SHORT COMMUNICATION

COTTON VIRESCENCE PHYTOPLASMA AND ITS WEED RESERVOIR IN MALI

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SUMMARY

A phytoplasma associated with cotton virescence disease was identified in several cotton plants showing virescence, stunting, yellowing and reddening in the cotton-growing areas of Yanfolila and Kolondjeba, in the Bougouni Region (south west Mali). The agent was detected by PCR with phytoplasma universal primer pairs and identified as a 16SrII-C subgroup member by RFLP of PCR fragments separately digested with *Mse*I, *Alu*I, *Hha*I and *Hpa*II restriction enzymes. The same phytoplasma was found in *Sida cordifolia*, a weed that can act as a reservoir. The spatial distribution of infected plants in a field was aggregated along the rows. A higher number of infected plants was present on the border rows, suggesting that primary infections are probably more important than secondary spread from cotton to cotton. The incidence of the disease in Mali cotton-growing areas fluctuates, but in some fields up to 50% of the plants were infected. We observed more infected plants in organic cotton. This can be due to the use of neem as insecticide, which is ineffective against leafhopper adults that spread the disease.

Key words: 16SrII phytoplasma, *Gossypium hirsutum, Sida cordifolia*, epidemiology, Western Africa.

With an annual production of 600,000 tons, Mali and Burkina Faso are the main cotton producing countries in Sub-Saharan Africa. In this region, cotton is the main export commodity and is strongly vulnerable to the fluctuation of the market. In both countries, cotton growers use mainly conventional agricultural techniques but, in the last ten years, at least 4000 small-size cotton farms started to grow organic cotton. In Mali, the areas of Yanfolila and Kolondjéba (Bougouni Region, south-west Mali) are the main cotton-growing areas.

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Cotton diseases and pests in Western Africa are described in a compendium (Vaissayre and Cauquil, 2000). Among arthropod-borne diseases, cotton virescence associated with phytoplasmas, has been recorded from Burkina Faso (Delattre, 1965) and, later, from Ghana and Ivory Coast (Desmidts *et al*., 1973). The main symptoms are virescence, yellowing and reddening of the leaves and general stunting of the plant. Infected plants are unproductive. The economic importance of the disease is variable, but in several sites up to 30% of the plants were affected between 1960 and 70 (Desmidts *et al*., 1973). The leafhopper *Orosius cellulosus* (Lindberg), breeding mainly on *Sida cordifolia* L. and *Mitracarpus scaber* Zucc. (Desmidts and Laboucheix, 1973), has been identified as the vector of the cotton virescence phytoplasma and two species of the genus *Sida*, family Malvaceae, act as phytoplasma reservoir (Laboucheix *et al*., 1973). The past epidemiological studies relied on symptom and electron microscope observations and on transmission experiments, but the lack of molecular tools hampered further characterization.

In the early 2000s, farmers in the area of Yanfolila reported important damages to cotton production due to a virescence-inducing disease. Thus, this study was aimed at identifying and characterizing the disease agent in southern Mali, and provide some insights in its epidemiology.

During 2003-2006, field surveys were carried out from July to October in the cotton growing-region of southern Mali. In 2003 and 2004, nine cotton plants (*Gossypium hirsutum* L.) showing virescence, yellowing, reddening and stunting symptoms, were collected in different organic- and conventionally-grown cotton fields at Yanfolila, Kolondjeba and Bougouni. The samples were wrapped in dry, soft paper and sent immediately to the laboratory for molecular diagnosis. Plants without symptoms were also collected in each location. Cotton seedlings, grown under controlled conditions in the greenhouse, were used as further negative controls.

In 2006, surveys for alternative hosts of cotton virescence phytoplasma were carried out on weeds collected nearby infected cotton fields. Symptomatic plants were sent to the laboratory for diagnosis. Plants without

symptoms were also collected.

