Phytopathologia Mediterranea (2014) 53, 1, 328–332
DOI: 10.14601/Phytopathol_Mediterr-14113

SHORT NOTES

Identification of three potential insect vectors of *Xylella fastidiosa* in southern Italy

Toufic ELBEAINO[1], Thaer YASEEN[1], Franco VALENTINI[1], Issam Eddine BEN MOUSSA[1], Valerio MAZZONI[2] and Anna Maria D'ONGHIA[1]

[1] CIHEAM - Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (BA), Italy
[2] Research and Innovation Center, Foundation Edmund Mach, S. Michele all'Adige, via Mach 1, 38010 (TN), Italy

Summary. In order to identify potential vectors of *Xylella fastidiosa* in olive orchards in Puglia (southern Italy), Hemiptera insects were collected from October to December, 2013, in olive orchards with high incidences of *X. fastidiosa* associated with "rapid decline" symptoms. The study focused on species in the *Auchenorrhyncha* (sharpshooter leafhoppers and froghoppers or spittlebugs), a group that includes known vectors of *X. fastidiosa.* Adults of three species, i.e. *Philaenus spumarius* L. (Aphrophoridae), *Neophilaenus campestris* Fallén (Aphrophoridae) and *Euscelis lineolatus* Brullé (Cicadellidae) were captured, from which total DNA was extracted and assayed by PCR using three sets of specific primers designed for *X. fastidiosa* detection. Results of PCR showed that 38 out of a total of 84 tested insects were positive for *X. fastidiosa,* i.e. eight (of 20) *P. spumarius*, 14 (of 18) *N. campestris* and 16 (of 46) *E. lineolatus*. PCR amplicons of the RNA polymerase sigma-70 factor gene from six specimens (two of each insect species) were sequenced. The sequences obtained were 99.3–99.4% identical. BlastN analyses demonstrated these sequences to be similar to those of *X. fastidiosa* isolates from olive OL-X and OL-G reported from Puglia, whereas they displayed distant molecular identity (always less than 98%) with *X. fastidiosa* subspecies from other countries. The detection of *X. fastidiosa* in *P. spumarius* and, for the first time, in *N. campestris* and *E. lineolatus* (which, unlike the others, is a phloem feeder), indicates potential vectoring roles of these insects for the spread of the bacterium in Puglia. Further investigations and specific infectivity trials are required to definitively determine the roles of these insects as effective vectors of this pathogen.

Key words: olive trees, *Philaenus spumarius, Neophilaenus campestris, Euscelis lineolatus*, rapid decline.

Introduction

Xylella fastidiosa is a xylem-inhabiting, vector-borne, Gram-negative bacterium that infects numerous host plant species, on which it can be latent or induce mild to severe symptoms. The main diseases caused by *X. fastidiosa* include Pierce's disease of grapevine (Davis *et al*., 1978), citrus variegated chlorosis (Chang *et al*., 1993), coffee leaf scorch (Li *et al*., 2001), pecan leaf scorch (Sanderlin and Heyderich-Alger, 2000), phony peach (Wells *et al*., 1983), plum leaf scald (Raju *et al*., 1982), and almond leaf scorch (Mircetich *et al*., 1976). *Xylella fastidiosa* has also been shown to be the causal agent of leaf scorch diseases in landscape plants such as oleander (Purcell *et al*., 1999), mulberry (Hernandez-Martinez *et al*., 2006) and oak (Barnard *et al*., 1998). Less clear seems the etiological role of *X. fastidiosa* subsp. multiplex on Californian olive trees exhibiting leaf scorch and/or branch dieback symptoms (Krugner *et al*., 2014). The bacterium was known to occur only in the American continents and in some limited areas of Asia (Taiwan). The recent detection of *X. fastidiosa* in Puglia (southern Italy) (Saponari *et al*., 2013), has demonstrated that the pathogen is present in the European Union, and is relevant for the entire European region (Council Directive 2000/29/EC, Annex I-section II).

