Natural Occurrence of Five Seedborne Cowpea Viruses in Pakistan

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ABSTRACT

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A total of 151 cowpea leaf samples with viruslike symptoms were collected from 13 districts of Punjab and North-West Frontier Province (NWFP) of Pakistan during the summers of 1990 and 1991. Desiccated samples were tested by direct antigen coating (DAC) or double antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) for the presence of seven viruses known to be seedborne in cowpea: blackeye cowpea mosaic (BlCMV) and cowpea aphid-borne mosaic (CABMV) potyviruses, cucumber mosaic (CMV) cucumovirus, cowpea mosaic (CPMV) and cowpea severe mosaic (CSMV) comoviruses, cowpea mottle carmovirus (CPMoV), and southern bean mosaic sobemovirus (SBMV). One or more seedborne viruses were detected in 47% (71 of 151) of the symptomatic plant samples. The viruses detected and the percent incidence were: CABMV, 29%; SBMV, 21%; CSMV, 17%; BlCMV, 8%; and CPMoV, 4%. Neither CMV nor CPMV was detected. Seven cowpea seed lots representing two commercial seed types and seven locations in Punjab and NWFP were collected for tests of the same seven seedborne viruses. Only CABMV was seed-transmitted, and only in four of seven seed lots, at frequencies of <1 to 7%. Likewise, although leaf samples collected from plants of a local Punjab cultivar contained four ELISA-detectable seedborne viruses (BlCMV, CABMV, CSMV, and SBMV), only CABMV was transmitted through seeds (7% frequency) from these plants. None of the seven seedborne viruses tested for were detected in 80 of 151 virus-symptomatic samples, suggesting infection with other viruses. This is believed to be the first record of the natural occurrence of BlCMV, CABMV, CPMoV, CSMV, and SBMV in Pakistan and the first report of CPMoV outside of Africa.

Cowpea (*Vigna unguiculata* (L.) Walp. subsp. *unguiculata*), an important soil-improving legume crop, is grown in Pakistan as a vegetable, as food grain, and as fodder. Cowpeas are grown as both spring and summer crops. Because cowpeas are drought-resistant, they are widely grown in the nonirrigated areas of Punjab and North-West Frontier Province (NWFP). With the recent introduction of short-season cultivars, the crop is becoming more important to both irrigated and nonirrigated areas.

Previously, only a whitefly (*Bemisia tabaci* (Gennadius))-transmitted yellow mosaic virus (YMV) had been reported to naturally infect cowpea in Pakistan (1). However, several viruses have been reported to infect cowpea crops in areas of neighboring India and Iran where climatic conditions for cowpea cultivation are similar to those of central Pakistan. Reports include blackeye cowpea mosaic potyvirus (BlCMV) (12,13), cowpea aphid-borne mosaic potyvirus (CABMV) (14), cucumber mosaic cucumovirus (CMV) (15), cowpea severe mosaic comovirus (CSMV) (6), southern bean mosaic sobemovirus (SBMV) (17), and cowpea golden mosaic virus (12) from India and CABMV from Iran (10). It would therefore seem logical to expect such viruses in the cowpea crops of Pakistan.

At least 16 viruses are reported to be seed-transmitted in cowpea (5). Seedborne viruses have probably occurred for centuries in the cowpea-growing areas of the world. However, they may now be spreading to other parts of the world, through international seed commerce and/or germ plasm exchanges involving regions in which these viruses are indigenous (6,7). No prior information existed on the identity or distribution of viruses in cowpea in Pakistan. Our objectives in the present study were to ascertain the relative incidence of viruses known to be seedborne in cowpea and thereby to prioritize and initiate control measures (i.e., virus-free seed lots and resistance breeding) for the most significant viral diseases. A preliminary report of this investigation was presented in 1991 (3).

MATERIALS AND METHODS

Survey of cowpea crop and collections of samples. Thirteen districts comprising eight locations in Punjab and five in NWFP, representing 93 commercial cowpea fields or experimental plots, were surveyed for cowpea viruses during the summer seasons of 1990 and 1991. One hundred fifty-one leaf samples were collected from plants showing viruslike symptoms such as mosaic, mottle, and leaf distortion. The samples were placed individually in moistened paper towels, transported on ice to the laboratory, desiccated over magnesium perchlorate in vacuum desiccators, and subsequently assayed by ELISA for seven seedborne cowpea viruses. When sampled, the cowpea plantings ranged from seedling to podding stages, depending upon the sowing time at different localities or altitudes. Desiccated samples were brought or mailed to the Virology Laboratory, Department of Botany & Plant Pathology, Oregon State University, Corvallis, for detection and identification of viruses. Quarantine permits for this work were issued by the Oregon Department of Agriculture and the USDA Animal and Plant Health Inspection Service.

