Original Research Article

A survey of occurrence and identification of viruses infecting *Musa* species in Cameroon

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A B S T R A C T

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Banana and plantain are important staples cultivated in Cameroon. Their annual production is constrained by diseases especially those of viral etiology. Between September and June, 196 banana leaf samples with 137 (69.9 %) showing chlorotic, mosaic, stunting, streaking and heart-rot disease symptoms were collected from 68 farms in 32 villages in four banana-growing regions of Cameroon. The samples were indexed serologically by enzyme-linked immunosorbent assay, protein A antibody sandwich enzyme-linked immunosorbent assay, triple antibody sandwich enzyme-linked immunosorbent assay, polymerase chain reaction, biological assays, cloning and sequencing for the Musa viruses *banana streak Badnavirus, cucumber mosaic Cucumovirus*, *banana mild mosaic Potexvirus/Foveavirus* and *banana bunchy top Nanovirus*. Polyclonal antibody used for CMV assay was that of a cowpea isolate; that for BSV was of a Nigeria banana isolate and for BBTV and BanMMV. These assays confirmed that 34 samples (17.4 %) were infected 18 (9.2 %), 10 (5.1 %) and 6 (3.1 %) with CMV, BBTV and BSV, respectively. Plants of all four regions were infected with virus where the Southwest had the highest incidence i.e. 9 out of 26 samples (34 %), followed by the West region with 19.2 %. None of the samples tested positive for BanMMV while CMV and BSV were detected in samples from all four regions. BBTV was confined to the West region. Two samples were mix-infected with BSV and CMV and one with two isolates of BBTV (1 and 10) and CMV. Plant samples with the highest positive value for CMV were used for biological and biochemical tests. Chlorosis, mosaic, stunting and necrotic symptoms were recorded on 75 % and 60 % *Nicotiana glutinosa* and *Vigna unguiculata* (TVu 2657) test plants, respectively. All symptomatic plants tested positive for CMV 18 days post-inoculation. The expected band size of 600 bp was obtained on 1 % agarose gel electrophoresis after running immunocapture-reverse transcriptase-polymerase chain reaction using CMV antibody as trapping antibody and oligonucleotide primers specific for a 485 nucleotide sequence corresponding to the 3'-end coat protein and C-terminal non-coding region of RNA-3.

Introduction

Banana and plantain (*Musa* spp.) are the largest 'non woody' plants in the world that are by far the most important fruit crops in developing countries (Engelborgh *et al*., 2004). They are propagated vegetatively and grown both as staple food and major cash crop for local and export markets, throughout the tropical and sub-tropical regions of the world (Swennen and Jain, 2004). Together in the developing regions, they constitute the fourth most important global food commodity in terms of gross value of production after rice, wheat and maize (Frison *et al*., 1997). They are first in terms of fruit production and in addition, these two crops are endowed with such qualities as high rate of transformation of the fruit relative to other fruit types (Stover and Simmonds, 1987). Banana and plantain play an important role in the nutritional and economic status of millions of families in the tropical countries of Africa, Asia, Latin America and Oceania (Persley and de Langhe, 1999). World production of these crops is approximately 18 million metric tonnes with East, West and Central Africa accounting for over 6.1 mt (34%). Cameroon is ranked 4th in banana production in Africa and 3rd in plantain with annual production of 3 mt (FAO, 1998).

Production of these crops is limited by such biotic agents as insect and nematode pests but more importantly fungi, bacteria and viruses (Helliot *et al*., 2004). Viruses are assuming greater importance in *Musa* cultivation due to the current worldwide movement of its germplasm such as in-vitro plantlets throughout the tropical and sub-tropical regions. Also, intensification of production of these crops during the past twenty years has caused an increase in number of 'new' diseases that can cause a significant reduction in yield (Ortiz, 1995).

