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NEW OR UNUSUAL DISEASE REPORTS

First report of olive leaf scorch in Brazil, associated with *Xylella fastidiosa* subsp. *pauca*

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Summary. *Olea europaea* (L.) trees displaying leaf scorching symptoms, identical to those recently reported for olive trees colonized by *Xylella fastidiosa* in Southern Italy and also in Argentina, were observed in commercial orchards of two counties in Southeastern Brazil. PCR-based diagnosis using conserved primers for *X. fastidiosa* strains (RST31/33) and also specific to *X. fastidiosa* subsp. *pauca* (CVC1/272-2 int) were positive for all symptomatic tested samples (n = 8 of 9), but no template was obtained using twigs from asymptomatic trees (n = 20). Bacterial colonies were isolated from symptomatic tissues on culture medium and confirmed by PCR using the set of primers specific to *X. fastidiosa* subsp. *pauca*. Comparative sequence analyses of seven MLST loci amplified from one tripled passaged colony (MFG01) perfectly matched with sequences of alleles *leuA* #7, *petC* #6, *malF*#8, *cysG*#10, *holC*#11, *nuoL*#8, and *glfT*#8, the allelic profile of Sequence Type-ST16, which is represented by the strain COF0238 isolated from *Coffea arabica* (L.) in Brazil (<http://pubmlst.org/xfastidiosa/>). Phylogenetic analysis placed the ST16 into subspecies *pauca*, but genetically closer to ST11 and ST13, both obtained from *Citrus sinensis* (L.) trees with citrus variegated chlorosis. The results confirm the association of olive plants showing leaf scorching with the presence of *X. fastidiosa* subsp. *pauca*, and represent the first report of this bacterium in Brazilian olive orchards.

Key words: olive dieback, bacteria, natural infection.

Introduction

The dynamics of multi-host pathogens transmitted by vectors in multispecies host communities remains a major frontier for disease ecology. An example of this dynamism is observed in pathosystems involving *Xylella fastidiosa*, its multiple insect vectors and host plants. This xylem-inhabiting bacterium is the cause of some of the most important plant diseases that have emerged during the last few decades (Hopkins & Purcell, 2002). The pathogen is hosted by numerous plants species, from weeds to woody trees (Janse & Obradovic, 2010; <http://nature.berkeley.edu/xylella/control/hosts.htm>), although dis-

ease symptoms are not evident for most of them. Also numerous insects are the potential vectors for this bacterium, including xylem fluid-feeding leafhoppers (Hemiptera: Cicadellidae: Cicadellinae) and spittlebugs (Hemiptera: Cercopoidea). The insect subfamily Cicadellinae, which includes most of the known vectors, contains nearly 1950 species in different regions of the world (Redak *et al.*, 2004).

Despite the wide host range, most knowledge of *X. fastidiosa* genetic and genomic diversity is based on strains infecting commercial crops, allowing its classification into five different proposed subspecies, generally relating to the host plants colonized (and diseases caused): *X. f.* subsp. *fastidiosa* (causing Pierce's disease of grapevines and almond leaf scorch); *X. f.* subsp. *multiplex* (leaf scorch diseases of almond, peach, and oak); *X. f.* subsp. *sandyi* (ole-

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ander leaf scorch); *X. f.* subsp. *morus* (mulberry leaf scorch); and *X. f.* subsp. *pauca* (coffee leaf scorch – CLS, and citrus variegated chlorosis - CVC) (Schaad *et al.*, 2004; Scally *et al.*, 2005).

Previously restricted to the Americas (Hopkins & Purcell, 2002, Redak *et al.*, 2004), diseases caused by *X. fastidiosa* have been progressively reported in other regions of the world e.g. in Taiwan (Leu & Su, 1993, Su *et al.*, 2012), Yugoslavia (Berisha *et al.* 1998), Southern Italy (Saponari *et al.*, 2013) and Iran (Amanifar *et al.*, 2014). In the recent outbreak in Southern Italy, a novel *X. f.* subsp. *pauca* strain (CoDiRO), genetically different from the CVC and CLS strains, was strongly associated with severe leaf scorching symptoms in commercial olive trees [*Olea europae* (L.)] (Saponari *et al.*, 2013; Elbeaino *et al.*, 2014). Another recent association of olive plants with leaf scorching symptoms and the presence of *X. f.* subsp. *pauca* was reported in Argentina (Haelterman *et al.*, 2015), but the associated strain was different from that reported in Italy.

