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Recent status and distribution pattern of cotton leaf curl disease in Northwest India: an alarming situation in future cotton cultivation

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ABSTRACT

A survey was made to study cotton leaf curl disease (CLCuD) incidence in cotton growing areas of Haryana, Punjab and Rajasthan in Northwest (NW) India during the years of 2013 and 2014. The present study revealed higher overall CLCuD incidence of 77.5% with higher overall boll number reduction (BNR) of 36.9% in 2013 compared to incidence of 49.6% with 7.6% BNR in 2014 in Haryana. In Rajasthan the disease incidence of 55.9% and 21.6% BNR in 2013 when compared to 10.8% of incidence and 2.9% BNR in 2014 was recorded. The overall CLCuD incidence and BNR in cotton growing areas of Punjab were more or less similar for both the years of 2013 and 2014, where disease incidence of 54.1% with BNR 14.6% in 2013 and disease incidence 57.8% with BNR of 15.9% in 2014 was recorded. All the 11 Bt-cotton hybrids from the farmer's fields of Sri Ganganagar and Sirsa districts surveyed were highly susceptible to CLCuD in both the years; showing 100% disease incidence with BNR of 32.3-82.3% in 2013 and 49.2-100% with BNR of 8.7-17.4% in 2014. Infectivity study through whitefly (*Bemisia tabaci*) and polymerase chain reaction (PCR) of ORF V1 (CP gene) determined that CLCuD in NW India is caused by whitefly transmitted CLCuD-begomoviruses. Sequence analysis of CP gene indicated that at least three CLCuD-begomoviruses variants appeared in this cotton growing region. The increased CLCuD incidence with huge yield loss and occurrence of CLCuD-begomovirus variants reported in the present is an alarming situation for the profitable cultivation of cotton in north India.

Key words: Boll number reduction, Cotton, Cotton leaf curl disease (CLCuD)-begomovirus, Disease incidence, *Gossypium hirsutum*, Resistance, Whitefly

Cotton (*Gossypium hirsutum*) is a major agricultural commodity which plays a dominant role in Indian economy with 20% industrial production and 30% export value. India is the largest producer of cotton in the world accounting for about 18% of the world cotton production, occupying the largest area of 12.2 Mha under cotton cultivation constituting about 25% of the world area under cotton cultivation (Annonymus 2016). The Bt-cotton hybrids now account for 94.75% of the country's entire cotton cultivation. Cotton leaf curl disease (CLCuD) is one of the serious constraints in cultivation of cotton in 1.1 Mha areas in Northwest (NW) India (Briddon *et al*. 2001*,* Monga *et al*. 2005, Rajagopalan *et al*. 2012). CLCuD in the Indian subcontinent is caused by whitefly transmitted monopartite begomoviruses in association with betasatellite and alphasatellite molecules (Briddon and Stanley 2006, Kumar *et al*. 2010*,* Rajagopalan *et al*. 2012). The occurrence of CLCuD has been reported from almost all the cotton growing areas of NW India (Rishi and Chauhan 1994*,* Kumar *et al*. 2010, Monga *et al*. 2011a*,* Zaffalon *et al*. 2011*,* Rajagopalan *et al*. 2012, Godara *et al*. 2015). The CLCuD begomoviruses can also infect several alternate host as well as weeds which serve as source of primary inoculum (Nateshan *et al.* 1996*,* Sivalingam *et al.* 2004*,* Monga *et al.* 2005).

CLCuD was first reported from Indian Agricultural Research Institute (IARI), New Delhi in 1989 and from farmers fields in Sri Ganganagar, Rajasthan in 1993 (Ajmera, 1994*,* Varma *et al.* 1993). CLCuD-begomovirus species, *Cotton leaf curl Multan virus* (CLCuMuV), *Cotton leaf curl Kokhran virus* (CLCuKoV) and *Cotton leaf curl Alabad virus* (CLCuAlV) have been identified in India (Ahuja *et al.* 2007*,* Rajagopalan *et al.* 2012, Kumar *et al.* 2010). Cotton leaf curl Rajasthan virus (CLCuRV) and Cotton leaf curl Burewala virus (CLCuBuV) were identified as predominant viruses in cotton growing areas of NW India, but after recent report of Brown *et al*. (2015), these two viruses are now considered to be CLCuMuV-Rajasthan and

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CLCuKoV-Burewala strains, respectively.

CLCuD has emerged as a serious problem limiting cotton production in NW India in the current situation. Until 2004, CLCuMuV-Rajasthan was predominant, but since 2005-06, CLCuKoV-Burewala, a resistant breaking strain, reported first from Pakistan (Mansoor *et al.* 2003) appeared to be a predominant strain in NW India (Rajagopalan *et al.* 2012). Most of the cotton varieties resistant or tolerant to CLCuD earlier have become highly susceptible showing incidence of 1.0-97.0% with 53.6% boll number reduction (BNR) in particular field during the period of 2008-2010 in NW India and developed severe symptoms (Monga *et al.* 2011a and 2011b*,* Rajagopalan *et al.* 2012). During the growing seasons of 2009 and 2010, Rajagopalan *et al*. (2012) reported severe CLCuD epidemic in cotton fields of Bathinda, Abohar, Fazilka, Sri Ganganagar and the surrounding areas of Punjab and Rajasthan states were observed causing BNR up to 100% in some fields. A recent study based on a survey of CLCuD incidence in IARI, New Delhi cotton field for the last six years from 2009 to 2014 showed that overall disease incidence increased to as high as 15.92% in 2013 from 2.1% in 2009 (Godara *et al.* 2015). Of several cotton varieties evaluated against CLCuD in field condition in 2013 and 2014, none was found to be resistant, and in some cultivars showing incidence of 26.4-46.4% (Godara *et al.* 2015).