The presence of viruses on these crops affect not only yield but also fruit quality causing symptoms like unthrifty growth, streaking, heart rot, chlorosis and mosaic. Among the viruses that infect banana and plantain, *Banana bunchy top virus* (BBTV, *Nanovirus*) (Thomas and Dietzen, 1991) is of utmost importance as it is responsible for up to 70% yield loss in some *Musa* varieties, followed by *Banana bract mosaic virus* (BBrMV; *Potyvirus*). Other viruses which are also of importance are *Banana streak virus* (BSV; *Badnavirus*) (Lockhart, 1986), *Cucumber mosaic virus* (CMV *Cucumovirus*) (Pieterson and Thomas, 2001) and *Banana mild mosaic virus* (BanMMV), *Potexvirus/Foveavirus* (Gambley and Thomas, 2000). *Banana dieback virus* is recently identified on *Musa* in Nigeria (Hughes and Thomas, 1998).

Very little is known about the status of these viruses and may be other 'new' viruses infecting banana and plantain in the Southwest, Littoral, Northwest and West regions which are important banana-growing areas of Cameroon. We sought to determine prevalence and incidence of *Musa* viruses in these regions in order to assist in developing and establishing appropriate control measures for the virus diseases as well as assessing their importance with respect to plant quarantine measures and safe international movement of *Musa* germplasm.

Materials and Methods

The survey site

The survey was carried out between September and June. Using the geographical information system (GIS) equipment, the four regions of Cameroon surveyed lied within 5.61 E to 11.26 E and 5.10 N to 7.16 N with altitude ranging from 6 m to 2658 m

above sea level. The vegetation ranged from tropical forest to grassland and the site was generally densely populated with a total area of about 78.039 Km^2 (i.e. 20,248 km^2, 25,410 km^2, 18,611.7 km^2 and 13,770.5 km^2 for the Littoral, Southwest, Northwest and West regions, respectively) (Figure 1).

Sampling and virus identification

Leaf samples from naturally-infected *Musa* plants showing chlorotic, necrotic, unthrifty growth, streak, heart rot as well as asymptomatic symptoms (Figure 2) were randomly collected from 68 farms (i.e. 20, 35, 6, 7) and in 32 villages (8, 13, 5 and 6) from the Northwest, West, Littoral and Southwest regions, respectively in the four banana and plantain growing regions of Cameroon. The samples were placed in either labeled polythene sample bags or small bottles containing cotton on calcium chloride and further in an ice box and transported to the laboratory. Symptom type and the number of mats with symptom per farm were recorded. The proportion of the number of infected samples to the total number of samples collected was calculated to determine disease incidence.

The four viruses BSV, CMV, BBTV and BanMMV were indexed serologically to confirm their presence in the samples. Triple antibody sandwich indirect enzyme-linked immunosorbent assay (TAS-ELISA) tested for BSV and BBTV using rabbit polyclonal antibody for trapping and mouse monoclonal antibody for detection while Protein A antibody sandwich indirect enzyme-linked immunosorbent assay (PAS-ELISA) tested for CMV.

The CMV isolate was further characterized by biological assays, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), polymerase chain reaction (PCR) and sequencing.

Virus indexing

BBTV and BSV were indexed in field samples by TAS-ELISA (Ndowora *et al*., 1999), where polyclonal antibodies for BBTV and BSV were diluted separately at 1:1000 in coating buffer (1.59 g sodium carbonate; 2.93 g sodium hydro-carbonate (monobasic) in 1 litre of distilled water), pH 9.6 and 100 µl loaded each into of the wells of 'Cornings'microtitre' plates and incubated (GN, Mechanical Convection Incubator, Precision Scientific Group, Illinois, USA) at 37 ℃ for 2-3 hr. Approximately 1g each of banana leaf sample collected in survey was ground separately (using a sterile mortar and pestle) in 10ml phosphate buffered saline-Tween (PBS-T) grinding buffer (0.01 M sodium phosphate; 15 mM sodium chloride, pH 7.4 containing 0.05 % Tween 20) containing 1 % w/v sodium sulfite and 2 % polyvinyl pyrrolidone) and loaded into their corresponding plates at 100 µl/well and incubated as earlier described. Trapped BSV antigens were detected using a 1:4000 dilution of polyclonal antibody/goat anti-mouse alkaline phosphatase conjugate in PBS-T and monoclonal antibody for BSV. The reactions were visualized on addition of enzyme substrate (p-nitrophenyl phosphate, Sigma, USA) diluted at 0.02 g/20 ml in substrate buffer and dispensed at 200 µl per well and the absorbance (A_{405nm}) read off after an hour and overnight on incubation of the plates at room temperature. Confirmed virus-free tissue culture banana material served as negative control while BSV-infected material as positive control.