Extracts from desiccated tissue samples were prepared in appropriate buffers and processed by either direct antigen coating (DAC) or double antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA). Antisera to CPMV and CSMV were kindly provided by O. W. Barnett of Clemson University (now in the Department of Plant Pathology, North Carolina State University, Raleigh). Antisera to BlCMV and SBMV were obtained from C. W. Kuhn, Department of Plant Pathology, University of Georgia, Athens. Antisera to CMV, CABMV, and CPMoV were produced at Corvallis.

The DAC-ELISA was performed much as described by Hampton et al (6), except that a different antigen buffer was used for homogenizing tissue samples. The antigen buffer was selected following standardization comparisons and

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consisted of phosphate buffered saline (PBS), pH 7.2, containing 0.1% Tween 20 and 0.02 M sodium diethyldithiocarbamate (NaDIECA). Subsequent steps and reagents were in accordance with standardized procedures (6,9). Antisera requiring cross-absorption were diluted typically 1,000× with filtered extract from healthy cowpea tissues prepared in buffer and incubated 1 hr at 37 C immediately prior to loading microtiter plates. Absorbance (A_{405nm}) values were recorded on a Bio-Tek Auto ELISA reader Model EL 109, typically 90 min after addition of substrate (*p*-nitrophenyl phosphate).

The DAS-ELISA, including protocol and reagents, was conducted according to the methods of Clark and Adams (4). Purified antiviral IgGs and other reagents were used at concentrations providing optimal differentiation of extracts from healthy plants and plants infected with viruses homologous to the respective IgGs.

Evaluation of seed samples. The Pakistan cowpea seed samples (200–450 seeds per sample) were also collected from local markets and field-grown plants during the survey to detect and identify any viruses transmissible in these seeds. Seeds were germinated in 9-in. plastic pots and tested as seedlings for seedborne viruses. Seedling infection was first observed as viruslike symptoms on primary and/or first trifoliolate leaves, followed by virus detection/identification by ELISA. Occasional plants with anomalous or viruslike symptoms but ELISA-negative were further tested by homogenizing leaf tissues in buffer and mechanically inoculating 12-day-old cowpea plants with the tissue extract.

RESULTS

Seventy-one of 151 tissue samples contained ELISA-detectable seedborne viruses (Table 1). Neither CMV nor CPMV was detected in any of the samples. There were 119 virus detections among the 71 samples, indicating that some samples contained two or more viruses (Table 2). Of the five viruses detected, CABMV was the most prevalent, occurring in 44 of 151 samples (29%). The next most frequently detected viruses were SBMV (21% of the samples) and CSMV (17% of the samples), whereas BlCMV and CPMoV were detected in 8 and 4% of the samples, respectively.

The extent to which virus mixtures occurred was best illustrated among samples from the Sialkot District. One-half of the total 119 virus detections (Table 1) and all five viruses occurred among these 24 samples (Table 2). Each virus detected in these samples was accompanied by two or more other viruses. For instance, tissue samples No. 9, 12, and 13 from this district tested positive for all five viruses. Both CSMV and SBMV were detected only in infections with other viruses. All 12 Sialkot samples containing BlCMV were also infected with CABMV; however, samples No. 6, 15, 16, and 17 contained CABMV without BlCMV. Four of the six samples containing CPMoV originated in the Sialkot District. In each of these four samples, CPMoV was accompanied by one or four other viruses. Eight samples from symptomatic plants were negative for all seven seedborne viruses.

Commercial cowpea fields in the vicinities of Sialkot in Punjab and Mansehra in NWFP typically contained >80% plants with viruslike symptoms. Although five seedborne viruses occurred in tissues of symptomatic cowpea plants from Sialkot, only CABMV was seed-transmitted in a hand-picked seed lot from this area or from six other Pakistan seed lots tested for seed transmission of seven seedborne viruses (Table 3). Positive ELISA tests for seedborne CABMV were associated with striking seedling symptoms, including chlorotic leaf veins and/or dark-green leaf-midrib or vein banding. Such symptoms were reproducible when isolates were transferred to cowpea seedlings by mechanical inoculations. The highest observed rate of CABMV seed transmission (7%) occurred in seeds from a local Sialkot cultivar, the mother plants of which were sampled and found to contain BlCMV, CABMV, CSMV, and SBMV.

