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IDENTIFICATION OF SOME COWPEA ACCESSIONS TOLERANT TO *COWPEA MILD MOTTLE VIRUS*

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ABSTRACT
Five locally adapted cowpea accessions, as well as a susceptible control variety Ife Brown were evaluated under screen house conditions for resistance to *Cowpea mild mottle virus* (CPMMV). The experiments were arranged in completely randomised design with four replicates. Seedlings were inoculated at the three-leaf-stage, while uninoculated plants of each genotype served as negative controls. Virus concentration was quantified by enzyme-linked immunosorbent assay (ELISA). Disease incidence, severity, yields and agronomic traits were recorded. Generally, uninoculated control plants performed better than the inoculated. Three genotypes (IT07K-251-3-3, IT07K-299-4 and IT07K-299-6) were rated as partially tolerant, whereas the remaining ones were susceptible. The yield and growth attributes of the partially tolerant plants were not significantly (p>0.05) different from their healthy uninoculated counterparts. The most tolerant accession (IT07K-299-6) exhibited the lowest reduction in leaf area (0.7 %), plant height (7.3 %), seed weight per plant (2.3 %) and virus concentration (0.11 – 0.247). These results suggest that the partially tolerant accessions most likely contain genes that confer tolerance to CPMMV and could be useful in cowpea breeding for genetic improvement.

KEYWORDS: Area under disease progress curve, disease resistance, enzyme-linked immunosorbent assay, yield and morphological characters, yield reductions

INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp] is an important legume for human consumption in sub-Saharan Africa (SSA), Central Asia, and South America (Brito *et al*., 2012). Cowpea is rich in protein and essential amino acids that are deficient in cereals. It is consumed singly or as a complement to cereal food crops such as rice and maize and its haulm is extensively fed to livestock in form of fodder (Singh *et al*., 2003). Additionally, it contributes appreciably to improved soil fertility and plant growth by fixing atmospheric nitrogen into the soil. In West Africa, cowpea is second in importance after groundnuts, with Nigeria accounting for over 70 % of the total world production (Singh *et al*., 2000). Cowpea cultivation is widely adopted by millions of smallholder farmers in Nigeria partly owing to its compatibility with traditional cropping systems (Olufajo and Singh, 2000) where it is intercropped with cereals such as maize (Sikirou and Wydra, 2008), sorghum and millet. Increased interest in cowpea production is attributable to high demand from local and external markets, and the quest for foreign earnings (Sabiti *et al*., 1994). *Cowpea mild mottle virus* (CPMMV) (Thottappilly and Rossel, 1992) is one of the most damaging viruses affecting cowpea productivity. CPMMV was first observed in Ghana but is now found in practically all major cowpea-producing countries on the African continent. In Nigeria, CPMMV was first reported in 1980 (IITA, 1981). Incidences of CPMMV infection and yield losses vary with cowpea variety and virulence of the isolate. CPMMV is a virus with flexuous filamentous particles of approximately 650 nm in length. It is a member of the genus *Carlavirus*, currently classified in the family *Betaflexiviridae* (Tavasoli *et al*., 2009). Available records indicate that *Carlaviruses* can reduce yields of some crop species by 10 –15 % and, in mixed infections, can exacerbate the deleterious effects of other viruses (Brunt and Kenten, 1973). CPMMV infection can be transmitted by seeds of infected plants and several weeds. It is also disseminated by whitefly [*Bemisia tabaci* (Gennadius)] in a semi persistent manner (Tavasoli *et al*., 2009) and mechanical transmission has been confirmed. Symptoms induced on cultivated plants include necrotic lesions of the primary leaves, severe systemic chlorosis and necrosis on trifoliate leaves. Experimental host plants may display chlorotic local lesions or systemic mottle upon inoculation. Cowpea mild mottle virus disease can be controlled or prevented by cultural practices including the use of clean seeds, eradication of weed hosts and manipulation of time of planting. The insect vector of the virus can be controlled by insecticides (Reddy, 1991). However, the negative consequences such as human poisoning, high cost, soil contamination, inadequate knowledge of application by the farmers, as well as possibility of insect developing resistance to the chemicals have necessitated the need for stronger alternative management measures. Genetic resistance is highly valuable in the control of economically important plant virus diseases as it decreases or prevents replication or symptom expression. Cultivation of resistant or tolerant varieties is considered the best management approach because it is cost-effective, environment friendly and offers insurance against crop failure in the smallholders' cowpea fields. In Nigeria, CPMMV is considered to be of little or no significance as only a few crop genotypes are susceptible (IITA, 1981). It may however, serve as a threat to the production of other legumes such as groundnut and soybean which are often intercropped with cowpea (Brunt and Kenten, 1973). Availability of sources of CPMMV