The spatial distribution of plants with symptoms was recorded in one field at Djéguinéna, close to Yanfolila, on August-September 2004. The field, rectangular in shape, was surrounded by maize (north and west) and cotton (south and east) and consisted of 32 rows of about 60 plants each. Each single plant was observed on August 24th and September 10th 2004. The data were submitted to the analysis of doublets (Van der Plank, 1963). The expected number of random distributed doublets is estimated by the formula:

$$d_1 = [\mu (\mu-1)] / n$$

where (d_1 = n° of expected doublets, μ = total number of infected plants, n = total number of consecutive plants). A doublet consists of two adjacent infected plants, three subsequent infected plants are considered as two doublets and so on. For high values of n, standard error of d_1 is $\sqrt{d_1}$ (square root of d_1). When the number of doublets was significantly higher than $d_1 \pm 2\sqrt{d_1}$, the distribution of the infected plants was considered aggregated (Credi and Callegari, 1988).

Total DNA was extracted from 1.5 g of virescent petals and leaf veins following a phytoplasma enrichment protocol as detailed by Marzachì *et al.* (1999) and resuspended in 100 μl of sterile double distilled water (SDW). Total DNA was also extracted from four *Sida cordifolia* plants without symptoms as well as cotton seedlings. Two μl of total cotton DNA and 2 μl of a 1:100 dilution of *Sida* total DNA in SDW were used as templates in direct PCR driven with the phytoplasma-specific, universal primers P1/P7 (Schneider *et al.*, 1995), R16F2/R2 (Lee *et al.*, 1993) and fU5/rU3 (Lorenz *et al.*, 1995). Nested PCR assays were driven with R16F2/R2 (Lee *et al.*, 1993) and fU5/rU3 (Lorenz *et al.*, 1995), respectively. Reaction and cycling conditions were as detailed in the original papers. Fragments amplified with R16F2/R2 (Lee *et al.*, 1993) pairs were separately digested with *Mse*I (Invitrogen, USA), *Alu*I (Invitrogen, USA), *Hha*I (New England Biolabs GmbH, Germany) and *Hpa*II (Invitrogen, USA) at 37°C, and *Taq*I (Invitrogen, USA) at 65°C.

Faba bean phyllody (FBP, 16SrII-C), *Crotalaria saltiana* phyllody (CrP, 16SrII-C), and Tomato big bud (TBB, 16SrII-D) phytoplasmas, originally kindly provided by Dr. E. Seemüller (BBA, Institut für Pflanzenschutz im Obstbau, Dossenheim, Germany) and maintained in periwinkle in the collection of the Istituto di Virologia Vegetale, CNR, were used as reference isolates and positive controls in PCR experiments.

The P1/P7 phytoplasma-specific amplicon obtained from one of the symptomatic cotton plants collected in Kolondjeba was gel-isolated with the Wizard PCR Preps-DNA Purification System (Promega, USA), ligated into the pGEM-T plasmid following the manual of the pGEM-T Easy Vector System (Promega, USA), and transformed into DH5α competent cells (Invitrogen, USA), following a routine protocol. Two independent clones were sequenced on both strands with SP6 and T7 primers followed by the two internal primers 1cot3Fw (5'CTTGAGTTAGATAGAGGC3') and 1cot3rev (5'TTCAATCCGGACTGAGAC3'). Sequencing was performed using the capillary 3730 DNA Analyzer (ABI) (M-Medical s.r.l., Italy).

The complete cotton virescence ribosomal clone sequence was assembled after 2X sequencing coverage for each nucleotide position. Raw sequence data were manually edited with DNAman vers. 4.02 (Lynnon BioSoft). ClustalW software (http://www.ebi.ac.uk/clustalw/) was used for sequence homology analysis with the ribosomal sequences of the following phytoplasmas: FBP (X83432), Burkina Faso Cotton virescence (CoP, 16SrII-C, EF186827), CrP (16SrII-C, EF193355), TBB (16SrII-D, EF193359), Sesame phyllody phytoplasma (SEPN, 16SrII-A, EF193357), "*Candidatus* Phytoplasma aurantifolia", isolate LWB (16SrII-B, U15442), Alfalfa witches'-broom phytoplasma (AlfWB, 16SrII-D, AY169322).

