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Mixed Infections with Stunt-Associated Isolates of *Blackeye cowpea mosaic virus* from Arkansas and Georgia and Non-Stunt-Associated Isolates of *Cucumber mosaic virus* Cause Cowpea Stunt Disease

Hortense A. Diallo[1*] • Edwin J. Anderson[2] • Rose C. Gergerich[3]

[1] Université d'Abobo-Adjamé, UFR-SN, Laboratoire de Biologie et Amélioration des Productions Végétales, 02 B. P. 801 Abidjan 02, Ivory Coast
[2] Pioneer Hi-Bred International, Inc, 6900 N W 62nd Ave, Johnston, IA, 50131-0256, USA
[3] University of Arkansas, Department of Plant Pathology, 217 PTSC Bldg, Fayetteville, Arkansas, 72701, USA

Corresponding author: * attakyhortense@yahoo.com

ABSTRACT

'Coronet' cowpea (*Vigna unguiculata* (L.) Walp. subsp. *unguiculata*) plants mixedly infected with cowpea stunt-associated isolates of *Blackeye cowpea mosaic virus* (BlCMV) from Arkansas and Georgia ($BlCMV_{AR}$ and $BlCMV_{GA}$, respectively), and *Cucumber mosaic virus* (CMV) isolates that have not previously been associated with the disease in the field, resulted in plants displaying symptoms similar to cowpea stunt disease. Fresh weight analysis showed that all four combinations of mixed infections had similar effects on infected plants. Whereas in the trifoliolate leaves there was no difference in $BlCMV_{AR}$ and $BlCMV_{GA}$ accumulations in single versus mixed infections, in the stem there was a difference and that difference was significant. Although CMV strain VE 111 (originally isolated from bean in Iran, provided by Dr. Palukaitis) and CMV strain LeJ (originally isolated from bean in Japan, provided by Dr. Palukaitis) accumulations increased in the mixed compared to the single infections in the stems, in the trifoliolate leaves, CMV strain VE 111 accumulation was higher in the mixed infection while CMV strain LeJ accumulation was not. All these results not only suggest a specific interaction between CMV and BlCMV isolates in the cowpea stunt disease which varies with the plant part tested, but also show that symptom severity in cowpea stunt disease is not always positively correlated with increased CMV accumulation.

Keywords: ELISA, fresh weight, virus accumulation

INTRODUCTION

Cowpea stunt, a severe disease of cowpea, was first reported in Georgia, South Carolina and Alabama (Pio-Ribeiro *et al*. 1978, 1980). The disease is caused by two viruses, *Cucumber mosaic virus* (CMV) and *Blackeye cowpea mosaic virus* (BlCMV), which are mechanically and aphid transmitted as well as seed-borne. These viruses interact synergistically to cause a very devastating disease in cowpea. Yield losses of up to 86% have been attributed to cowpea stunt disease (Pio-Ribeiro *et al*. 1978). The disease has since been found in cowpea production areas in Arkansas where infected plants were stunted with small blistered leaves and did not flower (Anderson *et al*. 1994).

Cowpea stunt disease constitutes a serious problem because, although sources of resistance to BlCMV are known and present in some cowpea cultivars, no reliable source of CMV resistance has been found in cowpea. Gillaspie (2001) has reported on the identification of a cowpea Plant Introduction (PI) line with a very high level or resistance to BlCMV and moderate resistance to CMV in the USA.