resistance will be highly valuable to cowpea breeders trying to produce cultivars that are both virus-resistant and high-yielding. Undoubtedly, this will provide substantial relief to a significant proportion of food insecure populations in the region. Therefore, there is a critical need for continuous search for sources of resistant genes against the virus. The objective of this study was to evaluate the reactions of some locally adapted cowpea accessions to CPMMV.

MATERIALS AND METHODS

Virus source, identification and maintenance

The isolate of CPMMV used was obtained from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Virus identity was confirmed by antigen-coated plate enzyme-linked immunosorbent assay (ACP-ELISA) (Koenig, 1981). The virus was extracted by grinding infected leaves with cold carbonate buffer, pH 9.6 (0.015 M sodium carbonate plus 0.0349 M sodium bicarbonate per litre of distilled water), at a ratio of 1:10 (w/v) using cold sterilized mortars and pestles.

One hundred microlitres each of the virus sap, together with diseased, negative and buffer controls was loaded into duplicate wells of the polystyrene microtitre ELISA plate (Thermo Scientific "Nunc", Milford, MA). The plate was incubated at 37 ^{o}C for 1 h, washed thrice at three min intervals with phosphate buffered saline-Tween (8 g NaCl, 1.1 g Na_2HPO_4, 0.2 g KH_2PO4, 0.2 g KCl, 0.5 mL Tween-20, 1 L distilled water, pH 7.4) (PBS-T) and tap-dried. A solution of 3 % (w/v) dried nonfat skimmed milk in PBS-T was applied at the rate of 200 µl/well as a blocking solution. After incubating at 37 ^{o}C for 30 min the plate was emptied and tap-dried. This was followed by addition of 100 µl of the polyclonal antibody diluted (1:10, 000; v/v) in conjugate buffer [half strength PBS-T containing 0.05 % (v/v) Tween-20, 0.02 % (w/v) egg albumin, 0.2 % (w/v) polyvinylpyrolidone]. The polyclonal antibody raised against a Nigerian CPMMV isolate at the Virology and Molecular Diagnostics Unit, IITA, Ibadan was kindly provided by Dr. P. Lava Kumar (IITA, Ibadan). Healthy cowpea leaf ground with conjugate buffer (1:20) was incubated at 37 ^{o}C for 30 min. One hundred microlitres of the above was loaded into each well and the plate was incubated at 37 ^{o}C for 1 h. After washing three times100 µl of the goat anti-rabbit diluted with conjugate buffer (1:15,000) was added to each well and the plate was incubated at 37 ^{o}C for 1 h. The plate was washed and tap-dried again. Substrate was prepared using *p*-nitrophenyl phosphate dissolved in substrate buffer (97 ml diethanolamine, 1000 ml H_2O, pH 9.8) at the rate of 1 mg/ml and 100 µl of this was added to each well. The plate was incubated in dark at room temperature (37 ^{o}C). Absorbance readings were taken at 405 nm using a microplate reader (MRX, Dynex Technologies, Inc., USA) after 1 h. Values were accepted to be positive when the absorbance values were at least twice that of the mean for the negative control. Excess leaf tissue after serological analysis was maintained at 4 ^{o}C in sealed vials over anhydrous $CaCl_2$. This isolate was used for subsequent inoculations of the evaluated cowpea genotypes.

