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Early Events in Cowpea Stunt Disease: Symptomatology, Virus Concentration and Localization

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ABSTRACT

Cowpea (*Vigna unguiculata* (L.) subsp. *unguiculata*) cv. 'Coronet' seedlings inoculated with crude extracts from plants infected with *Cucumber mosaic cucumovirus* (CMV), *Blackeye cowpea mosaic potyvirus* (BlCMV), and a mixture of both extracts were observed for symptom development over a period of time. By eight days after inoculation, plants doubly infected with CMV and BlCMV developed more severe foliar symptom in addition to stem and petiole necrosis, characteristic of cowpea stunt disease. A time-course enzyme-linked immunosorbent assay (ELISA) conducted showed that at 15 days post-inoculation, the concentration of the non-*Potyvirus* increased in the mixed infection. Surprisingly, during early stages of infection (between six and eight days after inoculation), although plants mixedly-infected with CMV and BlCMV displayed more severe symptoms than either single infection, no enhancement of CMV accumulation occurred in the plant parts tested (i.e., primary leaves, first trifoliolate and stems). Therefore, increased symptom severity in the early stage of cowpea stunt disease was not correlated with an increase in CMV accumulation. Light microscopical studies indicated that the necrosis in the mixed infection was internal (pith and medullary ray) as well as external (epidermis and cortex). Electron microscopy studies showed that in the stems of mixedly infected plants, CMV and BlCMV particles were present in both epidermal and cortical cells. Most cortical cells were collapsed and densely stained. Cells adjacent to the collapsed areas showed increased vacuolation with large numbers of BlCMV particles, an increase in the number of hypertrophied mitochondria and abnormal chloroplasts. The increase in symptom severity observed during early infection was not due to the viruses infecting different tissues in single versus mixed infection.

Keywords: ELISA, microscopy, stem necrosis, symptom severity, virus accumulation

INTRODUCTION

Mixed infections by related or unrelated viruses have been recognized in nature for more than 70 years (Dickson 1925). During this time, several plant diseases have been reported to be caused by a mixture of viruses (Clark *et al*. 1980; Khan and Demski 1982; Ross 1968; Uyemoto *et al*. 1981). Often in mixed infections, the viruses involved interact synergistically to produce more severe symptoms than in the case of diseases caused by single infections (Cho *et al*. 2002; Kassanis 1963; Untiveros *et al*. 2007). One of the characteristics of synergistic interactions involving a *Potyvirus* is the dramatic increase in the level of the non-*Potyvirus* in the mixed infection when compared to that of the single infection, and this virus enhancement has been correlated with enhancement of disease severity (Rochow and Ross 1955; Goodman and Ross 1974; Calvert and Ghabrial 1983; Goldberg and Brakke 1987; Vance 1991; Vance *et al*. 1995). In cowpea, Taiwo *et al*. (2007) showed that mixed infection with *Cowpea aphid-borne mosaic potyvirus* (CABMV) and *Cowpea mottle carmovirus* (CMeV) resulted in plants displaying stronger symptoms as well as an increase in the CMeV concentration.

Cowpea stunt, a severe disease of cowpea was found to be caused by a synergistic interaction between two viruses: *Cucumber mosaic virus* (CMV) and *Blackeye cowpea mosaic virus* (BlCMV) (Pio-Ribeiro *et al*. 1978). The diseased plants are stunted with malformed leaves and stem and petiole necrosis. A study was conducted on cowpea stunt disease by Anderson *et al*. (1996) to determine if resistance to BlCMV based on visual symptoms was correlated with decrease in BlCMV accumulation and protection from cowpea stunt disease. It was found that on the inoculated leaves, at 5 days post-inoculation (dpi), even though symptoms were not very severe, CMV accumulation in the mixed infection was slightly higher than in the single infection. At 10 dpi however, no significant difference was observed in CMV accumulation in the inoculated leaves between the single and mixed infections although the plants displayed stronger symptoms. At 15 dpi when symptoms began to fade, CMV accumulation in both single and mixed infections declined but no significant difference was observed. Looking at the second trifoliolate leaves, Anderson *et al*. (1996) reported that, although CMV accumulation increased in the mixed infection, the difference between CMV accumulation in the single and mixed infections was not significant at 10 dpi. A significant difference in CMV accumulation between single and mixed infections was observed later during infection (15-20 dpi). It was concluded from that study that symptom severity observed in mixedly infected plants may not be due solely to increased CMV concentrations.

In another study conducted on cowpea stunt disease in Arkansas, it has been shown that, although plants mixedly infected with CMV and BlCMV developed very severe symptoms during early infection, CMV accumulation in the mixed infection was not significantly different from that of the single infection in the inoculated primary leaves, the first trifoliolate leaves as well as the stems (Diallo 1998). This result suggests that something other than the increase in CMV accumulation is responsible for the severe symptoms observed in the mixed infection. Similar findings were reported in the case of the synergistic interaction between *Cowpea chlorotic mottle comovirus* (CCMV) and the cowpea strain of *Southern bean mosaic sobemovirus* (SBMV),

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where despite the strong symptoms of the mixedly infected cowpea plants, no difference was found in SBMV and CCMV nucleoprotein concentration in single versus mixed infections (Kuhn and Dawson 1973). Kuhn and Dawson (1973) also suggested that viral infections may affect metabolic events which would then have an effect on the plant. Similar conclusions were also reached in other mixed infections causing necrosis (Lee and Ross 1972; Ross 1968). The possibility exists that in the early stages of the mixed infection, even though CMV accumulation was not higher than in the single infection, CMV or BlCMV or both viruses may have been able to infect cells which are not normally infected in the single infection. Carr and Kim (1983) demonstrated that in the mixed infection between *Bean golden mosaic geminivirus* (BGMV), a virus restricted to the phloem, and *Tomato mosaic tobamovirus* (TMV), BGMV was able to invade non-phloem tissue. Barker (1987) showed that in the mixed infection with *Potato leafroll luteovirus* (PLRV) and *Potato Y potyvirus* (PVY) in *Nicotiana clevelandii*, there was an increase in the proportion of leaf parenchyma protoplasts infected with PLRV when compared to the single infection where PLRV is normally phloem limited.

In mixed infections with unrelated viruses it has been shown that both viruses could be found within the same cell. Lee and Ross (1972) reported that *Soybean mosaic potyvirus* (SMV) inclusions and *Bean pod mottle comovirus* (BPMV) particles were found in the same cells of soybean plants.

Since symptom severity in the cowpea stunt disease occurs during early disease development (6-10 dpi), we decided to look more at the early events in the synergistic interaction between CMV and BlCMV. The objectives of this research were: 1) to determine the relationship between symptom severity during early cowpea stunt disease development and the accumulation of CMV and BlCMV in the stems which develop some necrosis, in the inoculated leaves, and also in the first trifoliolate leaves which are the only one present during this time period; and 2) to investigate the cause of stem and petiole necrosis occurring during early stages of the mixed infection by CMV and BlCMV.

MATERIALS AND METHODS

Virus and seed sources

The Arkansas isolates of cowpea stunt-associated CMV and BlCMV collected from a field in Columbia County, Arkansas in 1993 and separated into pure cultures (Anderson *et al*. 1994) were used in this study. Pure cultures of both viruses were maintained in the cowpea cultivar 'Coronet' (Brantley 1976). Seedlings of 'Coronet' were grown in the greenhouse in 7.5 cm clay pots containing Redi-Earth potting mixture (Grace Sierra, Milpitas, CA). The greenhouse temperature ranged from 20 to 24°C. Experiments were all conducted at the University of Arkansas.

