# Plant Pathology
## Journal

# Characterization of *Pepper yellow vein mali virus* in *Capsicum* sp. in Burkina Faso

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**Abstract:** The aetiology of a disease characterized by severe leaf curling and leaf distortion symptoms in peppers was studied in Burkina Faso. Surveys were conducted in fields planted with sweet pepper (*Capsicum annuum*) and hot pepper (*Capsicum frutescens*) to collect leaf samples and assess disease incidence. Collected samples were analyzed using biological tests (mechanical inoculation and whitefly transmission), triple-antibody-sandwich- enzyme linked-immunosorbent assay (TAS-ELISA), Polymerase Chain Reaction (PCR) and sequence analysis. Mechanical inoculation tests were negative while transmission by the whitefly *Bemisia tabaci* led to infection rates of more than 80%. TAS-ELISA and PCR tests indicated that symptoms observed were associated with begomovirus infections. Involved virus isolates showed pairwise nucleotide identity of more than 99% with *Pepper yellow vein mali virus* (PepYVMV) and formed with this virus a distinct clade in phylogenetic analyses. Altogether, these results indicated that PepYVMV was the causal agent of leaf curling symptoms in peppers.

**Key words:** Pepper, leaf curling, aetiology, *Bemisia tabaci* Genn., *Begomovirus*

## INTRODUCTION

Geminiviruses (family *Geminiviridae*) have emerged as economically important plant pathogens worldwide (Harrison and Robinson, 1999). The *Geminiviridae* family owns its name from a common feature shared by all virus member species which have twin or geminate particles. Most virus species in this family have bipartite genome, the two genomic components being called DNA-A and DNA-B both of which are circular and single-stranded DNA. DNA-A encodes functions necessary for viral replication and encapsidation, while genes on DNA-B are required for viral movement in plants (Lazarowitz, 1992). Some virus species have monopartite genomes and possess DNA-A component only. Part of monopartite begomoviruses have been found associated with a satellite molecule called DNAβ which function is not fully known. However, it was found that DNAβ plays an essential role in symptom induction by the begomovirus to which they are associated and in the increases of the level of viral nucleic acids in infected plants (Saunders *et al.*, 2000; Mansoor *et al.*, 2003).

The *Geminiviridae* family is composed of four virus genera (*Mastrevirus*, *Curtovirus*, *Begomovirus* and *Topocuvirus*) which have been distinguished in relation to host range, insect vector and genome organization (Stanley *et al.*, 2005). Begomoviruses have monopartite or bipartite genomes, are transmitted by the whitefly *Bemisia tabaci* Genn. and infect dicotyledonous plants. They formed the group of viruses formerly known as whitefly-transmitted geminiviruses (WTGs) (Harrison and Robinson, 1999). Common symptoms induced by begomoviruses include leaf-curling, mosaic, vein yellowing or more generalized leaf yellowing, often accompanied by stunting of infected plants and therefore severe yield losses. Besides their transmission by the same vector *B. tabaci*, begomoviruses exhibit close serological relationships among member species (Harrison and Robinson, 1999). This feature makes it possible to detect almost all begomoviruses with antibodies raised against a particular virus species of the genus. However, serology is not fully suitable for distinguishing between virus species. For this purpose, new molecular tools especially those targeting the viral genome had to be developed (Wyatt and Brown, 1996).

Early studies showed that tubers plants such as cassava (*Manihot esculenta* Crantz) and vegetables such as tomato (*Lycopersicon esculentum* L. Mill) and okra

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(*Abelmoshus esculentus* L. Moench) were severely affected by WTGs in different parts of Africa (Fargette *et al.*, 1987; Konate *et al.*, 1995; Thresh *et al.*, 1998). Since the last decade, with the emergence of the *Bemisia tabaci* in West Africa due to resistance to insecticides (Otoidobiga *et al.*, 2003), begomoviruses have become a major constraint to vegetable production in this region. Particularly, in Burkina Faso, new diseases most of which involved leaf curling symptoms appeared in peppers (*Capsicum* sp.). These symptoms resemble those induced by begomovirus infections and were clearly distinct from mosaic symptoms already reported to be caused by the *Pepper veinal mottle virus* in the country (Konaté and Traoré, 1999). Although pepper plantings were severely affected, the problem was not payed much attention and even the causal agent of observed the symptoms has not been clearly identified. The research described here was aimed at establishing the etiology of leaf curling symptoms commonly observed in pepper plantings in Burkina Faso.



