Tolerance to Blackeye Cowpea Mosaic Potyvirus Not Correlated with Decreased Virus Accumulation or Protection from Cowpea Stunt Disease

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ABSTRACT

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Progeny from a near-isogenic cowpea line responded heterogeneously when infected with a cowpea stunt–derived isolate of blackeye cowpea mosaic potyvirus (BlCMV). One group of plants developed a delayed, mild reaction to BlCMV while sister plants rapidly exhibited strong systemic mosaic symptoms. Conversely, enzyme-linked immunosorbent assay (ELISA) results indicated that BlCMV generally accumulated to the same levels and at the same rates in these two plant groups. Similar results were obtained for two commercial varieties that expressed different BlCMV symptoms. Symptom analyses and ELISA were used to demonstrate that one of these commercial varieties was highly resistant to this virus isolate. All genotypes responded with similar, mild reactions when inoculated with cowpea stunt–derived cucumber mosaic cucumovirus (CMV). Both symptoms and ELISA-detectable levels of CMV decreased as plants aged. Mixed infections with BlCMV and CMV resulted in severe cowpea stunt disease symptoms and high concentrations of CMV coat protein 20 days after inoculation in all plants that did not express extreme resistance to BlCMV. Interestingly, at early time points after inoculation, differences in symptom severity between singly and dually infected plants were not consistently correlated with significant differences in relative CMV concentrations. The results indicate that (i) resistance to BlCMV, as determined through visual observation, is not adequate when evaluating germ plasm for cowpea stunt disease resistance, and (ii) rapid development of severe symptoms on dually infected plants may not be due solely to increased CMV concentrations.

Cowpea stunt is a potentially devastating disease that poses an economic threat to U.S. cowpea (*Vigna unguiculata* (L.) Walp. subsp. *unguiculata*) production. The disease results from a synergistic interaction between cucumber mosaic cucumovirus (CMV) and blackeye cowpea mosaic potyvirus (BlCMV) (27). These viruses are transmitted individually or concomitantly via mechanical inoculation, aphids, and through seed (27).

Cowpea stunt was first reported in Georgia, Alabama, and South Carolina (25, 27) and, more recently, in Arkansas on plants exhibiting severe stunting, strong mosaic symptoms, leaf blistering, and little or no flowering (1). We believe that the disease is becoming an increasingly important problem in a number of other cowpea-producing states, including Louisiana, Mississippi, Oklahoma, and Texas, and major efforts have been focused on breeding for resistance.

While no reliable sources of resistance to CMV have been identified for incorporation into U.S. cowpea-breeding programs, a single recessive gene, *blc* (34), and other, less-characterized genes provide immunity or varying levels of resistance to a number of BlCMV strains (6,20,22,26). Unfortunately, little is known about whether resistance to BlCMV is due to an inhibition of virus replication or a restriction of virus movement, and only a few studies have been conducted to evaluate BlCMV-resistant plants for enhanced resistance to cowpea stunt disease (25,26). In addition, there are some disadvantages to using BlCMV-resistance genes, including the time required to backcross and select horticulturally desirable resistant progeny, the possibility that various strains of BlCMV may overcome the resistance (25), and the potential that resistance to BlCMV may not result in reduced cowpea stunt disease severity (23).

Varying levels of virus resistance have been engendered in transgenic plants expressing virus structural or nonstructural genes (13,18,35). Many of these efforts, especially those that involve the expression of a viral coat protein (CP) gene, have resulted in plants that are resistant, but not immune, to the homologous virus or some closely related viruses (7,29). Furthermore, the degree of resistance imparted by these methods is often sufficient to protect plants from economic losses under field conditions. These approaches are rapidly becoming accepted strategies for controlling viruses in agronomic and horticultural crops (14,15,31).

The long-range goal of our work is to better understand the molecular basis of cowpea stunt disease and to develop cowpea stunt-resistant cultivars through classical breeding and genetic engineering. A more complete characterization of virus/virus and virus/plant interactions will facilitate these efforts.

Excel (formerly, Ark 87-435) is a recently developed cowpea variety with an upright, determinant growth habit, excellent yield potential, and resistance to bacterial blight (*Xanthomonas campestris* pv. *vignicola*). A study was undertaken with progeny of single plant selections from this line that responded heterogeneously to BlCMV in order to determine whether resistance to BlCMV, based on visual symptom development, was correlated with a decrease in virus accumulation and protection from cowpea stunt disease.

MATERIALS AND METHODS

Virus and seed sources. Plants affected by cowpea stunt disease were collected in Columbia County, Arkansas, during the summer of 1993 and used as inoculum source material to obtain separate cultures of CMV and BlCMV (1,27). These cultures were maintained in cowpea cv. Georgia 21 under greenhouse conditions, with temperatures from 20 to 30°C and daylength ranging from 9 to 12 h. Seed of cowpea cvs. Coronet (8) and Pinkeye Purple Hull-BVR (PPH-BVR) (20) was provided by F. B. Cates (Western Seed Multiplication, Inc., Oglethorpe, GA).

