Seedborne Viruses in Preintroduction Cowpea Seed Lots and Establishment of Virus-Free Accessions

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ABSTRACT

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Seeds from 60 cowpea preintroductions from Botswana, India, and Kenya were increased at the University of California Riverside. Second generation seed were planted in insect-free greenhouses at two locations and resulting seedlings were assayed by direct antigen coated enzyme-linked immunosorbent assay (DAC-ELISA) for the presence of eight seedborne viruses. By visual selection and DAC-ELISA, 10 virus-free mother plants for each of the 60 accessions were established. The seedlots from these mother plants were subsequently planted in isolation plots at St. Croix, Virgin Islands. Seedlings from the St. Croix seed increase were observed and tested by DAC-ELISA and were found to have remained free from viruses during this field exposure. One or more of the following viruses were detected in 40 of the 60 preintroductions: 32 containing cowpea aphid-borne mosaic potyvirus (possibly blackeye cowpea mosaic potyvirus in some cases); 23 with cowpea severe mosaic comovirus; 22 with southern bean mosaic sobemovirus; seven with cucumber mosaic cucumovirus; and seven with cowpea mottle carmovirus. None were found to contain ELISA-detectable cowpea mosaic comovirus or cowpea mild mottle carlavirus. Twenty preintroductions were free of ELISA-detectable seedborne viruses. Virus-free experimental seed lots were produced for limited use by interested cowpea breeders.

Seedborne viruses are extremely important in the movement and utilization of crop germ plasm worldwide (5). Hampton et al (5) suggested that new accessions of germ plasm be assayed for seedborne viruses and that a first-generation seed increase should be accomplished in a greenhouse free of insects with several tests for seedborne viruses during the increase. Successive regenerations of seed should then be protected from recontamination with seedborne viruses (e.g., isolated from potentially infected plants). Two successful seedborne-virus elimination projects have been performed on food legume germ plasm collections and include pea seedborne mosaic virus (PSbMV) in pea and bean common mosaic virus (BCMV) in bean (4,7). In both cases the elimination of virus-infected plants, even with purported loss of genetic diversity, was preferred to uncontrolled germ plasm–borne viruses (1,4,7). To minimize the risk of virus spread when selecting virus-free accessions from BCMV-infected *Phaseolus* germ plasm, Klein et al (6) confined their greenhouse procedure to the winter-spring interval. Hampton et al (4) derived PSbMV-free nuclear stocks from all 2,700 available U.S. *Pisum sativum* germ plasm accessions. Two hundred entries of the nuclear stocks were experimentally increased without virus reinfection in field plots located within a one-mile-radius PSbMV-free zone.

Seedborne viruses in cowpea (*Vigna unguiculata* (L.) Walp. subsp. *unguiculata*) are a serious problem worldwide. At least 15 seed-transmitted viruses have been reported for this crop (2,9). Non-seedborne viruses also cause serious losses in cowpea seed yields (8). Hampton et al (3) tested cowpeas from developing countries against antisera to six seedborne viruses: cowpea aphid-borne mosaic potyvirus (CABMV), cowpea mild mottle carlavirus (CMMV), cowpea mosaic comovirus (CPMV), cowpea severe mosaic comovirus (CSMV), cucumber mosaic cucumovirus (CMV), and southern bean sobemovirus (SBMV). They found that one or more of the above viruses were present in 23 seed lots from nine countries. All of these viruses were found to occur in tropical Africa (10).

Seed lots from cowpea seeds imported from Botswana, India, and Kenya were planted in the greenhouse at the University of California Riverside (UCR), and observed by the research group of A. E. Hall for phenotypic uniformity and for any viruslike symptoms. On the basis of these observations, one or two representative asymptomatic mother plants were selected and grown to maturity. Resultant seeds were planted in field nurseries the following summers for further observations and evaluation. Seed lots included in the present study originated from these nursery plants.

In 1989-90, these UCR nursery plantings were critically examined for symptoms of seedborne viruses by R. O. Hampton, M. Bashir, and A. E. Hall. Numerous plants with viruslike symptoms were removed, returned to Corvallis, OR, and assayed by indirect enzyme-linked immunosorbent assay (ELISA) (3) for virus presence and identity. Many virus isolates were identified as typical CABMV. UCR nursery plants remaining after removal of symptomatic seedlings became the sources for each of 800 preintroduction seed lots (imported seed lots requiring tests for exotic seedborne viruses before introduction into the U.S. germ plasm collection) reposited at the Plant Genetic Resources Conservation Unit at Griffin, GA.

