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PLANT PATHOLOGY AND NEMATOLOGY

Resistance to Cotton Leaf Curl Virus (CLCuV) in a Mutant Cotton Line

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ABSTRACT

Cotton leaf curl virus (CLCuV) is one of the major biotic constraints of cotton production in Pakistan. Cultivation of resistant cotton genotypes is the most effective method of reducing yield losses due to CLCuV. PIM-76-8/5 is a new CLCuV-resistant line developed through the use of induced mutation. It exhibited a highly resistant response when artificially inoculated by grafting and produced yields significantly greater than the susceptible cultivar S-12. PIM-76-8/5 was field immune when naturally infected by the whitefly (*Bemisia tabaci* Genn.), the vector of CLCuV. At a few locations where a new strain of CLCuV has emerged and all of the previously developed resistant lines are now highly susceptible, PIM-76-8/5 gave a moderate to highly susceptible response. At locations with high levels of whitefly infestation or with artificial inoculation through grafting in this study, PIM-76-8/5 exhibited a high level of resistance against the old strain of CLCuV.

Cotton leaf curl virus (CLCuV) is one of the most destructive diseases of cotton (*Gossypium hirsutum* L.) in the Punjab area of Pakistan (Nelson et al., 1998). CLCuV is in the family Geminivirus, genus Begomovirus (Hameed et al., 1994) and is transmitted by the whitefly (*Bemisia tabaci* Genn.) (Nadeem et al., 1997; Nelson et al., 1998). The virus has single-stranded circular DNA (ssDNA) with two geminate particles. CLCuV disease is characterized by upward or downward curling of leaves. The leaf veins are thickened on the underside of leaves. In some infections, one or more cup-shaped outgrowths called "enations" appear on the underside of the leaf. When CLCuV is severe, infected plants are stunted (Hussain, et al., 1991; Brown, 2001).

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The disease was first observed in Pakistan near Multan on a few cotton plants in 1967. At that time, the disease was of minor importance and it did not attract serious attention. In 1988, only 60 hectares in the Multan district were damaged. Since 1988, the geographic distribution of CLCuV has increased greatly (Mahmood, 1999), and more than 7.7 million bales of cotton have been lost to CLCuV from 1988 through 2002 (Ahmad et al., 2002).

Development of a CLCuV-resistant cultivar is the most promising control option (Akhtar et al., 2003). The Nuclear Institute for Agriculture and Biology (NIAB) in Faisalabad, Pakistan, has initiated research to develop high yielding, CLCuV-resistant cotton cultivars with desirable fiber characteristics through the use of induced mutations. The CLCuV-resistant mutant PIM-76-8 was the result of an interspecific cross between NIAB-78 and REBA-288. The male parent pollen was irradiated with 10Gy of gamma rays. On average, PIM-76-8 produced yields 28.2 % higher than the standard CLCuV-resistant cultivar CIM-443 in micro-yield trials conducted at NIAB during 1998 and 1999 (Aslam and Elahi, 2000).

This study was conducted to determine the level of resistance of PIM-76-8 to CLCuV under field conditions at locations where CLCuV severity is normally very high. Single-plant progeny rows were evaluated for resistance through graft inoculation with CLCuV-infected plant material collected from different locations.

MATERIALS AND METHODS

Field evaluations. The response of 12- to 16-week-old plants of PIM-76-8 to infection by CLCuV under different environments in 26 producer fields in the cotton growing area of Punjab province was studied during August of 2000 and 2001. All of these locations were known for high incidence of CLCuV infection. The reactions of three resistant cultivars (CIM-473, CIM-446, and FH-900) and two susceptible cultivars (NIAB-Karshmia and NIAB-78) were also determined. Fifty to 300 plants (depending on the area under cultivation of the test cultivar) in each

location were randomly chosen. None of the plants were sprayed for whitefly control. Disease index (%) and reaction of the cultivars was determined using the disease scale described by Akhtar and Khan (2002) (Table 1).