ELISA data for both serological tests were used to compute the mean standard error ($\pm$ SE) of the absorbance (A_{405nm}) for each duplicate well (sample) and those that were two times greater than the mean of the healthy sample were considered positive

(Hughes *et al*., 2004). CMV and BanMMV were indexed in the samples by PAS-ELISA (Edwards and Cooper, 1972) similar to TAS- ELISA except that Microtitre plates (Cornings, Massachusetts, USA) were coated with 100µl of soluble protein antibody (Protein A) at 1µgml^{-1} in coating buffer before incubation. Antibody for both viruses was dispensed twice and also plates were incubated overnight after antigen was added. Lastly, the trapped CMV/BanMMV antigens were detected using a 1:4000 dilution of IgG goat anti-rabbit alkaline phosphatase conjugate in conjugate buffer.

Biological assay

Samples positive ($\geq$ x 5 A$_{405nm}$ of the healthy control) only for CMV by PAS-ELISA were bulk-ground in sterile mortar and pestle in 0.1 M phosphate buffer, pH 7.4, containing 0.1% 2-mercaptoethanol and 0.01 M L-cysteine and the sap mechanically inoculated onto the selected indicator plants *Nicotiana glutinosa and Vigna unguiculata* (10 plants per host species were raised under natural light in an insect-proof screened house at 24-31 °C). The leaves of carborundum-mesh-600 pre-dusted inoculated plants were rinsed almost immediately with water to wash off excess inoculum, while the control plants (3 test plants per host species) were rubbed only with the inoculation buffer. All plants were kept in an insect-proof screened house and the symptoms monitored regularly over a period of eight weeks and appropriately tested by PAS-ELISA.

Determination of the molecular weight of cucumber mosaic Cucumovirus coat protein by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The procedure (Leamnli, 1970) used a 10 % SDS-PAGE on a 4 % stacking (6.1 ml distilled water, 2.5 ml, 0.5 M tris-HCl pH 6.8, 100 µl 10 % SDS stock, 1.3 ml acrylamide, 50 µl of 10 % ammonium sulfate and 10 µl temed) and a 12.5 % separating gel (3.5 ml distilled water, 2.5 ml of 1.5 M tris-hydrochloric acid (HCL), pH 8.8, 100 µl of 10 % SDS stock, 4.0 ml acrylamide/bis, 50 µl of 10 % ammonium persulfate and 5 µl N, N, N', N' tetramethylethylenediamine (temed) in a Bio-Rad Mini Protean II electrophoresis cell.

A 1:3 dilution of partially-purified preparation of CMV/healthy banana tissue (Morrissey, 1989) and loading buffer were denatured (1.0 ml 0.5 M tris-HCL, pH 6.8; 8.0 ml glycerol; 4.0 ml distilled water; 0.04 ml mercaptoethanol; 1.6 ml 10 % (w/v) SDS and 0.2 ml bromothymol blue) and electrophoresed at 150 V for 45 minutes alongside low range protein molecular weight marker (M3913IVL, Marker Range 6,500-66,000, Sigma-Aldrich, Steinheim, Germany). The resolving gel was stained in 100 ml Coomasie solution (45 ml distilled water, 45 ml 100 % methanol and 0.25 g bromothymol blue) with gentle shaking for 3 hrs. then de-stained in 45 ml methanol, 45 ml distilled water and 10 ml 100 % glacial acetic acid in a 10 cm^3 plastic dish with shaking for 1 hr. when bands on the gel became conspicuous. The molecular weight of viral coat protein was estimated using a plot of logarithms of molecular weights of standard protein marker against the retention.