Studies conducted to compare the cowpea stunt disease viruses from Arkansas and Georgia both biologically and molecularly revealed that cowpea plants mixedly infected with viruses from both locations showed similar symptoms (Diallo 1998; Diallo *et al*. 2004). Furthermore, mixed infections with viruses from different locations also resulted in plants with characteristic cowpea stunt disease. Symptoms include strong mosaic, small and malformed leaves as well as stem and petiole necrosis. In addition, CMV and BlCMV from Arkansas and Georgia displayed a different accumulation pattern depending on the plant part tested and the number of days after inoculation (Diallo 1998). Nucleotide sequence comparison of the coat protein genes confirmed that the cucumovirus involved in cowpea stunt disease in Georgia and Arkansas was indeed CMV and that both isolates of CMV belong to subgroup I of CMV. These two CMV isolates differed by 8 nucleotides in their coat protein gene and 3′ untranslated region which resulted in two amino acids differences (Diallo *et al*. 2004). The potyvirus in the cowpea stunt disease was also confirmed to be BlCMV by nucleotide sequence comparison of the coat protein (CP) genes of the two viruses isolated from Arkansas and Georgia and other published sequences of BlCMV CP genes (Khan *et al*. 1993). The CP gene plus the 3′ untranslated regions (3′-UTR) of the Arkansas and Georgia isolates of BlCMV differed by only one nucleotide which in turn resulted in one amino acid difference (Diallo *et al*. 2004). Although CMV and BlCMV isolates from Arkansas and Georgia showed differences in their accumulation patterns and their genetic makeup, the disease they caused in mixed infections looked similar.

All mixed infections with the Arkansas and Georgia isolates of CMV and BlCMV resulted in cowpea stunt disease. The objective of this study was to determine if the typical cowpea stunt disease could be recreated when cowpea plants were mixedly infected with stunt-associated isolates of BlCMV and isolates of CMV not previously associated with cowpea stunt disease in the field.

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MATERIALS AND METHODS

Viruses and plants

The Arkansas isolate of BlCMV ($BlCMV_{AR}$), field-collected in Columbia County, Arkansas in 1994, and the Georgia isolate of cowpea stunt-forming BlCMV ($BlCMV_{GA}$) provided by Dr. A. G. Gillaspie, Jr., USDA-ARS Genetic Resources Unit, Univ. of Georgia, Griffin, GA were used in this study. CMV strain VE 111 and CMV strain LeJ (originally isolated from bean in Iran and Japan, respectively) not associated with the cowpea stunt disease, were provided by Dr. P. Palukaitis, Cornell University. The viruses were maintained by mechanical inoculation in cowpea cultivar Coronet (Brantley 1976) in the greenhouse with temperature ranging from 20-30°C. Seeds were sown individually in 7.5 cm clay pots containing Redi-Earth potting mixture (Grace Sierra, Miltipas, CA), and plants were fertilized once a week with Peter's Professional Fertilizer (15-16-17). All experiments were conducted at the University of Arkansas (Fayetteville) in the USA.

Single and mixed infections

The two primary leaves of cowpea plants (about eight days after planting) were mechanically inoculated with sap from 'Coronet' cowpea plants infected with the following viruses: CMV strain VE 111, CMV strain LeJ, $BlCMV_{AR}$, $BlCMV_{GA}$, CMV strain VE 111 + $BlCMV_{AR}$, CMV strain VE 111 + $BlCMV_{GA}$, CMV strain LeJ + $BlCMV_{AR}$ and CMV strain LeJ + $BlCMV_{GA}$, with 12 plants/treatment. Each plant sap was extracted in 0.05 M phosphate buffer (PB), pH 7.2. Sap from leaves of infected plants measured with a pipette and mixed with an equal volume of sap from healthy plants was used for the single virus inoculations. For inoculations to establish mixed infections, saps extracted from each virus-infected plant were mixed at equal volumes. Plants inoculated with sap from healthy cowpea leaves ground in 0.05 M phosphate buffer pH 7.2 were used as a control. All plant saps were maintained on ice. The mechanical inoculations were done by dusting carborundum (silicon carbide powder, grit 600; Buehler, Ltd., Lake Bluff, IL) on the primary leaves and gently rubbing the inoculum on the upper leaf surface. After inoculation, labeled plants were randomized on benches in the greenhouse maintained at 20 to 24°C with supplemental lightning to maintain a photoperiod of 14 h. Virus infection was monitored visually by weekly observation of symptoms and serologically by enzyme-linked immunosorbent assay (ELISA). Fresh weight of plants above the soil level was determined at 8 and 15 days post-inoculation (dpi). Data were analyzed using analysis of variance (ANOVA) and Fisher's Least Significant Difference test (LSD) at a significance level of $\alpha = 0.05$. This experiment was repeated twice.