By the end of 2014, symptoms identical to those reported for *X. fastidiosa*-infected olive trees in Southern Italy and in Argentina were observed in olive trees growing in the Mantiqueira Mountain Range region, located in the States of Minas Gerais (MG) and São Paulo (SP), in Southeastern Brazil. Commercial olive orchards are being established in this region as a new option for small farmers. Diseased trees were found in olive orchards of Maria da Fé (MG) and São Bento do Sapucaí (SP), which are approx. 130 km apart. The present study describes the diagnosis and identification of a *X. fastidiosa* strain associated with the diseased olive trees in these two localities.

Materials and methods

Plant samples

Tissues from olive trees (branches, twigs, and leaves) showing leaf scorching, as well as from symptomless trees, were collected in Maria da Fé (22°18'S/45°22'W) (MG) and São Bento do Sapucaí (22°40'S/45°40'W) (SP), Brazil, and brought to the laboratory at Centro de Citricultura Sylvio Moreira, Cordeirópolis, São Paulo, for analysis. Symptoms were observed for the olive varieties 'Ascolana', 'Grappolo', and 'Arbequina', but samples were collected only from the latter two varieties in Maria

da Fé and from variety 'Grappolo' in São Bento do Sapucaí. All of the symptomatic trees were 8 to 10 years old.

PCR-based diagnosis and bacterial isolation

For DNA extraction and bacterial isolation, we used leaves and twigs collected from non-symptomatic sections of symptomatic branches. Total DNA extraction was carried out by adding 200 mg of leaf petioles in 2 mL tubes with 5 mm stainless steel beads and 625 µL of buffer (100 mM Tris pH 8.0; 50 mM EDTA; 500 mM NaCl). Tissue was disrupted using TissueLyser II system homogenizer (Qiagen, Valencia, CA) at speed 30 Hz for 120 s; 725 µL of buffer 2 (CTAB 5 %; Sarcosyl 10 %; 10 mM B-mercaptoethanol) was then added to the tube, following a DNA purification protocol based on that of Murray & Thompson (1980). PCR amplification was performed in a 13 µL volume containing 1× master mix PCR (Dream Taq DNA polymerase), 10 pmol of each primer of sets RST31/RST33 (Minsavage *et al.*, 1993) and CVC1/272-2 int (Pooler & Hartung, 1995), and 3 µL of total DNA (100 ng/µL). Amplifications for both primer sets were conducted with the following setup: initial denaturation step at 95°C for 5 min, 36 cycles at 95°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min, with a final extension at 72°C for 5 min. The amplicons were separated on a 1% agarose gel. Negative (tissues from healthy olive plants grown in a vector-proof greenhouse provided by Agromillora Company - <http://www.agromilloraproducao.com.br/>) and positive (tissue from symptomatic CVC-infected citrus plants) controls were used throughout the procedure.

For bacterial isolation, twigs (6–8 cm long and 0.5–0.8 cm diameter) were surface disinfected with 2% bleach for 2 min and 70% alcohol for 2 min, followed by three washes in autoclaved water. Each twig was cut in the middle and the internal cut ends were squeezed with a pair of pliers, and the sap was blotted onto plates of BCYE agar (Wells *et al.*, 1981). These were incubated at 28°C and observed weekly for development of individual colonies (IC). Identification of IC as *X. fastidiosa* was based on *in vitro* fastidious growth, coloration and PCR using RST31/RST33 primers. Selected colonies were tripled cloned and stored in PW broth (Davis *et al.*, 1981) containing 50% glycerol at -80°C.

MLST amplification and sequencing

A fraction of cells extracted from one selected isolate obtained from olive variety 'Grappolo' (strain MFG01) was suspended in 50 µL of water, boiled and used for amplification of seven MLST loci (*leuA*, *petC*, *malF*, *cysG*, *holC*, *nuoL*, and *gltT*) by PCR, following the protocol and primers described by Yan *et al.*, (2010). After checking the amplification in agarose gel (1%), the amplicons were purified using the QIAquick PCR purification Kit (QIAGEN) and directly sequenced by the ABI 3730 sequencer (Life Technology). The forward and reverse sequences were assembled and primers trimmed by CLC Genomics Workbench platform (QIAGEN).

