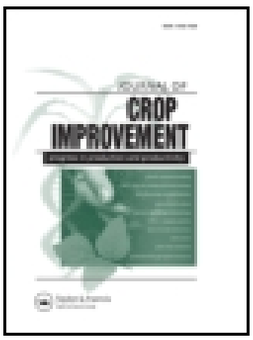

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Disease incidence and severity in cowpea lines evaluated for resistance to single and multiple infections of endemic viruses in Nigeria

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ABSTRACT

Cowpea (*Vigna unguiculata* (L.) Walp) is susceptible to several viruses in West Africa. Cowpea viral diseases are mainly controlled through the use of resistant cultivars. Co-infection with more than one virus is frequent in the fields and the resultant synergistic effect often compromises host resistance identified by screening against individual viruses under field or controlled conditions. In this study, eight improved cowpea breeding lines, identified as resistant to single infections and a susceptible cultivar (Ife Brown), were evaluated for their reactions to single and multiple infections of three viruses endemic in West Africa; viz., bean common mosaic virus-blackeye cowpea mosaic strain (BCMV-BlCM), southern bean mosaic virus (SBMV), and cucumber mosaic virus (CMV). Cowpea seedlings were inoculated with these viruses singly or in combination. Disease incidence and severity were recorded at weekly intervals for eight weeks. Virus infection was confirmed by enzyme-linked immunosorbent assay or reverse transcription-polymerase chain reaction. Systemic mosaic, vein-banding, and stunting were observed on inoculated plants. Mixed infection increased symptom severity and the highest severity was found in plants co-infected with CMV. Phenotyping against mixed-infections was more promising for estimating host resistance response in cowpea than single infections. Based on virus incidence and severity, lines IT97K-1069-6 and IT04K-405-5 were found to be resistant to SBMV, whereas IT99K-1060 and IT98K-503-1 were susceptible to the three viruses. IT-98 K-1092-1 was found to be resistant to BCMV and SBMV and tolerant to CMV under mixed inoculation scenario. Cowpea line IT-98 K-1092-1 is, thus, the best resistance source for use in virus resistance-breeding programs.

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Co-infection; cowpea; ELISA; host resistance; RT-PCR; West Africa

Introduction

Cowpea (*Vigna unguiculata* (L.) Walp) is the most important grain legume crop, which is widely cultivated for different uses in West Africa (Boukar et al. 2013). About 85.5% of the 12.49 million ha of global cowpea production

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area is in West Africa, accounting for 6.05 million tons of production annually (FAOSTAT 2018). Nigeria, Niger, and Burkina Faso are the top three cowpea-producing countries, collectively accounting for about 80% and 55% of the global cowpea-production area and production, respectively (FAOSTAT 2018). Average cowpea yield of about 567 kg/ha in West Africa is low because of a number of abiotic and biotic factors, especially infestation by insect pests, parasitic weed Striga, and diseases caused by bacteria, fungi, and viruses (Boukar et al. 2013). Viral diseases are known to reduce cowpea grain yields by 10% to 100%, depending on the time of infection and severity of infection in the field (Rachie 1985).

Cowpea is susceptible to more than 40 viruses worldwide and eight of these were frequently reported to infect cowpea in West Africa; these are bean common mosaic virus – blackeye cowpea mosaic strain (BCMV – BlCM, genus *Potyvirus*), cowpea aphid-borne mosaic virus (CABMV, genus *Potyvirus*), cowpea golden mosaic virus (CGMV, genus *Begomovirus*), cowpea mosaic virus (CPMV, genus *Comovirus*), cowpea mottle virus (CPMoV, genus *Carmovirus*), cowpea mild mottle virus (CPMMV, genus *Carlavirus*), cucumber mosaic virus (CMV, genus *Cucumovirus*) and southern bean mosaic virus (SBMV, genus *Sobemovirus*) (Taiwo 2003; Boukar et al. 2013). Infection by these viruses results in different types of mosaic symptoms, stunting, and reduction in yield. These viruses have a wide host range, comprising crops, weeds, and wild legumes; they are transmitted by insect vectors (aphids, beetles, or whiteflies) and all but CGMV are seed-transmitted in cowpea (Boukar et al. 2013; Odedara and Kumar 2016).

Occurrence of multiple infections in field crops is common, especially because the same vectors transmit multiple viruses of the same or different genera (e.g., *Aphis craccivora*) (DaPalma et al. 2010; Taiwo 2003). Co-infections by two viruses were reported to be prevalent; multiple infections caused by four or five viruses have also been reported in Nigeria (Shoyinka et al. 1997) and Uganda (Amayo et al. 2012). Multiple infections lead to a variety of host responses based on the nature of virus-virus interactions, viz., neutral (no adverse effect on viruses or host phenotypic response), antagonism (one virus suppressing another virus that may or may not change phenotypic response), and synergism (complementation between coinfecting viruses, often resulting in suppression of host resistance response, leading to severe symptoms). Synergistic interactions often lead to increased symptom severity and have caused the breakdown of resistance against single viruses in tomato, cucumber, and sweet potato (Syller 2012). Synergistic interaction of BCMV and CMV results in “cowpea stunt” disease, leading to a significant loss (86%) in cowpea productivity (Pio-Ribeiro, Wyatt, and Kuhn 1978; Eni et al. 2013).

Because of the complex epidemiology of cowpea-infecting viruses, use of host resistance is the most economical, convenient, and effective approach for cowpea virus disease control in smallholder farmer fields in West Africa. Although sources of resistance to some viruses have been reported in cowpea (Bashir et al. 1995; Singh and d'Hughes 1999; VanBoxtel et al. 2000), they were all selected by either screening against a single virus species under controlled conditions or in open fields exposed to natural infection. Such screening approaches have limitations. For instance, results from field screening under natural infections usually vary because of lack of control on variables, such as inoculum sources and vector activity necessary to ensure uniform infection. Neither approach has been effective in assessing precise phenotypic and genotypic response to multiple-virus infections. This study evaluated the phenotypic response of eight cowpea breeding lines to single and mixed infections of BCMV-BlCM, CMV, and SBMV by artificial inoculations under screenhouse conditions to demonstrate that phenotyping against mixed infections was better than that of single infections for estimating resistance response of crops frequently at risk of mixed infections under native farming conditions.

Materials and methods

Source of seeds and virus isolates

Seeds of eight improved breeding lines and a susceptible cultivar, Ife Brown (Table 1), evaluated in this study, were obtained from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. The eight breeding lines were resistant or tolerant to single inoculation of five cowpea viruses (CPMoV, CABMV, CPMMV, CPMV, and BCMV-BlCM), as revealed by an earlier screenhouse evaluation trial of 50 IITA improved breeding lines. The

Table 1. Characteristics of the cowpea genotypes evaluated in this study.

Genotype	Seed Color	Seed coat Texture[†]	Seed Size[‡]	Days to first Flowering	Days to 50% Maturity	Growth Habit[§]	Other Characteristics[¶]
IT98K-133-1-1	Brown	S	M	41	65	P	Early maturing
IT98K-1092-1	Black	S	M	43	67	S.E.	Striga resistant
IT97K-1069-6	Brown	S	M	44	68	S.E.	Medium maturing
IT98K-503-1	Cream	R	M	41	65	S.E.	Striga resistant
IT97K-1042-3	Brown	S	M	37	61	E	Early maturing
IT04K-405-5	Brown	S	L	47	72	P	Dual purpose
IT99K-1060	Brown	R	M	39	63	S.E.	Early maturing
IT99K-573-1-1	White	R	M	40	63	P	Striga resistant
Ife Brown	Brown	R	M	38	63	S.E.	Early maturing

[†]S, smooth; R, rough.
[‡]M, medium; L, large.
[§]P, prostrate; S.E., Semi-erect; E, erect.
[¶]Source, IITA Ibadan.

50 lines had been subjected to local multi-locational and international trials and possessed some important agronomic characteristics. An old cowpea cultivar "Ife Brown" from the same source was used as a susceptible check. The three virus isolates, i.e., BCMV-BlCM, SBMV, and CMV, used were maintained in the Virology and Molecular Diagnostic (VMD) Unit of IITA, Ibadan, Nigeria. These isolates were established, propagated, and maintained by mechanical inoculation on healthy, susceptible cowpea cv. Ife Brown and cv. TVu 76 in an insect-proof screenhouse.