Determination of relative virus accumulation in the first trifoliolate leaves and in the stems

At 8 and 15 dpi, plants were observed for symptom development and both the first trifoliolate leaves and the stems (area between the primary leaves and the soil level) were harvested. Sap was extracted from each of the samples using a tissue extractor (Erich Pollahne Co., Wennigsen, West Germany) and frozen at -70°C.

A modified version of the indirect enzyme-linked immunosorbent assay (ELISA) described by Bashir (1992) was used to determine the relative virus concentrations in the leaves and stems of plants singly and mixedly infected with CMV and BlCMV isolates from Arkansas and Georgia. Saps were extracted from the first trifoliolate leaves and stem of each plant and diluted 1:50 in antigen buffer [phosphate buffered saline (PBS), pH 7.4, 13 mM diethyldithiocarbamic acid]. The diluted sap was added to duplicate wells of microtiter plates (Immulon 1, Dynatech Laboratories, Inc., Chantilly, VA). Plates were incubated for 2 h at 37°C. Sap from healthy cowpea, diluted 1:50 in virus buffer (PBS, pH 7.4, 0.1% Tween-20, 2% polyvinylpyrrolidone (molecular weight 360,000) (PVP-360), 0.2% bovine serum albumin), was used to treat antisera to BlCMV (available in the laboratory) and CMV (obtained from R. O. Hampton, Oregon State University). Antisera were diluted 1:10,000 in the prepared sap solution and incubated for 1 h at room temperature. After 3 washes of 3 min each with PBS-Tween (PBS, pH 7.4, 0.1% Tween-20), and preabsorbed antisera were added to each well of the microtiter plates. Plates were incubated for 1 h and washed again with PBS-Tween. Anti-rabbit alkaline phosphatase conjugated IgG (Sigma, St. Louis, MO) diluted 1:10,000 in virus buffer was added to each well. The plates were incubated for 2 h and then washed. The substrate buffer (10% ethanolamine, 0.02% sodium azide, pH 9.8) containing 0.33 mg/mL of *p*-nitrophenyl phosphate (Sigma, St. Louis, MO) was added to each well. The working volume use to fill individual well of the plate was 200 µL for this test. Absorbance values were determined at 405 nm using a microplate reader (model 7500, Cambridge Technology Inc., Watertown, MA).

RESULTS

Fresh weight analysis

All four types of mixed infected plants showed severe symptoms at both 8 and 15 dpi. The trifoliolate leaves were small and presented a strong mosaic (**Fig. 1**). When comparing fresh weights at 8 and 15 dpi together (gross effect), CMV strain VE 111-infected plants weighed less than CMV strain LeJ and the healthy control (**Table 1**). There was, however, no significant difference between the weights of CMV strain LeJ-infected plant and the healthy control. There was no significant difference between plants inoculated with $BlCMV_{AR}$ and $BlCMV_{GA}$ and the healthy controls. Interestingly, no statistical difference was found between fresh weight of plants mixedly inoculated with CMV and BlCMV regardless of the source of the virus isolates involved (**Table 1**). Even though CMV strain VE 111 did affect plant fresh weight in the single infections while CMV strain LeJ did not, no significant differences were recorded in all four mixed infections (**Table 1**).

Single and mixed inoculations with stunt-causing isolates of BlCMV from Arkansas and Georgia and non-stunt-associated isolates of CMV in the stems and trifoliolate leaves: BlCMV accumulation

In the stems, at 8 dpi, $BlCMV_{AR}$ accumulated less in either mixed infections with CMV strain VE 111 or CMV strain LeJ when compared to the single infection (**Table 2**). Similar results were obtained at 15 dpi were $BlCMV_{AR}$ accumulated to half its ELISA value in the single infection (**Table 2**). The combined CMV (at 8 and 15 dpi) effect displayed the same accumulation pattern described at 8 and 15 dpi, meaning that $BlCMV_{AR}$ accumulation in the mixed infections was significantly lower than in the single infection. $BlCMV_{AR}$ in either single or mixed infections decreased between 8 and 15 dpi. Results showed that global $BlCMV_{AR}$ accumulation at 8 dpi was significantly higher than at 15