The response of six single plant progeny rows of PIM-76-8 (PIM-76-8/6, PIM-76-8/7, PIM-76-8/18, PIM-76-8/20, PIM-76-8/38, and PIM-76-8/5) and a resistant (CIM-473) and susceptible (S-12) standard to CLCuV using natural inoculation by whiteflies were determined during September in 2000 and 2001 at the Nuclear Institute for Agriculture and Biology (NIAB) in Faisalabad. Disease index (%) and reaction of the mutant lines and cultivars was recorded. No insecticides were applied to control the whiteflies. Similar studies were performed with single plant progeny rows of PIM-76-8/5 during 2001-02 and 2002-03 at a cotton experimental area of NIAB. Data from National Coordinated Varietal Trials (NCVT) and Director General Research trials (DGR) were also collected for PIM-76-8/5 (NIAB-98).

Graft inoculation studies. CLCuV inoculum for all graft inoculation studies was collected from naturally-infected cotton plants exhibiting characteristic leaf curl symptoms of CLCuV. The virus was maintained by grafting infected plants onto plants grown in a greenhouse of the commercial cultivar S-12, which is susceptible to CLCuV. Grafting was performed following the bottle leaf grafting method described by Akhtar et al. (2002c). Six-week-old plants were selected for graft-inoculation. A 1- to 2-cm long and 0.1- to 0.2-cm deep cut was made on the stem at the 2nd internode from the top of the plant. A CLCuV-infected branch with a 20-cm long growing tip was detached from a diseased plant. A cut similar to the one made on the test plant was made on this branch, and the corresponding cut surfaces were brought together and tied with parafilm. This stem was then placed in a 2-cm dia., 16-cm long test tube containing distilled water. The distilled water was changed daily for 5 d. After 5 d the tubes were removed, and plants were observed daily for symptom development.

In another study, four to six seeds of PIM-76-8 collected from different locations were sown in earthen pots under insect-free conditions during 2000-01. The commercial cultivars CIM-473 and S-12 were included as resistant and susceptible controls, respectively. Plants were thinned to one per pot 2 wk after germination and grafted for artificial inoculation. Clean tap water was used to irrigate the young seedlings throughout the study. The percentage of successful grafts, percentage of disease transmission, mean latent period (average time required for first disease symptoms to appear after grafting), and average disease severity at 90 d after grafting were recorded.

In a third study, six single plant progeny rows of PIM-76-8 (PIM-76-8/6, PIM-76-8/7, PIM-76-8/18, PIM-76-8/20, PIM-76-8/38, and PIM-76-8/5) were graft-inoculated in the field during the fall of 2000-01 to determine their reaction to CLCuV. CIM-473 and S-12 were included as resistant and susceptible standards, respectively. Ten plants in each replication were covered with insect proof cages made of very fine meshed cloth. Six weeks after emergence, one plant under each cage was inoculated by grafting. During 2001-02, seven plants of PIM-76-8/5

Table 1. Rating scale for cotton leaf curl virus symptoms

Symptoms	Disease rating [y]	Disease index (%) [z]	Disease reaction
Absence of symptoms.	0	0	Immune
Thickening of a few small veins or the presence of leaf enations on 10 or fewer leaves of a plant.	1	0.1- 1	Highly resistant
Thickening of a small group of veins.	2	1.1- 5	Resistant
Thickening of all veins but no leaf curling.	3	5.1-10	Moderately resistant
Severe vein thickening and leaf curling on the top third of the plant.	4	10.1 – 15	Moderately susceptible
Severe vein thickening and leaf curling on the half of the plant.	5	15.1 – 20	Susceptible
Severe vein thickening, leaf curling, and stunting of the plant with reduced fruit production.	6	>20	Highly susceptible

[y] Disease ratings that include enations are marked with an "E".

[z] The percentage disease index was calculated as follows: (sum of all disease ratings/total # of plants) x 16.66.

were graft-inoculated using the same methodology under field conditions. Data were recorded on the percentage of successful grafts, percentage of disease transmission, mean latent period (average time required for first disease symptom appearance after grafting), and average disease severity at 90 days after grafting. Disease severity and reaction of test lines and cultivars was recorded.