Cotton plants with symptoms of virescence, yellowing, reddening and stunting (Fig. 1A and B) were found

Fig. 1. A. Cotton plant showing virescence, yellowing, reddening and stunting. B. Detail of virescence.

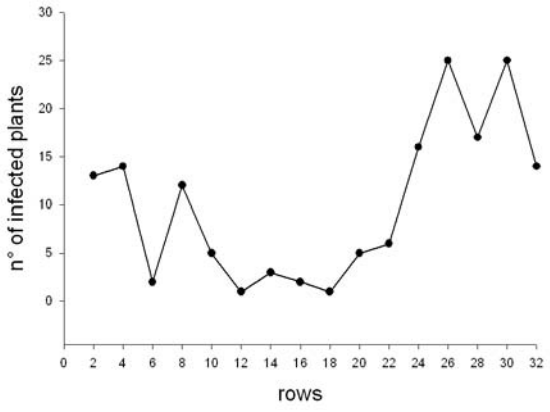

Fig. 2. Number of infected plants grouped for two adjacent rows, as recorded on September 10th in the Djéguinéna field. Row 1 (North) adjacent to cotton, row 32 (South) adjacent to maize.

in fields of Djéguinéna, Yanfolila, Kolondjeba and Bougouni, during 2003 and 2004 surveys. Symptoms were observed starting from beginning of August onwards, and in most cases symptoms involved all flowers except for the first basal ones. Few symptomatic plants (less than 5%) were present in both conventional and organic cotton fields at Kolondjeba and Bougouni. The disease incidence was higher at Yanfolila and Djéguinéna: in few organic fields in this area, up to 50% of the plants were infected.

Sida rhombifolia L. was the predominant weed bordering cotton fields at all sites, and *S. cordifolia* L. was also present. During July-August 2006, we found several *S. cordifolia* plants, at Djéguinéna and Kolondjeba in areas neighbouring infected cotton fields, showing severe dwarfing, yellowing, and very small leaves, and we sampled four plants for molecular detection. We never observed phytoplasma-specific symptoms on *S. rhombifolia* or other weed species.

In the Djéguinéna field (about 1,900 plants) 126 cotton plants showed symptoms on August 24th and 161 on September 10th. A higher number of infected plants was present in the border rows (Fig. 2). No obvious gradient of infected plants was recorded along the rows. Further observations, later in the season, were not carried out, and therefore the final incidence of the disease was not recorded. According to the Van der Plank (1963) doublet analysis, the distribution of the infected plants was aggregated along the rows (Table 1).

Amplicons of the expected size were obtained following direct amplification with the different phytoplasma-specific ribosomal primers of total DNAs extracted from 8 of the 9 symptomatic cotton and from 3 of the 4 symptomatic *S. cordifolia* plants (not shown). Phytoplasma-specific amplicons of the expected size were also obtained following nested PCR amplification with ribosomal primers of total DNAs from the remaining symptomatic cotton and *S. cordifolia* samples. Total DNA extracts of healthy cotton seedlings of *S. cordifolia* plants without symptoms, and of the two *S. rhombifolia* with yellowing, never produced phytoplasma-specific amplicons in direct and nested assays.

Digestion of 16SrF2/R2 amplicons from all symptomatic cotton plants with *Alu*I, *Hha*I, *Hpa*II, *Mse*I and *Taq*I endonucleases always produced patterns indistinguishable form those of the Faba bean phyllody and *Crotalaria saltiana* phyllody reference phytoplasma isolates (Fig. 3), both classified as 16SrII-C subgroup members according to Martini *et al.* (2007). Digestion of the same amplicons from *S. cordifolia* symptomatic plants produced the same results as amplicons from cotton (Fig. 3).