Plant inoculation

At approximately eight days after planting, the cowpea plants with fully expanded primary leaves were separated into four groups of 18 plants and dusted with carborundum (silicon carbide powder, grit 600; Buehler, Ltd., Lake Bluff, IL). The first group was inoculated with a crude extract from healthy Coronet cowpea leaves (control) prepared in 0.05 M phosphate buffer (pH 7.2). For the single-virus inoculations, the second and third groups of plants were inoculated with sap extracts from CMV-infected plants mixed with sap from healthy Coronet cowpea in ratio 1:1 (v/v) and BlCMV-infected plants mixed with sap from healthy Coronet cowpea in ratio 1:1 (v/v), respectively. The fourth group of cowpea plants was inoculated with a mixture of equal volumes of sap extracts from plants singly infected with CMV and BlCMV. Sap from infected cowpea plants were extracted in 0.05 M phosphate buffer (pH 7.2). Inoculation was done on the two primary leaves of each plant by gently rubbing the inoculum on the upper leaf surface. The plants were randomized on greenhouse benches. The greenhouse conditions were: 20 to 24°C and supplemental lighting to maintain a photoperiod of 14 h. The experiment was repeated three times.

Time course study of CMV and BlCMV accumulation in inoculated leaves, systemically infected leaves and stems

At different times after inoculation starting at day three and ending at day 15, plants were observed daily for symptom development. Inoculated leaves and stem portions between the primary leaves and the soil level were harvested at days 0, 3, 4, 6, 7, 8, 9, 10 and 15. The first trifoliolate leaves were harvested at days 6, 7, 8, 9, 10 and 15. Sap was extracted from inoculated primary leaves, first trifoliolate leaves and stems using a tissue extractor (Erich Pollahne Co., Wennigsen, West Germany), and frozen at -70°C. The experiment was repeated three times.

Relative virus accumulation in the different plant parts was determined using a modification of the indirect enzyme-linked immunosorbent assay (ELISA) procedure described by Bashir (1992). Samples were thawed and diluted 1:50 in antigen buffer [phosphate-buffered saline (PBS), pH 7.4, 13 mM diethyldithiocarbamic acid]. Diluted samples were added to duplicate wells (200 µl/well) of microtiter plates (Immulon I, Dynatech Laboratories, Inc., Chantilly, VA) and incubated for 2 h at 37°C. The polyclonal antibodies to CMV (obtained from R.O. Hampton, Oregon State University) and BlCMV were pre-absorbed with healthy cowpea sap (1:50 dilution of sap in virus buffer (PBS, pH 7.4, 0.1% Tween-20, 2% polyvinylpyrrolidone (molecular weight 360,000) (PVP-360), 0.2% bovine serum albumin) at 37°C for 1 h and then incubated at 37°C for another hour. Plates were washed three times for 3 min each in washing buffer (PBS-Tween). The pre-absorbed antisera were added to each well (200 µL/well) and the plates were incubated for 2 h at 37°C. After washing three times for 3 min each with washing buffer, anti-rabbit alkaline phosphatase conjugated IgG (Sigma, St. Louis, MO) diluted 1:10,000 in virus buffer was added to the wells (200 µL/well) and the plates were incubated for 2 h at 37°C. The plates were washed again and 200 µL of substrate buffer (10% diethanolamine, 0.02% sodium azide, pH 9.8) containing *p*-nitrophenyl phosphate (Sigma, St. Louis, MO) at a concentration of 0.33 mg/ml was added to each well. The plates were incubated for 20 min at room temperature and the absorbance values at 405 nm were determined using a microplate reader (model 7500, Cambridge Technology Inc., Watertown, MA).

The relationship between the relative CMV and BlCMV concentrations and days after inoculation was determined by fitting the linear and quadratic models to the data for CMV and BlCMV separately. The analysis of covariance techniques were used to determine if the coefficients in the regression models depended on the type of infection (single and mixed) or plant parts (inoculated primary leaves, first trifoliolate leaves and stems) or both. Single degree of freedom contrasts were used to determine significant differences among coefficients where appropriate.

Plant materials and sap inoculation

'Coronet' plants at the primary leaf stage were inoculated with CMV, BlCMV and CMV + BlCMV. For the single virus inoculation, the inoculum consisted of equal volumes of sap from virus-infected plants and sap from healthy plants. For the mixed infection the inoculum was made with equal volumes of sap from plants infected singly with CMV and BlCMV. Plants inoculated with healthy sap were used as controls. Plants were harvested 8 days after inoculation and processed for light and electron microscopy studies.

Viral infection and stem elongation

The stem portion below the primary leaves of cowpea plants at the primary leaf stage was marked in intervals of 0.6 cm each. Plants were then divided into four groups and each group was inoculated with one of the following virus treatments: CMV, BlCMV, CMV + BlCMV, and healthy sap. Eight days after inoculation, each interval on each plant stem was measured to determine any increase in

length. Interval increase will be compared between treatments to determine if virus infection has an effect on stem elongation. The data were analyzed as a split plot in which the whole plot structure was a randomized complete block with a 2 X 2 factorial treatment structure (presence-absence of CMV and presence-absence of BlCMV). Eight replications per treatment combination were used in each block. The split plot was positioned on the stem. The least significant difference test (LSD) at 0.05 was used to separate the means where appropriate.

Light microscopy

Hand-cut sections (approximately 0.5 mm) of stems (below the primary leaves) and petioles from plants infected with CMV, BlCMV and CMV + BlCMV, in addition to mock-inoculated plant stems were fixed in 2% paraformaldehyde. The unstained sections were mounted on a glass slide with a drop of glycerol and examined under a light microscope (Model BHT, Olympus Optical Instruments, Co., Tokyo, Japan) for gross localization of stem and petiole necrosis.

Electron microscopy

Four to five stems from plants infected with CMV, BlCMV and CMV + BlCMV along with mock-inoculated plant stems were excised (1-2 mm width) , fixed for a few days in a modified Karnovsky's fixative (Kim and Fulton 1984) consisting of 2% glutaraldehyde and 2% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.2. The tissues were washed twice with 0.05 M cacodylate buffer (pH 7.2) and post-fixed in 1% osmium tetroxide in cacodylate buffer for 2 h. Tissues were *en bloc* stained in 0.05% aqueous uranyl acetate at 4°C overnight and dehydrated in a series of ethanol and propylene oxide. The tissues were embedded in Spurr's low viscosity medium (Spurr 1969) and allowed to polymerize overnight at 70°C. Thin sections cut with a diamond knife and stained with uranyl acetate and lead citrate, were examined under a JEOL-100 CX transmission electron microscope. The experiment was repeated twice.

RESULTS

Symptom development

'Coronet' seedlings inoculated with CMV, BlCMV or a mixture of both CMV and BlCMV were observed for symptom development for 15 days after inoculation. Starting at 3 days after inoculation, plants inoculated with either CMV alone or a mixture of CMV and BlCMV showed chlorotic lesions on the primary leaves while there were no lesions on BlCMV-infected plants. Between 6 and 8 days after inoculation, plants infected with BlCMV showed chlorotic lesions on the primary leaves (**Fig. 1B**) while CMV-infected plants expressed vein clearing and mottling on the first trifoliolate leaves (**Fig. 1C**). At that same time period, plants infected with both CMV and BlCMV showed more severe symptoms than plants inoculated with either virus alone. The first trifoliolate leaves of these mixedly-infected plants were small, blistered and malformed while petioles and stems showed necrosis and were somewhat flattened (**Fig. 1D**) when compared to mock-inoculated healthy controls (**Fig. 1A**). The necrosis observed on stems and petioles was also present in tissues inside the stem (**Fig. 1D**).

After day eight, CMV symptoms on the first trifoliolate leaves became milder than those observed at earlier times. In contrast, BlCMV-infected first trifoliolate leaves continued to express strong symptoms after 8 days. Mixedly-infected plants also displayed stronger symptoms at 8 days post-inoculation than at earlier times and were stunted when compared to the singly-infected plants, and stem and petiole necrosis was also more pronounced.