Corresponding author: A.M. D'Onghia
Fax: +39 080 4606503
E-mail: donghia@iamb.it

ISSN (print): 0031-9465
ISSN (online): 1593-2095
www.fupress.com/pm

Some natural *Auchenorrhyncha* insect vectors (mainly sharpshooter leafhoppers and froghoppers or spittlebugs) contribute to its rapid spread, and adult insects remain infectious throughout their lives (Purcell and Finley, 1979). Many vectors of *X. fastidiosa* have been reported by Redak *et al*. (2004), and the following Hemiptera of the family *Cicadellidae* are particularly efficient: *Homalodisca vitripennis* Germar (glassy-winged sharpshooter), *Draeculacephala minerva* Ball (green sharpshooter), *Graphocephala atropunctata* Signoret (blue-green sharpshooter) and *Hordnia circellata* Baker for Pierce's disease; *Cuerna costalis* Fabricius, *H. insolita* Walker, *Oncometopia nigricans* Walker and *O. orbona* Fabricius for phony peach; *Acrogonia terminalis* Young, *Dilobopterus costalimai* Young and *Oncometopia fascialis* Signoret for citrus variegated chlorosis (Hill and Purcell, 1995; Hail *et al*., 2010). Putative vectors for Europe are considered to be *Cicadella viridis* L. (Cicadellidae) and *Philaenus spumarius* L. (Aphrophoridae) (Janse and Obradovic, 2010), even though the list of potential vectors should be extended to all the xylem-fluid feeders, as suggested by Purcell (1989).

Century-old olive trees infected by *X. fastidiosa* and exhibiting leaf scorch and "rapid decline" symptoms have been recently observed in Lecce province of Apulia (southern Italy), where the pathogen has been detected by PCR and ELISA in a large area (Saponari *et al*., 2013). Even though the etiological role of *X. fastidiosa* in the "rapid decline" disease in Italy remains to be demonstrated since other pathogens (fungi) and/or insects could also be involved, the wide spread of the bacterium in Lecce province prompted an investigation to identify local insects which may be involved in the transmission of the bacterium.

Materials and methods

Capture of insects

Four olive orchards with high incidence of *X. fastidiosa* were repeatedly visited from October to December, 2013, in Gallipoli area (Lecce province) for insect captures. Insects were also captured from one olive orchard located in an area where *X. fastidiosa* was absent. Two different collection methods were adopted: (i) three to five yellow sticky traps per orchard were placed outside the olive canopies; (ii) a sweeping net was used to manually trap the insects in the olive orchards both from the olive canopy and ground vegetation. Upon retrieval from the field after 20 d exposure, the sticky traps were placed into plastic bags and stored at 4°C. Once in laboratory, Hemiptera insects were removed from the traps by applying a solvent oil around each insect, and which was then washed in 95% ethanol and de-ionized water to remove any oil residue. The insects captured with nets were carefully collected (by aspiration) directly *in loco*, put in small tubes containing 95% ethanol and brought to the laboratory for identification.

Identification of insects

In this study, all leafhoppers and froghoppers were taken into consideration as potential vectors of the disease. Despite only xylem specialists have been proved to be effective vectors of *X. fastidiosa* (Almeida and Purcell, 2006) we examined also phloem feeders in that they can occasionally consume or get in contact with the xylem (Pompon *et al*., 2011).

The classification and nomenclature of captured insects were based on Ribaut (1952), Della Giustina (1989) and Holzingher *et al*. (2003). To accomplish identification, male genitalia of each insect were dissected and kept in KOH (10%) for 24 h; these were then mounted on glass slides in Berlese's liquid and observed under a stereoscopic microscope.