Sixty of the resulting seed lots from Botswana, India, and Kenya were evaluated before introduction into the U.S.

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Vigna germ plasm collection to determine the extent to which exotic seedborne viruses might have survived the preliminary, nonassay plant-selection procedure, and to assess the procedural requirements for establishing virus-free seed lots. Methods used and a summary of the viruses identified are given.

MATERIALS AND METHODS

Seed from the asymptomatic plants at UCR were planted, 100 seed per accession, in containers with Metro-Mix 220 (Grace Sierra Horticultural Products Co., Milpitas, CA), in insect-free greenhouses. Investigations were conducted in 1991–92 and 1992–93 between November and April to reduce risk of native insects entering the greenhouse. Ten of the nuclear seed lots were processed at Corvallis, OR; the remaining 50 were processed at Griffin, GA.

Plants were selected to identify approximately 10 virus-free mother plants in each seed lot. Cowpea seedlings were tested at the two- to three-leaf stage by direct antigen coated (DAC) ELISA for eight seedborne viruses. Seedlings with viruslike symptoms were excluded in the selection of candidate virus-free mother plants.

For viral assays, seedlings were sampled by removing a leaflet from each of the top three trifoliolate leaves of each plant. A disk of leaf tissue was then excised from each leaflet with a #13 cork borer. The three disks per sample were combined and triturated at a 1:10 dilution (w/v) in extraction buffer (0.15M NaCl, 0.036M Na_2HPO_4, 0.016M NaH_2PO_4, 0.013M sodium diethyldithiocarbamate, 0.003M NaN_3, pH 7.5) and further diluted to a final dilution of 1:100 in this buffer. Two-hundred-microliter aliquots of each sample extract were dispensed to two replicate microtiter plate wells for each of eight virus-specific DAC-ELISA assay plates (flat-bottom Immulon II plates, Dynatech Laboratories Inc., Chantilly, VA).

Polyclonal rabbit antisera were supplied by and used at the following dilutions: CABMV (1:2,000) and bean mild mosaic carmovirus (for detection of CPMoV) (1:500), R. O. Hampton, Corvallis, OR; CMMV (1:10,000), H. J. Vetten, Braunschweig, Germany; blackeye cowpea mosaic potyvirus (BlCMV) (1:2,000), CPMV (1:10,000), CSMV (1:10,000), CMV (1:4,000), and SBMV (1:10,000), O. W. Barnett, Clemson University, Clemson, SC.

For DAC-ELISA, plates were charged with plant extracts, incubated overnight at 4 C, and washed three times with phosphate-buffered saline–Tween 20 (0.0014M KH_2PO_4, 0.14M NaCl, 0.0077M Na_2HPO_4, 0.003M KCl, 0.5% w/v Tween 20 pH 7.5). The positive controls for each antiserum in each test were extracts of dried virus-infected leaf material homologous to each antiserum. Antisera were diluted at the indicated rates in healthy plant extract (i.e., diluted in 1:50 [w/v] mixture of healthy cowpea tissue and conjugate buffer [phosphate-buffered saline–Tween 20 + 2% w/v PVP Mol. Wt. 40,000 + 0.2% w/v grade 2 ovalbumin]) and the appropriate antiserum was pipetted into each well. The plates were then incubated 1.5 h at 37 C and washed three times. The secondary antibody, goat anti-rabbit antibody conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO), was diluted 1:1,500 in conjugate buffer, added to plates, and incubated 1.5 h at 37 C. After the final washing, the plates received alkaline phosphatase substrate, disodium p-nitrophenyl phosphate (Sigma) diluted to 0.0028 M concentration in 1.28 M diethanolamine, pH 9.8, containing 0.006 M NaN_3. After 1–2 h incubation at room temperature, p-nitrophenol absorption (A_{405}) values were recorded on a Dynatech MR 700 plate reader. Assays were considered positive when sample absorption values exceeded the mean value for healthy controls plus 2.5 times the standard deviation among wells containing these controls per plate.

Plants containing detectable virus were discarded. Approximately 10 plants containing no detectable viruses from each seed lot were repotted in larger containers and grown to maturity for seed production. Plants were resampled at flowering to ensure virus-free mother plants.