Progeny from 85 single plant selections (F6) of cowpea cv. Excel that appeared to be segregating for resistance to BlCMV were further screened for resistance to the BlCMV isolate recovered from plants expressing cowpea stunt. Sixteen to 20 seeds of each selection were planted in flats containing Redi-Earth 3CF potting mixture (Grace Sierra, Milpitas, CA). Plants were inoculated approximately 8 days after planting with a crude sap extract from

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BlCMV-infected plants prepared by grinding leaf tissue in 0.05 M phosphate buffer (pH 7.2) at an approximate ratio of 1:10 (wt/vol). Visual observations were made 21 days after inoculation. One BlCMV-resistant selection (Excel-68) and one BlCMV-sensitive selection (Excel-71) were chosen for further studies. Seed from selections Excel-68 and Excel-71 was propagated, harvested, and subsequently stored at 4°C.

Plant response to CMV, BlCMV, and mixed infections. Seed of each genotype (Coronet, Excel-68, Excel-71, and PPH-BVR) was planted in separate 6-inch pots containing potting mixture and maintained in the greenhouse. Seven to 8 days after planting, primary leaves were lightly dusted with Carborundum and gently inoculated with a crude sap extract from healthy, CMV-, BlCMV-, or dually infected cowpea cv. Georgia 21 plants. We have found that symptom development and virus CP accumulation patterns do not differ when plants are infected by combining inocula from CMV and BlCMV singly infected plants or by inoculating directly from dually infected plants (data not shown). Therefore, inoculum for mixed infections was derived from dually infected plants for the current study. Visual observations were made every day after inoculation and systemic symptoms were recorded every 2 days beginning 8 days after inoculation and continuing until 20 days after inoculation.

A visual scoring system of 0 to 5 was developed in which 0 represented no visual symptoms, 1 indicated a chlorotic or necrotic response on inoculated leaves, 2 represented faint vein clearing on leaves above the inoculated leaves (systemically infected leaves), 3 indicated more pronounced vein clearing, mosaic, mottling, and, in mixed infections, some stem or leaf malformations, 4 represented intense mosaic, mottling, some stunting, and, in mixed infections, more apparent stem and petiole deformation and bending, and 5 represented the most intense visual symptoms observed on dually infected plants, including severe stunting, leaf blistering and malformation, as well as stem and petiole necrosis.

At 20 days after inoculation, all aboveground plant tissues were harvested. Fresh weights were determined as a measure of plant stunting caused by each virus and virus combination.

Determination of relative virus accumulation. A modification of the indirect enzyme linked immunosorbent assay (ELISA) described by Bashir (5) was used to approximate relative concentrations of CMV and BlCMV in inoculated primary leaves and the second set of trifoliate leaflets at various times after inoculation. Inoculated leaves from different plants were harvested 5, 10, and 15 days after inoculation, while the trifoliate leaflets from separate plants were harvested 10, 15, and 20 days after inoculation. At days 10 and 15, inoculated and trifoliate leaves were taken from the same plants.

Plant sap, obtained by passing leaves through a tissue extractor (Erich Pollahne Co., Wennigsen, West Germany), was diluted 1:50 in antigen buffer (phosphate-buffered saline [PBS], pH 7.4, with 13 mM Na-diethyldithiocarbamic acid). Diluted sap (200 µl) was applied to duplicate wells of microtiter plates (Immulon 1, Dynatech Laboratories, Inc., Chantilly, VA), which were then incubated at 37°C for 2 h. Separate antisera (1:10,000) to CMV (provided by R. O. Hampton, Oregon State University) and BlCMV virions were cross-adsorbed with 1:50 dilutions of healthy cowpea sap extracts in antiserum buffer (PBS, pH 7.4, 0.1% [vol/vol] Tween 20, 2% [wt/vol] polyvinylpyrrolidone, 0.2% [wt/vol] bovine serum albumin) for 1 h prior to application to microtiter plate wells that had been washed three times with wash buffer (PBS, pH 7.4, 0.1% [vol/vol] Tween 20). Following a 2-h incubation at 37°C, plates were washed, and a goat anti-rabbit alkaline phosphatase conjugate (Sigma, St. Louis, MO) diluted in virus buffer (1:10,000) was applied. Plates were incubated at 37°C for 2 h and washed. Phosphatase substrate (Sigma, St. Louis, MO) was diluted to 0.33 mg/ml in 10% (vol/vol) diethanolamine, 0.02% (wt/vol) sodium azide, pH 9.8, and applied to microtiter plates. Plates were incubated for 60 min at room temperature, and absorbance values (405 nm) were determined by a microplate reader (model 7500, Cambridge Technology Inc., Watertown, MA).