Relative virus accumulation in inoculated primary leaves

A time course ELISA was conducted to evaluate the relative

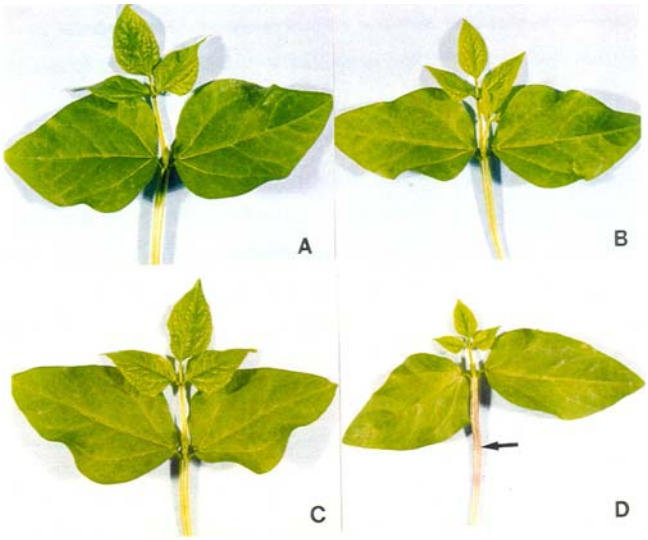

Fig. 1 Symptoms of single and mixed infections with CMV and BlCMV in 'Coronet' cowpea. (**A**) Healthy cowpea; (**B**) BlCMV-infected cowpea; (**C**) CMV-infected cowpea; (**D**) Cowpea infected with CMV and BlCMV.

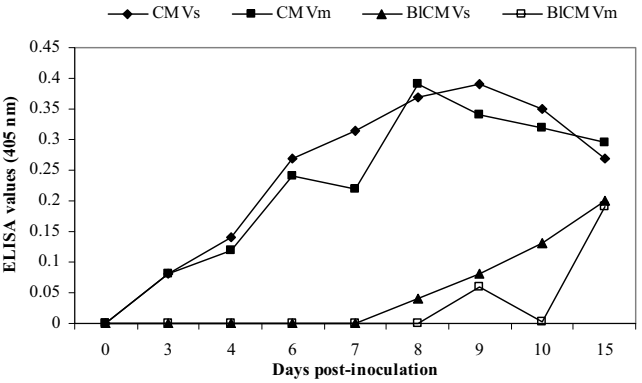

Fig. 2 Relative accumulation of CMV and BlCMV over time in primary leaves of 'Coronet' cowpea singly (s) and mixedly (m) infected. The experiment was repeated three times with nine plants/treatment each time.

CMV and BlCMV accumulation in relation to symptom development. Three days after inoculation, CMV was detected at low concentrations in plants inoculated with CMV alone and those mixedly inoculated with CMV and BlCMV (**Fig. 2**). There was no difference between relative CMV concentrations in single compared to mixed infections. BlCMV was not detected in either single or mixed infections at 3 days.

Four days after inoculation, even though CMV concentrations in single and mixed infections increased slightly when compared to CMV concentrations at 3 days post-inoculation, no difference was observed between CMV concentrations in single and mixed infections (**Fig. 2**). BlCMV was not detectable by ELISA in both single and mixed infections at 4 days post-inoculation.

From days four to eight after inoculation there was almost a four-fold increase in ELISA values representing CMV in both singly- and mixedly-inoculated plants (**Fig. 2**). There was no significant difference in CMV concentration at 8 days post-inoculation between single and mixed infections. BlCMV in the single and mixed infections was not detected until 8 days after inoculation and the accumulation level was very low.

Between 9 and 15 days post-inoculation, CMV concentration decreased slowly until day 15 in both the single and the mixed infections. No difference between CMV concentrations in single and mixed infection was observed for this time interval. BlCMV concentration in the single infection continued to increase until 15 days after inoculation. In con-

trast, BlCMV concentration in the mixed infection remained low until day 10 and then rapidly increased (20-fold) by day 15 after inoculation (**Fig. 2**). By 15 days post-inoculation, relative BlCMV accumulation in the single and mixed infections were almost identical. This experiment was replicated three times with similar result.

Relative virus accumulation in the first trifoliolate leaves

At 6 days after inoculation, relative CMV concentrations in the mixed infections were slightly higher than in the single infections (**Fig. 3**). These concentrations increased slightly from day six to day seven and then more rapidly from day seven to day eight. Up to 8 days after inoculation, there was no difference between CMV concentrations in both single and mixed infections.

After 8 days post-inoculation, the CMV concentration in both single and mixed infections decreased, more slowly in the case of the single infection (**Fig. 3**). Between 10 and 15 days after inoculation, CMV concentration in the mixed infection remained almost stable while a more rapid decrease occurred in the case of the single infection. Thus, by 15 days post-inoculation, the ELISA values for CMV in the mixed infections were approximately twice that of the single infections.

The relative BlCMV concentration in the single infection increased steadily from 8 to 15 days after inoculation while in the mixed infection the concentration stayed low until 10 days after inoculation before reaching a high concentration at 15 days after inoculation (**Fig. 3**).

Relative virus accumulation in stems

CMV was not detected in stems in either single or mixed infections until 6 days after inoculation (**Fig. 4**). Between 6 and 8 days after inoculation, CMV concentrations increased rapidly in the mixed infection, and more slowly between 8 and 10 days after inoculation. BlCMV was detected in both single and mixed infections starting at 8 days after inoculation. The BlCMV concentration increased until 15 days after inoculation with no significant difference between single and mixed infections. Between 10 and 15 days after inoculation, CMV concentrations decreased in both single and mixed infections, but with a more rapid decrease in single infections, resulting in lower concentrations in the single infections as was the case with the first trifoliolate leaves (**Fig. 4**).

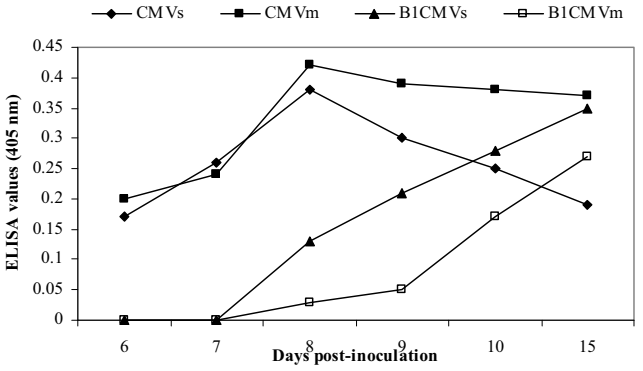

Fig. 3 Relative accumulation of CMV and BlCMV over time in trifoliolate leaves of 'Coronet' cowpea singly (s) and mixedly infected (m). The experiment was repeated three times with nine plants/treatment each time.

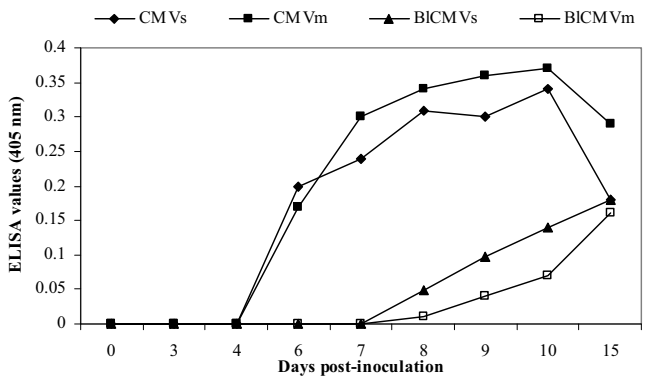

Fig. 4 Relative accumulation of CMV and BlCMV over time in stems of 'Coronet' cowpea singly (s) and mixedly (m) infected. The experiment was repeated three times with nine plants/treatment each time.