A 1991–92 test planting of 25 cowpea

Table 1. Enzyme-linked immunosorbent assay (ELISA) detection of seedborne viruses in preintroductions of 60 cowpea seed lots

UCR no.[a]	Origin	Antisera[b]					Total plants tested[c]
		CABMV/ BlCMV	CMV	CPMoV (BMMV)	CSMV	SBMV	
537	Kenya	6					15
538	Kenya	9					19
551	Kenya	3					15
569	Kenya	1					13
500	India	3					13
514	India	16					26
530	Kenya	8				1	26
503	India	6	1		2	3	13
508	India	20	5		9	6	26
504	India	13		1	3	4	25
529	Kenya	10	3		1	3	26
581	Kenya	1			1		15
584	Kenya	2			1		15
519	Kenya	4			2	2	39
525	Kenya	2			1	5	19
526	Kenya	10			1	4	26
532	Kenya	1			1	2	13
533	Kenya	4			10	1	26
534	Kenya	3			10	1	26
535	Kenya	1			12	1	26
552	Kenya	1			3	3	15
553	Kenya	1			1	1	15
562	Kenya	1			2	5	15
564	Kenya	3			1	9	21
585	Kenya	2			3	1	15
518	Botswana	4				5	19
536	Kenya	4				3	26
517	Botswana	4	11		3		26
505	India	3	7	8	5	1	44
999	Botswana	1		1			13
1013	Botswana	1					13
998	Botswana	1		1			13
542	Kenya		2				17
527	Kenya		1	1			13
1005	Botswana			1			13
1001	Botswana			1	1		13
566	Kenya				1		15
1004	Botswana				6		16
543	Kenya					4	15
563	Kenya					1	15

[a] University of California Riverside seed lots 531, 540, 544, 545, 546, 550, 555, 556, 558, 560, 567, 571, 573, 575, 576, 577 (from Kenya) and 1008, 1015, 1016, 1019 (from Botswana) are not listed because they were free of ELISA-detectable viruses.

[b] Cowpea aphid-borne mosaic potyvirus (CABMV)/ blackeye cowpea mosaic potyvirus (BlCMV), cucumber mosaic cucumovirus (CMV), cowpea mottle carmovirus (CPMoV) (bean mild mosaic carmovirus [BMMV] antiserum used), cowpea severe mosaic comovirus (CSMV), southern bean mosiac sobemovirus (SBMV). No seedlings tested positive for cowpea mosaic comovirus (CPMV).

[c] Plants were tested for presence of virus until approximately 10 virus-free mother plants were identified.

germ plasm accessions from another source (from the Griffin collection and representing diverse geographical origins) was grown at St. Croix, VI, as an experimental seed increase. Resultant plants remained healthy in appearance during seed production, and seedlings from these St. Croix plots were free from viruslike symptoms. This successful seed increase suggested that the St. Croix ecosystem had been a suitable environment for seed increase of *V. unguiculata* germ plasm accessions. Thus, virus-free *V. unguiculata* seed lots generated at Griffin/Corvallis were planted in 1992–93 and 1993–94 for seed increase at St. Croix. These seed-increase plots consisted of single rows of 20 plants per accession. After seed produced from these plots was harvested, 100 seeds of each accession were planted and seedlings examined by DAC-ELISA. Leaf disks from five seedlings were combined as pooled samples for the ELISA testing. (Preliminary tests indicated that one infected leaf could be detected when mixed with four healthy leaves.)

RESULTS AND DISCUSSION

Forty of 60 asymptomatic cowpea seed lots, representing distinct and valuable genotypes, contained one or more ELISA-detectable, seedborne viruses. The viruses detected in preintroduction seed lots are summarized in Table 1. Six seedborne viruses were found in the preintroduction seed lots. These viruses were: 32 preintroductions with CABMV (possibly BlCMV, the DAC-ELISA with polyclonal antisera does not clearly distinguish among isolates of CABMV and BlCMV); 23 with CSMV; 22 with SBMV; 7 with CPMoV; and 7 with CMV. Two of these (CABMV and CPMoV) have not been previously reported in the U.S. and BlCMV has not been reported in Africa. Tests for CMMV gave nonspecific reactions so were inconclusive, and no seedlings tested positive for CPMV. Only two seedlings, of the approximately 6,000 examined, developed viruslike symptoms under these environmental conditions and these two UCR 542 plants tested positive for CMV.