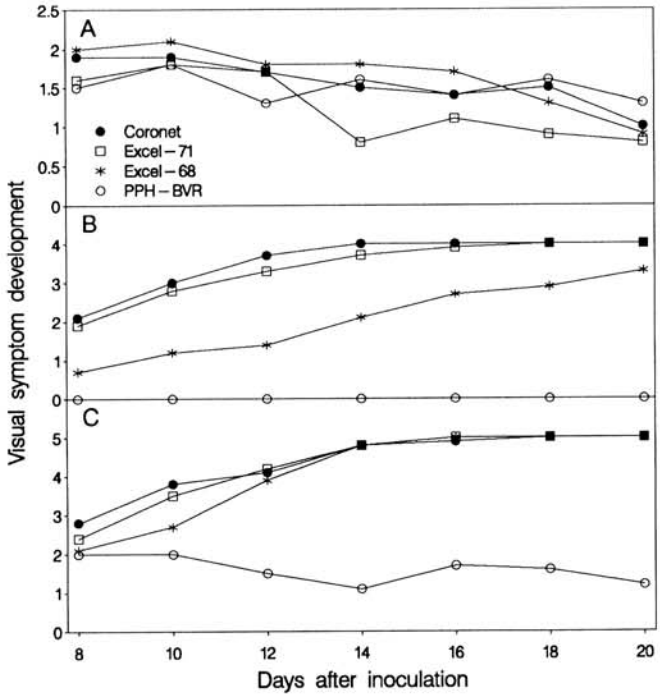

Fig. 1. Development of visual symptoms over time on four cowpea genotypes following inoculation with sap extracts from plants infected with (**A**) cucumber mosaic cucumovirus (CMV) alone, (**B**) blackeye cowpea mosaic potyvirus (BlCMV) alone, and (**C**) a mixture of CMV and BlCMV. The *y* axis represents a visual scoring system in which 0 = no symptoms, 1 = a chlorotic or necrotic response on inoculated leaves, 2 = faint vein clearing on systemically infected leaves, 3 = more pronounced vein clearing, mosaic, mottling (single infections), and/or some stem or leaf malformations (mixed infections), 4 = intense mosaic, mottling (single infections), and/or some stunting and more apparent stem and petiole deformation, bending, and some necrosis (mixed infections), and 5 = intense symptoms consisting of severe stunting, leaf blistering and malformation, and stem and petiole necrosis.

RESULTS

Visual symptom development. Approximately 5 days after inoculation with CMV, primary leaves of all cowpea genotypes expressed chlorotic lesions, while Coronet and Excel showed faint chlorotic lesions or ringspots and necrosis when inoculated with BlCMV or the virus mixture, respectively. Inoculated leaves of PPH-BVR showed no reaction to BlCMV but did express chlorotic lesions, typical of CMV infection, in response to the mixed inoculum.

Figure 1 illustrates the progression of symptom development on trifoliate leaves of Coronet, Excel-71, Excel-68, and PPH-BVR between 8 and 20 days after inoculation with either CMV, BlCMV, or the mixture. By 8 days after inoculation, trifoliate leaflets from all genotypes infected with CMV expressed mild vein clearing and mottling that generally faded in all systemically infected leaves as plants aged (Fig. 1A). Trifoliate leaflets of BlCMV-infected Coronet and Excel-71 expressed

vein clearing that increased in intensity, progressed up the plant, and developed into strong mosaic symptoms by 20 days after inoculation (Fig. 1B; Fig. 2). Conversely, Excel-68 was delayed in the expression of visual symptoms and developed only mild vein clearing and mottling by 20 days after inoculation with BlCMV (Fig. 1B; Fig. 2). PPH-BVR expressed no visual symptoms in response to BlCMV (Fig. 1B; Fig. 2).

Following inoculation with the virus mixture, Coronet, Excel-71, and Excel-68 did not differ in the development of severe cowpea stunt symptoms (Fig. 1C). By as early as 5 to 8 days after inoculation, these plants exhibited strong responses on inoculated leaves and stem malformations below the inoculated leaves. Symptoms rapidly intensified on inoculated leaves and were followed by leaf blistering on systemically infected leaves with further stem and petiole deformations and necrosis. Cowpea cv. PPH-BVR expressed mild, CMV-like symptoms (Fig. 1C).

Quantitation of disease phenotype differences. An analysis of total plant fresh weights 20 days after inoculation provided a semiquantitative way of measuring virus-induced plant stunting by demonstrating that single infections with CMV resulted in significantly reduced plant growth for all genotypes when compared with the respective mock-inoculated control plants (Table 1). Single infections by BlCMV caused reductions in plant fresh weight (Table 1), but these plants were generally not significantly stunted when compared with mock-inoculated plants. Fresh weights of PPH-BVR plants inoculated with the virus mixture were not different from mock-inoculated plants, but dually infected plants representing the other three genotypes were significantly stunted when compared with healthy plants (Table 1). When compared with mock-inoculated controls, the reductions in fresh weights of dually infected plants representing Coronet and Excel were always greater than the combined reductions induced by single infections. However, due to sampling variation, these differences were not statistically significant ($P = 0.01$).

Approximation of virus accumulation over time in inoculated leaves. To further characterize BlCMV resistance in Excel-68 and evaluate BlCMV and CMV accumulation patterns in single versus mixed infections over time, plant samples from susceptible and resistant genotypes were analyzed by ELISA at four time points after inoculation.

At 5 days after inoculation, when chlorotic lesions were barely detectable, CMV CP was readily detected in inoculated leaves from all genotypes (Fig. 3A). At this early sampling time, ELISA values for CMV in the inoculated leaves from dually infected plants were slightly higher than in plants infected with CMV alone, except in PPH-BVR (Fig. 3A).