RESULTS

Field evaluations. Response of PIM-76-8 to infection by CLCuV was recorded in 26 farmer's fields located in the Punjab during 2000-01. PIM-76-8 was field immune (no plants with symptoms) at 8 locations, highly resistant at 15 locations, and resistant at 3 locations (Table 2). The susceptible control exhibited

Table 2. Cotton leaf curl development in PIM-76-8 under natural infestation by the whitefly (*B. tabaci)* at 26 farm locations during 2000-2001

Location	PIM-76-8		Resistant control [w]		Susceptible control [x]	
	Disease index (%) [y]	Reaction [z]	Disease index (%) [y]	Reaction [z]	Disease index (%) [y]	Reaction [z]
Strain test, NIAB, Faisalabad	2.67	R	0.00*	FI	75.00*	HS
Multiplication, NIAB, Faisalabad	0.32	HR	0.00*	FI	75.00*	HS
NIAB Farm, Faisalabad	0.35	HR	0.00	FI	56.67	HS
PSC, Khanewal	2.62	R	0.90*	HR	74.40	HS
PSC, Khanewal (1.25 acre trial)	1.02	R	0.00	FI	77.38	HS
Chak No. 83/85, Khanewal	0.32	HR	----	----	56.67	HS
Zia Ahmad Khan Farm, Kamalia	0.55	HR	0.00	FI	60.41	HS
Ch. Afzal Farm, Kamalia	0.67	HR	0.00	FI	50.25	HS
Mian Asad-ur-Rehman Farm, Kamalia	0.00	FI	0.00	FI	53.70	HS
Mian Sajid-ur-Rehman Farm, Kamalia	0.32	HR	----	----	----	----
Ramdey Seed Corporation, T. T. Singh	0.00	FI	0.00*	FI	----	----
Rana Shaukat Farm, T. T. Singh	0.45	HR	0.00	FI	----	----
Haji Mukhtar A. Khitran, T. T. Singh	0.00	FI	0.00	FI	----	----
Umar Cotton Factory, T. T. Singh	0.00	FI	0.00*	FI	----	----
Rana Zulfiqar Farm, Harupa	0.00	FI	0.00**	FI	----	----
Mian M. Shafiq Farm, Harupa	0.00	FI	0.00	FI	----	----
Zaheer Seed Corporation, Burewala	0.00	FI	0.00	FI	----	----
Imam Farm Qatalpur, Khanewal	0.75	HR	0.90**	HR	----	----
Hassan Raza Farm, Multan	0.30	HR	0.00	FI	----	----
Prof. Riaz Hussain Khudi, Lodhran	0.00	FI	0.00**	FI	43.00	HS
Khursheed Ahmad Kanjoo, Multan	0.31	HR	0.21**	HR	----	----
Sharif Mumtaz Farm, Jhang	0.32	HR	0.00	FI	53.00	HS
Major Khursheed M., Bahawal-Nagar	0.32	HR	0.00	FI	21.00	HS
Farhat Ullah Khan, Mianchannu	0.44	HR	0.00	FI	----	----
Kot Sadat Farm, Burewala	0.50	HR	0.00	FI	----	----
Dr. Sher Muhammad Awan, Lodhran	0.33	HR	----	----	----	----

[w] The resistant control was CIM-473. Resistant controls designated with * or ** were FH-900 and CIM-446, respectively.

[x] The susceptible control was NIAB-K. Susceptible controls designated with * are S-12.

[y] The percentage disease index was calculated as follows: (sum of all disease ratings/total # of plants) x 16.66.

[z] FI = field immune, HR = highly resistant, R = resistant, HS = highly susceptible.

a highly susceptible response (disease indices of 21 to 75%) at the 12 locations where it was grown. The resistant controls were highly resistant at 3 locations and field immune at 20 locations. Although PIM-76-8 had a variable response to CLCuV, the disease index was low compared with the highly susceptible response of the susceptible controls at all locations where its was grown (Table 2).