Extraction of *Xylella fastidiosa* DNA and PCR

Insect heads of single adult specimens were removed as described by Bextine *et al*. (2004) and used for total DNA extraction using the commercial kit "DNeasy Plant Mini Kit", following the manufacturers' instructions (Qiagen). In parallel, 0.5 g of tissue from the midveins of infected olive leaves were used as *X. fastidiosa*-positive controls for the PCR assay using the same extraction kit. PCR assays were conducted with three sets of specific primers, targeting the RNA polymerase sigma-70 factor, the 16S rRNA and the hypothetical HL protein genes (Firrao and Bazzi, 1994; Minsavage *et al*., 1994; Francis *et al*., 2006), which generate PCR amplicons of size 733 bp, 404 bp and 221 bp, respectively. Each PCR mixture contained template DNA at 10 ng μL^{-1}, 1.25 U of Go Taq polymerase (Promega), 1 × Go Taq Flexi DNA Buffer, 0.2 mM dNTPs, 0.3 μM forward and reverse primers, all in a final reaction volume of 25 μL. The following thermocycling (IQ5Thermocycler, BioRad) programme was used: initial denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 40 s; with a final extension at 72°C for 7 min. Amplifications were confirmed on 1.2% agarose gel.

Cloning and sequence analyses

PCR amplicons were transformed in StrataClone^{TM} PCR Cloning vector pSC-A (Stratagene), subcloned into *Escherichia coli* DH5α or SoloPACK cells and custom sequenced (Primm). Four copies of each DNA clone were sequenced bi-directionally to eliminate any sequence ambiguity. The sequences obtained were cleaned from vector with the assistance of the DNA Strider 1.1 program (Marck, 1988). Sequence similarities were analyzed with BLASTN at the National Center for Biotechnology Information website (Altschul *et al*., 1990). Multiple sequence alignments were generated using the default options of CLUSTALX 1.8 (Thompson *et al*., 1997). Sequences obtained from the present study were deposited in GenBank.

Results and discussion

Identification of insects

A total of 84 adult insect specimens (Table 1), belonging to the taxon *Auchenorrhyncha*, were captured from the four infected olive orchards in Gallipoli, mainly using the sweeping net. The insects were identified as *P. spumarius* L. (Aphrophoridae), *Neophilaenus campestris* Fallen (Aphrophoridae) and *Euscelis lineolatus* Brullé (Cicadellidae). Specimens of the same insect species (32 *P. spumarius*, 15 *N. campestris* and 18 *E. lineolatus*) were also captured from one olive orchard located in Bari province, which is considered an *X. fastidiosa*-free area. *Philaenus spumarius* and *N. campestris* were easily distinguishable from one other based on rear leg morphology; for *P. spumarius* the number of apical spines was less than ten (7–8) both in tibia and tarsus, whereas the number was greater (11–12) in *N. campestris* (Figure 1). *Philaenus spumarius* is a cosmopolitan species and widely polyphagous (numerous herbs, shrubs and trees). This insect overwinters as eggs, and in Europe nymphs occur from April–May, according to the region, while adults appear 1 month

Table 1. Numbers of *Xylella fastidiosa*-positive Hemiptera insects captured from four infected olive orchards and one non infected orchard, using yellow sticky traps (YST) and sweeping net (SN).

Olive orchard	Capture mode	*Philaenus spumarius*		*Neophilaenus campestris*		*Euscelis lineolatus*		Infected/ captured
		♂	♀	♂	♀	♂	♀	
X. fastidiosa-infected area								
1	YST	2	0	0	1	1	3	19/33
	SN	4	3	4	4	3	8	
2	YST	0	1	2	0	2	0	15/21
	SN	2	2	0	4	5	3	
3	YST	0	1	0	0	0	3	1/14
	SN	1	1	0	1	2	5	
4	YST	1	0	0	0	0	2	3/16
	SN	0	2	0	2	7	2	
Infected/captured		2/10	6/10	4/6	10/12	8/20	8/26	38/84
X. fastidiosa-free area								
5	SN	13	19	5	10	8	10	65
Infected/captured		0/13	0/19	0/5	0/10	0/8	0/10	0/65