ELISA absorbance readings for CMV in primary leaves 10 days after inoculation remained relatively high and did not differ significantly between singly and dually infected plants of Coronet and PPH-BVR (Fig. 3A). In contrast to ELISA results, dually infected Coronet plants exhibited more severe symptoms than singly infected plants (Fig. 1A,C). ELISA detection of CMV CP in both Excel genotypes indicated more virus in the inoculated leaves of dually infected plants than in singly infected plants 10 days after inoculation (Fig. 3A).

By 15 days after inoculation, ELISA-detectable levels of CMV CP in the inoculated leaves of singly and dually infected plants began to decline (Fig. 3A). At the same time, symptoms faded on all leaves of singly infected plants, but inoculated leaves, adjacent petiole and stem tissues, and trifoliate leaves on dually infected plants showed more intense symptoms (Fig. 1A,C). Significant differences in ELISA values between singly and dually infected plants were observed only for Excel-71 at this sampling date (Fig. 3A).

BlCMV CP was detected at very low levels, or was not detected at all, in inoculated leaves of singly or dually infected plants at 5 days after inoculation (Fig. 3A). In addition, the ELISA values for BlCMV in Coronet, Excel-71, and Excel-68 were indistinguishable, indicating that virus replication was not inhibited in Excel-68.

ELISA values for BlCMV in singly and dually infected primary leaves of Coronet

Table 1. Total foliar fresh weights of four cowpea genotypes 20 days after mechanical inoculation with either cucumber mosaic cucumovirus (CMV), blackeye cowpea mosaic potyvirus (BlCMV), or a mixture of both

Cowpea genotype	Virus inoculum[x]	Fresh weight (g)[y]	Percent of mock
Coronet	Mock	32.2 a	100
	CMV	26.5 b	82
	BlCMV	27.8 ab	86
	Mixed	19.2 c	60
Excel-71	Mock	32.8 a	100
	CMV	26.3 b	80
	BlCMV	30.2 ab	92
	Mixed	20.2 c	62
Excel-68	Mock	34.2 a	100
	CMV	27.6 b	81
	BlCMV	27.6 b	81
	Mixed	19.8 c	58
PPH-BVR[z]	Mock	29.6 a	100
	CMV	21.9 b	74
	BlCMV	25.5 ab	86
	Mixed	26.2 ab	89

[x] Symptomatic leaves of CMV-, BlCMV-, or dually infected cowpea cv. Georgia 21 were used as virus inoculum source for primary leaves of young plants prior to the appearance of trifoliate leaflets (approximately 8 days after planting). Mock plants were inoculated with an extract from healthy cowpea cv. Georgia 21.

[y] Values are means of total fresh weights for all aboveground foliar tissue from a representative experiment in which 5 to 7 plants were harvested 20 days after inoculation. A two-factor analysis of variance was conducted in which factors were the presence or absence of CMV or BlCMV in the inoculum. Comparisons among individual means were made with *t* tests. Means within genotypes followed by the same letter are not statistically different at $P = 0.01$.

[z] Pinkeye Purple Hull-BVR.

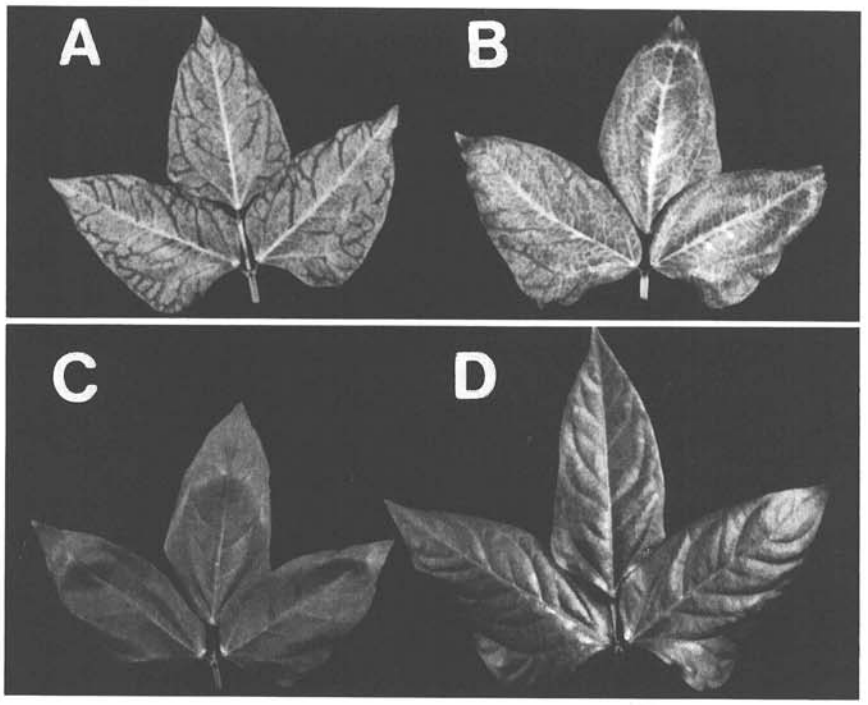

Fig. 2. Second trifoliate leaves from four cowpea genotypes expressing typical blackeye cowpea mosaic potyvirus–induced symptoms 20 days after inoculation. (**A**) Coronet, (**B**) Excel-71, (**C**) Excel-68, (**D**) Pinkeye Purple Hull-BVR.

and Excel increased significantly between 5 and 10 days after inoculation and did not differ significantly between singly and dually infected plants of the same genotype at the second harvest date, except in Excel-68 (Fig. 3A). Furthermore, BlCMV ELISA values for Excel-68 and Excel-71 were significantly different only for the mixed infection in Excel-68 ($P = 0.01$). No virus CP was detected in PPH-BVR (Fig. 3A).

