Relationship of Cowpea Seed-Part Infection and Seed Transmission of Blackeye Cowpea Mosaic Potyvirus in Cowpea

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ABSTRACT

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Blackeye cowpea mosaic potyvirus (BlCMV) was seedborne in four cowpea seed lots (two genotypes) at incidences of 0.4–50%. Fully mature seeds were hydrated and separated into testae and embryos. Each embryo was separated further into two cotyledons and the embryo axis. These were tested separately for BlCMV by DAS-ELISA and by bioassay on *Chenopodium amaranticolor*. Viruses occurred in cotyledons and/or embryo axes in Coronet 1985cs, Coronet 1986cs, and PI 517912 but only in the cotyledons in Coronet 1987. Viral antigen was found in or on the testae, but very little infectious virus was present. The germinability of cowpea seeds of five accessions that were hydrated, decorticated, and stored 12 wk ranged from 60 to 100%. Applicability of these data to the establishment of testing for seed-transmissible BlCMV is discussed.

Blackeye cowpea mosaic potyvirus (BlCMV) causes a mosaic disease of cowpea (*Vigna unguiculata* (L.) Walp. subsp. *unguiculata*) (12). Dual infection by BlCMV and cucumber mosaic cucumovirus results in severe stunting of cowpea (11). Blackeye cowpea mosaic virus was described by Anderson (2) and Lima et al (7). Transmission through cowpea seed (6,14,18) has been reported with frequencies as high as 30% (18). Sekar and Sulochana (13) reported that infectious BlCMV was present in cotyledons as well as in embryo axes in mature seeds of two infected cowpea cultivars, but the virus was absent from the seed coats.

An approach for detection of peanut stripe potyvirus (PStV) in peanut seed has been developed (3,10). This "intact-seed" ELISA involves sampling the distal end of a cotyledon with a razor blade, testing by ELISA, and growing out those seed that test negative for PStV. Our objective was to develop a similar test for decorticated cowpea seed.

Our research was undertaken to locate the virus in seeds of cowpea through infectivity and serological tests. Our purpose was to relate seed-part infection to BlCMV detection in seed and to BlCMV seed transmission so that a reliable seed test for BlCMV could be established. We examined the testa that is of maternal origin and the embryo that is isolated from the maternal tissue and consists of two cotyledons and the embryo axis.

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MATERIALS AND METHODS

Seed lots of cowpea lines Coronet 1985cs, Coronet 1986cs, Coronet 1987, Coronet RS-90, Coronet RSC-15, and PIs 353346 and 517912 were used in the virus distribution tests. Coronet 1985cs and 1986cs were from field increases of the commercial line that was naturally infected with BlCMV. Coronet 1987 was seed from plants mechanically inoculated in a field increase. When tested by whole-seed ELISA (5), Coronet RS-90 and Coronet RSC-15 showed a high percentage of infection. Seed lot PI 353346 had shown infection in previous seed regeneration plots at Griffin, Georgia, and PI 517912 was seed from known infected plants from increase plots. Samples of each seed lot were hydrated in deionized water for either 4 or 16 hr, and the testae were removed manually. Individual embryos were dissected with a razor blade so that there were four portions of each seed: testa, two cotyledons, and embryo axis. The seed portions were triturated separately with a mortar and pestle in seed antigen buffer (0.04 M KH_2PO_4, 0.46 M Na_2HPO_4, 0.14 M NaCl, 0.003 M NaN_3, 0.003 M KCl, 0.05% [v/v] Tween 20, pH 7.5) at the rate of 1 ml for each testa and cotyledon sample and 0.5 ml for each embryo axis.

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was performed with BlCMV IgG from polyclonal rabbit antiserum utilizing the same methodology as for PStV detection (10). The BlCMV IgG was prepared by precipitation of antiserum with 18% (w/v) sodium sulfate, dialysis, and adjustment to 1 mg/ml of protein with a titer of 1:2,000. Alkaline phosphatase-conjugated BlCMV IgG was suspended in conjugate buffer (0.137 M NaCl, 0.003 M NaN_3, 0.0015 M KH_2PO_4, 0.0077 M Na_2HPO_4, 0.0027 M KCl, 0.05% [v/v] Tween 20, 2% [w/v] polyvinylpyrrolidone 40,000 M_r, 0.2% [w/v] ovalbumin grade II, pH 7.4). Substrate consisted of 0.0028 M disodium *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) in 1.28 M diethanolamine and, 0.006 M NaN_3, pH 9.8. After 30–60 min of incubation, A_{405} values were recorded by a Dynatech MR 700 plate reader (Dynatech Laboratories Inc., Chantilly, VA). A sample was considered to be positive for BlCMV when it had an absorption value greater than the mean value plus 2.5 times the standard deviation for the healthy control. For infectivity testing, samples of seed parts were triturated with a mortar and pestle in 0.025 M potassium phosphate buffer, pH 7.2 (buffer volumes used were the same as those used for extraction of samples for serology above) and rubbed onto silicon carbide (600 grit)-dusted leaves of *Chenopodium amaranticolor* Coste & Reyn. plants with five fully expanded leaves. Leaves with local lesions were tested for BlCMV by DAS-ELISA.

