Diagnostics
Diagnostic

Candidatus *Phytoplasma aurantifoliae*

Specific scope

This standard describes a diagnostic protocol for Candidatus *Phytoplasma aurantifoliae* (Lime witches' broom phytoplasma).

Specific approval and amendment

Approved in 2005-09.

Introduction

The most characteristic symptom is the development of witches' brooms on affected acid lime (*Citrus aurantifolia*). This is the basis of which the name 'Witches' broom disease of lime' was given to the disorder in Oman (Bové, 1986). The disease was first observed in the Sultanate of Oman, and later was found to be present in United Arab Emirates (Garnier *et al*., 1991a), India (Ghosh *et al*., 1999) and Iran (Bové *et al*., 2000).

Identity

Name: Candidatus *Phytoplasma aurantifoliae*.
Synonyms: Lime witches' broom phytoplasma, Lime witches' broom MLO.
Taxonomic position: Bacteria, Firmicutes, Mollicutes, Acholeplasmatales, Acholeplasmataceae. On the basis of full-length 16S rDNA sequence and RFLP or putative restriction site analysis of nonribosomal DNA, Lime witches' broom is classified in the *Faba bean phyllody phytoplasma* group, according to Seemüller *et al*. (1998) or in phytoplasma group (16SrII), subgroup B according to Davis & Sinclair (1998) and Lee *et al*. (1998). It was the first phytoplasma to receive a 'Candidatus species' name (Zreik *et al*., 1995).
EPPO code: PHYPAF.
Phytosanitary categorization: EU annex designation II/A1.

Detection

Host plants and disease symptoms

The citrus cultivars found affected by *P. aurantifoliae* are: acid lime, Indian Palestine sweet lime (*Citrus limettioides*), sweet limetta and citron (*C. medica*). The major citrus commercial scion species such as sweet orange (*C. sinensis*), mandarin (*C. reticulata*), clementine (*C. clementina*) and grapefruit (*C. paradisi*) have not shown symptoms of infection in nature and they have not been experimentally infected by graft inoculation. *Poncirus trifoliata*, Troyer citrange (*P. trifoliata* × *C. sinensis*), Eureka lemon (*C. limon*), rough lemon (*C. jambhiri*), alemow (*C. macrophylla*), *C. excelsa*, *C. inchangensis*, *C. karna*, *C. hystrix*, Meyer lemon (*C. meyeri*) and Rangpur lime (*C. limonia*) have shown to be susceptible in graft-inoculation experiments. In contrast, the following species have not been infected with the *P. aurantifoliae* by grafting: *C. bergamia, C. deliciosa, C. halimii, C. junos, C. latifolia,* Cleopatra mandarin (*C. reshni*), *Fortunella margarita, Microcitrus australis, Severina buxifolia*.

In the field, affected trees show witches' brooms, which are characterized by their compactness and their very small, pale green leaves (Web Figs 1 and 2). Witches' brooms show many secondary thin shoots, developed from axillary buds that normally stay dormant, with shortened internodes. The bark of these shoots tends to fissure but this cracking cannot be attributed only to *P. aurantifoliae*. When the disease is in an advanced stage, the leaves dry, many witches' brooms appear and in four or five years the tree collapses (Web Fig. 3). No flowers or fruits are produced on witches' brooms and the ones produced on normal shoots are reduced in size.

P. aurantifoliae is localized in the phloem elements of infected plants and its distribution is irregular. Midribs of the smallest leaves contain the highest numbers of the pest. Disease symptoms are very characteristic but there has been some confusion with witches' broom symptoms of genetic origin in India (EPPO, 1997).

Identification

DAPI staining

Fluorescence microscopy with DAPI (4.6 diamidino-2-phenylindole 2 HCl) is a simple but non-specific technique for detecting phytoplasmas in infected plants. Leaf midribs are fixed for 2 h in 5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4, and sections 15–30 µm thick are cut with a freezing microtome. The sections are immediately transferred to DAPI solution (1 µg mL^{-1}) for 10 min at room temperature, washed in 0.1 M phosphate buffer and mounted on a microscope slide sealing with varnish. They are examined in a fluorescence microscope equipped with a high-pressure mercury lamp, 365 nm exciter filter and 450 nm emission filter. Samples containing phytoplasmas show high DAPI fluorescence in the sieve tubes due to the specific combination of this stain with DNA, while no fluorescence is seen in healthy plants.

Another disease of citrus, 'rubbery wood', affecting lime and lemon in India (Ahlawat, 1987), seems also to be associated with phytoplasmas but has different symptoms. If DAPI staining is used on asymptomatic plants, it is not possible to distinguish the two diseases.