Further characterization of CMV in ELISA positive samples was by IC-RT-PCR. Sterile 0.5 ml eppendorf tubes coated with 100 µl CMV IgG in coating buffer at 1:3000 dilutions were incubation at 37 °C for 2 hours. Overnight-incubated tubes loaded with 200 ul of sap from CMV positive tissue at a rate of 0.05 g/ml were used for a one

step RT-PCR. The primers used for RT and amplification of CMV RNA were oligonucleotide primers (CMV-1 (5' GCC GTA AGC TGG ATG GAC AA 3') and CMV-2 (5'TAG GAT AAG CTT GTT TCG CG 3') specific for a 485 nucleotide sequence (EU428827) corresponding to 3' end coat protein and C-terminal non-coding region of RNA-3. Samples were designed to amplify a 600-bp product.

The 12.5 ul RT-PCR reaction volume of 12.5 µl contained 10 pmoles of each primer, 2 units of *Taq* polymerase (Promega, M830B, Go Taq DNA Polymerase, Madison WI, USA), 25 nmoles of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$ and 2.5 µl of 5X green buffer (Promega, M891A, %X Green Buffer Go Taq, Flexi Buffer, Madison WI, USA), 20 units of Reverse transcriptase (Promega, M170B, M-MLV Reverse Transcriptase, Madison WI, USA) and prior to commencing the PCR cycling parameters, there was a 42 °C incubation for 15 mins., followed by 95 °C for 4.5 mins. denaturation. Tubes were made up to volume with sterile distilled water. Thirty-five reaction cycles was performed with periods of 95 °C/42 s, 56 °C/45 s, 72 °C/1 min. and the final cycle of 72 °C for 10 mins. All amplifications were carried out in a PTC-200, Peltier Thermal Cycler (MJ Research, Watertown, Massachusetts, USA). A 1 % agarose gel electrophoresis separated PCR products which were observed under ultra violet light (UV). A photograph of the gel was obtained with an 8.2 megapixel digital camera (Kodak, UK).

Results and Discussion

Field disease symptoms and virus indexing of banana and plantain

Field virus-like disease symptoms on banana

plants included chlorosis, streaking, stunting, heart rot, unthrifty growth, browning and mosaic (Figure 2). The symptoms were found in all the regions surveyed but were more common in the West relative to the other regions. The disease survey showed that four viruses were present in 196 samples collected with 34 (17.4 %) infected with these 4 viruses, 18 (9.2 %) with *Cucumber mosaic virus* (CMV), 10 (5.1 %) with *Banana bunchy top virus* (BBTV) and 6 (3.1 %) with *Banana streak virus* (BSV) tested by enzyme-linked linked immune-sorbent assay (ELISA).

None of the samples tested positive for *Banana mild mosaic virus* (BanMMV) (Table 1). The Southwest region had the highest infection rate of 34 % (i.e. 9 of 26 samples) followed by West region with 19.2 %. Two samples were mixed-infected with BSV and CMV and one with two isolates of BBTV (1 and 10) and CMV (Figure 3). While CMV and BSV were detected in plant samples from all the regions, BBTV was confined to the West region.

Biological assay by mechanical inoculation on herbaceous test plants

Symptoms of virus disease on herbaceous plants mechanically inoculated with banana sap from samples that tested positive by ELISA included chlorotic lesions, mosaic, stunting and necrosis.

