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Seed transmission of bean common mosaic virus - blackeye cowpea mosaic strain (BCMV-BlCM) threaten cowpea seed health in the Ashanti and Brong-Ahafo regions of Ghana

Adams F.K.[1]*, Kumar P.L.[2], Kwoseh C.[3] Ogunsanya P.[2], Akromah R.[3] and Tetteh R.[1]

[1]CSIR-Plant Genetic Resources Research Institute, P. O. Box 7, Bunso, Eastern region-Ghana
[2]International Institute of Tropical Agriculture (IITA), Oyo Road, PMB 5320, Ibadan, Nigeria
[3]Department of Crop and Soil Sciences, Faculty of Agriculture, Kwame Nkrumah University of Science and Technology, P.M.B., University Post Office, Kumasi, Ghana
*Corresponding Author Email : ffffulera@yahoo.com

ABSTRACT

Antigen-coated plate enzyme-linked immunosorbent assay (ACP-ELISA) and reverse transcription-polymerase chain reaction (RT-PCR) were used to detect the presence and seed transmissibility of bean common mosaic virus-blackeye cowpea mosaic (BCMV-BICM) in farm- retained cowpea seed lots obtained from 46 locations, including markets and farms in major cowpea growing areas in the Ashanti and Brong Ahafo regions of Ghana. In the grow-out tests, virus symptomatic plants were observed in seedlings of 19 of the 46 seed lots tested under insect-proof screen-house conditions. All the symptomatic plants tested positive to polyclonal antiserum raised against BCMV-BICM in ACP-ELISA. The seed transmission rates based on symptoms ranged from 0 to 37.8 %. RT-PCR with primer pair designed to amplify the potyvirus Cylindrical Inclusion (CI) region resulted in an expected 720 bp DNA segment in 19 seed lots as a further confirmation of virus in the seed lots. The remaining 27 lots were asymptomatic and tested negative to BCMV-BlCM in both ACP-ELISA and RT-PCR. The findings of this study revealed seed as the source of primary inoculum in the farmers' fields and may aid in the implementation of control strategies such as discouraging farmers from retaining their own seeds for subsequent sowing and encouraging them to take appropriate measures in obtaining virus-free cowpea seeds from other sources.

Key Words: Bean common mosaic virus-blackeye cowpea mosaic, Cowpea, vegetable legume, ELISA, Potyvirus, RT-PCR, virus detection virus-seed transmission

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp) is the most widely cultivated tropical vegetable legume in sub-Saharan Africa (SSA). It is predominantly produced by smallholder farmers because of its tolerance to drought and ability to thrive in zero or low input farming. It provides affordable protein for humans and animals in SSA, Asia, and Latin America (Bashir and Hampton 1993; Tarawali *et al*., 2002; Boukar *et al*., 2013) and also serves as a cover crop in soil nitrogen fixation and the control of erosion and weeds (Hutchinson and McGiffen, 2000). Cowpea has the potential to enhance food security and reduce poverty in West Africa, provided both socio-economic and biological constraints such as poor application of appropriate cultural technologies, infestation by weeds and insect pests, and infection by diseases are adequately tackled (Jackai and Adalla, 1997; Quin, 1997; Coulibaly and Lowenberg – DeBoer, 2002; Boukar *et al*., 2013).

In Ghana, cowpea is second to groundnut in terms of area under cultivation and quantity produced and consumed annually (Egbadzor *et al*., 2013). An average of 143,000 MT is produced annually on about 156,000 ha making Ghana the fifth-highest producer of cowpea in Africa (ICRISAT, 2012). The Guinea savannah zone of Ghana, which includes the Northern and Upper West regions, is the major production area in the country (Al-Hassan and Diao, 2007). Other

production areas include the Sudan savannah zone (Upper East region) and some districts in the transitional zones of Brong Ahafo and Ashanti regions (Haruna *et al*., 2018).

Bean Common Mosaic Virus – Blackeye Cowpea Mosaic (BCMV-BICM) is an important seed-borne virus reported in almost all cowpea growing areas worldwide (CABI/EPPO, 2010; Hema *et al*., 2014). Cowpea fields can suffer substantial yield losses from seed-borne pathogens (Bankole and Adebanjo, 1996). Sowing infected seeds increase germination failure, seedling mortality, and diseased plants, leading to lower yields. Additionally, diseased crops may increase seed infection levels in young plants (Manyangarirwa *et al*., 2009).