A sequence of about 1800 bp of the ribosomal operon of the cotton virescence phytoplasma from Mali (CoP_M) was deposited in GenBank with the accession number EF363314. Identity scores obtained comparing the 16S rDNA gene of CoP_M isolate were 99% with FBP and CrP, both members of the 16SrII-C subgroup, and LWB (16SrII-B). The 16S rDNA gene of CoP_M was also 99% identical to the cotton virescence isolate CoP (Martini *et al*., 2007) originally found in Burkina Faso by Cousin *et al*. (1969), and experimentally transmitted to periwinkle by Marcone *et al.* (1999). CoP was formerly classified as a 16SrII-F subgroup member (Khan *et al*., 2002), and later as a 16SrII-C member (Martini *et al*., 2007). Sequence identity of the 16S rDNA sequence of the CoP_M was 98% with SEPN, member of the 16SrII-A subgroup, TBB and AlfWB (16SrII-D).

Table 1. Number of doublets and results of the doublet test applied along and across the rows of the cotton field at Djéguinéna (total number of plants was 1,932).

Date	Infected plants (No.)	Expected pairs	Observed pairs	Distribution
Along rows				
August 24th	126	8.07	24	Grouped
September 10th	161	13.20	38	Grouped
Across rows				
August 24th	126	8.07	6	Random
September 10th	161	13.20	16	Random