As the high incidence and higher severity of CLCuD with considerable yield loss is common in NW Indian cotton growing areas, effort has been made to conduct extensive survey and study the disease incidence and yield losses caused by CLCuD-begomoviruses in the cotton growing regions of India and determine distribution of CLCuD begomoviruses and its natural variants.

MATERIALS AND METHODS

A survey was made to study CLCuD incidence in cotton growing areas of Rajasthan, Punjab and Haryana in NW India. Two cotton growing districts, Sri Ganganagar and Hanumangarh in Rajasthan, four districts Fazilka, Bathinda, Mansa and Faridkot in Punjab and four districts Sirsa, Fatehabad, Hisar and Rohtak of Haryana were undertaken for survey. Nine areas of Sri Ganganagar and seven areas of Hanumangarh districts in Rajasthan; 12 areas in Fazilka, 3 areas in Bathinda, 2 areas in Mansa and 1 area in Faridkot district in Punjab; and 7 areas in Sirsa, 6 areas in Fatehabad, 10 areas in Hisar and five areas in Rohtak districts in Haryana were surveyed for two successive years of 2013 and 2014.

The percent disease incidence was estimated using the standard method {(No. of plants infected/ No. of plant srandomly taken in a particular block) × 100}. The percent reduction in boll number (a measure of yield reduction) was estimated using the standard method {(Total numbers of bolls in 10 infected cotton plant randomly in the infected field /total numbers of bolls in 10 healthy cotton plant) × 100}. CLCuD incidence and BNR in 11 Bt-hybrid cottons grown in different farmer's field of Sri Ganganagar district of Rajasthan and Sirsa district of Haryana during 2013 and 2014 were estimated. Two to three twigs of cotton plant showing typical CLCuD symptoms were randomly selected and brought to laboratory for molecular analysis.

About five to seven cotton seeds were sown in earthen pots (25 cm-dia.) covered with muslin-cloth cage (microcage) in insect-proof greenhouse. Healthy whiteflies were maintained on healthy tobacco (*Nicotiana tabacum* cv. Xanthi and cv. White Burley), cotton (*G. hirsutum* cv. RST 9, bottle gourd (*Lagenaria siceraria* cv. Pusa Naveen), blackgram (*Vigna mungo* cv. KU-91) and mungbean (*V. radiata cv.* PS 16) plants in insect-proof greenhouse. Healthy whitefly colonies were given acquisition access period (AAP) for 18-24 h on source plants (infected cotton twigs or leaves). The CLCuD infected cotton plant (cv. H-1226) collected from field was used as source of virus inoculum. Eight to ten viruliferous whiteflies per plant were released on cotyledonary leaves of 4-4 days old healthy cotton test plants for inoculation access period (IAP) of 18-24 h. Inoculated plants were covered with micro-cage which could cover each plant individually. After IAP, the whiteflies were killed by spraying insecticide, imidacloprid @ 0.1-0.15%. The inoculated plants were kept in an insect-proof greenhouse for symptom observation up to 60 days of inoculation (DAI).

Symptomatic cotton samples (or isolates) were collected from different cotton growing areas of NW India and brought to laboratory for diagnosis through polymerase chain reaction (PCR) and molecular characterization based on ORF V1 (CP gene) of CLCuD-begomovirus. Total plant DNA from infected plants was extracted using CTAB method (Haible *et al.* 2006). About 80-100 mg of leaf tissue was ground with liquid N_2, homogenized with 1 ml of extraction buffer and total plant DNA was isolated. In order to confirm CLCuD-begomoviruses causing disease, specific primers, forward 5'-AATTATGTCGAAGCGAGCTG-3' and reverse 5'-TAATATCAATTCGTTACAGAG-3' targeting full length CP gene (ORF V1) described earlier (Kumar *et al.* 2010) were synthesized and used for PCR. The desired amplicons of CP gene were obtained and analysed in 1% agarose gel electrophoresis.

The PCR products of complete CP gene were purified using QIA Quick PCR purification kit and cloned into the T&A cloning vector system I according to the protocol used earlier (Biswas *et al.* 2012). Two clones of each isolate were sequenced using vector derived primers M13 Forward and M13 Reverse in an automatic sequencer. The consensus sequences were taken for further analysis. Multiple sequence alignment was performed with CLUSTAL X version 1.81 (Thomson *et al*. 1997). The sequence identity matrix was generated using Gene Doc version 2.6.002. The phylogenetic tree analysis was performed using Neighbor-joining method in MEGA 5.2 program (Tamura *et al.* 2011).

RESULTS AND DISCUSSION

Disease incidence and boll number reduction due to CLCuD in cotton growing areas in NW India

Surveys were made to study the incidence of CLCuD

and BNR in cotton by this disease in the cotton growing areas of Haryana, Punjab and Rajasthan of NW India for two successive years of 2013 and 2014. The CLCuD infected cotton plants showed typical downward or upward leaf curling or cupping, vein thickening and sometimes leafy enations. In field condition the disease symptoms generally appeared in infected cotton plant within 5-7 weeks after sowing of seeds. Early infection leads to severe leaf curl along with stunting of the plant with considerable BNR but late infection leads to mild symptoms with negligible BNR.