Virus treatments and inoculation procedures

Seeds of cowpea lines were grown in 8-inch (20-cm) plastic pots, each filled with about 4.5 kg sterilized sandy loam soil. The sown pots were maintained in an insect-proof screenhouse at IITA, Ibadan Station. Virus inoculum was prepared by grinding symptomatic cowpea leaves at 1:10 (w/v) ratio in a chilled sterilized mortar, using 0.05 M phosphate buffer (2.4 g KH_2PO_4, 5.4 g K_2HPO_4 and 0.04 (v/v) ml β-mercaptoethanol in one liter distilled water, adjusted to pH 7.5). Experiment included eight treatments comprising single and mixed infections of the three viruses: (i) BCMV-BlCM, (ii) SBMV, (iii) CMV, (iv) BCMV-BlCM + SBMV, (v) BCMV-BlCM + CMV, (vi) SBMV + CMV, (vii) BCMV-BlCM + SBMV + CMV, and (viii) control (no virus). For the mixed virus inoculation, single virus inoculum was mixed in 1:1 (v/v) ratio just before inoculation and applied to test plants. Test plants at the cotyledon leaf stage (6 to 8 days after planting (DAP)) were used for inoculation. Cotyledons (first leaves) of test plants were dusted with carborundum (600 mesh) to create micro-wounds and they were mechanically inoculated by applying freshly prepared inoculum. Experimental pots were arranged in an eight (virus treatments) by nine (cowpea genotypes) factorial experiment laid-out in a completely randomized design. Six seeds were sown and seedlings were thinned down to 4 per pot before inoculation. There were three replications, making 12 plants per cowpea line for each treatment. The cowpea plants were kept in an insect-proof screenhouse, where insecticide Lambdacyalothrin (Karate) at 4 ml per liter of water was sprayed weekly to control pest infestation.

Evaluation of virus disease incidence and severity

Viral disease incidence was determined by the ratio of the number of infected cowpea plants to the total number of inoculated plants, expressed as a percentage (Odedara et al. 2008). Disease severity was determined by taking weekly symptom severity scores for a period of eight weeks post-inoculation (WPI) using a symptom severity rating scale of 1–5, where 1 = no visible symptoms, 2 = mild mosaic or mottling on a few leaves, 3 = mosaic or

mottling on many leaves, 4 = severe mosaic on all leaves, puckering and mild stunting and 5 = severe mosaic, puckering, leaf distortion, severe stunting with necrosis or death of leaves or plants (Figure 1) (Bashir et al. 1995; Thottappilly and Rössel 1996; Shoyinka et al. 1997).

Virus detection by ELISA

At 5 WPI, all inoculated plants, including the asymptomatic plants, were tested for BCMV-BlCM, SBMV, and CMV using homologous antibodies available at IITA with Antigen Coated Plate-Enzyme-linked Immunosorbent Assay (ACP-ELISA), as described in Kumar et al. (2001). About 100 mg of tissue from the leaf apex was used for virus testing in a 96-well NUNC MaxiSorb (Nunc, Denmark) ELISA plate. Alkaline phosphatase (ALP)-labeled anti-rabbit antibodies were used to detect the immobilized antigen-antibody complex, and p-nitrophenylphosphate (Sigma, Gillingham, UK) was used as substrate. After 1 h of incubation, readings were taken at A405 nm in a Multiscan Plus ELISA plate reader (Labsystems, Helsinki, Finland). Optical density (absorbance at 405 nm (A405 nm)) values were used as proxy for semi-quantification of virus titer in single and mixed infections and depicted as +, ++, and +++ when the A405 nm reading was 2x, 3x, and >3x of the healthy control. Results of the samples that tested negative for virus in ELISA were tested for virus by RT-PCR for reconfirmation.

Virus detection using RT-PCR

Total RNA was extracted from 100 mg apical leaf tissues using a modified cetyltrimethyl ammonium bromide (CTAB) method described elsewhere (Abarshi et al. 2010). Quality of the extracted RNA was analyzed via agarose gel electrophoresis, as previously described (Kumar 2009), and total RNA concentration was estimated using NanoDrop (2000) spectrophotometer (Thermo Scientific Tegrant Corporation; Wilmington, Delaware, USA), as per the manufacturer's instructions.

RT-PCR was performed according to the procedure described by Kumar (2009). For BCMV-BlCM, cylindrical inclusion (CI) degenerates primer for the genus Potyvirus was used: CI-F, CGIVIGTIGGIWSIGGIAARTCIAC and CI-R, ACICCRTTYTCDATDATRTTIGTIGC. Specific primers used for SBMV were: SBMV-F, TGGTCCTTCGACGCAATCT and SBMV-R, GTCTGCTTCAGCTG CAG GACA and for CMV: CMV-F, GCCGTAAGCTGGATGGAC AA and CMV–R, TATGATAAGAAGCTTGTTTCGCG (Wylie et al. 1993). PCR amplification was performed in 12.5 µl reaction mixture comprising 10x PCR reaction buffer (supplied with Taq enzyme), 0.75 µl of 25 mM $MgCl_2$, 0.25 µl mixture of 10 mM deoxynucleotide triphosphates, 0.25 µl of respective primers, 0.3 units of

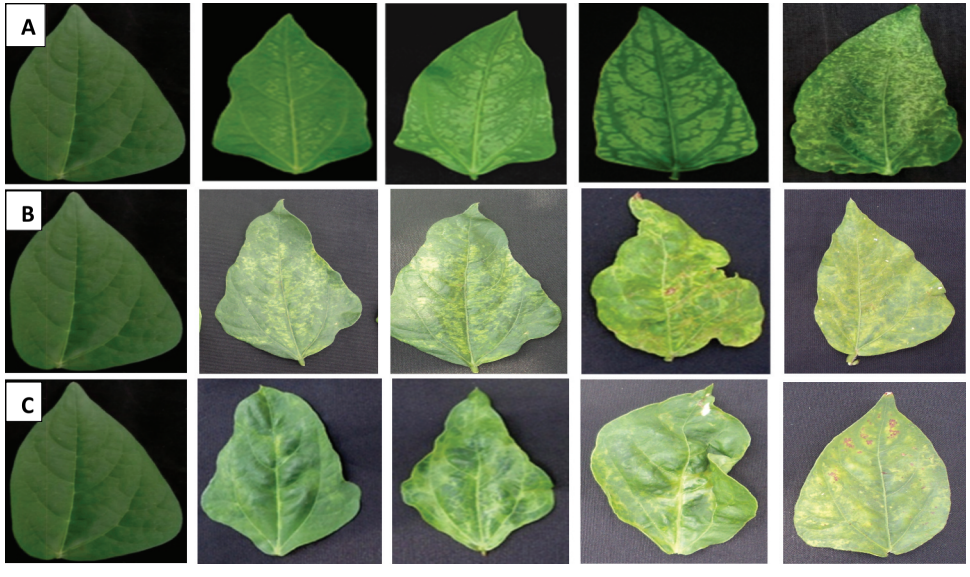

Figure 1. Disease severity rating scale 1–5 of (a) BCMV-BlCM symptoms, (b) SBMV and (c) CMV symptoms. Healthy control (1) and symptomatic leaves of virus infected Ife Brown (2 to 5).

Taq DNA polymerase (Promega Corporation, Madison, Wisconsin, USA), 12 units of Molony-murine leukemia virus (M-MLV) reverse transcriptase (RT) (Promega Corporation, USA), and 2.0 µl of 10 ng/µl total RNA and sterile distilled water. Amplification was performed with Applied Biosystems (GeneAmp® PCR System 9700) Cycler machine. Amplification of BCMV-BlCM RNA was done with one cycle of reverse transcription (RT) cDNA for 30 min at 42°C, initial denaturation at 94°C for 3 min, 40 cycles of amplification by denaturation at 94°C for 30 sec, primer annealing at 40°C for 30 sec, extension at 68°C for 1 min and final extension at 72°C for 10 min. SBMV RNA was amplified using one cycle of RT for 30 min at 44°C, initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 45 sec, annealing at 54°C for 45 sec and extension at 72°C for 45 sec and final extension at 72°C for 7 min. For CMV, RNA amplification was carried out by one cycle of RT for 10 min at 50°C, initial denaturation at 95°C for 5 min, followed by 35 cycles of amplification by denaturation at 95°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min and final extension at 72°C for 10 min. Amplified products were analyzed using 1.5% agarose gel stained with ethidium bromide under UV-illuminator using 100 bp DNA marker.

Classification based on phenotyping into resistant, tolerant, or susceptible

Cowpea lines were classified as resistant, tolerant, or susceptible on the basis of disease incidence, disease severity scores, and diagnostic confirmation for the presence or absence of virus by ACP-ELISA and/or RT-PCR. Plants without symptoms (severity score 1) and virus-negative in diagnostic assays were classified as resistant (R). Plants with severity score between >1 and 2 and positive for virus in diagnostic tests were classified as tolerant (T). Plants with severity scores 3, 4, and 5 and positive for virus in diagnostic tests were classified as moderately susceptible (MS), susceptible (S) and highly susceptible (HS), respectively.