Seedlings arising from tested seed lots sometimes produced mild symptoms similar to those induced by virus infections. Such plants, found by ELISA to be free of seven of 16 recognized seedborne viruses, were bioassayed on selected cowpea genotypes for mechanically transmissible agents. All such secondary assays were negative.

DISCUSSION

The present results indicate that BlCMV, CABMV, CPMoV, CSMV, and SBMV occur naturally in Pakistan-grown cowpea. This is believed to be the first report of these viruses in cowpea of Pakistan. Field occurrences of BlCMV, CABMV, and SBMV were previously reported in the bordering countries of Iran in 1975 (CABMV) (10) and India in 1974 (SBMV) (17) and 1988 (BlCMV and CABMV) (13).

Tissue samples were often infected with more than one virus—in some cases, with five viruses. Multiple viral infections have usually induced complex, variable symptoms, rendering field diagnosis difficult or impossible (11). In contrast, symptoms induced in seedlings by seedborne CABMV were characteristic and reproducible with pure isolates of CABMV.

Mixed viral infections have sometimes intensified symptoms, causing more serious crop losses than were caused by single viruses. For instance, whereas CMV infections are generally acknowledged to cause little yield reduction in many cowpea genotypes, losses of 10–42% resulted from mixed infections of BlCMV + CMV (stunt disease) (11). Likewise, coinfections by cowpea chlorotic mottle virus and SBMV were reported to cause greater cowpea yield losses than either virus alone (8). It is assumed that the mixed viral infections common to sampled fields in the Sialkot

Table 1. Viruses detected in cowpea leaf samples from cowpea-producing areas of Pakistan

Province / District	No. of samples tested	Samples containing ELISA-detectable viruses[a]				
		BlCMV	CABMV	CPMoV	CSMV	SBMV
Punjab						
Chakwal	7	...	...	...	...	...
Faisalabad	16	...	...	...	...	...
Gujranwala	5	...	...	...	...	...
Lahore	13	...	...	2[b]	5	5
Rawalpindi	13	...	3	...	...	...
Sargodha	8	...	...	...	...	...
Sialkot	24	12	16	4	12	15
Okara	2	...	...	...	...	...
North-West Frontier Province						
Balakot	7	...	...	...	1	1
Haripur	5	...	...	...	...	...
Mansehra	35	...	18	...	5	8
Noshera	4	...	...	...	...	...
Thahkot	12	...	7	...	2	3
Total	151	12	44	6	25	32

[a] Viruses included in tissue assays were blackeye cowpea mosaic potyvirus (BlCMV), cowpea aphid-borne mosaic potyvirus (CABMV), cowpea mottle carmovirus (CPMoV), cowpea severe mosaic comovirus (CSMV), and southern bean mosaic sobemovirus (SBMV). Neither cucumber mosaic cucumovirus (CMV) nor cowpea mosaic comovirus (CPMV) was detected among these 151 samples. Positive controls for these viruses were readily detected.

[b] Positive tests were based on A_{405} threshold (T) values, as previously reported (6). Separate values for T were derived for each plate as follows: T = average absorbance (A_{405}) values for tests of negative controls (extracts from healthy plants) plus five times the standard deviations among A_{405} values for two or more negative-control replicate wells. Sample wells concluded to contain a specific virus typically exceeded this threshold value many fold (see Table 2).

Table 2. Results from ELISA of extracts from virus-symptomatic cowpea leaf tissues collected in commercial fields of the Sialkot District of Punjab and tested against antisera/IgGs to seven viruses[a]