Haryana: Sirsa, Fatehabad, Hisar and Rohtak districts of Haryana were surveyed. Twenty fields in the year 2013 and 19 fields in 2014 covering seven cotton growing areas in Sirsa district were selected for survey (Table 1). The CLCuD incidence of 47.5-95.2% with BNR of 9.8-52.5% and incidence of 34.5-81.2% with BNR of 2.5-16.5% were estimated in 2013 and 2014, respectively in Sirsa. In this district, overall CLCuD incidence of 73.9% in 2013 and

Table 1 CLCuD incidence and yield loss in cotton growing areas of Northwest India during the years of 2013 and 2014

State	District	Area	2013			2014		
			No. of field surveyed	Disease incidence (%)	Boll number reduction (%)	No. of field surveyed	Disease incidence (%)	Boll number reduction (%)
Haryana	Sirsa	CICR-Sirsa	4	81.8	32.5	5	81.2	3.8
		Panjuwana	4	60.2	23.2	2	35.0	4.0
		Sahanwala	3	71.4	31.6	2	40.5	8.5
		Paniwala	2	47.5	9.8	2	35.5	2.5
		Odhan	3	67.5	26.7	4	54.3	5.25
		Bhawdin	2	95.2	52.5	2	34.5	10.0
		Moriwala	2	94.2	35.0	2	55.0	16.5
		Average		73.9	30.2		48.0	7.2
	Fatehabad	Aili Sardar	2	70.5	27.5	2	57.5	8.75
		Dilakhera	2	59.5	32.5	3	84.6	7.3
		Gilan Kheda	2	95.1	35.5	2	40.5	3.5
		Dhariapur	4	70.65	41.3	2	77.5	22.5
		Hanspur	4	97 .5	61.5	4	54.5	6.5
		Daulatpur	2	91.6	22.5	2	35.0	2.5
		Average		80.8	36.8		58.3	8.5
	Hisar	CCSHAU Farm	2	94.5	91.5	2	66.3	12.5
		Mudhal Sorkhi	2	100	55.0	2	27.5	10.0
		Hansi Gardi	1	80.0	36.6	3	50.3	2.3
		Ghana Kalan	2	94.5	85.0	2	44.7	5.0
		Dhanikutubpur (Hansi)	1	100	78.5	2	25.2	4.5
		Ludas	1	100	61.5	4	40.3	5.5
		Aryanagar	2	75.5	45.0	3	35.2	10.6
		Balsaman	4	49.5	38.75	2	25.1	6.5
		Gangwa	2	60 .0	43.4	2	54.3	5.5
		Agroha	2	50.5	40.0	2	64.5	10.0
		Average		80.5	57.5		43.4	7.2
	Rohtak	Bahu Jamalpur	3	43.3	2.6	2	50.1	7.8
		Kharkhara	1	90.2	15.2	4	30.4	5.2
		Bhahelba	1	50.3	7.5	2	45.4	7.5
		Bhaini-Maharajpur (Mahem)	1	100	69.6	2	59.1	10.6
		Madina	1	89.5	19.4	2	60.0	6.4
		Average		74.7	23.0		49 .0	7.5
		Overall in Haryana		77.5	36.9		49.6	7.6

Cond.

Table 1 (*Concluded*)

State	District	Area	2013			2014		
			No. of field surveyed	Disease incidence (%)	Boll number reduction (%)	No. of field surveyed	Disease incidence (%)	Boll number reduction (%)
Rajasthan	Sri Ganganagar	ARS, SKRAU	3	90.3	40.4	5	42.5	5.0
		Hindumalkot	2	79.7	14.6	4	7.0	2.5
		Dullapurikeri	2	70.2	12.5	2	5.2	2.5
		Fatuhi-1	2	34.8	22.5	2	5.9	3.0
		Fatuhi-2	2	79.7	27.5	2	7.5	2.5
		Fatuhi-3	2	34.9	18.8	2	2.9	0.5
		Kaliyan	2	97.5	71.2	2	17.5	5.3
		Udyog Nagar 1	2	75.1	35.0	2	25.0	5.7
		Indrapur	2	35.3	7.5	2	17.5	4.0
		Average		66.4	27.8		14.6	3.4
	Hanuman-garh	Pakka Sarna	2	60.2	16.0	4	2.5	0.5
		Jhandenwala	2	54.8	18.6	2	2.5	0.5
		Dhandelwala	2	70.0	12.5	2	7.5	1.5
		Hanumangarh Junction	2	44.8	10.0	2	6.5	2.5
		Chestian	4	62.5	32.4	2	7.5	2.5
		Shigawali	2	35.3	8.5	2	10.0	4.1
		Teen Chak	2	49.9	10.0	2	12.5	4.9
		Average		45.4	15.4		7.0	2.4
		Overall in Rajasthan		55.9	21.6		10.8	2.9
Punjab	Fazilka	Asman Kheda	2	45.5	8.5	2	47.5	8.5
		Gumjal	2	54.5	12.2	4	61.3	20.0
		Bakinwala	2	45.2	24.7	2	7.5	4.0
		Jhandwala	2	35.2	8.0	2	60.2	25.0
		Dharam Pura	2	60.1	15.3	2	35.4	8.5
		Kallar Khera	2	34.6	7.5	2	39.4	10.0
		Ghallu	2	25.2	2.5	2	67.5	27.5
		Danewala	4	29.7	3.8	4	45.2	8.0
		Bakeinwala	3	75.3	25.6	3	50.3	7.3
		Roopnagar	2	17.5	4.75	1	55.1	15.0
		Barikan	2	13.5	7.25	2	34.4	8.5
		Koel Khera	2	27.2	6.0	2	45.0	12.5
		Average		38.6	10.5		45.7	12.9
	Bathinda	Farmer field	3	27.5	9.0	4	45.0	12.75
		KVK Farm	2	40.0	12.5	4	62.2	17.5
		RRS Farm, PAU	4	53.75	3.5	4	36.9	3.0
		Average		40.4	8.3		48.0	11.7
	Mansa	Karandi	2	45.5	4.1	2	15.2	8.5
		Sangha	2	54.5	8.4	2	64.8	15.0
		Average		50 .0	6.2		40 .0	11.7
	Faridkot	RRS Farm, PAU	3	87.5	14.0	4	97.5	27.5
		Overall in Punjab		54.1	14.6		57.8	15.9