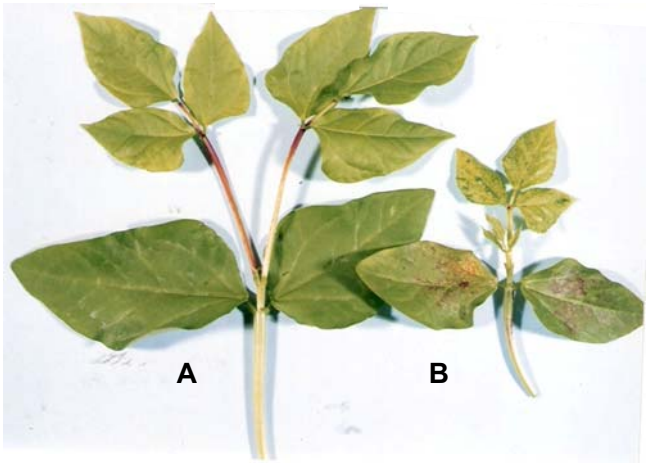

Fig. 1 Comparison between a healthy cowpea plant (A) and a plant inoculated with a mixture of CMV and BlCMV (B) 15 days after inoculation.

Table 1 Effect of single and mixed infections with stunt-causing isolates of BlCMV from Arkansas and Georgia and non-stunt-associated isolates of CMV on plant fresh weights (in grams).

Source of CMV	Source of BlCMV		
	Arkansas	Georgia	None
CMV strain VE 111	5.81 a	4.98 a	7.72 b
CMV strain LeJ	6.80 ab	5.78 a	9.12 c
None	8.91 c	7.87 bc	8.44 c

Means followed by same letter in columns and rows are not significantly different at α = 0.05 (LSD = 1.25). The experiment was repeated twice with similar results. The combined data is presented here.

Table 2 Effect of single and mixed infections with stunt-causing isolates of BlCMV from Arkansas and non-stunt-associated isolates of CMV in the stems: $BlCMV_{AR}$ accumulation.

Treatment	Mean absorbance (405 nm)		
	8 dpi	15 dpi	CMV effect
$BlCMV_{AR}$	0.29	0.18	A
$BlCMV_{AR}$ + CMV strain VE 111	0.20	0.08	B
$BlCMV_{AR}$ + CMV strain LeJ	0.21	0.08	B
Combined day effect	A	B	

Experiments were repeated twice with 6 plants/treatment and gave similar results. Same letters in column or row are not statistically different (α = 0.05).

Table 3 Effect of single and mixed infections with stunt-causing isolates of BlCMV from Georgia and non-stunt-associated isolates of CMV in the stems: $BlCMV_{GA}$ accumulation.

Treatment	Mean absorbance (405 nm)		
	8 dpi	15 dpi	CMV effect
$BlCMV_{GA}$	0.31	0.22	A
$BlCMV_{GA}$ + CMV strain VE 111	0.04	0.06	C
$BlCMV_{GA}$ + CMV strain LeJ	0.23	0.09	B
Combined day effect	A	B	

Experiments were repeated twice with 6 plants/treatment and gave similar results. Same letters in column or row are not statistically different (α= 0.05).

Table 4 Effect of single and mixed infections with stunt-causing isolates of BlCMV from Arkansas and non-stunt-associated isolates of CMV in the trifoliolate leaves: $BlCMV_{AR}$ accumulation.

Treatment	Mean absorbance (405 nm)	
	8 dpi	15 dpi
$BlCMV_{AR}$	0.25	0.35
$BlCMV_{AR}$ + CMV strain VE 111	0.26	0.35
$BlCMV_{AR}$ + CMV strain LeJ	0.23	0.27

Experiments were repeated twice with 6 plants/treatment and gave similar results. No statistical differences were found between single and mixed infections at either 8 or 15 days after inoculation (α = 0.05).

dpi.