Transmission to test plants

P. aurantifoliae can be transmitted from affected citrus plants to test plants of *Catharanthus roseus* by dodder (*Cuscuta campestris*). The symptoms on *C. roseus* are witches' brooms with very small leaves, and small flowers showing virescence.

Serological methods

Specific monoclonal antibodies (MAs) can be used to identify *P. aurantifoliae* by ELISA or immunofluorescence (IF).

Immunofluorescence (IF)

Sixteen hybridoma cell lines producing monoclonal antibodies to *P. aurantifoliae* have been obtained by Garnier *et al.* (1991a, b) and used in indirect immunofluorescence assays (Martin-Gros *et al.*, 1987). Longitudinal or transverse sections through leaf midribs are made with a freezing microtome and fixed onto microscope slides for 1 h at 50°C. Sections are incubated for 30 min at 37°C with 25 µL of specific monoclonal antibodies, then washed 3 times with PBS Tween and incubated as before with FITC-labelled anti-mouse IgGs. Sections are examined under an epifluorescent microscope with the following filters: BP455-490/FT510/LP420.

Double antibody sandwich ELISA (DAS-ELISA)

Monoclonal antibodies 7D5, 2H3, IE2 and 1D11 have been used for DAS-ELISA (Clarke & Adams, 1977) by Bové *et al.* (2000). ELISA microtiter plates are coated by filling the wells with 100 µL of specific gamma globulin (IgG) used at (10 µg mL^{-1}) in 0.05 M sodium carbonate buffer, pH 9.6 and incubated for 4 h at 37°C. The plates are then washed three times with phosphate-buffered saline plus Tween (PBS-T). Leaf midribs are homogenized in sample buffer (at 1 : 10 ratio) with mortar and pestle or in a plastic bag with a roller.

Samples are loaded onto the ELISA plate, two wells per sample and 100 µL per well, and incubated at 4°C overnight. Positive and negative controls should be included in the ELISA plate. Four washings as described above are followed by filling each well with 100 µL of alkaline phosphatase-labeled *P. aurantifoliae*-specific gamma globulin (IgG) diluted to 1 : 1000 in PBS-Tween-PVP containing 0.2% (w/v) ovalbumin. Incubation for 4 h at 37°C is required, then after three washes the wells are filled with 100 µL of freshly prepared substrate solution containing 1 mg mL^{-1} p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8. Following 1–2 h of incubation at room temperature, the plates are read at 405 nm. *P. aurantifoliae* is positively identified if the mean DAS-ELISA ($A405_{nm}$) value of sample exceeds a threshold value that is either at least twice the mean of the healthy control(s) or the mean of the negative control values plus 2 or 3 standard deviations; under these conditions the negative and positive controls should give $A405_{nm}$ values of < 0.07 and > 0.3, respectively.

PCR assay

Crude phloem tissue fractions of approximately 2.0 g are necessary to extract nucleic acids from plants. DNA extraction can be carried out by a simplified protocol of Maixner *et al.* (1995). *P. aurantifoliae* can be identified by PCR of 16S rDNA with universal primer and specific primer. 20–100 ng of DNA extracted from suspect plants is used as a PCR template with universal primer P1 (forward) (Weisburg *et al.*, 1989) and *P. aurantifoliae*-specific phytoplasma prier WB3 (reverse) (Zreik *et al.*, 1995). Amplification is performed in 50 µL reaction mixture containing each of the deoxynucleoside triphosphates at a concentration of 125 µM, each of the primers at a concentration of 0.5 µM, and 2.5 U of *Taq* polymerase. The PCR conditions are as follows: 30 cycles of 1 min at 95°C, 1 min at 53°C, and 1 min at 72°C, plus an additional cycle with a 10 min chain elongation step. Amplified DNA is analysed on 1% agarose gel. The specific *P. aurantifoliae* fragment is 1.0 kbp in size. Use of the specific PCR test allows *P. aurantifoliae* to be distinguished from other closely related phytoplasmas of the *Faba bean phyllody* group.

Conclusion

The presence of clear symptoms (witches' broom) gives strong presumptive evidence of identification. Laboratory confirmation can be obtained by observation of symptoms, combined with positive results of serological or molecular tests, or positive results with DAPI, or transmission to test plants, or, in the absence of symptoms, by positive results of both serological or molecular tests.

Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM7/– (in preparation).

Further information

Further information on this organism can be obtained from:
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Web Fig. 1: Close up of a witches' broom on a lime tree (*Citrus aurantifolia*).

Web Fig. 2: Lime shoot from a healthy tree (right); shoots from an affected tree (left);from right to left: shoots with early symptoms (leaves are still large), witches' broom before and after leaf drying.

Web Fig. 3: Lime trees affected by lime witches' broom phytoplasma

All pictures kindly provided by J.M. Bové - INRA Bordeaux (FR)