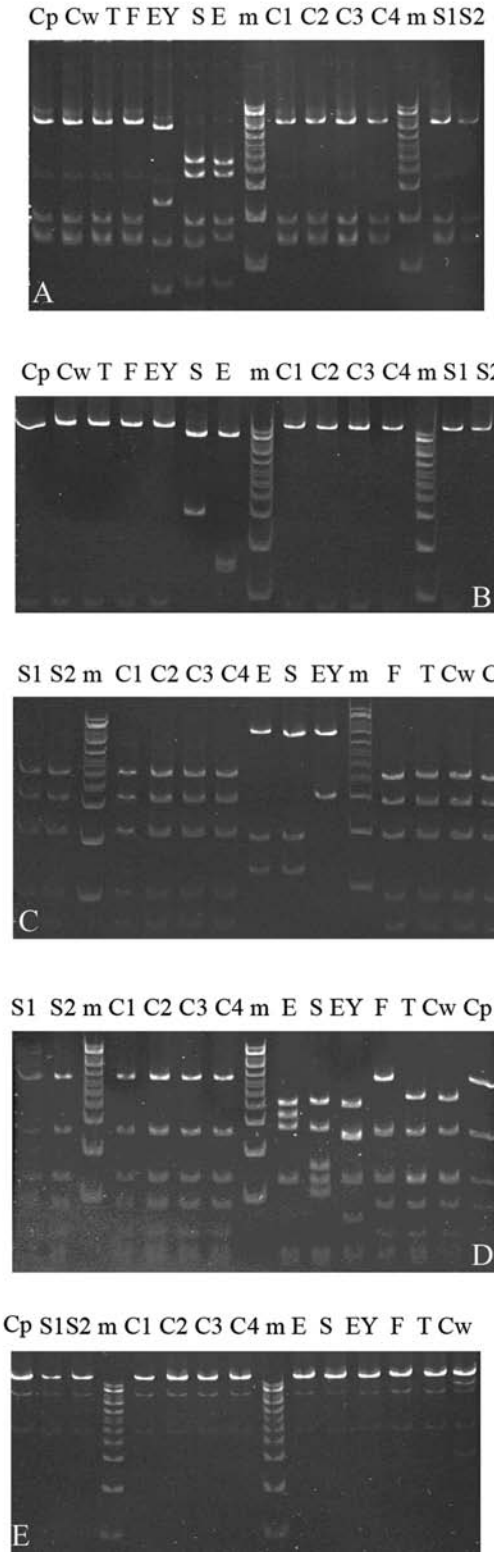

Fig. 3. Polyacrylamide gel electrophoresis of R16F2/R2 amplicons obtained from four symptomatic cotton (C1-C4) and two *Sida rhombifolia* (S1, S2) plants and European aster yellows (E), Stolbur isolate T2/92 (S), elm yellows isolate EY-1 (EY), faba bean phyllody (F), tomato big bud (T), crotalaria witches'broom (Cw), and crotalaria phyllody (Cp) periwinkle-maintained reference phytoplasma isolates digested with *Alu*I (Panel A), *Hba*I (Panel B), *Hpa*II (Panel C) *Mse*I (Panel D), and *Taq*I (Panel E). M: 1 kb DNA ladder (Invitrogen, UK), m: 50-1000 bp PCR ladder (Amersham Italia, Italy).

We have identified a phytoplasma of 16SrII-C taxonomic subgroup associated with cotton virescence disease in Mali. Several cotton samples, coming from different fields in different areas of southern Mali, were infected by the same phytoplasma. A phytoplasma belonging to the same taxonomic subgroup was found associated with virescent cotton originally collected in Burkina Faso (Marcone *et al*., 1999; Schneider *et al*., 1997), therefore, 16SrII-C phytoplasmas are definitely associated with cotton virescence disease in western Africa. In the past both cotton virescence and phyllody has been used to indicate the disease; we suggest the name "cotton virescence" since infected plants develop virescent flowers and phyllody has never been observed. We also identified the same phytoplasma in *S. cordifolia* plants next to the infected fields showing little leaf, dwarfing and yellowing symptoms. Both *S. cordifolia* and *S. rhombifolia* were reported as reservoir of the disease in Burkina Faso (Laboucheix *et al*., 1973), but so far in Mali we could only confirm the former species. *Sida* spp. are perennial plants that can maintain the pathogen over the years, in the absence of cotton during the dry season. At this time of the year only the root system of *Sida* plants survives but we have observed that after the first rainfalls the new sprouts of infected plants are already symptomatic.

In the early 70's the leafhopper *Orosius cellulosus* (Lindberg) has been reported as the vector (Laboucheix *et al*., 1973) and found to breed on *S. cordifolia*. These findings suggest an important role of this weed in the epidemiology of cotton virescence. Primary infections of cotton plants, due to incoming leafhoppers, are likely to be the most important. This hypothesis is supported by the grouped distribution of infected plants, possibly due to the short-range movement of immigrated infected vectors, and the border effect recorded in Djéguinéna field. Both grouped distribution of infected plants and border effect were reported for cotton virescence in Burkina Faso (Delattre and Joly, 1981). A secondary spread from cotton to cotton, although possible under controlled conditions (Laboucheix *et al*., 1973), has probably only a minor impact at the end of the growing season (Desmidts *et al*., 1973).

The incidence of the disease in Mali varies a lot, from few infected plants in most cases up to the heavily damaged fields we observed in Yanfolila area. A similar situation has been reported in Burkina Faso (Desmidts *et al*., 1973). The high incidence of the virescence recorded in some organic-grown cotton fields can be due to the use of neem known to be active against insect immatures only and therefore can not prevent spread of the disease by flying adults. The introduction of Bt cotton, and the consequent reduction of insecticide treatments against key pests such as bollworms, may increase the incidence of the disease also in non-organic fields. Control of the disease can be achieved by tillage and com-

plete removal of weeds that can host the phytoplasma. Also roguing of cotton infected plants is advisable, and largely practiced by Malian farmers.

So far, cotton virescence has been found only in Western Africa but this disease represents a threat to other cotton growing areas around the world since weeds of the genus *Sida*, potential reservoirs, are widespread and leafhoppers of the genus *Orosius* are present throughout Asia (from Middle to Far East), Australia and Pacific Region (Weintraub and Beanland, 2006).

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