CICR: Central Institute for Cotton Research, CCSHAU: Chaudhary Charan Singh Haryana Agricultural University, SKRAU: Swami Keshwanand Rajasthan Agricultural University, ARS: Agricultural Research Station, RRS: Regional Research Station, PAU: Punjab Agricultural University

48.0% in 2014, with overall BNR of 30.2% in 2013 and 7.2% in 2014, respectively, were observed. In the year 2013, the maximum disease incidence of 95.2% with maximum BNR of 52.5 % were estimated in the village Bhawdin, but in 2014, in the same village less disease incidence of 34.5% and minimum BNR of 10.0% were observed. During the year 2014, the maximum disease incidence of 81.2% was observed in the field of Central Institute for Cotton Research (CICR), Sirsa but BNR in this field was very less up to 3.8%; whereas, in Moriwala village the disease incidence was 55.0% but BNR was higher of up to 16.5%.

Sixteen fields in 2013 and 15 fields in 2014 covering six areas in Fatehabad district were surveyed (Table 1). The CLCuD incidence of 80.8% (range of 59.5-97.5%) in 2013 and 58.3% (range of 35.0-84.6%) in 2014 were observed. In this district, BNR was 36.8% in 2013 and 8.5% in 2014. In this district, the maximum disease incidence of 97.5% with BNR of 61.5% was recorded in Hanspur area in 2013. During 2014, the maximum incidence of 84.6% in Dilakhera and maximum BNR of 22.5% in Dhariapur were recorded.

In Hisar district, 19 fields in 2013 and 24 fields in 2014 covering 10 areas were surveyed. The CLCuD incidence was 80.5% (range of 49.5-100%) in 2013 and 43.4% (range of 25.1-66.3%) in 2014. The BNR of 57.5% (range of 36.6-91.5%) and 7.2% (range of 2.3-12.5%) were estimated in 2013 and 2014, respectively. Of 19 fields of Hisar district surveyed, five areas showed maximum disease incidence of 90-100% in 2013, but maximum of 64.5-66.3% was observed in two areas in 2014. The BNRs of 80% and above were recorded in CCS Haryana Agricultural University (CCSHAU) Farm (91.5%) and Ghana Kalan (85.0%) in 2013, but in 2014, maximum disease incidence of 66.3% with BNR of 12.5% was observed in CCSHAU Farm (Table 1).

Seven fields in 2013 and 12 fields in 2014 covering five areas of Rohtak district were surveyed (Table 1). The CLCuD incidence was 74.7% (range of 43.3-100%) in 2013 and 49.0% (range of 30.4-60.0%) in 2014. The BNR of 23.0% (range of 2.6-69.6%) in 2013 and 7.5% (5.2-10.6%) in 2014 was recorded. During 2013 overall disease incidence of 90% and above were recorded in three areas, Kharkhara, Bhaini-Maharajpur and Madina during the year 2013 and maximum BNR of 69.6% was recorded from Bhaini-Maharajpur, and 15.2% and 19.4% from Kharkhara and Madina of this district respectively. During the year 2014, the maximum disease incidence of 59.1-60.0% with BNR of 6.4-10.6% was recorded in Madina and Bhaini-Maharajpur.

In Rajasthan: Two cotton growing districts, Sri Ganganagar and Hanumangarh in Rajasthan were surveyed. Nineteen fields in 2013 and 23 fields in 2014 covering nine areas of Sri Ganganagar district were surveyed (Table 1). The disease incidence of 66.4% (range of 34.8-97.5%) with BNR of 27.8% (range of 7.5-71.2%) in 2013 and it was 14.6% incidence (range of 2.9-42.5%) with BNR of 3.4% (0.5-5.7%) in 2014 were estimated in Sri Ganganagar. In this district, higher incidence of 90.3% with BNR of 40.4% in Agricultural Research Station (ARS), Swami Keshwanand Rajasthan Agricultural University (SKRAU) and incidence of 97.5% with BNR of 71.2% in Kaliyan were recorded in 2013. But in 2014, the less incidence of 42.5% with less BNR of 5.0% was observed in ARS, SKRAU.

In Hanumangarh district, 16 fields covering seven areas were surveyed. The disease incidence of 45.4% (range of 35.3-70.0%) with BNR of 15.4% (range of 8.5-32.4%) in 2013 and incidence of 7.0% (range of 2.5-12.5%) with BNR of 2.4% (range of 0.5 to 4.9%) in 2014 were estimated (Table 1). In 2013, the maximum incidence of 60.2 to 70.0% with maximum BNR of 16.0-32.4% was recorded in the areas of Pakka Sarna, Dhandelwala and Chestian. During 2014, the maximum disease incidence of 12.5% with BNR of 4.9% was recorded in areas of Teen Chak.

In Punjab: Four districts, Fazilka, Bathinda, Mansa and Faridkot in Punjab were surveyed. In Fazilka district, 27 fields in 2013 and 28 fields in 2014 covering 12 cotton growing areas were surveyed (Table 1). The incidence was 38.6% (range of 13.5-75.3%) with BNR of 10.5% (range of 2.5-25.6%) in 2013 and incidence was 45.7% (range of 7.5 to 67.5%) with BNR of 12.9% (range of 4.0 to 27.5%) in 2014. In this district, the maximum disease incidence of 75.3% with BNR of 25.6% was recorded from Bakeinwala in 2013 but the maximum incidence of 67.5% with BNR of 27.5% was recorded from the fields of Ghallu in 2014.