Evaluation of the effect of viral treatments on yield parameters

The number of pods, pod length, number of seeds, and 100-seed weight per plant were recorded and used for estimating yield loss as per the formula given in Sarra (2005). Data on yield parameters were taken at 10 WPI and correlated with disease severity scores. Disease incidence, severity, and yield data were subjected to analysis of variance (ANOVA) using the PROC GLM statement of Statistical Analysis System, version 9.2 (SAS, 2008), and means were separated using Duncan's Multiple Range Test (DMRT).

Results and discussion

Disease incidence, symptoms, and severity

Incidence and symptom severity of BCMV-BlCM, SBMV, and CMV on cowpea depend on the virus type, genotype resistance status, and whether the plant is subjected to single or multiple infections. All three viruses resulted in systemic infection in the susceptible plants. BCMV-BlCM infection produced systemic foliar symptoms of mosaic, mottling, and vein banding on susceptible lines. SBMV produced chlorotic local lesions on inoculated leaves and systemic symptoms of mosaic, inter-veinal chlorosis, mottling, mild puckering, and leaf deformation, whereas CMV produced chlorotic local lesions that later resulted in abscission of inoculated leaves in highly susceptible cowpea genotypes. This progressed into systemic symptoms of mild mosaic, veinal, and mid-rib chlorosis, puckering, leaf distortion, and stunted growth (Figure 2). The three viruses incited severe symptoms in Ife Brown, whereas non-inoculated plants of the genotypes were symptomless. Symptomatic plants tested positive for homologous virus in ACP-ELISA and RT-PCR, whereas virus was not detected in uninoculated plants. Most

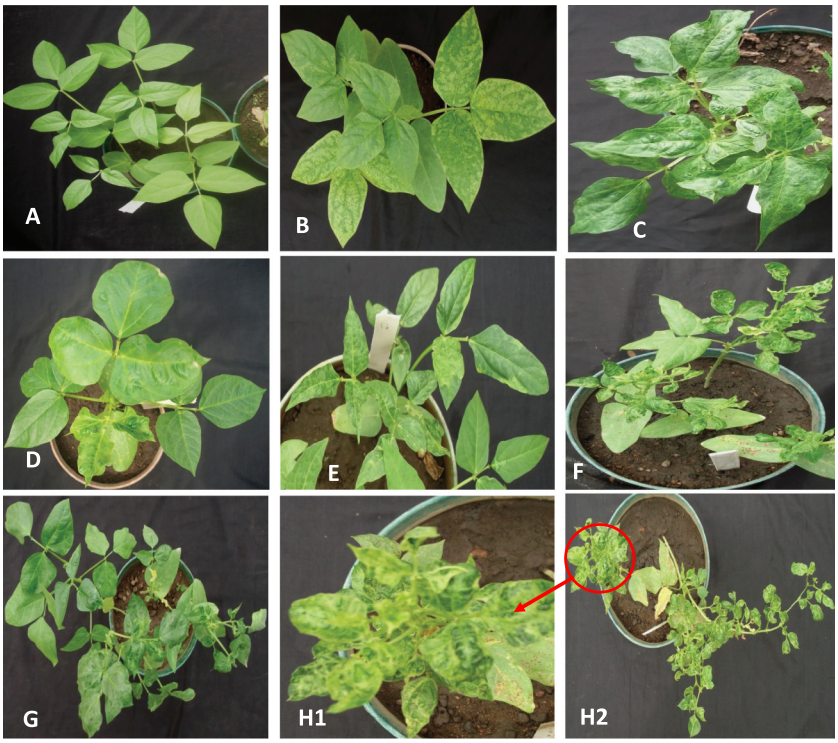

Figure 2. Symptoms induced by single and mixed infections of BCMV-BlCM, SBMV and CMV in cowpea: (a) = Healthy plant, (b) = BCMV-BlCM in Ife brown, (c) = SBMV in Ife brown, (d) = CMV in TVu 76, (e) = BCMV-BlCM + SBMV in IT99K-1060, (f) = BCMV-BlCM + CMV in IT99K-1060, (g) = SBMV + CMV in IT98K-503-1, H1 & H2 = BCMV-BlCM + SBMV + CMV in Ife brown.

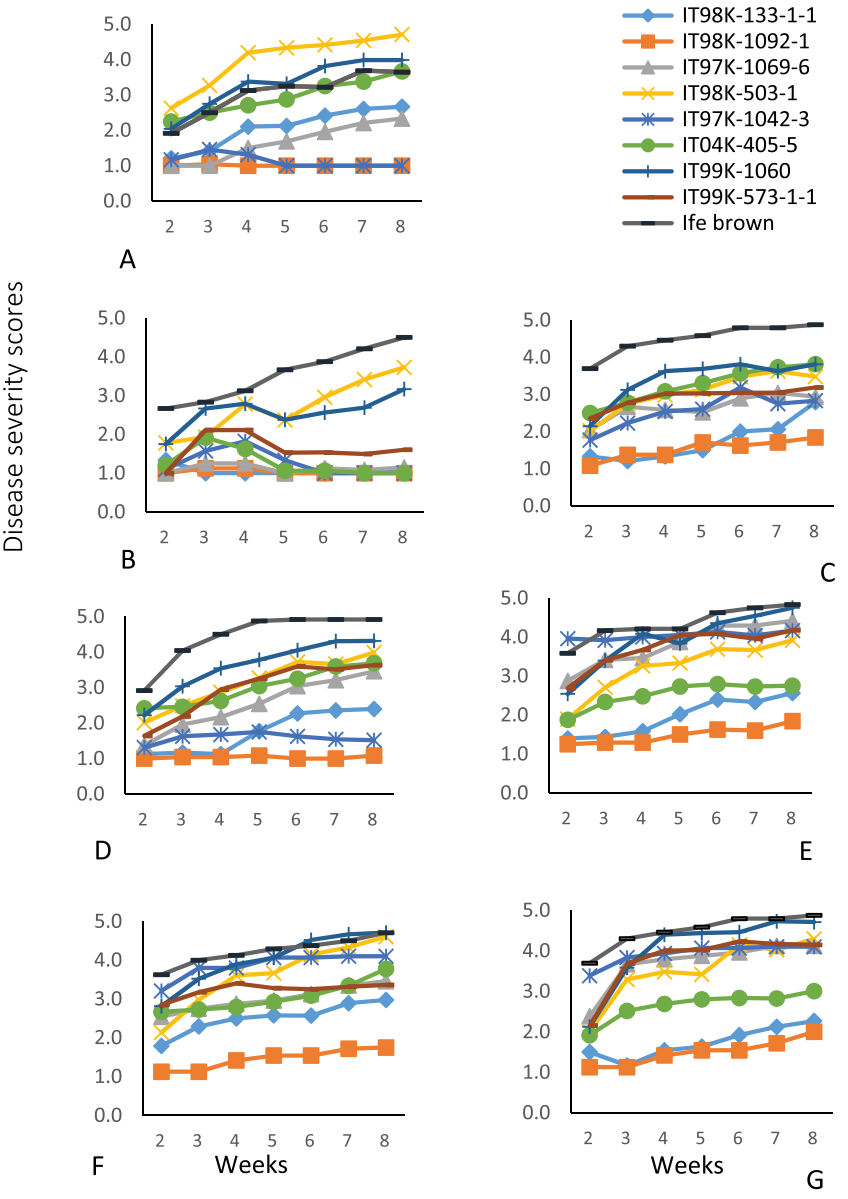

Figure 3. Change in symptom severity of eight virus inoculated cowpea breeding lines and one suscpetible control (Ife Brown) from 2 to 8 weeks post-inoculation. (a) BCMV-BlCM, (b) SBMV, (c) CMV, (d) BCMV-BlCM+SBMV, (E) BCMV-BlCM+CMV, (f) SBMV+CMV and (g) BCMV-BlCM+SBMV+CMV.

severe symptoms were observed at the fourth or fifth WPI in many of the test lines (Figure 3). The virus treatments and genotypes also significantly affected the cowpea yield parameters evaluated (Table 2).

Although, certain foliar symptoms, such as vein banding from BCMV-BlCM infection, puckering and leaf deformation by SBMV and veinal chlorosis, mid-rib chlorosis, and mild puckering by CMV, seem characteristic of

Table 2. Analysis of variance summary for cowpea yield parameters.