The first symptoms occurred 10 days after inoculation. Approximately 75 % of *Nicotiana glutinosa* and 60 % *Vigna unguiculata* (TVu 2576) test plants showed symptoms 18 days post inoculation (Figure 2 D). All the symptomatic *N. glutinosa* and *Vigna unguiculata* plants tested positive for CMV by PAS-ELISA.

Determination of the molecular weight of cucumber mosaic virus coat protein by immuno-capture reverse transcriptase-polymerase chain reaction

A well-defined band size approximately 29 K was obtained when a partial preparation (Morrissey, 1981) of this sample was run on Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3). A product of expected size (approximately 600-bp) was strongly amplified by one of the CMV positive samples while those for three amplifications were weak. No products were amplified from healthy banana plants (Figure 4).

A limited survey for *Musa* viruses in 1996-1997 in Southern Cameroon (Gauhl *et al.*, 1997) confirmed the presence of BSV on plantain samples and CMV on one asymptomatic sample. No banana sample was positive to any of these viruses. However, in this survey carried out in the wet season when it is known that virus symptoms are more apparent in *Musa* fields (Dahal *et al.*, 1998), typical BSV and CMV symptoms were observed in plants of both *Musa* species, more so findings with ELISA confirmed infection even though incidence was low (17.4 %) compared to 64 % for CMV reported in Nigeria (Dongo *et al.,* 2007).

Careful selection of virus-free planting material, use of resistant *Musa* varieties and farm sanitation may have been the reason for this discrepancy. This study contradicted the earlier hypothesis by Gauhl *et al*. (1997) that local *Musa* cultivars were resistant to BSV or less prone to symptom expression. During this time, no banana plant exhibited symptoms of virus disease but in our survey, 69 % of samples collected showed virus-like disease symptoms.

Symptomatic samples that tested negative to all antibodies may be due to genetic aberrations in the plant, low virus concentration in plant tissues that antibody could not detect, the diagnostic technique used was not sensitive enough for the virus concentration present in the sap or the conformational state of virus in tissues did not correspond with and could not be detected by the available antibody. Similar findings were reported on cassava that African cassava mosaic virus (ACMV) could not be detected in the lignified tissues of cassava stems except in young leaves of the new growth from same stems by the ELISA (Njock and Ndip, 2007), though these authors worked with a different virus-host system.

Not all exhibited on plants might have been induced by physical, chemical or other abiotic factors whose effects might be misconstrued for virus symptoms (Mathews, 1991). In addition, nutrient deficiency in plants induces abnormal coloration, discoloration and sometimes wilt, which sometimes can be misconstrued for virus infection. CMV was the most prevalent virus in this survey. This may be due to wide host range of this pathogen, also because of very active vectors of this virus and variable means of spread such as through the use of infected suckers.

Detection of two isolates of BBTV was a cause for concern because it is responsible for the most serious disease affecting banana worldwide. Yield losses caused by this pathogen can be as high as 70 % in some varieties. Since this virus was limited to West region, the initial control strategies required could quarantine and farm sanitation while in the long term breeding for resistance would be useful in managing the disease.

Table.1 Determination of the occurrence and distribution of *Banana streak*, *Cucumber mosaic*, *Banana mild mosaic* and *Banana bunchy top viruses* on banana samples by enzyme-linked immunosorbent assay between September and June

Region	Villages	Farms Visited	Samples Collected	Symptomatic Samples	Symptom Type	Number of Infected Plants (%)				
						BSV	CMV	BanMMV	BBTV-1	BBTV-10
Northwest	8	20	62	46	C, A, Sr, Ci	1	1	0	-	-
West	13	35	78	44	A, Bt, C, Ci, N,T, St	2	3	0	8	2
Southwest	6	7	26	21	C, A, Sr, N	2	7	0	-	-
Littoral	5	6	30	26	C, A, Sr, N, T	1	4	4	-	-
Total	**32**	**68**	**196**	**137**		**6**	**18**	**0**	**8**	**2**

A = asymptomatic; Bt = bunchy top; C = chlorosis; Ci = cigar leaf; N = necrosis; St = stunting; Sr = streaking; T = vein thickening