Seed source, sowing, sap inoculation and ELISA test

Five locally adapted cowpea accessions (IT04K-217-5, IT07K-251-3-3, IT07K-299-4, IT07K-299-6 and IT99K-1060), as well as a susceptible control (Ife Brown) obtained from the germplasm of IITA were evaluated under screenhouse conditions (28 – 39 ^{o}C), in two separate trials. The genotypes were arranged in completely randomised design with four replicates. Seeds were sown in 23-cm-diameter plastic pots filled with heat-sterilized loamy soil. Uninoculated control plants of each genotype were also established as described above. At the time of inoculation, the virus was recovered from the dehydrated leaf tissue by grinding in extraction buffer, pH 7.2 (0.1M sodium phosphate dibasic, 0.1M potassium phosphate monobasic, 0.01M ethylene diamine tetra acetic acid and 0.001M L-cysteine per litre of distilled water). A drop of 2-mercapto ethanol (β- mercapto ethanol) was added to the buffer just before inoculation. Grinding was done as earlier described. Seedlings were inoculated with the virus at the three-leaf-stage (10 days after sowing) by rubbing the upper surface of Carborundum-dusted (600 mesh) trifoliate leaves with the sap. Excess inoculum was washed off with distilled water and the plants were observed daily for symptoms expression. Plants were sprayed weekly with an insecticide (Cypermethrin 10% E.C.). At 6 WAI virus concentration in the leaves of the inoculated plants was analysed by ACP-ELISA as described above. For each genotype, three leaflets from the topmost (designated as leaf 1, 2 and 3) and lower (designated as leaf 4, 5 and 6) leaves were evaluated.

Data collection and analyses

Disease incidence, disease severity, number of leaf per plant, plant height and seed weight were recorded. Disease severity was assessed as percentage of leaf area exhibiting virus symptoms according to the rating scale of Arif and Hassan, (2002), where:

1 = no symptoms (apparently healthy plant);
2 = slightly mosaic leaves (10 – 30 %);
3 = mosaic (31 – 50 %) and leaf distortion;
4 = severe mosaic (51 – 70 %), leaf distortion and stunting;
5 = severe mosaic (>70 %), stunting and death of plants.

The disease severity data were subjected to Area Under Disease Progress Curve (AUDPC) (Shaner and Finney, 1977) for resistance class determination. Reductions in the yield and agronomic traits of the inoculated plants were analysed using analysis of variance (ANOVA) according to Gomez and Gomez (1984). Significant differences were separated by Student-Newman-Keuls (SNK) test at p=0.05. Statistical analyses were accomplished using statistical analysis system (SAS, 2008).

RESULTS

All the healthy uninoculated plants showed normal growth and development and there was no case of leaf discolouration. Conversely, those infected with the virus exhibited distinct variation in terms of leaf discolouration, growth and development. Typical leaf mottling symptom of CPMMV infection was first noted 10 days after inoculation and genotypic reactions were consistent in both trials. Although none of them was immune to infection symptoms expression varied among the genotypes. The genotypes IT07K-251-3-3, IT07K-299-4 and IT07K-299-6 were classified as partially tolerant, whereas the remaining ones were susceptible to infection (Fig. 1).