Fig. 1: Map of Burkina Faso showing locations of collection of leaf samples

## MATERIALS AND METHODS

**Leaf samples collection:** During the years 2005 and 2006, pepper fields were surveyed in five localities of Burkina Faso (3 to 10 fields per locality) in some of the country main vegetable crops producing areas (Fig. 1). Virus samples were collected in sweet pepper (*Capsicum annuum* L.) and hot pepper (*Capsicum frutescens*) field plantings. Leaves were collected from symptomatic plants and some leaves were also taken from symptomless plants to be used as negative controls. Leaf samples were put in small sampling plastic bags maintained on ice for two to three days. Upon arrival in the laboratory, they were immediately analysed or kept at -20°C for future processing.

**Plant inoculation:** All transmission tests were done in an insect-proof greenhouse with temperature between 25 and 30°C and 80-90% relative humidity. Seeds of sweet pepper (*C. annuum* cv. Yolo Wonder) and hot pepper (*C. frutescens* cv. JBF) were sown in steamed-sterilized soil. Emerged seedlings were transplanted in plastic pots 30 days after sowing and inoculated 10 days later. In a first experiment, leaves from infected pepper plants were ground in 1 M phosphate buffer, pH 8.0 and the sap was subsequently rubbed onto leaves of healthy seedlings. In a second experiment, viruliferous adult insects (*B. tabaci*) were allowed a 48 h acquisition-access period on diseased plants of both *C. annuum* and *C. frutescens* collected from the fields. Three diseased plants per species and from distinct localities were used. Then, 20 insects were placed
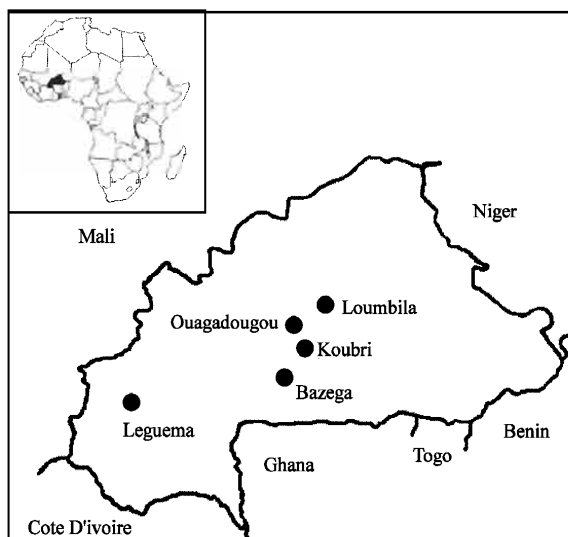
on healthy pepper seedlings maintained in individual transmission cages for an inoculation access-period of 48 h. Transmission tests from every diseased plant were done to five healthy seedlings of each of the two pepper species. Insects were subsequently removed and the plants were treated with a systemic pyrethroid insecticide. All inoculated plants were monitored for symptom development during 45 days post-inoculation.

**Seed transmission tests:** Seeds were collected form field-infected pepper plants (*C. annuum* and *C. frutescens*) and air-dried for three months. A total of 1000 seeds (500 seeds per pepper species) were subsequently sown in sterile soil. After germination, seedlings were maintained in the greenhouse for two months to monitor any development of symptoms.

**Serological tests:** Leaf samples were analyzed by triple antibody sandwich enzyme linked-immunosorbent assay (TAS-ELISA), as described in Zhou *et al.* (1997). The monoclonal antibodies (MAbs) to *African cassava mosaic virus* (ACMV) and *Okra leaf curl virus* (OkLCuV) were kindly provided by Dr. B.D. Harrison, Scottish Crop Research Institute, UK. Absorbance readings were recorded at 405 nm using a Metertech Σ960 automatic microplate reader.