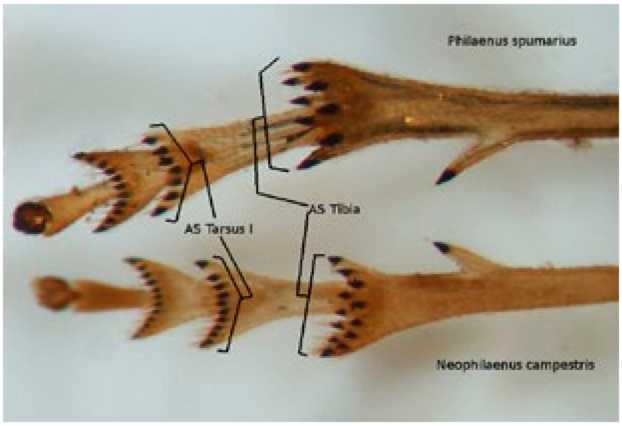

Figure 1. Distinction between adults of *Neophilaenus campestris* (below) and *Philaenus spumarius* (above) based on rear leg morphology. AS, apical spines.

later and can be found until November–December. *Neophilaenus campestris* is present throughout Europe and in many other Mediterranean countries, with a life cycle very similar to *P. spumarius.* It is very common in grasslands but can be also found on trees, probably used as temporary shelter on hot days. *Euscelis lineolatus* is common in Europe and in the sub-Mediterranean region; it overwinters as adults and is polyphagous (Mazzoni, 2005).

PCR and sequence analysis of *Xylella fastidiosa*

Results of PCR assays showed that *X. fastidiosa* was detected in 38 of 84 insects (45%) captured from the infected area, and in none of the 65 insects from the *X. fastidiosa*-free area. In particular, in the infected area eight of 20 (40%) were specimens of *P. spumarius*, 14 of 18 (78%) were *N. campestris* and 16 of 46 (35%) were *E. lineolatus*. No differences in infection were observed due to the sex of insects. All the three sets of primers gave identical results in PCR assays (no case of dissimilarity was observed), and efficiently detected *X. fastidiosa* in the insect specimens and in the infected olive leaves used as positive controls. The six sequences obtained were 99.3–100% identical at the nucleotide level, and three of the sequences (one from each insect) were deposited in GenBank (accession No. HG939491, HG939505, HG939506). BlastN analysis showed that all these sequences had the greatest nucleotide identity (99.6–99.7%) with *X. fastidiosa* DNA fragments OL-X4-5 (accession No. HG532021) and OL-G (accession No. HG532022) from olive of Puglia region. However, they shared 96–98% identity with homologous fragments of isolates from other countries, and in particular 98% with the strains of subspecies *fastidiosa* GB514 (CP002165 from Texas, USA) and M23 (CP001011 from California, USA), and 97% with the strain of subspecies *pauca* 9a5c (AE003849).

The detection of *X. fastidiosa* in these insects was not surprising, as much as the nucleotide differences found in the RNA polymerase sigma-70 factor gene, which was clearly distant from all sequences of *X. fastidiosa* reported in GenBank.

Based on our knowledge, this is the first report on *X. fastidiosa* detection in *N. campestris* and *E. lineolatus,* while the pathogen has previously been associated with *P. spumarius* (Severin, 1950; Guario *et al*., 2013). However, among these insects we expect that the ability to transmit *X. fastidiosa* is greater in *P. spumarius* and *N. campestris*, which are known to be xylem fluid feeders, than in *E. lineolatus* which is a phloem fluid feeding insect. As for the latter, our findings clearly indicate that also specialized phloem feeders such as leafhoppers, can come in contact with xylem vessels and become infected. Our contribution is the first documented evidence of this phenomenon in leafhoppers that needs further investigation, since up to date the role of phloem feeders in *X. fastidiosa* cycle and dissemination has been neglected. Furthermore it is worth to remind that *E. lineolatus* is already known as a vector for phytoplasmas [Aster Yellow (16SrI) and Stolbur (16SrXII)] (Landi *et al*., 2013).

