Detection of Phytoplasmas in Watercress and Onion Plants from Mauritius

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Abstract

Different phytoplasmas were detected and identified in watercress and onion plants from Mauritius. Symptoms observed on watercress were purple coloration of leaves, plant stunting, and reduced leaf size. In onion, witches’ broom and virescence were observed in the inflorescence. Disease incidence in watercress ponds ranged from 30 to 70%, while in onion seed production plots, it was around 10%. PCR/RFLP analyses as well as sequencing of 16S ribosomal gene enabled the identification of phytoplasmas belonging to two ribosomal groups, namely stolbur and aster yellows, from the two aforementioned crops.

Introduction

Watercress (Nasturtium officinale) is a popular green leafy vegetable in Mauritius. It is grown in waterways all over the island, on an area of around 14 ha, with an annual production amounting to about 200 tonnes, and an economic value approximating US $200,000. Diseases recorded so far on watercress in Mauritius are fungal leaf spots caused by Cercospora, Septoria, and Colletotrichum and leaf mosaic caused by Turnip mosaic virus (TuMV). In a survey performed in year 2000 in all watercress cultivation of the island, 81% out of 231 symptomatic leaf samples were tested positive for TuMV by DAS ELISA (12).

Onion is also an important crop in Mauritius, since it is a popular ingredient in the local cuisine. Annual onion production amounts to about 6,000 tonnes and imports can reach up to 11,600 tonnes. It is within government policy to boost seed production in Mauritius so as to promote onion cultivation and thus reduce imports. Major diseases recorded on onion so far in Mauritius include: purple blotch, pink root rot, rust, blast, Xanthomonas blight, root nematode, Phytophthora, onion yellow dwarf, and iris yellow spot viruses. To date, there are no official records of the presence of phytoplasmas in either watercress or onion in Mauritius.

Over the past year, some abnormalities were reported in watercress and onion. Symptoms observed on watercress were reduced leaf size, plant stunting, and purple leaf coloration; infected plants died, leaving empty patches in ponds (Fig. 1A). In onion seed production plots of variety ‘Bellarose,’ several plants were stunted and showed witches’ broom and phyllody symptoms in their inflorescence (Fig. 1B). These symptoms, frequently associated with abiotic stress factors in the past, were strongly suggestive of possible phytoplasma association.
Analyses to verify the presence of these prokaryotes in both onion and watercress were carried out using the polymerase chain reaction (PCR) assay with universal primers that amplify 16SrDNA sequences of phytoplasmas followed by molecular characterization of obtained amplicons.

**Sample collection and nucleic acids extraction.** Inflorescences from onion variety 'Bellarose' and watercress leaf samples were collected respectively from Richelieu and Carreau Esnouf in Mauritius (Fig. 2) in 2008. Asymptomatic samples from both species were also collected as negative controls. Onion samples originated from seed production plots, whereas watercress samples were from commercial privately owned ponds. Total nucleic acids were extracted from 1 g of pooled tissues following the protocol described by Prince et al. (25) (extraction method 1), and/or from 0.7 g of pooled tissues following a CTAB protocol (extraction method 2) (2). Nucleic acid from both extraction systems were dissolved in TE buffer, and maintained at -20°C. Before performing PCR assays nucleic acids were quantified and diluted in sterile distilled water to the final concentration of 20 ng/µl.
Fig. 2. Map of Mauritius showing in the dark areas where onion and watercress were sampled.

Reference phytoplasma strains. Phytoplasma reference strains maintained in collection in periwinkle [Catharanthus roseus (G.) Don.] (4) employed for phytoplasma identification were: koolsard aster yellows (KAY, ribosomal subgroup 16SrI-B), and stolbur from pepper from Serbia (STOL, ribosomal subgroup 16SrXII-A); moreover, 'Candidatus Phytoplasma japonicum' maintained in naturally infected Hydrangea from Japan (27) was also employed.

16S ribosomal DNA. Detection of phytoplasmas was done using PCR assays with phytoplasma universal primer pair P1/P7 (8,28) in direct PCR reaction, followed by nested PCR by R16F2/R2 primers (18). Further nested PCR reactions on P1/P7 amplicons were performed with R16(I)F1/R1 primer pair, specific for phytoplasmas belonging to aster yellows (16SrI) (20), faba bean phyllody (16SrII) (32), and stolbur (16SrXII) (21) groups.