**Nucleic acid extraction, PCR and sequencing:** Total nucleic acids were extracted from two selected leaf samples using the CTAB method as described by Doyle and Doyle (1987). Polymerase Chain Reaction (PCR) tests

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Table 1: List of begomoviruses from Genbank database used in phylogenetic analyses

| Virus/Isolate | Abbreviation | Accession No. |
|---|---|---|
| *Pepper yellow vein Mali virus* | PepYVMV | AM691548 |
| *Tobacco leaf curl Zimbabwe virus* | ToLCZV | AM701756 |
| *Tomato curly stunt virus* | TomCuSV | AF261885 |
| *Tomato leaf curl Madagascar virus* | TomLCMaV | AJ865338 |
| *Tomato yellow leaf curl Mali virus _Ethiopia* | TYLCMV Eth | DQ358913 |
| *Tomato yellow leaf curl virus Mild _Spain* | TYLCVMild | AF071228 |
| *Tomato yellow leaf curl Mali virus* | TYLCMV | AY502934 |
| *Tomato yellow leaf curl virus Iran* | TYLCVIran | AJ132711 |
| *Tomato yellow leaf curl virus Cucumber* | TYLCVCu | EF433426 |
| *South African Cassava mosaic virus ZW* | SACMVZW | AJ575560 |
| *South African Cassava mosaic virus M12* | SACMVM12 | AJ422132 |
| *East African Cassava mosaic virus Zanzibar* | EACMVZan | AF422174 |
| *East African Cassava mosaic virus K12* | EACMVK12 | AJ717561 |
| *Okra yellow crinkle virus Mali* | OkYCVM | EU024118 |
| *Mimosa yellow leaf curl virus* | MiYLCV | DQ641695 |
| *Ageratum enation virus* | AgENV | AJ437618 |
| *Tomato yellow leaf curl virus* from USA | TYLCVUSA | EF110890 |
| *Tomato yellow leaf curl virus* from Jordan | TYLCVJor | EF054893 |
| *Tomato yellow leaf curl virus* from Uganda | TYLCVUg | DQ127170 |
| *Tomato yellow leaf curl virus Sardinia* | TYLCVSar | L27708 |

were performed using degenerate primers designed for amplification of the core region of begomoviruses coat protein located on DNA-A. Two pairs of primers (P1 and P2) were used. Primer pair P1 consisted of primers PTYv369 (5'-ACGCCCGCTCGAAGGTTCG-3') and PTYc1023 (5'-CGTCAGCCATATACAATAACAA GGC-3'. Primer pair P2 comprised primers V324 (5'-GCC(CT)AT(GA)TA(TC)AG(AG)AAGCC(AC)AG-3')and C889 (5'GG(AG)TT(ATG)GA (GA)GCATG(TCA)GTAC ATG-3'). PCR conditions were those described in Navot *et al.* (1991) and in Wyatt and Brown (1996), respectively for primers pairs P1 and P2. To check amplifications, 10 μL of PCR products were electrophoresed in 1% agarose gels, subsequently stained with ethidium bromide and viewed under UV transillumination. PCR products were sequenced using the *Taq* terminator sequencing kit (Applied Biosystems) and analyzed on an Applied Biosystems 373A sequencer. To get adequate sequence accuracy, two readings per base (in the 3' to 5' and 5' to 3' directions) were done (Fargette *et al.*, 2004). Sequences obtained were assembled by Seqman (DNASTAR) and a blast search (www interface) was performed to retrieve homologous sequences. The database accession numbers of the begomovirus sequences retrieved are indicated in Table 1. Then, the whole corpus of sequences was aligned by Clustal W (Thompson *et al.*, 1994) and the phylogenetic software Paup (Swofford, 2000) was used to analyse phylogenetic relationships between begomoviruses in the corpus.