In Bathinda district, 9 fields in 2013 and 12 fields in 2014 covering three cotton growing areas were surveyed. The CLCuD incidence was 40.4% (range of 27.5-53.7%) with BNR of 8.3% (range of 3.5-12.5%) in 2013 and it was 48.0% incidence (36.9-62.2%) with BNR of 11.7% (3.0-17.5%) in 2014.In this district, during the season of 2013, the maximum incidence was 53.7% in the field of RRS, Punjab Agricultural University (PAU), but BNR was minimum up to 3.5% in this area.In the field of Krishi Vigyan Kendra (KVK), PAU, the disease incidence was 40.0% but BNR was higher up to 12.5%. In 2014, the maximum incidence of 62.2% with BNR of 17.5% was recorded in KVK, PAU.

In Mansa district, 4 fields in 2013 and 4 fields in 2014 covering two areas were surveyed. The CLCuD incidence of 50.0% with BNR of 6.2% in 2013 and incidence of 40.0% with BNR of 11.7% in 2014 were estimated. In Faridkot district, three fields in 2013 and four fields in 2014 in the Farm of RRS, PAU were surveyed. The CLCuD incidence of 87.5% with BNR of 14% in 2013 and disease incidence of 97.5% with BNR of 27.5% in 2014 were estimated.

The CLCuD incidence and boll number reduction caused by CLCuD begomovirus varied in cotton growing areas and districts of Rajasthan, Punjab and Haryana in NW India. The overall CLCuD incidence and BNR was generally higher in the year 2013 compared to the year 2014. In the year 2013, high CLCuD disease incidence and BNR in all the districts of Haryana were observed; incidence of 73.9% in Sirsa, 80.8% in Fatehabad, 80.5% in Hisar and 74.7% in Rohtak district with BNR of 30.2%, 36.8%, 57.5% and 23.0%, respectively. However, during the year 2014, moderate disease incidence of 43.4-58.3% with BNR of 7.2-8.5% for all the four districts was observed. The

present study revealed higher overall CLCuD incidence of 77.5% with higher overall BNR of 36.9% in 2013 compared to incidence of 49.6% with 7.6% BNR in 2014 in cotton growing areas of Haryana.

The present study showed higher overall disease incidence of 66.4% with BNR of 27.8% in Sri Ganganagar district, whereas less incidence of 45.4% with less BNR of 15.4% in Hanumangarh district of Rajasthan in the year 2013. But the disease incidence was observed to be very low of 7.0-14.6% with minimum BNR of 2.4-3.4% in both the districts of Rajasthan in 2014. In Rajasthan, the overall disease incidence of 55.9% and BNR of 21.6% in 2013 were very high when compared to incidence of 10.8% and BNR of 2.9% in 2014.

In 2013, CLCuD incidence and BNR were high in the cotton growing areas of Haryana and Rajasthan and it might be due to appearance or dominance of virulent strains CLCuD-begomoviruses and its mixed infection. High population of insect vector whiteflies and its efficiency for CLCuD transmission might be another factor for occurrence of increased disease incidence in this region.

CLCuD incidence and BNR in cotton growing areas of Punjab is more or less similar in both the years of 2013 and 2014. The disease incidence of 38.6-87.5% with BNR of 6.2-14.0% in 2013 and incidence of 40.0-97.5% with BNR of 11.7-27.5% BNR in 2014 were recorded. However, in Punjab overall disease incidence of 54.1% and BNR of 14.6% in 2013 and the disease incidence of 57.8% and BNR of 15.9% in 2014 were similar in both the years; it might be due to the prevalence of similar CLCuD-begomoviruses or strains and/or similar vector or vector population density responsible for transmission in both the years.

Even though in some areas CLCuD incidence was very high, the yield losses are less. For an instance, during the year 2014 in CICR Farm in Sirsa, the disease incidence was as high as 81.2% but the BNR was very less up to 3.8%, whereas in Bhawdin of Sirsa district, Haryana, the incidence is comparatively less of 34.5% but the BNR is rather high up to 10.0%. Similarly, during the year 2013, RRS Farm of PAU, Bathinda, the disease incidence was as high as 53.7% but the BNR was very less up to 3.5%, whereas disease incidence of 27.5% with high BNR of 9.0% was observed in farmer's fields of Bathinda. The elevated CLCuD incidence with minimum yield losses might be due to appearance of disease at later stage of crop growth showing less severity, as crop loss is related with the time of infection of virus. Infection of cotton by CLCuD and early stages of plant growth causes remarkable crop damage (Akhtar *et al*. 2003). If the CLCuD-begomovirus infects at late stage when plants are aged and mature enough, it can tolerate the attack of the disease and the crops give good yield even in the presence of CLCuD symptoms in the plant, even with severe leaf curling at top (Ali *et al*. 1995).

During the cropping season of 2009, higher CLCuD incidence with severe symptoms in the cotton fields of Abohar and Bathinda in Punjab, and Sri Ganganagar of Rajasthan have been reported (Rajagopalon *et al.* 2012). The CLCuD in cotton fields in the areas of Hisar, Sirsa and Dabawali regions of Haryana appeared during the 2010, but it was noticed to be almost free from this disease in these areas until 2009. During the year of 2010, the disease incidence was quite high in the Abohar, Bathinda and Sri Ganganagar areas (50-100%) showing 100% disease incidence in all the fields of Abohar surveyed, but it was sporadic in the areas of Fazilka and Hanumangarh districts showing a range of disease incidence from 0 to 30% (Rajagopalon *et al.* 2012).