Seed transmission offers an effective means of introducing viruses into crop fields at an early stage, giving randomized foci of primary infections throughout the season, which serves as the primary inoculum source for further virus spread by insect vectors (Booker *et al*., 2005). Viruses may persist in cotyledons and embryo axes of matured seeds for long periods (Sekar and Sulochana, 1988), enabling scope for long distances virus spread through contaminated seed lots. The role of farmer-saved seeds in transmitting cowpea diseases was analyzed in northern Nigeria (Biemond *et al*., 2013), and seed to plant transmission of seed-borne pathogens in farmer-saved cowpea was investigated in Zimbabwe (Manyangarirwa *et al*., 2009). These studies have shown that farmer-saved cowpea seeds were heavily infected, with a range of seed- and soil-borne pathogens. The latter emphasizes the negative influence on germination and potential crop losses. Infections caused by seed-borne viruses reduce seed quality and the potential yield of crops. Booker *et al*. (2005) reported seed transmission rates from less than 1 to 100% depending on the virus and host. Yield reductions from expected 2500kg/ha to 50kg/ha were also reported in fields infected with BCMV-BICM in India (Puttaraju *et al*., 2000a). Further, cowpea varieties inoculated with BCMV-BICM at the primary leaf stage showed 92-100% infection at first trifoliate leaf (http://cropgenebank.sgrp.cgiar.org/ Date accessed: 16/07/2019). The virus is readily transmitted mechanically and in a non-persistent manner by the aphids *Aphis craccivora*, *A. gossypii,* and *Myzus persicae* (Orawu, 2007).

A survey conducted on cowpea fields in the Forest and Transitional zones of Ghana revealed the presence of BCMV-BlCM among other six viruses, namely, cowpea aphid-borne mosaic virus (CABMV, genus Potyvirus), cowpea mottle virus (CPMoV, genus Carmovirus), southern bean mosaic virus (SBMV, genus Sobemovirus), cowpea mild mottle virus (CPMMV, genus Carlavirus), cowpea yellow mottle virus (CYMV, genus Comovirus) and cucumber mosaic virus (CMV, genus Cucumovirus) with BCMV-BICM being the most prevalent (Adams *et al*., 2020). According to the study, farmers in the Forest and Transitional zones of the Brong-Ahafo and Ashanti regions adopt production practices such as high cropping density as a result of random sowing methods, recycling of seeds from season to season, the closeness of fields to each other with different planting and pesticide application periods as well as preference for and cultivation of susceptible local cowpea cultivars which increases the incidence and severity of viruses on fields in those areas (Amaza *et al*., 2010; Adams *et al*, 2016).

During the 2015 growing season, viral disease symptoms, similar to those caused by the BCMV-BlCM, were observed on cowpea fields in the Ashanti and Brong Ahafo regions of Ghana. Seeds collected from farmers in these areas were mostly shriveled. This study was conducted to confirm the virus identity in the symptomatic plants observed in the farmers' fields and virus seed transmission in the seeds lots harvested from the 46 farmers' fields and seed markets in Ghana.

MATERIALS AND METHODS

Seed sample collection

A total of forty-six (46) cowpea seed lots were collected from randomly selected farms and markets in the Amantin-Atebubu (17 lots), Ejura-Sekyeredumasi (13 lots), and Nkoranza (16 lots) districts. Seed lots were obtained from 24 farm locations (15 in Amantin-Atebubu, and 9 in Ejura-Sekyeredumasi) and 22 market locations (16 in Nkoranza, 2 in Amantin-Atebubu, and 4 in Ejura-Sekyeredumasi) (Table 1). Seeds sourcing from farmers was done by selecting cowpea farms separated by at least 0.5 Km in each district. In each farm, seed lots were obtained by collecting and bulking seeds from 30 plants randomly selected in an 'X' transect,

with 15 plants per diagonal axis. For market-sourced seeds, lots were obtained by randomly collecting seeds from different market women during the main market days in each district. Seed samples were kept in labeled sample bags with naphthalene balls. A GPS device was used to record coordinates and altitudes of the field and market locations.