## RESULTS

**Description of field symptoms:** The most prominent symptoms were severe distortions of the leaves of infected plants (Fig. 2). There was an overall upward curling of the leaves especially when the plants were infected at an early stage. When infection happened in late stages, symptoms were always present on top leaves whereas basal leaves maintained a relatively normal shape. In addition to leaf curling, leaf distortions also included formation of several swellings with dark green margins resembling mosaic symptoms (Fig. 2a). At the whole plant scale, infected plants were stunted, giving a stacked aspect to the leaves. Moreover, infected plants produced smaller fruits with irregular maturity. Most of the time, disease incidence reached 100% in pepper fields surveyed, regardless of the pepper species and the localities of surveys. However, marked differences were noticed in disease severity, indicating discrepancies in time of disease establishment between fields.

**Transmission tests:** No plant inoculated mechanically developed any disease symptom. Inoculated plants showed aspects similar to that of healthy uninoculated plants. By contrast, in transmission test through the whitefly *B. tabaci*, inoculated seedlings of sweet and hot peppers showed symptoms which were similar to those observed on field infected plants. Transmission tests were highly successful as infection rates were between 80 and 100%, regardless of the virus source and the tested plants. In almost all cases, infected plants become stunted and produce no fruit. When seeds from diseased plants were sown, emerged plantlets remained healthy throughout the experiment, indicating the absence of seed transmission.

**Begomovirus detection by serology:** MAbs directed to ACMV and OkLCuV reacted with all tested symptomatic samples in TAS-ELISA, indicating virus detection in these

Table 2: Detection of begomoviruses in infected pepper leaf extracts by TAS-ELISA using monoclonal antibodies (MAbs)[a]

| Leaf extracts[b] | Plant species | MAbs against ACMV | | | | | MAbs against OkLCuV | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | SCR 12 | SCR 14 | SCR 16 | SCR 25 | SCR 53 | B1 | B3 | C12 | C5 | C1 |
| S1 | *C. annum* | 5 | 2 | 3 | 0 | 0 | 0 | 5 | 0 | 1 | 0 |
| S2 | *C. annum* | 5 | 2 | 3 | 0 | 0 | 0 | 5 | 0 | 1 | 0 |
| S3 | *C. frutescens* | 2 | 2 | 2 | 0 | 0 | 0 | 5 | 0 | 1 | 0 |
| S4 | *C. annum* | 5 | 2 | 3 | 0 | 0 | 0 | 5 | 0 | 1 | 0 |
| S5 | *C. annum* | 5 | 2 | 3 | 0 | 0 | 0 | 5 | 0 | 1 | 0 |
| S6 | *C. frutescens* | 2 | 2 | 3 | 0 | 1 | 0 | 3 | 0 | 0 | 0 |
| S7 | *C. frutescens* | 2 | 1 | 2 | 0 | 0 | 0 | 4 | 0 | 1 | 0 |
| S8 | *C. frutescens* | 2 | 2 | 3 | 0 | 0 | 0 | 3 | 0 | 1 | 0 |
| S9 | *C. frutescens* | 2 | 3 | 3 | 0 | 0 | 0 | 5 | 0 | 1 | 0 |
| S10 | *C. annum* | 5 | 3 | 3 | 0 | 0 | 0 | 5 | 0 | 1 | 0 |
| OkLCuV-infected okra | Okra | 2 | 2 | 1 | 0 | 5 | 4 | 5 | 3 | 3 | 5 |
| ACMV-infected cassava | Cassava | 5 | 5 | 5 | 3 | 4 | 0 | 5 | 0 | 1 | 0 |
| Healthy controls | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a]: MAbs were directed to *African cassava mosaic virus* (ACMV) and *Okra leaf curl virus* (OkLCuV), respectively. Figures in the table are reaction scores that represent intensity of reactions as follows: 5 (A405 nm> 1.8), 4 (1.21-1.8), 3 (0.61-1,2), 2 (0.31-0.6), 1(0.15-0.3), 0 (<0.15), [b]: Leaf extracts of okra and cassava infected by OkLCuV and ACMV respectively, were used as positive controls. Healthy controls included leaf extracts of *C. annum*, *C. frutescens*, cassava and okra, respectively