Disease incidence and boll number reduction in CLCuD infected Bt-hybrid cotton in NW India

The CLCuD incidence and BNR of 11 Bt-cotton hybrids from different Private Seed Companies of India grown in farmers fields of Sri Ganganagar district, Sirsa district and ARS, SKRAU, Sri Ganganagar were studied (Table 2). All the Bt-hybrids were found to be susceptible in both the years. All the Bt-cotton hybrids showed 100% disease incidence with BNR of 32.3-82.3% in the year 2013. But, higher BNR of 68.9-82.3% was observed in four Bt- cotton hybrids, KCH-59, KSCH-209, MRC- 7361 and VICH-310. In 2014, the Bt-cotton hybrids showed 49.2-100% disease incidence with BNR ranging from 8.7 to 17.4%. Similar pattern of disease incidence and BNR as observed in Bt-cotton hybrids was observed in all the four non-Bt cotton varieties, Bihani-161, F-2228, LH-2108, and MR-786 grown in the farmer's field in both the years of 2013 and 2014. All these non-Bt cotton varieties showed higher incidence of 90-100% with BNR of 32.3-71.0% in 2013 and incidence of 49.2-86.4% with BNR of 8.2-19.5%.

The Bt-cotton hybrids were introduced in NW India during the year of 2005 and after that a large number of Bt-cotton hybrids are introduced for cultivation in this region. Mostly all of the Bt-cotton hybrids are recently observed to be highly susceptible to CLCuD (Akram *et al*. 2013, Godara *et al*. 2015, Akhtar *et al*. 2015). Akram *et al*. (2013) reported that Bt-cotton hybrids are more susceptible host for whiteflies than non-Bt cottons in Pakistan resulting in higher disease incidence in Bt-hybrid cotton. Akhtar *et al*. (2015) in Pakistan evaluated 75 Bt-cotton hybrids against CLCuD under high inoculum pressure in the field condition and none of them was found to be disease free. Recently, 12 Bt-cotton hybrids have been evaluated against CLCuD-begomovirus in greenhouse condition through whitefly inoculation in India, and all were found to be highly susceptible showing 67-100% disease incidence with typical CLCuD symptoms (Godara *et al.* 2015). The present study showed similar pattern of disease incidence and yield losses for both the Bt-hybrid and non-Bt cottons. The previous (Akram *et al*. 2013, Godara *et al*. 2015, Akhtar *et al*. 2015) and the present study concluded that the susceptibility of Bt-cotton hybrids to CLCuD is almost similar to the susceptibility of the non Bt-cottons.

Infectivity test of CLCuD through whitefly

Six CLCuD infected samples/isolates from different

Table 2 CLCuD incidence and yield loss in Bt-hybrid cotton in cotton growing areas of Northwest India during 2013 and 2014

Cotton genotype	Seed source	2013		2014		Symptoms observed
		Disease incidence (%)	% boll number reduction	Disease incidence (%)	% boll number reduction	
Bt Hybrid cotton						
Bioseed- 6488	Shriram	100	52.5	65.3	16.5	Uc, Vt
Bunty 2113-2 (BGII)	Bioseeds	100	41.4	69.4	17.4	Dc, Uc, Vt
JKCH-1947 (BII)	JK Seeds	100	32.3	78.3	8.7	Uc, Vt
KCH-59	Jadoo	100	71.0	78.0	9.8	Vt, LE, LH
KSCH-209 (BGII)	Kohinoor Seeds	100	68.9	82.0	11.5	Dc, Uc, Vt, Less LE
MRC-7361 (BGII)	Mahyco	100	70.8	100	12.5	Uc, Vt
NCS-459 (BG-II) Sumo	Nuziveedu Seeds	100	53.8	49.2	8.75	Dc, Uc, Vt
PCH-877 BGII	Parbhat Seeds	100	38.4	65.8	9.8	Uc,Vt
RCH-653	Rasi Seeds	100	36.8	56.8	9.7	Dc, Uc, Vt, LE
SP-7017 (BGII)	Bayer Crop Science	100	53.8	70.2	14.2	Uc, Vt
VICH-310 (BGII)	Vikram Seeds	100	82.3	65.8	10.6	Uc, Vt
Non-Bt cotton (variety)						
Bihani-161	Bihani Seeds Pvt Ltd	90	40.2	78.0	8.9	Dc, Uc, Vt
F-2228	RS, PAU, Faridkot	90	71.0	49.2	18.4	less Dc, less Uc, Vt
LH-2108		100	70.8	65.8	8.2	Dc, Uc, Vt
MR-786	MR Seeds Pvt. Ltd	95	32.3	86.4	19.5	Dc, LE, Uc, Vt
Control/Check experiment						
RCH-134 (BGII) (Bt-Hybrid)*	Rasi Seeds	100	83.3	100	80.9	Uc, LE, Vt
RS-2013 (variety)*	ARS, SKRAU, Sri Ganganagar	87.5	38.4	83.6	16.8	Dc, Uc, Vt
RST-9 (variety)*		100	73.4	100.0	66.3	Dc, Uc, Vt
HS-6 (variety)*	CCS HAU, Hisar	100	75.5	78.0	68.8	Dc, LE, Uc, Vt

*Susceptible cultivars grown at ARS, Sri Ganganagar and presently not used by farmers; Uc: Upward leaf curling, Dc: Downward leaf curling, Vt: Vein thickening, LE: Leaf enation, LH: Leaf hardening; RS: Regional Station, PAU: Punjab Agricultural University, ARS: Agricultural Research Station, SKRAU: Swami Keshwanand Rajasthan Agricultural University

cotton growing areas of Haryana, Punjab and Rajasthan were randomly collected and pathogenicity of these isolates was tested through whitefly inoculation on the healthy susceptible cotton variety RST-9 in greenhouse condition. All the isolates produced typical CLCuD symptoms in the inoculated plants within 8-25 days after inoculation. About 60-100% of inoculated plants were found to be infected by CLCuD. All the isolates showed similar types of symptoms in the inoculated cotton variety (Table 3). These findings concluded that the leaf curl disease in NW India is efficiently transmitted by insect vector whiteflies.