At 15 days after inoculation, BlCMV ELISA values for inoculated leaves of singly and dually infected plants were approximately the same as those measured at the 10-day sampling point (Fig. 3A). There were no significant differences in ELISA values between singly and dually infected plants within genotypes and no difference ($P = 0.01$) between Excel-68 and Excel-71, except for the dually infected Excel-71 sample (Fig. 3A). Again, BlCMV was not detected in PPH-BVR–inoculated leaves (Fig. 3A).

Approximation of virus accumulation over time in trifoliate leaves. Second trifoliate leaflets harvested on the tenth day after inoculation were small (approximately 2.5 cm long). Although intense differences in symptoms were observed between CMV- and dually infected plants (Fig. 1), ELISA values for trifoliate leaves were not significantly different for any cultivar at this harvest date (Fig. 3B). At 15 days after inoculation, CMV ELISA values for second trifoliate leaves from singly infected plants were surprisingly low, except in PPH-BVR, where the virus concentration was similar to what had been detected at the 10-day sampling point (Fig. 3B). As with inoculated leaves, these data were in agreement with the decrease in symptom intensity on CMV-infected plants (Fig. 1).

In stark contrast to the drop in ELISA-detectable CMV CP in trifoliates from singly infected plants was the finding that CMV ELISA values for trifoliates from dually infected plants at 15 days after inoculation had increased significantly in all genotypes except PPH-BVR (Fig. 3B). In genotypes that were not highly resistant to BlCMV, the CMV ELISA readings were significantly (11.8 to 40.5 times) higher for dually infected plants (Fig. 3B).

At 20 days after inoculation, ELISA values for CMV in trifoliate leaves from singly infected plants, as well as dually infected PPH-BVR, had dropped to near background levels (Fig. 3B) while the detectable levels of virus antigen in dually infected plants, except PPH-BVR, remained nearly as high as they had been on day 15 and ranged from approximately 16 to 75 times higher than values recorded for single infections (based on a low value of 0.012 to a high value of 0.909) (Fig. 3B).

Ten days after inoculation, ELISA values for BlCMV in second trifoliate leaves from Coronet and Excel were relatively high and not significantly different when compared across genotypes ($P = 0.01$), except when dually infected plants of Excel-68 and Excel-71 were compared (Fig. 3B). Within genotypes, the only significant difference observed was between singly and dually infected Excel-68 plants (Fig. 3B).

At 15 and 20 days after inoculation, ELISA values for BlCMV in trifoliates from singly or dually infected plants within genotypes were not significantly different, except in Coronet and Excel-68 at 15 and 20 days after inoculation, respectively (Fig. 3B). When compared across genotypes, BlCMV ELISA values for dually infected Coronet and all Excel treatments were not significantly different ($P = 0.01$). Generally, BlCMV ELISA values for second trifoliate leaves remained relatively unchanged throughout the time course study (Fig. 3B).

The results presented here are representative of three separate experiments. While significant differences in relative accumulation levels of CMV and BlCMV CPs were detected in some CMV-inoculated and BlCMV-inoculated and trifoliate leaves, significant differences were consistently observed only when comparing trifoliates from CMV- and dually infected plants not highly resistant to BlCMV at 15 and 20 days after inoculation (Fig. 3A,B).

Inoculation of single leaves of *Chenopodium quinoa* with 1:10 and 1:20 dilutions of ELISA extracts and subsequent determination of the number of lesions that formed on each leaf corroborated the method of approximating virus accumula-