Source of variation	d.f.	Pod no.	Pod length	Seed no.	100-seed wt.
Genotypes (G)	8	2.84**	116.08**	135.67**	148.70**
Virus treatment (T)	7	3.38**	102.05**	34.07**	230.02**
G × T	56	0.52**	15.67**	8.83**	18.67**
Error	144	0.31	8.36	4.90	11.15

**, Significant at $p < 0.01$

each of the viruses on cowpea, the three viruses also produced common systemic foliar symptoms of mosaic, chlorosis, and stunting. This implies that symptoms induced on plants by different virus infections cannot be used to identify specific viruses but diagnostic tools have to be used for effective identification. This limitation of symptom-based detection of viruses was further confirmed by the latent infections of CMV observed in cowpea line IT98K-1092-1, on which symptomless plants tested positive via ELISA and RT-PCR. The latency of CMV has also been reported in other crops, such as bell pepper (*Capsicum annuum*) (Garcia-Ruiz and Murphy 2001) and spinach (*Spinacia oleracea*) (Bos, Huttinga, and Maat 1980). Field surveys of cowpea viruses, thus, require adequate diagnostics as a result of this overlapping symptom and the possibility of latent infections.

Virus disease incidence and severity differed significantly ($p < 0.01$) among the eight cowpea lines evaluated (Tables 3 and 4). Incidence ranged from 0% to 100% in BCMV-BlCM and SBMV, and 70.8% to 100% for CMV (Table 3), and the rate of incidence followed a similar trend as did disease severity. Apart from Ife Brown, incidence and severity of BCMV-BlCM infection at 8 WPI were significantly higher ($p < 0.01$) in cowpea lines IT98K-503-1 (100% and 4.7 ± 2.7) than in IT99K-1060 (81.3% and 4.0 ± 0.6), which were also high in IT04K-405-5 (100% and 3.7 ± 1.0) and IT99K-573-1-1 (87.5% and 3.7 ± 0.6). However, BCMV-BlCM infection did

Table 3. Disease incidence (%)[†] of BCMV-BlCM, SBMV, and CMV infections in inoculated cowpea genotypes eight weeks post-inoculation[‡].

Genotype	Bl	SB	CM	Bl+SB	Bl+CM	SB+CM	Bl+SB+CM
IT98K-133-1-1	95.8ab[¶]	4.2 c	95.8ab	79.2b	81.3a	100.0a	100.0a
IT98K-1092-1	0.0d	0.0 c	70.8 c	8.3d	75.0a	62.5b	66.7b
IT97K-1069-6	66.7 c	14.6 c	100.0a	100.0a	100.0a	100.0a	100.0a
IT98K-503-1	100.0a	87.5a	87.5ab	93.8ab	87.5a	100.0a	93.8a
IT97K-1042-3	0.0d	0.0 c	87.5ab	50.0 c	91.7a	87.5a	91.7a
IT04K-405-5	100.0a	0.0 c	95.8ab	95.8a	72.3a	87.5a	81.3ab
IT99K-1060	81.3bc	100.0a	100.0a	100.0a	100.0a	100.0a	100.0a
IT99K-573-1-1	87.5ab	47.9b	81.8bc	89.6ab	91.7a	94.3a	89.6a
Ife Brown[§]	100.0a	100.0a	100.0a	100.0a	100.0a	100.0a	100.0a

[†]Values (expressed in %) are means of two trials and three reps.
[‡]Bl, BCMV-BlCM; SB, SBMV; CM, CMV.
[§]Susceptible check.
[¶]Means followed by the same letter in each column are not significantly different ($p = 0.01$) according to Duncan's multiple range test.

Table 4. Resistance classes (CL)[†] of cowpea genotypes following BCMV-BlCM, SBMV, and CMV infections determined by mean disease severity (DS) rating scores and enzyme-linked immunosorbent assay (ELISA).

Genotype	BCMV-BlCM			SBMV			CMV		
	DS	ELISA[‡]	CL	DS	ELISA	CL	DS	ELISA	CL
IT98K-133-1-1	2.7 ± 0.5d[¶]	+++	S	1.0 ± 0.1 c	-	R	2.8 ± 0.3bc	+	S
IT98K-1092-1	1.0 ± 0.0e	-	R	1.0 ± 0.0 c	-	R	1.8 ± 0.3d	++	T
IT97K-1069-6	2.3 ± 0.5d	+++	S	1.1 ± 0.1 c	-	R	3.0 ± 0.4bc	++	S
IT98K-503-1	4.7 ± 2.7a	+++	HS	3.7 ± 1.0b	+++	HS	3.5 ± 1.0ab	++	HS
IT97K-1042-3	1.0 ± 0.0e	-	R	1.0 ± 0.0 c	-	R	2.6 ± 0.3 c	++	S
IT04K-405-5	3.7 ± 1.0 c	+++	HS	1.0 ± 0.0 c	-	R	3.8 ± 0.5a	++	HS
IT99K-1060	4.0 ± 0.6bc	+++	HS	3.2 ± 1.0b	++	S	3.8 ± 0.5a	++	HS
IT99K-573-1-1	3.7 ± 0.6 c	+++	HS	1.6 ± 0.4 c	++	T	3.2 ± 0.5abc	+++	HS
Ife Brown[§]	4.6 ± 0.4ab	++	HS	4.5 ± 0.4a	+++	HS	3.8 ± 0.6a	+++	HS

[†]R, resistant; T, tolerant; S, susceptible; HS, highly susceptible.

[‡]-, ELISA negative (ELISA plate reading at A_{405nm} Absorbance); +, ++ and +++ = A405 nm reading was 2x, 3x and >3x of the healthy control, which indicate ELISA positive, moderately positive and highly positive, respectively.

[§]Susceptible check.

[¶]Means (data represent means of two trials and three reps) followed by the same letter in each column are not significantly different (p = 0.01) according to Duncan's multiple range test.

not produce symptoms in IT97K-1092-1 and IT98K-1042-3; both symptomless lines tested negative to BCMV-BlCM by ELISA and RT-PCR. SBMV infection was most pronounced in IT98K-503-1 (87.5% and 3.7 ± 1.0) and IT99K-1060 (100% and 3.2 ± 1.0), whereas three genotypes (IT98K-1092-1, IT97K-1042-3, and IT04K-405-5) were symptomless and tested negative for the virus in the diagnostic assays. CMV inoculation produced the least incidence and symptom severity (75% and 1.8 ± 0.3) on IT98K-1092-1, whereas infection was high in all other genotypes, and all the cowpea genotypes tested CMV positive by ELISA (Table 4). We found that under single infections, incidence and severity of the three viruses were high in IT98K-503-1 and IT99K-1060, which is an indication of high susceptibility of the lines to BCMV-BlCM, SBMV, and CMV. Moderate symptom reactions of IT98K-133-1-1 and IT97K-1069-6 to BCMV-BlCM imply their moderate susceptibility to the virus. Incidence, severity, and detection of CMV in all the test lines denote lack of resistance to CMV in the evaluated cowpea lines. Despite that, very low incidence and severity of CMV on IT98K-1092-1, together with lack of reduction in the plant's vigor and yield traits, are indications of IT98K-1092-1 being tolerant to CMV infection.

Mixed infections caused severe symptoms on IT04K-405-5, IT97K-1069-6, and IT99K-573-1-1, which showed susceptibility to one or two of the viruses under single infections, and produced a combination of symptoms shown by each of the viruses under single infections. For instance, while vein-banding was not observed on plants infected with SBMV, co-infection of BCMV-BlCM + SBMV produced vein-banding, mosaic, and severe mottling (Figure 2(e)). Co-infections involving CMV produced the most severe symptoms,

including abscission of inoculated leaves, reduction in size of the leaf lamina, stunted growth, few or no pods, and premature death in highly susceptible lines, IT99K-1060 and IT98K-503-1. Mixed infections generally resulted in increased disease severity and virus titer, depending on host genotype. Incidence and severity were high in plants co-infected with the three viruses compared with single infections (Tables 3, 4 and 5; Figure 2(e-h)). Dual infections involving CMV (BCMV-BlCM + CMV and SBMV + CMV) caused defoliation of the first trifoliate leaves in IT99K-1060 and Ife Brown 7 DPI to 9 DPI. Unlike other cowpea lines, IT98K-1092-1 showed some tolerance to mixed infections by producing moderate symptoms (Figure 3; Table 5). The higher infection severity from coinfections involving CMV than single infections indicated the presence of synergistic virus-virus interactions.