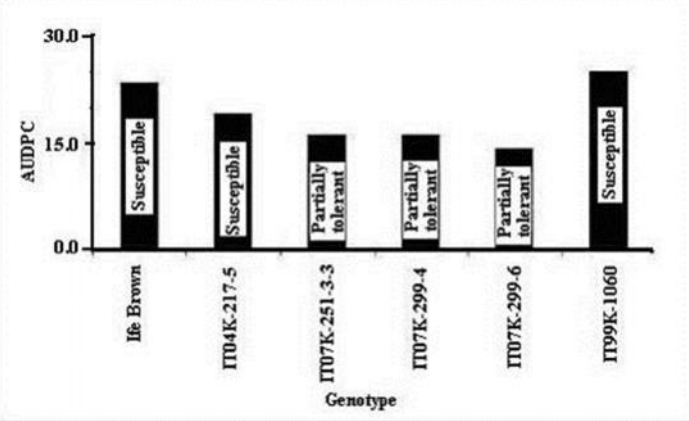

FIGURE 1: Area Under Disease Progress Curve (AUDPC) and resistant classes of cowpea genotypes infected with *Cowpea mild mottle virus* 6 weeks after inoculation in a screenhouse

In addition to chlorosis, the leaves of susceptible plants were distorted. In the partially tolerant ones symptom expression was less marked compared to the susceptible genotypes. Amongst those classified as partially tolerant symptom severity was mildest in IT07K-299-6 while IT99K-1060 showed the greatest typical symptoms of the disease. Prior to inoculation all the plants produced equal number of leaves. Similarly, the number of leaves per plant remained uniform one week post inoculation. Within the healthy uninoculated plants the highest number of leaf per plant came from IT07K-299-6 while Ife Brown and IT99K-1060 produced the lowest. With the exception of IT07K-299-6, genotypic variation in leaf number became noticeable from two weeks post inoculation and this continued till the end of evaluation. Although generally, there was no significant (p>0.05) difference in mean leaf number between uninoculated and infected plants, slightly higher values were observed in the former. Considering only those infected with the virus, the highest and lowest leaf number was observed in IT07K-299-6 and IT99K-1060, respectively (Fig. 2A).

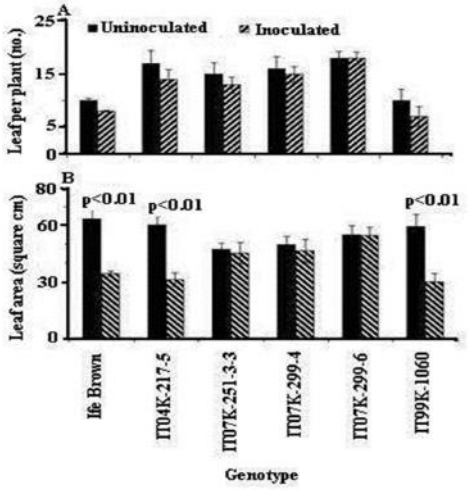

FIGURE 2: Mean number of leaves per plant (A) and leaf area (B) from uninoculated and *Cowpea mild mottle virus*-inoculated cowpea genotypes 6 weeks after inoculation in a screenhouse
Bars are means ± standard deviation

The genotypes exhibited strong variation in leaf area development. Substantial differences were found within the healthy uninoculated plants as well as those infected with CPMMV. In the susceptible genotypes, leaf area of healthy uninoculated was significantly (p<0.05) higher than the inoculated plants. In contrast, the difference in leaf area between uninoculated and infected plants was not significant in the tolerant ones (Fig. 2B). The leaves of inoculated Ife Brown and IT99K-1060 were conspicuously narrow and twisted. Reductions in leaf area varied significantly among the inoculated plants such that the lowest leaf area reduction came from the most tolerant accession IT07K-299-6, whereas one of the susceptible genotypes IT04K-217-5 suffered the greatest (Fig. 2C). Plant height was impaired by the virus and the magnitude of effects varied with genotypes. Irrespective of the genotype, uninoculated plants exhibited normal growth and were taller than those infected with CPMMV. The differences which became evident from the second week after inoculation were sustained for the rest of the study period. Some plants of the susceptible genotypes were stunted, with poor growth appearance and short

internodes. These observations were evident in the susceptible genotypes (Fig. 3A). Plant height reduction was most pronounced in the susceptible check (Ife Brown), whereas IT07K-299-6 exhibited the lowest. However, the height reduction recorded in Ife Brown was not significantly different from those in IT04K-217-5 and IT99K-1060. Plant height reductions were also at par among the partially tolerant genotypes (Fig. 3B).