Sequencing analysis

Nucleotide sequences of all seven MLST loci were submitted to analysis in the website *Xylella fastidiosa* MLST databases (<http://pubmlst.org/xfastidiosa/>) following the orientations described by Nunney *et al.* (2013) and also presented in the web site. The phylogenetic reconstruction was achieved based on maximum likelihood (ML) with 1,000 bootstraps using Jukes-Cantor distance run by MEGA (Tamura *et al.*, 2013). For the reconstruction, we downloaded only the sequences of seven MLST loci which belong to the sequence type (ST) representing the type strains of *X. f.* subsp. *pauca*, according to *X. fastidiosa* MLST database (<http://pubmlst.org/xfastidiosa/>) and ran together with the sequences obtained in this work. All the sequences obtained were deposited at GenBank under the accession numbers KT357607 to KT357613, respectively for the alleles *leuA*, *petC*, *malF*, *cysG*, *holC*, *nuoL*, and *gltT*.

Inoculations into olives cuttings

The *X. fastidiosa* subsp. *pauca* strains 9a5c (ST13), PLS8 (ST71), and MFG01 (ST16) were inoculated by pin-pricking into 20 cm tall olives plants, and the infectivity verified by PCR at 60 d from inoculation. Briefly, colonies of the strains grown in PW medium were suspended in PBS at OD ≈ 1, and 10 µL was dropped onto the main stem of each olive plant. The drop was punctured few times using entomological needle #1. This procedure was repeated twice at 2 week intervals, so a total of 30 µL of inoculum was applied to each plant. Two plants were inoculated with PBS as controls, and five plants were inoculated

for each bacterium strain. Two plants inoculated with strain MFG01 died before 60 d after first inoculation as consequence other than *X. fastidiosa* infection.

Results and discussion

The diseased olive trees found in commercial orchards of the Mantiqueira Mountain Range region in Brazil displayed branches that were either entirely desiccated, or had basal and apical leaves expressing different degrees of scorching, starting at the leaf tips. Additional symptoms included pale green leaves, partial defoliation and death of shoots and branches (Figure 1). The symptoms were identical to those recently described for olive plants infected with *X. fastidiosa* in Southern Italy, and in Argentina (Saponari *et al.*, 2013; Haelterman *et al.*, 2015).

PCR-based diagnosis using either conserved primers (RST31 / RST33) for all subspecies of *X. fastidiosa* or primers specific to *X. fastidiosa* subsp. *pauca* (CVC1 / 272-2 int) confirmed the presence of this bacterium in eight of nine twig samples collected from symptomatic branches, but no template was obtained using twigs from asymptomatic trees (n = 20). Positive amplifications by the CVC1/272-2 int primer set is a strong indication that *X. f.* subsp. *pauca*, known to infect sweet orange and coffee trees, is the bacterium present in the olive plants showing leaf scorch. Successful isolations resulting in bacterial colonies with characteristics described for *X. fastidiosa* e.g. *in vitro* fastidious growth and white colour, were obtained on BCYE medium. Identification was confirmed by positive amplifications with specific primers to *X. f.* subsp. *pauca*.

Blast analysis of MLST sequences from strain MFG01 using the *Xylella fastidiosa* MLST Databases (<http://pubmlst.org/xfastidiosa/>) revealed that sequences perfectly matched with sequences of alleles *leuA* #7, *petC* #6, *malF*#8, *cysG*#10, *holC*#11, *nuoL*#8, and *gltT*#8. This allelic profile results in the Sequence Type-ST16 (Nunney *et al.*, 2012), represented by the strain type COF0238 isolated from coffee trees in Brazil in the early 2000's (<http://pubmlst.org/xfastidiosa/>). Phylogenetic analysis confirmed the ST16 into *X. f.* subsp. *pauca*, but showed that isolates were genetically closer to ST11 and ST13, both obtained from sweet orange trees with CVC, when compared to sympatric ST14 from coffee trees (Figure 2). Despite of this closer genetic relationship with the CVC strains (ST11 and ST13), no successful infection were obtained by