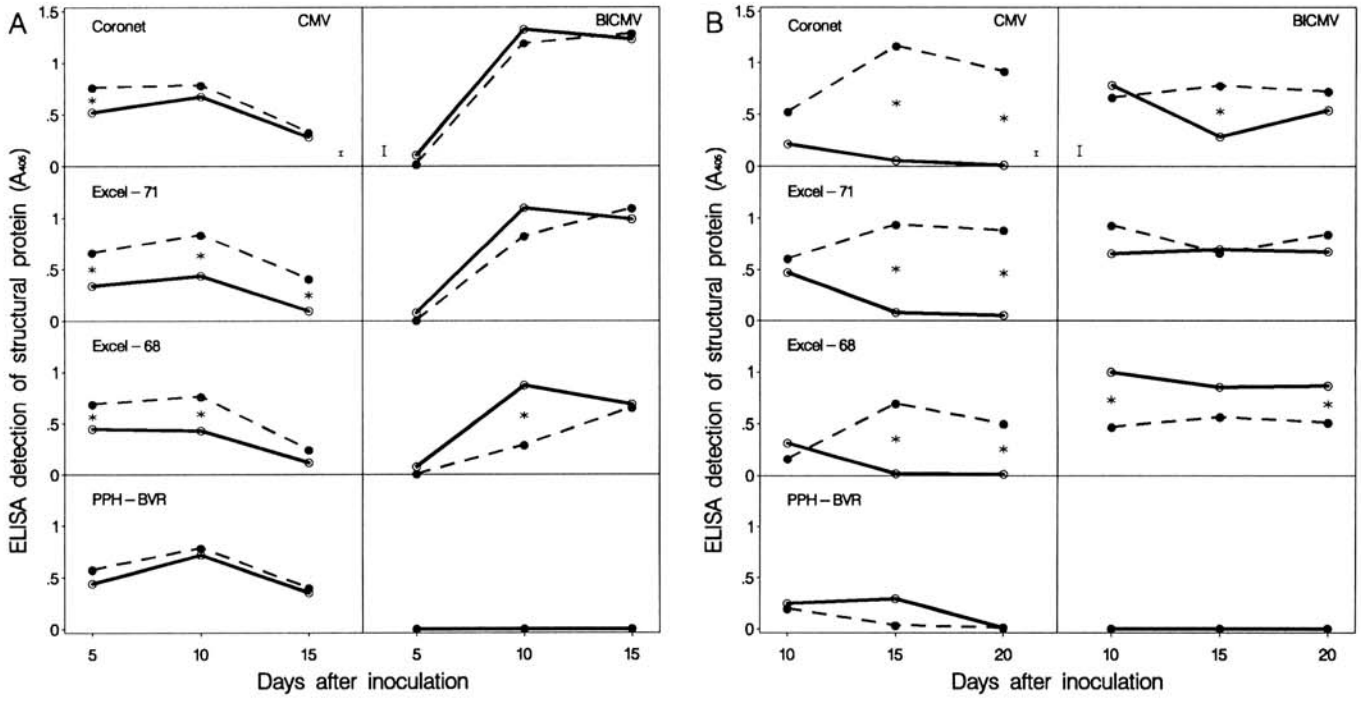

Fig. 3. Relative accumulation of virus over time, based on enzyme-linked immunosorbent assay (ELISA) detection of virus coat protein, in primary leaves and second trifoliate leaflets of cowpea genotypes inoculated singly (-O-) or doubly (-●-) with cucumber mosaic cucumovirus (CMV) and blackeye cowpea mosaic potyvirus (BlCMV). **(A)** The accumulation of CMV and BlCMV in the inoculated leaves of the cowpea cultivars indicated in the left panels. **(B)** the accumulation of CMV and BlCMV in the second set of trifoliate leaflets of the cowpea cultivars indicated in the left panels. Vertical bars in the top panels indicate standard errors of the means for all values in graphs below them. ELISA values for each genotype at each sampling point were subjected to a three-factor analysis of variance in which the three factors were genotype, inoculum, and harvest day. Means were compared by multiple *t* tests. For each genotype, at each sampling date, values for single versus mixed infections that were significantly different at $P = 0.01$ are indicated by an asterisk (*).

tion patterns by measuring the amount of ELISA-detectable virus CP. Lesion numbers always supported ELISA results, even in cases in which very large differences in CMV ELISA values for single versus mixed infections were recorded (data not shown). In addition, all singly and dually infected leaf samples were evaluated, by ELISA, for the presence of both viruses. Singly infected plants never contained the heterologous virus.

DISCUSSION

A number of plant viruses interact synergistically to cause severe, economically important diseases (3,24,28). Unfortunately, information concerning the mechanisms of virus synergy and characteristics of host responses to single and mixed infections that might be helpful in developing resistance, especially against cowpea stunt disease, is currently lacking.

Striking differences in BlCMV symptoms expressed by near-isogenic progeny from Excel could not be correlated with differences in plant fresh weights, nor in rates or levels of BlCMV accumulation based on ELISA and local lesion assays. While it is not clear why Excel-68 consistently expressed milder symptoms than near-isogenic plants, it appears that delayed symptom expression was not due to an inhibition of virus replication or a restriction of virus movement in this BlCMV-tolerant genotype. These results also help to explain why Excel-68 was not protected from the synergistic effects of CMV and BlCMV in mixed infections in which the ability of BlCMV to replicate and move systemically resulted in the synergistic interaction with CMV. Conversely, PPH-BVR was shown to express an extremely high level of resistance to this strain of BlCMV, and these plants were consequently protected from the effects of the mixed infection.

Not surprisingly, these results demonstrate that protection from cowpea stunt synergism can be achieved by preventing the establishment of a BlCMV infection. Because no source of CMV resistance has yet been identified, it is important that cowpea breeders use germ plasm containing genes that provide a high degree of resistance against the most prevalent BlCMV isolates for the production of cowpea stunt–resistant plants. Furthermore, evaluation of germ plasm for resistance to BlCMV based on symptom expression is not adequate for assessing resistance to single or mixed infections.