With $BlCMV_{GA}$ accumulation in the stem, similar results were obtained at both 8 and 15 dpi as in the case with $BlCMV_{AR}$. $BlCMV_{GA}$ accumulation was lower in either mixed infection than in the single infection at both 8 and 15 dpi. However, the decrease in $BlCMV_{GA}$ accumulation at 8 dpi in the mixed infection with CMV strain LeJ was less than that observed in the case of the mixed infection with CMV strain VE 111 (**Table 3**). The combined CMV effect indicated that $BlCMV_{GA}$ accumulation in the single infection was higher than that of the mixed infections. Additionally $BlCMV_{GA}$ accumulated significantly higher in the mixed infection with CMV strain LeJ than with CMV strain VE 111. In general $BlCMV_{GA}$ accumulation in the stem was higher at 8 dpi than at 15 dpi (**Table 3**). In summary, in the stems, $BlCMV_{AR}$ and $BlCMV_{GA}$ had the same effect except that $BlCMV_{GA}$ accumulated more in the mixed infection with CMV strain LeJ than with CMV strain VE 111 while there was no difference between those mixed infections with $BlCMV_{AR}$.

In the trifoliolate leaves, at both 8 and 15 dpi, there was no statistically significant difference in $BlCMV_{AR}$ accumulation in the single infection versus either mixed infection (with CMV strain VE 111 and CMV strain LeJ) (**Table 4**). However, there appeared to be an increase in $BlCMV_{AR}$ accumulation from 8 to 15 dpi, but that difference was not significant (**Table 4**).

Table 5 Effect of single and mixed infections with stunt-causing isolates of BlCMV from Georgia and non-stunt-associated isolates of CMV in the trifoliolate leaves: $BlCMV_{GA}$ accumulation.

Treatment	Mean absorbance (405 nm)		
	8 dpi	15 dpi	CMV effect
$BlCMV_{GA}$	0.33	0.38	A
$BlCMV_{GA}$ + CMV strain VE 111	0.27	0.23	B
$BlCMV_{GA}$ + CMV strain LeJ	0.33	0.39	A

Experiments were repeated twice with 6 plants/treatment and gave similar results. Same letters in column are not statistically different (α = 0.05).

There was no day effect on $BlCMV_{GA}$ accumulation in the trifoliolate leaves. $BlCMV_{GA}$ accumulation patterns were similar at 8 and 15 dpi. However CMV did have an effect on $BlCMV_{GA}$ accumulation. Results showed that at 8 and 15 dpi $BlCMV_{GA}$ accumulation in the mixed infection with CMV strain LeJ was similar to that of the single infection but higher than that of the mixed infection with CMV strain VE 111 (**Table 5**). The combined CMV effect indicated that there was a significant difference between $BlCMV_{GA}$ accumulation in the single infection and mixed infection with CMV strain VE 111 but not with CMV strain LeJ (**Table 5**). Except for the fact that $BlCMV_{GA}$ accumulated less in the mixed infection with CMV strain VE 111, $BlCMV_{GA}$ and $BlCMV_{AR}$ had similar effect in the trifoliolate leaves.

Single and mixed inoculations with stunt-causing isolates of BlCMV from Arkansas and Georgia and non-stunt-associated isolates of CMV in the stems and trifoliolate leaves: CMV accumulation

In the stems, at 8 dpi, CMV strain VE 111 accumulation was enhanced in the mixed infections with $BlCMV_{AR}$ and $BlCMV_{GA}$ when compared to single infection (**Table 6**). However, at 15 dpi, CMV strain VE 111 accumulation in either mixed infection (with $BlCMV_{AR}$ or $BlCMV_{GA}$) was more than 2 times higher than in the single infection. Combined data showed that there was a significant difference in CMV strain VE 111 accumulation between single and mixed infections (**Table 6**). There was a significant decrease in CMV strain VE 111 accumulation between 8 and 15 dpi.

Similar results were obtained with CMV strain LeJ. Its accumulation was enhanced in the mixed infections compared to the single infection at both 8 and 15 dpi (**Table 7**). The combined BlCMV effect showed that the difference in CMV strain LeJ accumulation between single and mixed infections was statistically significant (**Table 7**). Except in the

Table 6 Effect of single and mixed infections with stunt-causing isolate of BlCMV from Arkansas and Georgia and a non-stunt-associated isolate of CMV in the stems: CMV strain VE 111 accumulation.