Relationship between the relative virus concentrations, the type of infection and the number of days after inoculation

For CMV, the relative virus concentration was a quadratic function of days after inoculation in which only the constant terms depended on the plant part tested (primary leaves, first trifoliolate leaves and stems) (**Table 1**). Since CMV accumulation did not depend on infection type, data for single and mixed infections were combined for this analysis. The intercepts for stems and trifoliolate leaves were not significantly different but, the intercept for the primary leaves was significantly higher than that of the stems and the first of trifoliolate leaves (**Table 1**). This means that CMV accumulation over time was much greater in the primary leaves than the first trifoliolate leaves and stems which have similar accumulation patterns.

For BlCMV, the relative virus concentration was a linear function of days after inoculation in which only the constant terms depended on both the infection type (single and mixed) and the plant part tested (**Table 2**). Within each plant part, the intercepts for single and mixed infections were significantly different, with the intercepts for single infections higher than those of the mixed infections (**Table 2**). This means that BlCMV accumulation over time in the single infections was higher than in the mixed infections. Within each infection type, the intercepts for primary leaves and stems were not significantly different but were significantly lower than the intercept for the trifoliolate leaves (**Table 2**). With regard to infection type, this indicates that BlCMV accumulated over time in the primary leaves and stems at similar levels but that the accumulation level was lower than that of the trifoliolate leaves.

Table 1 Relationship between relative CMV concentrations in primary leaves, first trifoliolate leaves and stems and number of days after inoculation.

	Regression coefficients		
	Constant	**Days**	**Days^2**
Primary leaves	-0.250 ± (0.050)* a	0.119 ± (0.011)	-0.06 ± (0.001)
Trifoliolate leaves	-0.289 ± (0.056) b		
Stems	-0.295 ± (0.056) b		

Standard errors are given in parentheses. Constant terms followed by the same letter are not significantly different at p = 0.05. The coefficients for days and days^2 are the same for all plant parts.

Table 2 Relationship between relative BlCMV concentrations, infection types and plant parts.

Infection type	**Plant part**	**Regression coefficients**	
		Intercept	**Slope**
Mixed	Primary leaves	-0.240 ± (0.031)* a	-0.027 ± (0.002)
	Stems	-0.224 ± (0.030) a	
	Trifoliolate leaves	-0.149 ± (0.030) c	
Single	Primary leaves	-0.180 ± (0.030) b	
	Stem	-0.164 ± (0.029) b	
	Trifoliolate leaves	-0.089 ± (0.029) d	

Standard errors are given in parentheses. Intercepts followed by the same letter are not significantly different at p = 0.05. The slope is the same for all infection types and plant parts.

Microscopy study of stem and petiole

Symptoms description

Uninfected plants and plants infected singly with either BlCMV or CMV did not develop apparent necrosis in stems (**Figs. 1A-C**). Stems of the mixedly-infected plants however showed necrosis (**Fig. 1D**).

Stem elongation

To determine whether or not mixed virus infection affected the elongation of the stem below the primary leaves since part of that area became necrotic, stems of young cowpea plants, after being marked at regular 0.6 cm intervals and then inoculated with CMV, BlCMV, and CMV + BlCMV, were measured after an incubation period of 8 days in the greenhouse to detect any increase in length (**Fig. 5A-D**). The measurements for the first five intervals on the cowpea stem (below the primary leaves from top to bottom) were recorded. These intervals will be referred to as intervals A-E, A being the topmost interval. The largest increase in the stem was observed in the stem portion closest to the primary leaves (interval A). Going down the stem, the increase in the interval became less and less, with no increase in the stem portions below the cotyledons and a few cm above the cotyledon (**Table 4**). The increase in length in different stem sections for each treatment is shown in **Table 3**. Analysis of the data indicated that BlCMV had no effect on stem elongation, while CMV did have an effect (**Table 4**). There was a significant difference in intervals A-C between CMV-infected plants (CMV singly and mixedly infected) and plants not infected with CMV (healthy and BlCMV-infected plants) (**Table 4**). No difference in elongation was found in interval D among all treated plants. It is important to note that intervals A-D where an increase was observed (for all treatments) corresponded to the area where necrosis occurred in the stems of mixedly infected plants.

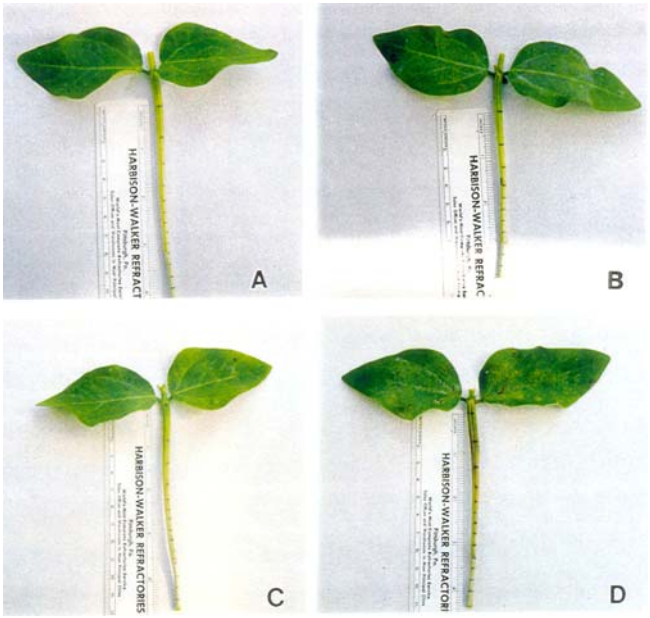

Fig. 5 Effect of different virus treatments on stem elongation eight days after inoculation. Control plant (**A**); BlCMV-infected plant (**B**); CMV-infected plant (**C**); plant mixedly infected with CMV and BlCMV showing necrosis in the first four intervals (**D**).

Table 3 Effect of virus infection on stem elongation (cm).

Treatment	Stem intervals[a]				
	A	B	C	D	E
Healthy	2.56	1.56	1.18	0.83	0.60
BlCMV	2.30	1.48	1.08	0.82	0.60
CMV	2.06	1.36	1.06	0.88	0.60
CMV+BlCMV	2.01	1.42	1.04	0.79	0.60

[a]The first five intervals on each stem were designated intervals A through E starting at the top of the stem below the primary leaves.
The experiment was repeated twice with a total of 16 plants/treatment. Values in the table represent the mean obtained from the 16 plants in both experiments.

Table 4 Effect of CMV infection on stem elongation (cm).

Position on stem	CMV	
	Absence[a]	Presence[b]
A	2.43	2.03
B	1.52	1.39
C	1.19	1.05
D	0.82	0.83

The first five intervals on each stem were designated intervals A through E starting at the top of the stem below the primary leaves. Values in the table represent the mean obtained from 32 plants in both experiment.
[a]Absence = healthy and BlCMV infected plants.
[b]Presence = CMV singly and mixedly infected plants.
LSD to compare position at same level of CMV = 0.12.
LSD to compare elongation at different level of CMV = 0.14.

Localization of stem necrosis (thick sections)

In order to determine the localization of the necrotic areas in stems of the mixedly infected plants, approximately 0.5 mm cross sections of stems (below the primary leaves) made from plants infected with CMV, BlCMV and CMV + BlCMV, were observed with a light microscope. Sections of stems from healthy plants were included as a control. Sections of BlCMV-infected stems did not show any necrosis (**Fig. 6A**), therefore were similar to the sections from the healthy stem sections (Data not shown). Most CMV-infected stem sections did not develop necrosis, and thus were similar in appearance to the BlCMV-infected or the healthy sections (**Fig. 6A**). Occasionally, the stems of CMV-infected plants did become necrotic. These necrotic areas had a brownish appearance and individual cells were not distinguishable. Sections of these stems showed that cells affected by the necrosis were limited to the pith and the medullary ray cells (**Fig. 6B**). Since this type of necrosis is located in the interior of the stem, it will be referred to as "internal necrosis". Sections of plant stems mixedly infected with CMV and BlCMV not only developed internal necrosis similar to those in CMV-infected stems, but also showed necrosis in the epidermis and cortex of the stem (**Figs. 6B, 6C**). This type of necrosis will be referred to as "external necrosis". CMV and BlCMV in the single infections did not cause any necrosis in the petiole (data not shown). Mixed infection with these two viruses, however, caused only internal necrosis in the petiole (data not shown).