In the tests of the 60 seed lots increased in St. Croix in 1992–93 or in 1993–94, a subsample of 100 seeds from each accession were planted, their seedlings tested by DAC-ELISA, and all viral assays were negative. Thus, all nuclear seed lots were concluded to have remained free of ELISA-detectable viruses during the seed increase on St. Croix.

This procedure is therefore considered successful in producing virus-free germ plasm. Loss of diversity in selecting seed-mother plants is irrelevant in this study since preintroductions were derived from only one or two plants per accession during the screening procedures at UCR prior to our tests. Significant losses of diversity were reported for beans and peas in virus-elimination programs (1,7). Hampton et al (4) suggest that these reports failed to consider the loss of diversity during routine increases or the loss of resources through disuse because of viral contamination. Predominant self-pollination of cowpeas produces largely homogeneous populations, thus also moderating diversity loss in landrace collections. Likewise, we consider introduction of virus-free accessions into collections preferrable to including genotypes that could cause contamination.

Seed numbers generated in the greenhouse for each nuclear seed source and those resulting form the St. Croix field increases are listed in Table 2. Although seed yields at each stage were variable among lines, seed quantities were sufficient to enable the Plant Genetic Resources Conservation Unit to provide pathogen-free seed to both private and public cowpea breeders. Moreover, the respective breeding programs, other users, and U.S. agriculture will have been safeguarded from inadvertent introduction of serious exotic pathogens (CABMV and CPMoV). We believe our objectives were accomplished in this investigation and that they are consistent with two important USDA/ARS mandates: to provide free access to valuable crop germ plasm and simultaneously to assure safety from germ plasm–borne pathogens.

These results confirm the importance of seedborne viruses in disease dissemination via germ plasm exchanges in *Vigna* germ plasm (2,3,5). Results from the present germ plasm exchange indicate that selection of asymptomatic plants is an unreliable method for obtaining virus-free mother plants. Preintroductions from Botswana probably originally contained most of the total seedborne-virus contamination; however, the incidence of vector-mediated secondary spread in the Riverside field plots is unknown.

Because of the possibility of virus escape or movement in greenhouses during the summer months (6), and because attempts to grow virus-free cowpeas in fields near Griffin have typically reintroduced seedborne viruses, our field-plot seed increases were conducted at St. Croix. Tests during the summer of 1993 at Griffin indicated that virus-free cowpeas can be increased in screenhouses with 30-mesh screening, but insufficient space in such facilities was available for this purpose. Tests of alternative seed-increase locations in the southwestern and southeastern U.S. isolated from cultivated legumes are planned.

Table 2. Numbers of virus-free seed generated in greenhouse and field (St. Croix) plantings

UCR line no.[a]	Greenhouse increase	Field increase	UCR line no.[b]	Greenhouse increase	Field increase
500	334	7,883	537A[c]	400	5,626
503	422	3,810	537B[c]	181	6,423
504	232	2,249	538	756	3,735
505	111	3,292	540	655	8,822
508	134	5,562	542	1,043	5,874
514	177	524	543	879	2,172
517	146	141	544	1,841	5,593
518	197	6,552	545	710	2,433
519	141	2,001	546	629	4,156
525	248	4,269	550	950	2,070
526	184	3,925	551	393	1,702
527	76	6,184	552	786	8,067
529	154	9,144	553	812	6,658
530	118	4,765	555	517	6,271
531	597	5,302	556	751	6,084
532	497	6,404	558	1,569	3,642
533	183	7,128	560	1,271	7,338
534	155	3,403	562	693	5,245
535	80	6,759	563	683	3,073
536	118	9,481	564	904	5,892
998	175	5,580	566	963	7,715
999	39	4,885	567	1,137	10,074
1001	216	7,245	569	1,057	5,341
1004	119	5,949	571	954	4,715
1005	179	5,051	573	541	7,483
1008	285	8,937	575	315	3,630
1013	158	5,620	576	808	10,236
1015	121	849	577	450	7,717
1016	218	6,739	581	457	3,530
1019	155	3,009	584	971	7,585
			585	1,038	8,138

[a] Preintroductions processed in greenhouses in winter 1991–92 and in St. Croix field plots in 1992–93: UCR (University of California Riverside) 550–536 at Griffin, GA, and UCR 998–1019 at Corvallis, OR.

[b] Preintroductions processed in greenhouse at Griffin in winter 1992–93 and at St. Croix 1993–94.

[c] UCR 537 was separated into samples A and B based on pod and seed color.

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