We also observed that mixed infection seemed to influence virus titer of coinfecting viruses. For instance, BCMV-BlCM was detected in IT98K-133-1-1 under single infection, but not in same plants co-inoculated with SBMV either with ELISA or RT-PCR (Tables 4 and 5). In the same vein, BCMV-BlCM titer was lower in genotypes IT98K-503-1 and IT04K-405-5 co-inoculated with SBMV compared with single infection of BCMV-BlCM (Tables 4 and 5). Another indication of virus-virus interactions among the co-infecting viruses is the differences observed in virus detection under mixed vis-à-vis single infections in line IT98K-133-1-1. Results from this study showed synergy in the dual infection of BCMV-BlCM + CMV and in SBMV + CMV. The mechanisms involved in virus-virus interactions, which may be synergistic or antagonistic, include cross-protection, mutual exclusion, recombination among the viruses, and gene silencing; some of which usually result in development of a new variant of virus (DaPalma et al. 2010; Rentería-Canett et al. 2011; Syller 2012). There is also a helper-dependent interaction, where a virus (helper) performs a complementary function to the other (dependent) to facilitate its cell-to-cell and long-distance transport, or transmission by vectors (Syller 2012). This study showed absence of synergy in BCMV-BlCM + SBMV coinfection. However, the mode of interaction in BCMV-BlCM + SBMV observed in line IT98K-133-1-1 is not clear. From the absence of BCMV-BlCM in plants co-infected with SBMV, which was detected under single BCMV-BlCM infection, recombination of the two viruses is suspected. As a result of the different intra-host virus-virus interactions observed among viruses under multiple infections, plant virus indexing for quarantine certification and other germplasm health screening works need to consider the possibility of virus interactions and the effects on the accuracy of screening results. Although synergistic interaction has been reported between BCMV-BlCM and CMV (Pio-Ribeiro, Wyatt, and Kuhn 1978; Fajimi 2019), that observed between SBMV and CMV is novel in cowpea. This synergy from SBMV + CMV coinfection has the potential to

Table 5. Resistance classes (CL)[†] of cowpea genotypes following mixed infections of BCMV-BICM (BI), SBMV (SB), and CMV (CM) determined by disease severity (DS) and enzyme-linked immunosorbent assay (ELISA).

Genotype	BI+SB				BI+CM				SB+CM				BI+SB+CM				
	ELISA[‡]				ELISA				ELISA				ELISA				
	DS	BI	SB	CL	DS	BI	CM	CL	DS	SB	CM	CL	DS	BI	SB	CM	CL
IT98K-133-1-1	2.4 ± 0.3 c[¶]	-	-	R	2.6 ± 0.5bc	+	++	S	3.0 ± 0.9 c	++	++	S	2.8 ± 0.5bc	+	-	++	S
IT98K-1092-1	1.1 ± 0.2d	-	-	R	1.8 ± 0.3 c	-	++	T	1.8 ± 0.5d	-	+++	T	2.0 ± 0.7bc	-	-	+++	T
IT97K-1069-6	3.5 ± 1.2b	++	-	S	4.4 ± 0.9a	+	+++	HS	3.5 ± 0.6 c	-	++	HS	4.3 ± 0.8a	++	-	+++	HS
IT98K-503-1	4.0 ± 0.9b	++	+++	HS	3.9 ± 1.0a	+	+++	HS	4.6 ± 0.5a	+++	++	HS	4.3 ± 0.7a	+	+++	+++	HS
IT97K-1042-3	1.6 ± 0.1d	-	-	S	4.3 ± 0.7a	-	++	S	4.3 ± 0.8ab	-	++	S	4.3 ± 1.1a	-	-	++	S
IT04K-405-5	3.7 ± 0.9b	++	-	S	2.8 ± 1.1b	+	+++	S	3.8 ± 0.8bc	-	+++	S	3.2 ± 0.6b	+	-	+++	HS
IT99K-1060	4.3 ± 0.6ab	++	++	HS	4.8 ± 0.3a	++	+++	HS	4.7 ± 0.4a	+++	++	HS	4.9 ± 0.1a	++	+++	++	HS
IT99K-573-1-1	3.6 ± 0.3b	+++	+++	HS	4.2 ± 0.6a	+	+++	HS	3.4 ± 0.6 c	+	++	S	4.2 ± 0.4a	+	++	++	HS
Ife Brown[§]	4.9 ± 0.1a	++	+++	HS	4.8 ± 0.2a	++	+++	HS	4.7 ± 0.5a	+++	+++	HS	4.9 ± 0.1	++	+++	+++	HS

produce a severe pathological response in the form of a devastating reduction in cowpea productivity in West Africa, where both viruses are endemic.

Cowpea resistance, tolerance, and susceptibility to single and multiple infections

Development of resistant cultivars is universally considered the most effective method to control viral diseases in cowpea (Hampton, Thottappilly, and Rössel 1997). This is important since many popular landraces and commercial cowpea cultivars are susceptible to viral diseases. We identified sources of single and multiple resistances and tolerance to viruses in the evaluated cowpea lines. Based on virus incidence, severity, and serological or RT-PCR detection results, cowpea line IT98K-1092-1, when singly infected with each of the three viruses, was resistant to BCMV-BlCM and SBMV, and tolerant to CMV. The absence of incidence, severity, and detection of viruses demonstrated the line's ability to resist intra- and inter-cellular movement or multiplication of BCMV-BlCM and SBMV. Mild and moderate symptoms of CMV infection persisted in this line under dual and triple infection, respectively (Figure 3) and only CMV was detected from the dual and triple infections by ACP-ELISA (Table 5) and RT-PCR (Figure 4). Lines IT97K-1069-6 and IT04K-405-5 were resistant to SBMV but susceptible to CMV and BCMV-BlCM when infected singly or under mixed infection conditions (Table 4, 5 and Figure 3). However, lines IT99K-1060 and IT98K-503-1 were highly susceptible to the three viruses under single and multiple infections (Table 3, 4 and Figure 3). The new sources of single resistance to SBMV (IT97K-1069-6 and IT04K-405-5) and multiple resistance to BCMV-BlCM + SBMV (IT98K-1092-1) identified from this study should be important in breeding programs aimed at developing virus-resistant cowpea varieties, which can provide effective viral disease management. Such cowpea varieties can result in improved cowpea productivity for the farmers, and food and nutritional security for millions of people in West Africa, for whom cowpea is a major source of dietary protein (Kareem and Taiwo 2007). Disease-resistant crop varieties are also critical for managing and adapting to the progressive climate change, which modifies host susceptibility and mechanisms of plant infection (Elad and Pertot 2014; Hellin, Bellon, and Hearne 2014). Tolerance to CMV observed in line IT98K-1092-1 is also very important in cowpea viral disease management. This tolerance has been of a considerable benefit in some crops (e.g., in the control of CMV in cucumber) (Hull 2002).

Results from evaluation of IT97K-1042-3, IT99K-573-1-1, and IT98K-133-1-1 were somewhat unexpected because host responses under single-virus infections differed considerably from those under mixed infections. Line IT97K-1042-3, under single infections, showed resistance to BCMV-BlCM

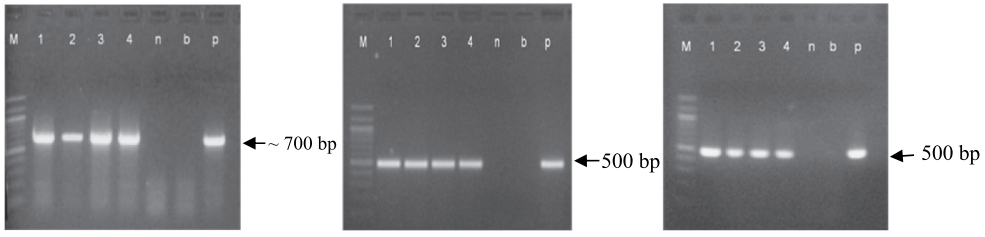

Figure 4. (ac): Detection of (a) BCMV-BlCM, (b) SBMV and (c) CMV in cowpea by RT-PCR; M, DNA size marker (100 bp ladder; Promega); lanes 1–4 = extracts of 4 virus infected samples (a = BCMV-BlCM, b = SBMV and c = CMV); n, uninfected cowpea sample; b = no template control; p = virus positive control.

and SBMV, but it was susceptible to CMV. However, mixed infection of BCMV-BlCM and SBMV resulted in mild symptoms at 50% disease incidence, indicating an apparent loss of resistance under mixed infections (Tables 3, 4 and 5). As observed in IT98K-133-1-1, neither BCMV-BlCM nor its viral partner (SBMV) was detected in IT97K-1042-3 by the diagnostic assays under dual infections.