Figure.1 Map of Africa and Cameroon showing the four regions surveyed between September and June
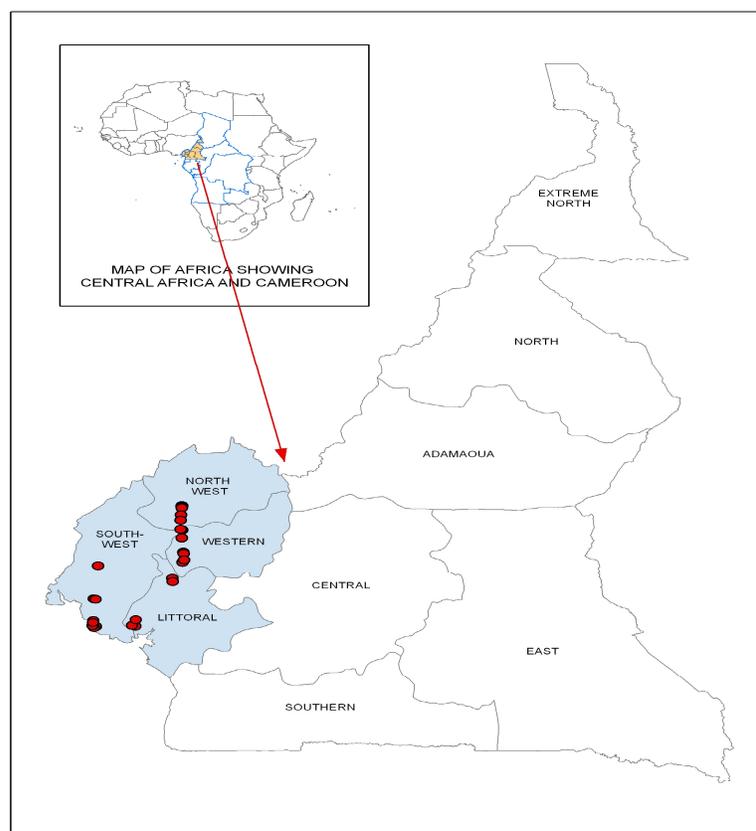
Figure.2 Virus symptoms: (A) necrotic streak by *Banana streak virus* on a banana leaf; (B) chlorosis on a banana leaf and (C) unthrifty banana plant growth characteristic of *Cucumber mosaic virus* infection; (D) *Vigna unguiculata* herbaceous test plant showing stunted and chlorotic leaves after sap inoculation with *cucumber mosaic virus*