To determine the percent seedborne infection, seed from each lot were planted in Metro Mix 220 (Grace Horticultural Products, Cambridge, MA) in metal trays in the greenhouse. Each test consisted of 100 seeds from each lot, with 50 seeds per tray. The youngest fully expanded leaf of each seedling was tested (at 1:10 dilution [w/v] as determined by standardization tests) by DAS-ELISA at the second or third trifoliolate leaf stage. To determine whether the virus was on the surface of the embryo or only within the embryo, samples (100 seeds each) were taken from seed lots of Coronet 1986cs and PI 517912. Seeds were hydrated in tap water for 4 hr, the seed coats were removed, the embryos (cotyledons and embryo axes together) were washed in running tap water for 2 hr to remove any virus particles on the surface (procedure established for pea seedborne mosaic potyvirus [PSbMV] in pea seed by R. O. Hampton, *personal communication*), and DAS-ELISA was performed on the embryos for BlCMV.

Length of storage tests of decorticated seed were undertaken to establish a reli-

able intact-seed test for BlCMV seed transmissibility. Seed from virus-free lots of California Blackeye, Texas Pinkeye, and PIs 354581, 487474, and 527263 representing widely different seed types were hydrated overnight in deionized water. Testae were removed and the decorticated seeds were placed in a drying room at 21 C and 20% RH for 5 days, then stored at 4 C and 20% RH along with a control set of untreated seed. A random sample of 25 seeds from each accession was planted immediately following testae removal and after 1, 2, 4, 8, and 12 wk of storage. Seed were planted into 25.4-cm pots in Metro Mix 220 with 25 seeds per pot in the greenhouse. Plants were counted 2 wk after seeding.

RESULTS

Blackeye cowpea mosaic virus was seed transmitted in four of the seven cowpea seed lots tested (Table 1). The incidence of seed transmission of virus ranged from 0.4 to 50%.

Viral antigen was present in the testae (Table 2), but there was little infectious virus present in the testae (Table 3). The incidence of virus and the incidence of viral antigen varied between the embryo axis and the cotyledons within an infected seed. The incidence of positive BlCMV tests for the embryos (cotyledons and embryo axis) of two cultivars was similar in serological and biological assays (2.9 and 2.0% for Coronet 1985cs and 36.7 and 40.4% for PI 517912, respectively). The incidence of positive BlCMV tests for Coronet 1986cs was higher in serological (17.1%) than in biological (3.7%) tests. The serological results for Coronet 1987 in which the virus was found only in the cotyledons (Table 2) differed from those for the other seed lots.

Table 1. Incidence of blackeye cowpea mosaic potyvirus seed transmission among seven selected cowpea seed lots, as determined by DAS-ELISA of seedlings[a]

Seed lot[b]	No. of seed planted/ no. germinated	No. of infected seedlings
Coronet 1985cs	100/89	1[c]
Coronet 1986cs	100/88	5
Coronet 1987	500/453	2
Coronet RS-90	300/224	0
Coronet RSC-15	300/238	0
PI 353346	100/92	0
PI 517912	100/34	17

[a] Seedlings were tested by ELISA at the second or third trifoliolate leaf stage. The youngest fully expanded leaf was ground in antigen buffer at 1:10 dilution for testing.
[b] Seed lots selected for testing had a positive virus test in a whole-seed ELISA determination or were from fields or plants with a high rate of infection.
[c] A sample was positive when the A_{405} was higher than the mean value plus 2.5 times the standard deviation for the healthy control.

Results of ELISAs of washed embryos indicated the presence of BlCMV within the embryo. The percentages of BlCMV-positive embryos within washed and unwashed samples were 9 and 7%, respectively, for Coronet 1986cs and 43 and 39%, respectively, for PI 517912.