It is not known whether protection from cowpea stunt can be obtained through partial resistance to either BlCMV or CMV. Since the mild BlCMV symptoms observed on Excel-68 were not associated with decreased virus accumulation, this question remains unanswered. Theoretically, it should be possible to engender partial resistance to these viruses in transgenic plants by expressing either, or both, of the CMV and BlCMV CP genes. A major portion of our efforts is currently focused on developing a transformation and regeneration system for cowpea in order to accomplish this task, preferably in a BlCMV-resistant genotype (4).

Mechanistic studies on other synergistic virus interactions have suggested that 1.3- to 10-fold increases in the replication and accumulation of a nonpotyvirus in a mixed infection increases disease severity (10,17, 21,28). Our results represent the first time similar studies have been conducted for cowpea stunt–diseased plants and agree with those previously presented for other systems. In the present study, however, distinctly more severe symptoms observed at early sampling points (5 and 10 days after inoculation) on dually infected plants were not always correlated with significantly higher CMV titers at these sampling times. It was only at later sampling points (15 to 20 days after inoculation) that CMV ELISA values and local lesion infectivity assays indicated virus titers that were much higher in dually infected plants than in singly infected plants. Furthermore, the rapid deformation and necrosis of stems and petioles on dually infected plants indicated that one or both of these viruses were replicating or accumulating in vascular tissues that do not express overt symptoms during single infections. Preliminary results in our laboratory support this suggestion. If verified, this finding will agree with previous studies showing that one virus in a mixed infection can facilitate infection of normally uninfected tissues by another virus (2,11,16,30) and help us gain insight into the early events in virus synergy that contribute to severe symptom establishment.

Recent work to elucidate the molecular determinants of potato potexvirus X (PVX) and potato potyvirus Y (PVY) synergism has revealed that the 5′ terminal portion of the potyvirus genome influences synergy (32). This portion of the genome and/or its protein products may play a role in mediating PVX replication or stimulating genome amplification (12,32,33). However, it has also been suggested that this region contains sequences that might bind single-stranded RNA, function in unfolding virus RNA during replication, and mediate cell-to-cell and long-distance movement (9,19). In the case of cowpea stunt, these potyvirus sequences may complement CMV infection and/or accumulation in the vasculature, resulting in petiole and stem deformation and necrosis.

Currently, we are examining the early events in CMV and BlCMV mixed infections in cowpea in order to determine what factors, other than increased levels of CMV, contribute to enhanced disease severity. These studies center on evaluating the possibility that BlCMV may facilitate more rapid and extensive CMV infection of plant vascular tissues.

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LITERATURE CITED

1. Anderson, E. J., Kline, A. S., Kim, K. S., Goeke, S. C., and Albritton, C. W. 1994. Identification of cowpea stunt disease in south central Arkansas. Arkansas Farm Res. 43:14-15.
2. Barker, H. 1986. Invasion of non-phloem tissue in *Nicotiana clevelandii* by potato leafroll luteovirus is enhanced in plants also infected with potato Y potyvirus. J. Gen. Virol. 68:1223-1227.
3. Barker, H. 1989. Specificity of the effect of sap-transmissible viruses in increasing the accumulation of luteoviruses in co-infected plants. Ann. Appl. Biol. 115:71-78.
4. Barker, H., Webster, K. D., Jolly, C. A., Reavy, B., Kumar, A., and Mayo, M. A. 1994. Enhancement of resistance to potato leafroll virus multiplication in potato by combining the effects of host genes and transgenes. Mol. Plant-Microbe Interact. 7:528-530.
5. Bashir, M. 1992. Serological and biological characterization of seed-borne isolates of blackeye cowpea mosaic and cowpea aphid-borne mosaic potyviruses in *Vigna unguiculata* (L.) Walp. Ph.D. thesis, Oregon State University, Corvallis.
6. Bashir, M., and Hampton, R. O. 1992. Biological characterization of pathotypes of blackeye cowpea mosaic and cowpea aphid-borne mosaic potyviruses. (Abstr.) Phytopathology 82:1103.
7. Beachy, R. N., Loesch-Fries, S., and Tumer, N. E. 1990. Coat protein-mediated resistance against virus infection. Ann. Rev. Phytopathol. 28:451-474.
8. Brantley, B. B. 1976. Coronet, a new southern pea variety. Ga. Agric. Exp. Stn. Res. Rep. 220.
9. Brantley, J. D., and Hunt, A. G. 1993. The N-terminal protein of the polyprotein encoded by the potyvirus tobacco vein mottling virus is an RNA-binding protein. J. Gen. Virol. 74: 1157-1162.
10. Calvert, L. A., and Ghabrial, S. A. 1983. Enhancement by soybean mosaic virus of bean pod mottle virus titer in doubly infected soybean. Phytopathology 73:992-997.
11. Carr, R. J., and Kim, K. S. 1983. Evidence that bean golden mosaic virus invades non-phloem tissue in double infections with tobacco mosaic virus. J. Gen. Virol. 64:2489-2492.
12. Carrington, J. C., and Freed, D. D. 1990. Cap-independent enhancement of translation by a plant potyvirus 5′ nontranslated region. J. Virol. 64:1590-1597.
13. Fitchen, J. H., and Beachy, R. N. 1993. Genetically engineered protection against viruses in transgenic plants. Ann. Rev. Microbiol. 47:739-763.
14. Fox, J. L. 1994. USDA likely to okay Asgrow's engineered squash. Bio/Technology 12:761-762.
15. Fuchs, M., and Gonsalves, D. 1995. Resistance of transgenic hybrid squash ZW-20 expressing the coat protein genes of zucchini yellow mosaic virus and watermelon mosaic virus 2 to mixed infections by both potyviruses. Bio/Technology 13:1466-1473.
16. Fuentes, A. L., and Hamilton, R. I. 1991. Sunn-hemp mosaic virus facilitates cell-to-cell spread of southern bean mosaic virus in a nonpermissive host. Phytopathology 81:1302-1305.