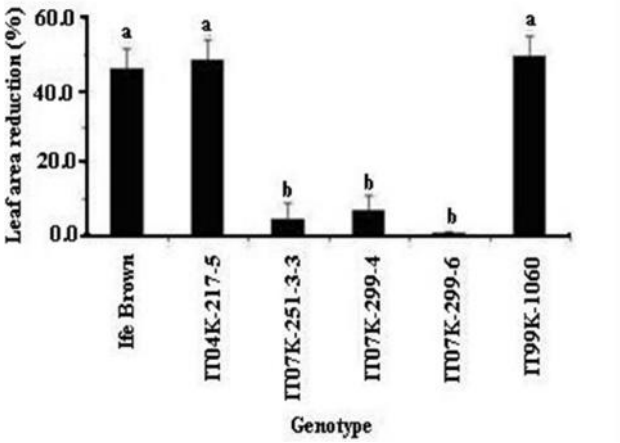

FIGURE 2C: Reductions in leaf area of cowpea genotypes infected with *Cowpea mild mottle virus* 6 weeks after inoculation in a screenhouse

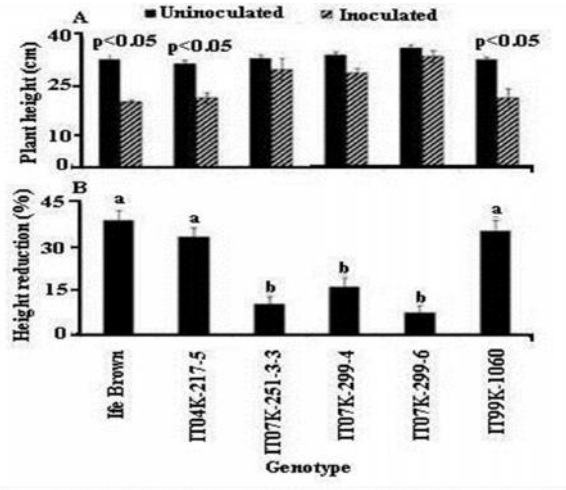

FIGURE 3: Plant heights of uninoculated and *Cowpea mild mottle virus*-inoculated cowpea genotypes (A) and height reduction after sap inoculation (B) in a screenhouse

Bars are means ± standard deviation; Bars with the same letter do not differ significantly (p=0.05) according to Student-Newman-Keuls (SNK) test

TABLE 1: Analysis of accumulation of *Cowpea mild mottle virus* (CPMMV) by antigen coated plate enzyme-linked immunosorbent assay (ACP-ELISA) in topmost and lower trifoliate leaves of cowpea plants 6 weeks after inoculation in a screenhouse

	Ife Brown		IT04K-217-5		IT07K-251-3-3		IT07K-299-4		IT07K-299-6		IT99K-1060	
[a]Leaf	[b]Abs	[c]Rxn	Abs	Rxn	Abs	Rxn	Abs	Rxn	Abs	Rxn	Abs	Rxn
1	0.352	++	0.352	++	0.113	-	0.113	-	0.110	-	0.361	++
2	0.357	++	0.352	++	0.113	-	0.113	-	0.111	-	0.370	++
3	0.358	++	0.358	++	0.115	-	0.119	-	0.116	-	0.395	++
4	0.476	++	0.362	++	0.289	+	0.311	+	0.247	+	0.478	++
5	0.476	++	0.363	++	0.289	+	0.311	+	0.247	+	0.479	++
6	0.478	++	0.364	++	0.289	+	0.313	+	0.247	+	0.479	++
DC	0.718											
HC	0.117											
BC	0.111											

DC, HC, and BC = Diseased, Healthy, and Buffer control, respectively

[a]Leaf: 1, 2, and 3 = right, middle and left leaflet, respectively of the topmost trifoliate leaves; 4, 5, and 6 = right, middle and left leaflet of the lower trifoliate leaves