Grow-out test

From each sampled seed lot, 100 seeds were sown in trays filled with two liters of steam-sterilized topsoil in an insect-proof screen house. Cowpea seedlings were visually examined for any symptoms. The total number of plants germinated and the number of symptomatic plants was counted in each try to estimate the percent symptomatic plants. At the three-week stage, apical leaves of both symptomatic and asymptomatic plants were sampled for BCMV-BlCM indexing by antigen-coated plate enzyme-linked immunosorbent assay (ACP-ELISA). Symptomatic and asymptomatic plants were tested separately. In the case of asymptomatic plants, ten apical leaves, one from each plant, were collected, and they were together as one composite sample for virus indexing. This was repeated for all the seed lots.

ACP-ELISA for BCMV-BICM detection

To test each plant, a sterile cork borer was used to obtain 5 mm diameter pieces of all leaves in each of the 46 groups of leaf samples. About 100 mg of leaf tissue from each sample was grounded in the carbonate coating buffer (0.015 M Na_2CO_3 and 0.0349 M $NAHCO_3$) with DIECA at 100 mg/ml buffer (1:10 w/v). One hundred microlitres of the extract were added to each well of a microtitre plate. Infected, healthy plant sap and buffer were used as controls. Plates were incubated in a humid chamber for 1 hour at 37°C and then washed with three changes of phosphate-buffered saline with Tween 20 (PBS-Tween 20), allowing three minutes for each wash. Plates were emptied and tapped dry on a layer of paper towel. Wells were blocked with 200 µl of 3% dried skimmed milk in PBS-Tween 20. Plates were incubated at 37°C for 30 minutes, and then tapped dry. Healthy cowpea leaf extract in PBS-TPO (1:10 w/v) was used to cross-adsorption of the BCMV-BICM antiserum at 1:5000 µl. The mixture was incubated at 37°C for 30 minutes. One hundred microlitres of the cross-adsorbed antisera was dispensed in each well and plates were incubated at 37°C for 1 hour. Plates were washed and tapped dry as described above. One hundred microlitres of goat anti-rabbit alkaline phosphatase (ALP) conjugate diluted in conjugate buffer (Ovalbumin, Polyvinyl Pyrrolidone and PBS-Tween 20) (1: 15,000) were dispensed into each well and incubated for 1 hour at 37°C. Plates were washed and tapped dry.

One hundred microlitres of 0.5 mg ml^{-1} p-nitrophenyl phosphate substrate in substrate buffer (diethanolamine and distilled water) were added to each well and incubated in a dark room for 1 hour. Absorbance values were measured, and plates were kept in a refrigerator at 4°C overnight. Quantitative measurements of the p-nitrophenyl substrate conversion resulting in yellow colour were made by determining the absorbance at 405 nm (A405) in an ELISA plate reader at 1 and 6 hours. The mean absorbance readings of negative controls were determined, and twice the values were used as the positive thresholds.

Reverse-transcription polymerase chain reaction (RT-PCR)

The RT-PCR protocol described by Gillaspie *et al*. (2001) was used for the detection of BCMV-BlCM in the 46 seed lots to confirm the ACP-ELISA result. Total nucleic acid was extracted using the Cetyl Trimethyl Ammonium Bromide (CTAB) method described by Dellaporta *et al*. (1983). Cylindrical inclusions forward (CI-F; 5'- CGI VIG TIG GIW SIG GIA ART CIA C-3') and reverse (CI-R; 5'-ACI CCR TTY TCD ATD ATR TTI GTI GC-3') primers designed by Ha *et al*. (2008) were used for RT-PCR amplification and the RT-PCR products were resolved on a 1.5% agarose gel along with 100 bp DNA ladder as a size marker (Cat Nos N0467S, Quick-load, Biolabs Inc., Ipswich, MA, USA). The gel was viewed under a UV trans-illuminator (BioRad Gel Doc XR, California, USA), and the virus-specific band in the samples were identified based on the presence of an expected amplicon size of 720bp.

RESULTS

Among the 46 seed lots of cowpea subjected to a grow-out test in the screen-house, 19, made up of six lots obtained from Atebubu-Amantin, five from Ejura-Sekyeredumasi, and eight from Nkoranza, showed mottling and mosaic (Fig. 1) on leaves.