Fig. 2: Field symptoms of severe distortions (a) and upward curling (b) of the leaves of diseased peppers
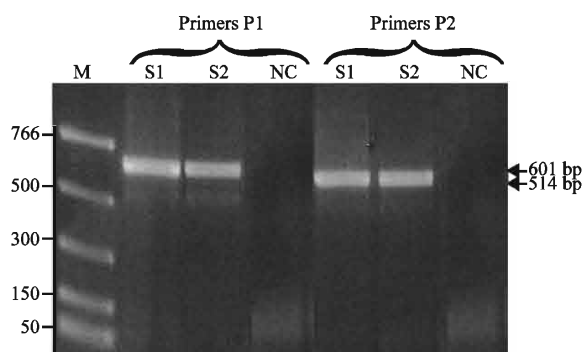


Fig. 3: PCR-amplified fragments from four virus samples (S3, S4, S6 and S10) visualized in ethidium-bromide-stained 1% agarose gel. PCR amplification was done using primers pairs P1 (PTYv369/PTYc1023) and P2 (V324/C889) both directed to the core region of begomoviruses. M: PCR markers (sizes in bp); S3 and S6: infected *Capsicum frutescens* cv. JBF; S4 and S10: infected *Capsicum annuum*; NC: negative controls (healthy *C. frutescens* and *C. annuum*, respectively). Expected fragments from each pair of primers are indicated by arrows

samples (Table 2). Reactions of respective healthy leaf extracts were all negative and allowed clear distinction from those of extracts from diseased leaves. As expected, OkLCuV-infected leaf extract reacted more strongly with anti-OkLCuV MAbs than anti-ACMV MAbs and vice versa. Both OkLCuV-infected and ACMV-infected extracts reacted differently from any of the symptomatic pepper leaf extracts. Half of the MAbs (SCR12, SCR14,

SCR16, B3 and C5) reacted with most pepper leaf extracts. In contrast, no pepper leaf extract reacted positively with four MAbs (SCR25, B1, C1 and C12) while extract S6 weakly reacted with SCR53. Overall, pepper extracts could be divided in three main groups according to their serological reaction profiles. Group 1 included samples S1, S2, S4, S5 and S10 which reacted strongly with MAbs SCR12 and B3. Samples in group 2 (S3, S7 and S9) reacted strongly with MAb B3 but weakly with SCR12. In the last group (S6 and S8), samples reacted weakly with both MAbs B3 and SCR12.
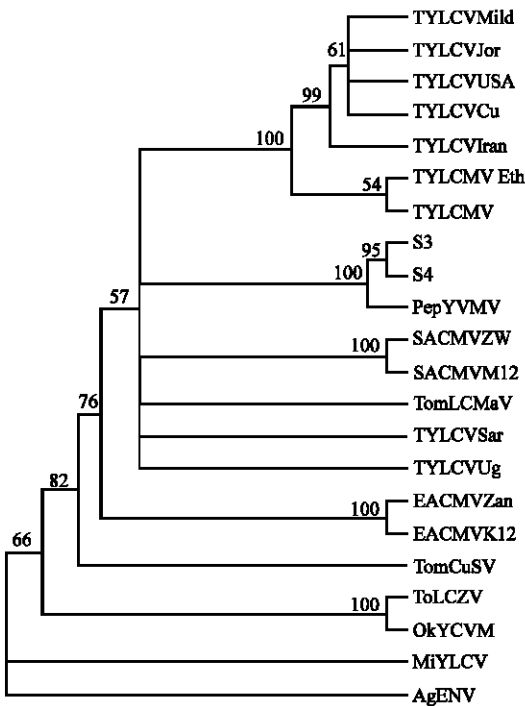
Fig. 4: Phylogenetic tree showing relationships among pepper-infecting begomoviruses and other representative begomoviruses based on multiple alignments of nucleotide sequences of the coat protein core region. The tree was constructed by the neighbour-joining method as implemented in PAUP (Swofford, 2000). Bootstrap supports of the nodes are indicated as percentages (from 1000 replicates) branches with bootstrap values less than 50% were collapsed

**PCR amplification and sequence analysis:** Two fragments of 601 and 550 bp were amplified using the two sets of degenerate primers P1 (PTYv369/PTYc1023) and P2 (V324/C889), respectively (Fig. 3). Normally, primer set P2 was the one chosen to be used in all PCR tests. Unfortunately, no amplification was obtained from samples S3 and S6 when primer set P2 was used. Therefore, primer set P1 was tested and successfully used for DNA amplification in these two samples.