The response of six single plant progeny rows of PIM-76-8 to CLCuV was assessed through natural inoculation by *B. tabaci* under field conditions 2000-01. All of the progeny and CIM-473 were field immune (free from leaf curl symptoms), while the susceptible control (S-12) was highly susceptible with a disease index of 89.90 % (Table 3).

About 3617 plants of PIM-76-8/5 during 2001-02 and more than 8552 during 2002-03 under conditions of natural inoculation by whiteflies were observed. Only one plant from more than ten thousand plants of PIM-76-8/5 displayed symptoms, while the susceptible control had a disease index of 92.52 and 97.85% during 2001-02 and 2002-03, respectively (Table 4). These results indicate that PIM-76-8/5 is highly resistant to CLCuV.

Performance of PIM-76-8/5 and NIAB-98 was also assessed for CLCuV infection in the National Coordinated Variety Trials (NCVT) at 12 locations and in Director General Research Trials (DGR) at 17 locations during 2002-03 (Table 5). PIM-76-8/5 was field immune under 9 NCVT trials and 12 DGR locations. It was highly resistant at NIAB in Faisalabad, moderately susceptible at Mailsi, and highly susceptible at CRS-Vehari in NCVT trials with a 0.3, 14.8 and 68% disease index, respectively. In DGR trials, it was highly resistant at NIAB, resistant at CCRI in Multan, moderately resistant at Mailsi, susceptible at Kassowal, and highly susceptible at CRS-Vehari. CIM-473, the resistant control, was field immune or highly resistant at most locations. At two locations in the NCVT trials and three locations in the DGR trials, CIM-473 was highly susceptible.

Graft inoculation studies. Based on the results from the observations in the farmer's fields, seeds of PIM-76-8 were collected from 9 (3 field immune, 3 highly resistant and 3 resistant locations) of the 26 locations for artificial inoculation through grafting. All of the grafts were successful and none of the plants were immune. Seeds collected from location VI gave a highly resistant response with minor disease severity (Table 6). At 26 days after inoculation, a few small, scattered veins were thickened on all the plants from location VI, and plants, including the resistant control CIM-473, from eight other locations gave resistant responses with a thickening of small veins within 22 to 24 days after grafting. The susceptible cultivar S-12 displayed severe vein thickening, leaf curling, and stunting 15 days after grafting (Table 6).

Table 3. Cotton leaf curl development in single plant progeny rows (SPPR) of PIM-76-8 at NIAB in Faisalabad during 2000-2001

SPPR	Natural infection by whiteflies			Graft inoculation			
	Plants inspected (#)	Disease severity [x]	Reaction[y]	Plants grafted (#)	Latent period [z]	Disease severity 90 d after grafting	Reaction[y]
PIM-76-8/6	62	0	FI	5	24	2	R
PIM-76-8/7	14	0	FI	5	23	2E	R
PIM-76-8/18	37	0	FI	5	24	2	R
PIM-76-8/20	38	0	FI	5	23	2	R
PIM-76-8/30	44	0	FI	5	24	2	R
PIM-76-8/5	74	0	FI	5	27	1	HR
CIM-473	35	0	FI	5	21	2E	R
S-12	70	6E	HS	5	15	6E	HS

[x] None of the plants was infected, except for S-12. For S-12, 68 plants were infected with a disease index of 89.90. Disease severity based on a scale of 0 to 6, where 0 = no symptoms and 6 = severe vein thickening, leaf curling, stunting, and reduced fruiting. E indicates enations were present on the leaves.

[y] FI = field immune; HR = highly resistant; R = resistant; HS = highly susceptible

[z] Grafting success and disease transmission were 100%. Latent period is the time between grafting and the first appearance of symptoms.