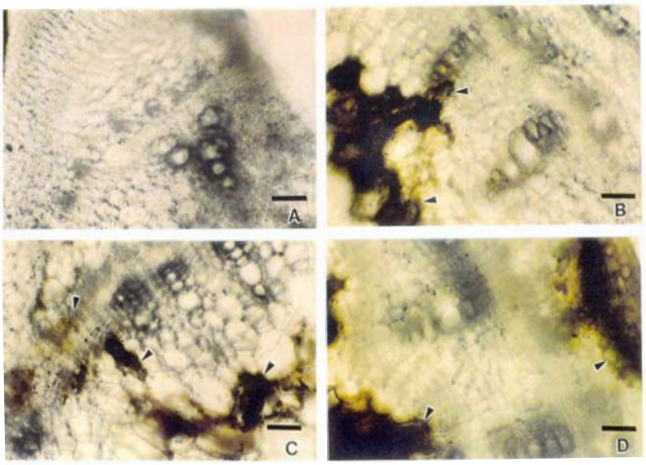

Fig. 6 Localization of necrosis in thick sections of cowpea stems. Stem of healthy or BlCMV- or most CMV-infected stem sections (**A**); stem of CMV-infected plants exhibiting occasional internal necrosis (**B**); stem of mixedly infected stem showing internal necrosis (**C**); stem of mixedly infected plant showing not only internal necrosis but also external necrosis in the epidermis and cortex (**D**). Arrowhead indicates necrotic areas. Bar = 115 µm.

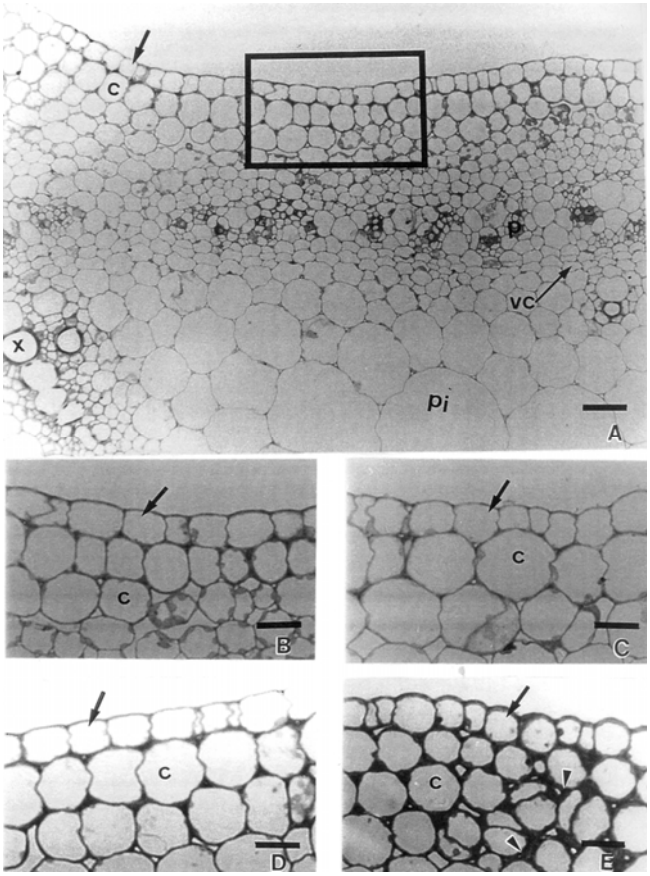

Fig. 7 Light micrographs of semi-thin sections of cowpea stem with emphasis on the epidermis and cortex. Semi-thin section of stem from a healthy plant showing the normal arrangement of cells as well as their structure at low magnification (bar = 160 μm) (**A**); higher magnification of the boxed area in A showing epidermal and cortical cells of control stem (**B**); semi-thin section of BlCMV-infected stem similar to control (**C**); semi-thin section of CMV-infected stem similar to control (**D**); semi-thin section of stem mixedly infected with CMV and BlCMV showing intercellular spaces filled with densely stained material (**E**). Arrow indicates epidermal cells. vc, vascular cambium; c, cortical cells; pi, pith; x, xylem. Arrowhead indicates necrotic cell. Bar = 80 μm for B, C, D, and E.

Light microscopy study of semi-thick sections of stems: epidermis and cortex

All epidermal and cortical cells of stems from BlCMV-infected stems (**Fig. 7C**) appeared similar to the cells in the healthy cowpea stem (**Figs. 7A, 7B**). BlCMV seemed to have no effect on the structure of these cells. Infection by CMV alone also did not have any effect on the epidermal and cortical cell structure (**Fig. 7D**). In the mixed infection, however, these two types of cells were somewhat malformed when compared to singly infected or healthy stems (**Figs. 7E, 8**). The cell walls of these cells appeared thicker and the intercellular spaces were filled with densely stained material.

Light microscopy study of semi-thick sections of stems: vascular cambium

No apparent malformation was observed in the vascular cambium cells of BlCMV-infected stems (**Fig. 9B**) which appeared similar to the vascular cambium cells of the healthy control (**Fig. 9A**). However, the vascular cambium cells of CMV and mixedly infected stems were malformed, irregular and compressed (**Figs. 9C, 9D**). No apparent difference was found in the vascular cambium cells between CMV and mixedly infected stems.

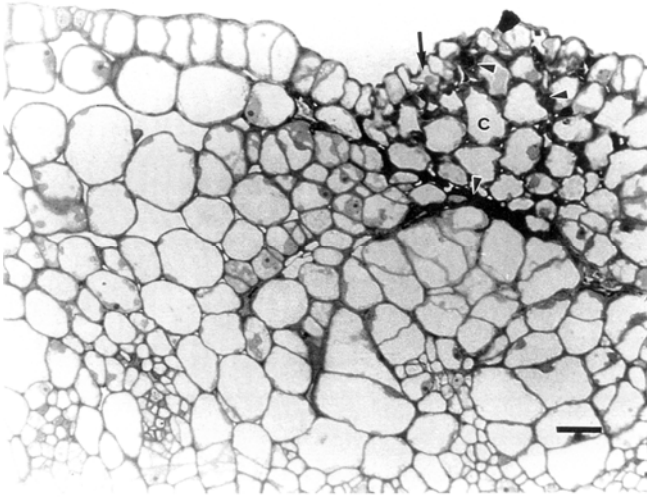

Fig. 8 Light micrograph of semi-thin section of cowpea stem from plants mixedly infected with CMV and BlCMV. Collapsed epidermal and cortical cells showing intercellular spaces filled with densely stained material (arrowhead). Arrow indicates epidermal cells. c, cortical cells. Bar = 80 μm.