Each 25 µl PCR reaction mix contained 20 ng template DNA, 2.5 µl 10× PCR buffer, 0.8 U Taq polymerase (Sigma-Aldrich Co., St. Louis, MO), 0.2 mM dNTPs, 1.5 mM MgCl₂ and 0.4 µM of each primer. Asymptomatic samples were run as negative controls in each PCR reaction. One µl of amplicon from direct PCR diluted 1:30 in sterile distilled water, was used as template in nested PCR reactions. Thirty-five PCR cycles were performed under the following
conditions: 1 min (2 min for the first cycle) for denaturation at 94°C, 2 min for annealing at 50°C, and 3 min (10 min for the last cycle) for primer extension at 72°C. Six µl of PCR products were separated in 1% agarose gel, stained with ethidium bromide, and photographed under UV at 312 nm using a transilluminator.

Identification of detected phytoplasmas was done using RFLP analyses with TruI, HhaI, and TaqI (Fermentas, Vilnius, Lithuania) restriction enzymes on amplified ribosomal DNA sequences. Visualization of RFLP products was performed in a 5% polyacrylamide gel, stained with ethidium bromide, and visualized with UV transilluminator.

For better molecular identification longer 16S rDNA sequences were obtained with further nested amplification on P1/P7 amplicons with F1/B6 primers (9) on samples WatM-1, WatM-2, and OnM-2 (Table 1) and the amplified products (about 1,700 bp) were purified using Qiagen PCR Purification Kit (Qiagen GmbH, Hilden, Germany, EU), and then sequenced in both directions with primers F1, 350R (24), and B6 from Eurofins MWG Operon (Ebersberg, Germany). These sequences were aligned with those of representative strains of the genus 'Candidatus Phytoplasma' (14) retrieved from GenBank and aligned using CLUSTAL X and BioEdit (13,31). The obtained sequences of samples WatM-1 (1,625 bp), WatM-2 (1,513 bp), and OnM-2 (1,524 bp) were deposited in the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) NCBI under accession No. GU129975, GU129976, GU129974, respectively.

Maximum parsimony analysis using the close neighbour interchange algorithm was performed with MEGA version 4 (30) to construct a phylogenetic tree from the aligned 16S ribosomal sequences and related sequences from 39 phytoplasma strains (Table 1). The initial tree was created by random addition for 10 replications. Bootstrap analysis was also performed and replicated 1,000 times for estimation of stability and support for the clades. Acholeplasma laidlawii (a cultivable Mollicute, phylogenetically related to phytoplasmas) was designated as the out-group to root the tree.

Virtual RFLP analyses on R16F2/R2 amplicons were performed using p-DRAW32 program version 1.0 to verify consistency of sequences with RFLP results obtained.

Table 1. Phytoplasma 16S rDNA sequences retrieved from GenBank, including strains from watercress and onion from Mauritius, employed for phylogenetic analysis.