Treatment	Mean absorbance (405 nm)		
	8 dpi	15 dpi	BlCMV effect
CMV strain VE 111	0.21	0.08	B
CMV strain VE 111 + $BlCMV_{AR}$	0.32	0.15	A
CMV strain VE 111 + $BlCMV_{GA}$	0.34	0.19	A
Combined day effect	A	B	

Experiments were repeated twice with 6 plants/treatment and gave similar result. Same letters in column are not statistically different (α = 0.05).

Table 7 Effect of single and mixed infections with stunt causing isolates of BlCMV from Arkansas and Georgia and a non-stunt-associated isolate of CMV in the stems: CMV strain LeJ accumulation.

Treatment	Mean absorbance (405 nm)		
	8 dpi	15 dpi	BlCMV effect
CMV strain LeJ	0.30	0.05	B
CMV strain LeJ + $BlCMV_{AR}$	0.36	0.35	A
CMV strain LeJ + $BlCMV_{GA}$	0.39	0.34	A
Combined day effect	A	B	

Experiments were repeated twice with 6 plants/treatment and gave similar results. Same letters in column or row are not statistically different (α = 0.05).

Table 8 Effect of single and mixed infections with stunt causing isolates of BlCMV from Arkansas and Georgia and a non-stunt-associated isolate of CMV in the trifoliolate leaves: CMV strain VE 111 accumulation.

Treatment	Mean absorbance (405 nm)		
	8 dpi	15 dpi	BlCMV effect
CMV strain VE 111	0.18	0.12	B
CMV strain VE 111 + $BlCMV_{AR}$	0.30	0.31	A
CMV strain VE 111 + $BlCMV_{GA}$	0.38	0.36	A

Experiments were repeated twice with 6 plants/treatment and gave similar results. Same letters in column are not statistically different ($\alpha = 0.05$).

Table 9 Effect of single and mixed infections with stunt causing isolates of BlCMV from Arkansas and Georgia and a non-stunt-associated isolate of CMV in the trifoliolate leaves: CMV strain LeJ accumulation.

Treatment	Mean absorbance (405 nm)		
	8 dpi	15 dpi	BlCMV effect
CMV strain LeJ	0.18	0.15	AB
CMV strain LeJ + $BlCMV_{AR}$	0.19	0.24	A
CMV strain LeJ + $BlCMV_{GA}$	0.15	0.10	B

Experiments were repeated twice with 6 plants/treatment and gave similar results. Same letters in column are not statistically different ($\alpha = 0.05$).

case of the single infection where CMV strain LeJ accumulation was greatly reduced at 15 dpi (1/5 of its value at 8 dpi), CMV strain LeJ accumulation in the mixed infections remained the same at both 8 and 15 dpi (**Table 7**). The combined day effect indicated that CMV strain LeJ accumulated significantly more at 8 than 15 dpi. The most striking difference between the effect of CMV strain LeJ and CMV strain VE 111 in the stems was that while CMV strain LeJ accumulation in the mixed infections with $BlCMV_{AR}$ or $BlCMV_{GA}$ remained the same between 8 and 15 dpi, CMV strain VE 111 accumulation in the mixed infections decreased during the same period.

At 8 and 15 dpi, in the trifoliolate leaves, CMV strain VE 111 in both mixed infections with $BlCMV_{AR}$ and $BlCMV_{GA}$ accumulated more than in the single infection (**Table 8**). Between 8 and 15 dpi, CMV strain VE 111 accumulation in the single infection decreased while in the mixed it remained almost the same. There was no day effect on CMV strain VE 111 accumulation in the trifoliolate leaves. The combined data however showed that there was a significant difference between CMV strain VE 111 accumulation in single versus mixed infections (**Table 8**).