Sequences of PCR-amplified fragments from samples S3 and S4 were compared to those of 20 begomoviruses retrieved from Genbank database. Sequence identity was 99.8% between S3 and S4. Of begomoviruses included in the analysis, only *Pepper yellow vein Mali virus* (PepYVMV) had particularly high sequence identity with samples S3 and S4 (99.0 and 99.2%, respectively). Sequence identity between the two samples and other begomoviruses ranged from 72.6 and 84.8%. Phylogenetic

analyses indicated that both samples S3 and S4 clustered with PepYVMV and their clade was clearly distinct from any other clade (Fig. 4).

**DISCUSSION**

Begomoviruses are known to induce a diversity of symptoms in the plants they infect (Harrison and Robinson, 1999). However, leaf curling and other leaf distortions seem to be the most frequent symptoms associated with infections caused by begomoviruses (Paximadis *et al.*, 1999; Wang *et al.*, 2004; Seal *et al.*, 2006). Severe leaf curling symptoms were observed in recent years in peppers in Burkina Faso but no virus associated with such symptoms was reported. In this study, mechanical inoculation tests of the causal agent were unsuccessful. Because whiteflies were the most frequent insects observed in affected pepper fields, transmissions through these insects were attempted. Unlike in mechanical inoculation tests, disease symptoms were readily induced through transmissions by whiteflies, as infections rates were higher than 80%.

Virus transmission by *B. tabaci* is somewhat specific. Particular genes involves in the ability of this species to transmit plant viruses have been reported (Liu *et al.*, 1998). However, more than 111 plant viruses including begomoviruses, criniviruses, carlaviruses and ipomoviruses are transmitted by the insect (Jones, 2003). Therefore, virus transmissibility by *B. tabaci* cannot be used as the only criterion for virus identification. In this study, leaf samples taken from diseased pepper plants were positive in TAS-ELISA using antibodies raised against begomoviruses. Added to transmission by *B. tabaci*, this result indicated that leaf samples were infected by viruses which belong to the *Begomovirus* genus.

Although transmission by *B. tabaci* and serological tests, especially using monoclonal antibodies are two useful criteria for identification of begomoviruses, they are not suitable for taxonomic demarcation of begomovirus species (Brown *et al.*, 2001; Fauquet *et al.*, 2003). The core CP gene was successfully amplified from diseased pepper leaf samples by PCR. This result is consistent with transmission and serological data and confirms the begomoviral aetiology of the disease observed in peppers. Moreover, analysis of sequences obtained from PCR-amplified fragments indicated high pairwise nucleotide identity (>99%) with *Pepper yellow vein mali virus* (PepYVMV). No other begomovirus species had pairwise nucleotide identity exceeding 85%. Nucleotide identity not less than 89% has been advocated

as the threshold for demarcating begomovirus species (Fauquet *et al.*, 2003). Therefore, begomoviruses identified in diseased pepper leaf samples belong to the PepYVMV species (Vadivukarasi *et al.*, 2007).

Altogether, the results obtained in this study indicated that leaf curling symptoms observed in sweet and hot pepper in Burkina Faso were caused by PepYVMV. However, Zhou *et al.* (2008) recently proposed the name tomato yellow leaf crumple virus (ToYLCrV) instead of PepYVMV because of marked discrepancies in symptoms caused by the virus in pepper and tomato. They reported that first symptoms induced by the virus were upcurled leaves and yellow vein symptoms in pepper. But, subsequently, surveys in a few West African country including Burkina Faso, Benin, Ghana, Niger, Senegal and Togo indicated that the virus was mostly associated with yellow leaf crumple symptoms in tomato. In the present study, the virus incidence in tomato was not assessed. However, high incidences observed in pepper fields indicated that the virus is widely spread in pepper.

## ACKNOWLEDGMENTS

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