[b]Abs = Absorbance values (405 nm) recorded 1 hour after incubation with substrate. Values are average of two wells each

[c]Rxn = Reaction: - = negative; + = positive (absorbance of the healthy control ×2); ++ = positive (absorbance of the healthy control ×3)

Genotypic differences for seed weight per plant were found within the healthy and inoculated plants. The trend was as observed in plant height. In the susceptible genotypes the seed weights per plant from uninoculated plants were significantly heavier than the CPMMV-inoculated (Fig. 4A). The inoculated plants of Ife Brown, IT04K-217-5 and IT99K-1060 produced tiny and unmarketable seeds. Within the healthy uninoculated plants, the highest seed weight was found in IT07K-299-6, while the lowest came from Ife Brown. Reductions in seed weights were not significant among the partially tolerant genotypes but the lowest was found in IT07K-299-6. On the other hand, the most susceptible IT99K-1060 suffered the greatest reduction in seed weight. The value obtained in Ife Brown was statistically similar to that in IT04K-217-5 (Fig. 4B).Virus accumulation varied considerably among the inoculated plants. A common phenomenon was the detection of higher virus concentration in the lower leaves, compared to the topmost ones. Plants from the susceptible genotypes showed higher absorbance values compared to the tolerant ones. In the former virus content was consistently high and all the leaves were ELISA-positive. Virus titre was highest in the lower leaf of IT99K-1060. As for the tolerant ones, virus concentration was lower in the topmost compared to the lower leaves. Additionally, only the lower leaves were ELISA-positive. Even in those that were positive virus titre was much lower compared to the lower leaves of the susceptible genotypes. Of the three partially tolerant accessions IT07K-299-6 exhibited the lowest range of virus concentration (Table 1).

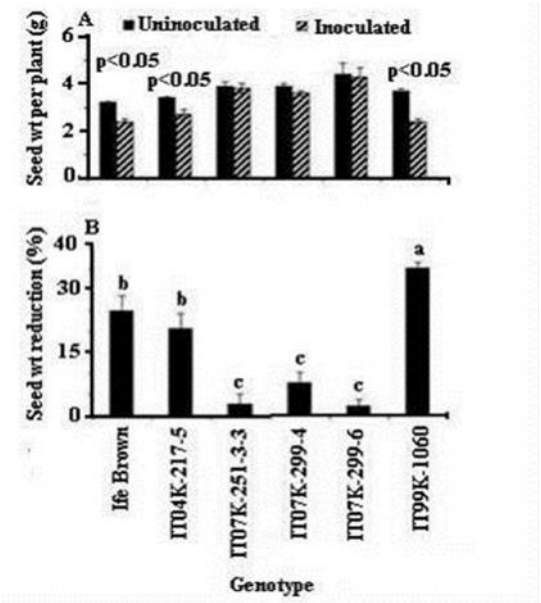

FIGURE 4: Seed weight per plant from uninoculated and *Cowpea mild mottle virus*-inoculated cowpea genotypes (A) and seed weight reduction after inoculation (B) in a screenhouse