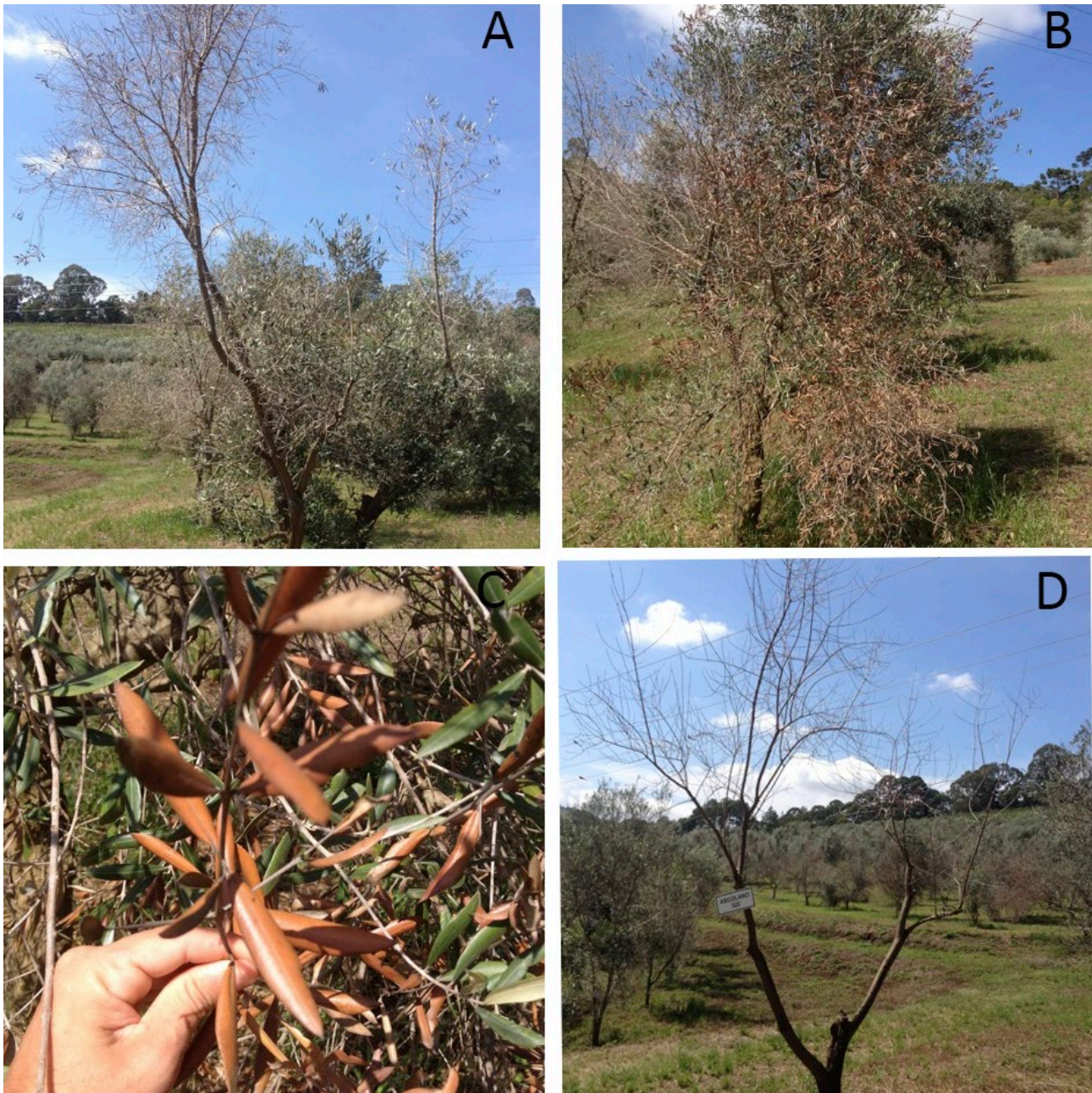


Figure 1. Symptoms observed on olive trees in Maria da Fé, Minas Gerais State, Brazil. A. Leaf drop. B. Leaves showing severe scorching. C. Detail of leaf scorching. D. Plant death and (background) olive trees showing leaf scorching spreading through the orchard.