Germination of decorticated seed of five cowpea accessions ranged from 60 to 100% after 12 wk of storage: 60% for California Blackeye, 68% for Texas Pinkeye, 92% for PI 487474, and 100% for PIs 354581 and 527263.

DISCUSSION

The failure to demonstrate any seed transmission in seed lots Coronet RS-90 and Coronet RSC-15, which had a high percentage of seeds with viral antigen by the whole-seed ELISA test, indicates that this test method does not accurately select for seed transmissibility of BlCMV in cowpea. The testae of these cowpea lines must contain noninfectious viral antigen (Tables 2 and 3). A similar situation has been described for PSbMV in mature pea seed, in which testae must be removed for accurate estimation of potential seed transmission by a "whole-embryo" ELISA (8,16). Our results demonstrate the efficacy of this approach for estimating seed transmission potential of BlCMV in cowpea seed. The whole-seed ELISA approach has worked well for seed transmissibility estimations with bean common mosaic potyvirus in bean (5), lettuce mosaic potyvirus in lettuce (4), and soybean mosaic potyvirus in soybean (9). The lack of viral antigen in the testae of mature peanut seed with PStV (3,17) and with peanut mottle potyvirus (1) indicates that the whole-seed ELISA test would work to estimate seed transmissibility for these virus-host combinations also.

Varma et al (15) found that at least 100 ng of blackgram mottle virus (BMoV) was necessary in the embryonic axis of blackgram seed for the resulting seedling to become systemically infected. Although BMoV is not a potyvirus and the host is different, the same type of phenomenon could be operative for BlCMV in cowpea seed, and all ELISA-detectable BlCMV or even infectious BlCMV may not result in seed transmissibility. On the basis of our results (Tables 1–3), this would be a very small portion of the seed tested.

The results of tests in which decorticated seeds were washed show that the ELISA-detectable virus is within the embryo and not on the outside. In our tests (Tables 2 and 3), BlCMV was not distributed evenly within the embryos of all infected seed. Those seed with virus in only the embryo axis or in only one cotyledon could reduce the accuracy of a test that relies on sampling just a portion of one cotyledon. A positive

Table 2. Incidence of ELISA-detectable blackeye cowpea mosaic potyvirus (BlCMV) antigen in hydrated, dissected seed parts of cowpea

Seed lot	Number tested	Number of seed with BlCMV-positive parts[a]: Testa only	Testa + any other seed part[b]	One cotyledon only	Two cotyledons only	Embryo axis + at least one cotyledon	Embryo axis only
Coronet 1985cs	105	59	2	0	0	1	0
Coronet 1986cs	105	27	3	1	1	10	3
Coronet 1987	683	110	2	11	1	0	0
Coronet RS-90	105	11	0	0	0	0	0
Coronet RSC-15	105	14	0	0	0	0	0
PI 353346	102	19	0	0	0	0	0
PI 517912	98	0	0	5	3	14	14

[a] Data in all columns are exclusive of data in other columns.
[b] Other seed parts with ELISA-detectable BlCMV: Coronet 1985cs, embryo axis and one cotyledon = 1, one cotyledon only = 1; Coronet 1986cs, embryo axis and one cotyledon = 2, one cotyledon only = 1; Coronet 1987, one cotyledon only = 2.

Table 3. Incidence of blackeye cowpea mosaic potyvirus (BlCMV) in hydrated, dissected seed parts of cowpea, based on *Chenopodium amaranticolor* bioassays

Seed lot	Number tested	Number of seed with BlCMV-positive parts[a]: Testa only	Testa + any other seed part[b]	One cotyledon only	Two cotyledons only	Embryo axis + at least one cotyledon	Embryo axis only
Coronet 1985cs	101	0	0	0	0	1	1
Coronet 1986cs	107	1	0	0	0	1	3
PI 517912	99	0	3	4	5	20	8

[a] Data in all columns are exclusive of data in other columns.
[b] Other seed parts testing positive for virus: PI 517912, embryo axis and at least one cotyledon = 2, one cotyledon only = 1.

intact-embryo ELISA result is needed to reliably determine the incidence of infected seed and subsequently to reduce the incidence of seed transmission during regeneration of the cowpea germ plasm collection. Furthermore, in our tests, decorticated cowpea seed stored for 12 wk still germinated at acceptable levels. We propose sampling decorticated seed for virus infection before drying and storage so that many seed could be tested over a 12-wk period before planting. Although a lower germination rate with some cultivars may slightly reduce genetic diversity, we feel that this loss would be offset by the enhanced potential for production of virus-free seed stocks.

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