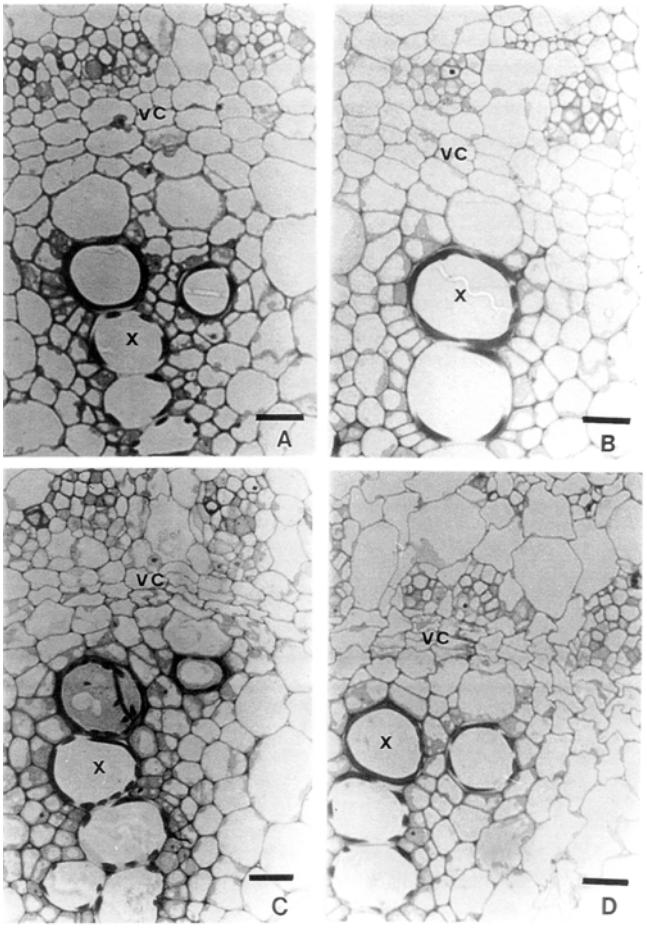

Fig. 9 Light micrographs of semi-thin sections of cowpea stem with emphasis on the vascular cambium. Control cambium (**A**); stem from BlCMV-infected plant showing intact cambium (**B**); stem from CMV-infected plant showing malformed and compressed vascular cambium cells (**C**); stem from plants mixedly infected with CMV and BlCMV also exhibiting malformed and compressed vascular cambium cells (**D**). vc, vascular cambium; x, xylem. Bar = 80 μm.

Light microscopy study of semi-thick sections of stems: medullary ray

No malformation or irregularity was observed in the medullary ray cells of stems from plants infected with BlCMV

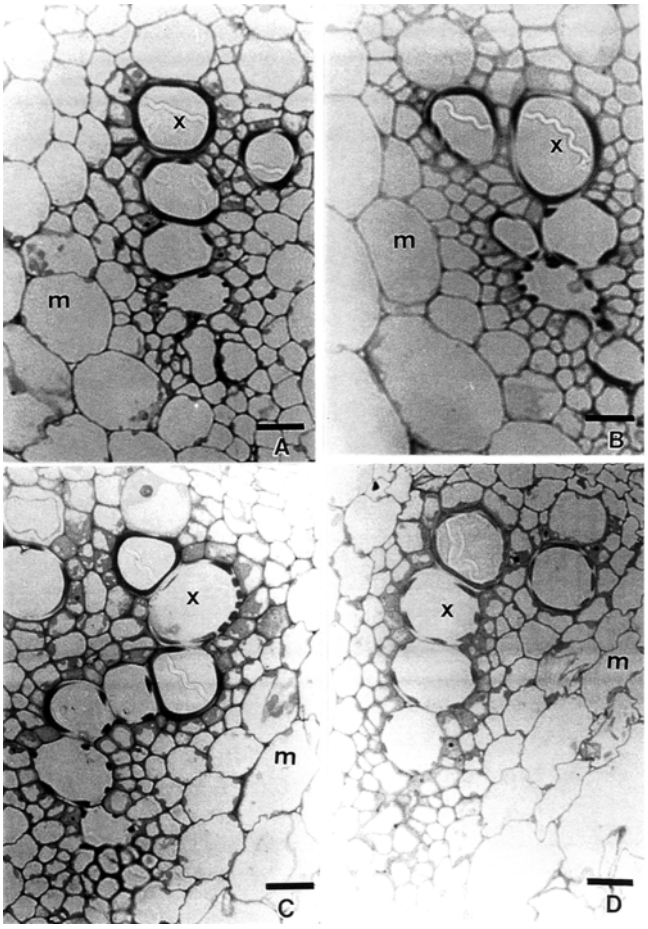

Fig. 10 Light micrographs of semi-thin sections of cowpea stem with emphasis on the medullary ray. Healthy control with normal medullary ray (**A**); stem from BlCMV-infected plant with normal medullary ray (**B**); stem from CMV-infected plant with malformed medullary ray cells (**C**); stem from a plant mixedly infected with CMV and BlCMV showing malformed medullary ray cells (**D**). m, medullary ray; x, xylem. Bar = 80 µm.

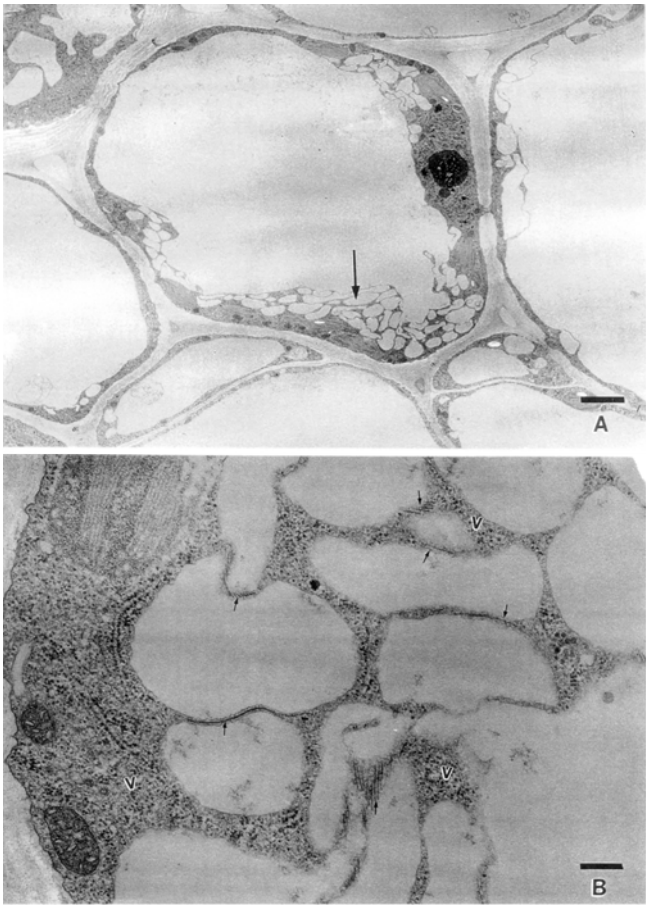

Fig. 11 Transmission electron micrographs of ultrathin sections of cortical stem cells from a plant mixedly infected with CMV and BlCMV. Cortical cell exhibiting abnormal vacuolation (bar = 2.5 µm) (**A**); vacuoles in cortical cell associated with BlCMV and CMV particles (bar = 312 nm) (**B**). Large arrow indicates vacuole; small arrow, BlCMV particles; v, CMV particles.

(**Fig. 10B**) compared to healthy plants (**Fig. 10A**). Medullary rays cells in stems of plants infected with CMV and plants mixedly infected with CMV and BlCMV showed some irregularity and malformation in their structure (**Fig. 10C, 10D**). They appeared compressed. No apparent differences were found in medullary rays of plants infected with CMV and plants infected with CMV and BlCMV.