inoculating the 9a5c (ST13) or another sequence type (ST71) of *X. f.* subsp. *pauca* in small olive plants under screen house conditions, but 100% infection (three out of three inoculated plants) was obtained with

the MFG01 strain (ST16) from olive trees (Figure 3). Haelterman *et al.* (2015) reported the presence of *X. f.* subsp. *pauca* in olive plants with leaf-scorching symptoms in Argentina. The nucleotide sequence of the

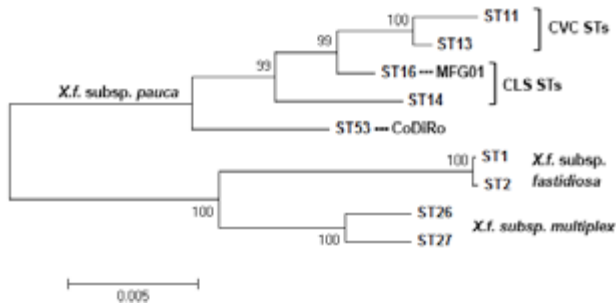


Figure 2. Maximum likelihood tree for concatenate sequences of seven MLST loci from Sequence Types (ST) of *Xylella fastidiosa* subsp. *pauca*, *X. fastidiosa* subsp. *fastidiosa*, and *X. fastidiosa* subsp. *multiplex* present on the MLST database, plus the sequences obtained in the present study the MFG01 strain. Numbers in the nodes are the bootstrap results. CLS = coffee leaf scorch; CVC = citrus variegated chlorosis.



Figure 3. Gel electrophoresis of RST 31/33 primers in olive samples inoculated with different stains of *Xylella fastidiosa* subsp. *pauca*. L = (GeneRuler 1Kb ladder marker - Fermentas). P and N represent positive and negative controls. Lanes 1 and 2 represent olive plants inoculated with PBS; 3 to 7 represents plants inoculated with *X. fastidiosa* strain 9a5c (ST13); 8 to 10 represent plants inoculated with strain MFG01 (ST16) isolate from olive plants with leaf scorch symptoms; 11 to 15 represent olive plants inoculate with strain PLS8 (ST71) from plum. Bands represent samples infected by *X. fastidiosa* (positive samples).

rpoD gene showed 100% of homology with 9a5c type strain from CVC. However, sequences of *rpoD* gene are highly conserved among CVC and CLS strains of *X. fastidiosa* (Wendland *et al.*, 2003). This indicates that another methodology such as MLST should be used to better clarify the origin of *X. fastidiosa* infecting olive trees in Argentina. MLST analysis has been successfully used for identification of subspecies of *X. fastidiosa* (Yan *et al.*, 2010; Nunney *et al.*, 2012), and groups of strains within the same subspecies (e.g. CVC and CLS strains) (Almeida *et al.*, 2008).

This note represents the first report of *X. fastidiosa* subsp. *pauca* infecting olive trees with symptoms of

leaf scorching in Brazil, but indicate that a different sequence type of the bacterium (ST16) is associated with the disease. In Italy the outbreak is associated with ST53 of *X. fastidiosa*, the same ST found infecting oleander and coffee plants in Costa Rica (Nunney *et al.*, 2014). *X. fastidiosa* present in olive plants in Brazil was possibly introduced from coffee plants via leafhopper vectors, considering the proximity of coffee plantations to olive orchards in the affected region. The finding of diseased olive trees with positive diagnosis for the same ST16 in two localities distant 130 km from each other (Maria da Fé and São Bento do Sapucaí), indicate that the bacterium is spreading through orchards in the Mantiqueira Mountain Range region.

Although *X. fastidiosa* has not yet been proved as the causal agent of the new disorder in olives, a strong correlation between leaf scorching symptoms and presence of this bacterial pathogen is evident as reported for three distant regions (Southern Italy, Argentina, and now Brazil). Further pathogenicity tests with different strains of *X. f. subsp. pauca* (STs 11, 13, 14, and 16 – see Nunney *et al.*, 2012) are in development in Brazil, and these should provide information about the susceptibility of olive plants to a broad spectrum of *X. fastidiosa*.

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