Table 4. Response of single plant progeny rows of PIM-76-8/5 (NIAB-98) to cotton leaf curl virus infection through natural inoculation by whiteflies at NIAB

Location	2001-2002			2002-2003		
	Plants evaluated	Disease index (%) [x]	Reaction[y]	Plants evaluated	Disease index (%) [z]	Reaction [y]
1	123	0	FI	2871	0	FI
2	140	0	FI	950	0.07	HR
3	135	0	FI	1695	0	FI
4	269	0	FI	126	0	FI
5	250	0	FI	792	0	FI
6	262	0	FI	366	0	FI
7	267	0	FI	572	0	FI
8	387	0	FI	---	---	-
9	448	0	FI	---	---	-
10	595	0	FI	---	---	-
11	211	0	FI	---	---	-
12	260	0	FI	---	---	-
Strain test	270	0	-	266	0	FI
Fertilizer trial	-	-	-	914	0	FI
S-12	89	92.52	HS	92	97.85	HS

[x] None of the plants were infected, except for S-12. For S-12, 85 plants were infected and disease severity was 6E. Disease severity based on a scale of 0 to 6, where 0 = no symptoms and 6 = severe vein thickening, leaf curling, stunting, and reduced fruiting. E indicates enations were present on the leaves.

[y] FI = field immune; HR = highly resistant; R = resistant; HS = highly susceptible.

[z] One plant at location 2 was infected with a disease severity of 4E. For S-12, 90 plants were infected with a disease severity of 6E.

Table 5. Response of PIM-76-8/5 (NIAB-98) to cotton leaf curl virus infection under field conditions in National Coordinated Varietal Trials (NCVT) and Director General Research Trials (DGR) trials during 2002-2003

Location	NCVT Trials				DGR Trials			
	PIM-76/8-5		CIM-473 (resistant control)		PIM-78/8-5		CIM-473 (resistant control)	
	Disease index (%)[y]	Reaction[z]	Disease index (%)[y]	Reaction[z]	Disease index (%)[y]	Reaction[z]	Disease index (%)[y]	Reaction[z]
NIAB, Faisalabad	0.3	HR	0	FI	0.61	HR	0	FI
CRS, Faisalabad	0	FI	0	FI	0	FI	0	FI
CRSS, Jhang	0	FI	0	FI	0	FI	0	FI
CRS, Sahiwal	0	FI	0	FI	0	FI	0	FI
CRSS, Khanewal	0	FI	0	FI	0	FI	0	FI
CCRI, Multan	0	FI	0	FI	1.5	R	1.0	HR
CRS, Multan	0	FI	0	FI	0	FI	0	FI
CRS, Vehari	68.0	HS	80	HS	88	HS	97	HS
Mailsi	14.8	MS	67.9	HS	10.0	MR	60	HS
CRS, Rahim Yar Khan	0	FI	0	FI	0	FI	0	FI
CRS, Bahawalpur	0	FI	0	FI	0	FI	0	FI
CRSS, Dera Ghazi Khan	0	FI	0	FI	0	FI	0	FI
Rajana	-	-	-	-	0	FI	0	FI
T. T. Singh	-	-	-	-	0	FI	0	FI
Kassowal	-	-	-	-	20.0	S	30	HS
Lodhran	-	-	-	-	0	FI	0	FI
CRSS, Sargodha	-	-	-	-	0	FI	0	FI

[y] The percentage disease index was calculated as follows: (sum of all disease ratings/total # of plants) x 16.66.

[z] FI = field immune; HR = highly resistant; R = resistant; MS = moderately susceptible; HS = highly susceptible.

Table 6. Response of local selections of PIM-76-8 to cotton leaf curl virus infection from graft inoculation during 2000-2001

Location [x]	Latent period [y]	Disease severity after 90 d [z]	Reaction
I	23	2	Resistant
II	23	2	Resistant
III	22	2	Resistant
IV	24	2E	Resistant
V	24	2	Resistant
VI	26	1	Highly resistant
VII	23	2	Resistant
VIII	23	2	Resistant
IX	23	2	Resistant
CIM-473	18	2E	Resistant
S-12	15	6E	Highly susceptible

[x] CIM-473 is the resistant control, and S-12 is the susceptible control.