Electron microscopy of epidermal and cortical cells

Epidermal and cortical cells of the stems of plants singly infected with either CMV or BlCMV contained particles of respective viruses, indicating that these cells are infected (data not shown). Cells infected with BlCMV also contained potyvirus characteristic pinwheel inclusions (data not shown). Cortical cells of mixedly infected stems exhibited extensive vacuolation (**Fig. 11A, 11B**). The cytoplasmic strands bordering these vacuoles were mostly composed of BlCMV particles (**Fig. 11B**). CMV particles were also found in the same cells. Electron-dense aggregates were observed in the cytoplasm of many cortical cells in the stems of mixedly infected plants (**Fig. 12A, 12B**). In these cells, large numbers of mitochondria were found (**Fig. 12A, 12B**) along with disrupted or malformed chloroplasts (**Fig. 12A**). Large numbers of cortical cells, especially those above the vascular bundles, were collapsed (data not shown). These collapsed cells appeared compressed, forming a layer of tightly packed necrotic cells. The cytoplasm of these cells appeared electron-dense with the organelles no longer distinguishable. These collapsed cells corresponded to the cells showing necrosis in the light microscopy studies. Vacuoles in necrotic areas contained dense material composed of pinwheel inclusions and CMV particles (data not shown). CMV and BlCMV particles were found in the phloem and medullary ray cells of stems from plants singly and mixedly infected.

DISCUSSION

Mixed infections with related or unrelated viruses are not uncommon in nature. These mixed infections are often characterized by an increase in symptom severity which is related to an increase in the concentration of one of the viruses involved. Examples of such mixed infections include the synergistic interactions between *Bean pod mottle virus* and *Soybean mosaic virus* (Calvert and Ghabrial 1983), *Potato viruses X* and *Y* (Rochow and Ross 1955; Goodman and Ross 1974), *Maize chlorotic mottle* and *Maize dwarf mosaic viruses* (Goldberg and Brakke 1987) and *Sweet potato chlorotic stunt virus* (*Crinivirus*) with Carla-, Cucumo-, Ipomo- and Potyviruses (Untiveros *et al*. 2007). Contrary to previous reports on the enhancement of the non-*Potyvirus* in the synergistic interactions involving a *Potyvirus*, our experiments showed that in the case of early cowpea stunt disease (6-8 days post-inoculation), the accumulation of CMV in single infections and in all the tissues tested (inoculated primary leaves, first trifoliolate leaves and stems) was not significantly different from that of the mixed infections. The accumulation of CMV was therefore not directly correlated with the increase in symptom severity but probably occurred only after increased symptom severity. Although

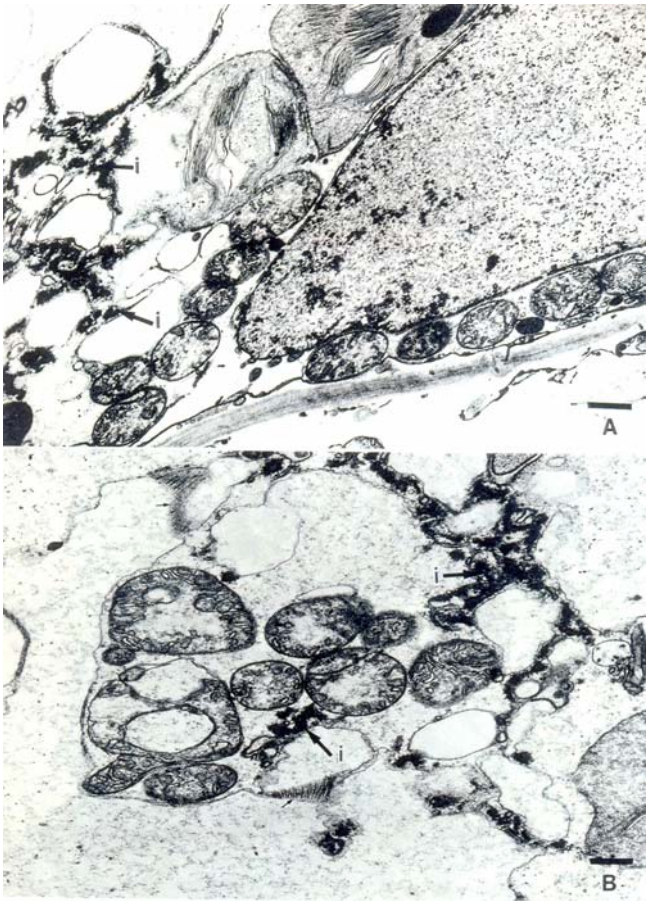

Fig. 12 Transmission electron micrographs of ultrathin sections of cortical stem cells from a plant infected with CMV and BlCMV. Stem cell exhibiting increased number of mitochondria, virus-specific inclusions (i), and disrupted chloroplasts (bar = 600 nm) (**A**); stem cell with virus-specific inclusions (i) and several mitochondria of which a few are vacuolated (bar = 500 nm) (**B**). Small arrow indicates BlCMV particles.

CMV concentration in mixedly infected stems and trifoliolate leaves was higher than that of singly infected plants at 15 days post-inoculation, that difference was not significant in this study. Anderson *et al*. (1996), in a study looking at cowpea stunt-associated virus accumulation, reported that in the inoculated primary leaves, at 5 days post inoculation, the relative CMV concentration was significantly higher in the mixed infection compared to the single infection. However, between 5 and 10 days post inoculation, CMV accumulation in both single and mixed infections remained almost at the same level and then decreased after 10 dpi with still no significant difference observed. In the present paper, CMV accumulation levels in both single and mixed infections continued to increase until day eight after inoculation and then decreased, and most importantly, no significant difference was observed at any time. Despite the slight difference in the accumulation pattern in both studies, the conclusions were in general the same: there was no statistical difference between CMV accumulation in the single and mixed infections.

Regarding the trifoliolate leaves, Anderson *et al*. (1996) found that there was an increase in CMV accumulation in the mixed infections between days 10 and 15 after inoculation, and only a slight decrease from days 15 to 20 while in the single infections, CMV accumulation decreased from days 10 to 20. Significant differences were observed at 15 and 20 days after inoculation, but not at day 10. In this paper, however, CMV accumulation in both single and mixed infections increased from 6 to 8 days after inoculation and then decreased thereafter, slightly for the mixed infections while more pronounced in the single infections. Interestingly, no significant differences were observed in the relative CMV concentrations between single and mixed infections at any time. The results obtained up to 10 days after inoculation agree with reports by Anderson *et al*. (1996) in the sense that there were no significant differences between CMV accumulation in the single and mixed infection during that time period. However, the big difference between the present study and that reported by Anderson *et al*. (1996) is that in our study no significant differences were observed in CMV accumulation at 15 days post inoculation (dpi) in the singly and mixedly infected plants. The difference in results could be explained by the fact that different types of leaf samples were used for the 15 dpi sampling. Whereas in the study by Anderson *et al*. (1996) the systemically-infected leaves tested were the second set of trifoliolate leaves, this study used the first trifoliolate leaves since the objective of the study was to focus mostly on the early events of the cowpea stunt disease. Although no difference was observed in the CMV accumulation between single and mixed infections in our study, it is possible that differences could have been detected in the second trifoliolate leaves as previously reported (Anderson *et al*. 1996). It has been shown that virus accumulation varies with leaf position (Calvert and Ghabrial 1983). In that study, it was reported that in the synergistic interaction between *Bean pod mottle virus* (BPMV) and *Soybean mosaic virus* (SMV), the accumulation level of BPMV in the mixed infection was greatly increased in all the trifoliolate leaves except the first which were tested at 13 dpi. There was therefore no significant difference in BPMV titer in the single and mixed infection in the first trifoliolate leaves, whereas that difference was significant for all other trifoliolate leaves. Another possible explanation for the difference in the results presented here and those described by Anderson *et al*. (1996), is the inoculum composition. In their experiments the inoculum for the mixed infections was sap extracted directly from mixedly infected plants. Although it was stated that inoculum from mixedly infected plants and inoculum made by mixing inocula from singly infected plants produced similar symptoms development and resulted in similar virus accumulation, the exact composition of the inoculum made using singly infected plants was not indicated.