Fig. 1. Mottle mosaic symptoms of seed-borne BCMV-BlCM on grow-out cowpea plants in a screen house

All the symptomatic plants of 19 seed lots also gave positive reactions to BCMV-BlCM in ACP-ELISA (Table 1). BCMV-BlCM transmission based on symptoms among the lots ranged from 0 to 37.8 % (Table 2). Some of the infected seed lots recorded low germination rates. For instance, of the 100 seeds of each seed lot planted, 51 of "Nkoranza-14" and 30 of "Amantin-15," which were positive to BCMV-BICM, germinated. All asymptomatic plants tested negative to BCMV-BlCM in ACP-ELISA (Table 1).

All the 19 seed lots that had symptomatic plants and tested positive to BCMV-BlCM have also tested positive to the virus in RT-PCR (amplified a 720 bp amplicon) (Fig. 2). Amplification was not detected in

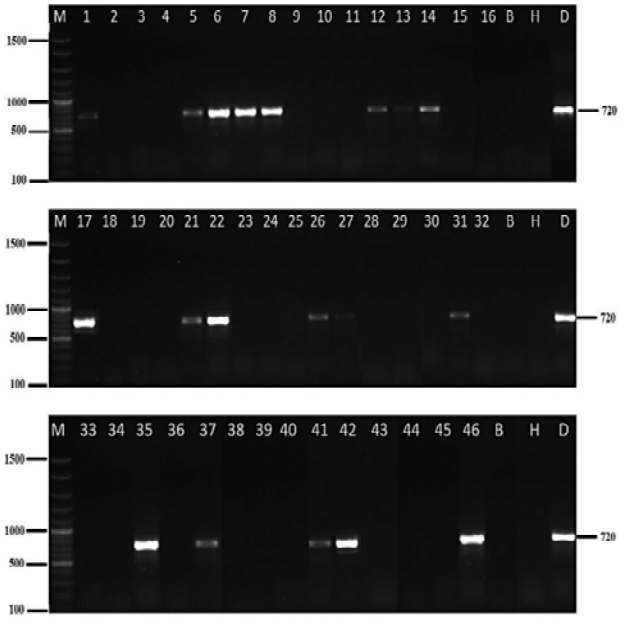

Fig. 2. Agarose gel electrophoresis showing amplification of RT-PCR products

Key; M = DNA marker, H = Healthy control, B = Buffer, D = Positive control Nkoranza samples: 1-16; Amantin samples: 17-33; Ejura samples: 34-46

the remaining 27 asymptomatic seed lots (Fig. 4), confirming the results obtained using BCMV-BlCM antiserum in ACP-ELISA.

DISCUSSION

Grow-out tests, ACP-ELISA and RT-PCR have confirmed BCMV-BlCM seed transmission in the 19 of 46 seed lots assessed in this study. Aliyu *et al*. (2012) previously detected BCMV-BICM among other seed-borne viruses infecting cowpea in Nigeria, using ACP-ELISA. Like the results obtained in this study, several authors (Hampton *et al*., 1997; Shanker *et al*., 2009; Ittah and Binang, 2012) have at various times proved seed transmission of the virus. Shanker *et al*. (2009) reported BCMV-BlCM as a serious pathogen on cowpea worldwide, to which field plants succumb to infections from virulent strains. Booker *et al*. (2005) also reported the detrimental effect of the virus on cowpea production, causing stunting and plant deformation in the early growth stage and not allowing the plants to reach their full potential. Mottling and interveinal chlorosis observed on the primary leaves of the plants in the grow-out test were consistent with symptoms reported to be associated with infections caused by BCMV-BlCM (Aliyu *et al*., 2012).

The BCMV-BlCM incidence in farmers' fields and the corresponding seed transmission rates were given in Table 3. Some seed lots obtained from markets recorded seed transmission rates as high as 36.3% (Nkoranza-6) in the grow-out test. Some seed lots collected from farmers' fields with high BCMV-BlCM incidences recorded zero seed-transmission (Amantin-2, -7, -13, -14 and Ejura-3) while a few other lots recorded very low seed transmission values (Amantin-1, -11, Ejura-8 and -13). Amantin-6, Ejura-9, Amantin-5, and Ejura-4 recorded 100, 90, 87 and 83% field incidences, respectively, with corresponding seed transmission rates of 21.3, 37.8, 16.7 and 16.3%, respectively (Table 3).