[y] Latent period is the time between grafting and the first appearance of symptoms.

[z] Grafting success and disease transmission were 100%. Disease index based on a scale of 0 to 6, where 0 = no symptoms and 6 = severe vein thickening, leaf curling, stunting, and reduced fruiting. E indicates enations were present on the leaves.

Graft inoculation of the single plant progeny rows was successful. All of the plants were symptomatic. Of the progeny tested, only progeny PIM-76-8/5 showed minor symptoms at 27 d after inoculation. CIM-473 showed a resistant response with a disease severity of 2 at 21 to 24 d after grafting, and S-12 displayed a highly susceptible response with a disease severity of 6 at 15 d after grafting (Table 3).

Seven plants of PIM-76-8/5 were grafted for artificial inoculation with CLCuV under field conditions during 2001-02. Grafting success and disease transmission were 100%. All the grafted plants exhibited a highly resistant response with a disease severity of 1, except plant number 5 with a disease severity of 1 > 2. The latent period for all material in the test required a minimum of 27 d, except S-12 which required 15 d. The disease severity of the inoculated plants was the same at harvest as at 90 d (Table 7).

DISCUSSION

This study suggests that PIM-76-8/5 has a potential for the management of CLCuV in cotton growing areas of Punjab in which the old strain of the virus is present. All of the material tested through graft inoculation was susceptible to CLCuV infection with varying levels of symptom expression, but it grew well and produced a normal yield at most locations. Akhtar et al. (2000; 2001; 2002b; 2003; 2004) have also observed that all the genotypes exhibiting resistance in the field are susceptible to infection by CLCuV under graft inoculation to which they respond with varying levels of symptom development. The susceptible control S-12 showed a high level of infection through artificial inoculation and under field conditions produced no yield.

These experiments found that PIM-76-8/5 is a mutant with a high level of resistance to the old strain of CLCuV. It exhibited resistance in most of the locations in which it was tested, but it exhibited a high level of susceptibility at a few locations. Un-

Table 7. Response of single plant progeny rows of PIM-76-8/5 (NIAB-98) to cotton leaf curl virus infection through graft inoculation during 2001-2002

Plant no. [w]	Latent period [x]	Disease severity after 90 d [y]	Reaction	Growth of the grafted plants [z]
1	27	1	Highly resistant	Good
2	27	1	Highly resistant	Good
3	27	1	Highly resistant	Good
4	27	1>2	Highly resistant -resistant	Good
5	28	1	Highly resistant	Good
6	27	1	Highly resistant	Good
7	27	1	Highly resistant	Good
S-12	15	6E	Highly susceptible	Poor

[w] S-12 is the susceptible control.

[x] Latent period is the time between grafting and the first appearance of symptoms.

[y] Disease severity is based on a scale of 0 to 6, where 0 = no symptoms and 6 = severe vein thickening, leaf curling, stunting, and reduced fruiting. E indicates enations were present on the leaves.

[z] Grafting success and disease transmission was 100%. Good = growth was comparable to non-inoculated plants; poor = plants were stunted.

fortunately, a new resistance-breaking strain of virus that is capable of exhibiting a high level of disease development on all known germplasm has emerged in these areas (Akhtar et al., 2002a; Mansoor et al., 2003). Zhou et al. (1998) has suggested that four variants of CLCuV exist in the field. Multiple infections of cotton plants by CLCuV and other whitefly-transmitted geminiviruses are prevalent in other cotton growing areas. The chances of recombination among the geminiviruses does exist and could lead to the emergence of new more virulent and resistance-breaking variants (Shah et al., 1999). Continued efforts are needed to develop resistant germplasm to these constantly evolving viruses.

From these studies it is evident that a new CLCuV strain has emerged and a search for new source(s) of resistance is needed. Demonstrating susceptibility to CLCuV was most efficient by using artificial inoculation. The results obtained from both grafting and natural infection by whiteflies in the evaluation of germplasm for resistance to CLCuV should be considered, but emphasis should be given to highly resistant material selected through grafting.

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