<table>
<thead>
<tr>
<th>GenBank #</th>
<th>Phytoplasma strain</th>
<th>Origin</th>
<th>Disease</th>
<th>Ref. #</th>
</tr>
</thead>
<tbody>
<tr>
<td>M30790</td>
<td>'Candidatus. Phytoplasma asteris'</td>
<td>Michigan (US)</td>
<td>Aster yellows</td>
<td>14</td>
</tr>
<tr>
<td>U15442</td>
<td>'Ca. P. aurantifolia'</td>
<td>Oman</td>
<td>Lime witches broom</td>
<td>14</td>
</tr>
<tr>
<td>L04682</td>
<td>'Ca. P. pruni'</td>
<td>California (US)</td>
<td>X disease</td>
<td>14</td>
</tr>
<tr>
<td>U18747</td>
<td>'Ca. P. palmae'</td>
<td>Florida (US)</td>
<td>Palm lethal yellowing</td>
<td>14</td>
</tr>
<tr>
<td>X80117</td>
<td>'Ca. P. cocostanzianae'</td>
<td>Tanzania</td>
<td>Palm lethal yellowing</td>
<td>14</td>
</tr>
<tr>
<td>Y14175</td>
<td>'Ca. P. cocosnigerianae'</td>
<td>Nigeria</td>
<td>Palm lethal yellowing</td>
<td>14</td>
</tr>
<tr>
<td>AF122910</td>
<td>'Ca. P. ulmi'</td>
<td>New York (US)</td>
<td>Elm yellows</td>
<td>14</td>
</tr>
<tr>
<td>AF305240</td>
<td>'Ca. P. ziziphi'</td>
<td>South China</td>
<td>Jujube witches broom</td>
<td>14</td>
</tr>
<tr>
<td>X76560</td>
<td>'Ca. P. vitis'</td>
<td>France</td>
<td>Flavescence dorée</td>
<td>14</td>
</tr>
<tr>
<td>AY390261</td>
<td>'Ca. P. trifolii'</td>
<td>Canada</td>
<td>Clover proliferation</td>
<td>14</td>
</tr>
<tr>
<td>AF092209</td>
<td>'Ca. P. fraxini'</td>
<td>New York (US)</td>
<td>Ash yellows</td>
<td>14</td>
</tr>
<tr>
<td>AF086621</td>
<td>'Ca. P. luffae'</td>
<td>Taiwan</td>
<td>Luffa witches broom</td>
<td>14</td>
</tr>
<tr>
<td>AF515637</td>
<td>'Ca. P. phoenicium'</td>
<td>Lebanon</td>
<td>Almond witches broom</td>
<td>14</td>
</tr>
<tr>
<td>AJ542541</td>
<td>'Ca. P. mali'</td>
<td>Italy</td>
<td>Apple proliferation</td>
<td>14</td>
</tr>
</tbody>
</table>

(continued)
Disease status. Disease incidence varied from 40 to 70% across watercress ponds; the surveillance program started in January 2009, revealed that these symptoms are present on all three locally grown watercress varieties ('Cresson Filant,' 'Brede Doux,' and 'Cresson Constance'), and 60% of sites surveyed were affected. Disease incidence of symptomatic onion plants did not exceed 10% on variety 'Bellarose,' the only onion variety presently cultivated for seed production in Mauritius.

16S Ribosomal DNA. All symptomatic samples from both species examined were positive in direct and/or nested PCR analyses. Selected samples from both species were also examined with RFLP analyses and results are summarized in Table 2. The asymptomatic samples of onion and watercress were negative in all
PCR experiments performed such as the water control, while all the reference strains employed were positive.

Table 2. Results of detection and identification of phytoplasmas in selected samples from Mauritius by two different extraction procedures.

<table>
<thead>
<tr>
<th>Samples acronyms</th>
<th>Extraction 1 RFLP results</th>
<th>Extraction 2 RFLP results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onion</td>
<td>OnM-1 performed 16SrI-B</td>
<td>performed -</td>
</tr>
<tr>
<td></td>
<td>OnM-2 performed -</td>
<td>performed 16SrXII*</td>
</tr>
<tr>
<td>Water-cress</td>
<td>WatM-1 performed 16SrI-B</td>
<td>Not performed</td>
</tr>
<tr>
<td></td>
<td>WatM-2 Not performed</td>
<td>performed 16SrXII-A</td>
</tr>
<tr>
<td></td>
<td>WatM-3 performed 16SrI-B</td>
<td>Not performed</td>
</tr>
</tbody>
</table>

− negative results in PCR/RFLP analyses.
* not classified at subgroup level.

Phytoplasma identification was achieved in all symptomatic samples of onion, but by using different extraction methods. In particular, phytoplasmas were identified in some samples when extraction method 1 was employed, while the phytoplasmas in other samples were only detected by using extraction method 2 as shown for selected samples in Table 2. Two different phytoplasmas were identified by RFLP analyses in symptomatic onion (Table 2), i.e. aster yellows and stolbur (Fig. 3).