Disease diagnosis by polymerase chain reaction

Nine samples (or isolates), S-9 from CICR, Sirsa; S-11 from Panjwana, Si-14-1from farmers field of Sirsa, Si-17 from Moriwala, Sirsa; Uf-1 from CCSHAU, Hisar; Hi-3 from Ghanakalan, Hisar; Rh-4 from Kharkhara, Rohtak; Sa-3 from Sahanwala of Haryana state and Ma-14-3 from Manza, Punjab were collected, and CLCuD-begomovirus infection was detected through PCR using specific primer pairs KS1F and KS1R targeting CP gene of CLCuD-begomovirus. The complete CP genes (750 nt) were amplified from all the samples tested and observed in 1% agarose gel electrophoresis. These results confirmed that the leaf curl disease in NW India is caused by CLCuD-begomovirus.

Molecular characterization of CLCuD-begomovirus based on cloning and sequencing of CP gene

The complete CP gene (750 nt) of CLCuD-begomovirus isolates amplified in the present study by PCR were purified and cloned in T&A cloning vector. Two clones from each isolate were sequenced and the consensus sequences were taken for sequence analysis. The CP gene of the present CLCuD-begomovirus isolates were analysed and compared with 23 other CLCuD-begomovirus isolates retrieved from NCBI database (Fig 1 and 2). The present isolates shared 80-100% nucleotide (nt) identity among themselves, and 78-100% nt identity among other isolates. The present isolates S-9, Rh-4, Uf-1, Sa-3, Si-17 and Ma-14-3 were similar showing 97-100% nt identity among themselves. The isolates S-11 and Hi-3 were similar showing 94% nt identity between them. The isolate Si-14-1 was distinct as

Table 3 Infectivity test of CLCuD field isolates of Northwest India through whitefly inoculation in greenhouse condition

CLCuD source	Source of inoculums (in field)	Inoculated cultivar (in greenhouse)	Days taken to appear symptom after inoculation	No. pl infected/ No. of pl inoculated (%)	Symptoms induced
Sirsa (CICR)	H-1236	RST-9	20-25	6/10 (60)	Uc, Vt
Fatehabad (Dilakhera)	RST-9	RST-9	20-25	10/12 (83.3)	Dc, Uc, Vt
Fazilka (Gumjal)	H-117	RST-9	11-20	8/10 (80)	Dc, St
Bhatinda (KVK farm)	P-31	RST-9	8-19	9/10 (90)	Dc, Uc
Sri Ganganagar (ARS, SKRAU)	Rasi-134 Bt	RST-9	14-21	7/10 (70)	LE, Uc
Hanumangarh (Pakka Sarna)	SP-7007 Bt	RST-9	15-21	10/10 (100)	Dc, LE, Uc

*Dc: Downward curling, Uc: upward curling, St: stunting, LE: leaf enation, Vt: vein thickening; plants 4-7 days after germination were inoculated with 5-10 viruliferous whitefly per plant

it had 80% nt identity with other present isolates.

In the phylogenetic analysis, the present isolates fell into three genogroups; the isolates, S-9, Rh-4, Uf-1, Sa-3, Si-17 and Ma-14-3 fell into first group along with many isolates of CLCuMuV. The isolates S-11 and Hi-3 fell into second group along with many isolates or strains of CLCuMuV and CLCuAlV, and the isolate Si-14-1 fell into third group along with the Burewala and -Shahdadpur strain of CLCuKoV (Fig 1).

In the present study, CLCuD-affected cotton samples were collected from different cotton-growing areas of NW India and the infection of cotton by CLCuD-begomovirus was confirmed by PCR, The genetic variability of the causal virus was determined by cloning and sequencing analysis of the full-length CP gene. At least three CLCuD-begomovirus variants causing CLCuD in cotton were identified based on CP gene analysis. However, analysis of CP gene is not enough to recognize the new or resistance-breaking or virulent strains. Thus, cloning and sequencing of complete virus genome is necessary to determine the specific CLCuD-begomovirus variant occurring in NW India.

The present study reports the high incidence of CLCuD with high yield losses in the current situation in NW Indian cotton growing areas and both the Bt-hybrid cotton and non-