Low germination rates recorded in some infected seed lots may be attributed to infection by the BCMV-BICM. Ittah *et al*. (2010) reported in a previous study that seed-borne viruses such as BCMV-BICM, CABMV, CMeV, and SBMV may cause some infected cowpea lines to lose their

Table 1. ACP-ELISA result for BCMV-BlCM seed transmission

Samples	BCMV-BlCM	Samples	BCMV-BlCM	Samples	BCMV-BlCM
Nkoranza 1	3.175*	Amantin 1	2.658*	Ejura 1	0.287
Nkoranza 2	0.253	Amantin 2	0.349	Ejura 2	2.658*
Nkoranza 3	0.225	Amantin 3	0.367	Ejura 3	0.204
Nkoranza 4	0.222	Amantin 4	0.288	Ejura 4	3.370*
Nkoranza 5	3.438*	Amantin 5	3.383*	Ejura 5	0.345
Nkoranza 6	3.446*	Amantin 6	2.851*	Ejura 6	0.282
Nkoranza 7	3.645*	Amantin 7	0.247	Ejura 7	0.281
Nkoranza 8	3.445*	Amantin 8	0.416	Ejura 8	3.457*
Nkoranza 9	0.139	Amantin 9	0.373	Ejura 9	3.285*
Nkoranza 10	0.249	Amantin 10	3.396*	Ejura 10	0.224
Nkoranza 11	0.193	Amantin 11	2.962*	Ejura 11	0.282
Nkoranza 12	3.174*	Amantin 12	0.568	Ejura 12	0.283
Nkoranza 13	3.381*	Amantin 13	0.316	Ejura 13	3.322*
Nkoranza 14	3.275*	Amantin 14	0.471		
Nkoranza 15	0.517	Amantin 15	3.140*		
Nkoranza 16	0.285	Amantin 16	0.410		
		Amantin 17	0.419		
Positive control	OUT		OUT		OUT
Negative control	0.268		0.36		0.36
Buffer	0.21		0.28		0.28

*Absorbance value (A405 nm) is >2x of negative control regarded as the virus positive.
"OUT" indicates an out-of-range value (A405 >4)

Table 2. Seed transmission rates of BCMV-BlCM among accessions

Seed transmission rate (%)	Number of seed lots
0	27
0.1 - 5.0	6
5.1 - 10	0
10.1 - 20	4
20.1 - 30	4
30.1 - 37.8	5

ability to germinate. Fawole *et al*. (2006) also analyzed the effect of seed-borne fungi infection of cowpea seed on germination rate and found reduced germination rate because of infection by the fungi. Further, Manyangarirwa *et al*. (2009) reported that farmer-produced cowpea seeds were heavily infected with a range of seed- and soil-borne pathogens in Zimbabwe, emphasizing the negative influence on germination. However, in contrast to the above findings, Biemond *et al*. (2013) found that natural infection of cowpea seeds with some seed-borne pathogens increased germination.

Although BCMV-BICM has been previously detected in cowpea seeds in Ghana (Zettler and Evans, 1972), according to the literature available, most previous detections were limited to grow-out test, host range, and reactivity to polyclonal antibodies. Ojuederie *et al*. (2009) suggested stringent screening methods such as RT PCR to be used in screening for the presence of seed-borne viruses in addition to ELISA, which employs reactivity to polyclonal antibodies since samples which appear negative with the latter could be positive when tested with RT PCR. The study conforms with the above recommendation as BCMV-BlCM was assessed with ACP-ELISA, and the results were confirmed with RT-PCR.

BCMV-BlCM was identified to be seed-borne in cowpea collected from farms and markets in

Table 3. BCMV-BlCM incidence in farmers cowpea fields and respective seed transmission rates observed in grow-out test