If these insect species have effective roles in transmission of *X. fastidiosa* in Puglia, this needs to be confirmed by further investigations and specific infectivity trials. These should aim to demonstrate the ability of insects to transmit the bacterium from one plant to another. Nevertheless, the control of any of these potential vectors will be very difficult due to their polyphagous nature, which involves both wild and cultivated plants.

Acknowledgements

Grateful thanks are expressed to Dr. G. Cataldi for the helpful collaboration in the field activity.

Literature cited

Almeida R.P.P. and A.H. Purcell, 2006. Patterns of *Xylella fastidiosa* colonization on the precibarium of sharpshooter vectors relative to transmission to plants. *Annals of Entomological Society of America* 90, 884–890.

Altschul S.F., F. Stephen, W. Gish, W. Miller, E.W. Myers and D.J. Lipman, 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215, 403–410.

Barnard E.L., E.C. Ash, D.L. Hopkins and R.J. McGovern, 1998. Distribution of *Xylella fastidiosa* in oaks in Florida and its association with growth decline in *Quercus laevis*. *Plant Disease* 82, 569–572.

Bextine B., S.J. Tuan, H. Shaikh, M. Blua and T.A. Miller, 2004. Evaluation of methods for extracting *Xylella fastidiosa* DNA from the glassy-winged sharpshooter. *Journal of Economic Entomology* 97, 757–763.

Chang C.J., M. Garnier, L. Zreik, V. Rossetti and J.M. Bové, 1993. Culture and serological detection of the xylem-limited bacterium causing citrus variegated chlorosis and its identification as a strain of *Xylella fastidiosa*. *Current Microbiology* 27, 137–142.

Davis M.J., A.H. Purcell and S.V. Thomson, 1978. Pierce's disease of grapevines isolation of the casual bacterium. *Science* 199, 75–77.

Della Giustina W., 1989. Homoptères Cicadellidae - *Faune de France* 73(3), 1–350.

Firrao G. and C. Bazzi, 1994. Specific identification of *Xylella fastidiosa* using the polymerase chain reaction. *Phytopathologia Mediterranea* 33, 90–92.

Francis M., H. Lin, J. Cabrera-La Rosa, H. Doddapaneni and E.L. Civerolo, 2006. Genome-based PCR primers for specific and sensitive detection and quantification of *Xylella fastidiosa*. *European Journal of Plant Pathology* 115, 203–213.

Guario A., F. Nigro, D. Boscia and M. Saponari, 2013. Disseccamento rapido dell'olivo, cause e misure di contenimento. *L'Informatore Agrario* 46, 51–54.

Hail D., F. Mitchell, I. Lauzière, P. Marshall, J. Brady and B. Bextine, 2010. Detection and analysis of the bacterium, *Xylella fastidiosa*, in glassy-winged sharpshooter, *Homalodisca vitripennis*, populations in Texas. *Journal of Insect Science* 10, 168.

Hernandez-Martinez R., T.R. Pinckard, H.S. Costa, D.A. Cooksey and F.P. Wong, 2006. Discovery and characterization of *Xylella fastidiosa* strains in southern California causing mulberry leaf scorch. *Plant Disease* 90, 1143–1149.

Hill B.L. and A.H. Purcell, 1995. Acquisition and retention of *Xylella fastidiosa* by an efficient vector, *Graphocephala atropunctata*. *Phytopathology* 85, 209–212.

Holzinger W.E., I. Kammerlander and H. Nickel (ed.), 2003. *The Auchenorrhyncha of Central Europe (Fulgoromorpha, Cicadomorpha excl. Cicadellidae)*. Volume I. - Brill Publishers, Leiden, The Netherlands, 673 pp.

Janse J.D. and A. Obradovic, 2010. *Xylella fastidiosa*: Its biology, diagnosis, control and risks. *Journal of Plant Pathology* 92 (S1), 35–48.

Krugner R., M.S. Sisterson, J.C. Chen, D.C. Stenger and M.W. Johnson, 2014. Evaluation of olive as a host of *Xylella fastidiosa* and associated sharpshooter vectors. *Plant Disease* (in press) doi:http://dx.doi.org/10.1094/PDIS-01-14-0014-RE.