From the study conducted by Anderson *et al*. (1996), it was concluded that, during early cowpea stunt disease, the enhancement in symptom severity observed in the mixed infections was not consistently correlated with an increase in the CMV titer in the mixed infection. By having more sampling points during that early development of the cowpea stunt disease in order to focus more on events taking place during that period, and also by testing the stems which showed very strong necrosis in the mixed infections between 6 and 8 dpi, this study supports the same conclusion: disease severity in the early cowpea stunt disease was not due to an increase in CMV accumulation in either the primary inoculated leaves, the first trifoliolate leaves or the stems. Since in the cowpea stunt disease synergism there was no difference in CMV accumulation during early infection, it is possible that the increase in symptom severity may be due in part to something other than enhancement of CMV concentration. In addition, the determination of CMV accumulation in the stem tissues which became necrotic in the mixed infections did not reveal significant differences between CMV accumulations in the single and mixed infections. This result further supports the idea that symptom severity in early cowpea stunt disease is not due to an increase in CMV accumulation.

When comparing CMV accumulation over time in the primary leaves, first trifoliolate leaves and stems, it was found that CMV accumulated to the same level in the first trifoliolate leaves and stems, however, that accumulation was lower than that of the primary leaves. This is not surprising since the primary leaves were the ones inoculated in these experiments. One interesting aspect of this study is the accumulation pattern of BlCMV. It has been generally accepted that in synergistic interactions involving a *Potyvirus*, the concentration of that virus does not differ signifi-

cantly in the mixed and single infections. In the present study it appeared that BlCMV concentration in the single infections was higher than that of the mixed infection and that result did not depend on the plant part tested. However, at 15 days post-inoculation, BlCMV concentration in the mixed and single infections became similar, as reported in other synergistic systems. The statistical analysis showed that, when considering each plant part separately, BlCMV accumulation in the single infections was higher than in the mixed infection. This is interesting because it is a report of a type of repression of the *Potyvirus* accumulation in a synergistic interaction between plant viruses. Additionally, for each infection type, BlCMV accumulation in the primary leaves and stems were similar but, lower than that of the trifoliolate leaves.

This phenomenon of enhancement of the non-*Potyvirus* accumulation has also been found and reported in the case of the cowpea stunt disease where, during later stages of infection, CMV accumulation in the second trifoliolate leaves increases greatly in the mixed infection compared to the single infection (Anderson *et al*. 1996). Since one of the characteristics of the disease, the appearance of stem necrosis was usually observed early during infection, this study also focused on the stem in order to gain an understanding of early events leading to the increase in symptom severity.

In previous studies (Diallo 1998; Diallo *et al*. 2004) it was reported that although plants mixedly infected with CMV and BlCMV displayed very strong symptoms including stem necrosis, no significant difference was found in CMV accumulation in the stem between single and mixed infections. The suggestion from these studies was that either or both viruses in the mixed infection might be able to infect tissues which were not infected in the single infections, as has been previously been reported to occur with other viruses (Barker 1987; Carr and Kim 1983). In the synergistic interaction between *Potato leafroll luteovirus* (PLRV) and PVY in *Nicotiana clevelandii*, Barker (1987) showed that the concentration of PLRV was increased and that PLRV which is normally phloem-limited was able to invade non-phloem tissue in the mixed infection. Similar results were obtained by Carr and Kim (1983) when working on mixed infections with TMV and BGMV, where the latest normally restricted to the phloem, was able to move out of this tissue. Karyeija *et al*. (2000) hypothesized that *Sweet potato chlorotic stunt virus* (SPCSV) is able to enhance the multiplication of *Sweet potato feathery mottle virus* (SPFMV) in tissues other than where it usually occurs. The possibility of CMV and/or BlCMV or both to invade in the mixed infections cells which are not normally infected in the single infection was investigated. In the cowpea stunt disease, however, fluorescent microscopy studies indicated that CMV and BlCMV infected the same tissues in stems in both single and mixed infections (data not shown). Murphy and Bowen (2006) showed that in mixed infection of CM and *Pepper mottle virus* (PepMoV) in pepper, symptom severity increased due to the presence of both viruses in developing and emerging tissues. The findings in our study were also confirmed by electron microscopy observations.

The stem necrosis associated with mixed infection of CMV and BlCMV developed in the portion of the stem about 1-2 cm below the primary leaves. This is the area of the stem that is elongating during the time that stem necrosis develops. Whereas BlCMV did not have an effect on stem elongation, CMV in single and mixed infections did affect stem elongation. This result shows the critical role of CMV in the cowpea stunt disease.

In a study conducted by Fukumoto *et al*. (2003), it was concluded that either CMV alone or mixed with *Watermelon mosaic virus* induced stem necrosis in pea plants. With light microscopy it was observed that, in the mixed infection resulting in cowpea stunt disease, the stem necrosis was not only internal (pith, medullary ray) as seen sometimes in the case of the single infection with CMV, but also external (epidermis and cortex). The pith, medullary ray and vascular cambium stem cells were malformed and compressed in both CMV and mixedly infected plants. Electron microscopy examination of these cells revealed no difference between CMV and mixedly infected plants in terms of cell structure or virus localization. This study was therefore focused on the effect of virus infection on epidermal and cortical cells. Plants singly infected with CMV and which developed stem necrosis always outgrew the necrosis and recovered, while plants mixedly infected with CMV and BlCMV became more necrotic, suggesting that the external necrosis may play a critical role in the development of severe symptoms in these mixedly infected plants.

In the stems of mixedly infected infected plants, some of the epidermal and cortical cells were irregular in shape, degraded, and densely stained. A decrease in cytoplasmic volume might have caused the disintegration of the cytoskeleton, resulting in cell collapse. Since these necrotic cells provided no clues as to what had caused the necrosis, cells adjacent to the necrotic cells were examined. Cells close to these collapsed cells contained both CMV and BlCMV, and were greatly vacuolated. These vacuoles were found to contain large numbers of BlCMV particles and virus-specific densely stained crystalline inclusions. Similar crystalline inclusions were reported in *Vicia faba* infected with *Bean yellow mosaic virus* (Weintraub and Ragetli 1966). The formation of the vacuoles is probably an attempt by the cells to remove all the debris created by the necrosis. In necrotic areas, the vacuoles were filled with aggregates comprising pinwheel inclusions, CMV particles and some densely stained material. In the cells infected mixedly with combinations of *Tobacco mosaic virus*-U1 (TMV-U1)+PepMoV and *Pepper mild mottle virus* (PMMoV) PMMoV+PepMoV, the virus particles and inclusions of the two different viruses were found simultaneously in the same cytoplasm (Kim *et al*. 2005).

Chloroplast alteration was also found in the cortical cells of stems from mixedly infected plants. However, CMV single infection has been reported to cause such alteration in tobacco leaf cells (Ehara and Misawa 1975). All these results together indicate that the necrosis of epidermal and cortical cells plays a detrimental role in the cowpea stunt disease, and that it is the presence of both viruses together in these cell types which caused the strong necrosis observed during early stages (eight days after inoculation) of the mixed infection by CMV and BlCMV. In mixed infections with *Turnip mosaic virus* (TuMV) (*Potyvirus*) and *Ribgrass mosaic virus* (RMV) (*Tobamovirus*) occurring in cabbage, particles of both viruses made a nonagon-like rings (NLR), with one TuMV surrounded by nine RMV particles (Cho *et al*. 2002). The two viruses were found to be compacted in the central part of the spiral aggregates (SA). Different cytopathological structures are induced in mixed infections involving unrelated viruses Martin *et al*. (2004). With CMV and BlCMV, eight BlCMV encircle one CMV icosahedron and formed an octagonal arrangement of what was called mixed virus particle aggregates (MVPAs).

During early infection (6-8 days post-inoculation) in the cowpea stunt disease, plants showed severe foliar symptoms as well as stem and petiole necrosis. However, relative virus accumulation did not show any increase in CMV accumulation as expected in synergistic interactions involving a *Potyvirus*, even in the stem of infected plants. The microscopy studies, an important part of our work, complement the other aspects by showing the localization of the CMV and BlCMV particles in the single and mixed infections.

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