Under single infections, IT99K-573-1-1 and IT98K-133-1-1 were susceptible to BCMV-BlCM and CMV but tolerant to SBMV. However, the tolerance was lost under mixed infection with the other two viruses. Considering the influence of multiple viral infections on the host response, our results provide a strong evidence that screening for virus resistance under single infections is not effective in cowpea since host resistance or tolerance observed under such conditions is not always stable under mixed infections. Contrarily, many of the previous host resistance screenings in cowpea (Bashir et al. 1995; VanBoxtel et al. 2000; Gillaspie 2006; Lima et al. 2011; Mbeyagala et al. 2014; Tettey et al. 2018) did not consider plant's resilience to multiple infections. Such host resistance identified through field screening using natural infections by insect vectors or infector rows, or screenhouse evaluation with only single virus inoculation might be easily overcome by multiple viral infections. The synergy from multiple infections usually compromises host resistance selected by screening against individual viruses since coinfection is a natural phenomenon in field crops. In this study, where plants were screened with both single and mixed infections under controlled conditions, our findings demonstrate that phenotyping against mixed infections is a better approach compared to single infections for evaluating resistance response in cowpea. However, one should be aware of the possibility of within host virus-virus interactions under multiple-virus screenings. Also, coinfection evaluated in this study was limited to triple infection. This is because screening under multiple infections beyond three viruses may not be effective under experimental conditions, as this may likely affect the ability of resistant plants to withstand such high density of the coinfecting viruses. Besides, although multiple infections of four viruses have been observed, dual and triple infections are the most common in cowpea fields (Shoyinka et al. 1997).

Effect of viral diseases on yield parameters

The interaction between cowpea genotype and viral treatment for cowpea yield parameters was highly significant ($p < 0.01$) (Table 2). This indicates that cowpea genotypes respond differently to each of the virus treatments with regard to their effects on the number of pods per plant, pod length, number of seeds per pod, and 100-seed weight. Virus infections resulted in different levels of reductions in the cowpea yield parameters evaluated (Table 6). Cowpea genotypes with no viral treatment generally had higher yields compared to their inoculated counterparts. The resistant line IT98K-1092-1

had the highest pod length, number of seeds per pod, and 100-seed weight, which were not different from that of healthy plants under single, dual, or triple infections and, with reduction only in the number of pods per plant. This line also had the least mean yield loss attributable to viral infections (Table 7). These reactions to viral infections further support the resistance and tolerance status of IT98K-1092-1 to the viruses.

In the single-infection scenario, both IT98K-1092-1 and IT97K-1042-3, when infected with BCMV-BlCM and SBMV, did not show a significant reduction in yield parameters other than number of pods per plant (Table 6). Meanwhile, unlike BCMV-BlCM and SBMV, CMV infections caused significant reductions in the number of seeds per pod and other yield parameters in all the genotypes, except IT98K-1092-1, where only number of pods was affected. The results indicated that CMV was the most aggressive with respect to incidence and reduction of yield traits, followed by BCMV-BlCM; SBMV was the least aggressive virus. This is contrary to previous reports, where CABMV, BCMV-BlCM, and SBMV were considered to be more prevalent and economically important cowpea viruses than CMV (Shoyinka et al. 1997). Field surveys of cowpea viruses have previously revealed a high frequency of occurrence of strains of CMV, especially in dual infections (Eni et al. 2013; Orawu, Obuo, and Omadi 2015).

Double infections did not cause yield reduction in IT98K-1092-1 in all parameters studied, except that number of pods was affected by BCMV-BlCM + CMV and SBMV + CMV (Table 6). Double infections involving CMV (BCMV-BlCM + CMV and SBMV + CMV) resulted in significant reductions in yield parameters in all the cowpea lines, except IT98K-1092-1. The BCMV-BlCM + SBMV did not reduce number of pods, pod length, and number of seeds in IT98K-133-1-1 and the four yield-related traits in IT98K-1092-1 and IT97K-1042-3. The response of IT98K-1092-1 to BCMV-BlCM + SBMV with respect to yield parameters is in line with its resistance to the two viruses under single and dual infections. The results confirm the absence of synergy between BCMV-BlCM and SBMV. The relatively high level of reduction in the yield parameters, produced by BCMV-BlCM + CMV and SBMV + CMV, in most of the lines supports the synergistic interaction between the dual infections involving CMV. This high reduction in cowpea yield traits caused by the synergy between SBMV + CMV calls for consideration in virus disease management. This is important because of the fast-evolving nature of viruses, and since rapid changes occurring within farming systems, including crop intensification and the anthropogenic climate change, usually make field conditions increasingly conducive for vectors and viral disease establishment (Legg et al. 2019). There is a need for proactive development of resilient cowpea varieties with multiple resistances to the two viruses, which will be helpful in Nigeria, where dual viral infections, especially those involving CMV, are prevalent in cowpea fields (Shoyinka et al. 1997; Eni et al. 2013).

Table 6. Effects of genotype and viral treatment on yield parameters of cowpea.

Genotype	Virus treatment	Pod no./ plant	Pod length (cm)	Seed no./pod /plant	100-seed wt. (g)
IT98K-133-1-1	None	1.75 ± 0.00a‡	14.44 ± 55a	11.59 ± 0.95a	14.76 ± 1.27a
	BCMV	1.55 ± 0.69ab	13.05 ± 1.94ab	12.00 ± 1.57a	10.88 ± 1.31bc
	SBMV	1.20 ± 0.21abc	12.54 ± 1.51ab	10.06 ± 1.89ab	12.21 ± 3.0ab
	CMV	1.15 ± 0.22bc	12.89 ± 1.37ab	9.53 ± 2.16abc	8.56 ± 1.42 c
	BCM+SBM	1.50 ± 0.35abc	14.30 ± 0.94a	9.36 ± 2.09abc	9.90 ± 2.02bc
	BCM+CMV	0.95 ± 0.21 c	9.72 ± 4.42bc	7.70 ± 3.64bc	9.90 ± 2.12bc
	SBM+CMV	1.07 ± 0.69bc	7.49 ± 4.53 c	6.31 ± 3.09 c	9.00 ± 2.98 c
	BC+SB+CM	1.20 ± 0.11abc	9.79 ± 2.87bc	8.75 ± 2.02abc	8.52 ± 2.18 c
IT98K-1092-1	None	2.4 ± 0.34a	9.21 ± 0.76a	8.43 ± 0.9a	12.98 ± 2.46a
	BCMV	2.00 ± 0.36abc	9.61 ± 0.43a	8.58 ± 1.38a	13.04 ± 2.25a
	SBMV	1.50 ± 0.18 cd	9.13 ± 0.61a	8.23 ± 1.80a	10.63 ± 1.75a
	CMV	1.30 ± 0.21d	9.46 ± 0.50a	9.14 ± 1.82a	11.85 ± 0.86a
	BCM+SBM	2.20 ± 0.57ab	10.20 ± 0.70a	10.32 ± 1.34a	13.41 ± 2.69a
	BCM+CMV	1.65 ± 0.42 cd	9.31 ± 0.59a	9.66 ± 1.34a	11.90 ± 0.72a
	SBM+CMV	1.46 ± 0.19 cd	9.21 ± 0.67a	8.43 ± 1.49a	12.98 ± 1.02a
	BC+SB+CM	1.70 ± 0.59bcd	8.97 ± 1.00a	10.04 ± 0.98a	10.84 ± 2.43a
IT97K-1069-6	None	2.40 ± 0.49a	13.50 ± 0.92a	10.33 ± 1.04a	16.42 ± 1.12a
	BCMV	1.50 ± 0.31b	11.93 ± 1.02a	6.53 ± 2.38bc	10.90 ± 4.20bc
	SBMV	1.35 ± 0.28bc	12.18 ± 1.36a	6.27 ± 1.10bc	13.34 ± 0.71ab
	CMV	1.40 ± 0.29bc	11.22 ± 0.58a	6.34 ± 1.08bc	12.80 ± 1.08ab
	BCM+SBM	1.35 ± 0.22bc	10.39 ± 2.9a	7.31 ± 2.18b	13.04 ± 2.63ab
	BCM+CMV	0.75 ± 0.43 c	5.33 ± 3.36b	4.10 ± 2.58 c	7.64 ± 5.27 c
	SBM+CMV	0.93 ± 0.63bc	6.13 ± 2.28b	4.98 ± 1.06bc	10.04 ± 1.11bc
	BC+SB+CM	0.95 ± 0.78bc	7.07 ± 4.56b	5.11 ± 3.42bc	7.30 ± 5.38 c
IT98K-503-1	None	2.58 ± 0.69a	10.75 ± 0.75a	7.90 ± 1.65a	15.38 ± 2.31a
	BCMV	0.85 ± 0.65b	3.14 ± 2.22b	2.42 ± 1.63b	5.19 ± 3.99d
	SBMV	0.80 ± 0.64b	4.70 ± 2.32b	2.67 ± 1.81b	9.64 ± 1.10bc
	CMV	0.95 ± 0.84b	3.96 ± 3.10b	3.09 ± 3.00b	6.76 ± 2.96 cd
	BCM+SBM	0.95 ± 0.74b	5.13 ± 4.45b	3.44 ± 3.19b	9.52 ± 0.83bc
	BCM+CMV	1.20 ± 0.69b	5.34 ± 2.90b	4.43 ± 2.71b	8.98 ± 2.88bc
	SBM+CMV	1.25 ± 0.75b	5.91 ± 2.99b	3.88 ± 1.89b	10.18 ± 1.26b

(*Continued*)

Table 6. (Continued).