Sample	Absorbance (A_{405}) values recorded after 90-min incubation of enzyme substrate						
	BlCMV	CABMV	CMV	CPMoV	CPMV	CSMV	SBMV
1	0.027[b]	0.012	0.001	0.001	0.012	0.003	0.004
2	0.005	0.004	0.034	0.040	0.031	0.025	0.007
3	0.021	0.023	0.032	0.040	0.023	0.007	0.061
4	1.707[c]	0.302	0.012	0.034	0.012	2.577	0.385
5	0.012	0.004	0.041	0.007	0.004	0.017	0.001
6	0.007	0.414	0.002	0.021	0.011	0.031	0.019
7	0.653	1.212	0.017	0.018	0.002	2.023	1.021
8	0.753	1.009	0.009	0.015	0.001	1.315	1.209
9	0.320	1.070	0.034	0.697	0.007	2.227	1.715
10	0.771	0.494	0.028	0.003	0.002	0.521	1.315
11	1.112	0.574	0.009	0.013	0.001	0.424	3.000
12	0.410	0.721	0.018	0.208	0.008	0.509	1.871
13	0.917	1.116	0.042	1.061	0.021	1.324	0.405
14	0.352	0.644	0.061	0.035	0.012	1.118	2.710
15	0.007	0.753	0.049	0.010	0.001	2.721	1.111
16	0.005	0.670	0.003	2.110	0.010	0.015	0.003
17	0.007	1.295	0.051	0.071	0.005	0.945	1.105
18	0.723	1.140	0.019	0.002	0.013	0.002	0.661
19	0.812	1.065	0.051	0.004	0.009	2.210	1.117
20	0.417	0.613	0.047	0.011	0.011	0.005	0.779
21	0.004	0.012	0.006	0.005	0.010	0.005	0.001
22	0.012	0.006	0.005	0.012	0.010	0.006	0.011
23	0.002	0.001	0.041	0.051	0.004	0.001	0.021
24	0.001	0.024	0.071	0.011	0.002	0.002	0.041
Homologue	1.204	1.004	2.002	1.021	2.510	2.217	1.021
Heterologue[d]	0.012	0.003	...	...	...	...	...
Healthy[e]	0.003	0.006	0.003	0.012	0.002	0.001	0.002

[a] Viruses included in tissue assays were blackeye cowpea mosaic potyvirus (BlCMV), cowpea aphid-borne mosaic potyvirus (CABMV), cucumber mosaic cucumovirus (CMV), cowpea mottle carmovirus (CPMoV), cowpea mosaic comovirus (CPMV), cowpea severe mosaic comovirus (CSMV), and southern bean mosaic sobemovirus (SBMV). DAC-ELISA with diluted crude antiserum was used for assays of CPMV and CSMV; remaining viruses were assayed by DAS-ELISA.

[b] A_{405} values derived as averages of data for at least two replicate test wells.

[c] A_{405} values indicating virus detection (underlined) were based on threshold (T) values for each microtiter plate. See statistical derivation of T values in Table 1.

[d] Heterologues included BlCMV for CABMV and CABMV for BlCMV, to illustrate DAS-ELISA distinction between these two potyviruses.

[e] Tissue extracts from healthy cowpea plants.

Table 3. Seed transmissions of cowpea aphid-borne mosaic potyvirus (CABMV) in cowpea seed lots from local markets or from field-grown mother plants in Punjab and North-West Frontier Province

Location	Seed type	Transmission	
		Frequency[a]	%
Punjab			
Chakwal city (Chakwal)	Medium, white	0/285	0
Narowal (Sialkot)	Small, brown	2/200	1.0
Pattokey (Lahore)	Small, brown	11/431	2.6
Texla (Rawalpindi)	Medium, white	2/238	0.8
Sialkot	Small, brown	9/127[b]	7.0
North-West Frontier Province			
Manghora (Swat)	Medium, white	0/215	0
Khaza Khaila (Swat)	Medium, white	0/185	0

[a] Number of seedlings showing symptoms and reacting positively by ELISA/total seedlings examined.

[b] Seeds collected from virus-infected field-grown plants.

District of Punjab were compounding yield losses there.

The average farm yield of local Pakistan cultivars (450 kg/ha) represents only about 20% of that produced in experimental plots of advanced selections (2,200 kg/ha). Viral diseases may be responsible for much of this yield disparity.

Prior to this study, CPMoV had been reported only from Nigeria (16). This is therefore the first report of this virus outside of Africa and the first report on the continent of Asia. Because CPMoV is seedborne (16), the virus can probably be expected to occur in other cowpea-producing regions of the world.

Eighty cowpea tissue samples, mostly from the Lahore, Faisalabad, Okara, Gujranwala, and Sialkot districts of Punjab, were negative in all virus assays. Symptoms on these plants may have been induced by whitefly-transmitted YMV (not seed-transmissible), reported in Punjab by Ahmad (1), or by other viruses not included in our tests. Further detection and identification of viruses infecting cowpea crops in Pakistan is planned.

We hereby propose the generation of virus-free seed stocks for commercial cowpea production in Pakistan, as an interim disease control measure, with simultaneous initiation of virus-resistance breeding, utilizing virus-resistant cowpea genotypes identified during these investigations (2).

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