DISCUSSION & CONCLUSION

Cowpea is a staple food source for millions of people in sub-Saharan Africa. However, productivity is partly constrained by several viruses including CPMMV. The use of CPMMV-tolerant cultivars has been cited as one of the major strategies, among an array of options, to increase cowpea yields. However, the potential success is premised on the availability of sources of resistance and the incorporation of the resistance genes into the local germplasm to develop resistant cultivars. The morphological and yield reductions observed in the inoculated plants underscore the adverse effect of CPMMV infection on cowpea. This result agrees with the report by El-Hassan *et al*. (1997) who documented that the virus induced serious yield losses in groundnut fields. The fact that all the inoculated plants exhibited typical leaf mottling symptom of CPMMV implies that none of the cowpea genotypes evaluated was immune to the disease. However, the differences exhibited by the various genotypes could have resulted from the variation in their genetic architecture. It is a common phenomenon that a series of physiological changes are triggered as soon as a virus is introduced into a host plant such that susceptibility or resistance depends largely on the genetic background of the invaded plant. Disease severity was lowest in IT07K-299-6 probably because of the presence of resistance genes. Conversely, the greatest disease severity recorded in the genotype IT99K-1060 reveals its vulnerability to CPMMV infection. In line with the result herein, the tendency of the youngest leaves to exhibit mild symptom at advanced growth stage has been reported in some maize genotypes infected with *Maize streak virus* (MSV) (Salaudeen *et al*., 2010). In the partially tolerant genotypes virus content was lower in the topmost leaflets compared to the lower ones possibly due to the fact that the latter were the first point of contact following inoculation and replication of the virus. This observation is supported by Fraser (1990). Virus translocation from the region of inoculation is facilitated by cell-to-cell movement of its particles following replication and establishment (Fraser, 1990). Systemic and upward movement of virus particles in the partially tolerant genotypes could have been constrained by host factors. This is in agreement with reports by Lazarowitz and Beachy (1999).

The lower number of leaves observed in the inoculated plants reveals the deleterious impact of CPMMV disease on the tested cowpea genotypes. This corroborates the earlier work of Kareem and Taiwo (2007) who reported fewer leaves from cowpea plants infected with *Cowpea mottle virus* (CMeV). Significant greater leaf area was observed in uninoculated plants of some genotypes as a result of adverse effect of CPMMV infection on the inoculated ones. Because the variation observed in leaf area reduction varied with resistance classes it could be postulated that there was a positive correlation between the symptom severity and agronomic performance. The low leaf area reduction in the partially tolerant genotypes suggests strong expression of CPMMV tolerance gene(s) and leaf size. Leaf number and area are important morphological attributes when selecting cowpea for improvement and /or cultivation because of their direct relationship with photosynthesis. These in turn influence haulm and grain yields. The serological test showing negative reactions of some topmost leaves is similar to the observations reported by Ariyaratne *et al*. (1996) for symptomless leaves of *Capsicum* plants infected with *Tobacco etch virus* (TEV). Moreover, the detection of higher virus concentration in the lower leaves indicates a positive correlation between visual symptoms scoring and serological test. A similar phenomenon was encountered by Thomas *et al*. (2000) in some potato lines infected with *Potato leafroll virus*. The highest virus concentration detected in IT99K-1060 could be attributed to its poor genetic background and vulnerability to CPMMV infection. Conversely, virus accumulation was lowest in IT07K-299-6 probably because of the inherent CPMMV-tolerance gene (s). The lowest virus titer found in the most partially tolerant cowpea genotype (IT07K-299-6) is

similar to the previous finding of Pilowsky and Cohen (1974) when some tomato plants were challenged with TYLCV.

Circumstances where uninoculated plants were significantly taller than the CPMMV-inoculated resulted from deleterious impact of the virus. However, the nonsignificant height difference between uninoculated and infected plants in the partially tolerant genotypes indicates that the latter really possess CPMMV resistance genes. This finding corroborates that reported by Kareem and Taiwo (2007) who noted nonsignificant height difference between healthy cowpea plants and those inoculated with SBMV at 10 days after sowing. As observed in this study, Pio-Ribeiro *et al*. (1978) elucidated that height reduction arises from combined effects of reduced internode and stunting in diseased plants. In addition, it could be speculated that height reduction was very conspicuous in the susceptible genotypes due to cumulative effects of reduced leaf number and area induced by the pathogen. In the partially tolerant genotypes, seed weights of the CPMMV-inoculated plants were similar to their healthy counterparts because of the mild impacts of the virus on the yield and yield components. Conversely, vulnerability of the susceptible ones could be attributed to the combined negative effects of the pathogen on their morphological characters. Although none of the accessions was immune to infection, IT07K-251-3-3, IT07K-299-4 and IT07K-299-6 most likely contain genes that confer tolerance to CPMMV and could be useful in cowpea breeding programmes.

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