Genotype	Virus treatment	Pod no./ plant	Pod length (cm)	Seed no./pod /plant	100-seed wt. (g)
	BC+SB+CM	1.25 ± 0.73b	4.30 ± 2.73b	3.44 ± 2.10b	10.52 ± 0.83b
IT97K-1042-3	None	2.60 ± 0.14a	12.77 ± 0.34a	8.93 ± 1.13a	13.78 ± 2.61a
	BCMV	2.15 ± 0.38ab	11.98 ± 1.47a	6.94 ± 1.23ab	12.28 ± 0.96a
	SBMV	1.85 ± 0.68b	11.84 ± 1.27a	6.39 ± 1.41ab	11.06 ± 1.83ab
	CMV	1.80 ± 0.45b	10.33 ± 1.90a	6.39 ± 1.07bc	10.27 ± 1.17abc
	BCM+SBM	2.40 ± 0.29ab	10.88 ± 0.46a	7.48 ± 1.87ab	11.08 ± 1.11ab
	BCM+CMV	0.40 ± 0.40 c	3.76 ± 3.46b	2.45 ± 2.43 c	6.51 ± 6.00bc
	SBM+CMV	0.07 ± 0.67 c	6.30 ± 5.85b	4.68 ± 4.33bc	5.64 ± 5.14 c
	BC+SB+CM	0.65 ± 0.62 c	4.20 ± 4.53b	3.02 ± 3.32 c	5.94 ± 5.43bc
IT04K-405-5	None	1.60 ± 0.58a	15.56 ± 1.02a	13.13 ± 0.68a	21.10 ± 1.38a
	BCMV	1.68 ± 0.63a	12.83 ± 2.05abc	9.03 ± 2.51bc	15.40 ± 1.26bc
	SBMV	1.48 ± 0.29a	14.43 ± 2.06ab	11.81 ± 2.28ab	16.96 ± 3.35b
	CMV	1.36 ± 0.32a	11.592.16bcd	9.15 ± 3.40bc	13.78 ± 4.19bc
	BCM+SBM	1.40 ± 0.38a	12.78 ± 1.00abc	11.00 ± 1.98abc	12.84 ± 3.11 c
	BCM+CMV	1.00 ± 0.39a	8.89 ± 3.31d	7.77 ± 3.29 c	9.25 ± 1.44d
	SBM+CMV	1.15 ± 0.45a	11.69 ± 2.75bcd	10.03 ± 1.93abc	13.26 ± 1.69 c
	BC+SB+CM	1.35 ± 0.29a	10.72 ± 0.88 cd	9.59 ± 2.30bc	12.18 ± 1.64 cd
IT99K-1060	None	2.60 ± 0.75a	10.51 ± 0.79a	7.23 ± 1.72a	15.90 ± 3.88a
	BCMV	1.35 ± 0.52b	5.34 ± 4.87bc	2.02 ± 2.34b	9.06 ± 5.42bc
	SBMV	1.10 ± 0.52b	7.53 ± 2.88b	3.80 ± 1.34b	11.65 ± 2.64ab
	CMV	1.25 ± 0.79b	5.86 ± 3.60bc	2.95 ± 1.21b	10.92 ± 2.38ab
	BCM+SBM	1.05 ± 0.75b	5.24 ± 3.44bc	3.50 ± 2.02b	8.64 ± 5.54bc
	BCM+CMV	0.90 ± 0.72b	5.53 ± 3.71bc	3.21 ± 1.92b	8.12 ± 1.64bc
	SBM+CMV	0.95 ± 0.97b	4.49 ± 3.89bc	2.66 ± 1.86b	11.16 ± 2.16ab
	BC+SB+CM	0.50 ± 0.64b	2.44 ± 2.43 c	2.76 ± 2.55b	4.16 ± 3.40 c
IT99K-573-1-1	None	2.35 ± 0.60a	14.16 ± 1.56a	8.36 ± 2.74a	20.06 ± 2.12a
	BCMV	1.65 ± 0.34ab	7.87 ± 5.09bc	3.70 ± 2.93b	15.32 ± 1.07b
	SBMV	1.35 ± 0.65b	8.98 ± 2.18b	4.24 ± 1.83b	15.44 ± 0.99b
	CMV	1.10 ± 0.34b	7.66 ± 2.83bc	4.38 ± 1.32b	13.24 ± 2.52bc
	BCM+SBM	1.45 ± 0.78b	9.16 ± 1.80b	5.17 ± 1.08b	15.52 ± 0.82b

(*Continued*)

Table 6. (Continued).

Genotype	Virus treatment	Pod no./ plant	Pod length (cm)	Seed no./pod /plant	100-seed wt. (g)
	BCM+CMV	0.95 ± 0.86b	3.65 ± 3.61 c	2.36 ± 2.26b	10.88 ± 1.42 c
	SBM+CMV	1.45 ± 0.69b	7.78 ± 2.78bc	4.04 ± 0.25b	15.10 ± 2.43b
	BC+SB+CM	1.10 ± 0.58b	5.40 ± 3.03bc	3.29 ± 1.50b	12.66 ± 2.00 c
Ife Brown[†]	None	2.15 ± 0.45a	9.74 ± 0.48a	7.38 ± 1.55a	15.16 ± 1.07a
	BCMV	1.10 ± 1.15b	0.68 ± 1.00d	0.41 ± 0.89d	6.96 ± 6.35b
	SBMV	1.00 ± 0.53b	5.19 ± 1.42b	4.44 ± 1.54b	10.13 ± 0.97b
	CMV	1.00 ± 0.50b	4.41 ± 2.58bc	3.61 ± 2.23bc	8.95 ± 3.36b
	BCM+SBM	0.25 ± 0.25b	2.29 ± 2.22 cd	2.15 ± 2.30bcd	7.48 ± 4.29b
	BCM+CMV	0.35 ± 0.52b	1.90 ± 2.31 cd	1.50 ± 1.95 cd	5.46 ± 4.98b
	SBM+CMV	0.80 ± 0.77b	3.06 ± 2.42bcd	2.80 ± 2.19bcd	6.54 ± 4.30b
	BC+SB+CM	0.60 ± 0.76b	2.14 ± 2.46 cd	1.93 ± 2.12bcd	4.78 ± 5.52b

[†]Susceptible check.

[‡]Means (values represent means of three reps with four plants per rep) followed by the same letter in each column for each cowpea genotype are not significantly different ($p = 0.01$) according to Duncan multiple range test.

Table 7. Comparative percent loss in cowpea yield traits under single and mixed viral infections.