Landi L., N. Isidoro and P. Riolo, 2013. Natural phytoplasma infection of four phloem-feeding Auchenorrhyncha across vineyard agroecosystems in central-eastern Italy. *Journal of Economic Entomology* 106(2), 604–13.

Li W.B., W.D. Pria, C. Teixeira, V.S. Miranda, A.J. Ayres, C.F. Franco, M.G. Costa, C.X. He, P.I. Costa and J.S. Hartung, 2001. Coffee leaf scorch caused by a strain of *Xylella fastidiosa* from citrus. *Plant Disease* 85, 501–505.

Marck C., 1988. DNA Strider: a "C" programme for the fast analysis of DNA and protein sequences on the Apple Macintosh family computers. *Nucleic Acids Research* 16, 1829–1836.

Mazzoni V., 2005. Contribution to the knowledge of the Auchenorrhyncha (Hemiptera Fulgoromorpha and Cicadomorpha) of Tuscany (Italy). *Redia* 88, 85–102.

Minsavage G.V., C.M. Thompson, D.L. Hopkins, M.V.B.C. Leite and R.E. Stall, 1994. Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. *Phytopathology* 84, 456–461.

Mircetich S.M., S.K. Lowe, W.J. Moller and G. Nyland, 1976. Etiology of almond leaf scorch disease and transmission of the casual agent. *Phytopathology* 66, 17–24.

Pompon J., D. Quiring, C. Goyer, P. Giordanengo and Y. Pelletier, 2011. A phloem-sap feeder mixes phloem and xylem sap to regulate osmotic potential. *Journal of Insect Physiology* 57, 1317–1322.

Purcell A.H., 1989. Homopteran transmission of xylem-inhabiting bacteria. *Advances in Disease Vector Research* 6, 243–266.

Purcell A.H. and A. Finlay, 1979. Evidence for noncirculative transmission of Pierce's disease bacterium by sharpshooter leafhoppers. *Phytopathology* 69, 393–395.

Purcell A.H., S.R. Saunders, M. Hendson, M.E. Grebus and M.J. Henry, 1999. Causal role of *Xylella fastidiosa* in oleander leaf scorch disease. *Phytopathology* 89, 53–58.

Raju B.C., J.M. Wells, G. Nyland, R.H. Brlansky and S.K. Lowe, 1982. Plum leaf scald isolation and culture and pathogenicity of the casual agent. *Phytopathology* 72, 1460–1466.

Redak R.A., A.H.Purcell, J.R.S. Lopes, M.J. Blua, R.F. Mizell and P.C. Andersen, 2004. The biology of xylem fluid-feeding insect vectors of *Xylella fastidiosa* and their relation to disease epidemiology. *Annual Review of Entomology* 49, 243–270.

Ribaut H., (1952): Homoptères Auchénorhynches. II. (Jassidae). *Faune de France* 57, 1–474.

Sanderlin R.S. and K.I. Heyderich-Alger, 2000. Evidence that *Xylella fastidiosa* can cause leaf scorch disease of pecan. *Plant Disease* 84, 1282–1286.

Saponari M., D. Boscia, F. Nigro and G.P. Martelli, 2013. Identification of DNA sequences related to *Xylella fastidiosa* in oleander, almond and olive trees exhibiting leaf scorch symptoms in Apulia (Southern Italy). *Journal of Plant Pathology* 95, 659–668.

Severin H.H.P., 1950. Spittle-insect vectors of Pierce's disease virus. II. Life history and virus transmission. *Hilgardia* 19, 357–376.

Thompson J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins, 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25, 4876–4882.

Wells J.M., B.C. Raju and G. Nyland, 1983. Isolation culture and pathogenicity of the bacterium causing phony disease of peach *Prunus persica*. *Phytopathology* 73, 859–862.

Accepted for publication: April 5, 2014