17. Goldberg, K.-B., and Brakke, M. K. 1987. Concentration of maize chlorotic mottle virus increased in mixed infections with maize dwarf mosaic virus, strain B. Phytopathology 77:162-167.
18. Harms, C. T. 1992. Engineering genetic disease resistance into crops: Biotechnological approaches to crop protection. Crop Prot. 11: 291-306.
19. Klein, P. G., Klein, R. R., Rodriguez-Cerezo, E., Hunt, A. G., and Shaw, J. G. 1994. Mutational analysis of the tobacco vein mottling virus genome. Virology 204:759-769.
20. Kuhn, C. W., Brantley, B. B., Demski, J. W., and Pio-Ribeiro, G. 1984. 'Pinkeye purple hull-BVR', 'white acre-BVR', and 'corona' cowpeas. Hortic. Sci. 19:592.
21. Lee, Y.-S., and Ross, J. P. 1972. Top necrosis and cellular changes in soybean doubly infected by soybean mosaic and bean pod mottle viruses. Phytopathology 62:839-845.
22. Miller, J. C., Jr., and Scheuring, D. C. 1994. 'Texas pinkeye purple hull' cowpea. Hortic. Sci. 29:926-927
23. Murphy, J. F., and Kyle, M. M. 1995. Alleviation of restricted systemic spread of pepper mottle potyvirus in *Capsicum annuum* cv. Avelar by coinfection with a cucumovirus. Phytopathology 85:561-566.
24. Niblett, C. L., and Claflin, L. E. 1978. Corn lethal necrosis - a new virus disease of corn in Kansas. Plant Dis. Rep. 62:15-19.
25. Pio-Ribeiro, G., and Kuhn, C. W. 1980. Cowpea stunt: Heterogeneous and differential reactions of cowpea cultivars. Phytopathology 70:244-249.
26. Pio-Ribeiro, G., Kuhn, C. W., and Brantley, B. B. 1980. Cowpea stunt: Inheritance pattern of the necrotic synergistic reaction. Phytopathology 70:250-252.
27. Pio-Ribeiro, G., Wyatt, S. D., and Kuhn, C. W. 1978. Cowpea stunt: A disease caused by a synergistic interaction of two viruses. Phytopathology 68:1260-1265.
28. Rochow, W. F., and Ross, A. F. 1955. Virus multiplication in plants doubly infected by potato viruses X and Y. Virology 1:10-27.
29. Stark, D. M., and Beachy, R. N. 1989. Protection against potyvirus infection in transgenic plants: Evidence for broad spectrum resistance. Bio/Technology 7:1257-1262.
30. Taliansky, M. E., de Jager, C. P., Wellink, J., van Lent, J. W. M., and Goldbach, R. W. 1993. Defective cell-to-cell movement of cowpea mosaic virus mutant N123 is efficiently complemented by sunn-hemp mosaic virus. J. Gen. Virol. 74:1895-1901.
31. Tricoli, D. M., Carney, K. J., Russell, P. F., McMaster, J. R., Groff, D. W., Hadden, K. C., Himmel, P. T., Hubbard, J. P., Boeshore, M. L., and Quemada, H. D. 1995. Field evaluation of transgenic squash containing single or multiple virus coat protein gene constructs for resistance to cucumber mosaic virus, watermelon mosaic virus 2, and zucchini yellow mosaic virus. Bio/Technology 13:1458-1465.
32. Vance, V. B., Berger, P. H., Carrington, J. C., Hunt, A. G., and Shi, X. M. 1995. 5′ proximal potyviral sequences mediate potato virus X/potyviral synergistic disease in transgenic tobacco. Virology 206:583-590.
33. Verchot, J., and Carrington, J. C. 1995. Evidence that the potyvirus P1 proteinase functions *in trans* as an accessory factor for genome amplification. J. Virol. 69:3668-3674.
34. Walker, C. A., Jr., and Chambliss, O. L. 1981. Inheritance of resistance to blackeye cowpea mosaic virus in cowpea [*Vigna unguiculata* (L.) Walp.]. J. Am. Soc. Hortic. Sci. 106:410-412.
35. Wilson, T. M. A. 1993. Strategies to protect crop plants against viruses: Pathogen-derived resistance blossoms. Proc. Natl. Acad. Sci. USA 90:3134-3141.