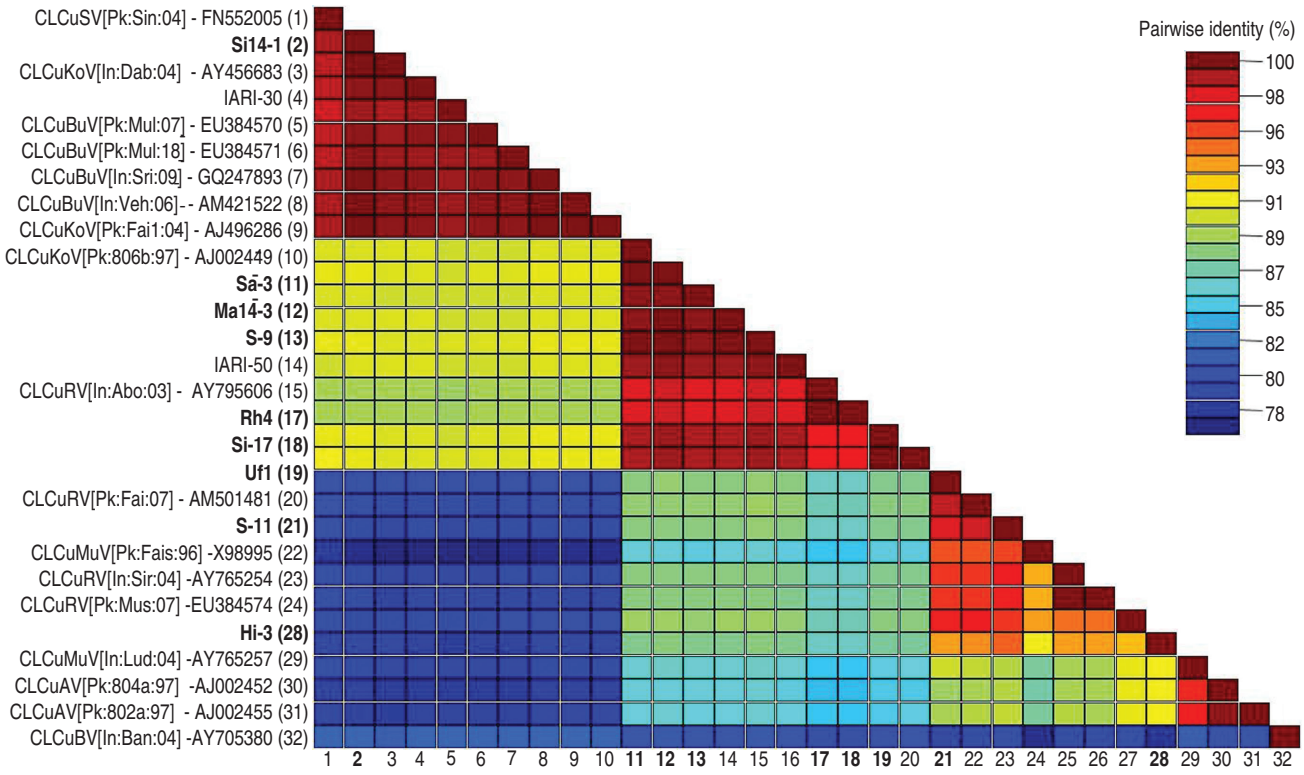

Fig 1 Pairwise identity matrix of ORF V1 (CP gene) of the present CLCuD-begomovirus isolates from northwest India and other CLCuD-begomovirus from India, Pakistan and China. The preset isolates are highlighted with bold letter.

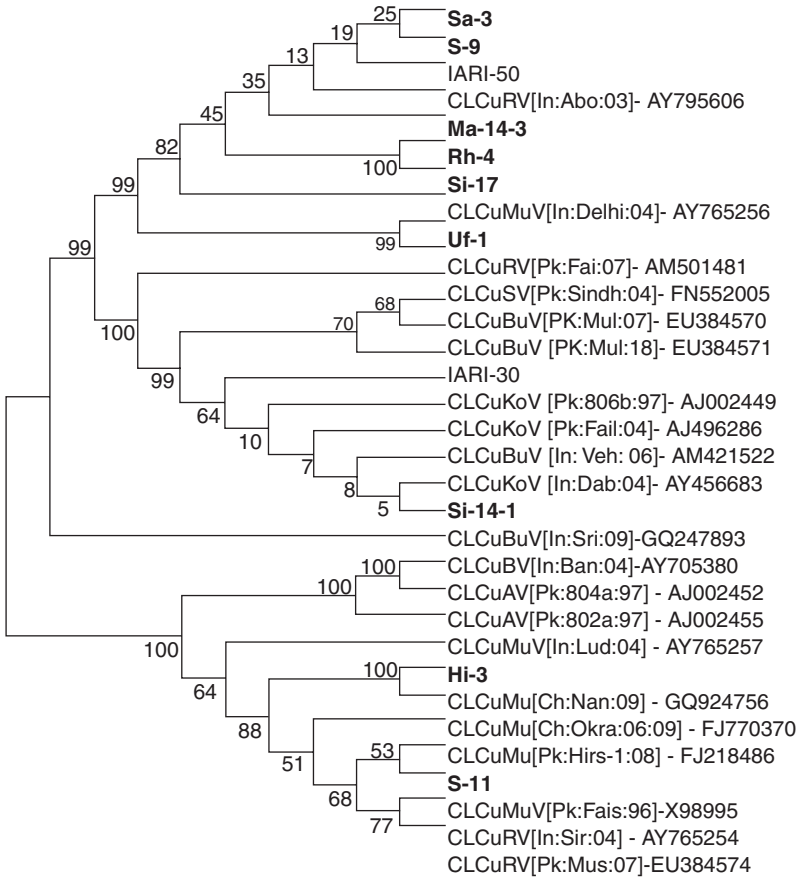

Fig.2 Phylogenetic relationships of the present CLCuD-begomovirus isolates with other CLCuD-begomoviruses based on nucleotide sequences of ORF V1 (CP gene). The tree was generated using the Neighbor-joining method in MEGA 5.2 (software). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The preset isolates are highlighted with bold letter

Bt cotton are highly susceptible to CLCuD. Present study determined occurrence of at least three CLCuD-begomovirus variants causing CLCuD in NW India. This present findings are alarming to profitable cultivation of cotton and also indicate the possibility of severe disease epidemic in near future which cannot be overlooked. The yield losses in cotton is not necessarily related with the higher incidence of CLCuD rather depends on a number of variable factors like cotton varieties, environmental conditions, time of virus infection, presence of virulent begomovirus strains, mixed infection and population of efficient whitefly vector. The increased CLCuD incidence in NW India is due to lack of resistance in cotton cultivars or the occurrence of virulent or resistant breaking strains and their mixed infection along with the presence of whitefly vectors. Further, the begomoviruses can infect several alternate or weed hosts which serve as inoculum source for new infection. Therefore, a better understanding of virus–vector-host interactions is essential to develop a long term strategy for management of CLCuD in India.

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