Figure.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the coat protein of *Cucumber mosaic virus* (1) and protein molecular weight low range marker (2)
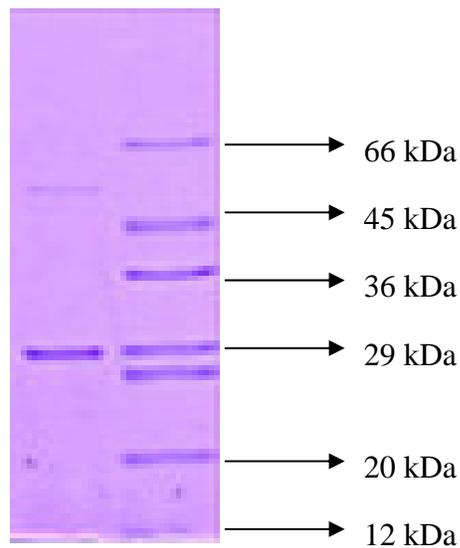
Figure.4 Agarose gel electrophoresis of immunocapture-reverse transcriptase-polymerase chain reaction products derived from banana leaf samples that tested positive by ELISA for *Cucumber mosaic virus* (lanes 5 to 9), healthy banana (lane 4) and banana samples marginally positive (less than 1.5 times mean of healthy sample (lanes 2 and 3) for CMV by ELISA and protein molecular weight low range marker (lane 1)
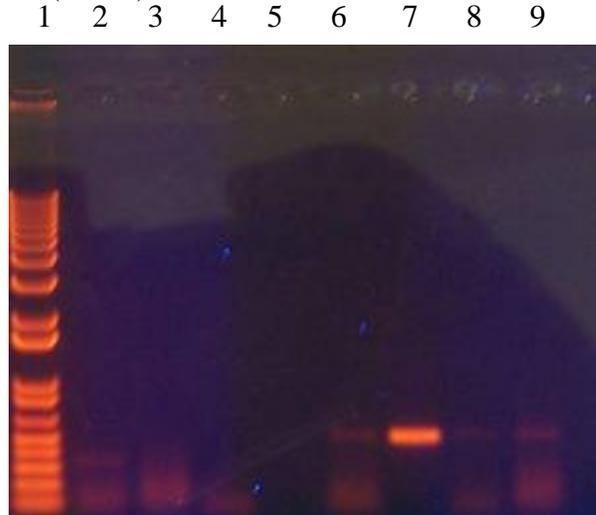
Figure.5 Number of mix-infected banana samples surveyed in September to June.
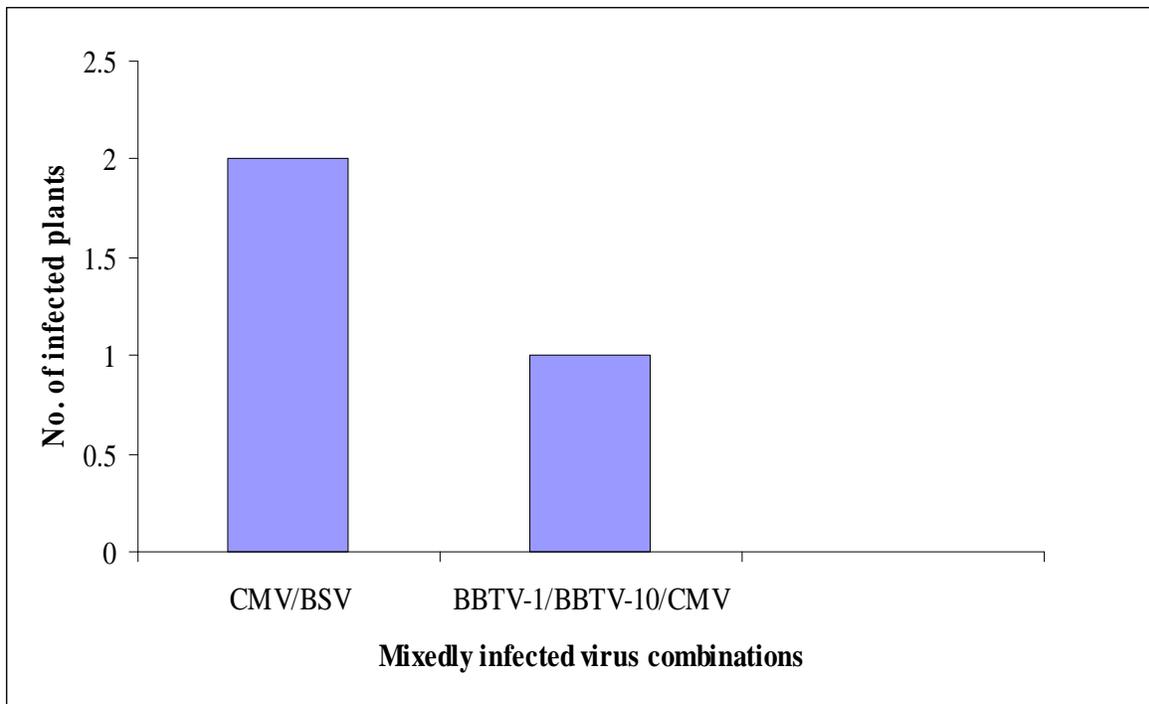
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