![Polyacrylamide gels showing the RFLP profiles of 16S rRNA gene sequences amplified with primers R16F2v/R16R2 (A) and R16(1)F1/R1 (B) from reference phytoplasma strains and from watercress and onion from Mauritius (acronyms as in Table 2). TruI was used for restriction endonuclease digests in both gels. Phytoplasma acronyms: KAY, koolsard aster yellows (16SrI-B); Ca. P. japonicum, 'Candidatus Phytoplasma japonicum'; STOL, stolbur (16SrXII-A). PhiX174, marker ΦX174HaeIII digested.](image-url)
In all the symptomatic samples of watercress, phytoplasma presence was identified (see examples in Table 2) and RFLP analyses allowed the identification of aster yellows and stolbur phytoplasmas (Fig. 3). In a number of samples, represented in Table 2 by sample WatM1, aster yellows phytoplasma was identified, while in other samples (i.e., WatM2) stolbur phytoplasma was identified. In a few samples, represented by WatM3, both phytoplasmas were identified in mixed infection only after RFLP analyses on 16R(1)F1/R1 amplicon, while only aster yellows phytoplasma was identified on 16RF2/R2 amplicon of the same samples. Moreover, both real (Fig. 3) and virtual (data not shown) RFLP analyses showed that phytoplasma strain identified in sample OnM2 was distinguishable from the other stolbur strains either from onion or watercress for the presence of a TruI restriction site in position 804. Virtual RFLP analyses confirmed the presence of this difference also when this sequence was compared with several other stolbur phytoplasma strains present in the GenBank (data not shown), indicating uniqueness of this among described stolbur strains.

Phylogenetic comparison of the 16SrRNA genes of phytoplasma samples detected and sequenced from selected watercress and onion samples in Mauritius with 39 representative strains of the genus ‘Candidatus Phytoplasma’ confirmed that the OnM-2 and WatM-2 phytoplasmas are most closely related to the stolbur phytoplasmas, and to other members of the 16SrXII group, while the strain WatM-1 is closely related to the aster yellows phytoplasmas (Fig. 4) corroborating data obtained by RFLP analyses.

A rather high incidence of phytoplasma was noted in watercress; this is likely because watercress is a perennial crop, and in many regions of Mauritius it is continuously harvested from ponds all year round. Moreover, the same varieties are grown season after season using cuttings from the previous years. Furthermore, the exchange of cuttings is a common practice between growers from different areas of the island, thus increasing the possibility of disease spread. This is confirmed from the high number of infected ponds.

Recent reports of severe outbreaks of aster yellows phytoplasma associated disease in watercress in Hawaii were characterized by symptoms only partly similar to those reported here, although death of infected plants was observed in both places (7). Phytoplasma vector transmission in Hawaii was described, and Macrosteles sp. was demonstrated as an experimental vector under greenhouse conditions (6).

Phytoplasma infections in onion have been reported worldwide (Japan, Italy, Canada, and USA), but until now only aster yellows-related (16SrI-B) phytoplasmas were identified (16,22,24,35). Stolbur phytoplasmas were however reported infecting leek in Italy together with aster yellows phytoplasmas in mixed infection (5) indicating the susceptibility of Liliaceae to this phytoplasma as well.

Phytoplasmas belonging to two different ribosomal groups (16SrI and 16SrXII) were identified from both onion and watercress; however, the symptomatology associated with single phytoplasmas was indistinguishable in the two species concerned. This is a phenomenon common for several plant diseases, especially for those associated with phytoplasmas either in herbaceous or in woody host plants (4).
These phytoplasmas are being reported for the first time in onion and watercress in Mauritius and this could represent a severe threat to agriculture in this small island. The fact that mixed infection of aster yellows and stolbur phytoplasmas in watercress sample WatM3 was identified only using specific primer pair 16R(I)F1/R1 is probably related to increased sensitivity/specificity of this group-specific primer pair, but indicates that both phytoplasmas are spreading in an efficient way either by unknown insect vectors or by cuttings (watercress).

Phylogenetically while the watercress aster yellows (WatM1) phytoplasma clusters with strains belonging to 16SrI-B subgroup, both stolbur phytoplasmas identified in onion (OnM2) and watercress (WatM2) cluster with 16SrXII-A subgroup.
Following the identification of phytoplasmas from watercress, a disease surveillance program was initiated across major watercress ponds of Mauritius in January 2009, in order to monitor the incidence and spread of the disease across the island, as well as to verify the presence of insects vectoring the disease. Further monitoring is also in progress to rogue out all symptomatic onion plants in order to reduce the potential of further spread of the two phytoplasmas over the island.

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Literature Cited