Cowpea Seed lots	Seed source	Total sown	Total germinated	Total symptomatic	% Field incidence	Transmission rate (%)
Nkoranza 1	Market	100	53	18	*	34
Nkoranza 2	Market	100	42	0	*	0
Nkoranza 3	Market	100	93	0	*	0
Nkoranza 4	Market	100	58	0	*	0
Nkoranza 5	Market	100	63	14	*	22.2
Nkoranza 6	Market	100	80	29	*	36.3
Nkoranza 7	Market	100	91	28	*	30.8
Nkoranza 8	Market	100	65	10	*	15.4
Nkoranza 9	Market	100	36	0	*	0
Nkoranza10	Market	100	71	0	*	0
Nkoranza11	Market	100	89	0	*	0
Nkoranza12	Market	100	87	18	*	20.7
Nkoranza13	Market	100	80	19	*	23.8
Nkoranza14	Market	100	51	16	*	31.4
Nkoranza15	Market	100	68	0	*	0
Nkoranza16	Market	100	50	0	*	0
Amantin 1	Farm	100	60	1	63	1.7
Amantin 2	Farm	100	68	0	50	0
Amantin 3	Farm	100	52	0	30	0
Amantin 4	Farm	100	42	0	33	0
Amantin 5	Farm	100	78	13	87	16.7
Amantin 6	Farm	100	94	20	100	21.3
Amantin 7	Farm	100	63	0	70	0
Amantin 8	Farm	100	69	0	38	0
Amantin 9	Farm	100	82	0	38	0
Amantin 10	Farm	100	96	12	87	12.5
Amantin 11	Farm	100	100	4	60	4
Amantin 12	Farm	100	73	0	45	0
Amantin 13	Farm	100	76	0	87	0
Amantin 14	Farm	100	65	0	57	0
Amantin 15	Market	100	30	1	*	3.3
Amantin 16	Farm	100	83	0	30	0
Amantin 17	Market	100	33	0	*	0
Ejura 1	Farm	100	42	0	43	0
Ejura 2	Market	100	86	2	*	2.3
Ejura 3	Farm	100	62	0	63	0
Ejura 4	Farm	100	80	13	83	16.3
Ejura 5	Farm	100	82	0	30	0
Ejura 6	Market	100	72	0	*	0
Ejura 7	Farm	100	71	0	17	0
Ejura 8	Farm	100	92	1	50	1.1
Ejura 9	Farm	100	90	34	90	37.8
Ejura 10	Market	100	66	0	*	0
Ejura 11	Market	100	57	0	*	0
Ejura 12	Farm	100	63	0	40	0
Ejura 13	Farm	100	80	1	53	1.3

***Denotes unknown (seed lots sourced from markets)**

Nkoranza, Amantin, and Ejura. A study conducted by Biemond *et al*. (2013) showed that farmer-produced cowpea seeds were heavily infected with a range of seed- and soil-borne pathogens. Transmission rates based on symptoms ranged from 0 to 37.8 %. Ladipo (1977) and Ng and Hughes (1998) estimated that the rate of seed-transmission of virus in cowpea may range from 0 to 90%, which aligns with the seed transmission rates observed in this study and a previous study by Zettler and Evans (1972) that showed the frequency of seed transmission of BCMV-BlCM at about 30.9% in cowpea. Seed transmission rates of BCMV-BlCM did not necessarily correspond with infection levels observed in fields from which the collections were made. Although some lots obtained from fields with high disease incidence recorded correspondingly high transmission rates in the grow-out test, others recorded either zero or very low rates.

Low transmission rates of BCMV-BlCM in seed lots obtained from fields with high disease incidences could be due to several reasons, including infection after flowering to the presence of virus in seed coat but not in the embryos (Gupta *et al*., 1985). Shanker *et al*. (2009) showed that sowing cowpea seeds with the incidence of BCMV-BlCM as low as less than 1% might result in significant virus spread with a major influence on grain yield. Puttaraju *et al*. (2004b) also reported a 65-100% BCMV-BlCM transmission resulting from sowing cowpea seeds with about 4-10% infection rate. Thus, even with the relatively low seed transmission rates observed in the current study, there is cause for concern. According to Shanker *et al*. (2009), a threshold level below 2% infection for cowpea seeds is recommended as suitable to avoid the risk of economic losses due to the spread of BCMV-BlCM in cowpea.

Seed-borne viruses can present a challenge to managing viral diseases in the fields and complicate the transfer of seeds by trade and other methods of seed exchange between farmers (Manyangarirwa *et al*., 2009; Ittah *et al*., 2010; Biemond *et al*., 2013). Recycling farmers' seeds for subsequent planting, as in the present study areas, may result in high virus incidence and significant yield loss (Owolabi *et al*., 1988).

In conclusion, this study demonstrated a high risk of seed-borne virus threat in the farmer-saved seed. It showed a need to improve awareness among farmers and extension agents about the risk of seed-borne virus infections and discourage farmers from reusing their seeds for long periods, particularly those harvested from infected fields. This study also calls for an increase in the supply of certified seed production that will serve as a sustainable solution to reduce the risk of BCMV-BlCM threat to cowpea production in Ghana.

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