In the case of CMV strain LeJ accumulation in the trifoliolate leaves, by looking at the data it appeared that at 8 dpi there was no difference CMV strain LeJ accumulation in the single versus mixed infections (**Table 9**). At 15 dpi however, the strain LeJ of CMV seemed to accumulate more in the mixed infection with $BlCMV_{AR}$ than the single and mixed infection with $BlCMV_{GA}$ (**Table 9**). The days after inoculation did not have an effect on CMV strain LeJ accumulation. The combined BlCMV effect indicated that CMV strain LeJ accumulation in the mixed infections was significantly different; however there were no significant differences between CMV strain LeJ accumulation in the single vs. mixed infection (**Table 9**).

DISCUSSION

In this study, we show that by combining stunt-causing isolates of BlCMV from Arkansas and Georgia with CMV isolates not previously associated with the stunt disease, inoculated cowpea plants showed characteristic cowpea stunt disease symptoms. The possibility exists that mixed infections involving even one virus which had already been associated with cowpea stunt disease is sufficient to induce stunt disease. *Cucumber mosaic virus* has a very wide host range, infecting more than 750 plant species in 365 genera and 85 families (Douine *et al*. 1979). The fact that CMV has such a wide host range and that cowpea stunt could occur with CMV isolates that had not previously been associated with the disease, show how great the chance of the disease occurrence is and therefore how important it is to get a better understanding of the synergistic interaction between these two viruses. Furthermore, it has been reported that in cowpea, mixed infections with two or more viruses exist in nature, with infections caused by two being the most prevalent (Shoyinka *et al*. 1997). Different combinations of double infections involving *Cowpea aphid-borne mosaic potyvirus* (CABMV), *Cowpea mottle carmovirus* (CMeV) and *Southern bean mosaic sobemeovirus* (SBMV), plants displayed more severe symptoms height reductions and yield losses were observed (Taiwo and Akinjogunla 2006; Kareem and Taiwo 2007).

One of the characteristics of synergistic interactions involving a potyvirus which is generally accepted is the enhancement of the non-potyvirus titer in the mixed infection whereas the potyvirus titer remains stable. Recently, Taiwo *et al*. (2007) showed that mixed infection in cowpea plants with CABMV and CMeV lead to stronger symptoms and an increase in the CMeV concentration. Our experiments were designed to test if these findings also apply to the case of mixed infections with stunt-causing isolates of BlCMV and CMV isolates not associated with cowpea stunt. In the trifoliolate leaves, CMV strain VE 111 accumulation increased in the mixed infection with $BlCMV_{AR}$ or $BlCMV_{GA}$ compared to the single infection. However, when CMV strain LeJ was mixedly inoculated with these two isolates of BlCMV, no enhancement of CMV accumulation was observed even though all plants mixedly infected showed similar cowpea stunt disease symptoms. This result not only shows the importance of the type of CMV involved, but also clearly indicates that the synergistic interaction with potyviruses cannot always be explained by the enhancement of the non-potyvirus titer in the mixed infection. Furthermore, the result agrees with findings in the mixed infections with the Arkansas isolates of CMV and BlCMV (Diallo 1998) where no increase in the CMV titer was observed at 8 dpi in the first trifoliolate leaves. Anderson *et al*. (1996) suggested that the rapid development of severe symptoms in plants doubly infected with CMV and BlCMV may not be due solely to the increase in CMV concentration. Indeed, they found that severe symptoms observed early during infection (5-10 days post-inoculation) was not always correlated with higher CMV concentration in the plant tissues tested.

In the stem, CMV strain VE 111 and CMV strain LeJ accumulations greatly increased in mixed infections with $BlCMV_{AR}$ and $BlCMV_{GA}$ when compared to the single infection, supporting previous findings that symptom severity in the synergistic interaction with potyviruses could be due to an increase in non-potyvirus titer (Rochow and Ross 1955; Calvert and Ghabrial 1983; Goldberg and Brakke 1987; Vance 1991; Vance *et al*. 1995). This result also shows the importance of testing different plant parts at different time periods in the cowpea stunt disease.

