminiviruses by acting as reservoirs (alternate hosts) of the virus during the non-cropping seasons¹⁸. Detectable hybridization was seen in the blots that contained DNA from infected tobacco,

China rose, *Sida* sp., ageratum, xanthium, okra, hollyhock and amaranthus (Fig. 3). Hybridization signal was not obtained in the blot with DNA isolated from xanthium and amaranthus. The results strongly suggest that the geminivirus that infects, tobacco, ageratum, hollyhock, *Sida* sp., Chinrose and okra are closely related to CLCuV. The observations as well as the positive detection of CLCuV using specific geminiviral probe prepared from DNA-A of CLCuV indicate and confirm that the mentioned weeds act as alternate hosts of CLCuV geminivirus.

Therefore, nucleic acid based tests appear to be the sensitive method and could especially useful in screening cotton germplasm in cotton breeding programmes in detecting the presence of leaf curl

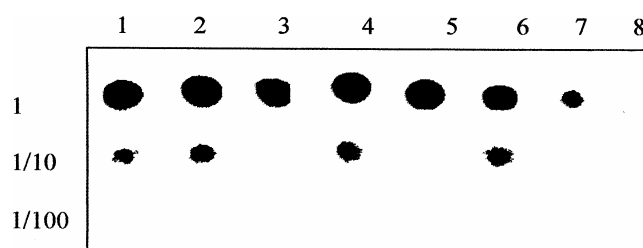


Fig. 1—Detection of CLCuV in test hosts by nucleic acid spot hybridization using CLCuV DNA A probe of glasshouse grown cultivars. Lane 1 HS-6, 2 H-1098, 3 F-846, 4 H-182, 5 H-777, 6 LH-1556 & 7 RST-9

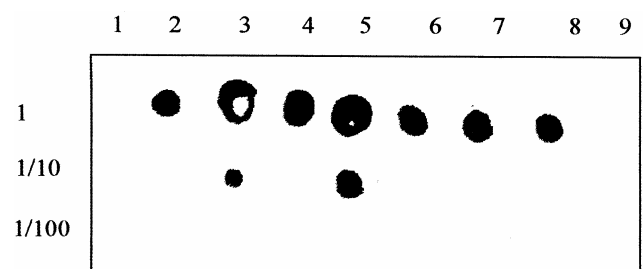


Fig. 2—Detection of CLCuV in test hosts by nucleic acid spot hybridization using CLCuV DNA A probe of field samples. Lanes 1, 2 & 3 Hisar, Lanes 4, 5 & 6 Dabwali, Lanes 7, 8, & 9 Sirsa

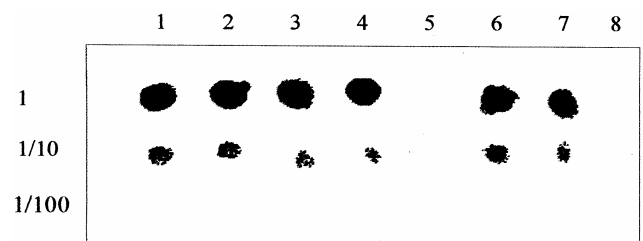


Fig. 3—Detection of CLCuV in test hosts by nucleic acid spot hybridization using CLCuV DNA A probe. Lane 1 Tobacco, 2 *H. rosa-chinensis*, 3 *Sida* sp., 4 *A. conyzoides*, 5 *Xanthium*, 6 Okra, 7 Hollyhock & 8 Amaranthus

disease in the infected plants even before the expression of symptoms. This technique has practical application for monitoring the viruliferous whitefly population and forecasting the leaf curl disease.

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