		Loss (%)				
Genotype	Viral treatment	Pod no. /plant	Pod length /plant (cm)	Seed no./ pod/plant	100-seed wt. (g)	Mean
IT98K-133-1-1	BCMV	11.4	9.6	0.0	26.3	11.8
	SBMV	31.4	13.2	13.2	17.3	18.8
	CMV	34.3	10.7	17.8	42.0	26.2
	BCM+SBM	14.3	1.0	19.2	32.9	16.9
	BCM+CMV	45.7	32.7	33.6	32.9	36.2
	SBM+CMV	38.9	48.1	45.6	39.0	42.9
	BC+SB+CM	31.4	32.2	24.5	42.3	32.6
IT98K-1092-1	BCMV	16.7	0.0	0.0	0.0	4.1
	SBMV	37.5	0.9	2.4	18.1	14.7
	CMV	45.8	0.0	0.0	8.7	13.6
	BCM+SBM	8.3	0.0	0.0	0.0	2.1
	BCM+CMV	31.3	0.0	0.0	8.3	9.9
	SBM+CMV	39.2	0.0	0.0	0.0	9.8
	BC+SB+CM	29.2	2.6	0.0	16.5	12.1
IT97K-1069-6	BCMV	37.5	11.6	36.8	33.6	29.9
	SBMV	43.8	9.8	39.3	18.8	27.9
	CMV	41.7	16.9	38.6	22.0	29.8
	BCM+SBM	43.8	23.0	29.2	20.6	29.2
	BCM+CMV	68.8	60.5	60.3	53.5	60.8
	SBM+CMV	61.3	54.6	51.8	38.9	51.6
	BC+SB+CM	60.4	47.6	50.5	55.5	53.5
IT98K-503-1	BCMV	67.1	70.8	69.4	66.3	68.4
	SBMV	69.0	56.3	66.2	37.3	57.2
	CMV	63.2	63.2	60.9	56.0	60.8
	BCM+SBM	63.2	52.3	56.5	38.1	52.5
	BCM+CMV	53.5	50.3	43.9	41.6	47.3
	SBM+CMV	51.6	45.0	50.9	33.8	45.3
	BC+SB+CM	51.6	60.0	56.5	31.6	49.9
IT97K-1042-3	BCMV	17.3	6.2	22.3	10.9	14.2
	SBMV	28.8	7.3	28.4	19.7	21.1
	CMV	30.8	19.1	28.4	25.5	25.9
	BCM+SBM	7.7	14.8	16.2	19.6	14.6
	BCM+CMV	84.6	70.6	72.6	52.8	70.1
	SBM+CMV	97.3	50.7	47.6	59.1	63.7
	BC+SB+CM	75.0	67.1	66.2	56.9	66.3
IT04K-405-5	BCMV	0.0	17.5	31.2	27.0	18.9
	SBMV	7.5	7.3	10.1	19.6	11.1
	CMV	15.0	25.5	30.3	34.7	26.4
	BCM+SBM	12.5	17.9	16.2	39.1	21.4
	BCM+CMV	37.5	42.9	40.8	56.2	44.3
	SBM+CMV	28.1	24.9	23.6	37.2	28.4
	BC+SB+CM	15.6	31.1	27.0	42.3	29.0
IT99K-1060	BCMV	48.1	49.2	72.1	43.0	53.1
	SBMV	57.7	28.4	47.4	26.7	40.1
	CMV	51.9	44.2	59.2	31.3	46.7
	BCM+SBM	59.6	50.1	51.6	45.7	51.8
	BCM+CMV	65.4	47.4	55.6	48.9	54.3
	SBM+CMV	63.5	57.3	63.2	29.8	53.4
	BC+SB+CM	80.8	76.8	61.8	73.8	73.3
IT99K-573-1-1	BCMV	29.8	44.4	55.7	23.6	38.4
	SBMV	42.6	36.6	49.3	23.0	37.9
	CMV	53.2	45.9	47.6	34.0	45.2
	BCM+SBM	38.3	35.3	38.2	22.6	33.6

(*Continued*)

Table 7. (Continued).

		Loss (%)				
Genotype	Viral treatment	Pod no. /plant	Pod length /plant (cm)	Seed no./ pod/plant	100-seed wt. (g)	Mean
	BCM+CMV	59.6	74.2	71.8	45.8	62.8
	SBM+CMV	38.3	45.1	51.7	24.7	39.9
	BC+SB+CM	53.2	61.9	60.6	36.9	53.1
Ife Brown	BCMV	48.8	93.0	94.4	54.1	72.6
	SBMV	53.5	46.7	39.8	33.2	43.3
	CMV	53.5	54.7	51.1	41.0	50.1
	BCM+SBM	88.4	76.5	70.9	50.7	71.6
	BCM+CMV	83.7	80.5	79.7	64.0	77.0
	SBM+CMV	62.8	68.6	62.1	56.9	62.6
	BC+SB+CM	72.1	78.0	73.8	68.5	73.1

Triple infection (BCMV-BlCM + SBMV + CMV) caused premature death of some of the highly susceptible genotypes and a marked reduction in the yield parameters in most of the genotypes, except in IT98K-1092-1 and IT98K-133-1-1. It produced greater reduction in yield parameters than single and double infections, with the exception of BCMV-BlCM + CMV, which caused severe losses that are similar to, and in some lines greater than, those from triple infections (Tables 6 and 7). Such production of similar or more devastating losses in yield traits by dual infections than triple infections has been reported for CABMV-, CPMoV-, and SBMV-infected cowpea (Nsa and Kareem 2015). Despite the different genetic yield potentials among the cowpea genotypes, most of the susceptible cowpea lines (IT99K-1060, IT98K-503-1, and IT99K-573-1-1) generally produced lower yield-related traits than the resistant counterparts, though some susceptible lines did not show significant yield losses. The low or absence of reduction in the yield traits observed under triple infections in IT98K-1092-1 supports its multiple resistance status. To our knowledge, this is the first report of multiple sources of resistance to BCMV-BlCM and SBMV in cowpea. Disease severity of the viruses was negatively correlated with the yield parameters evaluated (Table 8).

Multiple viral infections in cowpea usually produce synergistic interactions that cause higher disease severity and yield reduction than those from single infections (Amayo et al. 2012). This study shows that multiple infections can breakdown host resistance developed using single-virus screenings. This significant observation is very useful in breeding programs aimed at developing resistance to viruses in cowpea. The higher rates of reduction in yield parameters caused by BCMV-BlCM + CMV infections compared to single and other dual infections and similar to triple infection, further confirm a strong synergy between BCMV-BlCM and CMV with respect to the yield of cowpea, which was hardly influenced by SBMV. Such synergy has been reported to cause "Cowpea stunt" disease in Georgia, USA, which resulted in a nearly complete yield loss (Pio-Ribeiro, Wyatt, and Kuhn 1978).

Table 8. Pearson's correlation coefficients between virus severity and cowpea yield parameters.

Virus		Severity	Pod no.	Pod length	Seed no.	100-seed wt.
		(1)	(2)	(3)	(4)	(5)
BCMV-BlCM	1	-	-	-	-	-
	2	−0.87**	-	-	-	-
	3	−0.70*	0.73*	-	-	-
	4	−0.63*	0.61*	0.90***	-	-
	5	−0.47ns	0.79**	0.72*	0.56ns	-
SBMV	1	-				
	2	−0.79**	-			
	3	−0.88**	0.71*	-		
	4	−0.71*	0.50ns	0.83**	-	
	5	−0.41*	0.40ns	0.68*	0.51ns	-
CMV	1	-	-	-	-	-
	2	−0.41ns	-	-	-	-
	3	−0.47*	0.58*	-	-	-
	4	−0.81*	0.40ns	0.88***	-	-
	5	−0.20ns	0.40ns	0.46ns	0.33ns	-

*, **, *** significant at P ≤ 0.05, P ≤ 0.01 and P ≤ 0.001, respectively; ns, not significant.

These co-infections, involving BCMV-BlCM and CMV, already observed in Northern Nigeria (Eni et al. 2013), have the potential to reach an epidemic situation since the same insect vector transmits both viruses. Thus, the cowpea line IT98K-1092-1 identified as resistant to BCMV-BlCM and tolerance to CMV might be of great importance for proactively managing the disease.

Conclusion and recommendations

This study shows that cowpea virus disease incidence, severity, and resultant effects on yield parameters vary according to the type of virus, whether single or mixed infections and the genotype's resistance status. Multiple infections of BCMV-BlCM, SBMV, and CMV resulted in higher disease incidence, severity, and devastating reduction in cowpea yield traits compared to single infections. Synergy observed between BCMV-BlCM and CMV and between SBMV and CMV caused severe symptoms and high reduction in yield parameters. Multiple infections produced intra-host virus-virus interactions, which overcame host resistance derived using single virus screening. The study demonstrates that phenotyping against mixed infections is more promising for estimating host resistance response in cowpea than single infections. Also, only field screening of germplasm for virus host resistance might be ineffective because of the difficulty in ensuring uniform mixed infections among all the test plants, which is required to detect genotypes with multiple resistance that can withstand mixed infections that are commonplace in field crops. We recommend that a breeding program for developing virus-resistant cowpea varieties should complement field-screening with screenhouse evaluation under both single and mixed infections, for an effective and durable host resistance response.

Since BCMV-BlCM, SBMV and CMV are endemic in Nigeria, causing economic yield loss in cowpea, single resistance to SBMV identified in lines IT97K-1069-6 and IT04K-405-5, which was stable under mixed infection, should be useful in developing virus-resistant cowpea varieties. As cowpea lines with multiple virus resistance provide a broader and probably more stable resistance than could be expected from single resistance source, line IT98K-1092-1 with multiple resistances to BCMV-BlCM, SBMV, and tolerance to CMV, which was stable under single or mixed infection scenario, should be of great benefit in transferring multiple virus resistance to susceptible but high-yielding cowpea varieties. This is important for producing varieties with durable resistance to multiple virus infections that proffer a lasting solution to losses in cowpea yield and productivity.

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No potential conflict of interest was reported by the authors.

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