One interesting aspect of this study is that, in all four types of mixed infections, increase in symptom severity including stem necrosis at 8 dpi, was correlated to enhancement of CMV accumulation in the stems as in the case of the mixed infections with CMV_{GA} and either $BlCMV_{AR}$ or $BlCMV_{GA}$, and contrary to what found in the mixed infections involving CMV_{AR} (pers. comm.). The results clearly indicate that in the cowpea stunt disease, symptom severity in the stem is not solely due to an increase in the CMV accumulation, and that enhancement of CMV accumulation depends on the CMV isolate used in the study. The results this study and previous ones revealed that CMV accumulation patterns in the mixed infections varied with the virus isolates used and the plant parts tested. Therefore, for results in studies of synergistic interactions to be conclusive, several virus isolates need to be tested not only in leaves but also in other plant parts.

In the trifoliolate leaves, whereas the source of CMV did not have an effect on $BlCMV_{AR}$, it did have an effect on $BlCMV_{GA}$ accumulation. $BlCMV_{GA}$ only decreased in the mixed infection with CMV strain VE 111 compared to the

single infection. This result further supports the idea of specific interaction between viruses involved in the mixed infection. In the stems, $BlCMV_{AR}$ and $BlCMV_{GA}$ accumulation in the mixed infections were significantly lower than in the single infection, agreeing with previous findings (Diallo 1998).

In the cowpea stunt disease where accumulation patterns were also determined in the stems, it was found that in addition to the enhancement of CMV in the mixed infections, BlCMV accumulation decreased in the mixed infections. This is the first report of such phenomenon in the stems of plant mixedly infected. The fact that BlCMV accumulation decreased in the stems of all mixedly infected plants may or may not contribute directly to the overall increase in symptom severity. However, it is important to note that something other than the increase in the non-potyvirus accumulation may contribute to the enhancement of symptom severity, and this study showed that synergism occurs in the absence of an increase in the non-potyvirus accumulation.

Carr and Kim (1983) concluded that a possible explanation for the increase in symptom severity in synergistic interactions could be that either virus in the mixed infection or even both are able to invade cells which are not normally infected in the single infection. In their study it was shown that in the mixed infection with *Tobacco mosaic virus* (TMV) and *Bean golden mosaic virus* (BGMV) which is phloem-limited, more non-phloem cells became infected in the mixed infections. Barker (1987) also showed that although *Potato leafroll luteovirus* (PLRV) seemed to be restricted to phloem cells and a few parenchyma cells in *Nicotiana clevelandii*, more parenchyma cells became infected in the mixed infection with PVY. The possibility of that phenomenon occurring in the cowpea stunt disease needs to be investigated. Martin *et al*. (2004), showed different cytopathological structures are induced in mixed infections involving unrelated viruses. In the case of CMV and BlCMV, eight BlCMV encircle one CMV icosahedron, leading to an octagonal arrangement of what they refer to as mixed virus particle aggregates (MVPAs).

The research conducted here is applicable in Africa where cowpea constitutes an important food and fodder legume, at least in the sub-humid tropics. Similar studies have been conducted on cowpea viruses on the African continent even though the viruses under investigation in our study (CMV and BlCMV) are different from the ones reported in the mixed infections in cowpea (Taiwo and Akinjogunla 2006; Kareem and Taiwo 2007; Taiwo *et al*. 2007). Even though CMV and BlCMV have been found to infect cowpea in Nigeria, BlCMV had a low occurrence rate, while CMV had a localized importance (Taiwo 2003). Knowing how devastating the mixed infection with these two viruses was in the USA, efforts must be made to prevent them from coming together on cowpea plants.

CONCLUSIONS

The results of this study clearly show that cowpea stunt disease can occur even with CMV isolates that have not been previously involved in the disease. This is an important finding. Indeed, considering the wide host range of CMV, as well as its modes of transmission, this disease could cause great losses in cowpea production wherever this crop is grown. A management strategy usually recommended for all seed-borne viruses, is the used of virus-free seeds. However, a practical and economical way of controlling plant virus diseases is through the use of resistant varieties/cultivars if available. It is therefore necessary to develop through classical breeding, cowpea stunt-resistant cultivars that could be used by growers in their respective regions.

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